"DIFFERENTIAL REGULATION OF MULTIDRUG RESISTANCE GENE(S) IN THE CLINICAL ISOLATES OF PATHOGENIC YEAST *CANDIDA ALBICANS*"

Thesis submitted in partial fulfillment of the requirement for the award of degree of

Doctor of Philosophy

by

Raman Manoharlal

SCHOOL OF LIFE SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067
INDIA

September, 2008

SCHOOL OF LIFE SCIENCE
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067

Certificate

This is to certify that this thesis titled **"Differential regulation of multidrug resistance gene(s) in the clinical isolates of pathogenic yeast *Candida albicans*"** submitted to Jawaharlal Nehru University, New Delhi, in fulfillment of the requirement for the award of the degree of doctor of philosophy, embodies original research work carried out by **Raman Manoharlal** at School of Life Sciences, Jawaharlal Nehru University, New Delhi under our guidance and has not been submitted in part or full for any degree or diploma of this or any other university.

Prof. Rajendra Prasad
School of Life Sciences
Jawaharlal Nehru University,
New Delhi-110067
(Supervisor)

Prof. R. K. Kale
School of Life Sciences
Jawaharlal Nehru University,
New Delhi-110067
(Dean)

September, 2008

SCHOOL OF LIFE SCIENCE
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067

Declaration

The research work entitled **"Differential regulation of Multidrug resistance gene(s) in the clinical isolates of pathogenic yeast *Candida albicans*"** presented in this thesis embodies the results of original work carried out by me at the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work has not been submitted in part or full for any degree or diploma of this or any other university.

Raman Manoharlal

(Candidate)

September, 2008

Dedicated to....

Goddess Saraswati

whose benedictions complete the code of my life

Abstract

Toxic compounds such as drugs are used to treat many diseases by killing the harmful target cells, which can be either foreign pathogenic organisms or the tumor cells of the patients. However, both prokaryotic and eukaryotic cells can acquire the ability to become resistant to toxic compounds through the phenomenon of Multidrug (MDR) or Pleiotropic drug resistance (PDR). MDR is characterized by the cross-resistance of cells to a large number of structurally and functionally unrelated cytotoxic compounds. The pathogenic yeast, *Candida albicans* can also acquire MDR, making it a valuable tool in the study of this phenomenon so that we may gain insights into the mechanisms behind MDR in pathogenic fungi and in higher eukaryotes. A common feature underlying acquisition of MDR in azole resistant clinical *C. albicans* isolate is the over-expression of genes encoding plasma membrane ATP-binding cassette (ABC) drug efflux pump, *CDR1* (*Candida* drug resistance 1) that causes the expulsion of drugs from within the cell and, as a result, survival in the presence of these drugs.

Research to overcome this problem in candidiasis chemotherapy has been focused mainly on two areas: (1) development of Cdr1p modulators/inhibitors, (2) advanced and sensitive detection of *CDR1* mRNA or Cdr1p and its expression regulation. However, recent understanding of the transcriptional regulation of efflux pump *CDR1*, provides new and effective avenues for the development of broad-spectrum fungicides in contrast to indirect intervention *via* inhibition of the energy supply for drug efflux by modulators/inhibitors.

The main focus of this study was, however, in processes different from the classical transcriptional control as majority of studies to date focus on the transcriptional regulation of *CDR1* and relevance of the post-transcriptional events associated with it are poorly understood. Evidences from our present studies suggests that post-transcriptional regulation in the form of increased *CDR1* mRNA stability in addition to its enhanced transcription activation is also a key mechanism, indicating that steady-state level of *CDR1* mRNA as determined by both its synthesis as well as degradation plays an important role in determining the azole resistance in clinical *C. albicans* isolates.

In essence, it is tempting to suggest that *CDR1* 3'UTR may function as a destabilizing element as (1) 3'UTRs have been characterized as important determinants of mRNA decay, (2) several *trans*-acting factors (RNA-binding proteins) have been shown to bind these regions and play a important role in the modulation of the half-life of corresponding transcript. Therefore,

understanding the biological function and importance of these regions in the context of *CDR1* mRNA will provide insights on the molecular mechanism of mRNA stability in the development of MDR of *C. albicans*. Our present study reveals that differential RNA-protein interaction(s) and poly(A) tail length of *CDR1* 3'UTR rather than *cis*-acting sequences within the *CDR1* 3'UTR itself are important determinant of observed differential mRNA decay in azole-susceptible and -resistant isolates.

Since enhanced transcriptional activation and mRNA stability are the key mechanisms for *CDR1* over-expression in azole-resistant clinical isolates, thereby new therapeutic approaches in combating MDR can be devised to effectively down-regulate *CDR1* by targeting its mRNA.

Acknowledgments

I express my heartfelt gratitude to my Supervisor, **Prof. Rajendra Prasad** for his guidance, suggestive criticism, infectious unending energy, 'never say die' attitude, hard work, organized nature and. exemplary administrative skills. I consider myself fortunate in being a part of his research group, which gave me a chance to know myself and learn the values of dedication, hard work and perseverance from him.

My special thanks to **Prof. Joachim Morschhäuser** (Wurzburg, Germany) for his valuable suggestions, discussions and critiques were of essence to the progress of this work. I am greatly indebted to the **Reviewers and Editors** for their scientific comments in improving the quality of this research work.

With a deep sense of gratitude, I wish to express my sincere thanks to **Dr. Gauranga Mukhopadhyay** (SCMM, JNU), **Prof. Chinmay K. Mukhopadhyay** (SCMM, JNU), **Dr. Suman K. Dhar** (SCMM, JNU), **Prof. Shyamal Goswami** (SLS, JNU), **Dr. K. Natarajan** (SLS, JNU), **Prof. P.C. Rath** (SLS, JNU) and **Prof. Uttam K. Pati** (CBT, JNU) for their excellent suggestions in experimental design and interpretation whenever I asked for.

I am greatly indebted to the former dean **Prof. P.K. Yadava** and current dean **Prof. R.K. Kale** for providing excellent facilities in the School.

I also appreciate the help rendered by Central Instrument Facility staff **B.A. Khan, A.C. Alexander, S.P. Sharma, S.K. Mishra, Rajendra, Pankaj, Aslam** and **Balwant** for extending their help in day to day work.

Meenu Mam of SLS office deserves special mention for her friendly and caring attitude throughout these years.

A special mention must go to **Mr. Shankar** and **Ms. Pratima** for their constant help and timely assistance every now and then in all official affairs. I would also like to mention the names of the technical staff members **Tiklal, Yadavji, Sharmaji, Nareshji, Sukhendu, Surendra, Gagan, Vijay, Vijender** and **Reema** for their untiring efforts to make things easy to work in the lab. Ever smiling **Sanjay** and **Suneeta** are simply unforgettable.

Financial assistance in the form of JRF and SRF from Council of Industrial and Scientific Research (CSIR) is gratefully acknowledged.

I extend my warm thanks to **Vibha mam**, for providing the homely environment and sharing wonderful parties. Time spent with naughty **Mohit** will be cherished a lot.

Warm regards to all my seniors **Neerja**, **Suneet**, **Sudhakar**, **Ali**, **Nagendra**, **Preeti**, **Nivedita**, **Annu**, **Tulika**, **Gopal**, **Aparna**, **Dola**, **Parul**, **Sardhi**, **Nandini**, **Sudhanshu**, **Versha**, **Ritu** , **Absar**, **Sunil** and **Ruchi** for the useful discussions. I would like to give my sincere thanks to 'cool and calm' **Naseem** for being very supportive and introducing me to the world of Candida. The time spent with beautiful **Manisha**, Untiring **Nidhi** and lively **Hina** is quite memorable. **Monika** and **Sanjiveeni** deserve special thanks for being extremely supportive, unconditional in every possible way, patient enough to tolerate my bad tempers. Other labmates **Jyoti**, **Shipra**, **Antresh**, **Sarmishtha**, **Saif**, **Ajeet**, **Ashutosh**, **Indresh**, **Manpreet**, **Kalpana**, **Lamuk Vikram**, **Sangeeta**, **Rakhi**, **Deepti** and **Haris** needs a worth mentioning for making the lab atmosphere lively. It's a great pleasure to have friends like **Vipin bhaiya**, **Amit**, **Sumit**, **Renju**, **Master Chandan Singh**, **Nandlal**, **Rashid Anwar Rafique**, **Ishtiyaq** and batchmates like **Ramesh**, **Parveen**, **Naveen**, **Ratnakaran**, **Abhinay**, **Manoj**, **Manish**, **Ankush**, **Kulbhushan** and **Swapnil**.

My special thanks to ever-ready **Shiteshu** who was always there for sharing my ups and downs during all these years.

Special word of thanks to **Jyotsna** for her ever-helping attitude extended unmitigated motivation and care in every possible way. Her guidance, encouragement, healthy discussions, unconditional help and support helped me in all my endeavors.

I can't move on without my special gratitude to 'ideal' **Sachin Ramesh Tendulkar** for constant source of inspiration and being a positive control throughout my life.

My final word goes to my parents, for their absolute confidence in me. They have always been there for me and lent me unconditional support and

inspiration throughout. They have respected all my decisions and stood by me always. It is their endless encouragement, affection and blessings that keep me going.

Last but not least, thanks to the Great almighty, whose blessings helped me be what I am....

Raman

Abbreviations

List of abbreviations

µg	microgram (10^{-6} grams)
aa	amino acid(s)
ABC	ATP Binding Cassette
AIDS	Acquired ImmunoDeficiency Syndrome
AP-1	Activator Protein-1
AR	Azole Resistant
ARE	Azole responsive element
AS	Azole Susceptible
ATP	Adenosine 5'-triphosphate
AUG	initiation codon
AURE	Adenylate- and Uridylate-rich (AU-rich) element
BARC	Bhabha Atomic Research Center
BCS	Bathocuprioinedisulphonic acid
BEE	Basal Expression Element
bp	base pair(s)
BRE	Benomyl Responsive Element
Ca	*Candida albicans*
CAD	Cadmium resistance
CaMDR1	*Candida albicans* multidrug resistance 1
CAP	*Candida* activation protein (transcription factor)
cDNA	complementary DNA (DNA complementary to RNA)
CDR	*Candida* drug resistance
Cdr1p	*Candida* drug resistance protein
CE	Cytoplasmic Extracts
CFTR	Cystic fibrosis transmembrane conductance receptor
CM	Crude membrane
Conc.	Concentration
CPE	Cytoplasmic Polyadenylation Element

DEPC	DiEthylPyroCarbonate
DMSO	DiMethylSulphOxide
DNA	Deoxyribonucleic acid
DRE	Drug-Responsive Element
DTT	Dithiothreitol
E-743	Ecteinascidin-743
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
EMSA	Electrophoretic Mobility Shift Assay
ER	Endoplasmic Reticulum
ERG	Ergosterol biosynthetic enzymes
FACS	Fluorescence Activated Cell Sorting
FCR1	Fluconazole Resistance 1
FCY	Flucytosine resistance
FLR1	Fluconazole resistance 1
ΔG	Gibbs free energy
GFP	Green Fluorescent Protein
H	hour
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HFAR	High-Frequency Azole Resistance
HIV	Human Immunodeficiency Virus
HRE	H_2O_2 Responsive Element
HSE	Heat Shock Element
HSF	Heat Shock Transcription Factor
HSP	Heat Shock Protein
IPTG	Isopropyl thio-β-D-galactoside
IRES	Internal Ribosome-Entry Sequence
kb	kilobase pair (s)
kcal/mol	kilocalories per mole
kDa	kilo Dalton

lacZ	ß-galactosidase
MALDI	Matrix-Assisted Laser Desorption/Ionization
MB	Molecular Biology
MDR	Multi Drug Resistance
MDT	MultiDrug Transporter
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
min	minutes
ml	millilitre (10^{-3} litres)
mM	millimolar (10^{-5} molar)
mRNA	messenger ribonucleic acid; messenger-RNA
MRR1	Multidrug Resistance Regulator 1
NBD	Nucleotide Binding Domain
NDT80	Non DiTyrosine 80
ng	nanogram (10^{-9} grams)
nM	nanomolar (10^{-9} molar)
NRE	Negative Regulatory Element
NS-SNP	Non-Synonymous Single Nucleotide Polymorphism
nt	nucleotide(s)
N-terminal	amino terminal
OPC	Oropharyngeal candidiasis
ORF	Open Reading Frame
p	protein
P450$^{14\alpha dm}$	P450 14 α-demethylase
PABP	Poly(A)-Binding Protein
PAGE	PolyAcrylamide Gel Electrophoresis
PAS	PolyAdenylation Signals
PAT	PolyAdenylation Test
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PDR	Pleiotropic Drug Resistance
PDREs	Pleiotropic Drug Resistance Elements
P-gp	P-glycoprotein
PM	Plasma membrane
PMSF	Phenylmethylsulphonyl fluoride
Poly(A) tail	polyadenylate residues at the 3'end of an mRNA
Py	polypyrimidine tracts
R6G	Rhodamine6G
RACE	Rapid Amplification of cDNA Ends
R-EMSA	RNA-Electrophoretic Mobility Shift Assay
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAP	RNA polymerases
rpm	revolutions per minute
RT	Reverse Transcriptase
Sc	*Saccharomyces cerevisiae*
SD ura⁻	Synthetic defined uracil⁻ media
SDS	Sodium Dodecyl Sulfate
sec	seconds
SLS	Sodium Lauryl Sulfate
SRE	Steroid Response Element
SRR	Steroid Responsive Region
S-SNP	Synonymous Single Nucleotide Polymorphism
STRE	Stress Response Element
$t_{1/2}$	half-life
TAC1	Transcriptional Activator of *CDR* genes
TEMED	N, N, N, N'-TEtraMEthyleneDiamine
TF	Transcription factor
TMD	Transmemberane domain
tRNA	transfer Ribonucleic Acid

TRO	Transcription Run-On
TSP	Transcription Start Point
Tup1	Thymidine uptake 1
UAS	Upstream Activating Sequences
uORF	Upstream Open Reading Frame
Upc2	Uptake control 2
URS	Upstream Repressing Sequences
UTP	Uridine 5'-triphosphate
UTR	UnTranslated Region
UV	Ultraviolet light
v/v	volume per volume
w/v	weight per volume
wt	wild type
X g	times 'g' (unit gravitational field)
X-GAL	5-bromo-4-chloro-3-indolyl- ß-D-galactopyranoside
yAP	Yeast activation protein
YCF	Yeast cadmium factor
YEF	Yeast elongation factor
YEPD	Yeast Extract Peptone Dextrose

Index

Index

List of Figures

Introduction

Materials and Methods

Results and Discussion

SECTION-I

SECTION-III

List of Tables

Introduction

Materials and Methods

Results and Discussion

SECTION-II

"What is research?
.....but a blind date with knowledge"

Introduction

Introduction

1. INTRODUCTION

Fungal infections in humans range from superficial and cutaneous to deeply invasive and disseminated forms (Table 1). Though these pathogenic fungi have a worldwide distribution, particular species may predominate in certain geographical areas. Among them infections due to *Candida* and *Aspergillus* spp. are the most common. Essentially, this rise in the infections is observed to occur predominantly in people whose immune system is compromised (due to AIDS, organ transplantation or cancer chemotherapy), who have been treated with broad-spectrum antifungal drugs, or who have been subject to invasive procedures (like catheters and prosthetic devices).

Table 1. Common fungal pathogens* 

FUNGI	TYPE	SITES OF INFECTION
Aspergillus fumigatus	filamentous	lungs(aspergillosis), systemic
Candida albicans	dimorphic	lungs, systemic candidiasis
Cryptococcus neoformans	true yeast	meningitis
Histoplasma capsulatum	dimorphic	lung infections
Coccidiodes immitis	dimorphic	lung infections
Blastomyces dermatidis	dimorphic	lung infections
Paracoccidiodes brasiliensis	dimorphic	lung infections
Rhizopus arrhizus	filamentous	lungs,rhinocerebral, systemic

*Compiled from Marichal *et al.*, 1999.

1.1 *Candida* and Candidiasis 

The history of Candidiasis dates back to the fourth century BC when Hippocrates described oral apthae in two patients with severe underlying disease in his book *Epidemics*. The genus *Candida* is composed of an extremely heterologous group of organisms that mainly grow as yeast. *C. albicans* is the species most frequently associated with fungal infections in humans (Vanden Bossche *et al.*, 1998). *C. albicans* accounts for majority of the systemic infections with mortality rates ranging from 50% to 100%. This pathogen is the most common cause of

septicemia in western hospitals, and accounts for development of oropharyngeal and clinical thrush in >80% of the HIV-infected population. *Candida* spp. rank fifth among causes of nosocomial bloodstream infections. Not only are the bloodstream infections with *C. albicans* and non-*albicans* species occurring more frequently, but the relative proportion due to non-*albicans* species is increasing, including species such as *C. glabrata*, *C. krusei*, *C. parasilopsis*, *C. tropicalis*, *C. lusitaniae* and (Table 2).

Table 2. Species commonly causing invasive Candidiasis*

SPECIES	FREQUENCY
Candida albicans	50%
Candida tropicalis	15-30%
Candida glabrata	15-30%
Candida parasilopsis	15-30%
Candida krusei	~1%
Candida lusitaniae	~1%

* From: http://www.doctorfungus.org/index.htm

Common risk factors for blood-stream candidiasis include extremes of age (low-birth-weight infants and the elderly), immunosupression, malignancy with leukopaenia, major surgery, or trauma, exposure to multiple antibacterial agents, central venous catheterization as well as parenteral nutrition (Odds, 1988). Other factors contributing to development of *Candida* infection are listed in Table 3.

Table 3. Factors contributing to development of *Candida* infection in hosts*

- ✓ Exposure to the fungus
- ✓ Inoculum delivered during the exposure
- ✓ Virulence of the fungus
- ✓ Host's immune status
- ✓ Underlying disease
- ✓ Immunosuppressive therapy
- ✓ Prior exposure to the fungus

* Compiled from Odds, 1988.

Although a potential pathogen, *C. albicans* is present as a commensal organism in many, if not most, healthy individuals, where it may be found on the skin and in the oral cavity, gastrointestinal tract and vagina. *C. albicans*, the principal infectious agent of human infection, is oval yeast of 2-6 μM in diameter. It exists as diploid, asexual polymorphic yeast with various biochemical abilities, both assimilative and fermentative, but lacks any proper sexual stage as well as carotenoid pigments. This medically significant fungus has the ability to undergo phenotypic switching and has 8 chromosomes (Odds, 1988; Prasad, 1991).

1.2 What is antifungal drug resistance?

From a clinical perspective, drug resistance may be defined as the persistence or progression of an infection despite appropriate drug therapy. The clinical outcome of treatment depends not only on the susceptibility of the pathogen to a given drug but also on many other factors including pharmacokinetics, drug interactions, immune status, and patient compliance, as well as several specific conditions such as the occurrence of biofilms on surfaces of catheters and prosthetic valves (White *et al.,* 1998). Antifungal drug resistance has been classified as either primary, when a fungus is resistant to a drug prior to any exposure, or secondary, when an initially sensitive fungus becomes resistant after exposure to the drug (Rex *et al.,* 1995; White *et al.,* 1998).

The therapeutic options for treating fungal infections, often caused by the emerging new pathogens whose incidence has increased due to the AIDS pandemic and use of immunosuppressive drugs in transplant and cancer patients, are limited by the relatively low number and structural variety of antifungals (Kolaczkowski & Goffeau, 1997). Only a few classes of antifungal agents are available to treat these infections. Figure 1 depicts the sites of action of some of the common antifungals. Most commonly used antifungals inhibit the ergosterol biosynthetic pathway and chiefly includes azoles, allylamines and morpholines and others such as polyenes and 5-FC impair membrane barrier function and macromolecule synthesis respectively (Vanden Bossche *et al.,* 1989; Williams and Wilkins, 2004).

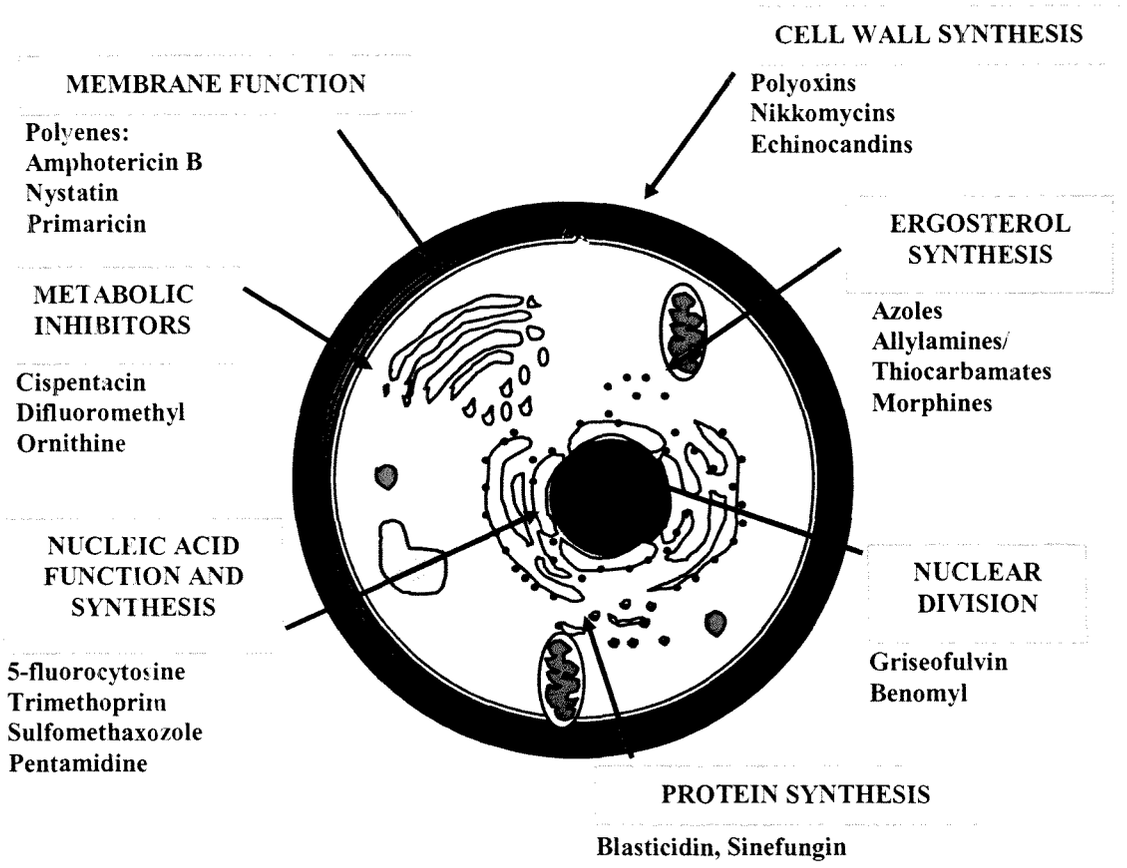


Figure 1. Site and mechanism of action of different classes of drugs on a typical fungal cell. (**Courtesy**: Adopted from Georgopapadakou and Walsh, 1994).

1.4 Multidrug resistance (MDR)

In the 1990s, drug resistance has become an important problem in a variety of infectious diseases including human immunodeficiency virus (HIV) infection, tuberculosis, and other bacterial infections which have profound effects on human health. At the same time, there have been dramatic increases in the incidence of fungal infections, which are probably the result of alterations in immune status associated with the AIDS epidemic, cancer chemotherapy, and organ and bone marrow transplantation. The rise in the incidence fungal infections has exacerbated the need for the next generation of antifungal agents, since many of the currently available drugs have undesirable side effects, are ineffective against new or reemerging fungi, or lead to the rapid development of resistance. Although extremely rare 10 years ago, antifungal drug resistance is quickly becoming a major problem in certain populations, especially those infected with HIV, in whom drug resistance of the agent causing oropharyngeal candidiasis is a major problem (Graybill, 1988; Vanden Bossche *et al.*, 1994).

For instance, 33% of late-stage AIDS patients in one study had drug-resistant strains of *C. albicans* in their oral cavities (Law *et al.,* 1994). *C. albicans* cells have evolved elaborate molecular mechanisms to protect themselves from injuries caused by environmental exposure to toxic compounds of different structure and functions. One of these mechanisms termed multidrug resistance (MDR) or pleiotropic drug resistance also operates in a wide variety of cell types including bacteria, protozoan, fungi and mammalian cells (Balzi and Goffeau, 1994 and 1995; Gottesman *et al.*, 1995 and 1996).

MDR can develop after sequential or simultaneous exposure to all the different drugs to which the cell or microorganism is resistant. However it may also develop surprisingly before exposure to many of the compounds to which the cell or microorganism is found to be resistant. It generally involves a network of membrane-associated transporters acting as multidrug efflux pumps and transcription factors regulating the expression of these pumps. These multidrug efflux systems present a disturbing clinical threat, since the acquisition of such a single system by a cell may decrease its susceptibility to a broad spectrum of chemotherapeutic drugs. The development of drug resistance is a frequent impediment to the effective treatment of infectious and malignant diseases (Van denbroucke-Grauls, 1993; Sternberg, 1994; Peters, 1996). A primary goal in the study of chemotherapy is to understand how cells can become drug resistant by lowering the intracellular concentration of drugs or altering the ability of the drugs to affect their targets. Mechanisms of multidrug resistance are opportunistic in their manipulation of the normal pathways of cellular homeostasis. Several alterations in the cell take place resulting in drug resistance.

A drug-resistant, pathogenic fungus is an organism, according to Kerridge (1985 and 1986) that will grow and produce clinical symptoms of disease in the presence of the drug at the maximal concentration, at the site of infection.

From a mycological viewpoint different classifications of resistance are made.

1.4.1 Intrinsic resistance

A species is regarded as intrinsically resistant when it is not included in the normal spectrum of a given antifungal compound. Primary or innate resistance is characteristics of any species and is not dependent upon previous exposure to the drug. For example, *Candida krusei* can be regarded as intrinsically resistant to fluconazole and *C. parapsilosis* to Amphotericin B.

1.4.2 Acquired resistance

Acquired resistance is found in isolates belonging to a species which is normally susceptible to the compound. Alternately, resistance can develop in previously susceptible isolates during or after antifungal drug exposure (Klepser *et al.*, 1997). Acquired resistance is also termed as secondary resistance and is most familiar in the setting of HIV-infected patients with oropharyngeal candidiasis due to *C. albicans* who receive multiple courses of azole antifungal therapy.

1.4.3 Selective resistance

When a patient is colonized with multiple species or strains, during treatment the most sensitive isolates are eradicated favoring the growth and selection of less sensitive or resistant isolates. This type of resistance is called selective resistance.

1.4.4 Phenotypic resistance

This occurs when a strain develops a progressive increase in resistance during continued incubation in the presence of antifungal compound.

1.4.5 Genotypic resistance

It is a feature of the clone and is inherited by daughter cells.

1.5 Mechanisms of resistance to antifungals

Although the molecular basis of drug resistance in *Candida* is not very clear, evidence accumulated so far suggests that MDR is a multifactorial phenomenon where a combination of mechanisms could contribute to drug resistance (White, 1997a; Franz *et al.*, 1998a; Lopez-Ribot *et al.*, 1998). A schematic representation of the known molecular mechanisms of antifungal drug resistance in pathogenic fungi is depicted in Figure 2 and Table 4.

Azoles are most commonly used to treat the fungal infection. But how azoles enter susceptible fungal cells is not known, although their relative hydrophobicity may facilitate entry by passive diffusion. Once inside the cells, azoles interact with the 14α-DM enzyme in the ergosterol biosynthetic pathway, allowing precursors to be incorporated into newly synthesized regions of plasma membrane (Barrett-Bee and Dixon, 1995). Not all of the azole entering the cell remains there, because two low-level active efflux systems, the ABC transporters and the major facilitators, pump free drug from the cell.

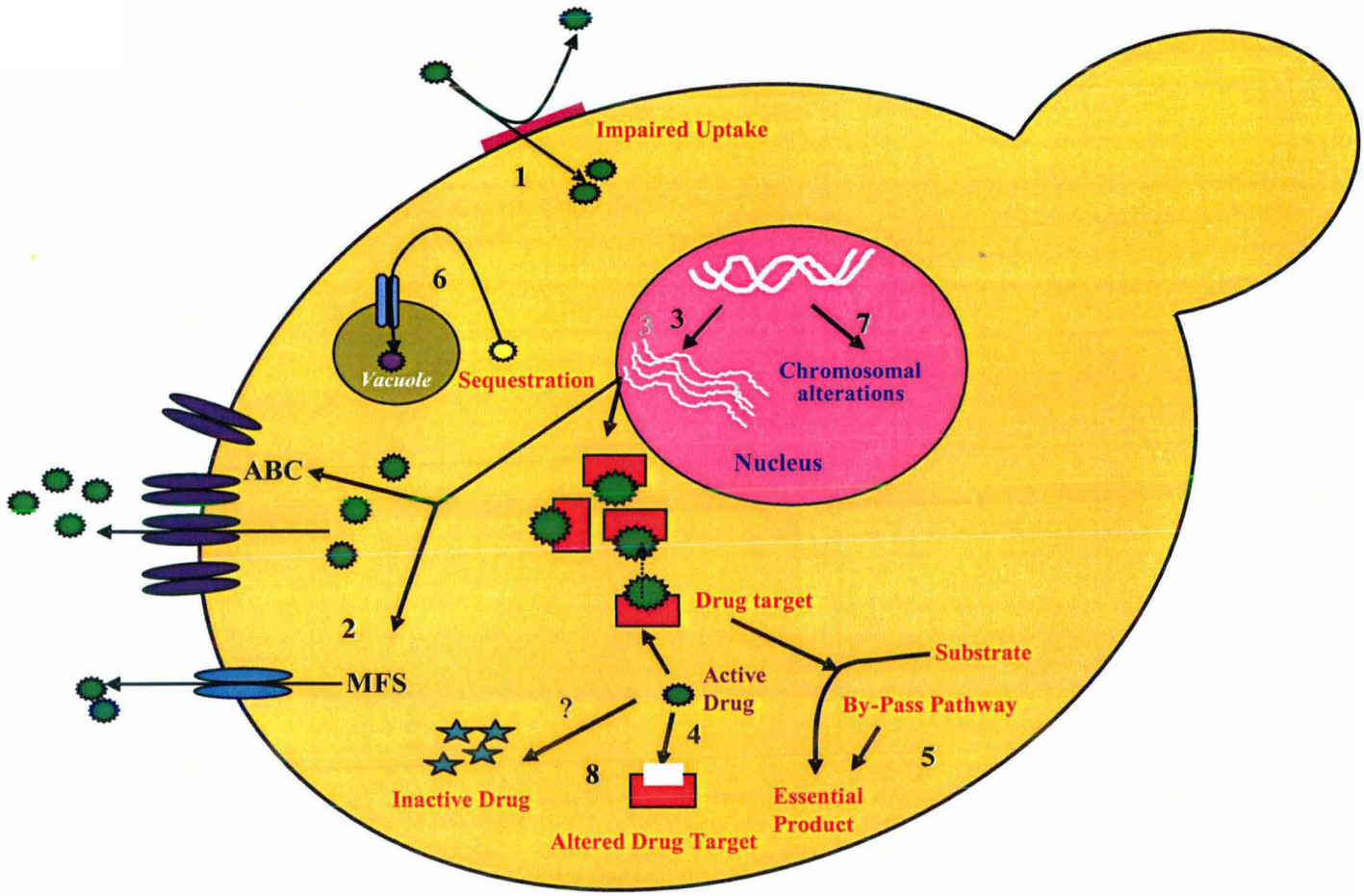


Figure 2. Mechanisms of azole resistance in fungi.

(1) changes in cell wall/plasma membrane leading to impaired azole uptake.
(2) efflux of drugs mediated by the ABC or the MFS class of efflux pumps.
(3) over-expression of the drug target or of the efflux pumps.
(4) mutation in the drug target (p45014DM) does not allow the drug to bind due to low affinity.
(5) activation of alternate pathways such as Δ5,6-desaturase.
(6) sequestration of the drug into an organelle-like vacuole by organellar pump.
(7) chromosome alterations or changes in chromosome number as a means to maintain more copies of the required gene.
(8) modification of azoles (CYP52) to an inactive form.
(**Courtesy**: modified from Prasad *et al.*, 2000).

Table 4. Molecular Mechanisms leading to antifungal resistance*

MOLECULAR MECHANISMS OF ANTIFUNGAL RESISTANCE
Alterations in drug import by change in sterol components of the plasma membrane
Alterations in intracellular drug processing
Modification
Degradation
Genetic changes in target enzyme, *ERG11* including:
Point mutations
Over-expression
Gene amplification (leads to over-expression)
Gene conversion or mitotic recombination
Alterations in other enzymes in ergosterol biosynthetic pathway
Increased efflux of drug due to:
ABC transporters (*CDR* genes)
Major Facilitator (*MDR1* gene)

* Courtsey: White *et al.*, 1998.

Although the molecular basis of drug resistance in *Candida* is not very clear, accumulated evidences suggests that MDR is a multi-factorial phenomenon where various different type of resistance mechanisms are known to be present in different supplementing combinations (White *et al.*, 1998). These chiefly include reduction in the import of drug into the cell; modification or degradation of the drug once it is inside the cell; changes in the interaction of the drug with the target enzyme; changes in other enzymes in the same biosynthetic pathway; and an increased efflux of the drug from the cell (Prasad *et al.*, 2000a and 2002) . Some of the well-known mechanisms of resistance in *Candida* are discussed below which have been broadly divided into azole and non-azole mediated resistance.

1.5.1 Mechanisms of azole resistance

Azole antifungal agents are by far the most commonly used drugs to treat infections caused by *C. albicans*. The target for azole antifungal agents in yeast is a cytochrome P-450. This enzyme is involved in the 14α-demethylation of lanosterol which is an important step in the biosynthesis of ergosterol. Azole drugs interact in the active site of the target enzyme by binding to the heme moiety, acting as competitive inhibitors of the substrate. Figure 2 depicts molecular mechanisms of azole resistance that are commonly present in the pathogenic yeast *C. albicans* which include increased over-expression of multidrug transporters, mutations in

lanosterol demethylase that reduce azole binding and increased expression of *ERG11* (Bossche *et al.*, 1987; Vanden Bossche *et al.*, 1994a and 1994b; Vanden Bossche and Koymans, 1998; Marichal, 1999). Additional known mechanisms have also been demonstrated to participate in azole resistance in *C. albicans* clinical isolates. These include decreased import, alterations in other enzymes in ergosterol biosynthetic pathway, chromosomal alterations, modification or degradation of the drug and transcriptional regulators. Nevertheless, other unknown mechanisms of azole resistance may still be found in clinical isolates. In the following sections these mechanisms have been discussed in detail.

1.5.1.1 Permeability resistance

Defects in drug import are a common mechanism of drug resistance. Many hydrophilic drugs, for example the anticancer antimetabolite methotrexate, cannot easily diffuse through the plasma membrane and have to use specific transporters for this purpose. Alterations in these transporters often lead to reduced influx of drugs. In the case of methotrexate, resistance is found to be associated with alterations in the folate transporter (Skovsgaard *et al.*, 1994).

Decreased toxicity can also be caused due to changes in the lipid composition of the membranes leading to a decrease in permeability. The decreased permeability and fluidity of the membranes result from *cis-* to *trans*-isomerization of their saturated fatty acids. Resistance to the polyene systemic antifungal such as amphotericin B, which interacts with membrane ergosterol to form pores in membrane results in most cases, from defects in the ergosterol biosynthetic pathway, leading to a decreased ergosterol levels in the plasma membrane (Kelly *et al.*, 1997). Several studies have demonstrated that when the ergosterol component of the plasma membrane is eliminated or reduced in favor of other sterol components such as 14α-methyl sterols, there are concomitant permeability changes in the plasma membrane and a lack of fluidity (Bossche *et al.*, 1987). These changes may lower the capacity of azole drugs to enter the cell. Most amphotericin B resistant isolates contain abnormally low number of ergosterol molecules in their plasma membrane thereby limiting the number of available binding sites for the polyene and thus preventing the membrane damage. Existence of other mechanisms of polyene resistance not linked to sterol alterations has also been suggested (Joseph-Horne and Hollomon, 1997). Another kind of permeability

barrier in *C. albicans* involves formation of biofilms, which are large masses of cells. They have taken center stage with the increasing recognition of their role in human infections. In the protected microenvironment of biofilms, the pathogens are more resistant to antimicrobial therapies (Reynolds and Fink, 2001).

However reduction of membrane permeability due to changes in the membrane biophysical properties is not a very efficient way of resistance unless it is accompanied by another resistance mechanism, such as active efflux or enzymatic activation, which often is the case.

1.5.1.2 Efflux mediated resistance

In addition to an alteration or an over-expression of 14α-lanosterol demethylase involved in sterol biosynthesis, azole resistance in *C. albicans* is also elicited by other mechanisms like the reduced intracellular drug accumulation of drugs due to drug efflux pumps. The most intriguing aspect of these transporters generally relates to their lack of specificity, wherein a single transporter can recognize a variety of unrelated xenobiotics. It is apparent from a host of studies that drug transporters of yeasts, similar to its homologues in mammalian system, are capable of transporting substrates that are structurally diverse. It is suggested that ABC transporters are primarily involved in protection against exogenous toxic compounds, whereas MFS transporters may have evolved primarily as a mechanism to secrete endogenous toxic metabolites (Del Sorbo *et al.*, 2000). The characterization of ABC proteins e.g. *CDR1* (Prasad *et al.*, 1995), *CDR2* (Sanglard *et al.*, 1997), and of *CaMDR1* (Fling *et al.*, 1991; Ben-Yaacov *et al.*, 1994), a MFS transporter, as efflux pumps of *C. albicans*, and their over-expression in certain instances of azole resistant isolates has confirmed that these transporters represent another mechanism involved in the MDR scenario of *C. albicans*.

1.5.1.2.1 ABC transporters

The most intensively investigated MDR ABC transporters in this respect are the mammalian ABC transporter protein P-gp. Since the substrates of P-gps and many related transporters are hydrophobic they tend to accumulate in membranes. Several lines of evidence suggest that P-gp somehow can directly recognize and export from the cell membrane the variety of compounds to which it mediates resistance. Studies on mechanisms of MDR transporter

functioning have proposed that P-gp recognizes the drugs as they enter the membrane lipid phase in the manner of a hydrophobic vacuum cleaner model (Raviv *et al.*, 1990; Kolaczkowski and Goffeau, 1997). Another idea is that the transporter is essentially a "flippase" that detects drug within the inner leaflet of the plasma membrane and "flips" it into the outer leaflet (from which it can diffuse away from the cell) or directly into the extracellular space Their substrate specificity profiles include a huge variety of hydrophobic compounds ranging from peptides and steroid hormones to anticancer drugs. Resulting from the explosion of sequence information, it is now known that ATP-binding cassettes (ABC)-ATPases constitute one of the largest and most highly conserved superfamilies and are found in all organisms organisms (Higgins, 1992 and 1993; Ambudkar and Gottesman, 1998; Holland and Blight, 1999). The fact that these transporters are structurally and functionally conserved throughout evolution indicates that these proteins could be involved in performing some indispensable functions in the cell. Some of the important physiological functions attributable to ABC proteins known to date include roles in absorption, excretion, signal transduction, bacterial pathogenesis and most importantly in drug and antibiotic resistance (Kerridge, 1986; Prasad *et al.*, 2000a and 2000b) .

The drug efflux pump encoded by *CDR1* of *C. albicans* was the first ABC efflux pump implicated in conferring resistance to cycloheximide in a *PDR5* disruptant hypersensitive strain of *S. cerevisiae* (Prasad *et al.*, 1995). *CDR1* encodes a 1501 amino acid long protein (169.9 kDa) whose predicted structural organization is characterized by two homologous halves, each comprising a hydrophobic region with a set of six transmembrane stretches, preceded by a hydrophilic nucleotide binding fold (Figure 3). The structure is identical to that of the *S. cerevisiae* ABC proteins Pdr5p and Snq2p. It mirrors the architecture of the yeast a-mating pheromone transporter Ste6, as well as mammalian drug resistance P-glycoprotein (P-gp or *MDR1*) and cystic fibrosis factor CFTR (Dassa and Bouige, 2001). The significance of inversion of domains in some of the ABC drug transporters is not understood and may be related to their physiological functions. Cdr1p is remarkably similar to Pdr5p of *S. cerevisiae*. The similarity is not limited to only the ATP binding motif but is conserved along the entire length of the protein. Despite the high homology between *CDR1* and *PDR5*, and their encoded products, some functional features tend to distinguish them. For example, while

single or low copies of *CDR1* are sufficient to increase drug resistance in *S. cerevisiae*, multiple copies of *PDR5* are required to yield a similar level of drug resistance.

That efflux pumps other than *CDR1* could be contributing to drug resistance became apparent after isolation of its close homolog *CDR2*. *CDR2* exhibits 84 % identity with Cdr1p and confers resistance to fluconazole and several other drugs (Sanglard *et al.*, 1997). In spite of close identity between *CDR1* and *CDR2,* their flanking promoter regions exhibit considerable divergence. Interestingly the promoter of both the genes, though a close homolog of *PDR5*, lacks the characteristic *PDR1/PDR3* transcription factor binding sites, which further suggests divergence in the regulation between close homologs of the two yeasts (Prasad *et al.*, 1995).

Since azole resistance appears to be a multi-factorial phenomenon this led to search of more homologs of known *CDRs*. Using PCR-based cloning, other homologs of *CDR1* and *CDR2* namely *CDR3, CDR4*, and *CDR5*, were identified (Alarco *et al.*, 1997; Balan *et al.*, 1997; Franz *et al.*, 1998a and 1998b; Sanglard *et al.*, 1998a and 1999). Cdr3p and Cdr4p show the highest homology to Cdr1p and Cdr2p; however, compared to Cdr1p and Cdr2p which are > 90% similar, Cdr3p and Cdr4p are only 75% similar to Cdr1p and Cdr2p. Interestingly over-expression of *CDR3* and deletion of *CDR3* and *CDR4* could only affect drug susceptibilities (Alarco *et al.*, 1997; Franz *et al.*, 1998a and 1998b). Why some of the known Cdrp's are unable to elicit a multidrug resistance phenomenon is not yet clear.

1.5.1.2.2 Major facilitators

The major facilitator (MFS) proteins that are involved in symport, antiport, or uniport of various substrates, have been found to be ubiquitously present from bacteria to higher eukaryotes. These proteins are proton motive force (PMF)-dependent antiporters which efflux out substrates in exchange of one or more H^+ ion. The MFS has originally been defined as a superfamily of permeases that are characterized by two structural units of six transmembrane spanning α-helical segments, linked by a cytoplasmic loop (Figure 4).

These transporters have been classified into five distinct clusters or families of membrane transport proteins within the MFS involved in (i) drug resistance, (ii) sugar uptake, (iii) uptake of Krebs cycle intermediates, (iv) phosphate ester/phosphate antiport and (v) oligosaccharide uptake (Saier and Reizer, 1991; Marger and Saier, 1993; Paulsen *et al.*, 1996). On the basis of hydropathy and phylogenetic analyses, MFS drug efflux proteins

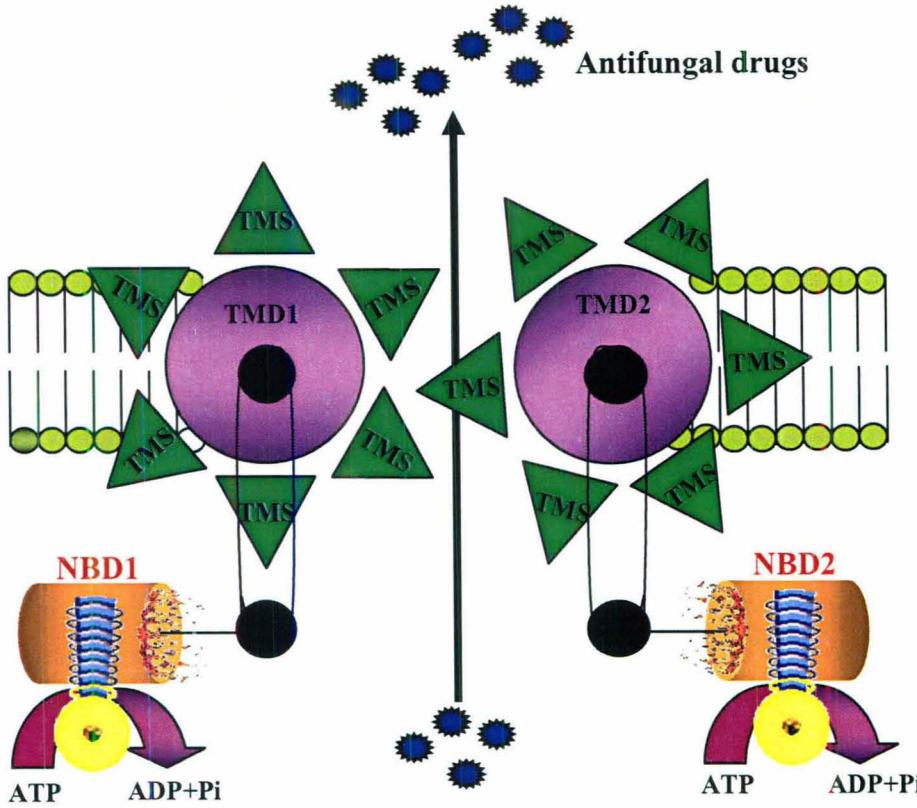


Figure 3. A diagrammatic representation of an ABC transporter of yeast.

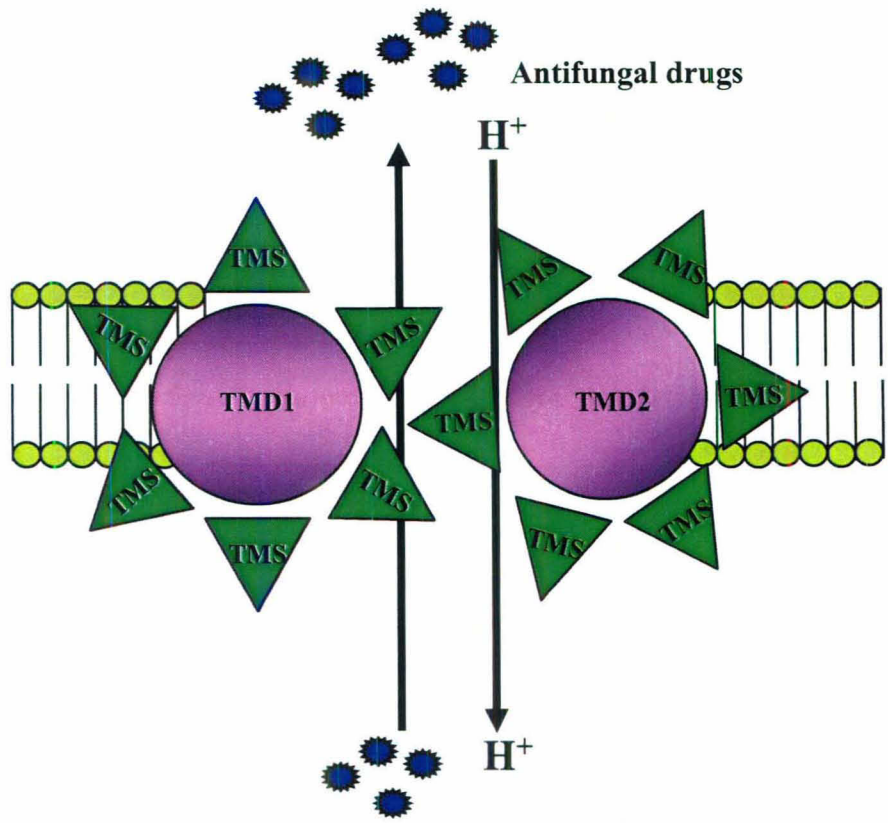


Figure 4. A diagrammatic representation of an MFS transporter of yeast.

which have more than 100 members, can be divided into two distinct groups containing either 12 or 14 TMS (Trans Membrane Segments) (Paulsen *et al.*, 1996).

CaMDR1 (previously known as BEN^r) and *FLU1* are the two MFS genes identified in *C. albicans*. *CaMDR1* was initially identified as a gene, which conferred resistance to tubulin binding agent benomyl and tetrahydrofolate reductase inhibitor methotrexate (Fling *et al.*, 1991; Ben-Yaacov *et al.*, 1994).

Correlation, not cause. It is important to note that the efflux pumps (discussed above), including the *CDR* genes and the *MDR1* gene, show an increased expression that correlates with resistance in clinical isolates of *C. albicans*. Mutant strains of *C. albicans* and *S. cerevisiae* with disruptions in these genes are hyper-susceptible to azole and other drugs, and over-expression of these genes in *S. cerevisiae* can cause an increased resistance. However, there is no definitive proof that over-expression of these genes in *C. albicans* results in a drug-resistant phenotype.

1.5.1.3 Molecular alterations in other ERG genes

Modifications in the ergosterol biosynthetic pathway are likely to generate cross resistance to a large number of unrelated drugs. In addition to the alterations in lanosterol demethylase, a common mechanism of resistance is an alteration in other enzymes of the same biosynthetic pathway. A defective lanosterol demethylase will result in the accumulation of 14α-methylfecosterol and 14α-methylergosta-8,24(28)-dien-3-β,6-α diol. Presence of 14α-methylfecosterol can modify the membrane function and fluidity. Cells with this sterol product have an increased sensitivity to oxygen-dependent microbicidal systems of the host. This diol causes growth arrest in *S. cerevisiae* but is tolerated in *C. albicans*. The toxic effects of this diol is eliminated by a mutation in *ERG3*. To date *ERG1*, *ERG2*, *ERG3*, *ERG4*, *ERG7*, *ERG6*, *ERG11*, *ERG24*, *ERG25* and *ERG26* have been cloned from *C. albicans*, *ERG3* and *ERG11* have been cloned from *C. glabrata*, and *ERG11* has been cloned from *C. krusei* (Burgener-Kairuz *et al.*, 1994). The *ERG3* and *ERG11* genes of *C. glabrata* have been studied in some detail. The double deletion mutant of *ERG3* and *ERG11* in *C. glabrata* has been shown to be aerobically viable, accumulates 14-α-methylfecosterol and lanosterol and is resistant to azoles and AmB (Geber *et al.*, 1995).

Another well characterized mechanism of azole resistance in both *S. cerevisiae* and *C. albicans* is conferred by loss-of-function mutations in sterol $\Delta^{5,6}$-desaturase (Kelly *et al.*, 1997). A defect in this enzyme leads to the accumulation of 14α-methylfecosterol instead of 14α-methylergosta-8, 24(28)-dien-3-β,6-α diol. Accumulation of sufficient amounts of 14α-methylfecosterol compensates for ergosterol in the membranes and thus contributes to azole resistance in *C. albicans* (Vanden Bossche and Koymans, 1998; White *et al.*, 1998; (Ghannoum and Rice, 1999). Because of the lack of ergosterol in their membranes, these isolates were cross-resistant to AmB. In *S. cerevisiae*, the $\Delta^{5,6}$-desaturase defect can also suppress the lethality resulting from the inactivation of *CYP51A1* and is also linked to acquisition of resistance to azole derivatives (Kelly *et al.*, 1997). *C. glabrata* strains produced under laboratory conditions lacking functional *ERG3* and *CYP51A1* genes showed similar phenotypes (Geber *et al.*, 1995).

Azoles also inhibit the cytochrome P450-catalyzed Δ^{22}-desaturase enzyme. Δ^{22}-destaurase (*CYP61* and also *ERG5*) and its homologues have also been identified in *C. albicans* and *Schizosaccharomyces pombe*. It has been hypothesized that an increase of this non-essential enzyme would capture sufficient azole to prevent the inhibition of the 14α-demethylation reaction. So far, however, no biochemical evidence has been presented to support this hypothesis (Marichal *et al.*, 1999).

1.5.1.4 Molecular alterations of ERG11

As discussed above the predominant target site of the azole drugs is the heme protein, lanosterol demethylase also called *CYP51A1* (encoded by *ERG11*) which cocatalyzes cytochrome P450 dependent 14α-demethylation of lanosterol (Ghannoum and Rice, 1999). Inhibition of 14α-demethylation leads to the depletion of ergosterol and accumulation of sterol precursors, including 14α-methylated sterols (lanosterol, 4, 14-dimethylzymosterol, and 24-methylenedihydrolanosterol), resulting in the formation of plasma membrane with altered structure and function.

Several lines of evidence implicate a modification in the quantity or quality of 14a-demethylase in the expression of resistance to azole antifungal agents. Several genetic alterations like point mutations, over-expression of the gene, gene amplification and gene conversion have been identified in *ERG11* of *C. albicans*.

Point mutations

Alteration of the affinity of azole derivatives for *CYP51A1*, which has been described in different post-treatment yeast isolates of *C. albicans* (Bossche, 1994) is another important mechanism of resistance. A survey of resistant and sensitive clinical *C. albicans* isolates has identified different point mutations that are associated with azole-resistant isolates. Sequence data identified Y132H (tyrosine 132 is replaced by a histidine), T135A (threonine 315 is replaced by alanine) or R476K (arginine 476 is replaced by lysine) mutations that decrease the affinity of the target for an azole derivative. It has been shown that R467K alone is sufficient to cause azole resistance (Marichal, 1999).

Another significant change observed in the *ERG11* gene of the resistant isolate was reported by White, 1998, namely loss of allelic variation in the *ERG11* promoter and in the downstream *THR1* gene (which encodes homoserine kinase, which is involved in threonine synthesis). Point mutations in *ERG11* have been developed in laboratory strains also that result in azole resistance. Although these changes may account for resistance development, they are not the only factors involved (Ghannoum and Rice, 1999).

Over-expression

Over-expression of *CYP51A1* as a resistance factor has been mentioned for a few *C. albicans* and *C. glabrata* isolates and has been implicated as a mechanism of resistance to azole antifungals (Vanden Bossche and Koymans, 1998; Ghannoum and Rice, 1999). Over-expression of *CYP51A1* in *C. albicans* resistant isolates has been measured and was up to three times higher than could be measured in azole susceptible isolates, thus probably accounting for a minor effect on the development of resistance. However, some *C. albicans* strains have up to a 10-fold increase in *CYP51A1* mRNA. Over-expression of *CYP51A1* can be achieved by gene amplification, as shown in a *C. glabrata* isolate resistant to azole derivatives (Marichal *et al.*, 1997). Hybridization experiments on chromosomal blots indicate that this increase in copy number is due to amplification of the entire chromosome containing the *CYP51A1* gene. Azole dependent upregulation of *ERG11* has been described in several different clinical isolates of *C. albicans* (Sanglard *et al.*, 1995; Albertson *et al.*, 1996; White *et al.*, 1997). But it is difficult to assess the contribution of *ERG11* over-expression to a resistant phenotype, since these limited cases of over-expression have always accompanied other alterations associated with resistance including mutations in *ERG11* and over-

expression of efflux pumps. Hence over-expression of the target enzyme plays only a limited role in clinical resistance to the azoles.

Azole dependent up-regulation is not limited to *ERG11* and also involves upregulation of other *ERG* genes upstream and downstream of *ERG11*. Likewise *ERG11* upregulation was also not limited to azole drugs and *ERG11* was also inducible by drugs that target other enzymes of the ergosterol biosynthetic pathway (Morita and Nozawa, 1985; Barrett-Bee and Dixon, 1995). These data suggest a common global *ERG* upregulation e.g. in response to ergosterol depletion.

1.5.1.5 Nucleo-cytoplasmic interactions as determinants of drug resistance

Another class of proteins which are involved in pleiotropic drug resistance in yeast are the transcription regulators (Balzi and Goffeau, 1994 and 1995). In *S. cerevisiae* several regulatory proteins (Pdr1p, Pdr3p, and Yap1p) controlling expression of multiple drug resistance genes of the ABC transporters and MF superfamilies are known and mutations in these regulators that result in upregulation of their respective target genes have been identified. Various genetic regulators connecting *PDR* regulators to drug pumps have been uncovered. As a first example, the regulators *PDR1, PDR3, PDR7* and *PDR9* have been shown to control transcription of the multidrug transporter gene *PDR5*, encoding an ABC protein (Balzi and Goffeau, 1995). yAP1 targets include the cadmium resistance ABC transporter gene *YCF1*, as well as a number of genes involved in response to oxidative stress such as *GSH1*, encoding γ-glutamylcysteine synthase, *TRX2*, one of the two genes encoding for thioredoxin, and *GLR1*, encoding glutathione reductase. Several polymorphic mutant alleles of *PDR1* gene which display altered drug resistance profiles have been isolated (Choi *et al.*, 1988; Subik *et al.*, 1986; Carvajal *et al.*, 1997).

Recently functional homologs of these regulators have also been found in *C. albicans*. Over-expression of YAP1 homologue *CAP1* in *S. cerevisiae* resulted in resistance of the transformants against fluconazole and other drugs that was mediated by the transcriptional activation of the *FLR1* gene encoding a major facilitator homologous to the *C. albicans CaMDR1* gene (Alarco *et al.*, 1997). Over-expression of a mutated form of *CAP1*, but not wild type *CAP1* in CAI4 resulted in activation of the *CaMDR1* gene and concomitantly resistance against fluconazole and several other drugs, suggesting the possibility that similar

mutations might also be responsible for *CaMDR1* activation in fluconazole resistant clinical *C. albicans* isolates. On the other hand, disruption of the *CAP1* gene in the *CaMDR1* over-expressing FR2 strain did not suppress but further increased the level of *CaMDR1* expression, and it was therefore recognized as a negative regulator *CaMDR1* gene. Similarly the *C. albicans* transcriptional regulator *FCR1* was identified by functional complementation of *pdr1/pdr3* mutant (Talibi and Raymond, 1999). Over-expression of *FCR1* in this strain resulted in fluconazole resistance that was mediated by the transcriptional activation of the ABC transporter *PDR5*. In contrast disruption of *FCR1* in *C. albicans* resulted in a hyperresistance against fluconazole, demonstrating that similarly to *CAP1*, *FCR1* behaved as a transcriptional activator when overexpressed in *S. cerevisiae* but acted as a negative regulator of drug resistance in *C. albicans*. The transcriptional targets of *FCR1* have not been reported.

So far, none of the transcriptional regulators of drug resistance identified in *C. albicans* has been shown to be involved in the development of fluconazole resistance in clinical isolates and it was suggested that mutations in the regulatory region of the multiple drug resistance genes themselves may be responsible for their over-expression in resistant isolates. This lack of knowledge about the molecular changes leading to activation of multiple drug resistance genes in clinical *C. albicans* strain is due to the fact that wild type *C. albicans* strains is not easily accessible to genetic manipulation. However, using MPA^R technique (mycophenolic acid). Wirsching *et al.* (2000) have shown that *CaMDR1* regulation in fluconazole resistant isolates that over-express *CaMDR1* involves trans-regulatory factor. It is likely that such a mechanism is of general relevance and may be present in other fluconazole resistant *C. albicans* strains. The unraveling of this unexpectedly large pleiotropic network for multidrug resistance control should have implications for the development of new fungicides.

1.5.2 Mechanisms of non-azole mediated resistance

1.5.2.1 Resistance to 5-flucytosine

Primary resistance to 5-FC is a common phenomenon. Resistance may occur due to the deficiency or lack of enzymes involved in the uptake or metabolism of 5-FC, or may be due to the deregulation of the pyrimidine biosynthetic pathway, whose products can compete with the fluorinated metabolites of 5-FC (Kerridge and Whelan, 1984). Detailed

investigations on the molecular mechanisms of resistance to 5-FC have shown that intrinsic resistance in fungi can be due to a defect in cytosine permease (with the exception of *C. albicans*), while acquired resistance results from a failure to metabolize 5-FC to 5-FUTP and 5-FdUMP, or from the loss of feedback control of pyrimidine biosynthesis.

1.5.2.2 Resistance to polyenes

Polyene resistance has not been a major clinical problem to date, although polyene resistant isolates have been isolated and characterized. Acquired resistance to AmB is often associated with alteration of membrane lipids, especially sterols. Most polyene resistant clinical *Candida* isolates have a greatly reduced ergosterol content in their membranes (Hitchcock *et al.*, 1987; White *et al.*, 1998). There is evidence to suggest that alterations in the membrane structure or in the sterol-to-phospholipid ratio in the membrane may be associated with resistance. Recently, clinical isolates of *C. albicans* resistant to AmB were described lacking ergosterol and accumulating 3-β-ergosta-7,22-dienol and 3-β-ergosta-8-enol, typical for a defect in the sterol $\Delta^{5,6}$ desaturase system (Kelly *et al.*, 1997). Such a defect is known in laboratory yeasts (*S. cerevisiae*) harbouring a defect in the $\Delta^{5,6}$ desaturase gene *ERG3* (Kelly *et al.*, 1994; Geber *et al.*, 1995).

1.5.2.3 Resistance to allylamines

Squalene epoxidase (product of the *ERG1* gene) is the target enzyme of the allylamines naftifine and terbinafine (Favre *et al.*, 1999). Both are used mainly to treat dermatophytosis. The gene encoding squalene epoxidase (*ERG1*) has been cloned in *S. cerevisiae*. Deletion of this gene affected viability of *S. cerevisiae* during aerobic growth. Resistance of yeasts to allylamines has been reported only rarely however, the potential to develop resistance by the action of multidrug efflux transporters does exist. For example, the over-expression in *S. cerevisiae* of the *C. albicans CDR1* and *CDR2* genes, and of the *CaMDR1* gene, can confer resistance to terbinafine (Sanglard *et al.*, 1997), showing that this compound is a substrate for these transporters. Moreover, deletion of the *CDR1* gene in *C. albicans* renders cells hypersusceptible to the same drug (Sanglard *et al.*, 1996). Several *C. albicans* isolates resistant to azole antifungal agents are also less susceptible to terbinafine. Since such isolates are resistant to azole derivatives by the mechanism of multidrug efflux transporter gene over-

expression, the cross-resistance to terbinafine could perhaps be explained by this phenomenon.

1.5.2.4 Resistance to morpholines

Amorolfine is the only morpholine derivative in clinical use. It inhibits at least two enzymes in the post lanosterol ergosterol biosynthesis pathway, Δ^{14}-reductase (product of the *ERG24* gene) and $\Delta^{8,7}$-isomerase (product of the *ERG2* gene). Acquired resistance to morpholine derivatives has not been reported so far in yeast pathogens and this is probably due to the limited use of this antifungal for the treatment of superficial fungal infections. However, resistance to morpholine derivatives in *S. cerevisiae* can be by the over-expression of the *ERG24* or *ERG4* (sterol C-24 (28) reductase) genes (White *et al.*, 1998). Moreover, recent work has pointed out that amorolfine, like terbinafine, could be a substrate of multidrug efflux transporters of the ABC-family. This was concluded from results showing that: (i) over-expression of the *C. albicans CDR1* and *CDR2* genes in *S. cerevisiae* could render cells resistant to amorolfine (Sanglard *et al.*, 1997), (ii) *C. albicans* multidrug transporter mutants were hypersusceptible to this antifungal agent (Sanglard *et al.*, 1996), and (iii) *C. albicans* clinical isolates resistant to azole antifungal agents over-expressing the *CDR1* and *CDR2* genes were less susceptible to amorolfine (D. Sanglard, *unpublished results*). Therefore, the potential for developing resistance to this agent do exist.

1.5.2.5 Resistance to 1,3-β-glucan synthase inhibitors

In *S. cerevisiae*, β-(1,3)-glucan synthase is a multienzyme complex with two subunits encoded by the *FKS1* and *FKS2* genes. Deletion of both genes in this yeast results in a lethal phenotype (Douglas *et al.*, 1997; Kelly *et al.*, 2000; Douglas, 2001). Resistance to these compounds is possible, since spontaneous mutants resistant to the echinocardin L-733560 have been isolated *in vitro* in *S. cerevisiae* (Douglas *et al.*, 1997; Douglas, 2001) and in *C. albicans* (Kurtz *et al.*, 1996). The mechanism of resistance in these two yeast species is thought to be a lower affinity of echinocandin to the β-(1,3)-glucan synthase produced in these mutants. Resistance to echinocandin may not be relevant in clinical situations, since it was shown that *C. albicans* resistant mutants exhibited attenuated virulence in animal experiments (Kurtz *et al.*, 1996).

1.6 Strategies to combat MDR

Considerable evidence has accumulated indicating that the multidrug efflux pumps (mainly *CDR1/CDR2* and *CaMDR1*) plays a role in the development of simultaneous resistance to multiple antifungal drugs in clinical *C. albicans* isolates. In recent years, research to overcome this barrier in treatment of fungal infections has been focused mainly on two areas: **(1)** advanced and sensitive detection of multidrug efflux pumps mRNA or protein and its expression regulation **(2)** development of inhibitors/modulators of these multidrug efflux pumps. While the studies dealing with development of modulators/inhibitors have an indirect intervention *via* inhibition of the energy supply for drug efflux, recent understanding of the transcriptional regulation of plasma membrane efflux pumps provides effective and direct avenue for the development of broad-spectrum fungicides. Thereby discerning the MDR regulatory network could help us to hit back the problem of MDR.

In the following section, an overview of regulation for controlling expression of MDR genes is presented.

1.7 Regulation of MDR genes

The term '*gene expression*' refers to all processes that are needed to convert the genetic information contained in a gene to produce a functional protein. Regulation of gene expression in an eukaryotic cell is a series of complex control mechanism, starting with the activation and nuclear transport of the transcription factors, transcription in the nucleus, followed by modification and splicing of the transcript, export of the mRNA from the nucleus to the cytoplasm, cytoplasmic distribution of the mRNA, and finally, translation to protein (Figure 5). Subsequently the activity, multimerization and degradation of the functional protein are likewise tightly regulated.

1.7.1 Regulation of Human multidrug resistance type 1 gene (h*MDR1*)

The h*MDR1* gene encodes a 1,280 amino acid protein, termed P-glycoprotein (P-gp). P-glycoproteins were first discovered by Juliano and Ling in 1976 as large cell membrane proteins which are over-expressed in cancer cells and conferred resistance to a diverse array of hydrophobic drugs. P-glycoprotein (P-gp) is a 170 kDa ATP-dependent membrane-bound transporter that is known to confer resistance to a variety of structurally unrelated, clinically

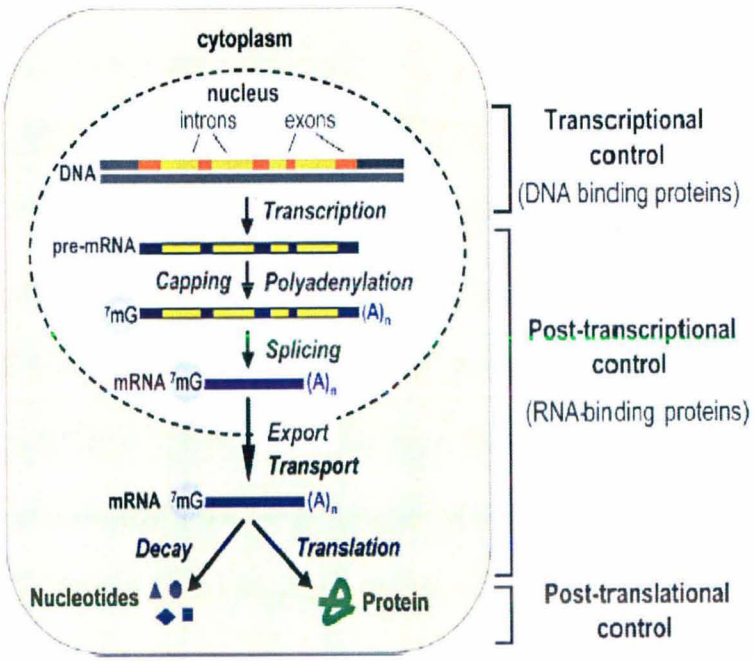


Figure 5. Steps at which eukaryotic gene expression can be controlled.
Gene expression is a multi-step process in which the information contained in genomic DNA is converted *via* mRNA to protein. *See text for detail.*

important antineoplastic agents (Childs and Ling 1994; Fardel *et al.* 1996; Sharom, 1997). P-gp is encoded by the *mdr1* genes, which includes *MDR1* in humans and *mdr1a* and *mdr1b* in rodents.

Over-expression of the *mdr1* gene product(s) has been implicated as a primary mechanism of tumor drug resistance (Riordan *et al.*, 1985; Shen *et al.*, 1986; Lum and Gosland, 1995), particularly in tumors arising from tissues which normally express P-gp (e.g., the liver, kidney, intestine and blood-brain barrier). Several observations suggest a role for the *MDR1* gene in both intrinsic, transiently induced or acquired drug resistance (Figure 6). Amongst the higher eukaryotes though the expression of P-gp has been frequently examined, regulatory mechanisms of this gene product are complex and still poorly understood.

Evidence from numerous studies indicate that expression and activity of P-gp can be controlled either pre- or post-transcriptionally by a myriad of environmental influences. For instance, protein kinase C activators which increase P-gp activity and drug resistance have been found to enhance *mdr1* gene expression *via* both transcriptional and translational pathways (Chaudhary and Roninson, 1992). Modulation in protein stability, plasma membrane incorporation, mRNA stability and processing gene transcription and gene amplification have each been reported for P-gp (Shen *et al.,* 1986; Gottesman *et al.,* 1995; Sharom, 1997). Of these, alterations in P-gp expression that occur at the level of mRNA are perhaps the most frequently observed. The increase in *MDR1* mRNA levels in tumor cells can occur *via* gene amplification of the *MDR1* gene during selection of cells for resistance to a single agent, enhanced *MDR1* transcription rates, transcriptional induction by transcription factors and upon prolonged cellular exposure to several cytotoxic drug agents and by stabilization of the *MDR1* mRNA.

1.7.1.1 Gene rearrangement

A t(4q;7q) translocation in an adriamycin-selected human cell line resulted in a hybrid mRNA containing sequences from both *MDR1* and a novel gene normally located on chromosome 4. This gene rearrangement resulted in the activation of *MDR1* expression mediated by promoter sequences found within the translocated chromosome 4 DNA. Additional gene rearrangements have been identified in other MDR cell lines and in some

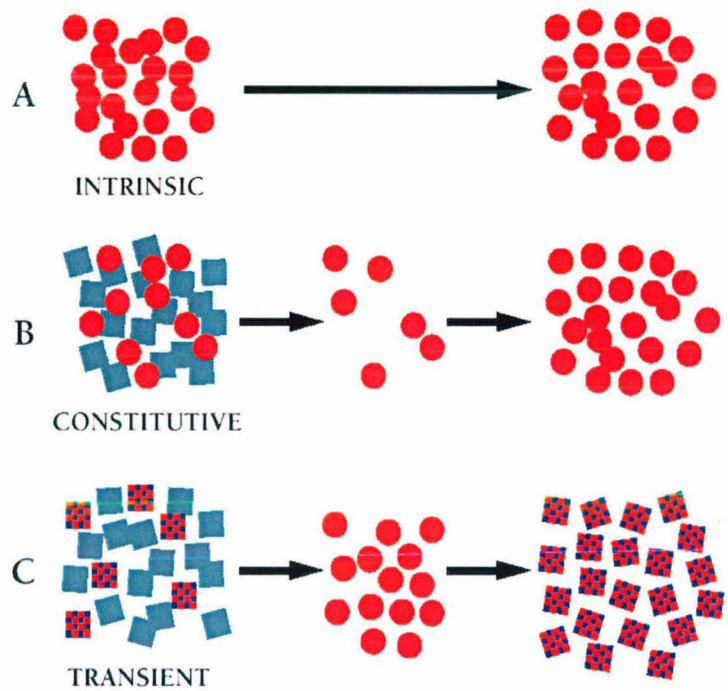


Figure 6. Modes of drug resistance in tumor cells.
Tumor cell populations are indicated by colored geometries. *Black arrows* indicate the passage of time over which drug is administered.
(A) Intrinsic drug resistance. The tumor is predominantly resistant to drug treatment, perhaps due to inherent properties of the tumor cells (*red circles*).
(B) Constitutive (acquired) resistance. Within the population of tumor cells there exists, in addition to drug-sensitive cells (*blue squares*), a subpopulation of cells that, by virtue of mutation, have acquired an MDR phenotype (*red circles*). These MDR cells survive initial therapy and repopulate as a resistant tumor.
(C) Transient (acquired) resistance. A subset of cells (*red checked squares*) within the tumor cell population are able to survive drug treatment temporarily by over-expressing the *MDR1* gene (**Courtesy**: Scotto and Johnson, 2001).

patient tumors, suggesting that this may be a frequent mechanism for over-expression of otherwise silent P-gp genes in acquired drug resistance.

1.7.1.2 Gene amplification

Several studies have identified amplified *MDR1* DNA sequences from multidrug resistant cell lines (Meyers *et al.,* 1985; Fojo *et al.,* 1987; Scotto *et al.,* 1988). The elevated 4.5-kb *MDR1* messenger RNA levels during selection of human KB carcinoma cells as well as other multidrug-resistant sublines of human leukemia and ovarian carcinoma cells selected in colchicine, vinblastine, or adriamycin is associated with amplification of the *MDR1* DNA sequences (Shen *et al.,* 1986). Increase in *MDR1* mRNA expression can also precede gene amplification (Shen *et al.,* 1986).

1.7.1.3 Transcriptional regulation

1.7.1.3.1 Cis-element and cognate binding factors

The *MDR1* gene contains at least two promoter regions (Ueda *et al.,* 1987a; 1987b; Rothenberg *et al.,* 1989). Ueda *et al.* (1987a; 1987b) demonstrated that *MDR1* transcription can be initiated from major downstream promoter (at positions -136 to -140) or from a minor upstream promoter (at positions -155 to -180) which was found to be the case in colchicines-, but not doxorubicin- or vinblastine-selected KB cells. The major downstream promoter is used in most of the normal tissues.

MDR1 belongs to a group of protein-encoding genes that lack a consensus TATA box within the proximal promoter region and this has been associated with the diversity of transcription sites in eukaryotic cells (Maniatis *et al.,* 1987). Instead, basal transcription is directed by an initiator (Inr) sequence that encompasses the transcription start site (+1) and most likely acts as the position of nucleation of the RNA Pol II preinitiation complex (Figure 7). The first identification of an Inr element in the *MDR1* promoter came from *in vitro* studies, which showed that sequences from -132 to +82 were sufficient for accurate initiation and deletion of sequences downstream of +5 decreased elongation of correctly initiated transcripts to undetectable levels (Cornwell, 1990). Subsequently, transient transfection studies indicated that sequences between -6 and +11 were sufficient for proper initiation of transcription *in vivo* (Van Groenigen *et al.,* 1993; Madden *et al.,* 1993). Like most other "TATA-less" genes,

the *MDR1* promoter also includes both an inverted CCAAT element (-82 to -73) (Goldsmith *et al.,* 1993) and a GC-rich element (-56 to -43) that have been shown to interact with members of the Sp1 family of transcription factors (Cornwell and Smith, 1993a; Thayer *et al.,* 1996; Sundseth *et al.,* 1997). Transfection analyses of promoter constructs mutated in one or both of these elements indicate the involvement of the two elements in the constitutive (*i.e.*, operative under normal growth conditions) expression of *MDR1* in some cell lines.

A second GC-rich element has been identified (-110 to -103) that is unable to interact with Sp1 and may be the site for an unidentified protein complex (Cornwell and Smith, 1993a). Interestingly, immediately downstream and overlapping this GC-rich region is an inverted MED-1 element (Multiple start site Element Downstream 1; iMED; Figure 7), which was first described in the hamster *MDR1* homolog, *pgp1*, and shown to be involved in the activation of that gene in drug-resistant cells (Thayer, 1999).

Functional disruption of iMED, either through mutation of the element or the use of a transcriptional decoy to sequester iMED-binding proteins, led to a decrease in constitutive *MDR1* transcription in human neuroblastoma (Thayer, 1999) and leukemia (Marthinet *et al.,* 2000) cell lines; whether this element is also involved in induction of *MDR1* transcription in resistant cells is under investigation. Consensus binding sites for AP-1, CEBP and Y-box proteins in the 5' flanking region of *MDR1* genes have also been identified (Silverman and Schrenk, 1997). Potential transcriptional regulatory elements found within the *MDR1* gene includes several heat shock consensus elements, evidenced by an enhancement of *MDR1* mRNA after heat shock treatment and a phorbol ester response element (Angel *et al.,* 1987; Chin *et al.,* 1990a). The mouse and hamster *mdr* promoter DNA sequences differ significantly from that of the human. This difference is generally thought to be the reason for the enhanced responsiveness of the rodent promoters to certain kinds of environmental stresses. The murine promoters contain both TATA and CAAT boxes as well as putative SP-1, AP-1 and AP-2 sites (Hsu *et al.,* 1990; Raymond *et al.,* 1990; Ikeguchi *et al.,* 1991; Cohen *et al.,* 1991).

Studies show that there is more than one *mdrla* promoter, which, in combination with alternative polyadenylation sites, may account for the multiplicity of *mdrla* transcripts observed in mouse cells (Hsu *et al.,* 1990). In addition, elevated P-gp expression has been observed in the gravid mouse uterus which suggests hormonal influence on expression

(Arceci *et al.,* 1988 and 1990). Interestingly, the *mdr1 b* promoter which drives P-gp expression in the adrenal and secretory glands of the endometrium, contains a progesterone response element (Cohen *et al.,* 1991).

1.7.1.3.2 Transcriptional Inducers of hMDR1

By far, the most studied regulation of *MDR1* is at the level of transcriptional induction. From the earliest studies of P-glycoprotein function and regulation, it has been suggested that P-gp is a '*stress-response gene*' since its activity can be modulated by environmental adversity (Scotto and Egan, 1998). *MDR1* mRNA level is have been show to be enhanced by various factors, including hormones and environmental circumstances. Induction is seen after heat shock, treatment by heavy metal (Chin *et al.,* 1990a), differentiation agents such as sodium butyrate and retinoic acid, or chemotherapeutic agents (Scotto and Egan, 1998), or in response to carcinogens in the liver (Fairchild *et al.,* 1987; Burt and Thorgeirsson, 1988; Bradley *et al.,* 1992). Treatment with cytotoxic agents (Fardel *et al.,* 1996a), ultraviolet radiation, or partial hepatectomy has also been shown to increase *MDR1* expression in both rodent and human cell lines (Thorgeirsson *et al.,* 1987; Burt and Thorgeirsson, 1988; Mickley *et al.,* 1989; Chin *et al.,* 1990b; Marino *et al.,* 1990; Uchiumi *et al.,* 1993; Chaudhary and Roninson, 1993). Cadmium chloride and sodium arsenite can also induce *MDR1* mRNA (Chin *et al.,* 1990a and 1992) and the induction was found to be sensitive to actinomycin D, therefore it requires synthesis of RNA (Chin *et al.,* 1990b). The effects of the increase in *MDR1* expression seen following exposure to anti-neopiastic agents, including those which are not P-gp substrates, persisted for several weeks following removal of the drug (Chaudhary and Roninson, 1993). There have been reports that non-specific protein kinase inhibitors block drug-mediated *MDR1* induction. However, recent work by Parissenti et *al.* (1999) showed no apparent change in P-gp levels or cellular sensitivity by inhibition of PKA activity to block *MDR1* gene expression in adriamycin-resistant MCF-7 breast cancer cells. MDR-CAT reporter gene constructs have demonstrated that mutant Ras as well as both mutant and wild type p53 genes may stimulate the *MDR1* promoter (Chin *et al.,* 1992; Zhou and Kuo, 1998). There are also suggestions of transcriptional regulation of *MDR1* by the c-Raf signal transduction pathway (Cornwell and Smith, 1993b).

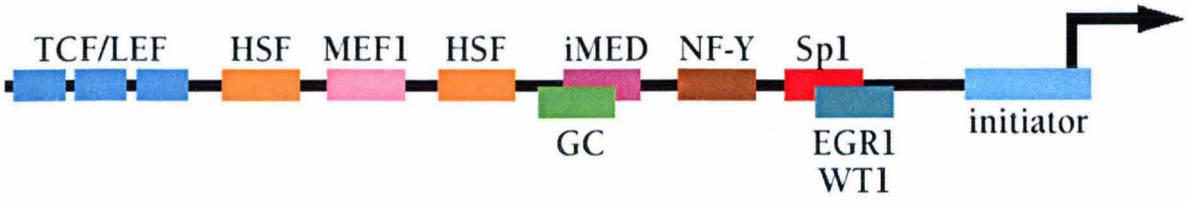


Figure 7. Untranslated 5' regulatory region of the human *MDR1* gene showing promoter elements.
All elements and DNA-binding factors involved in the transcriptional regulation of *MDR1* represented in the figure are discussed in the text. Arrow over the initiator window indicate the major transcription start sites. *See text for detail.* (**Courtesy**: Scotto and Johnson, 2001).

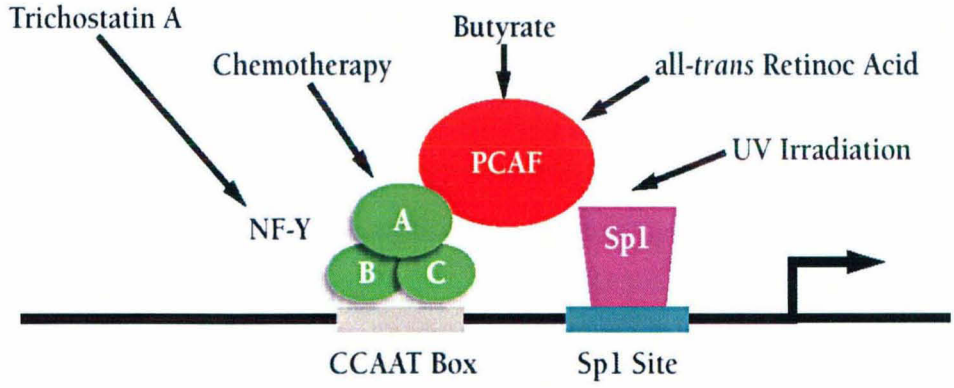


Figure 8. Schematic of the *MDR1* enhanceosome, which includes the transcription factors NF-Y, Sp1, and PCAF.
See text for detail. (**Courtesy**: Scotto and Johnson, 2001).

Furthermore, there are also evidences that *MDR1* mRNA may be co-induced by 2,3,7,8-TCDD, which is also a potent inducer of the CYP4501 gene family (Burt and Thorgeirsson, 1988). In addition, treatment of human colon cancer cell lines with differentiating agents such as dimethyl sulfoxide or sodium butyrate has been show to elevate *MDR1* gene expression (Mickley *et al.,* 1989). Recent studies indicate that the signals from all of these divergent stimuli converge on a region of the *MDR1* promoter that we refer to as the *MDR1* enhanceosome (Jin, 1998; Hu *et al.,* 2000 and *unpublished work*) (Figure 8). This region includes binding sites for the trimeric transcription factor NF-Y and the Sp family of GC binding transcription factors. Together, these DNA-binding proteins recruit the histone acetyltransferase PCAF to the *MDR1* promoter, resulting in the acetylation of promoter-proximal histones and subsequent transcriptional activation that is likely mediated by further chromatin remodeling. Although the mechanism by which each agent transduces the signal that results in promoter activation has yet to be determined, the role of the *MDR1* enhanceosome in the regulation of transcription by a variety of stimuli makes it an attractive target for therapeutic intervention.

1.7.1.4 Post-transcriptional regulation: evidence for increased MDR1 mRNA stability

While much of the focus on understanding the normal regulation of *MDR1* gene expression in tissues is at the transcriptional level, there is growing evidence that suggests enhanced mRNA stability plays a key role in this regulation, most especially in those tissues which normally express the *MDR1* mRNA. The observed association between the localization of the *MDR1* gene product, P-gp, to the lumenal surface of hepatocytes, and the enhanced P-gp expression during rat liver carcinogenesis (Fairchild *et al.,* 1987; Thorgeirsson *et al.,* 1987; Nakatsukasa *et al.,* 1992; Bradley *et al.,* 1992) which correlated with the process of tumor progression (Bradley *et al.,* 1992; Nakatsukasa *et al.,* 1992) has prompted studies on the regulation of P-gp in the liver as a model system to identify possible triggers whereby the P-gp system is dysregulated. Several important studies using the rat liver system led to the realization that mRNA stability was involved in P-gp over-expression. First, results by Lee *et al.* (1993) using gene specific probes generated fiom the 3'UTRs of each rat P-gp gene demonstrated that only one member of the P-gp gene family, class II P-gp, was strongly induced when hepatocytes were grown in primary culture. In addition, this over-expression

paralleled that of the cytoskeletal genes: actin and tubulin and suggested a possible common regulatory mechanism in primary rat hepatocytes. Second, the class II P-gp, which is expressed at a very low level in normal liver, has also been shown to be over-expressed in several models of rat liver carcinogenesis. By nuclear run on assays and mRNA decay studies, it was demonstrated that an increased mRNA stability is the major mechanism involved in the increased expression of class II P-gp. Moreover, studies using various drugs also indicate that the integrity of the cytoskeleton is important for the maintenance of high expression of class II P-gp. Disruption of the cytoskeleton in cultured hepatocytes with cytochalasin D did not affect the transcriptional activity of the class II P-gp gene but rapidly destabilized its mRNA. It is possible that an association between class II P-gp RNA and cytoskeletal elements may underlie the mechanism that regulates class II P-gp mRNA stability (Lee *et al.,* 1993). Perhaps the strongest evidence which supports mRNA stability came from the follow-up studies by Lee *et al.* (1993) where they directly measured the in *vivo* mRNA half-life and performed nuclear run analysis of class II P-gp mRNA and a diverse group of unrelated genes which they have previously determined to be over-expressed in primary monolayer of adult rat hepatocytes during culturing (Lee *et al.,* 1993). Interestingly, in 1998 their published results showed that these same transcripts were also over-expressed and stabilized in primary liver cancers and transplantable tumors (Lee *et al.,* 1998). Their findings led them to suggest that increased mRNA stability is a primary mechanism contributing to over-expression of P-gp and other genes in rat liver tumors. Although this study focused on the rat counterpart of *MDR1*, it adds support to the knowledge that *MDR1* mRNA stability, although it still remains less characterized, may be a potential mechanism of *MDR1* mRNA over-expression in human liver tumors. An important point emerged from this study is the observed global mRNA stabilization of other genes besides *MDR1*. Does stabilization of the mRNA represent a characteristic mechanism for up-regulation of P-gp and other genes in rat liver tumors? If so, does this same mechanism operate in the human liver system? However, to date, no current knowledge of the human *MDR1* mRNA stabilization in *vivo* has been shown. However, several liver cancer cell lines, such as the human hepatoma line (HepG2) are now available to address this issue.

1.7.1.5 Determinants of MDR1 mRNA Stability/decay

1.7.1.5.1 Cis-elements: RNA sequences

Specific *cis*-elements contribute to the steady-state level of mRNA by promoting degradation or stabilization (Klausner *et al.,* 1993; Ross 1996). Sequences that function as determinants to promote *MDR1* mRNA degradation have been identified in all regions of the transcript, such as the 5' Untranslated region (5' UTR), the protein coding region, and the 3' Untranslated region (3'UTR) (Figure 9). Among these, best characterized regions of *MDR1* mRNA stability are located in the 3'-untranslated region. In fact, 3'UTR can direct mRNA decay independent to that of the remaining regions of the mRNA (Ross, 1996). 3'UTRs consist of sequence motifs which were found to be important in cellular processes such as sub-cellular localization, translation efficiency and mRNA stability (Ross, 1996). Interestingly, length of 3'- as well as 5'-UTR varies as a function of species (Figure 10). The 3'UTR of the human multidrug resistance type 1 (h*MDR1*) mRNA is approximately 380 nucleotides long and is very AU-rich (70%). The *MDR1* 3'UTR shares sequence similarities to the 3'UTRs of rapidly degraded mRNAs. By far, the best characterized mRNA decay determinants are adenylate-uridylate (AU) sequences in the 3'UTR. A large number of unstable mRNAs have been shown to have AU-rich sequences contained in their 3'UTRs (Rajagopalan and Malter, 1997; Maurer *et al.,* 1999; Bloch, 1999).

However, not all sequences have comparable destabilizing effects on reported mRNAs. *MDR1* mRNA contains AU sequences which are sirnilar to rapidly degrading mRNAs of the *c-myc, cfos* and the lymphokines. A destabilizing function for *MDR1* has been proposed (Marino *et al.,* 1990; Chen and Shyu, 1994) and previously published work has provided the first test of this hypothesis. Other studies suggested that the *MDR1* mRNA decays with a half-life of 10 hours or longer (Muller *et al.,* 1995).

Previously published work of Prokipcak *et al.* (1999) has shown that the *MDR1* 3'UTR does not behave as a classic ARE *cis*-element *i.e.* it does not function as an active mRNA destabilizer in human hepatoma cell line, HepG2. *MDR1* mRNA has an intermediate half life (8 h) compared to that of the *c-myc* (30 min) or GAPDH mRNA (> 24 hr). Furthermore, this half-life was prolonged (>20 h) upon exposure to cycloheximide, a protein synthesis inhibitor, suggesting the requirement of protein synthesis. Using ß-globin chimeric mRNAs, whereby the 3'UTR of *c-myc* or *MDR1* was fused to the ß-globin coding region, it was

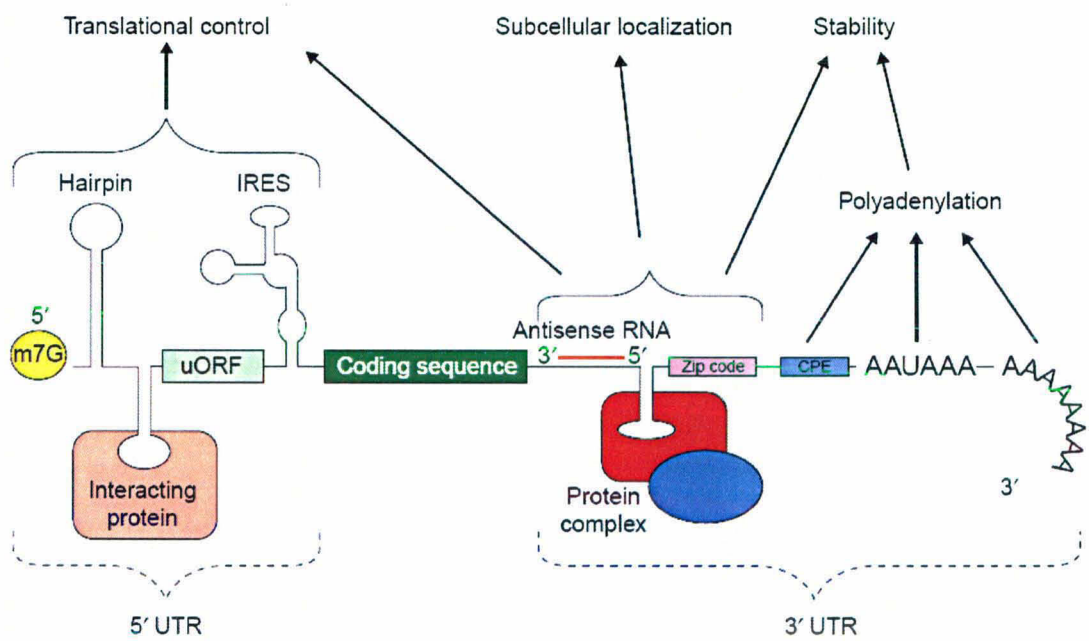


Figure 9. The generic structure of a eukaryotic mRNA, illustrating some post-transcriptional regulatory elements that affect gene expression.
Abbreviations (from 5' to 3'): UTR, UnTranslated Region; m7G, 7-methyl-Guanosine cap; hairpin, hairpin-like secondary structures; uORF, upstream Open Reading Frame; IRES, internal ribosome entry site; CPE, Cytoplasmic Polyadenylation Element; AAUAAA, polyadenylation signal (**Courtesy**: Mignone *et. al.*, 2002).

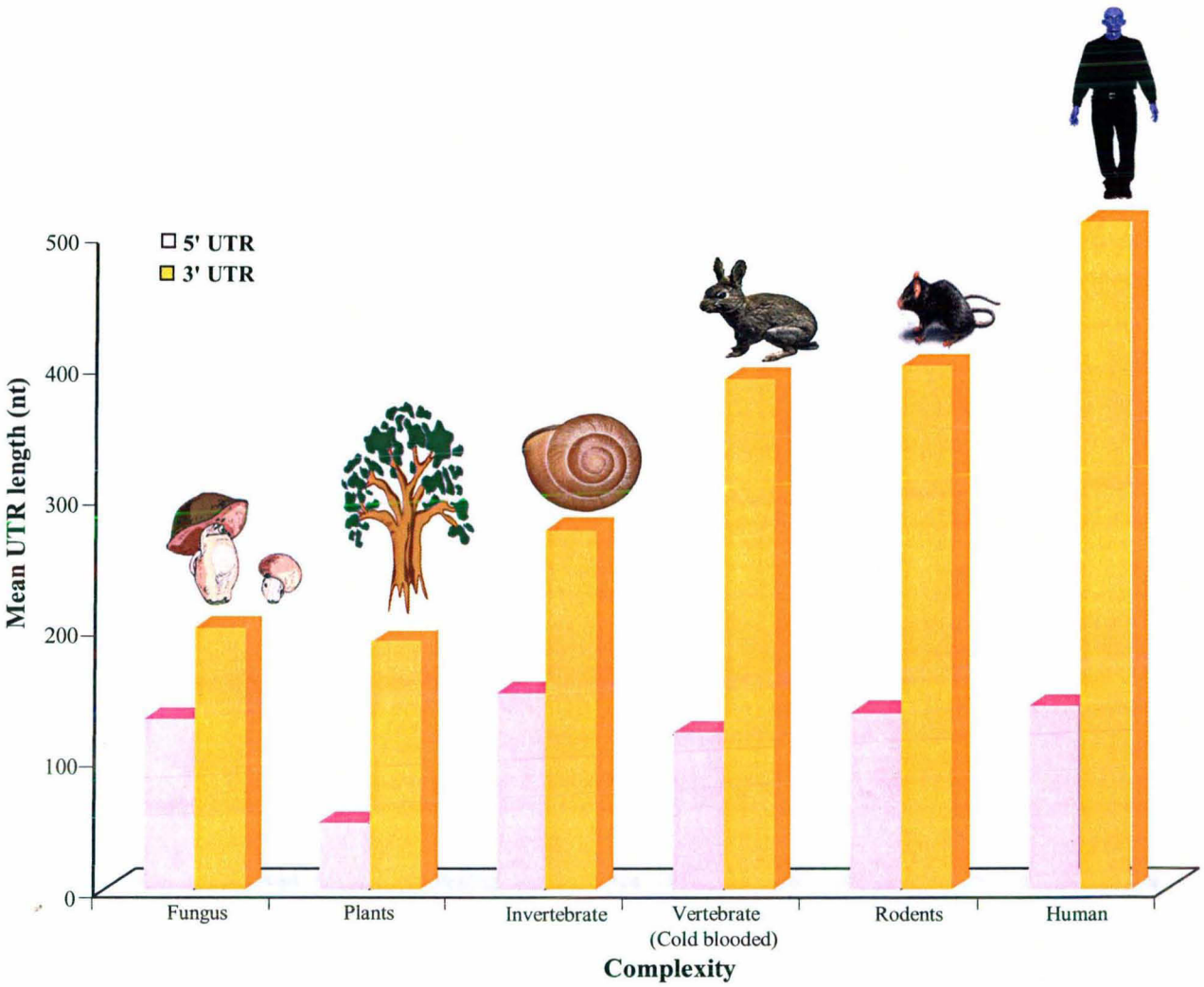


Figure 10. Length of 5′- and 3′-UnTranslated Regions (UTRs) as a function of species. Length of mean 5′ -and 3′-UTRs from various classes and species. Data are compiled from the UTR database. (**Courtesy**: modified from Pesole *et al.*, 2002).

demonstrated that the *c-myc* 3'UTR, as predicted, destabilized the globin mRNA. However, the *MDR1* 3'UTR had no effect on the stability of the globin mRNA.

1.7.1.5.2 Trans-acting factors: RNA-binding proteins

The *cis* determinants discussed above play a role in determining the stability or decay of most mRNAs. However, a number of studies have show that although these elements may target an mRNA for degradation there are cases whereby a stimulus (such as a drug, hormone, intracellular factors) can in fact modulate the inherent mRNA half-life (Williams *et al.*, 1993; Tian *et al.*, 1998; Bloch, 1999; Blaxall *et al.*, 2000; Liu *et al.*, 2000). In majority of the studies to date, it has been shown that RNA-binding proteins form the primary means of regulating this half-life by their ability to bind to these elements or structures (Siomi and Dreyfuss, 1997). The nuclear RNA processing reactions mediated by interactions between proteins and relatively short *cis*-acting elements can be found almost anywhere along the RNA. However, an overriding majority of these interactions which regulate mRNA expression in the cytoplasm is regulated *via* the 3'UTRs (Cusack, 1999).

Competition analysis showed that the *MDR1* 3'UTR had a five fold lower affinity for AU-binding proteins which are known to interact with *c-myc* AU-rich 3'UTR (Prokipcak *et al.*, 1999). Overall the data suggest an association between the affinity of AU-binding proteins to the 3'UTR and the degree of *MDR1* mRNA stability. Thus, stability of the *MDR1* mRNA is the result of the interaction between specific *cis*-elements in its 3'UTR and *trans*-acting factors (RNA-binding proteins). The uniqueness of this interaction may contribute in part to the observed differences in mRNA half-life between *MDR1* and other genes bearing similar *cis* elements.

1.7.2 Regulation of MDR in *S. cerevisiae*

Molecular mechanism underlying the up-regulation of PDR genes in the development of multidrug resistance is well described in the baker's yeast *S. cerevisiae,* wherein several drug extrusion pumps like *PDR5* (Pleiotropic Drug Resistance), *SNQ2* (Sensitivity to 4-Nitroquinoline N-oxide) and *YOR1* (Yeast Oligomycin Resistance), etc. have been implicated in the development of drug resistance. In *S. cerevisiae* MDR is known as PDR (Pleiotropic Drug Resistance). Three networks of genes, *PDR* (Pleiotropic Drug Resistance), *YRR* (Yeast

Reveromycin A Resistance) and *YAP* (Yeast AP-1 like factor) are mainly involved in multidrug resistance. More recently a repressor of drug resistance 1 (*RDR1*) has also been identified. Over-expression of yeast multidrug extrusion pumps results from over-production, genetic alterations (YAP network) and or spontaneous point mutations (PDR network) in regulatory factors governing their expression. Some drug efflux transporters are also induced in the Msn2p-dependent manner (Wolfger *et al.,* 2004). Msn2p is the Cys_2His_2 Zn finger protein acting as a regulator in the general stress response pathway (Martinez-Pastor *et al.* 1996). The major determinants of multidrug resistance in *S. cerevisiae* involve the transcriptional regulator *PDR1* and *PDR3*, which control the expression of ABC pumps, MFS transporters and other genes. Spontaneous dominant mutations which activate the master transcription regulators Pdr1p and Pdr3p result in spectacular multidrug resistance phenotypes (Balzi & Goffeau, 1995) which either actively extrude drugs out of the cell (Kolaczkowski & Goffeau, 1997) or modify their passive diffusion into the cell by altering the lipid composition of the cell bilayer.

1.7.2.1 The PDR network

The *PDR1* and *PDR3* genes encode highly homologous transcription factors involved in the control of multidrug resistance in the yeast *S. cerevisiae.* Both genes belong to the family of Gal4p-like proteins characterized by the N-terminal DNA binding domain of the $Zn(II)_2$-Cys_6 binuclear cluster DNA-binding motif with the consensus sequence of $CysX_2CysX_6CysX_{5\text{-}12}CysX_2CysX_{6\text{-}8}Cys$ (Delahodde *et al.,* 1995). They recognize the consensus sequence 5′TCCGCGGA3′ designated as PDRE (PDR Responsive Element) present in the promoters of target genes. The target genes code for the ATP binding cassette (ABC) transporters (*PDR5, SNQ2, YOR1, PDR10, PDR11, PDR15, BAT1*) and the transporters (*HXT9, HXT11, FLR1*) belonging to the major facilitator super-family (MFS) (Figure 11). Their over-expression due to mutations either in the *PDR1* or *PDR3* genes results in the increased efflux of cytotoxic compounds from the cells and establishment of multidrug resistance. The PDR network is currently known to comprise 10 transcription factors regulating about 70 different target genes. In this network, the Pdr1p transcription factor has the largest set (about 30) of potential targets.

Despite their structural (36% identity, 57% similarity over the entire amino acid sequence) and global functional similarities, Pdr1p and Pdr3p play distinct roles. Disruption of *PDR1*

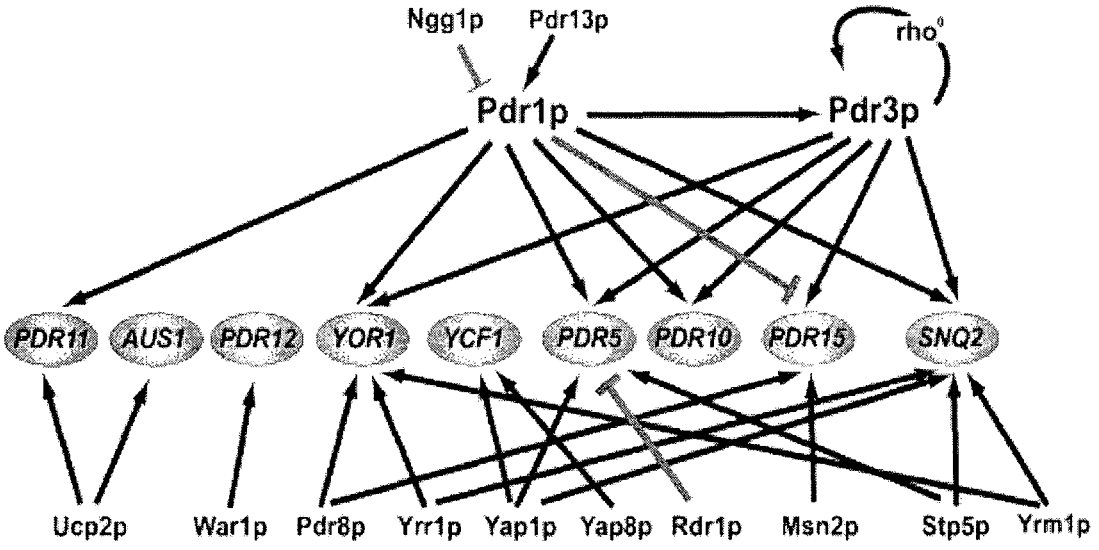


Figure 11. The PDR network.
Genes in the centerline represent target genes of transcriptional regulators depicted above and below. Note that the cartoon only lists genes of the ABC family. The yeast PDR network also contains non-ABC genes whose function has not established in many cases (*see text for details*). Red lines indicate a negative regulatory impact, while black lines ending with an arrow indicate positive regulation (**Courtesy**: Jungwirth and Kuchler, 2006).

has a more pronounced effect on drug sensitivity than disruption of *PDR3*. However, sensitivity to a set of drugs, such as rhodamine 6G or diazoborine (Kolaczkowska & Goffeau, 1999) is more affected by the inactivation of the *PDR3* gene. The role of *PDR1* is well documented by genetic experiments showing that the *PDR1* locus is associated with resistance to more than 20 structurally unrelated inhibitors of both cytoplasmic and mitochondrial function (Kolaczkowska & Goffeau, 1999) or with permeability to small molecules like estradiol. Single point mutations in the *PDR1* locus (*pdr1-2, pdr1-3, pdr1-6, pdr1-7, pdr1-8*) increase to various extents the mRNA level of downstream target genes, such as *PDR5*, *SNQ2*, *YOR1, PDR10, PDR15* and the newly identified *PDR16*. Similarly, *PDR3* mutant alleles (*pdr3-2* to *pdr3-10*) have been shown to activate the expression of the *PDR5*, *SNQ2*, *PDR15* and *PDR3* genes (Kolaczkowska and Goffeau, 1999).

Recent DNA microarray analysis has shown that the transcription levels of several soluble proteins (which functions not obviously related to cellular efflux of drugs) are also regulated by *PDR1/PDR3* genes. This signifies the universal role of Pdr1p and Pdr3p in regulating varied physiological functions of *S. cerevisiae.* Remarkably, Pdr1p and Pdr3p can positively or negatively regulate expression of target genes, implying that additional factors modulate their activity (Martens *et al.,* 1996; Saleh *et al.,* 1997).

1.7.2.1.1 Molecular mechanism of PDR network activation upon exposure to antifungal drug

In *S. cerevisiae,* mutation of a single nucleotide in the genes encoding the transcription factors Pdr1p or Pdr3p could initiate multi drug resistance; these factors control the expression of several ABC efflux pumps (Carvajal *et al.,* 1997; Nourani *et al.,* 1997; DeRisi *et al.,* 2000). Rapid and transient activation of drug-efflux genes is also observed8 when fungi are treated with high concentrations of antifungal drugs. Thakur *et al.* (2008) find that various antifungal drugs and other xenobiotics (alien substances) can bind directly to a hydrophobic domain (XBD) in Pdr1p and Pdr3p, and that this binding leads to drug-dependent activation of drug-efflux genes. Thakur *et al.* (2008) make another intriguing observation: deletion of the gene encoding the Gal11 protein decreases drug-dependent expression of Pdr5p and other drug-efflux pumps. Gal11p is one of the 20 subunits of the yeast Mediator complex, which recruits the RNA polymerase II machinery for the transcription of specific sets of genes. Thakur and colleagues (2008) show that the activation

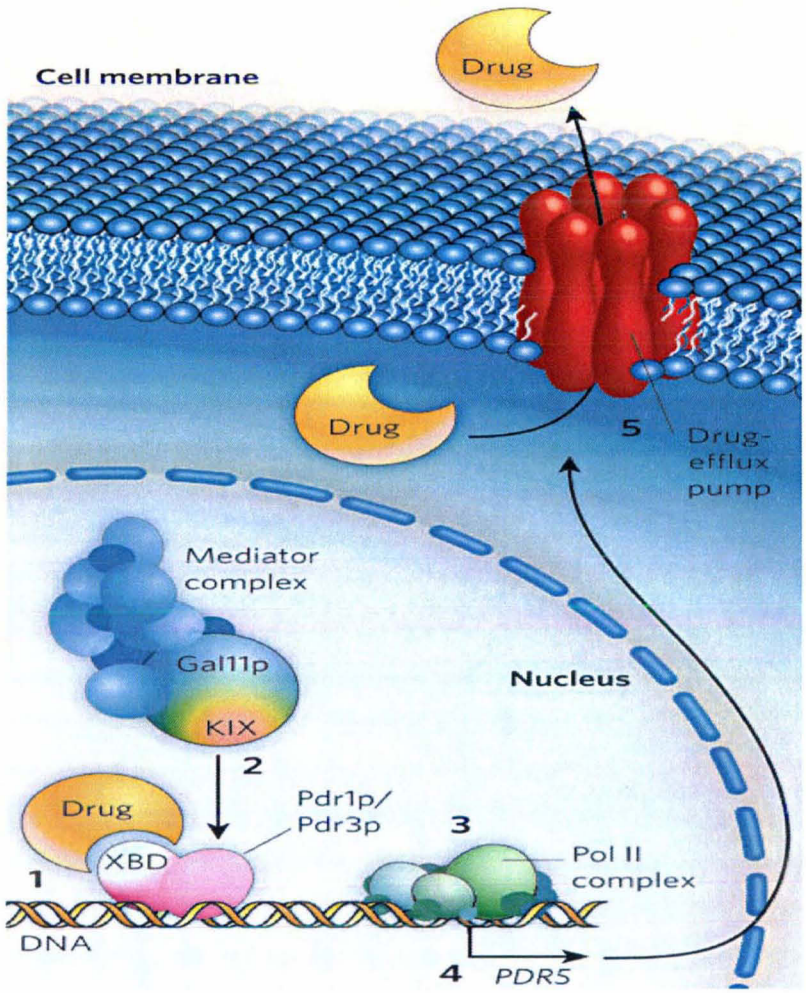


Figure 12. Transcriptional activation of PDR network upon exposure to antifungal drug.

(1) Within a fungal cell, an antifungal drug might bind to the XBD domain of the transcription factors Pdr1p/Pdr3p, which associate with the promoter sequence for drug-efflux genes such as *PDR5*.

(2) This would allow the binding of the Gal11p subunit of the Mediator complex through its KIX domain to the drug-Pdr1/Pdr3p-DNA complex. The Mediator complex would then facilitate.

(3) recruitment of RNA polymerase II (Pol II) and

(4) increased transcription of *PDR5*. *PDR5* is one of a family of genes that encode drug-efflux pumps such as ABC transporters.

(5) At the cell membrane, these pumps allow the efflux of the antifungal drug, thus diminishing the fungal cell's susceptibility to the toxic compound.

(**Courtesy**: Goffaeu, 2008).

domain of Pdr1p/Prd3p at their carboxyl termini interacts with the KIX domain of Gal11p, and that this interaction is enhanced by antifungal drugs (Figure 12).

More over, they show that the yeast KIX domain is structurally similar to its mammalian counterpart, which can also bind to yeast Pdr1p. Mammalian KIX domains interact with specific transcription factors known as nuclear receptors, which affect cholesterol biosynthesis and other aspects of lipid homeostasis. Similarly, in yeast, several aspects of lipid homeostasis depend on Pdr1p/Pdr3p (DeRisi *et al.,* 2000), and this process is now shown to be regulated by the KIX domain (Thakur *et al.*, 2008). With the discovery of the involvement of new molecular domains (XBD in Pdr1p/Pdr3p and KIX in Gal11p) in the pathway leading to antifungal-drug resistance, Thakur and colleagues (2008) argue that there is now reason to redirect the long-standing search for small molecules that inactivate resistance (Lamping *et al.*, 2007). But finding such small molecules to prevent drug-Pdr1p/Pdr3p interaction might be difficult, because this interaction is of limited specificity. Nonetheless, the successful use of the oestrogen-receptor antagonist tamoxifen in the treatment of breast cancer indicates that small molecules antagonizing oestrogen-PXR interaction could be found. The similarity between drug-Pdr1p/Pdr3p interaction and oestrogen-PXR interaction gives reason to believe that drug-Pdr1p/Pdr3p interaction could also be blocked with small molecules. And a broad screen, which is not restricted to the blockage of drug-Pdr1p/Pdr3p interaction but can detect any inhibitor molecule that reduces Pdr1/Pdr3p activity, has already been reported by Kozovská and Subik (2003).

1.7.2.1.2 Other PDR regulators

Pdr1p is post-transcriptionally regulated by Pdr13p, which is the unique yeast member of the Hsp70 protein family (Kolaczkowska and Goffeau, 1999). Pdr13p regulates the function of Pdr1p but not of Pdr3p (Figure 11). Over-production or gain-of-function mutation (S295F) of Pdr13p elevates Pdr1p wild-type activity. Yeast cells carrying inactivated *PDR13* gene exhibit cold-sensitive growth defect. Phenotype analysis of the strain lacking the *PDR13* gene shows its possible connection with the general-stress response system. In the *pdr13* knockout strain, the induced expression of several genes containing STRE elements being under the control of the general stress response factors Msn2p and Msn4p, is observed. Tested genes include *CIT1* (cytoplasmic catalase), *HSP12* (heat shock protein) as well as

CUP1 (metalothionine). *PDR6* gene has recently been shown to be a member of an important RanBP7/importin-ß/Cse1p superfamily of Ran GTP-binding proteins. Since these proteins are involved in nuclear transport, Pdr6p is implicated to be a novel candidate protein involved in the regulation of nuclear transport of transcription factors involved in multidrug resistance (Capieaux *et al.,* 1991; Gorlich *et al.,* 1997; Pennisi, 1998). Two loci, *PDR7* and *PDR9*, both localized on chromosome II, were reported to affect the transcription of the *PDR5* gene (Dextar *et al.,* 1994). Mutants of both genes, isolated as spontaneous suppressors of the hypersensitive *pdr1* knockout derivative restore resistance to cycloheximide and sulfometuron methyl. Since these genes have not yet been identified within the sequence of chromosome II, it is difficult to predict their role within the PDR network.

Genomic approaches uncovered another zinc finger regulator, Pdr8p, as involved in the PDR network (Hikkel *et al.,* 2003). Pdr8p mediates resistance to ketoconazole and oligomycin, operating mainly through Yrr1p and its respective target genes. Recently another transcription factor, Rdr1p (Repressor of drug resistance 1; YOR380W), belonging to the Gal4 family of zinc cluster proteins has been identified to be a repressor of *PDR5* gene *via* PDRE, a *cis*-regulatory element also shared by *PDR1/PDR3* (Hellauer *et al.*, 2002). Δ*rdr1*/Δ*rdr1* strain has increased resistance to cycloheximide, as expected from the over-expression of *PDR5*. In addition, the activity of a *PDR5-lacZ* reporter is increased in Δ*rdr1* strain. Also it has been shown that Rdr1p interacts with the PDREs and mutations known to reduce binding of Pdr1/Pdr3p abolished the induction observed in the Δ*rdr1* strain (Hellauer *et al.,* 2002). Heterodimers of Rdr1p with Pdr1p or Pdr3p compete with Pdr1p/Pdr3p for binding to PDREs (Hellauer *et al.,* 2002). Akache and Turcotte (2002) have done by the phenotypic analysis of 33 genes encoding yeast zinc cluster proteins in order to better understand their functional role in MDR phenomenon. In this endeavor, they have identified that deletion mutants of nine such genes- *RDS1*, *UPC2*, *STB5*, *ECM22, HAL9*, *YIL130W*, *YKL222C*, *RDS2*, *and RDS3,* are either resistant or sensitive to at least one drug. Of these deletion of *STB5* greatly decreased RNA levels of *SNQ2* and *PDR16* (and to lesser extent *PDR5*), thereby suggesting that Stb5p activates the expression of MDR genes (Akache *et al.,* 2004). Stb5p activates transcription by binding to PDREs, the same elements found to be critical for Pdr1p and Pdr3p activity. Stb5p acts predominantly within a Pdr1p heterodimer,

while no interactions occur with Pdr3p or Yrr1p, the latter is only present as a homodimer (Akache and Turcotte, 2002; Akache *et al.*, 2004).

Recently, yet another novel zinc finger regulator, War1p, was identified as the main modulator of weak acid stress adaptation through transcriptional control of PDR12 (Kren *et al.*, 2003). War1p controls a rather small regulon (Schuller *et al.*, 2004), forms homodimers, is activated by stress-triggered hyper-phosphorylation (Frohner *et al., unpublished observations*), and decorates a cis-acting weak acid response element in the *PDR12* promotor (Kren *et al.*, 2003, Schuller *et al.*, 2004). Ecm22p and Umc2p are members of the $Zn(II)_2Cys_6$ transcription factors involved in regulation of membrane sterol homeostasis (Crowley *et al.*, 1998; Vik and Rine, 2001) through control of ABC transporters. Based on genome-wide transcriptional profiling of a Upc2-1 gain-of-function mutant strain that exhibits aerobic sterol influx, *PDR11* and *AUS1* were identified as major determinants required for uptake of free sterols under conditions of impaired ergosterol biosynthesis (Wilcox *et al.*, 2002).

The ABC transporter Pdr15p is induced upon various stresses, including heat shock, high osmolarity and weak acid stress in an Msn2p-dependent manner (Wolfger *et al.*, 2004). The Cys_2His_2 zinc finger protein Msn2p, a master regulator of general stress response pathway, modulates a large set of genes in response to a variety of different environmental stimuli (Gasch *et al.*, 2000). Interestingly, Pdr15p stress induction requires Msn2p (Wolfger *et al.*, 2004), but bypasses upstream components of the HOG pathway (Seet and Pawson, 2004), suggesting the existence of a novel as yet undisclosed HOG signaling branch converging at the downstream Msn2p regulator (Wolfger *et al.*, 2004).

Moreover, Pdr15p levels are strongly elevated in cells undergoing diauxic shift, while expression of Pdr5p almost completely disappears (Mamnun *et al.*, 2004; Wolfger *et al.*, 2004). Thus, Pdr15p and Pdr5p may perform non-overlapping functions related to detoxification in different growth phases or under certain metabolic conditions.

1.7.2.2 YRR1 and YRM network

Yrr1 was recently identified as a Zn_2Cys_6 transcription factor involved in control of the PDR phenomenon (Cui *et al.*, 1998). It was isolated by reveromycin A resistance screening (Cui *et al.*, 1998). Gain-of-function mutations in its activation domain confer high level resistance to

the cell cycle inhibitor reveromycin A, to the DNA-damaging agent 4-nitroquinoline-*N*-oxide and to oligomycin (Zhang *et al.*, 2001; Le Crom *et al.*, 2002). *YRR1* deletion leads to hypersensitivity to 4-nitroquinoline-N-oxide (4-NQO) and oligomycin but not to cycloheximide (Cui *et al.*, 1998). *SNQ2* gene is involved in *YRR1*-mediated NQO resistance, as suggested by observations that *SNQ2* confers resistance to 4-NQO (Decottignies *et al.*, 1998; Servos *et al.*, 1993). Promoter deletions have also demonstrated that Yrr1p interacts with the promoter region of *SNQ2* (Zhang *et al.*, 2001). *Yrr1* affects oligomycin resistance by activating *YOR1* expression *via* a region in the *YOR1* promoter that is very similar, but not identical, to the PDRE elements recognized by *Pdr1/Pdr3* and *RDR1* (Zhang *et al.*, 2001). Thus, *YRR1* is involved in complex PDR network regulation, directly activating *SNQ2* and *YOR1*, both of which are common targets of Pdr1p, Pdr3p and another regulator of MDR, Yap1p. As Pdr1p activates Yrr1p (Zhang *et al.*, 2001), it seems highly likely that *YRR1* plays a major role in PDR regulation network. An intricate interplay of cross-regulation links *YRR1, PDR1,* and *PDR3*. *PDR1* regulates *PDR3* and *YRR1*, both of which regulate their own expression (Delahodde *et al.*, 1995; Zhang *et al.*, 2001), *see* Figure 13. In addition, different post-translational processes, including nuclear targeting or heterodimer formation (Mamnun *et al.*, 2002), may be involved in different regulatory pathways.

Genome-wide analyses have established that the sets of target genes directly regulated by these three transcription factors display considerable overlap (DeRisi *et al.*, 2000; Devaux *et al.*, 2001; Le Crom *et al.*, 2002). Most of these co-regulated genes encode for proteins involved in the structural organization of the plasma membrane. These proteins include ABC and MFS transporters, together with proteins involved in the control of specific steps in lipid biosynthesis. Accordingly, the drug resistance phenotypes conferred by the gain-of-function mutations for the three transcription factors reflect changes in plasma membrane composition. Thus, discrete changes in plasma membrane properties can be accomplished through sophisticated cross-regulations between several transcription factors, not all of which have been identified (Akache & Turcotte, 2002; Moye-Rowley, 2002).

YRM1 (Yeast Reveromycin resistance Modulator) encodes another $Zn(II)_2Cys_6$ regulator acting as a specific inhibitor of Yrr1p. In the absence of Yrr1p, Yrm1p activates the transcription of most genes regulated by Yrr1p, reflecting the high level of complexity of the regulatory processes controlling drug resistance phenotypes in yeast (Lucau-Danila *et al.*,

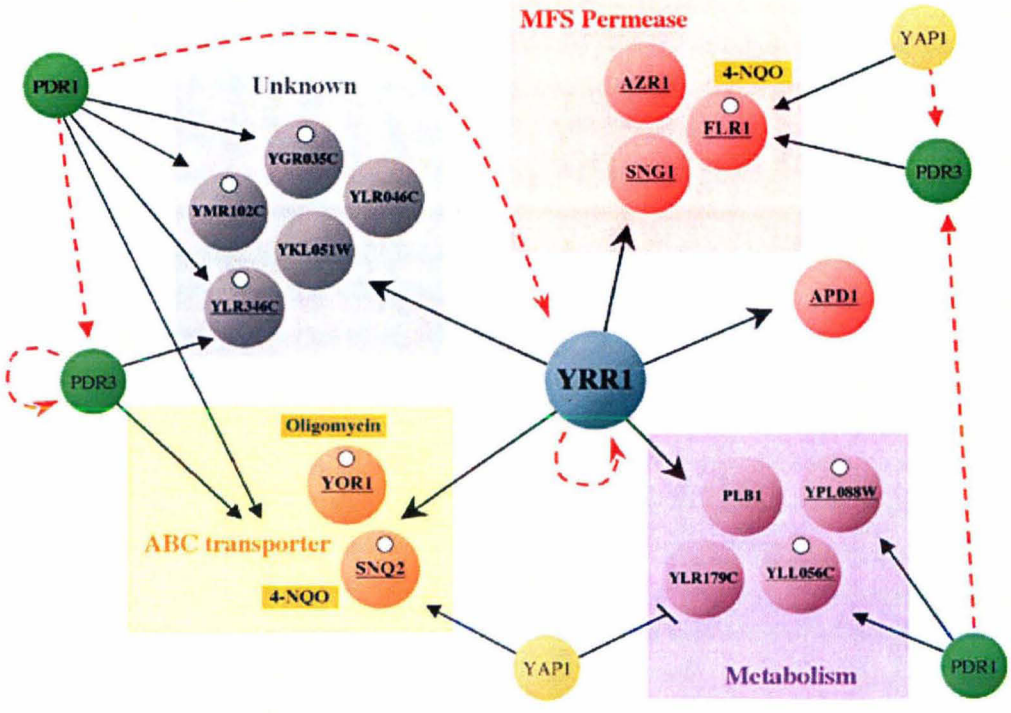


Figure 13. The *YRR1* regulation network is linked to the PDR network.
All the regulated genes found to be activated by Yrr1p fall into the three main characterized functional groups: MFS permease, ABC transporter, and metabolic genes. For all these genes, the consensus PDRE sequence is shown as an *open circle*. Most of these genes are also regulated by Pdr1p, Pdr3p, and Yap1p. These transcription factors control the expression of other transcription factors (*dotted arrows*) or, in some cases, their own expression (*YRR1* and *PDR3*). Finally, the most common drugs used (4-NQO and oligomycin) are marked next to the genes associated with specific resistance.
(**Courtesy**: Le Crom *et al.*, 2002).

2003). *YRM1* is homologous (41% identities) to *YRR1*. The degree of similarity is higher in the zinc finger domain, but the two proteins display considerable similarity throughout the entire length of their sequences. The sequences in the Cys regions are quasi-identical suggesting that the two factors recognize similar DNA binding motifs.

Microarray experiments were carried out to determine whether *Yrm1* was a transcription factor and to identify the corresponding direct target genes. The situation turned out to be more complex than expected: *Yrm1* acts as a transcription factor and interacts with the promoter of the target genes only in the absence of *Yrr1*. The sets of target genes directly regulated by *YRR1* or *YRM1* are similar, but not identical (Figure 14). Alternative promoter occupancy thus plays a key role in this cross-regulation adding a new degree of complexity to the cell drug response (Akache & Turcotte, 2002).

1.7.2.3 YAP network

Yap1p, the first member of the family of Yaps to be described, was initially identified by its ability to bind and activate the SV-40 AP-1 recognition element (ARE: TGACTAA). Based on its ARE binding capacity, this factor was purified as 90Kda protein and the corresponding gene was cloned by screening a λgt11 library with a monoclonal antibody against Yap1p (Harshman *et al.,* 1988). Subsequently this gene was also found in multi-copy transformants resistant to the iron chelators 1,10-phenanthroline and 1-nitroso-2-naphtol (Schnell *et al.,* 1992) as well as to a variety of drugs including 4-nitroquinoline-N-oxide, N-methyl-N-nitro-N-itrosoguanine, triaziquone, sulphomethuron methyl and cycloheximide, the locus being historically designated PAR11SNQ3/PDR4 (Hertle *et al.,* 1991; Hussain and Lenard, 1991; Haase *et al.,*1992). Yap1p have also been implicated in ABC gene regulation, although their precise role in PDR or stress response has not been unraveled so far (Moye-Rowley, 2003; Rodrigues-Pousada *et al.,* 2004). Yap1p decorates promoters of SNQ2 and YCF1 to modulate their expression (Moye-Rowley *et al.,* 1989; Wemmie *et al.,* 1994).

Notably, another member of the b-ZIP family, Yap8p, also participates in arsenite detoxification by regulating Ycf1p expression (Wu *et al.,* 1993; Bobrowicz *et al.,* 1997). Interestingly, recent studies revealed a link between Yap1p and the aging process, because over-expression of YAP1 significantly improves survival during chronological aging in yeast cells (Herker *et al.,* 2004), possibly through Ycf1p expression regulation (Jungwirth *et al.,*

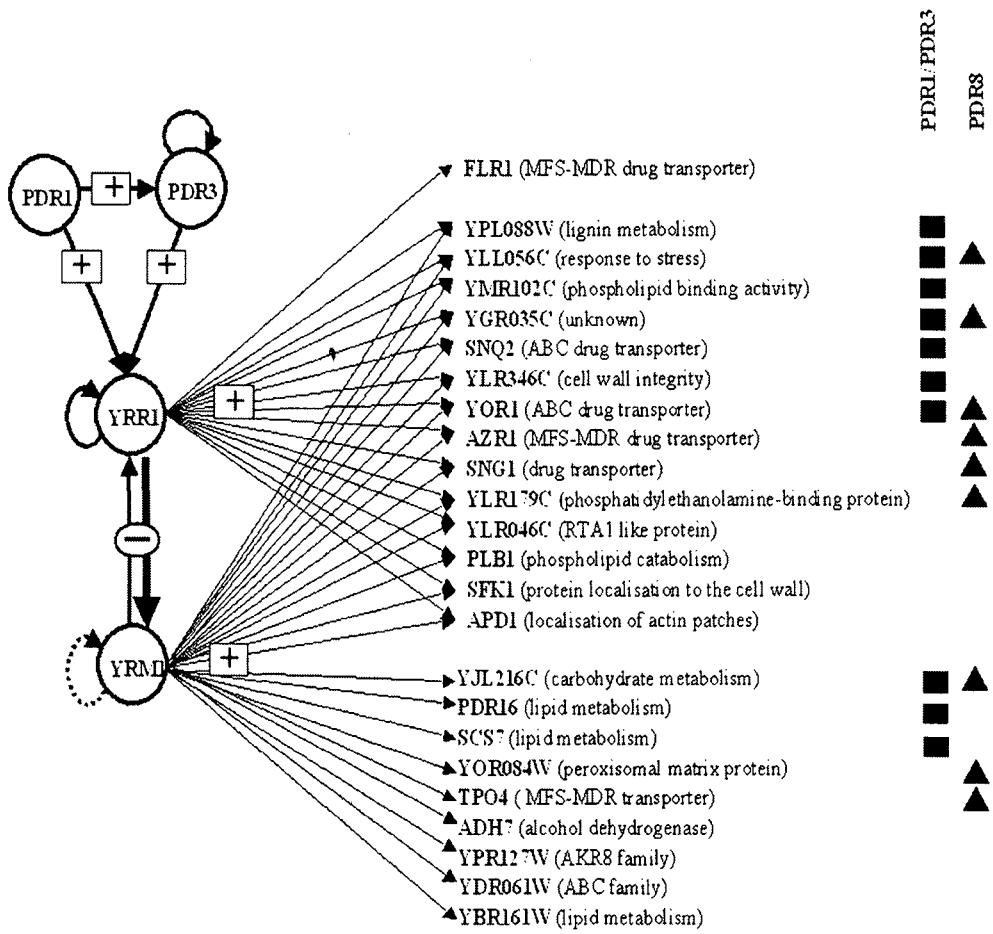


Figure 14. The yeast multidrug resistance phenomenon is controlled by a pair of paralogous transcription factors, which are themselves interconnected.
The *square plus* indicates transcriptional regulation whereas the *circle minus* indicates post-transcriptional regulation. *PDR1/PDR3* were the first example of paralogous transcription factors acting on the drug resistance phenotype to be identified. They positively regulate a set of genes, most of which encode for plasma membrane proteins (3, 4), but they also regulate the expression of *YRR1*. The target genes directly regulated by *YRR1/YRM1* and by *PDR1/PDR3* are indicated by a *black square* (*right*). The genes directly regulated by *PDR8* and by *YRR1/YRM1* are indicated by *black triangles*. Note that *FLR1* is regulated by *YRR1* only.
(**Courtesy**: Lucau-Danila *et al.*, 2003).

unpublished data). Besides *YAP1*, a second gene, *YAP2*, conferring resistance to 1,10-phenanthroline in transformed cells over-expressing a multicopy yeast library, was also described (Bossier *et al.*, 1993). This gene encodes a 45 KDa protein that also binds the ARE *cis*-acting element. Sequence homologies identified it as *CAD1*, due to the acquisition of cadmium resistance in cells over-expressing a multi-copy genomic library (Hirata *et al.*, 1994). Later, it was shown that these cells gain resistance to cycloheximide (Bossier *et al.*, 1993; Wu *et al.*, 1993). The sequence of the *YAP1* and *YAP2* genes revealed the presence of a bZip family domain in the N-terminus homologous to the true budding yeast AP-1 factor Gcn4p and to c-Jun in mammalian counterpart. Six more factors of b-Zip family *YAP3-8* homologous to the *YAP1* and *YAP2* was identified by sequence alignment. The Yap family binding site was thus subsequently characterized as TTAC/GTAA (Fernandes *et al.*, 1997) for Yap1p-Yap4p. So far the corresponding binding site for Yap5p-Yap8p has not been characterized, although incase of Yap8p it appears to be TTAATAA (Wysocki *et al.*, 2004).

1.7.2.3.1 YAP network and its interaction with PDR

Yap1p seems to be involved in stress-dependent activation of certain PDR genes, which suggests a functional interplay between YAP and PDR networks in *S. cerevisiae* (Wu *et al.*, 1993). The *PDR5* and *SNQ2* genes, possess, in addition to PDRE, *YAP* response elements (YRE) in their promoters (Miyahara *et al.*, 1996; Fernandes *et al.*, 1997). The presence of a functional *YAP1* gene was reported to be required for the expression of *SNQ2* stimulated by external stress conditions (Wolfger *et al.*, 1997). Thus, at least one *PDR* target gene, *SNQ2*, could be under the control of the general stress response pathway *via* Yap1p.

On the other hand, a *S. cerevisiae* strain overproducing Cap1p, the homologue of Yap1p from *C. albicans* or its truncated version, not only shows dramatically enhanced mRNA level of the MFS transporter-*FLR1* but also slightly elevated level of the *PDR5* transcript. Thus, it seems that Yap1p could contribute to the control of 4-NQO and cycloheximide resistance *via* genes encoding ABC transporters, such as *SNQ2* and *PDR5* and/or MFS transporters (Figure 15). The *YAP1*-mediated diazoborine and 4-NQO resistance are mainly dependent on the *PDR3* gene (Wendler *et al.*, 1997). Furthermore the double pdr1pdr3 knockout strain is hypersensitive to diazoborine even when YAP1 is over-expressed, demonstrating a requirement of intact *PDR1* and *PDR3* loci for YAP1-mediated resistance to that drug

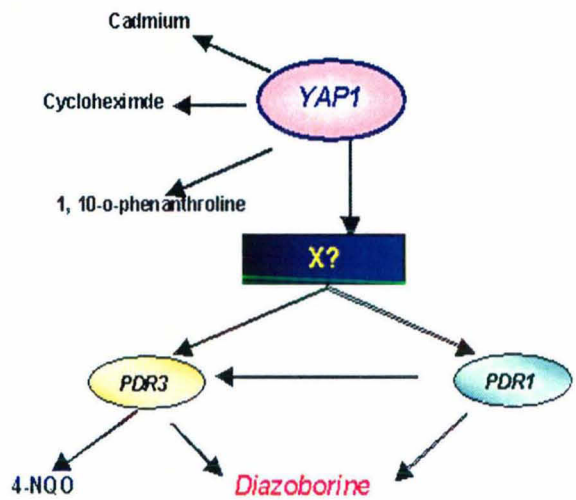


Figure 15. A schematic diagram showing the cross talk between the YAP1 and PDR networks in *S. cerevisiae.*

Yap1-mediated resistance to 1,10-phenanthroline, cycloheximide, and probably cadmium is independent from *PDR1* or *PDR3*. In contrast, Yap1-mediated resistance to diazaborine and 4-NQO is exerted mainly *via PDR3*. The contribution of *PDR1* to diazaborine resistance may be either direct or indirect through *PDR3*. (**Courtesy**: Wendler *et al.*, 1997).

Table 5: Trans-regulatory factors controlling the PDR in *S. cerevisae.*

TFs	Description	Target genes	Functions
Pdr1	$Zn(II)_2Cys_6$	*PDR3, PDR5, PDR10,PDR15, PDR16, IPTG1, SNQ2, YOR1, HXT9, HXT11*	Activation of PDR genes
Pdr3	$Zn(II)_2Cys_6$	*PDR3, PDR5, PDR15, PDR10, SNQ2, YOR1, HXT9, HXT11*	Activation of PDR genes
Pdr8	$Zn(II)_2Cys_6$	*PDR15, YOR1*	Regulation of PDR
Pdr13	HSP 70 homologue	*PDR1*	Post–translational Pdr1p regulation
Stb5	$Zn(II)_2Cys_6$	*PDR5, SNQ2*	Regulation of PDR, Pdr1p-heterodimer
War1	$Zn(II)_2Cys_6$	*PDR12*	Regulation of weak acid stress response
Ecm22/ Upc2	$Zn(II)_2Cys_6$	*PDR11, AUS1*	Regulation of sterol biosynthesis
Rdr1	$Zn(II)_2Cys_6$	*PDR5, PDR15, PSDR16, PHO84, YOR049C*	Repressor of drug resistance genes
Yrr1	$Zn(II)_2Cys_6$	*SNQ2, YOR1, FLR1, AZR1, SNG1, YLLo56C, YLR346C, YRM1*	Activation of PDR and repressor of *Yrm1*
Yrm1	$Zn(II)_2Cys_6$	*YRR1, SNQ2, YOR1, AZR1, SNG1, PDR16, TPO4*	Activation of PDR and repressor of *Yrm1*
Yap1/ Yap8	bZip	*PDR5, SNQ2, YOR1, YCF1, TRX2, GSH1, GLR1, FLR1, ATR1*	Oxidative stress and heavy metal stress response
Msn2	Cys_2His_2	*PDR15*	Regulation of general stress response
Ngg1	——	*PDR1,PDR3*	Inhibition of PDR1p activity

(Wendler *et al.,* 1997). This, together with the observation that Yap1p indirectly affects diazoborine resistance suggests the existence of a Yap1p-dependent activation of Pdr3p (but much less of Pdr1p) rather than direct Yap1p binding to the gene(s) responsible for multidrug resistance.

1.7.2.3.2 Complex interplay among MDR regulators in S. cerevisiae

The Gal4p family of yeast zinc cluster proteins comprises regulators of MDR genes in *S. cerevisiae* as described above. These zinc cluster proteins contribute differently to the regulation of specific PDR genes. For example, induced-expression of *FLR1*, a gene involved in PDR (Alarco *et al.,* 1997), is dependent on Pdr3p but not Pdr1p (Brôco *et al.,* 1997). Conversely, a deletion of Pdr1p greatly diminishes expression of *PDR5* while removal of Pdr3p has marginal effects (Mahé *et al.,* 1996). Moreover, deletion of *PDR1* or *PDR3* results in increased or decreased expression of *PDR15* (a homologue of *PDR5*), respectively (Wolfger *et al.,* 1997). This pattern of regulation is further complicated by the fact that Pdr3p undergoes positive auto-regulation (Delahodde *et al.,* 1995), while expression of *YRR1* is under the control of Pdr1p/Pdr3p and itself (Zhang *et al.,* 2001). Growth conditions also modulate Pdr3p expression (Mamnun *et al.,* 2004). Moreover, activity of Yrr1p is negatively regulated by over-expression of the zinc cluster protein Yrm1p (Lucau-Danila *et al.,* 2003). The observation that zinc cluster proteins form various combinations of dimers will be invaluable in better understanding the complex regulation of PDR genes. Different combinations of *PDR1* or *PDR3* homo- and hetero-dimers may regulate the expression of different genes, which might help explain how these two proteins act differently. For example, Pdr1p and Pdr3p bind as homo- or hetero-dimers to pleiotropic drug response elements (PDREs) found in promoters of target genes. Pleiotropic drug response elements (PDREs) present in the promoters of their target genes, ABC transporters, as well as in the *PDR3* promoter have been shown to be important in the regulation of these genes. Pdr1p, Pdr3p, Stb5p, and Rdr1p all act through this element, with Pdr1p, Pdr3p, and Stb5p able to bind to an everted repeat CCGCGG. Characterization of PDREs in the *PDR3* promoter indicates its auto-regulation. Even though they act through the same element, all these zinc cluster proteins may perform different functions.

To better understand the interplay among these activators, Akache *et al.*(2004) performed native co-immunoprecipitation experiments using strains expressing tagged zinc cluster

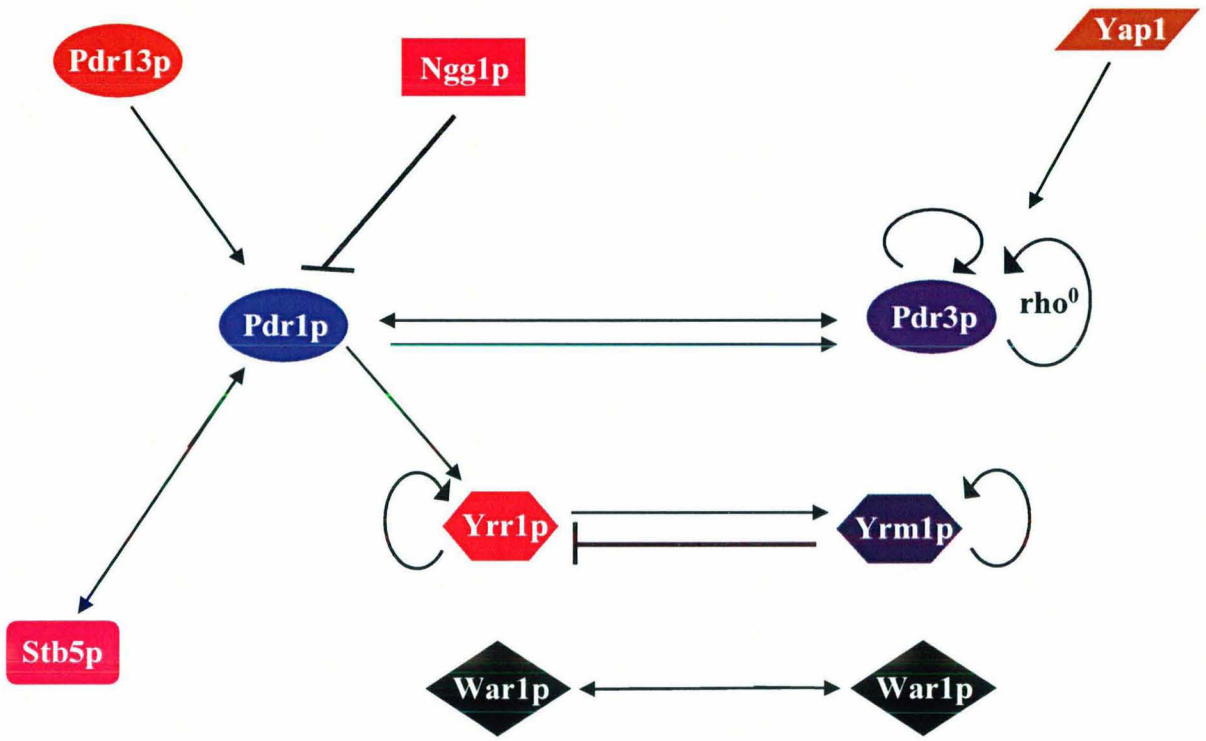


Figure 16. A schematic diagram of interactions among zinc cluster PDR regulator of *S. cerevisiae*.

Arrows indicate zinc cluster protein interactions identified. rho^0 denotes petite mutant. *See text for detail* (**Courtesy**: modified from Akache *et al.*, 2000).

proteins from their natural chromosomal locations as well as glutathione S-transferase pull-down assays and reveals that Stb5p was found predominantly as a Pdr1p heterodimer and shows little homodimerization. No interactions of Stb5p with Pdr3p or Yrr1p was detected. In contrast to Stb5p, Yrr1p is only detected as a homodimer. Importantly, the purified DNA binding domains of Stb5p and Pdr1p were also bound to a PDRE as heterodimers *in vitro*. These results indicates that the DNA binding domains of Pdr1p and Stb5p are sufficient for heterodimerization. The interactions among these various transcriptional activators are summarized in Figure 16.

In conclusion, these results demonstrate that the four zinc cluster protein activators of MDR genes do not act individually. Instead, there is existence of a complex interplay among these activators as they form various populations of homo- and hetero-dimers (Figure 16). There may be differences in the binding specificity or activity of each of these populations, allowing for a very specific and varied expression of genes involved in PDR. Pdr1p was the only protein able to show multiple interactions indicating that it is similar to mammalian nuclear receptor RXR in its ability to recruit various partners. With at least four different zinc cluster proteins regulating the expression of *PDR* genes *via* different PDREs, the cellular ability to respond to drugs is much more adaptable and flexible to fine tune the regulation of MDR genes.

1.7.3 Regulation of MDR in *C. albicans*

C. albicans also harbors Pdr5p-like ABC pumps *i.e. CDR1*, *CDR2* and Flr1p-like MFS transporters *i.e. CaMDR1*. Hence it would appear that there would be some similarity in the transcription regulatory factors governing multidrug resistance between *C. albicans* and *S. cerevisiae*. However this does not appear to be the case as reports show that regulation of expression is quite different in both the strains. In *C. albicans* at least two mechanisms lead to clinical fluconazole resistance: alteration of the drug target (lanosterol 14-α demethylase) as reviewed in a previous section and a decrease of drug accumulation associated with over-expression of multidrug ABC transporters, such as *CDR1*, *CDR2* or the MFS transporter BEN^r/*CaMDR1*, see Figure 2 (Fling *et al.*, 1991; Sanglard *et al.*, 1995 and 1997). The latter mechanism seems to be responsible for an early response developing rapidly after exposure to fluconazole, whereas genetic alterations in target genes and/or trans-acting regulatory

factors are also observed in long term treatment (Franz *et al.,* 1998a). Several *trans*-acting factors of *C. albicans* identified by functional complementation in *ΔPDR1/ΔPDR3 S. cerevisiae* strains were unable to regulate the MDR genes expression in *C. albicans* (Talibi and Raymond, 1999). Yeast to hyphal transition has been associated to the virulence of *C. albicans* and several regulatory pathways involved in yeast to hyphal transition (*EFG1, CPH1, TUP1, NRG1*) has been established, however no direct evidence for the evolvement of these regulatory pathways in *CDR1* regulation has been established yet.

1.7.3.1 ERG11

Mechanisms of resistance to the azole antifungal agents that have been described for *C. albicans* include increased expression of the *ERG11* gene encoding 14-α lanosterol demethylase. Mutations in *ERG11* that interfere with the ability of the azole to bind to its target can also confer resistance (White, 1997b; Franz *et al.,* 1998a; Sanglard *et al.,* 1998b; Kelly *et al.,* 1999a and 1999b; Lamb *et al.,* 2000; Kakeya *et al.,* 2000; Perea *et al.,* 2001). Previous studies have shown that *ERG11* transcript levels vary depending on the growth state of the culture in both *S. cerevisiae* and *C. albicans* (Turi and Loper, 1992; Krishnamurthy *et al.,* 1998; Henry *et al.,* 2000; Lyons and White, 2000) and in response to azole antifungals (*i.e.*, fluconazole, itraconazole, and ketoconazole), wild-type *C. albicans* strains over-express *ERG11* and other genes involved in ergosterol biosynthesis (De Backer *et al.,* 2001; Copping *et al.,* 2005; Liu *et al.,* 2005). In a previous study of transcriptional regulation of the *ERG11* promoter, the full-length *C. albicans ERG11* promoter was fused to the luciferase reporter gene (*Rluc*) from *Renilla reniformis* (P_{Rluc}) shows maximal induction under azole drug pressure, but not during logarithmic growth but as cells approach stationary phase, probably because the cells have exhausted sterol stores, such as lipid droplets, at this time (Song *et al.,* 2004). This effect was observed for several azole drugs as well as for fenpropimorph and terbinafine, suggesting that it is related to ergosterol depletion more than any specific intermediate in the ergosterol pathway. The high levels of azole induction as cells approach stationary phase were observed only when the cells were grown under continuous drug pressure. The *ERG11* promoter activity was shown to parallel nascent sterol synthesis and to be inversely proportionate to total ergosterol levels. Overall, it appears that the *ERG11* promoter is activated in response to sterol depletion in the cell, regardless of the mechanism of that depletion (Song *et al.,* 2004). Subsequent 5'-deletions and linker scan mutations of

ERG11 promoter localized the region important for azole induction to a segment from bp -224 to -251 upstream of the start codon, specifically two 7-bp sequences separated by 13 bp, see Figure 17A (Oliver *et al.*, 2007). These sequences form an imperfect inverted repeat and has homology to the sterol response element (SRE; TCGTATA) from *S. cerevisiae* and was regulated by the transcription factor Upc2p, which has been implicated in sterol regulation in both *S. cerevisiae* and *C. albicans* (Oliver *et al.*, 2007). This region was recognized by the transcription factor Upc2p and functions as an enhancer of transcription, as it can be placed upstream of a heterologous promoter in either direction, resulting in the azole induction of that promoter (Oliver *et al.*, 2007). This promoter constructs are not azole inducible in the *Δupc2/Δupc2* homozygous deletion, demonstrating that Upc2p controls the azole induction of *ERG11*. These results identify an azole-responsive enhancer element (ARE) in the *ERG11* promoter that was controlled by the Upc2p transcription factor. No other ARE was present in the *ERG11* promoter. The activity of the ARE was *UPC2* dependent, suggesting that Upc2p regulates *ERG* genes through direct binding to these ARE (Oliver *et al.*, 2007). Thus, this ARE and Upc2p were necessary and sufficient for azole induction of *ERG11*.

Regarding the *C. albicans* transcription factor Upc2p (Uptake control 2), which regulates sterol metabolism, has been identified and characterized (MacPherson *et al.*, 2005; Silver *et al.*, 2004; White and Silver, 2005) based on its homology to the known *S. cerevisiae* factors Upc2p and Ecm22p. Gene deletion of *UPC2* in *C. albicans* results in significant increases in drug susceptibility to azoles and other ergosterol biosynthesis inhibitors (EBIs) (Silver *et al.*, 2004; MacPherson *et al.*, 2005; White and Silver, 2005). The *Δupc2/Δupc2* homozygous deletion strain also shows reduced accumulation of radio-labeled cholesterol and reduced cellular ergosterol levels. Northern blot analysis of mRNA hybridized with several genes in the ergosterol pathway, including *ERG11*, demonstrated that azole induction of these genes did not occur in the *Δupc2/Δupc2* strain (Silver *et al.*, 2004). Finally, the DNA binding domain (DBD) of the *C. albicans* Upc2p was shown to bind to a region of the *ERG2* gene containing a sequence with homology to the sterol response element (SRE) previously identified in *S. cerevisiae* (MacPherson *et al.*, 2005). This suggests that Upc2p is an important transcription factor in the cell's response to azole drugs because of its role in the regulation of sterol metabolism.

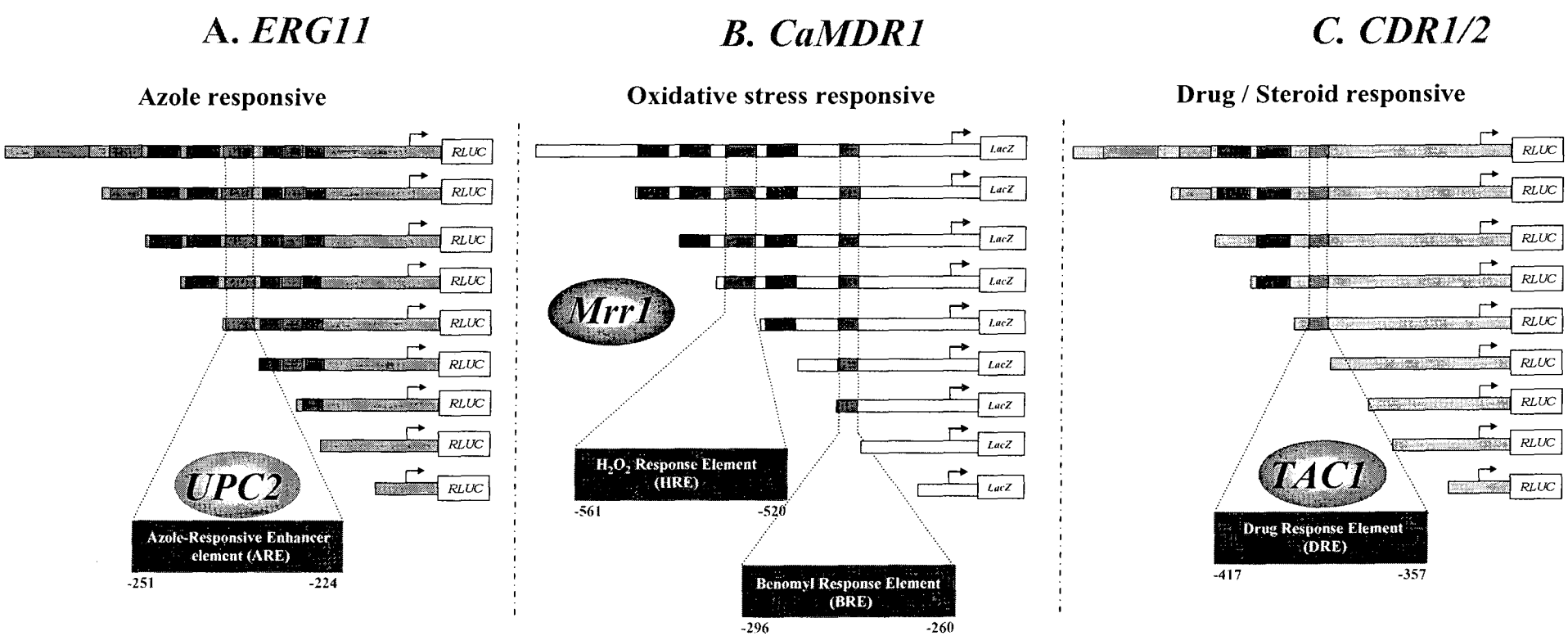


Figure 17. 5'-deletion analysis of MDR genes of *C. albicans*.
Identified *cis*-acting elements and cognate binding factor are shown **(A)** *ERG11*; **(B)** *CaMDR1* and **(C)** *CDR1/2*. *See text for details.*

Recent genome-wide location profiling identifies the *in vivo* transcriptional targets of Upc2p, which includes 12 genes involved in ergosterol biosynthesis (*NCP1*, *ERG11*, *ERG2* and others), 18 genes encoding ribosomal subunits (*RPS30*, *RPL32*, *RPL12*, and others), 3 genes encoding drug transporters (*CDR1*, *MDR1*, and *YOR1*), 4 genes encoding transcription factors (*INO2*, *ACE2*, *SUT1* and *UPC2*), and 6 genes involved in sulfur amino acid metabolism (*MET6*, *SAM2*, *SAH1*, and others) (Znaidi *et al.*, 2008). Bioinformatics analyses suggested that Upc2p binds to the DNA motif 5'-VNCGBDTR that includes the previously characterized Upc2p binding site 5'-TCGTATA (Znaidi *et al.*, 2008). Northern blot analysis showed that increased binding correlates with increased expression for the analyzed Upc2p targets (*ERG11*, *MDR1*, *CDR1*, *YOR1*, *SUT1*, *SMF12* and *CBP1*). The analysis of *ERG11*, *MDR1*, and *CDR1* transcripts in wild-type and Δ*upc2*/Δ*upc2* strains grown under Upc2p-activating conditions (lovastatin treatment and hypoxia) showed that Upc2p regulates its targets in a complex manner, acting as an activator or as a repressor depending upon the target and the activating condition (Znaidi *et al.*, 2008). Taken together, these results indicate that Upc2p is a key regulator of ergosterol metabolism and contribute to azole resistance by regulating the expression of drug efflux pump-encoding genes as well as its own transcription by an auto-regulatory mechanism in addition to ergosterol biosynthesis genes. More recently, using genome-wide gene expression profiling, it has been shown that *UPC2* and other genes involved in ergosterol biosynthesis were coordinately up-regulated with *ERG11* in a fluconazole-resistant clinical isolate compared with a matched susceptible isolate from the same patient (Dunkel *et al.*, 2008). Sequence analysis of the *UPC2* alleles of these isolates revealed that the resistant isolate contained a single-nucleotide substitution in one *UPC2* allele that resulted in a G648D exchange in the encoded protein (Dunkel *et al.*, 2008). Introduction of this mutated allele into a drug-susceptible strain resulted in constitutive up-regulation of *ERG11* and increased resistance to fluconazole (Dunkel *et al.*, 2008). Thus it has been shown for the first time that a gain-of-function mutation in *UPC2* leads to the increased expression of *ERG11* and imparts resistance to fluconazole in clinical isolates of *C. albicans* (Dunkel *et al.*, 2008). More recently, UPC2 promoter was cloned upstream of a luciferase reporter gene (*Rluc*) by Hoot *et al.* (2008). P_{CaUPC2}-*Rluc* activity was induced in response to ergosterol biosynthesis inhibitors and in response to anaerobicity. Under both conditions, induction correlates with the magnitude of sterol depletion (Hoot *et al.*, 2008). Azole

inducibility in the parental strain was approximately 100-fold, and in a Δ*upc2*/Δ*upc2* homozygous deletion strain was 17-fold, suggesting that, in addition to auto-regulation, *CaUPC2* transcription is controlled by a novel, Upc2p-independent mechanism(s) (Hoot *et al.,* 2008). Curiously, basal P_{CaUPC2}-*Rluc* activity was fivefold higher in the deletion strain, which may be an indirect consequence of the lower sterol level in this strain, or a direct consequence of repression by an auto-regulatory mechanism (Hoot *et al.,* 2008). These results suggest that transcriptional regulation of *CaUPC2* expression is important in the response to antifungal drugs, and that this regulation occurs through Upc2p-dependent as well as novel Upc2p-independent mechanisms (Hoot *et al.,* 2008).

1.7.3.2 CaMDR1

Up-regulation of *CaMDR1* confers resistance to fluconazole in clinical *C. albicans.* Understanding the transcriptional control of *CaMDR1*, by both *cis-* and *trans*-acting effectors, is therefore important for determining how azole resistance and transport mechanisms are regulated in *C. albicans*. *CaMDR1* expression is controlled by at least two regulatory promoter *cis*-acting regions as reported recently by Harry *et al.* (2002). Deletion of one of these regions abolished benomyl-induced *MDR1* expression in a *C. albicans* laboratory strain indicating that this region affects the activities of corresponding transcription factors. Recently, Riggle and Kumamoto (2006) exploited the P_{CaMDR1}-yEGFP reporter system to identify MDRE (Multidrug Response Element) which is 80 bp upstream of TATA box. Finer deletion of MDRE abolishes its *trans*-activation and factor binding ability thereby indicating its importance in *CaMDR1* transcription activation. Efforts of other group (Hiller *et al.*, 2006; Rognon *et al.*, 2006) have also led to the identification of various other *cis*-acting elements such as H_2O_2 Responsive Element (HRE; -561 to -520 with respect to the ATG start codon) and Benomyl Responsive Element (BRE; -296 to -260 with respect to the ATG start codon) in *CaMDR1* promoter region mediating its up-regulation in AS isolates and its constitutive activation in AR isolates, see Figure 17B. Two potential binding sites (TTAG/CTAA) for the bZip transcription factor Cap1p (*Candida* AP-1 protein) lie within the HRE and inactivation of *CAP1* abolished the transient response to H_2O_2 (Rognon *et al.*, 2006). Sequence analysis of the *CaMDR1* regulatory region in two pairs of matched fluconazole-susceptible and resistant isolates did not reveal any promoter (*cis*-) mutations in

the resistant isolates. However, integration of P_{CaMDR1}-*GFP* (*CaMDR1* promoter from the fluconazole-susceptible strain CAI4) to an ectopic locus into the genome of the clinical resistant isolates exhibited a fluorescent phenotype, whereas transformants of the corresponding susceptible isolates did not express the *GFP* gene (Wirsching *et al.,* 2000). These results demonstrate that the *CaMDR1* promoter was activated by *trans*-regulatory factor(s) that was mutated in fluconazole-resistant isolates, resulting in deregulated, constitutive *CaMDR1* expression and this is likely to be true also for other fluconazole-resistant *C. albicans* isolates and other genes encoding efflux pumps as also reported by Riggle and Kumamoto (2006).

Trans-acting factors regulating *CaMDR1* were reported recently. One of the cellular MDR determinants in *C. albicans* is the *CAP1* gene encoding an AP-1 like regulatory protein, [homologous to the *YAP1* (Yeast activator protein)], was cloned by functional complementation of *ΔPDR1/ΔPDR3* drug susceptible strain of *S. cerevisiae* (Alarco *et al.,* 1997). Deletion of *CAP1* in *C. albicans* results in hypersensitivity to a limited number of drugs and oxidants, indicating its involvement in multidrug resistance and oxidative stress response (Alarco & Raymond 1999). The pleiotropic phenotype of Cap1p is likely to result from modulation of target genes including *CaYCF1*, *CaTRX2*, *MDR1* (a MFS), *CaGSH1*, *CaGLR1* and possibly the *CIP2* gene, which has a putative YRE element in its promoter (Park *et al.,* 1998; Alarco & Raymond 1999). The truncated *CAP1* gene, lacking the C-terminus, over-expresses the *CaYCF1*, *CaTRX2*, *CaTRR1* and *MDR1* genes and results in hyper-resistance phenotype. The observation that the hyperactive truncated Cap1p does not display increased resistance to ketoconazole and fluphenazine, two well known substrates of the Cdr1p ABC transporter, suggests that the *CDR1* gene is not likely to be a target for Cap1p. Cap1p acts as a positive regulator of the *FLR1* gene expression in *S. cerevisiae.* However, inactivation of Cap1p in a *C. albicans* wild type strain as well as in a fluconazole resistant strain does not affect susceptibility of those cells to fluconazole and other antifungal agents like cerulenin or brefeldin A (Alarco *et al.,* 1997; Alarco & Raymond, 1999). Furthermore, the disruption of *CAP1* in *C. albicans* results in up-regulation of the *CaMDR1* gene, indicated that in *C. albicans* Cap1p acts as a negative transcription factor of Ca*MDR1* expression and that it is not responsible for the stable fluconazole resistance phenotype (Alarco and Raymond, 1999).

Recently, by genome-wide gene expression analysis Morschhäuser *et al.* (2007) identified a zinc cluster transcription factor, designated as *MRR1* (Multidrug Resistance Regulator 1), that was coordinately up-regulated with *MDR1* in drug-resistant, clinical *C. albicans* isolates. Inactivation of *MRR1* in two such drug-resistant isolates abolished both *MDR1* expression and multidrug resistance. Subsequent sequence analysis (Morschhäuser *et al.,* 2007) reveals the existence of homozygous hyperactive *MRR1* alleles (P683S exchange in one isolate and a G997V substitution in the other isolate) causing the *MDR1* over-expression in resistant isolates. Introduction of these mutated alleles into a drug-susceptible *C. albicans* strain resulted in constitutive *MDR1* over-expression and multidrug resistance. Many of the Mrr1p target genes encode oxido-reductases, whose up-regulation in fluconazole-resistant isolates may help to prevent cell damage resulting from the generation of toxic molecules in the presence of fluconazole and there by contribute to drug resistance (Morschhäuser *et al.,* 2007).

1.7.3.3 CDR1 / CDR2

Candida drug resistance gene 1 (*CDR1*) was cloned by functional complementation of a *PDR5* null mutant strain of *S. cerevisiae.* Introduction of the *CDR1* gene into this cycloheximide and azole susceptible *PDR5* null mutant of *S. cerevisiae* resulted in drug resistant phenotype to the drugs tested (Prasad *et al.,* 1995). Northern blotting analyses revealed enhanced level of *CDR1* transcript in azole resistant clinical isolates of *C. albicans* and its *in vitro* activation was demonstrated by elevated temperature, human steroid hormones and drugs including azoles (Krishnamurthy *et al.,* 1998). Several elements of *CDR* genes are important for the regulation of *CDR1* and *CDR2*, see Figure 18. *In-silico* analysis revealed the presence of several putative stress inducible *cis*-regulatory elements (HSE, MDR-NF1/YB-1, AP-1 etc) in the *CDR1* promoter (Table 6).

Both SRE1 and SRE2 were specific for steroids, as they did not respond to other drugs, such as cycloheximide, miconazole and terbinafine. *In silico* analysis of SRE1/2 with the promoter sequence of MDR (*CDR2* and *PDR5*) and non-MDR (*HSP90*) steroid responsive genes revealed a similarity with respect to conservation of three 5 bp stretches (AAGAA, CCGAA and ATTGG) (Karnani *et al.,* 2004). Using a *CDR1*/luciferase fusion in an integrative plasmid, de Micheli *et al.* (2002) analysed the upstream regulatory region of *CDR1*. A basal

Table 6: Putative *cis*-acting elements identified in *CDR1* promoter.

Sequence Name	Consensus sequence	Sequence present *	Comments
1. General promoter and enhancer element			
TATA box	TATATT	TATATA (-59)	involved in basal machinery
Inverted CCAAT box	ATTGG	ATTGG(-603)	involved in basal machinery
CCAAT box	CCAAT	CCAAT	involved in basal machinery
2. Specific promoter and enhancer element			
ERE	TGACCT	TGACCC (-884)	Estrogen receptor binding site
PRE	AGAACA	AGAACA (-465)	Progesterone receptor binding site
HSEI	TTCCCGAA	TTCCCGAA (-259)	Heat shock factor binding site
HSEII	TTCTTGAA	TCTTGAA (-128)	Heat shock factor binding site
PhRE	TGTAAACA	TGTAAAGC (-979)	Pheromone responsive
GRE	TGTTCT	TGTTCC (-994,-340)	General stress response element
pHRE	TGGGGT	TGGG-T (-596,-378)	pH response element
YAP1	TTACTAA	TTACTCA (-166,-1137)	Oxidative stress response
AP-1	TGACTC	TGACCC (-884)	UV stress
CPH1	TGAAACA	TGAAACT	Involved in morphogenesis
MDR-NF-1	CTGATTGG	CTGATTGAG (-271)	Drug responsive

* Position shown in parenthesis are relative to transcription start point (TSP) of *CDR1*.

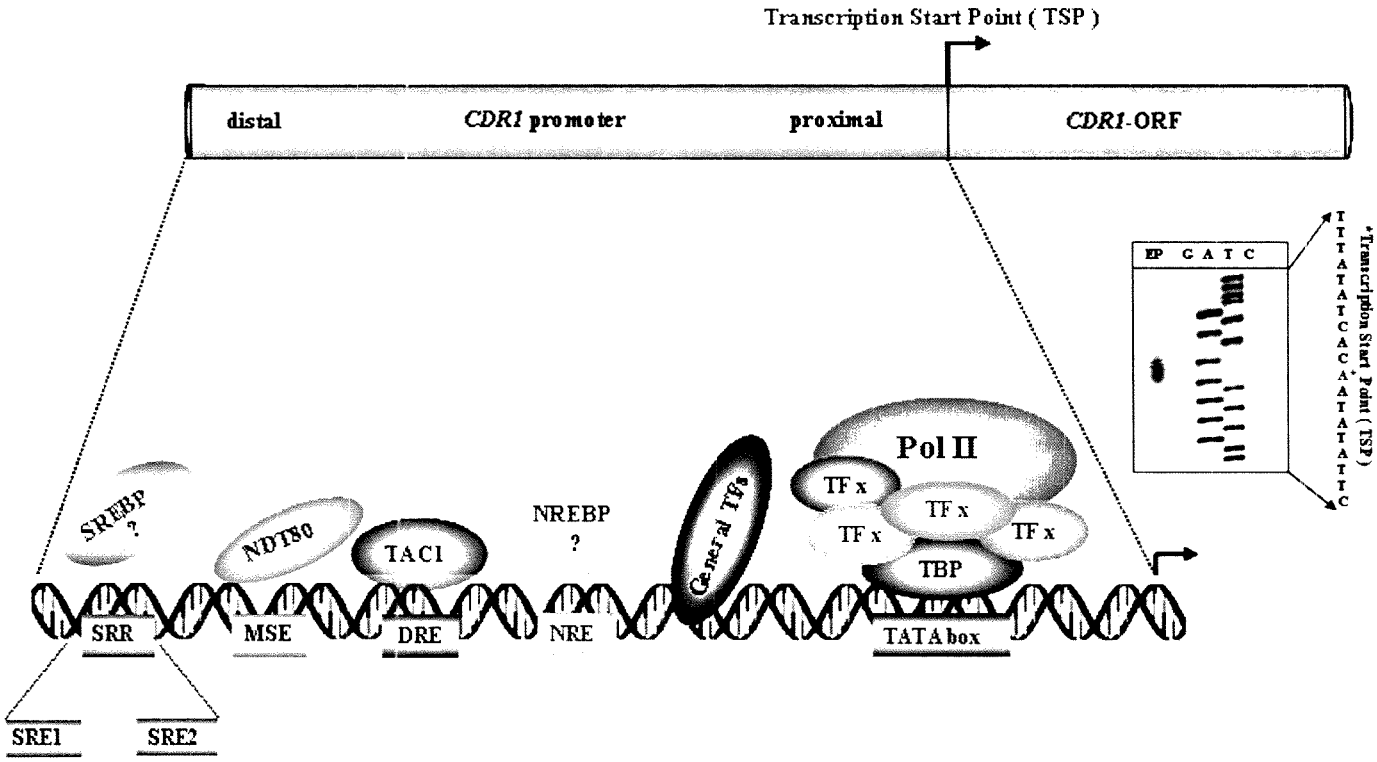


Figure 18. Untranslated 5'-regulatory region of the *CDR1* gene showing identified promoter elements and cognate binding factors.

Positions are relative to the TSP (Transcription Start Point), which is represented by the *bent arrow*.

Abbreviations:

NRE : Negative Regulatory Element; NREBP: Negative Regulatory Element Binding Protein
DRE : Drug Responsive Element; TAC1 : Transcription Activator of *CDR* genes
SRR : Steroid Responsive Region; SREBP : Steroid Responsive Element Binding Protein
SRE1 : Steroid Responsive Element 1; SRE2 : Steroid Responsive Element 2
MSE : Mid Sporulation Element; CaNDT80 : Transcription Activator of *CDR* genes

Inset blot shows the TSP that has been mapped by primer extension analysis as depicted by *asterisk*.
EP : Extension product; GATC : Sequencing marker (**Courtesy**: Puri *et al.*, 1999).

response element (BRE) has been identified between nt -860 and -810 in the *CDR1* promoter. The BRE regulates basal expression of *CDR1* (de Micheli *et al.*, 2002). Another BRE (located between -243 and -234) and a negative regulatory element (NRE) located within the -289 region have been reported in *CDR1* (Puri *et al.*, 1999; Gaur *et al.*, 2004). *In silico* analysis reveals that NRE (-ccaaCTGATTGAaact-) harbors ATTGA which shows significant homology with MDR-NF1/YB-1 binding site (ATTGG) in human *MDR1* promoter (inverted CCAAT) that is known for its involvement in basal as well as stress induced expression of human *MDR1* (Hu *et al.* 2000). A purified ~55 kDa nuclear protein was specifically found to bind to NRE, this report describes purification of the first transacting protein of *CDR1* promoter which may control its basal expression (Gaur *et al.*, 2004). Recently, a common drug responsive element called DRE1/2, which was located between -397 to -376 bp and -303 to -280 bp in *CDR1* promoter (from TSP) was identified, see Figure 17C. (de Michelli *et al.*, 2002). DRE sequence (5'-CGGAA/TATCGGATA-3') was present in the promoters of both *CDR1* and *CDR2* and has been found to be crucial for the up-regulation of these genes in azole-resistant strains as well as for the transient up-regulation of both genes in the presence of different drugs such as ß-estradiol, progesterone, or fluphenazine in azole-susceptible strains (de Michelli *et al.*, 2002). However, sequential or internal deletion of DRE did not completely abolish the stress responsiveness of *CDR1* promoter (de Michelli *et al.*, 2002). Recently, Looi *et al.* (2005) has identified the several hotspot point mutations in *CDR1* promoter of the resistant clinical isolates compared to the susceptible isolates at 39, 49 and 151 nucleotides upstream from the ATG start codon.

Trans-acting factors regulating *CDR1* and *CDR2* were reported recently. Three *C. albicans* genes, designated *FCR* (fluconazole resistance), have been isolated by their ability to complement the fluconazole hypersensitivity of a *S. cerevisiae* mutant lacking the transcription factors Pdr1p and Pdr3p. Over-expression of any of the three *FCR* genes in the *Δpdr1Δpdr3* mutant resulted in increased resistance of the cells to fluconazole and cycloheximide and increased expression of drug efflux transporter *PDR5*, whose transcription is under the control of Pdr1p and Pdr3p. Deletion of *PDR5* in the *Δpdr1Δpdr3* strain completely abrogated the ability of the three *FCR* genes to confer fluconazole resistance, demonstrating that *PDR5* is required for *FCR*-mediated fluconazole resistance in *S. cerevisiae* (Talibi and Raymond 1999). *FCR1* gene (*CaO19.6817*) encodes a putative 517-

amino-acid protein with an N-terminal Zn_2C_6-type zinc finger motif homologous to that found in fungal zinc cluster proteins, including *S. cerevisiae* Pdr1p and Pdr3p. Interestingly, inactivation of *C. albicans* Fcr1p, (ortholog of Pdr1p/Pdr3p of *S. cerevisiae*) results in fluconazole resistance (Talibi & Raymond 1999). *C. albicans Δfcr1/Δfcr1* knock-out derivative displays increased resistance to fluconazole and to drugs known to be transported by Cdr1p and Cdr1p. This hyper-resistance could be reversed to wild-type levels by re-introduction of *FCR1* into the *Δfcr1/Δfcr1* mutant indicating Fcr1p as repressor of *CDR1* in *C. albicans* (Talibi and Raymond, 1999). However, one cannot exclude that hyperactive, gain-of-function alleles of Fcr1p (and/or Fcr2p, Fcr3p) could result in constitutive expression of a set of genes implicated in fluconazole resistance and MDR similarly, as in *S. cerevisiae.* Alternatively, other regulatory factors or alterations in *cis*-acting DNA sequences of the Yap1p target genes *FCR1* or *CAP1*, could contribute to the development of MDR. Therefore, detailed characterization of Fcr1p, its target genes as well as the molecular mechanisms leading to aberrant up-regulation of *MDR1* and *CDR1* genes is needed for the understanding of multidrug resistance in *C. albicans* and fluconazole resistance in clinical isolates (Kolaczkowska & Goffeau, 1999). According to a recent report by Shen *et al.* (2007), Fcr1p inhibits development of fluconazole resistance in *C. albicans* by abolishing *CDR1* function. Chen *et al.* (2003, *unpublished data*) has also identified another regulator of *C. albicans* termed as Repressor of Efflux Pump (REP2). Over-expression of *REP2* leads to six fold increase in the β-galactosidase activity of the P_{CDR1}-*lacZ* construct in *S. cerevisiae.* Furthermore, *Δrep2/Δrep2* mutant of *C. albicans* appears to be more susceptible to antifungal drugs than the parental strain. Quantitative real-time PCR reveals the decreased *CDR1* expression in *Δrep2/Δrep2* mutant in the presence of azole. Recently, Chen *et al.* (2004) described another potential activator of *CDR1* identified by screening of a *C. albicans* genomic library expressed in a *S. cerevisiae strain*, which contained a P_{CDR1}-*lacZ* fusion. This factor, *CaNDT80* (Non DiTyrosine 80), is a homolog to a meiosis-specific transcription factor in *S. cerevisiae* (Chen *et al.,* 2004). Deletion of *CaNDT80* in *C. albicans* conferred hypersensitivity to azoles and decreased the inducible expression of *CDR1.* Interestingly, the global repressor, Thymidine uptake 1 (*Tup1*) acts as a negative regulator of *CDR1* expression (Murad *et al.*, 2001; Yang *et al.*, 2006). A recent genome wide location profiling (ChIP-chip) show that a TF of the zinc cluster family, Uptake control 2 (*Upc2*) which regulates *ERG*

genes, also targets *CDR1* (Znaidi *et al.*, 2008). Recently, another transcription factor belonging to the family of zinc-finger proteins with a Zn_2Cys_6 motif namely *TAC1* (Transcriptional activator of *CDR*) was identfied (Coste *et al.*, 2004). Tac1p is a 980 amino-acid protein of the Zn_2-Cys_6 family, it contains a typical DNA binding domain and an activation domain rich in acidic residues at the C-terminus. Tac1p binds to the DRE, which contains two CGG triplets typical of the DNA-binding sites of Zn_2Cys_6 transcription factors (Coste *et al.* 2004). Tac1p is responsible for transient up-regulation of both *CDR* genes in azole-susceptible strains in the presence of inducers (Coste *et al.*, 2004). Till now, only Tac1p has been experimentally proved to function as a transcriptional activator of *C. albicans* drug efflux transporter genes, such as *CDR1* and *CDR2* (Coste *et al.*, 2004).

Interestingly, *TAC1* locus is on chromosome 5 and is located close to (within ~14 kb) the mating-type-like (MTL) locus where *ERG11* is also located. Previous studies reported a strong correlation between homozygosity at the mating-type locus and azole resistance in a number of clinical isolates (Rustad *et al.*, 2002). In a previous study, Coste *et al.* (2004) showed that a clinical azole-resistant strain (DSY296) that is homozygous at the mating-type locus contains a *TAC1* allele that is sufficient to confer fluconazole resistance to a laboratory strain lacking *TAC1*. This type of allele was defined as '*hyperactive*' because it caused constitutive high expression of *CDR1* and *CDR2* in a *Δtac1/Δtac1* mutant. In contrast, *TAC1* alleles of the matched azole-susceptible clinical strain (DSY294) or of a laboratory strain (CAF2-1), which are strains heterozygous at the mating-type locus, were not able to confer azole resistance to a *Δtac1/Δtac1* mutant. These alleles were defined as '*wild-type*' alleles. Analysis of *TAC1* alleles of matched azole-susceptible and azole-resistant clinical *C. albicans* reveals a single point mutation (N977D) in both hyperactive *TAC1* alleles, which is sufficient to confer hyperactivity as measured by up-regulation of *CDR1* and *CDR2* and levels of drug resistance (Coste *et al.*, 2006). These hyperactive alleles carrying this mutation are co-dominant with other wild-type alleles such that only strains homozygous for hyperactive alleles show high expression levels of *CDR1* and *CDR2*. Later it has been shown that homozygosity at MTL accompanies the acquisition of *TAC1* homozygosity by either through mitotic recombination between copies of chromosome 5 or through the presence of extra copies of chromosome 5 harboring a gain-of-function *TAC1* allele and loss of

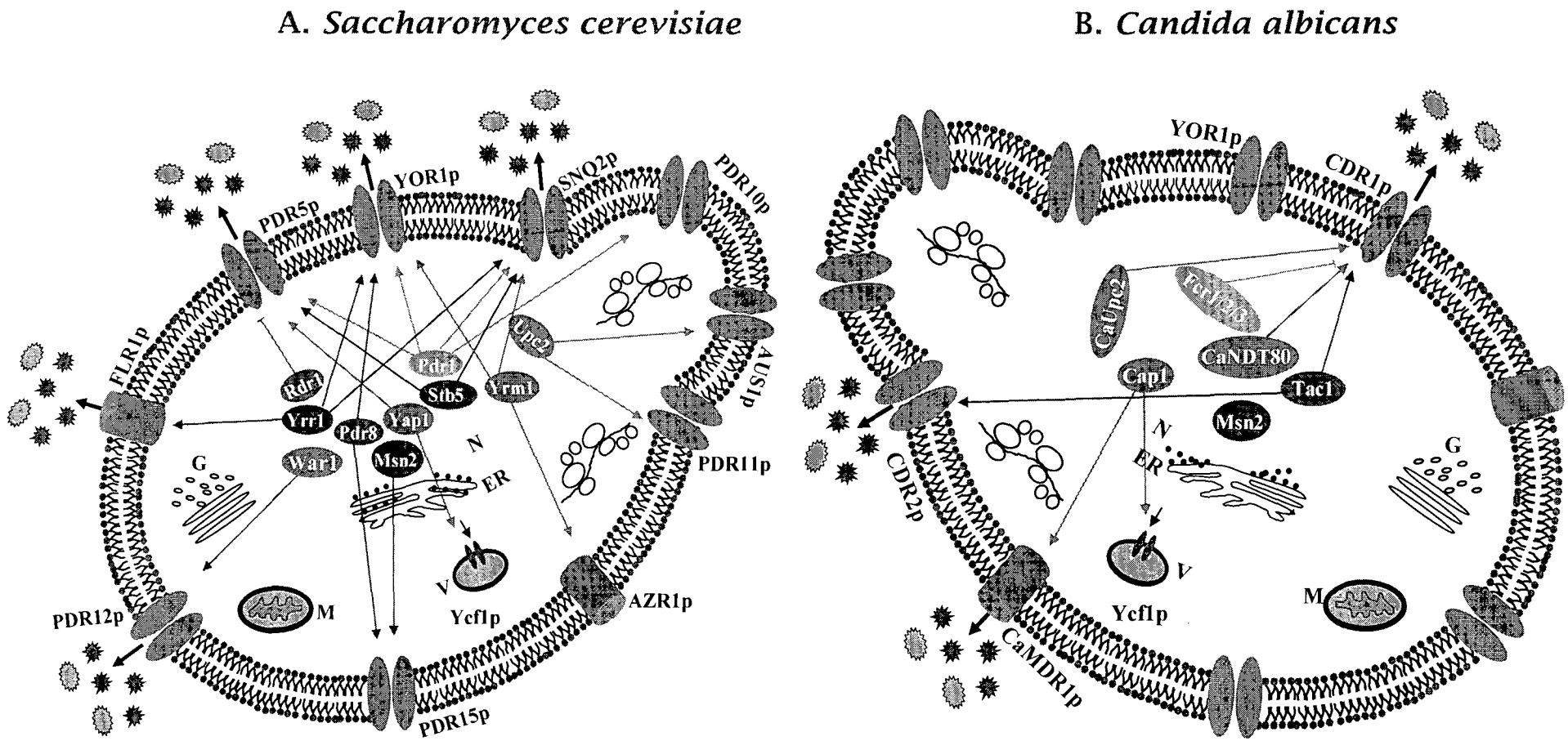


Figure 19. MDR regulatory circuit.

Identified transcription factor(s) factor and corresponding target genes of MDR regulatory circuit are shown. *See text for details.*
(A) *S. cerevisiae* **(B)** *C. albicans.*
Abbreviations: *N-Nucleus; ER-Endoplasmic reticulum; V-Vacuole; M-Mitochondrion; G-Golgi bodies.*

chromosome 5 with the wild-type *TAC1* allele (Coste *et al.,* 2006), but MTL homozygosity itself does not contribute to the azole resistance phenotype (Coste *et al.,* 2006).

Recently, genome-wide location (ChIP-chip) analysis (a procedure combining chromatin immuno-precipitation with hybridization to DNA intergenic microarrays) eight genes were identified, whose expression was modulated in the four azole-resistant clinical isolates in a *TAC1*-dependent manner and whose promoters were bound by Tac1p, qualifying them as direct Tac1p targets: *CDR1*, *CDR2*, *GPX1* (putative glutathione peroxidase), *LCB4* (putative sphingosine kinase), *RTA3* (putative phospholipid flippase) and orf19.1887 (putative lipase), as well as *IFU5* and orf19.4898 of unknown function (Liu *et al.,* 2007). In a separate study, it was shown that *PDR16*, encoding a putative phosphatidylinositol transfer protein contributing to clinical azole resistance, is also a target of Tac1p (Saidane *et al.*, 2006; Znaidi *et al.*, 2007). Interestingly, all of these Tac1p-regulated genes contain a putative DRE in their promoters. These results show that Tac1p binds under non-activating conditions to the promoters of its targets, including to its own promoter. Role of Tac1p in regulating lipid metabolism (mobilization and trafficking) and oxidative stress response in *C. albicans* has also been proposed (Liu *et al.,* 2007).

Therefore, observations from these studies suggest that multiple *cis*-elements and cognate binding activators/ repressors regulate the expression of MDR network in *S. cerevisiae* and *C. albicans* (Figure 19).

1.8 Aims and scope of the present work

The most well-characterized and frequently over-expression of the multidrug resistance protein in azole resistant clinical *C. albicans* isolate is *Candida* drug resistance 1 protein (Cdr1p), encoded by the *CDR1* gene, belonging to ATP-binding cassette (ABC) family (Figure 20). Research to overcome this barrier in treatment has been focused mainly on two areas: (1) advanced, sensitive detection of *CDR1* mRNA or Cdr1p and its expression regulation (2) development of Cdr1p modulators/inhibitors.

Keeping in view the importance of *CDR1* in development of drug resistance, in this study we undertook the first possibility to dissect the molecular mechanisms of *CDR1* expression in matched set of isolates, *i.e.* sensitive and resistant versions of the same strain, as determined by RFLP or karyotyping (Figure 21). *CDR1* gene is not detectably expressed in azole-

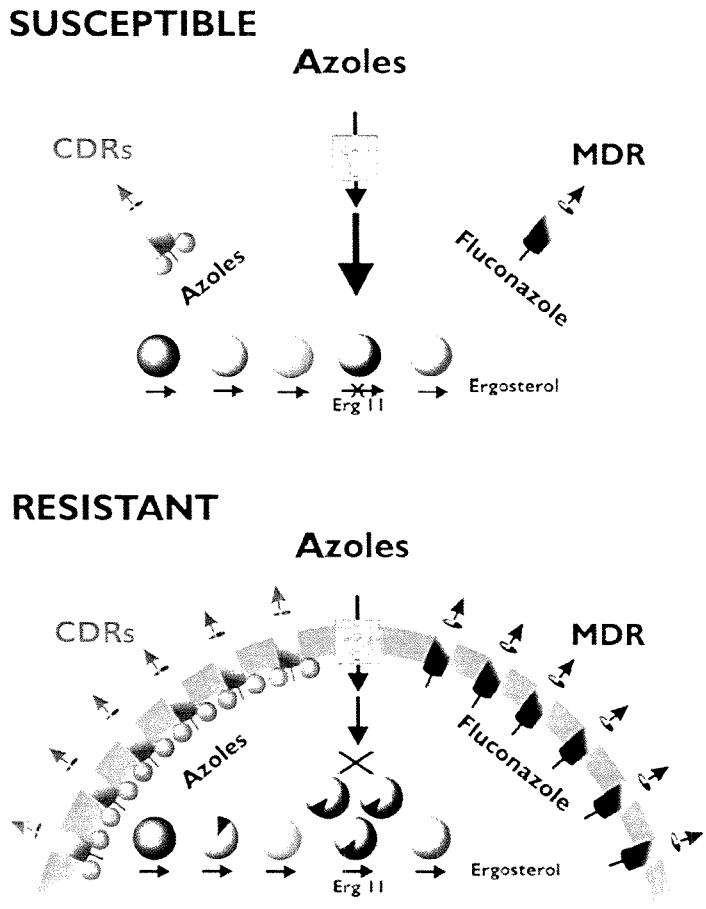


Figure 20. Molecular mechanisms of azole resistance in clinical isolates of *C. albicans*. In a susceptible cell, azole drugs enter the cell through an unknown mechanism, perhaps by passive diffusion. The azole then inhibits Erg11 (*red circle*), blocking the formation of ergosterol. Two types of efflux pumps are expressed at low levels. The CDR proteins are ABC transporters with both a membrane protein (*green tubes*) and two ABC domains (*green circle*). The MDR protein is a MFS with a membrane pore (*red tubes*). In a "model" resistant cell, the azoles also enter the cell through an unknown mechanism. The azoles drugs are less effective against Erg11 for two reasons; the enzymes has been modified by specific point mutations (*dark circle in pink circle*) and the enzyme is over-expressed. Modification in other enzymes in the ergosterol biosynthetic pathway contribute to azole resistance (*dark slices in blue spheres*). The sterol components of the plasma membrane are modified (*dark orange of membrane*). Finally, the azoles are removed from the cell by over-expression of the *CDR* genes (ABC transporters) and MDR (MFS). The *CDR* genes are effective against many azole drugs, while MDR appears to be specific for fluconazole.

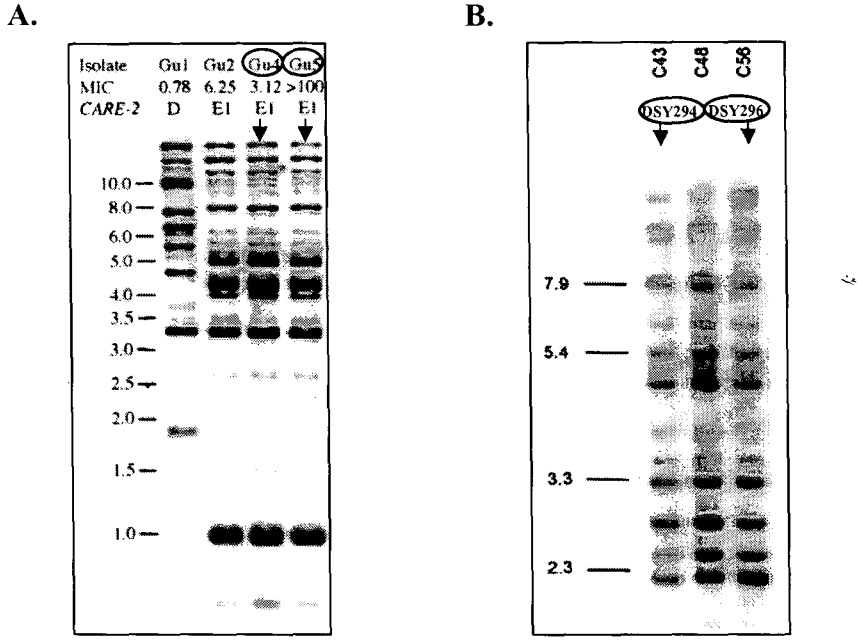


Figure 21. CARE-2 hybridization pattern of multiple successive oral *C. albicans* isolates (used in this study) from two different HIV$^+$-AIDS patients.

DNA Fingerprinting patterns shows no alteration in genomic organisation in matched AS and AR isolates. The positions of reference molecular size markers (in kb) are shown to the left of each blot. **(A)** Gu4 *versus* Gu4 and **(B)** DSY294 *versus* DSY296.

(**Courtesy**: Franz *et al.*,1999 and Boerlin *et al.*, 1996.)

susceptible isolates but is strongly activated after the development of azole resistance and also up-regulated by transient drug inductions (Krishnamurthy *et al.,* 1998). *CDR1* over-expression correlates well with increasing azole MICs in resistant clinical isolates. The comparison of *CDR1* gene expression in matched azole susceptible and -resistant isolates has proved to be a powerful tool to identify the resistance mechanisms of clinical *C. albicans* isolates. This is required because *C. albicans* is mostly clonal; that is, it grows vegetatively without sexual reproduction, indicating that mating is not a common event (Pujol *et al.,* 1993). Most strains of *C. albicans* contain a large number of balanced lethals (recessive null mutations) in which the cell relies on one functional copy of the gene for activity (Whelan and Soll, 1982). These recessive lethal mutations could not persist in a population that mated frequently. The clonal nature of *C. albicans* makes it imperative that matched sets of sensitive and resistant versions of a single strain should be characterized when determining the molecular mechanisms of resistance.

Specific aims:

Regulating gene expression from DNA to protein is a complex multi-stage process with multiple control mechanisms (Figure 5). Transcriptional regulation has been considered the major control point of protein production in eukaryotic cells; however, there is growing evidence of pivotal post-transcriptional regulation for many genes. The mRNA level of particular gene is undoubtedly determined by the balance between synthesis and degradation. This has prompted us to do extensive investigations to elucidate the possibility as well as mechanism controlling mRNA stability/turnover along with transcriptional control of *CDR1*. Over-expression of the *CDR1* drug efflux pump has been demonstrated by the increased mRNA levels which can be due to:

1. Increased *CDR1* transcription from endogenous copies of gene due to mutation(s) in the *CDR1* promoter or alterations in the levels or activity of corresponding transcriptional activator(s) that interact with the *CDR1* promoter that might result in deregulated *CDR1* expression and ultimately increased *CDR1* mRNA level in AR isolates.

2. Molecular changes associate with possible *CDR1* mRNA decay *i.e.* increased half-life of the *CDR1* mRNA as a result of a mutation(s) in the 3'UTR of the mRNA.

The main aim of the present study was to characterize the possibility of above said mechanism of differential expression regulation of *CDR1* in two different clinical matched pairs of AS and AR isolates *viz.* Gu4 *versus* Gu5 and DSY294 *versus* DSY296 as well as between uninduced and transiently induced resistance (IR) of AS isolates *viz.* Gu4 *versus* Gu4-IR and DSY294 *versus* DSY294-IR.

The work in the thesis is embodied in two chapters:

1. Transcriptional activation and increased mRNA stability contribute to over-expression of *CDR1* in azole-resistant *C. albicans.*

2. Differential RNA-protein interaction and poly(A) tail length contributes to increased *CDR1* mRNA stability in azole resistant clinical isolates of *C. albicans.*

In the emerging scenario of increased incidence of development of drug resistance in *C. albicans*, it is pre-requisite to understand the molecular basis leading to MDR. Hence, a complete knowledge of physiological regulation of MDR is a key to design strategies for the prevention of MDR and improve the therapeutic efficacy of drugs widely used to combat these fungal infections. Our following study would pave the way for further investigation, if *CDR1* dependent transcriptional and post-transcriptional processes can become potential novel targets for a new class of antifungal agents.

"Inventing is a combination of brains and materials, the more brains you use, the less material you need"

Materials and Methods

Materials and Methods

2. MATERIALS AND METHODS

2.1 MATERIALS

RNAse A, Sephadex G-50, DEPC (Diethyl Pyrocarbonate), Phenol, Agarose, Rubidium Chloride, Calcium Chloride Sodium dodecyl sulphate (SDS), MOPS (3-[N-morpholino]-2-hydroxypropane sulphonic acid), Formamide, Trizma base, EDTA, Boric acid, glycerol, 2-mercaptoethanol, DMSO, TEMED, ammonium persulphate, Urea, acrylamide, bis-acrylamide, Brilliant Blue G, Coomassie Blue, BSA, Bathocuproienedisulfonate (BCS), Sorbitol, and protease inhibitors: PMSF, leupeptin, pepstatin A, aprotinin were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Megaprime DNA labeling system and PCR amplification kit were procured from Amersham Pharmacia Biotech, USA and Roche, respectively. Molecular weight markers, restriction Endonuclease, DNA-modifying enzymes, Taq DNA polymerase, ultra pure deoxyribonucleotides (dATP, dGTP, dCTP and dTTP) for PCR and ribonucleotides (ATP, CTP, GTP and UTP) for TRO and *in vitro* transcription were purchased from New England Biolabs (NEB Inc), USA. Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RT), proteinase K, T7 promoter primer, Sp6 promoter primer, IPTG, X-GAL were obtained from MBI Fermentas. Proof-reading *Pfu* DNA polymerase and Phusion^{TM} high fidelity DNA polymerase (catalogue # F530S) were obtained from MBI Fermentas and New England Biolabs (NEB Inc), USA respectively. All PCR amplifications were done in a eppendorf thermal cycler. Radio-labeled [α-^{32}P] dATP, [α-^{32}P] dATP, [?-^{32}P]dATP, [5,6-^{3}H] uridine, [α-^{32}P]UTP and [^{14}C]arginine were obtained from Amersham Biotech and Bhabha Atomic Research Center (B.A.R.C.), India. Riboprobe® *in vitro* transcription systems (catalogue # P1460) and pGEM®T-Easy vector system II used for T/A DNA cloning purpose was purchased from Promega, Madison, WI. Plasmid backbone pBS-KS (+) used for cloning purpose was purchased from Stratagene. High molecular weight protein marker was obtained from Amersham Biosciences, Buckinghamshire, U.K. Zymolyase 100T was from Seikagaku Corporation, Japan. Nourseothricin was obtained from Werner Bioagents (Jena, Germany). Thiolutin (CP-4092) was a generous gift from Pfizer, Inc., Groton CT. Polyclonal anti-Cdr1p antibody was custom synthesized from Covance Research Products Inc, PA, USA. PM-ATPase antibody were kindly provided by R. Serrano. Glass beads (0.45mm diameter)

were obtained from Braun Co.,Germany. Media chemicals -YEPD broth (Yeast extract Peptone Dextrose), YEPD agar, LB broth and LB agar were obtained from HiMedia (Mumbai, India) and Difco, BD Biosciences, NJ, USA. Ethanol was obtained from Merck Chemicals, Mumbai, India. All other routinely used Molecular Biology (MB) grade chemicals (Tris, sodium chloride, glycine, potassium acetate, sodium carbonate, magnesium chloride, sodium hydroxide pellets, methanol, glacial acetic acid) used in this study were obtained from Qualigens, India. α-amanitin was procured from Boehringer Mannheim GmbH, Mannheim, Germany. Fluconazole was kindly provided by Ranbaxy Laboratories, New Delhi, India. Ketoconazole and itraconazole were provided by Dupont (Wilmington, Del.). Fluphenazine, progesterone, ß-estradiol, cycloheximide, miconazole, Rhodamine6G (R6G), 1, 10-phenanthroline, actinomycin D, terbinafine, and clotrimazole were purchased obtained from Sigma Chemical Co. (St. Louis, MO), USA. Bacterial plasmid DNA extraction kits and Yeast Genomic DNA extraction, Gel elution and PCR purification kit were obtained from were obtained from Qiagen, Germany.

2.2 RESISTOTYPING

At least two different methods were used for characterizing the drug resistance profile of the yeast strains used in this study. These can be listed as follows:

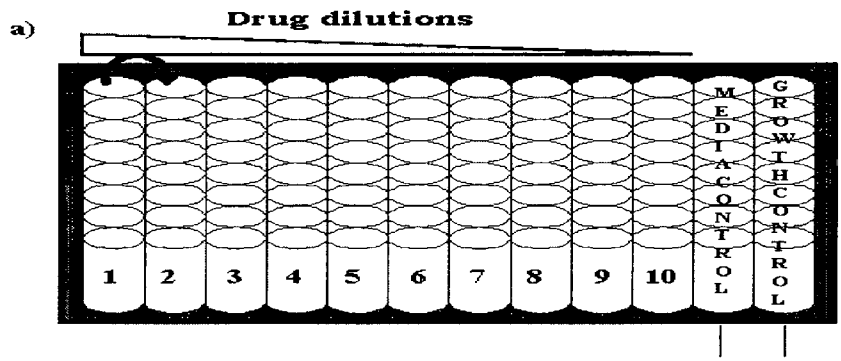
1. Spot test
2. Antifungal susceptibility testing using NCCLS method M27A

Following is a detailed description of these different methods and the drugs along with their respective solvents used in this study are listed in Appendix V.

2.2.1 Spot test. *C. albicans* strains were grown overnight on YEPD plates at 30°C. Cells were then suspended in normal saline to 0.1 OD (A_{600}). Five microlitres of five fold serial dilutions of each yeast culture were spotted on to YEPD plates in the absence (control) and presence of the drugs. Growth differences were recorded following incubation of the plates for 48 h at 30°C (Mukhopadhyay *et al.,* 2002). All clinical *C. albicans* isolates were tested for fluconazole, itraconazole, clotrimazole, ketoconazole, miconazole, terbinafine and cycloheximide. Growth was not affected by the presence of the any solvents used for the drugs.

2.2.2 Antifungal susceptibility testing using NCCLS method M27A. The relative azole susceptibility of *Candida* isolates used in this study was essentially determined

using NCCLS 27A method (Martins and Rex, 1997; Rex *et al.*, 1997; Espinell-Ingroff *et al.*, 1998) by modified microdilution test (Talibi and Raymond, 1999) in YEPD. Cells were grown for 48 h at 30°C to obtain single colonies which were resuspended in a 0.9 % normal saline solution to give an optical density at 600nm (OD_{600}) of 0.1. The cells were then diluted 100 folds in 0.9% saline. The diluted cell suspensions were added to round bottomed 96-well microtiter plates (100 µl/well) in wells containing equal volumes of medium (100 µl/well) with different concentrations of drugs. Drug free control was also included. The plates were incubated at 30^0C for 48 h. The Minimum inhibitory concentration (MIC) end point results were evaluated both visually and by monitoring the cell density at OD_{620} in a micro-plate reader (Bio-Rad model 550, USA) set at 620 nm. MIC_{80} was recorded as the lowest concentration of the drug, which gave > 80 % inhibition of growth compared to that of the drug-free growth control. Figure 22 depicts the scheme for microdilution based susceptibility testing.



b) Visual scoring of growth

0	Optically clear (no turbidity)
1	Slightly hazy (some turbidity)
2	prominent decrease in turbidity
3	slight reduction in turbidity
4	no reduction in turbidity

Figure 22. Antifungal susceptibility testing.
(A) Scheme: From a stock solution of drug, doubling dilutions of drugs are prepared in RPMI or YEPD media [see *'Methods'*] to which 0.1 ml of inoculum is added at a final concentration of 2×10^3 cells/ ml. Well 12 of each row is the growth control and contain inoculum without drug. Column 11 has no drug/ no inoculum and represents the medium control. **(B)** The MIC may be determined by the eye by scoring turbidity or may be checked spectrophotometrically at A_{619} using a microtiter plate scanner.

2.3 STRAINS, PLASMIDS AND CULTURE MEDIA

Escherichia coli DH-5α was used as a host for plasmid constructions and propagation. *E. coli* was cultured in LB (Luria-Bertani) broth or on LB plates, supplemented with ampicillin (100 µg/ml) for the transformants. All the bacterial strains were grown at

37°C. *C. albicans* strains used in this study were maintained on YEPD. When grown on solid media, 2.5% (*w/v*) bacto-agar was added to the media. The detailed composition of the respective media used is given in Appendix I. All strains were stored as frozen stocks with 15% glycerol at -80°C. Before each experiment, *C. albicans* strains were freshly revived on YEPD plates from corresponding glycerol stock. All the yeast strains were grown at 30°C. Tables 9 and 10 show a complete list of all the plasmids and strains used in this study.

2.4 OLIGONUCLEOTIDES

Oligonucleotides used for probe preparation (Southern/Northern blotting), Sequencing, Site directed mutagenesis, DNA-EMSA and for making different clones were commercially custom synthesized from Sigma-Aldrich Company, USA. Table 8 shows a complete list of all the oligonucleotides used in this study.

2.5 MOLECULAR BIOLOGY METHODS

2.5.1 Preparation of gene fragments and gene probes

Genomic DNA from *C. albicans* isolates was extracted as described ahead and used as a template for amplification of the gene fragments to be used for cloning and probe purpose. PCR was carried out with a high-fidelity *Pfu* DNA polymerase with the gene specific primers (Listed in Table 8). Amplicons were purified with a QIA quick PCR purification/Gel elution kit (Qiagen, Germany) and were used subsequently as per their requirement.

2.5.2 Plasmid constructions

The recombinant transcriptional/translational fusion constructs harboring *GFP/lacZ* reporter under control of the *CDR1* promoter for the integration into the genome of clinical *C. albicans* isolates were constructed as follow:

2.5.2.1 Construction of pCPG1: Substitution of a *C. albicans* adapted *GFP* reporter (Morschhäuser *et al.,* 1998) for the *lacZ* reporter in pCPL1 (*described ahead*) generated pCPG1. The *GFP* reporter gene down-stream to the transcriptional control of *CDR1* promoter was cloned in pCPL1 by exploiting convenient restriction site. A 5.4 Kb vector back bone having *CDR1* promoter was taken out by digesting vector pCPL1 with

Table 8. List of the primers used for PCR amplification of gene specific regions.

S. No.	PRIMERS	DIRECTION	SEQUENCE (5'--------3') [a]
CDR1 ORF primers for probe amplification			
1.	**KM1**	F	CTTTTCCACTGGTAACTACT
2.	**KM2**	R	CAATAAACCTGCTGACGAG
CDR1 promoter primers			
3.	**CDR1F**	F	*GATC*GGGCCCTCGTTACTCAATAAGTAT
4.	**CDR1R**	R	*AGCT*CTCGAGTTCTTTTTGACCTTTTAAAG
5.	**CDR1F-RML**	F	GCTTCGCCTCAACTTCTTATAAAGTTTTGAAAG
6.	**CDR1R-RML**	R	CGTGGTATTCATTAATGGAATGGTTTTCTGAAG
CDR1 downstream region primers			
7.	**CDR29**	F	*AATT*CTGCAGTTTGTTTTTTGACATGGTGGTATC
8.	**CDR30**	R	*TCGTG*CCGCGGAACACTTTTTCTTTCTAATTATAA
9.	**CDR32** (CT-CDR1-F)	F	*ATTT*GGTACCATACATTAAATTTGCTGGTGGG
10.	**CDR31**	R	*GTTT*GGATCCTTTCTTATTTTTTTTCTCTCTGTTACCC
11.	**CT-CDR1-R**	R	*GTTT*CTCGAGTTTCTTATTTTTTTTCTCTCTGTTACCC
Sequencing primers			
12.	**T7**	F	TAATACGACTCACTATAGGG
13.	**SP6**	F	ATTTAGGTGACACTATAG
14.	***lacZ*-F-RML**	F	GCATTCGAATTCAGTGCTTTGCG
15.	***lacZ* -R-RML**	R	ACTGTATCCTACGAAGTTACCATTGACCC
16.	**T_{ACT1}-F-RML**	F	CTGGAAATCTGGAAATCTGG
Site directed mutagenesis primers			
17.	**A-del pCPL52**	F	GAGAGAAAAAAAAATAAGAAACTCGAGATGAACATGACTGA AAAAATTCAAAC

18.	**A-del pCPL52**	R	GTTTGAATTTTTTCAGTCATGTTCATCTCGAGTTTCTTATTTTTTTTCTCTC
19.	**A-*Xho*I del pCPL52**	F	GTAACAGAGAGAAAAAAAATAAGAAAATGAACATGACTGAAAAAATTCAAAC
20.	**A-*Xho*I del pCPL52**	R	GTTTGAATTTTTTCAGTCATGTTCATTTTCTTATTTTTTTTCTCTCTGTTAC
21.	***lacZ*-F-PacI**	F	GATTGAACCACTAAATTAGTTAATTAAGGATCTAAATTCTGGAAATCTGGAAATCTGG
22.	***lacZ*-R-*Pac*I**	R	CCAGATTTCCAGATTTCCAGAATTTAGATCCTTAATTAACTAATTTAGTGGTTCAATC
23.	***lacZ*-F-*Mlu*I**	F	GATTCATCCCATTCATTCCATCATAAAATACGCGTCGTCAAAACTAGAGAATAATAAAG
24.	***lacZ*-R-*Mlu*I**	R	CTTTATTATTCTCTAGTTTTGACGACGCGTATTTTATGATGGAATGAATGGGATGAATC
RT-PCR primers for 3'-RACE of CDR1			
25.	**CT-CDR1-F**	F	*ATTT*GGTACCATACATTAAATTTGCTGGTGGG
26.	**CT-CDR1-R**	R	*GTTT*CTCGAGTTTCTTATTTTTTTTCTCTCTGTTACCC
27.	**Oligo(dT)$_{18}$ AP**	R	*GATCAAA*-CTCGAG-CCGCGG-TCTAGATTTTTTTTTTTTTTTTTTG[b]
28.	**AP-RML**	R	*GATCAAA*CTCGAG-CCGCGG-TCTAGA
29.	**Oligo(dC$_9$T$_6$) AP**	R	*GATCAAA*CTCGAG-CCGCGG-TCTAGACCCCCCCCCTTTTTT
CDR1 3'UTR primers			
30.	**Gu4/DSY294/296-UTR-F-*Pac*I**	F	*GATC*TTAATTAAATTAAACAGTTTGTTTTTTGACATGG
31.	**Gu5-UTR-F-*Pac*I**	F	*GATC*TTAATTAAATTTAAACAGTTTGTCTTTTGACATGG
32.	**Gu4/DSY296-UTR-R-*Mlu*I**	R	*GATC*ACGCGTAAAAACAGGTATAAATATATTTATG
33.	**Gu5/DSY294-UTR-R-*Mlu*I**	F	*GATC*ACGCGTCAAATTACTAATATTATTAATATTAAAAAAC
ACT1 specific primers			
34.	**ACT23**	F	GACATAACAATGGTACCGTATAATTC
35.	**ACT34**	R	CGGACGATATGGGCCCAATCTGGCATCAC
36.	**CT-ACT1-F**	F	GTTAGAAAAGAATTATACGG

37.	**CT-ACT1-R**	R	GAAACATTTGTGGTGAACAATGG

[a] Restriction sites introduced into the primers are underlined, while the flanking bases are written in italics.

[b] Oligo$(dT)_{18}$ AP primer possessing an additional and non specific nucleotide at 3'-end is written in bold.

Table 9. List of plasmids used in this study.

S. No.	PLASMIDS	DESCRIPTION	SOURCE
1.	**pBS-KS (+)**	Plasmid Bluescript II KS+ vector	Stratagene
2.	**pGEM®T-Easy**	Plasmid backbone used for T/A DNA cloning purpose	Promega
3.	**pUC18**	Small, high copy number plasmid	MBI, Fermentas
4.	**pS12-35**	Plasmid harboring the whole *CDR1* gene	Prasad *et al.* (1995)
5.	**pACT1**	Plasmid harboring *ACT1* gene of *S. cerevisisae*	Gift by Anand Bachhawat
6.	**pSAT1**	Plasmid harboring *caSAT1* marker	Dr. Joachim Morschhäuser, Germany
7.	**pMPG2**	Plasmid harboring P_{MDR1}-*GFP* fusion for *ACT1* locus integration	Dr. Joachim Morschhäuser, Germany
8.	**pLACZ6**	Plasmid harboring *lacZ* reporter gene	Dr. Joachim Morschhäuser, Germany
9.	**pADH1G3**	Plasmid harboring *GFP* reporter and *caSAT1* marker	Dr. Joachim Morschhäuser, Germany
10.	**pCPG1**	Plasmid harboring P_{CDR1}-GFP fusion for ACT1 locus integration	Gaur *et al.* (2005)
11.	**pCPG3**	Plasmid harboring P_{CDR1}-GFP fusion for *CDR1* locus integration	This study
12.	**pCPG2**	Plasmid harboring *CDR1*-GFP fusion for *CDR1* locus integration	This study
13.	**pCPL1**	Plasmid harboring P_{CDR1}-*lacZ* fusion for *ACT1* locus integration (SC5314 P_{CDR1})	Gaur *et al.* (2005)
14.	**pCPL4**	Plasmid harboring P_{CDR1}-*lacZ* fusion for *ACT1* locus integration (Gu4 P_{CDR1})	Gaur *et al.* (2005)
15.	**pCPL5**	Plasmid harboring P_{CDR1}-*lacZ* fusion for ACT1 locus integration (Gu5 P_{CDR1})	Gaur *et al.* (2005)
16.	**pCPL51**	Plasmid harboring P_{CDR1}- *lacZ* fusion for *CDR1* locus integration	This study
17.	**pCPL52**	Plasmid harboring *CDR1-lacZ* fusion for *CDR1* locus integration	This study
18.	**pCPL62**	Plasmid harboring *CDR1-lacZ* fusion for *CDR1* locus integration ('A' deleted)	This study
19.	**pCPL62**	Plasmid harboring *CDR1-lacZ* fusion for *CDR1* locus integration ('A + *Xho*I' deleted)	This study
20.	**Gu4-3'UTR-RT**	Plasmid harboring 3'RACE product of Gu4 isolate	This study
21.	**Gu5-3'UTR-RT**	Plasmid harboring 3'RACE product of Gu5 isolate	This study
22.	**DSY294-3'UTR-RT**	Plasmid harboring 3'RACE product of DSY294 isolate	This study
23.	**DSY296-3'UTR-RT**	Plasmid harboring 3'RACE product of DSY296 isolate	This study
24.	**Gu4-3'UTR**	Plasmid harboring *CDR1* 3'UTR of Gu4 isolate	This study
25.	**Gu5-3'UTR**	Plasmid harboring *CDR1* 3'UTR of Gu5 isolate	This study
26.	**DSY294-3'UTR**	Plasmid harboring *CDR1* 3'UTR of DSY294 isolate	This study

27.	**DSY296-3'UTR**	Plasmid harboring *CDR1* 3'UTR of DSY296 isolate	This study
28.	**pCPL53**	Plasmid harboring P_{CDR1}-*lacZ*-T_{ACT1} with *PacI* site introduced	This study
29.	**pCPL54**	Plasmid harboring P_{CDR1}-*lacZ*- T_{ACT1} with *PacI*/*Mlu*I sites introduced	This study
30.	**pCPL54-Gu4-3'UTR**	Plasmid harboring P_{CDR1}-*lacZ-CDR1* 3'UTR of Gu4 isolate	This study
31.	**pCPL54-Gu5-3'UTR**	Plasmid harboring P_{CDR1}-*lacZ-CDR1* 3'UTR of Gu5 isolate	This study
32.	**pCPL54-DSY294-3'UTR**	Plasmid harboring P_{CDR1}-*lacZ-CDR1* 3'UTR of DSY294 isolate	This study
33.	**pCPL54-DSY296-3'UTR**	Plasmid harboring P_{CDR1}-*lacZ-CDR1* 3'UTR of DSY296 isolate	This study
34.	**pGu4-poly'A'**	Plasmid harboring poly 'A-tailed' RT-PCR product of Gu4 isolate	This study
35.	**pGu5-poly'A'**	Plasmid harboring poly 'A-tailed' RT-PCR product of Gu5 isolate	This study
36.	**pDSY294-poly'A'**	Plasmid harboring poly 'A-tailed' RT-PCR product of DSY294 isolate	This study
37.	**pDSY296-poly'A'**	Plasmid harboring poly 'A-tailed' RT-PCR product of DSY296 isolate	This study
38.	**pGu4-FPZ-poly'A'**	Plasmid harboring fluphenazine inducible poly 'A-tailed' RT-PCR product of Gu4 isolate	This study
39.	**pGu4-Pro-poly'A'**	Plasmid harboring progesterone inducible poly 'A-tailed' RT-PCR product of Gu4 isolate	This study
40.	**pGu4-ß-Est-poly'A'**	Plasmid harboring ß-estradiol inducible poly 'A-tailed' RT-PCR product of Gu4 isolate	This study
41.	**pDSY294-FPZ-poly'A'**	Plasmid harboring fluphenazine inducible poly 'A-tailed' RT-PCR product of DSY294 isolate	This study
42.	**pDSY294-Pro-poly'A'**	Plasmid harboring progesterone inducible poly 'A-tailed' RT-PCR product of DSY294 isolate	This study
43.	**pDSY294-ß-Est-poly'A'**	Plasmid harboring ß-estradiol inducible poly 'A-tailed' RT-PCR product of DSY294 isolate	This study

Table 10. List of *C. albicans* strains used in this study.

S. No.	STRAIN	GENOTYPE / DESCRIPTION	SOURCE
1.	**SC5314**	*URA3/URA3*	ATCC
2.	**Gu4**	Fluconazole-Susceptible Clinical isolate	Dr. Joachim Morschhäuser, Germany
3.	**Gu4G1**	P_{CDR1}-GFP integrated at *CDR1* locus	This study
4.	**Gu4G2**	P_{CDR1}-*CDR1*-GFP integrated at *CDR1* locus	This study
5.	**Gu4L1**	P_{CDR1}-*lacZ* integrated at *ACT1* locus	This study
6.	**Gu4L2**	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
7.	**Gu4L3**	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study
8.	**Gu5**	Fluconazole-Resistant Clinical isolate	Dr. Joachim Morschhäuser, Germany
9.	**Gu5G1**	P_{CDR1}-GFP integrated at *CDR1* locus	This study
10.	**Gu5G2**	P_{CDR1}-*CDR1*-GFP integrated at *CDR1* locus	This study
11.	**Gu5L1**	P_{CDR1}-*lacZ* integrated at *ACT1* locus	This study
12.	**Gu5L2**	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
13.	**Gu5L3**	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study
14.	**DSY294**	Fluconazole-Susceptible Clinical isolate	Dr. Dominique Sanglard, Switzerland
15.	**DSY294G1**	P_{CDR1}-GFP integrated at *CDR1* locus	This study
16.	**DSY294G2**	P_{CDR1}-*CDR1*-GFP integrated at *CDR1* locus	This study
17.	**DSY294L1**	P_{CDR1}-*lacZ* integrated at *ACT1* locus	This study
18.	**DSY294L2**	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
19.	**DSY294L3**	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study
20.	**DSY296**	Fluconazole-Resistant Clinical isolate	Dr. Dominique Sanglard, Switzerland
21.	**DSY296G1**	P_{CDR1}-GFP integrated at *CDR1* locus	This study
22.	**DSY294G2**	P_{CDR1}-*CDR1*-GFP integrated at *CDR1* locus	This study
23.	**DSY296L1**	P_{CDR1}-*lacZ* integrated at *ACT1* locus	This study

24.	**DSY296L2**	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
25.	**DSY296L3**	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study
26.	**Gu4L2-CUN**	P_{CDR1}-*lacZ* -*CDR1* 3'UTR^N integrated at *CDR1* locus	This study
27.	**Gu4L2-CUS**	P_{CDR1}-*lacZ* -*CDR1* 3'UTR^S integrated at *CDR1* locus	This study
28.	**Gu5L2-CUN**	P_{CDR1}-*lacZ* -*CDR1* 3'UTR^N integrated at *CDR1* locus	This study
29.	**Gu5L2-CUS**	P_{CDR1}-*lacZ* -*CDR1* 3'UTR^S integrated at *CDR1* locus	This study
30.	**DSY294L2-CUN**	P_{CDR1}-*lacZ* -*CDR1* 3' UTR^N integrated at *CDR1* locus	This study
31.	**DSY294L2-CUS**	P_{CDR1}-*lacZ* -*CDR1* 3'UTR^S integrated at *CDR1* locus	This study
32.	**DSY296L2-CUN**	P_{CDR1}-*lacZ* -*CDR1* 3'UTR^N integrated at *CDR1* locus	This study
33.	**DSY296L2-CUS**	P_{CDR1}-*lacZ* -*CDR1* 3'UTR^S integrated at *CDR1* locus	This study

The superscript 'N' and 'S' in designated constructs stand for Native and Swapped *CDR1* 3'UTR respectively.

*Xho*I/*Pst*I, C-terminal *GFP* having *ACT1* termination and *SAT1* gene (~3.0 Kb) was excised by digesting vector pADH1G3 with *Nco*I/*Pst*I and ~250 bps of N-terminal *GFP* was obtained from pGFP68 by *Sal*I/*Nco*I digestion. The N-terminal *GFP* fragment, C-terminal *GFP* with *SAT1* gene and the *Xho*I/*Pst*I digested vector backbone was ligated to generate the final clone pCPG1 (Gaur *et al.*, 2005). *Kpn*I-*Sac*II linearized, gel-purified fragment containing the P_{CDR1}-*GFP* fusion, were used to transform the clinical *C. albicans* isolates. The flanking *ACT1* sequences served for genomic integration of the transcriptional reporter fusion at the *ACT1* locus and the dominant *caSAT1* marker (Reuss *et al.*, 2004) was used to select nourseothricin-resistant (Nou^R) transformants.

2.5.2.2 Construction of pCPG2: Plasmid pCPG2, which contains the *GFP* reporter gene fused in translational frame with the last codon of the *CDR1* ORF was constructed as follows. The 250 bp of C-terminal region of *CDR1* was amplified from SC5314 genomic DNA with the primers CDR32 and CDR31, digested at the introduced *Kpn*I and *Bam*HI sites, and ligated together with a *Bam*HI-*Pst*I fragment from pADH1G3 (Hiller *et al.*, 2006) containing *GFP*, the *ACT1* transcription termination sequence, and the *caSAT1* marker into the *Kpn*I/*Pst*I digested (2.9 kb) vector backbone of pCPL51 to generate the final clone pCPG2 (6.5 kb). *Kpn*I-*Sac*II linearized, gel-purified fragment (CT-*CDR1*-*GFP*-Ca*SAT1*-3'UTR) (*see* Figure 25), were used to transform the clinical *C. albicans* isolates. The flanking *CDR1* sequences in all these plasmids served for genomic integration of the reporter fusions at the native *CDR1* locus and the dominant *caSAT1* marker (Reuss *et al.*, 2004) was used to select nourseothricin-resistant (Nou^R) transformants.

2.5.2.3 Construction of pCPG3: To integrate the P_{CDR1}-*GFP* transcriptional fusion at *CDR1* locus, vector pCPG3 was constructed. A *CDR1* downstream fragment was then amplified from genomic DNA of strain SC5314 with the primers with the *Pst*I/*Sac*II digested plasmid vectors pCPG1 (*described above*) to generate pCPG3. CDR29 and CDR30. The PCR product was digested at the introduced *Pst*I and *Sac*II sites and ligated *Apa*I-*Sac*II linearized, gel-purified fragment containing the P_{CDR1}-*GFP* fusion (*see* Figure 25), was used to transform the clinical *C. albicans* isolates. The flanking *CDR1* sequences in all these plasmids served for genomic integration of the reporter fusions at the native *CDR1* locus and the dominant *caSAT1* marker (Reuss *et al.*, 2004) was used to select nourseothricin-resistant (Nou^R) transformants.

2.5.2.4 Construction of pCPL1: For construction of pCPL1, an *Xho*I-*Pst*I fragment from plasmid pMEP2LACZ2 (Biswas and Morschhäuser, 2005) containing the *Streptococcus*

thermophilus lacZ gene (Uhl and Johnson, 2001), the transcription termination sequence of the *C. albicans ACT1* gene, and the *URA3* selection marker was first ligated between flanking *ACT1* sequences of plasmid pMPG2 (Hiller *et al.*, 2006) to obtain pLACZ6. An *Xho*I-*Pst*I fragment containing the *caSAT1* selection marker (Reuss *et al.*, 2004) was then substituted for the *Sal*I-*Pst*I fragment containing the *URA3* marker to generate pLACZ7. A *Kpn*I-*Apa*I fragment containing 0.6 kb of the *ACT1* coding region was PCR amplified from genomic DNA of *C. albicans* strain SC5314 with the primers ACT23 (*Kpn*I and *Apa*I sites introduced into the primers during synthesis, Table 8). In addition, an *Apa*I–*Xho*I fragment containing 1196 bp of *CDR1* upstream sequences was amplified from SC5314 genomic DNA with the primers CDR1F and CDR1R (sites introduced into the primers during synthesis, Table 8). The *ACT1* fragment and the *CDR1* promoter fragment were ligated together into the *Kpn*I/*Xho*I digested vector pLACZ7 to generate pCPL1, which contains the *lacZ* reporter gene under the transcriptional control of the *CDR1* promoter (Gaur *et al.*, 2005). *Kpn*I-*Sac*II linearized, gel-purified fragment containing the P_{CDR1}-*lacZ* fusion, were used to transform the clinical *C. albicans* isolates. The flanking *ACT1* sequences served for genomic integration of the transcriptional reporter fusion at the *ACT1* locus and the dominant *caSAT1* marker (Reuss *et al.*, 2004) was used to select nourseothricin-resistant (Nou^R) transformants.

2.5.2.5 Construction of pCPL4/pCPL5: A 1196 bp *CDR1* promoter fragment was first PCR amplified from the genomic DNA of isolates Gu4/Gu5 with the primers CDR1F and CDR1R digested at the introduced *Apa*I and *Xho*I sites, 600 bp of 3'*ACT1* sequence was also amplified from Gu5 genomic DNA by using ACT23 and ACT34 primers and digested with *Kpn*I/*Apa*I restriction enzymes (sites introduced into the primers during synthesis), and ligated with *Kpn*I/*Xho*I digested pCPL1 to generate the clone pCPL5. *Kpn*I-*Sac*II linearized, gel-purified fragment containing the P_{CDR1}-*lacZ* fusion (*see* Figure 31), were used to transform the clinical *C. albicans* isolates. The flanking *ACT1* sequences served for genomic integration of the transcriptional reporter fusion at the *ACT1* locus and the dominant *caSAT1* marker (Reuss *et al.*, 2004) was used to select nourseothricin-resistant (Nou^R) transformants. Notably, plasmid pCPL1/pCPG1 harbors the *CDR1* promoter isolated from SC5314 genomic DNA.

2.5.2.6 Construction of pCPL51: To integrate the P_{CDR1}-*lacZ* transcriptional fusion at *CDR1* locus, vector pCPL51 was constructed. A *CDR1* downstream fragment was then amplified from genomic DNA of strain SC5314 with the primers CDR29 and CDR30. The PCR product was digested at the introduced *Pst*I and *Sac*II sites and ligated with the

*Pst*I/*Sac*II digested plasmid vectors pCPL5 (*described above*) to generate pCPL51. *Apa*I-*Sac*II linearized, gel-purified fragment containing the P_{CDR1}-*lacZ* fusion (*see* Figure 28), was used to transform the clinical *C. albicans* isolates. The flanking *CDR1* sequences in all these plasmids served for genomic integration of the reporter fusions at the native *CDR1* locus and the dominant *caSAT1* marker (Reuss *et al.*, 2004) was used to select nourseothricin-resistant (Nou^R) transformants.

2.5.2.7 Construction of pCPL52/pCPL62: To generate pCPL52, in which the *lacZ* reporter gene instead of *GFP* is fused in translational frame to the *CDR1* ORF, the C-terminal part of *CDR1* was PCR amplified from SC5314 genomic DNA with the primers CDR32 and CT-*CDR1*-R-RML, digested at the introduced *Kpn*I and *Xho*I sites, and ligated into the *Kpn*I/*Xho*I digested pCPL51 (*described above*). To ensure in frame translational fusion of the *CDR1* ORF with *lacZ*, pCPL62 was generated in which an additonal 'A' in pCPL52 before the *lacZ* ATG, was removed by site directed mutagenesis employing A-del pCPL52-F and A-del pCPL52-R primers. *Kpn*I-*Sac*II linearized, gel-purified fragment (CT-*CDR1*-*lacZ*-Ca*SAT1*-3'UTR, *see* Figure 28) were used to transform the clinical *C. albicans* isolates. The flanking *CDR1* sequences in all these plasmids served for genomic integration of the reporter fusions at the native *CDR1* locus and the dominant *caSAT1* marker (Reuss *et al.*, 2004) was used to select nourseothricin-resistant (Nou^R) transformants.

2.5.2.8 Construction of pCPL53/pCPL54: Plasmid pCPL51 (*described above*) harboring P_{CDR1}-*lacZ* was used for cloning of mapped *CDR1* 3'UTR in transcriptional fusion with heterologous *lacZ* reporter. For this purpose, sequences at the junctions of *lacZ*-T_{ACT1} and T_{ACT1}-*caSAT1* were mutated sequentially by introducing *Pac*I and *Mlu*I sites at these corresponding junctions (*lacZ*-T_{ACT1} and T_{ACT1}-*caSAT1*) using *lacZ*-F-*Pac*I-RML/*lacZ*-R-*Pac*I-RML and *lacZ*-F-*Mlu*I-RML/*lacZ*-R-*Mlu*I-RML primers (*see* Table 8) by QuikChange Site directed mutagenesis system (Stratagene, La Jolla, Calif.) to generate the plasmid pCPL53-RML (with *Pac*I site only) and pCPL54-RML (with both *Pac*I and *Mlu*I sites) respectively. Mapped *CDR1* 3'UTR fragment of each isolate was excised from their corresponding 3'UTR harboring pGEM® T-Easy vector clones (described ahead, *see* Table 8), by digestion at *Pac*I and *Mlu*I sites (sites introduced in primers during the synthesis), and further subcloned in *Pac*I/*Mlu*I digested pCPL54-RML to generate pCPL54-Gu4/Gu5-3'UTR-RML and pCPL54-DSY294/DSY296-3'UTR-RML (*see* Table 8). *Apa*I-*Sac*II linearized, gel-purified fragment containing the P_{CDR1}-*lacZ-CDR1* 3'UTR reporter fusion (*see* Figure 45), were used to transform the clinical *C.*

albicans isolates. The flanking *CDR1* sequences in these plasmids served for genomic integration of the transcriptional reporter fusions at the native *CDR1* locus, and the dominant *caSAT1* marker (Reuss *et al.*, 2004) was used to select nourseothricin-resistant (Nou^R) transformants. All constructs were confirmed by appropriate restriction digestion analysis as well as by sequencing.

2.5.3 DNA sequencing

Sequencing reaction: For denatured template preparation, 1.2 pmole of plasmid DNA (10 µg) was taken and 10 µl sterile water was added to make the volume to 20 µl. To this, 2 µl of 2 M NaOH and 1 µl of 4 mM EDTA was added and the mix was incubated at 37°C for 30 min. After the incubation, 3 µl of sodium acetate (pH 7.2), 7 µl of sterile water and 75 µl of 100 % ethanol was added and the contents were mixed by short centrifugation and kept at -20°C for overnight. The mix was centrifuged the next day at 15,000 rpm for 15 min and after decanting the supernatant the DNA pellet was washed with 200 µl of 70% ethanol. The pellet was dried in the speedvac after the wash. For annealing reaction, 1 pm of the primer, 2 µl of the reaction buffer and 7 µl of sterile water was added to the denatured DNA template. This was incubated at 80°C for 5 min and then at 37°C for 10 min for annealing the primer with the DNA template. For labeling reaction, a mix of 1 µl of 0.1 M DTT, 0.5 µl (10 µCi/µl) [α-^{32}P]dATP, 0.25 µl dGTP labeling mix, 1.75 µl dilution buffer and 0.25 µl of sequenase enzyme was prepared, mixed with the annealed DNA and incubated at 25°C for 5 min. For termination reaction, eppendorf tubes were labeled as G, A, T and C and kept in ice before adding the termination mix. 2.5 µl of ddGTP, 2.5 µl of ddATP, 2.5 µl of ddTTP and 2.5 µl of ddCTP were added to the respective labeled tubes. The tubes were prewarmed at 37°C for 2 min. 3.5 µl of labeling reaction was added to each tube and the tubes were incubated at 37°C for 5 min. To stop the reaction 4 µl of stop solution was added. The mix could be stored at -20°C till further use. The samples were heated at 90° C and chilled on ice before loading them on a sequencing gel.

2.5.4 Bacterial miniprep plasmid DNA isolation

3 ml of bacterial culture (harboring the desired plasmid) was harvested at 6,000 rpm for 5 min. Plasmid DNA was isolated using alkaline lysis method (0.2N NaOH and 1.0% SDS)

(Sambrook *et al.,* 1989). Contaminating proteins and *E.coli* genomic DNA were removed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1) twice. The DNA was precipitated with 2.5 volumes of chilled ethanol plus one-tenth volume sodium acetate (pH 5.2). Precipitated DNA pellet was subjected to 70% ethanol wash and then dried under vacuum and finally resuspended in 50 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA[pH 8.0]).

2.5.5 Yeast genomic DNA isolation

2.5.5.1 Protocol I. Yeast genomic DNA which was to be used as PCR template was prepared from overnight cultures of clinical *C. albicans* isolates grown in 10 ml of YEPD for 12-16 hours, harvested at 3000 rpm for 5 min and were resuspended in 1 ml of SOE (1 M Sorbitol and 0.1 M EDTA). SOE was then added to the cells to make final volume 500 µl and 20 µl of 10mg/ml solution of Zymolyase 100T was added and the tubes incubated at 37°C. The tubes were then turned upside down gently after every 15 min. The contents of the tube were then centrifuged at 2000 rpm for 1 min until the cells were just pelleted. The supernatant was discarded and the cells were resuspended by vortexing thoroughly for 1 min in 500 µl of 50 mM Tris, 20mM EDTA mix. 50 µl of 10 % SDS was added to the above suspension and incubated at 65°C for 30 min. Then, 200 µl of cold 5 M potassium acetate was added and the tubes were chilled on ice for 60 min after vortexing to mix the contents. After incubation on ice the cell suspension was centrifuged for 5 min at 4°C. The supernatant was recovered in fresh tubes after centrifugation and 1 volume of isopropanol was added. The tubes were inverted several times to get a pellet of DNA. Eventually centrifuging briefly pelleted the DNA down. The pellet was semi-dried in a speed vac and resuspended in 150µl of $T_{10}E_1$. The tubes were kept at 65°C till the DNA dissolved completely. 1.5µl of RNase A solution (10 mg/ml) was added and the tubes were incubated at 37°C for 30 min. The tubes were chilled on ice and then centrifuged for 5 min at 4°C. The supernatant was transferred to fresh tubes and the DNA was precipitated with 2.5 volumes of ethanol and 1/10 volumes of 3 M sodium acetate (pH 5.2). The DNA pellet was washed with 70% ethanol and dried in a speedvac. The DNA pellet was finally resuspended in 100-200 µl of $T_{10}E_1$ (10 mM Tris and 1 mM EDTA; pH 8.0) after the RNase treatment with subsequent phenol chloroform step. The DNA was stored in aliquots at -20°C until further use.

2.5.5.2 Protocol II (Miniprep). Yeast Genomic DNA was isolated as described by Hoffman and Winston (1987) with certain modifications. Yeast cultures (Wild type/ transformants) were grown overnight to saturation in 10 ml of YEPD media at 30°C. Cells were pellet down at 1,500 rpm for 2 min, resuspended in 0.5 ml of sterile distilled water and transfered to 1.5 ml tube. Again cells were pellet down at maximum speed (14,000 rpm) for 5 sec. Supernatant was discarded and pellet was briefly vortexed in the residual water. In the following order, 200 µl of yeast lysis buffer (Appendix III), 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3 g of acid-washed glass beads (Sigma) were added. Cells were lysed on cell disruptor for 10 min and spin for 5 min in a microfuge at 10,000 rpm at 4°C. Upper aqueous phase was transferred to a fresh 2 ml tube and 10 µl of 4M ammonium acetate and 1.5 ml of 100% ethanol was added, and mixed by inversion. The DNA was allowed to precipitate for 5 minutes at room temp and again spin at maximum speed (14,000 rpm) for 5 min in a microfuge at 4°C. Supernatant was discarded and genomic DNA pellet was washed twice with 1ml of 70% ethanol. Pellet was semidried in a vacuum dryer for 5 min at 60°C and resuspended in 100 µl of $T_{10}E_1$ (10 mM Tris, 1 mM EDTA [pH 8.0]). 5 µl of 10 mg/ml RNase A (Sigma) was added and incubated at 37°C until the pellet is completly dissolved. Occasionally cellular debris remains in the samples, but it does not interfere with subsequent analyses. Expected genomic DNA yield ranges from 0.5-1 µg per µl of sample. Approximately 15-20 µl of this DNA was used for Southern analysis.

2.5.6 Yeast transformation procedures

2.5.6.1 LiAc method: The primary cultures of clinical *C. albicans* isolates were grown in 10 ml of YEPD medium overnight. From the saturated culture an aliquot was inoculated in 50 ml YEPD to an initial OD_{600} of 0.4. The culture was allowed to grow till an OD_{600} of 1.6 was reached. After the required OD_{600} was reached, the cells were harvested in a sterile 50 ml centrifuge tube at 5000 rpm for 5 min. The cells were resuspended in 1 ml of 100 mM LiAc (lithium acetate) and were transferred to a 1.5 ml eppendorf tube. The cells were pelleted at top speed and LiAc was removed with a micropipette. Finally, the cells were resuspended to a final volume of 500 µl in 100 mM LiAc. This cell suspension was vortexed and 50 µl samples were transferred to labeled microfuge tubes. After pelleting the cells and removing the LiAc, the basic transformation mix components were

added in the given order: 240 µl PEG (50% *w/v*), 36 µl 1M LiAc, 15 µl of 10 mg/ml of salmon sperm DNA (denatured) + 65 µl of DNA (1-4 µg of linearized DNA fragment). The contents of the tube were mixed vigorously until the cell pellet had been mixed completely (usually takes about a minute) and was incubated at 30°C for 30 min. Heat shock was given to the cells at 42°C for 25 min after which the cells were centrifuged at 6000 rpm for 15s. The transformation mix was removed with pipette and the cells were resuspended in 1 ml of YEPD and allow it to grow at 30°C for 4-5 h. The cells were dissolved in minimum volume of YEPD and plated on the YEPD agar plates containing 200 µg/ml of nourseothricin for selection. Next day the colonies were streaked on YEPD plates containing 100 µg/ml of nourseothricin and also inoculated for genomic DNA isolation.

2.5.6.2 Electroporation: The cells were grown in 10 ml of YEPD media over night at 30°C for primary culture. For secondary, 1 and 5 µl of primary culture was inoculated in 50 ml of YEPD media and allow it to grow for 14 h (mid-log phase) and then harvested at 3000 rpm for 5 min after checking the OD_{600} (1.6-1.8) of the cells. The pellet was resuspended in 8 ml of ice cold sterile MQ water, 1 ml of 10× $T_{10}E_1$ (10mM Tris and 1 mM EDTA; pH 8.0) and 1ml of 1M LiAc. The cells were kept at 30°C for 1h shaking and then added 250 µl of 1 M DTT and kept it again at 30°C for 30 min in a shaking incubator. The cells were washed with ice cold MQ water twice at 3000 rpm for 5 min. The pellet was resuspended in 25 ml of 1M sorbitol and after centrifugation the supernatant was discarded and the pellet was dissolved in minimum volume of sorbitol left in the tube. 40µl of the electrocompetent cells were taken for electroporation using Biorad Genepulser XL^{TM} in 0.2 cm cuvette and mixed with the 5 µl (approximately 1 µg) linearized gel eluted DNA. The cells were dissolved in 1 ml of 1M sorbitol after giving electric pulse at 1.5 kV (Reuss *et al.*, 2004). After centrifugation the cells were dissolved in 1ml of YEPD and allow it to grow at 30°C for 4-5 h. The cells were dissolved in minimum volume of YEPD and plated on the YEPD agar plates containing 200 µg/ml of nourseothricin for selection (Reuss *et al.*, 2004). Nourseothricin resistant (Nou^{R}) transformants were picked and streaked on YEPD plates containing 100 µg/ml of nourseothricin and also inoculated for genomic DNA isolation. Single copy integration of each construct at the desired locus was confirmed by Southern hybridization with gene specific probes.

2.5.7 Southern blot hybridization

5-10 μg of genomic DNA or total yeast DNA was digested with the appropriate enzyme (usually 10-20 U of enzyme) and loaded on 1 % agarose gel (Tris-acetate/ EDTA buffer). For the transfer of DNA from the agarose gel to the membrane, the gel was treated with 250 mM HCl for 10-15 min and then rinsed with water. It was then treated with denaturation solution (0.5 N NaOH, 1.5 M NaCl) twice for 15 min with gentle shaking and then rinsed with water. The gel was then neutralized in neutralization solution (0.5 M Tris-HCl, [pH 7.5], 3M NaCl). The DNA was blotted overnight from the gel by capillary transfer to the nylon membrane (Hybond-N^+, Amersham), using 20×SSC buffer. The DNA was UV crosslinked to the membrane as per the manufacturer's instructions and used for pre-hybridization. Pre-hybridization was done in a hybridization bottle at 65°C for 4h in the prehybridization solution (Appendix IV). Probes were [α-^{32}P]dATP labeled using the Megaprime DNA labeling system (Amersham Pharmacia Biotech) according to the manufacturer's instructions as shown below:

DNA Mix (D):

Template DNA	25-50 ng
Primers	5 μl
Water	to make up the final volume to 18 μl

The above contents are denatured for 10 min at 100°C.

Reaction Mix (R):

dCTP, dGTP and dTTP	4 μl each
Reaction buffer	5 μl

The denatured D and R are mixed and to this is added 2 μl Klenow and 3 μl [α-^{32}P] dATP and incubated at 37°C for 30 min and the radiolabeled probe purified using Sephadex G-50. For hybridization, 10^6cpm/ ml of radiolabeled probe was added and the membrane was left in hybridization solution overnight. After hybridization the unbound probe was removed by washing the membrane washed once for 5 min in 2× wash solution (2× SSC, 0.1% SDS) followed by washes in 1×SSC and 0.1× SSC at 65°C. Finally, the membrane was exposed on X-ray film with two intensifying screens containing cassettes at -80°C.

2.5.8 Fluorescence microscopy and flow cytometry

2.5.8.1 Fluorescence microscopy. The strains were grown overnight in YEPD liquid medium and aliquots were spotted on microscope slides. Fluorescence microscopy was performed with a Zeiss Axiolab microscope equipped for epifluorescence microscopy with a 50W mercury high-pressure bulb and the Zeiss fluorescein-specific filter set 09. Imaging scans were acquired with an Argon laser of 488 nm wavelength and corresponding filter settings for green fluorescent protein (GFP) and parallel transmission images.

2.5.8.2 Confocal microscopy. For Confocal microscopy, the cells were placed on the glass slides and directly viewed with a Bio-Rad confocal microscope (Radiance 2100, AGR, 3Q/BLD; Bio-Rad, UK) under a 100x oil immersion objective (Shukla *et al.*, 2003).

2.5.8.3 Fluorescence-activated cell sorter (FACS) analysis. FACS analysis was performed using a FACSCalibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with an argon laser emitting at 488 nm. Fluorescence was measured on the FL1 fluorescence channel equipped with a 530 nm bandpass filter. A total of twenty thousand events were counted at the low flow rate. Fluorescence data were collected using logarithmic amplifiers. Data analysis was performed with CellQuest software (Becton Dickinson Immunocytometry Systems). The mean fluorescence intensity was calculated using the histogram stat program (Shukla *et al.*, 2003). Averages and standard deviations were calculated from three independent experiments. Fluorescence averages were divided by the fluorescence of the parental strain (negative control) to yield fluorescence increase over control (FIOC) values.

2.5.9 ß-galactosidase assays

ß-galactosidase assays were performed as described earlier (Uhl and Johnson, 2001; Li *et al.*, 2004; Gaur *et al.*, 2005).

2.5.9.1 Qualitative assay. Qualitative assay were performed by visual screening of *lacZ* reporter harboring single copy integrated *C. albicans* transformants. 5 µl of (1×10^6) mid-exponential-phase cells for each sample were streaked onto the X-Gal-modified minimal media (XMM) plates. 1.70 g yeast nitrogen base without amino acids or ammonium sulfate, 20 g glucose, 5 g ammonium sulfate, and 20 g agar were dissolved in 930 ml H_2O for preparation of the plates. After autoclaving, 70 ml of 1M potassium phosphate, pH 7.0 and 2.0 ml of a 20 mg/ml X-Gal solution were added (Uhl and Johnson, 2001).

2.5.9.2 Quantitative assay. For quantitative liquid assay, duplicate samples of cells for three independent experiments were used. For this, 1 ml of exponential grown cell culture in YEPD was pelleted and resuspended in an equal volume of Z-buffer (Appendix VI) (Uhl and Johnson, 2001) and placed on ice. The OD_{600} was determined for each sample. 100 µl of resuspended cells were added to the Z-buffer to a final volume of 1.0 ml and permeabilized with 15 µl of 0.1% SDS and 30 µl chloroform by vortexing for 15 sec. Permeablized cells were equilibrated at 37°C for 15 min and then 0.2 ml of ONPG (4 mg/ml) (Appendix VI) was added incubated at 37°C till the hydrolyzed pale yellow product of O-nitrophenol is released. Reaction was stopped by addition of 0.5 ml of 1 M Na_2CO_3 and centrifuged for 5 min at 10,000×g to pellet down the cell debris. O.D. of the supernatant was measured at 420 nm. Units of activity were determined by the standard equation given by Ausubel *et al.* (1987) and is expressed in Miller Units per mg of protein. Miller units are arbitrary units.

$$U = \frac{1000 \times OD_{420}}{t \times v \times OD_{600}}$$

Where, t is time of reaction (in min) and v is volume of culture used in assay (in ml).

2.5.10 Measurement of growth rate

The strains were grown in YEPD media for 24 h and 5×10^6 cells (Optical density A_{600} = 0.1) were inoculated into the fresh media. Growth was followed for further 24-48 h and measured turbidometrically using Shimadzu UV 1201 spectrophotometer at 600 nm. Generation time was calculated as follows:

$$\text{Generation time (g)} = \frac{0.301\,(t_2-t_1)}{\log_{10} B_t - \log_{10} B_0}$$

Where,

g = generation time

B_t = optical density at time t_2

B_o = optical density at time t_1

t_1 = initial time

t_2 = final time and,

$$\text{Specific growth rate} = \frac{\ln 2}{d.t}$$

2.5.11 Growth curve *versus* ß-galactosidase activity

Cells were resuspended to an OD_{600} of 0.1 in YEPD broth and incubated in shaker at 200 rpm at 30°C. OD_{600} readings were taken at regular interval along with ß-galactosidase liquid assay as described above. A line graph was plotted to show the growth rate and histogram was plotted at the corresponding time point of growth to determine the ß-galactosidase reporter activity in Miller Units per mg of protein.

2.5.12 Protein sample prepration

2.5.12.1 Crude and plasma membrane protein isolation. The isolation of crude and plasma membrane for western blot analysis was carried essentially as described before (Sessions and Horwitz, 1983). Protein estimation was done by diluting appropriate aliquots to 100 µl with MQ water followed by addition of 1 ml of Bradford reagent and OD was recorded after 5 min at 595 nm (Bradford, 1976) as well as by Bicinchoninic (BCA) acid protein determination kit (sigma).

2.5.12.2 Whole cell extract prepration. Cell lysis and preparation of crude protein extract for Western blot analysis were performed by alkaline extraction procedure as described elsewhere (Hiller *et al.*, 2006) except that protocol was scaled up for a large amount of cells and that sample buffer excludes the bromophenol blue (as it interferes with the subsequent protein quantitation). Briefly, cells (5 OD_{600}) were resuspended in an eppendorf tube with 500 µl water and 150 µl of a solution containing 1.85 M NaOH and 7.5% ß-mercaptoethanol. This mixture was incubated on ice for 10 min. Proteins were next precipitated with 150 µl of a 50% trichloroacidic acid solution and the suspension was left on ice for another 10 min. Precipitated proteins were sedimented by a centrifugation step at maximal speed in a microfuge for 15 min. The sediment was resuspended in 50 µl of loading buffer (40 mM Tris-HCl [pH 6.8], 8 M urea, 5% SDS, 0.1 M EDTA, 1% ß-mercaptoethanol and 0.1 mg/ml bromophenol blue) and incubated at 37°C for 10 min. Non-solubilized material was cleared by a centrifugation step for 10 min. The whole cell extract was aliquoted and stored at -80°C. All centrifugation steps were carried out at 4°C. Protein content of whole cell extract preparation were estimated by Bradford's method (Bradford, 1976) and Bicinchoninic (BCA) acid protein determination kit (Sigma). Integrity of extract was also checked on the 8% SDS-PAGE.

2.5.12.3 Crude Nuclear Extract preparation. Nuclear extracts for *DNase*I foot-printing assay and EMSA were prepared from clinical *C. albicans* cells as described earlier (Gaur *et al.*, 2004) with slight modifications. For primary culture, *C. albicans* cells were grown in 10 ml of YEPD at 30°C. After 16 hours of growth, the culture was diluted (0.5%) into 100 ml of fresh media as a secondary culture and then grown to a density of 2×10^7 cells/ml. The cells were then harvested at 3000 rpm for 5 min. The cell pellet was weighed and resuspended in 2 volumes of Zymolyase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1M Sorbitol and 30 mM DTT). To this cell suspension 100 units of Zymolyase was added and incubated at 30°C for 2-3 hours. Sphaeroplast formation was checked by taking OD at 595 nm that upon addition of water became one-tenth of the initial absorbance of the cells. The cells were then harvested at 3000 rpm for 5 min and the pellet was resuspended in one volume of lysis buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgSO_4$, 1 mM EDTA, 10 mM potassium acetate, 1 mM DTT and 1 mM PMSF) and pellet resuspension was homogenized with 20 strokes of a tight-fitting glass Dounce homogenizer to lyse the cells. The lysate was centrifuged at 1000 rpm for 10 min and the supernatant was again centrifuged at 2000 rpm for 20 min to pellet the nuclei. The nuclear pellet hence obtained was resuspended in minimum volume of nuclear extraction buffer (20 mM HEPES [pH 7.0], 20% glycerol, 400 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF) and incubated on ice for 1h. This was followed by centrifugation at 8000 rpm for 20 min. The supernatant thus obtained was dialyzed in presence of a low salt buffer (20 mM NaCl) for 6-8 hours at 4°C. The dialyzed nuclear extract was aliquoted and stored at -80°C. All centrifugation steps were carried out at 4°C. Before using for *DNase*I footprinting assay and EMSA, the protein content of nuclear extract preparation were estimated by Bradford's method (Bradford, 1976) and Bicinchoninic (BCA) acid protein determination kit (Sigma). Integrity of nuclear extract was also checked on the 8% SDS-PAGE.

2.5.12.4 Crude whole cell extract preparation. Crude whole cellular protein preparation used for RNA-Electrophoretic Mobility Shift Assay (R-EMSA) was done with RNase free diethyl pyrocarbonate (DEPC) treated H_2O. *C. albicans* cells were grown in 10 ml of YEPD at 30°C. After 24 hours of growth, the culture was diluted (0.5%) into 100 ml of fresh YEPD media as a secondary culture and then grown to mid-logarithmically phase. Aliquot of yeast cells (~1×10^8 per ml) were then harvested at 3000 rpm for 5 min. The cell pellet was weighed and resuspended in 2 volumes of Zymolyase

buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 M Sorbitol and 30 mM DTT). To this cell suspension, 100 units of Zymolyase was added and incubated at 30°C for 2-3 hours. Sphaeroplast formation was checked by taking OD at 595 nm which upon addition of water became one-tenth of the initial absorbance of the cells. The cells were then harvested at 3000 rpm for 5 min and the pellet was resuspended in one volume of lysis buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgSO_4$, 1 mM EDTA, 10 mM potassium acetate, 1 mM DTT and 1 mM PMSF) and pellet resuspension was homogenized with 25 strokes of a tight-fitting glass Dounce homogenizer to lyse the cells. The lysate was centrifuged at 1000 rpm for 10 min and supernatant obtained was dialyzed in presence of a low salt buffer (20 mM NaCl) for 6-8 hours at 4°C. Protein content of the cellular extract preparation were estimated by Bradford's method and Bicinchoninic (BCA) acid protein determination kit (Sigma). Integrity of cellular extract was also checked on the 8% SDS-PAGE. The dialyzed cellular extract was aliquoted and stored at -80°C till further use.

2.5.13 Protein estimation

2.5.13.1 Bradford's[5] method. Soluble protein was determined by the Commassie blue method of Bradford (1976) using bovine serum albumin (BSA) as standard. This method involves the binding of Coomassie Brilliant Blue G-50 to protein, causing a shift in the absorption maximum at 595 nm, which is monitored. The colored reagent is prepared by dissolving 100 mg of Coomassie Brilliant Blue G-50 in 50 ml of 95% ethanol to which 100 ml of 85% (*v/v*) orthophosphoric acid was added, and the volume made up to 1 liter. Standard protein solution (BSA) was prepared containing 10 to 100 µg of protein in a 100 µl volume was dispensed into separate tubes. The volume in the test tube was adjusted to 100 µl by the addition of distilled water, 5 ml of coloring reagent was added to the test tubes and the contents were mixed by vortexing. The absorbance at 595 nm was measured after 2 minutes. A standard graph was made using 1 mg/ml BSA solution. The amount of protein in the sample was estimated by extrapolating the value of the absorbance of the sample from the standard graph.

2.5.13.2 Bicinchoninic acid assay (BCA). Protein samples were also quantified by Bicinchoninic acid (BCA) protein determination kit (Sigma) as per manufacturer's recommendation.

2.5.14 Protein gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). A 5-12% polyacrylamide gel was run at 80-120 V for 2 hours in Bio-Rad Mini Gel apparatus (Appendix II). The samples were prepared according to the method of Holzer and Hammes (1989). Protein aliquots were mixed with 5× samples loading buffer and incubated at 90°C for 10 min. After electrophoresis, the gel was visualized by silver staining (Blum *et al.*, 1987).

2.5.15 Western blot analyses

Total protein (approximately 20-40 μg) in the protein preprations were resolved by 10% (*w/v*) SDS-PAGE in a Mini-PROTEAN II electrophoresis cell (Bio-Rad) and was followed by transferred to nitrocellulose membranes by Western blotting using a Bio-Rad Mini Trans-Blot electrophoretic transfer cell according to the manufacturer's instructions. After transfer, membrane was stained by Ponceau reagent (0.25% Ponceau S in 40% methanol and 15% acetic acid) for 5 min to verify that protein extracts were evenly transferred. For Western blots, membranes were incubated with a 1:5000 dilution of monoclonal anti-GFP antibody (JL-8) (BD Biosciences, Clontech), 1:500 dilution of polyclonal anti-Cdr1p antibody or 1:1000 dilution of polyclonal anti-Pma1p (plasma membrane ATPase) antibody (Appendix IV). Immunoreactivity was detected using horseradish peroxidase-labeled secondary antibody with a dilution of 1:5000 (goat anti-mouse antibody for GFP and goat anti-rabbit antibody for Cdr1p and Pma1p) using the enhanced chemiluminescence assay system (ECL kit, Amersham Biosciences, Arlington Heights, IL, USA).

2.5.16 Transcription Run On (TRO) analysis

Transcription Run-On experiments were performed as described previously (Lyons and White, 2000; Garcia-Martinez *et al.*, 2004) with the following modifications. A schematic representation of TRO is shown in Figure 23. Briefly, cells were grown at 30°C in YEPD with agitation until the culture reached an OD_{600} of 1.0. An aliquot of yeast cells (6×10^8 per ml) was used to perform TRO. Transcriptional inhibitory potential of thiolutin was evaluated by treating the AS/AR isolates with an optimized thiolutin concentration (40 μg/ml) and either alone or along with *CDR1* inducer, fluphenazine (10 μg/ml) for 60 min. For *CDR1* induced conditions, cells were treated with inducers - fluphenazine (10 μg/ml), progesterone (1 mM) and β-estradiol (1 mM) for 30 min. The

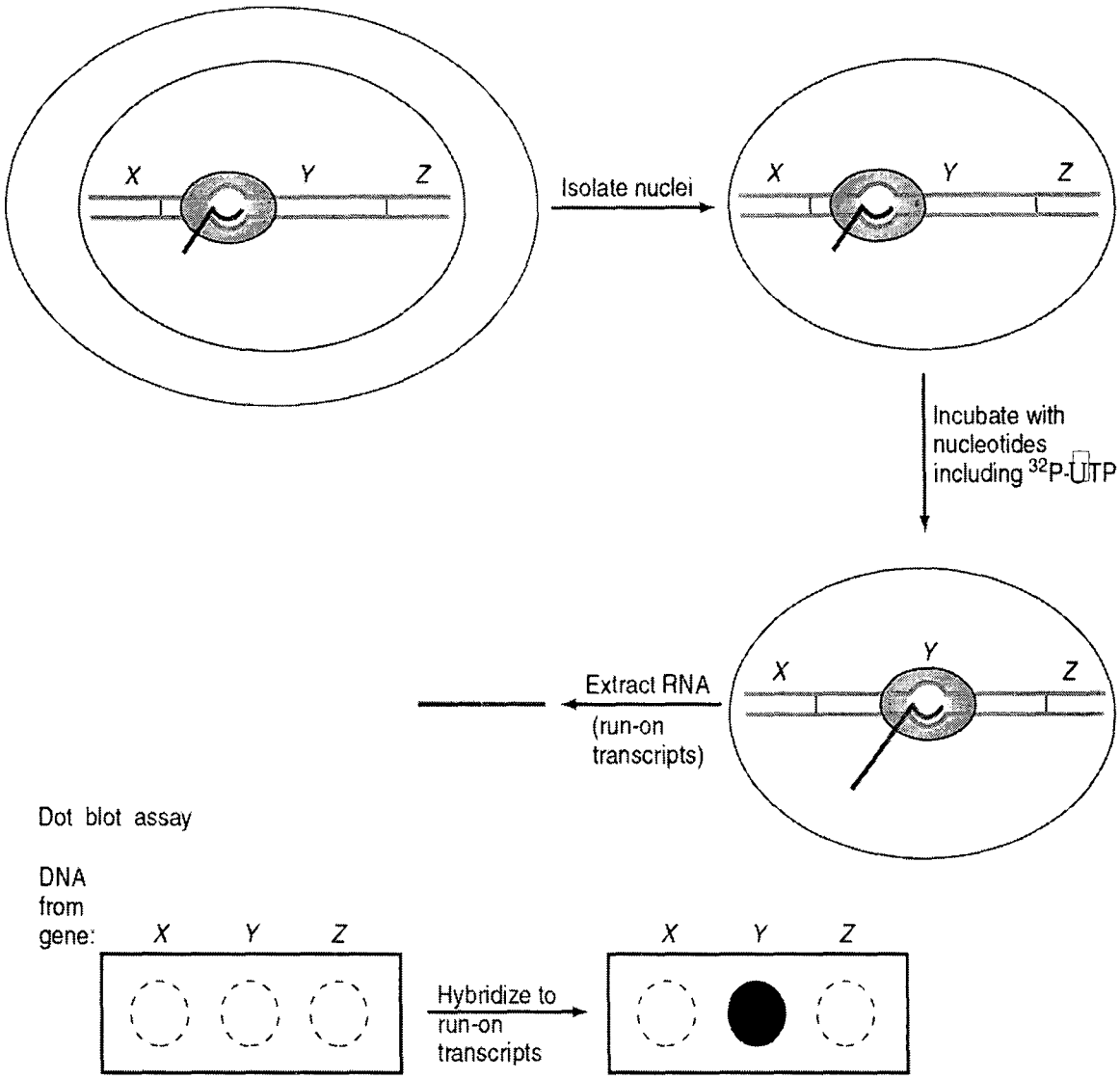


Figure 23. Schematic representation of the Nuclear run-on transcription assay.
Nuclear run-on transcription assay employed for the detection of transcription rate of particular gene is described. Briefly, nuclei was isolated from either uninduced or induced (at the indicated time after addition of inducers) as described under *Materials and Methods*. *In vivo* transcription was reinitiated, nascent RNA was labeled with [α-^{32}P]UTP to elongate already halted RNA transcripts. The total labeled nuclear run-on RNA were subsequently used as probes in reverse Northern hybridizations with the gene specific DNA (*X, Y and Z in this case*), which were immobilized by dot blotting on a charged nylon membranes (Hybond-N$^+$; Amersham Pharmacia Biotech) using a dot blot assembly apparatus. After removal of unhybridized RNAs, the labeled RNAs complementary to blotted DNA were revealed by PhosphorImager scanner (FLA-5000, FLA5000 Fuji phosphorimager) and hybridization signal intensities of nuclear RNA were quantified using densitometry scanning of phosphorimages. In the TRO scheme shown above, '*Y*' gene exhibited an increased transcription rate as compared with the '*X*' and '*Z*'.

final concentration used is given in *parenthesis*. Subsequently, untreated/treated cells were centrifuged for 5 min at 4,000×g and resuspended in 5 ml of cold TMN (10 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$; pH 7.4). The cells were again centrifuged for 5 min at 4,000×g and the cell pellet was resuspended in 900 µl of sterile cold DEPC treated water. Then the cell suspension was transferred to a fresh micro centrifuge tube containing 50 µl of 10% N-lauryl sarcosine sodium sulfate (sarkosyl) and was incubated for 20 min on ice. After the permeabilization step, cells were recovered by low-speed centrifugation at 6,000 rpm for 2 min at 4°C and the supernatant was removed. *In vivo* transcription was reinitiated by resuspending the permeabilized cell fraction in 120 µl of 2.5× transcription buffer (50 mM Tris-HCl [pH 7.7], 500 mM KCl, 80 mM $MgCl_2$), 16 µl of AGC mix (10 mM each of ATP, GTP and CTP), 6 µl of DTT (0.1 M), 1 U of RNase inhibitor per µl, 10 mM phosphocreatine, 1.2 µg of creatine phosphokinase per µl and 15 µl of [α-^{32}P]UTP (3000 Ci/mmol,10 µCi/µl). Cells were maintained on ice at all times. The final volume was adjusted to 300 µl with DEPC treated water and the mix was incubated for 15 min at 30°C to allow transcription elongation. The reaction was stopped by adding 1 ml of ice cold DEPC treated water to the mix. Cells were recovered by centrifugation to remove non-incorporated radioactive nucleotides. Total RNA was isolated using the Trizole reagent (Sigma) as per the manufacturer's specifications except that 200 µl of ice-cold acid-washed 0·4-0·6 mm diameter glass beads (Sigma, St. Louis, MO, USA) were also used for efficient and complete lysis of permeabilized cells. Isolated total labeled RNA was again precipitated by adding 2.5 M NH_4-acetate and an equal volume of isopropanol. The mixture was stored overnight at -20°C. To pellet the RNA, tubes were centrifuged at 14,000 rpm for 15 min in the micro-centrifuge. The isopropanol was removed, and the labeled RNA pellet was washed twice with 70% ethanol, dried and resuspended in 100 µl of DEPC treated water. This double precipitation of RNA was used to minimize DNA contamination. Total extracted RNA was spectrophotometrically quantified. An aliquot was used for specific radioactivity determination in a Tri-CARB 2900 TR Liquid scintillation analyzer (Packard instrument Co., Inc. USA). All of the *in vivo* labeled RNA of each isolate (~2-2.5×10^6 cpm) was subsequently used for reverse Northern hybridization with a dot blotted Nylon membrane (Hybond-N^+; Amersham Pharmacia Biotech) containing PCR amplified gene specific N-terminal *CDR1* sequences (nucleotides -242 to +256 from the TSP), plasmid pACT1 (positive control) and pBlueScript-KS(+) (negative control) as probes, as per the manufacturer's recommendation. Northern blots were scanned with a PhosphorImager scanner (FLA-

5000, FLA5000 Fuji phosphorimager). Signal intensities of hybridized nuclear RNA were quantified and subsequently normalized to the actin intensities using densitometry scanning.

2.5.17 mRNA chase assay

2.5.17.1 Screening of transcription inhibitors. To identify and determine the *in vivo* potency of various transcription inhibitors in clinical *C. albicans* isolates, inhibition in *de novo* biosynthesis of RNA was measured. Transcription inhibitors- actinomycin D, 1,10-phenanthroline, α-amanitin and thiolutin were added individually to a 5 ml of mid-log phase (OD_{600}~1.0) cultures grown in a defined SD-URA$^-$ media. Radio-labeled uridine ([5,6-^{3}H], 32 Ci/mmol) was added to a final concentration of 100 μCi/ml to each culture, incubated at 30°C for 60 min and subsequently inhibitors were added in a concentration dependent manner (*described in figure legend*). For thiolutin treatment, cells were treated with a 150 μM concentration of the copper chelator bathocuprioinedisulphonic acid (BCS) and incubated at 37°C for 10 min at 200 rpm. Subsequently, thiolutin was added along with 150 nM $CuSO_4$. Addition of BCS and $CuSO_4$ was found to enhance the response of the cells to thiolutin (Setiadi *et al.*, 2006). Transcription shut off was monitored 60 min after the inhibitor addition. For this purpose, 500 μl samples of each culture were collected and cold trichloroacetic acid (TCA) was added to a final concentration of 11.5% (*w/v*). After incubation on ice for 5 min, the samples were centrifuged at 13,000 rpm and the TCA-precipitated counts for each concentration were quantified in a in a Tri-CARB 2900 TR Liquid scintillation analyzer (Packard instrument Co., Inc. USA). Radioactive counts were normalized to that of solvent control of corresponding transcription inhibitors and plotted as % inhibition of radiolabeled uridine incorporation during *de novo* RNA synthesis with respect to untreated sample (*assuming 100% incorporation*) on a line graph.

2.5.17.2 Thiolutin treatment arrest transcription. Transcriptional inhibitory potential of thiolutin was evaluated by monitoring the R6G efflux as well as expression activity of indicator gene, *CDR1*. Glucose-mediated efflux of R6G (*described ahead*), fluorescence microscopy/flow cytometry, ß-galactosidase reporter and TRO assays, were carried out by standard protocols as described earlier except that AS/AR isolates were treated with an optimized thiolutin concentration (40 μg/ml) and either alone or along with *CDR1* inducer, fluphenazine (10 μg/ml) for 60 min. The final concentration used is given in parenthesis.

2.5.17.3 Total RNA isolation. For this, all experiments were done with DEPC treated H_2O and chemicals. Total RNA from clinical *C. albicans* isolates was isolated as described below.

2.5.17.3.1 Protocol I (TCES method). The cells were harvested at 2500 rpm for 10 min at 4°C and resuspended in 1 ml of ice cold DEPC MQ H_2O and divided into two eppendorfs. They were centrifuged for 1 min at 10,000 rpm and were then resuspended in 150 µl TCES and 150 µl phenol:chloroform:isoamyl alcohol. 0.3 ml of ice cold glass beads (0.5 mm) were added into the mixture and vortexed for 2-3 min. 200 µl TCES and 200 µl phenol: chloroform: isoamyl alcohol was added into the above mixture and vortexed for another min. The above mixture was centrifuged for 1 min. The upper clear phase was collected and precipitated it with 2.5 volumes of ice cold absolute ethanol for 1 h at -20°C. Tubes were centrifuged for 15 min at 4°C at highest speed. The pellet was resuspended in 250 µl DEPC MQ H_2O and heated at 56°C to dissolve the insoluble material like the carbohydrates. The mixture was centrifuged at highest speed for 15 min to remove insoluble materials. The supernatant was pooled in one tube and precipitated with $1/10^{th}$ vol. of Na-acetate and 2.5 volumes of ice cold absolute ethanol. The tube was kept at -20°C for minimum of 30 min and centrifuged for 15 min at 4°C. The pellet was washed with 70% ethanol and was dried in speedvac for 3 min after draining alcohol properly. Resuspended the pellet in 100 µl of 50% formamide and stored at -80°C till further use. The RNA content was measured at A_{260} (diluted 1000 times), [A_{260} = 40 µg of RNA/ ml].

2.5.17.3.2 Protocol II (TRI® Reagent method). Total RNA from clinical matched pair of *C. albicans* isolates was prepared from mid-logarithmically grown phase cells. In a standard preparation, 10 ml of cells, optical density at 600 nm (OD_{600}) of 1.0, were pelleted and washed with 10 ml of ice-cold H_2O and spun at 5000 rpm. The pellet was resuspended in 1.0 ml of TRI® Reagent (Sigma) and 0.3 ml of ice-cold acid-washed 0·4-0·6 mm diameter glass beads (Sigma, St. Louis, MO, USA) were added and vortexed for 5 min. Chloroform (0.2 ml) without isoamyl alcohol was added and the tubes were shaken vigorously for 15 s. The samples were incubated at room temperature for 15 min, followed by centrifuge at 12,000 × g for 15 min at 4°C. The upper colorless aqueous phase was transferred to a new tube and 0.5 ml of isopropanol was added. The tubes were incubated at room temperature for 10 min, followed by centrifugation at 12,000 × g for 10 min and the pellet washed with 75% ethanol and recentrifuged. The pellet was air

dried and resuspended in 100 µl of H_2O. For this preparation, all experiments were done with DEPC treated H_2O. DNA free RNA was prepared by treating total RNA with DNase RQ1 (promega). The OD_{260} and OD_{280} were measured, and the integrity of the total RNA was visualized by subjecting 2-5 µl of the sample to electrophoresis through a denaturing 1% agarose/2.2 M formaldehyde gel. The total RNA preparation isolated was stored at -80°C till further use.

2.5.17.4 Northern blot hybridization. For Northern analysis standard protocol was used (Sambrook *et al.*, 1989). The electrophoresed RNA was visualized on UV transilluminator. Northern transfer was performed overnight as given in standard laboratory protocol (Sambrook *et al.*, 1989) using Hybond^{TM}-N^+ nylon membrane (Amersham). RNA was fixed by UV cross linking (Amersham) as per the manufacturer's instructions. Membrane-bound RNA was stained with methylene blue before hybridization to check rRNA bands for equal loading. The pre-hybridization and hybridization was carried out as described in Southern blot hybridization except for the temperature which was 42°C for Northern hybridization. Membranes were pre-hybridized for at-least 30 min in a church buffer containing 7% SDS, 0.3 M Sodium phosphate buffer (pH 7.0) and 1 mM EDTA at 42°C. . The *CDR1/ACT1* probes used were generated by PCR amplification of plasmid pS12-35 (harboring the whole *CDR1* gene) and SC5314 genomic DNA. Gene specific primers used for preparing probes specific for *CDR1/ACT1* are listed in Table 8. The sizes of the amplified PCR products were checked by agarose gel electrophoresis and purified from the gel using the Gel elution kit (Qiagen, Germany). The probes were then radiolabeled with [α-^{32}P]dATP using Megaprime DNA labeling system (Amersham Pharmacia Biotech) for Northern blot hybridization. The purified labeled probes were added into the pre-hybridization buffer and left for hybridization for 12-16 hrs at 42°C. Quantification and Relative intensities (RI) of *CDR1* mRNA signals in Northern hybridizations were quantitated by exposure of the hybridized membrane in a FLA-5000 Fuji PhosphorImager system. The membranes were subsequently deprobed and rehybridized with the *ACT1* probe to monitor equal RNA loading and transfer.

2.5.17.5 Thiolutin Chase assay. In order to measure the *CDR1* mRNA half-life, a potent *in vivo* transcriptional inhibitor of *C. albicans*, thiolutin, was used as described earlier (Kebaara *et al.*, 2006; Setiadi *et al.*, 2006). AS and AR isolates of *C. albicans* were incubated with an optimized concentration (40 µg/ml) of thiolutin (*as described earlier*). For this purpose, cultures were treated with 150 µM of the copper chelator

Bathocuprioinedisulphonic acid (BCS) and incubated at 30°C for additional 10 min at 200 rpm. Transcription was subsequently shut off by the addition of 150 nM of $CuSO_4$ and 40 µg/ml of thiolutin. Addition of BCS and $CuSO_4$ was found to enhance the response of the cells to thiolutin (Setiadi *et al.*, 2006). For induction experiments, AS isolates were treated with inducers for 30 min, prior to thiolutin addition. Briefly, 100 ml of cells were grown to an OD_{600} of 1.0 at 30°C. Aliquots of cells were taken at the indicated times after transcriptional shutoff. Total RNA was isolated using Ambion's RiboPure^{TM}-Yeast RNA isolation kit (catalogue # 1926) as per the manufacturer's instructions. For Northern blots, approximately 20 µg of total RNA from the above samples were hybridized with probes derived from gene-specific sequences of the *CDR1* gene as described above. Hybridization signal intensity was quantified with a PhosphorImager scanner (FLA-5000, FLA5000 Fuji phosphorimager) and was normalized to the band intensity at time T_0 and plotted as a line graph.

2.5.18 Protein chase assay

2.5.18.1 Cycloheximide treatment arrest translation. For chase assay, effective cycloheximide concentration to inhibit the *de novo* protein synthesis in clinical matched AS and AR isolates was determined. For this purpose, cycloheximide was added to a 5 ml overnight mid-log phase grown cultures (OD_{600}~1.0) in a concentration dependent manner (0-100 mM). After 5 min of cycloheximide addition, radio-labeled [^{14}C] arginine (32Ci/mmol) was added to a final concentration of 100 µCi/ml to each culture and incubated further at 30°C. After 1 h of treatment, cells were washed with 0.05% tween-80 solution and subsequently resuspended in 100 µl of same solution. This suspension was added to 2 ml. of scintillation cocktail-O (SRL, Mumbai) and counts were measured in a Tri-CARB 2900 TR Liquid scintillation analyzer (Packard instrument Co., Inc. USA). Radioactive counts were plotted as a % inhibition of radiolabeled arginine incorporation during *de novo* protein synthesis with respect to that untreated sample (*assuming 100% incorporation*) on a line graph.

2.5.18.2 Cycloheximide Chase assay. Cdr1p degradation was studied using cycloheximide chase as described earlier (Gardner *et al.*, 2000) with certain modifications. Briefly, yeast strains were grown in 30 ml of YEPD media to an approximate optical density (OD_{600}) of 1.0-1.5. For chase experiment, cell growth is adjusted to approximately 10 A_{600}/ml. After adding cycloheximide with a final concentration of 75mM (determined empirically in a pilot scale experiment as described

earlier). Approximately 30 A_{600} of cells were withdrawn at the 0,30,60,90,120,180 and 300 min. after tanslational arrest by cycloheximide, resuspended in sodium azide solution (NaN_3) at a final concentration of 1mM (to instantly kill the cells), centrifuged and kept the pellet at -80 °C until processed further. One of the no-drug samples was also immediately lysed for the zero time point. Cell lysis and preparation of crude protein extract were performed by alkaline extraction procedure as described earlier. Briefly, aliquots of mid-log phase grown cells withdrawn after translational shutoff at the indicated times were lysed in solution containing 1.85 M NaOH and 7.5% ß-mercaptoethanol. Crude proteins isolated were precipitated with 50% trichloroacetic acid and sedimented. The sediment was resuspended in loading buffer (40 mM Tris-HCl [pH 6.8], 8 M urea, 5% SDS, 0.1 M EDTA, 1% ß-mercaptoethanol) and incubated at 37°C for 10 min. Non-solubilized material was cleared by a centrifugation step and solubilized Crude protein extracts were quantitated by Bicinchoninic acid (BCA) protein determination kit (Sigma). Approximately 20 and 30 µg protein for AR and AS isolates, respectively, in the lysates (~10-15µl of 100 µl final volume) were resolved by 10% (*w/v*) SDS-PAGE in a Mini-PROTEAN II electrophoresis cell (Bio-Rad) and followed by transfer to nitrocellulose membrane for Western blotting (*as described earlier*) using Bio-Rad Mini Trans-Blot electrophoretic transfer cell according to the manufacturer's instructions. Immunodetected Cdr1p signal intensity was quantified with a PhosphorImager scanner (FLA-5000, FLA5000 Fuji phosphorimager) and was normalized to the band intensity at time T_0 and plotted as a line graph.

2.5.19 Glucose mediated Rhodamine 6G efflux

R6G efflux was carried out by standard protocols as described previously (Shukla *et al.*, 2003). Approximately 10^7 cells from a culture grown overnight were inoculated in 50 ml of YEPD and grown for 3-4 h at 30°C. The cells were pelleted and washed three times with phosphate-buffered saline (PBS) without glucose. The cells were subsequently resuspended as a 2% cell suspension in PBS and pre-loaded with R6G to a final concentration of 10 µM under energy-depleted conditions and incubated for 2 h at 30°C. Cell samples were pre-incubated at 30°C with *CDR1* inducers, fluphenazine (10 µg/ml), progesterone (1 mM) and ß-estradiol (1 mM) alone or along with the transcription inhibitor, thiolutin (or a drug solvent control only) for 30-60 min before the addition of glucose. The final concentration used is given in parenthesis. The cells were then washed and resuspended in PBS with 2% glucose. An aliquot of 1 ml was taken after 30 min and

centrifuged at 9,000 ×g for 2 min. The absorbance of the supernatant was measured at 527 nm.

2.5.20 Reverse transcription-3'RACE-Nested PCR of *CDR1*

The nucleotide sequence of the oligonucleotide primers used for the reverse transcription-3'RACE-nested PCR were selected based on the published sequences of *CDR1* (GenBank accession number X 77589) (Prasad *et al.*, 1995). Total RNA isolated from each isolate using the Trizole reagent (Sigma) as per the manufacturer's specifications was enriched with poly(A)$^+$ (polyadenylated) mRNA using the Oligotex mRNA Mini Kit protocol (Qiagen) and used subsequently for performing the reverse transcription-3'RACE reaction as described elsewhere (Gerads and Ernst, 1998). For a typical 3' RACE reaction, cDNA synthesize from *ca.* 0.1 µg of poly(A)$^+$ RNA was placed in a 0.5 ml reaction tube with 1 µM of oligo(dT)$_{18}$ anchor primer stock and the volume was adjusted to 11 µl with DEPC treated water (Figure 42a, step 1). The mixture was incubated for 10 min at 70°C and chilled on ice for 1 min, after which the remainder of the reaction mixture was added from a master mix to the reaction tube in order for each reaction to contain a 1 mM concentration each of dATP, dCTP, dGTP and dTTP; 40U of RNase inhibitor (MBI, fermentas) in a buffer consisting of 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$ and 10 mM DTT. After brief mixing, the reaction was incubated for 10 min at 37°C followed by addition of 40U of M-MuLV reverse transcriptase (MBI, fermentas). Finally, the reaction was incubated at 37°C for 60 min and then stopped by heating at 70°C for 10 min followed by chilling on ice for 1 min. The synthesized cDNA was purified from unincorporated dNTPs, oligo(dT)$_{18}$ anchor primer and proteins by using Oligotex mRNA Mini Kit (Qiagen). Amplification of *CDR1* mRNA specific 3'-end of each isolate was performed (Figure 42a, step 2) using corresponding appropriate dilution of cDNA as template (determined empirically for *CDR1* gene to give a product in the linear range, generally 1:4) and 1 µM of each *CDR1* specific forward primer CT-*CDR1*-F (corresponding to position 4271-4293 in the *CDR1* genomic sequence) and reverse PCR-anchor primer as mentioned in Table 8. (*PCR parameters*: initial denaturation of 95°C for 5 min followed by 35 cycles denaturation at 95°C for 30 s, annealing at 55°C for 40 s, elongation at 72°C for 40 s and final extension at 72°C for 10 min). As a positive control, *CDR1* specific forward primer CT-*CDR1*-F and reverse primer CT-*CDR1*-R (corresponding to position 4475-4503 in the *CDR1* genomic sequence) was also used (*see* Figure 41). The negative control (without RT) established that the PCR products

generated in the RT-PCR were not due to genomic DNA contamination (*see* Figure 41). RT-PCR product of each isolate was electrophoresed on a 1.2% agarose gel in 1× TAE. The purified 3' RACE product (Qiagen PCR clean up kit) of each isolate was cloned directly in pGEM® T-Easy vector using T/A cloning kit (promega) as per the manufacturer's recommendations and sequenced (Figure 42a, step 3). The corresponding clone of each isolate was used as templates for a second Nested PCR amplification of *CDR1* (Figure 42a, step 4) with forward and reverse primer (UTR-F-*Pac*I and UTR-R-*Mlu*I, *see* Table 8) specific to each isolate (*PCR parameters*: initial denaturation of 95°C followed by 30 cycles denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s and final extension at 72°C for 10 min). The resulting PCR-amplified fragments (now exactly and specific to mapped 3'UTR *CDR1* of each isolates) was purified (Qiagen PCR clean up kit) and further subcloned in pGEM® T-Easy vector (Figure 42a, step 5a) generating *CDR1* 3'UTR specific clones of each isolate as mentioned in Table 9. All the cloned PCR amplified products were confirmed by appropriate restriction digestion analysis and sequenced further to determine the exact and precise *CDR1* 3'UTR (Figure 42a, step 5b).

2.5.21 Custom service nucleotide sequencing

Multiples of 3'UTR specific clone of each isolate were sequenced directly by extension from both sense and anti-sense strands using T7 promoter/T7 promoter primer and SP6 promoter/ SP6 promoter primer (*see* Table 8) by exploiting Big dye terminator chemistry and automated DNA sequencer (ABI Prism 3100). Reproducibility of the sequencing was confirmed by processing all samples at least twice.

2.5.22 Multiple sequence alignment, *in silico* analysis and computerized secondary structure predictions of *CDR1* 3'UTR

Multiple sequence alignment of mapped *CDR1* 3'UTR for both matched pair of isolates was done using the ClustalW (*version 1.83*) program (Thompson *et al.*, 1994). *In silico* analysis of 3'UTR for prediction of putative regulatory site was carried out by UTRscan (UTResources) program (Pesole and Liuni, 1999; Pesole *et al.*, 2002). We used *'mfold'* (*version 3.0*) algorithm created by Zucker *et al.* (1999) for *in silico* computer predictions of the secondary structure of 3'UTR by minimization of the free energy-based method Zucker *et al.* (1999). The folding temperature was fixed at 37°C and ionic conditions

were 1M NaCl without constraints, and no limit in the maximum distance between paired bases.

2.5.23 RNA-Electrophoretic Mobility Shift Assay (R-EMSA)

2.5.23.1 Crude whole cell extract preparation. Crude whole cellular protein preparation used for RNA-Electrophoretic Mobility Shift Assay (R-EMSA) was done with RNase free DEPC treated H_2O as described earlier.

2.5.23.2 Radiolabeling of RNA probes. To generate riboprobes, plasmid harboring mapped 3' UTR of each isolate (*see* Table 9) was linearized with *PacI*/*MluI* (depending upon the direction of cloning) for *in vitro* run-off transcription. Transcription reactions were usually performed at 37°C for 1 h in a final reaction volume of 20 µL containing 1 µg of a linearized DNA template, 2.5 mm of each rNTP, 50 µCi of [α-^{32}P] UTP (37 Ci/mmol; Amersham Biosciences), 10 mM DTT, 40 U of ribonuclease inhibitor, 1× transcription optimized buffer and 20 U of T7/Sp6 RNA polymerase as per manufacturer's recommendations (Promega). Finally, RNase-free DNaseI (5 units) was added to each reaction and the mixture was further incubated at 37°C for an additional 15 min to remove the DNA template. Labeled riboprobes were purified by Sephadex G-50 microcolumn filtration. An aliquot was used for specific radioactivity determination in a Tri-CARB 2900 TR Liquid scintillation analyzer (Packard instrument Co., Inc. USA) and used directly in RNA mobility-shift assays after dilution at ~ 50,000 cpm/µl.

2.5.23.3 R-EMSA. For R-EMSA, binding reactions were carried out with 30 µg of cellular extract and 1.5×10^5 cpm of [α-^{32}P]UTP body-labeled riboprobe (*as described above*) and incubated at room temperature for 30 min in 1× binding buffer (25 mM HEPES-KOH [pH 7.5], 50 mM KCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 10 µg of *E. coli* tRNA and 40 U of RNase inhibitor) in a final reaction volume of 30 µl to allow the formation of RNA-protein complexes (Thomson *et al.*, 1999). RNA-protein complexes were resolved at 200 V for 3 h at 4°C on pre-electrophoresed 5% non-denaturing (29:1 acrylamide: *bis*-acrylamide) PAGE gel in 0.5× TBE buffer (45 mm Tris-borate, 1 mm EDTA [pH 8.0]), vacuum-dried and quantified by scanning in a PhosphorImager scanner (FLA-5000, FLA5000 Fuji phosphorimager).

2.5.24 PolyAdenylation Test (PAT)-Poly(A) tail length analysis

A new and improved variation of 3' RACE polyadenylation test (PAT) involving G-tailing of mRNA (Kusov *et al.*, 2001), was employed to show both the presence of a polyA tail and its length as described diagrammatically in Figure 50a.

2.5.24.1 Guanylation of mRNA. The purified poly(A)$^+$-enriched mRNA (as described earlier) was used directly for polyguanylation using yeast poly(A) polymerase (PAP) (Catalog#E74225Y; Amersham Pharmacia Biotech/US Biochemicals) as per manufacturer's recommendations. To abolish the higher ordered secondary structure at the 3'-end of mRNA, samples (0.1 µg) were heated at 65°C for 5 min and immediately placed on ice. They were incubated for 1 h at 37°C with 600 U of PAP and 0.5 mM GTP in a 25 µl reaction mixture containing 20 mM Tris-HCl [pH 7.0], 50 mM KCl, 0.7 mM $MnCl_2$, 0.2 mM EDTA, 100 µg/ml BSA and 10% glycerol. Additional 300 U of PAP was further added and incubation was continued for an additional 1 h. The reaction was terminated by heat treatment at 65°C for 10 min, chilled on ice and kept at -80°C till further use.

2.5.24.2 RT-PCR. Polyguanylated mRNA (*as described above*) was applied directly for RT-PCR analysis using hot start conditions (65°C, 30 s). The first strand cDNA synthesis (RT, 42°C for 1 h) was primed by oligo(dC_9T_6)-anchor primer (1 µM). As specificity control, oligo$(dT)_{18}$-anchor primer was also used as described in Figure 41. The RT reaction was stopped by denaturation at 70°C for 10 min; the purified cDNA product (1:4 dilution) was used for PCR amplification with 1 µM of each *CDR1* specific forward primer (CT-*CDR1*-F/UTR-F-*Pac*I) and reverse anchor primer as mentioned in Table 8 (*parameters*: initial denaturation of 95°C for 5 min followed by 35 cycles denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 1 min and final extension at 72°C for 10 min). The PCR products were gel-purified (Gel extraction kit, Qiagen) and used for cloning into T/A cloning vector (pGEM® T-Easy) as recommended by the manufacturer (promega). Approximately 8-10 clones from at least three independent RT-PCR reactions of each isolate were sequenced. For induction experiments, PAT assay was carried out as described above except that AS isolates were treated with inducers for 30 min and the induced poly(A)$^+$-enriched mRNA fractions were used as template for polyguanylation and subsequent RT-PCR analysis.

2.5.25 Drug susceptibility and other functional parameters for induced AS isolates

The susceptibilities of AS isolates to different drugs in the presence of *CDR1* inducers-fluphenazine (FPZ), progesterone (PRG) and ß-estradiol (ß-EST) were tested by spot

assay, as described earlier. Glucose-mediated efflux of R6G, Northern, and ß-galactosidase assays, were carried out by standard protocols as described above except that each AS isolates were treated for 30 min with inducers-FPZ (10 μg/ml), PRG (1 mM) and ß-EST (1 mM). The final concentration used is given in parenthesis.

"Research is a very good word and the meaning is certainly plain, when results are still absurd, it literally means search again"

Results and Discussion

"Once you replace negative thoughts with positive ones,
you'll start having positive results"

Section-1

Results and Discussion

3.1 Transcriptional activation and increased mRNA stability contribute to over-expression of *CDR1* in azole-resistant *Candida albicans*

3.1.1 Review of literature

Resistance of the human fungal pathogen *Candida albicans* to azole antifungals is often caused by increased expression of genes encoding multidrug efflux pumps, the ATP-binding cassette (ABC) transporters *CDR1* and *CDR2* and/or the major facilitator *CaMDR1* (Prasad *et al.*, 1995; Sanglard *et al.*, 1995 and 1997; White, 1997; White *et al.*, 1998; Franz *et al.*, 1998; Lopez-Ribot *et al.*, 1998; Wirsching *et al.*, 2000; Sanglard and Odds, 2002; Akins, 2005; Riggle and Kumamoto, 2006). However, the molecular mechanisms leading to the constitutive over-expression of efflux pump encoding genes in drug-resistant, clinical *C. albicans* isolates are only beginning to be understood. Especially the regulation of *CDR1* expression has been studied by many groups (Krishnamurthy *et al.*, 1998; Puri *et al.*, 1999; de Micheli *et al.*, 2002; Gaur *et al.*, 2004; Karnani *et al.*, 2004; Coste *et al.* 2004; Chen *et al.*, 2004; Gaur *et al.*, 2005; Coste *et al.* 2006). Various unrelated stresses, including elevated temperature or the presence of drugs and steroids, induce a transient transcriptional activation of *CDR1* in drug-susceptible *C. albicans* strains (Krishnamurthy *et al.*, 1998). Several *cis*-elements in the *CDR1* upstream region that affect basal as well as induced *CDR1* expression have been identified. Puri *et al.* (1999) identified four Upstream Activating Sequences (UAS) and four Upstream Repressing Sequences (URS) in the 5' non-coding region of *CDR1*. A Basal Regulatory Element (BRE) and a Negative Regulatory Element (NRE), in the proximal region of the promoter, have also been characterized and implicated in the basal expression of *CDR1* (Gaur *et al.*, 2004 and 2005). A specific Steroid Responsive Region (SRR) in the distal part of the *CDR1* promoter, consisting of two progesterone responsive sequences and one β-estradiol responsive sequence has been shown to contribute exclusively to steroid responsiveness of *CDR1* (Karnani *et al.*, 2004). Another Basal Expression Element (BEE) in the *CDR1* upstream region and a Drug-Response Element (DRE), which is present in the upstream region of *CDR1* and *CDR2*, have been identified

by de Micheli *et al.* (2002) The DRE was found to mediate both the transient up-regulation of *CDR1* and *CDR2* by steroid hormones and drugs as well as their constitutive over-expression in a resistant strain (de Micheli *et al.*, 2002).

Coste *et al.* (2004) identified a transcription factor, *TAC1* (Transcriptional Activator of *CDR* genes), that binds to the DRE in the *CDR1* and *CDR2* promoters. Inactivation of *TAC1* resulted in the loss of fluphenazine-induced up-regulation of *CDR1* and *CDR2*, with little impact on basal expression levels, and also abrogated the constitutive over-expression of these efflux pumps in a drug-resistant strain (de Micheli *et al.*, 2002; Coste *et al.*, 2004; 2006). CaNdt80p, a homolog of the meiosis-specific transcription factor Ndt80p of *S. cerevisiae*, is another positive regulator of *CDR1*. Deletion of *CaNDT80* abolished the induced expression of *CDR1* and increased the sensitivity of *C. albicans* to antifungals (Chen *et al.*, 2004). Interestingly, the global repressor CaTup1p acts as a negative regulator of *CDR1* expression (Murad *et al.*, 2001; Yang *et al.*, 2006).

Although transcriptional regulation is considered to be the key step accounting for complex basal and induced patterns of *CDR1* expression, the possibility of post-transcriptional control of *CDR1* expression, which could also affect drug resistance, still remains to be explored. The high amounts of Cdr1p, which correlate with high *CDR1* mRNA levels, in azole-resistant *C. albicans* strains may not only be due to increased *CDR1* transcription but could also be caused by increased stability of its mRNA and protein. It is therefore of interest to compare (i) *CDR1* transcription initiation rates, (ii) *CDR1* mRNA stability and (iii) Cdr1 protein stability in drug-susceptible and *CDR1* over-expressing, drug-resistant *C. albicans* strains. In this study, we have addressed these issues by exploiting two pairs of matched azole-susceptible (AS) and *CDR1* over-expressing, azole-resistant (AR) isolates. By using transcriptional and translational reporter gene fusions, transcriptional run-on, thiolutin and cycloheximide chase assays, we demonstrate that *CDR1* over-expression in *C. albicans* is caused by an increase in its transcriptional initiation rate and by increased mRNA stability.

3.1 2 Results

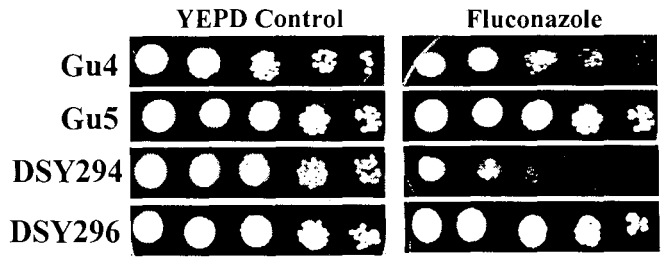
3.1.2.1 Expression of transcriptional and translational *GFP* reporter fusions in AS and AR isolates.

A.

Strains	Phenotype	*MIC_{80} (μg/ml)
Gu4	Susceptible	4
Gu5	Resistant	> 128
DSY294	Susceptible	0.25
DSY296	Resistant	128

* Fluconazole

B.



C.

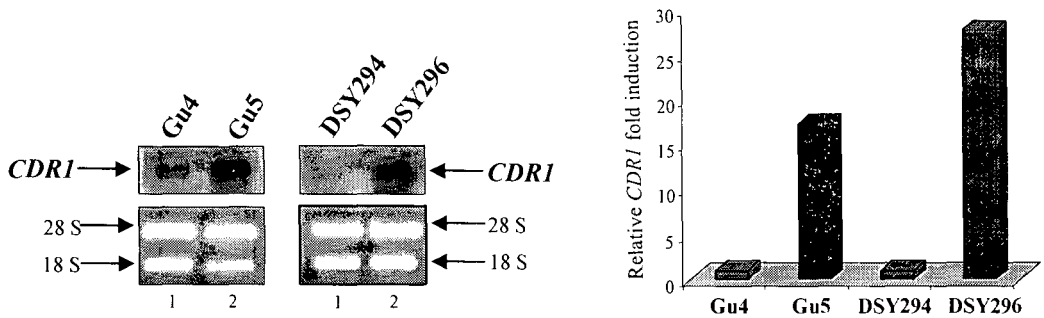


Figure 24. Drug resistance and *CDR1* expression profiling of clinical AS and AR isolates.

Drug resistance profiles of the clinical matched pair *C. albicans* isolates were determined by the **(A)** Broth microdilution and **(B)** Spot assays. The MIC_{80} (defined as the lowest drug concentration that gave >80% inhibition of growth compared to the growth of the drug-free controls) was determined by the broth microdilution method and was evaluated both visually and by reading the absorbance at 620 nm in a microplate reader. In the spot assay, 5 μl of fivefold serial dilutions of each yeast culture (A_{600}=0.1) was spotted onto YEPD plates in the absence (control) and the presence of the fluconazole (2 μg/ml). **(C)** Expression levels of *CDR1* mRNA in AS and AR isolates. Total RNA from the both the matched pair of isolates was prepared by TRI®Reagent (Sigma). The RNA samples were electrophoresed, stained with ethidium bromide to monitor equal loading of the RNA and subsequently blotted onto a charged nylon membrane. The blot was hybridized with [α-^{32}P]dATP labeled *CDR1* specific probe. After hybridization, relative intensities of *CDR1* mRNA signals were quantitated by exposure of the hybridized membrane in a PhosphorImager scanner (FLA-5000, FLA5000 Fuji phosphorimager).

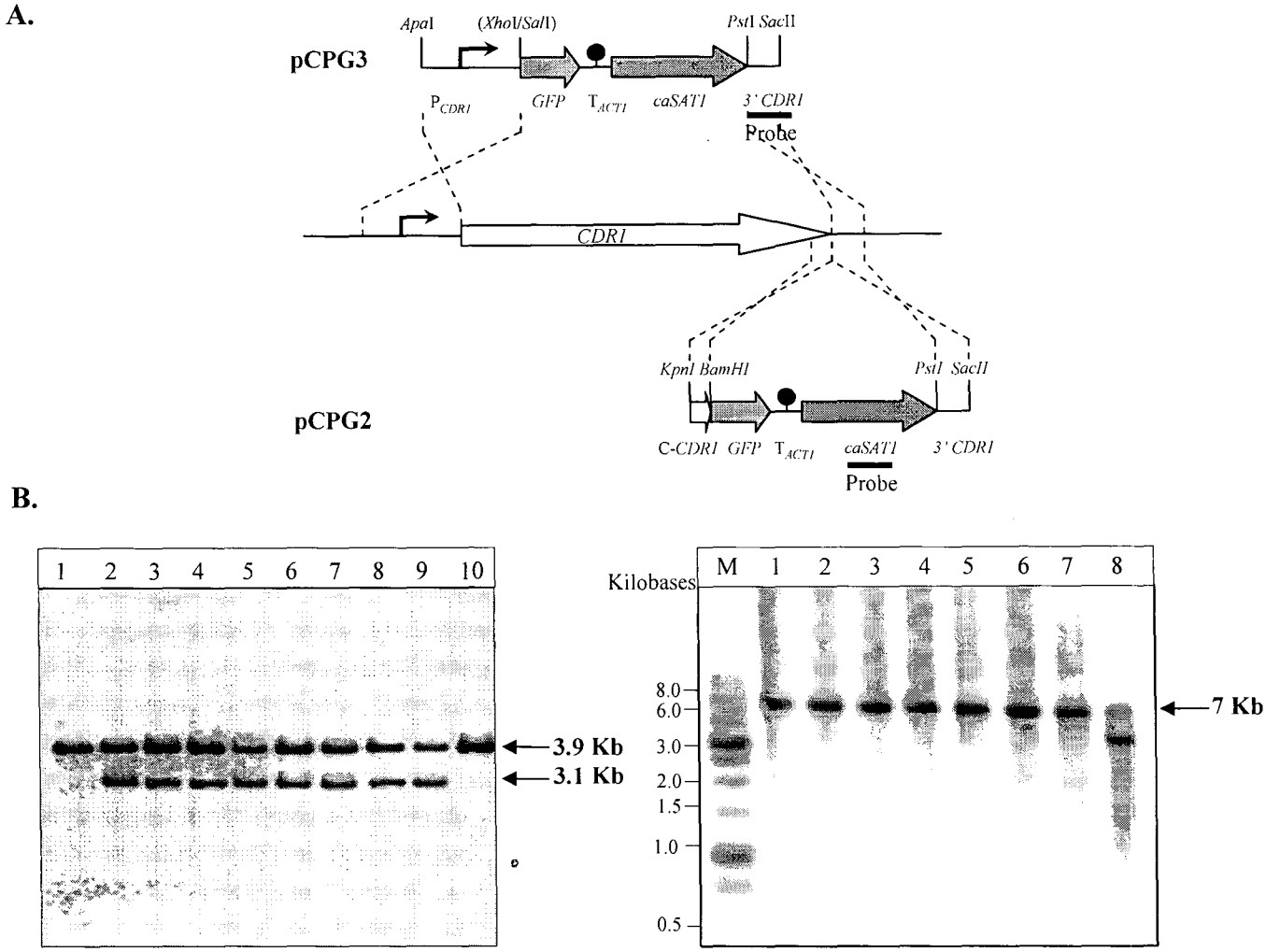


Figure 25. Schematic depiction of *GFP* reporter fusion constructs and their integration in AS and AR isolates.

(A) Structure of the DNA cassettes which were used to integrate the transcriptional (P_{CDR1}-*GFP*, *top*) and translational (P_{CDR1}-*CDR1-GFP*, *bottom*) *GFP* reporter fusions into the *CDR1* locus of the clinical *C. albicans* isolates (*middle*). The *CDR1* and *GFP* coding regions are represented by *white and green arrows* respectively, the *caSAT1* marker by the *grey arrow*, and the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the *filled circle*. *CDR1* upstream and downstream regions are represented by *solid lines*, the *CDR1* promoter (P_{CDR1}) is symbolized by the *bent arrow*. Only relevant restriction sites are shown. **(B)** Chromosomal DNA from *C. albicans* transformants was isolated as described under *Material and Methods*. Genomic DNA (~10 µg) of P_{CDR1}-*GFP* (*left blot*: *Lanes* 2,3-Gu4G1; *Lanes* 4,5-Gu5G1; *Lanes* 6,7-DSY294G1; *Lanes* 8,9- DSY296G1; *Lanes* 1,10-control SC5314 strain) and P_{CDR1}-*CDR1-GFP* (*right blot*: *Lanes* 1,2-Gu4G2; *Lanes* 3,4-Gu5G2; *Lanes* 5,6-DSY294G2; *Lanes* 7,8-DSY296G2) reporter transformants were digested with *Bgl*II, separated on a 1% (*w/v*) agarose gel, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Probe labeling, hybridization, washing and signal detection were performed as described previously. Single copy integration of each construct at the desired locus was confirmed with gene specific probe indicated by a *thick line*. The size of the hybridizing fragments are indicated on right side of the each blot. *Lane M*, nucleotide size marker (*1 kb Marker*).

To analyze the molecular basis of *CDR1* upregulation in azole-resistant, clinical *C. albicans* isolates in more detail, we employed two matched pairs of azole-susceptible (AS) and azole-resistant (AR) isolates. The resistant isolates Gu5 and DSY296, which were obtained after fluconazole therapy of oropharyngeal candidiasis in two different HIV positive patients, were shown by DNA fingerprinting to be highly related to the susceptible isolates Gu4 and DSY294, respectively, which were recovered from earlier infection episodes in the same patients (Sanglard *et al.*, 1995; Boerlin *et al.*, 1996; Franz *et al.*, 1999). It was recently shown that a mutation in the transcription factor *TAC1* is responsible for *CDR1* and *CDR2* upregulation in DSY296 (Coste *et al.*, 2006). However, it has not yet been explored if other mechanisms contribute to the over-expression of the efflux pumps in these isolates.

We compared the expression of two different *GFP* reporter fusions in these isolates, one in which *GFP* was expressed from the *CDR1* promoter (P_{CDR1}-*GFP*) and in another where *GFP* was fused in frame to the last codon of the *CDR1* ORF and expressed from the *CDR1* promoter (P_{CDR1}-*CDR1-GFP*) (*see Materials and Methods* and Figure 25A). The reporter fusions were integrated at the native *CDR1* locus and two transformants of each of the four parental strains were used for further analysis (Figure 25B). The reporter strains were designated as Gu4G1 (P_{CDR1}-*GFP*), Gu4G2 (P_{CDR1}-*CDR1-GFP*); Gu5G1 (P_{CDR1}-*GFP*), Gu5G2 (P_{CDR1}-*CDR1-GFP*); DSY294G1 (P_{CDR1}-*GFP*), DSY294G2 (P_{CDR1}-*CDR1-GFP*); DSY296G1 (P_{CDR1}-*GFP*) and DSY296G2 (P_{CDR1}-*CDR1-GFP*).

Expression of the P_{CDR1}-*GFP* transcriptional and P_{CDR1}-*CDR1-GFP* translational fusions in cells grown to the mid-exponential phase (OD_{600} ~1.0) was detected by epifluorescence microscopy (Figure 26A) and quantified by FACS analysis (Figure 26B). As expected, the fluorescence intensities of the AR reporter fusion strains were higher than those of the corresponding AS strains (2.5-fold for Gu5G1 *versus* Gu4G1, 19-fold for Gu5G2 *versus* Gu4G2; 6-fold for DSY296G1 *versus* DSY294G1, and 80-fold for DSY296G2 *versus* DSY294G2), confirming the previously reported increased *CDR1* transcript and Cdr1p protein levels in the AR isolates (Sanglard *et al.*, 1995; Franz *et al.*, 1999; Coste *et al.*, 2006). Interestingly, however, expression of the P_{CDR1}-*CDR1-GFP* translational fusion resulted in much lower fluorescence than expression of the P_{CDR1}-*GFP* transcriptional fusion in AS isolates (6-fold for Gu4G2 *versus* Gu4G1 and 13-fold for DSY294G2

A.

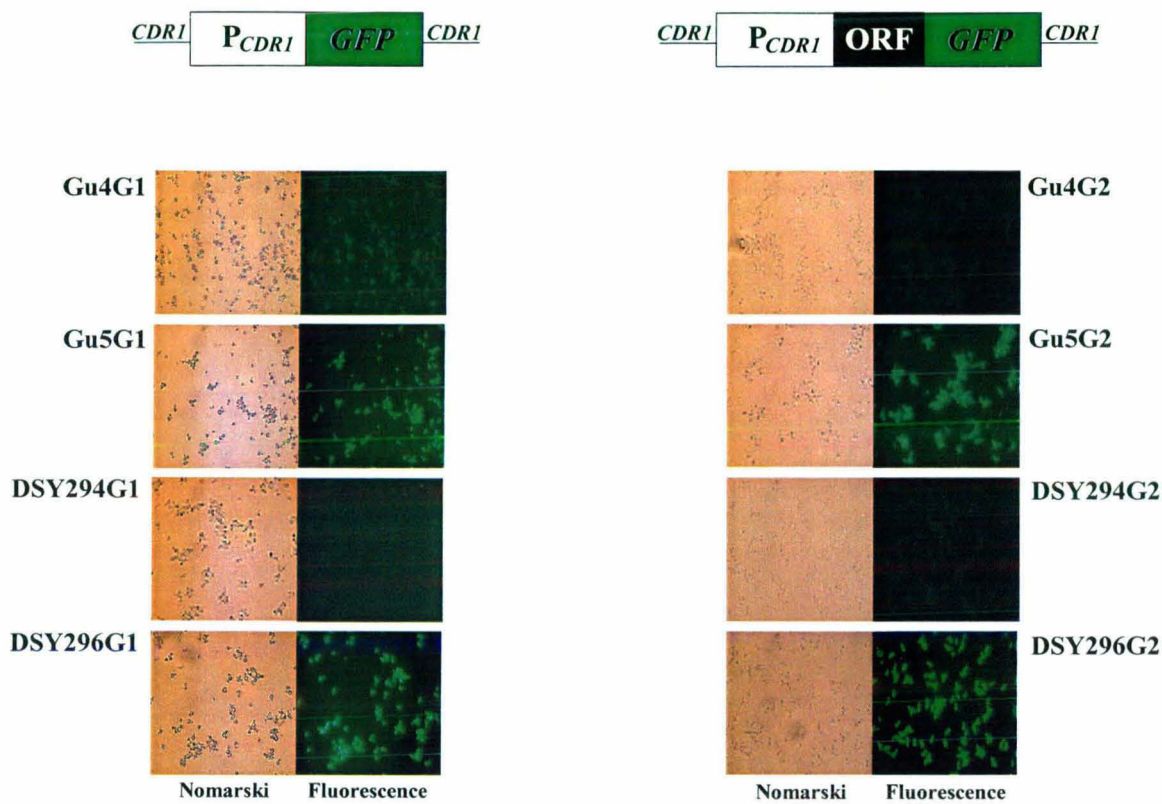


Figure 26. Expression of *GFP* reporter fusion integrants in AS and AR isolates.
(A) Nomarski and corresponding fluorescence micrographs of transformants containing the chromosomally integrated P_{CDR1}-*GFP* (left) and P_{CDR1}-*CDR1-GFP* (right) reporter fusions.

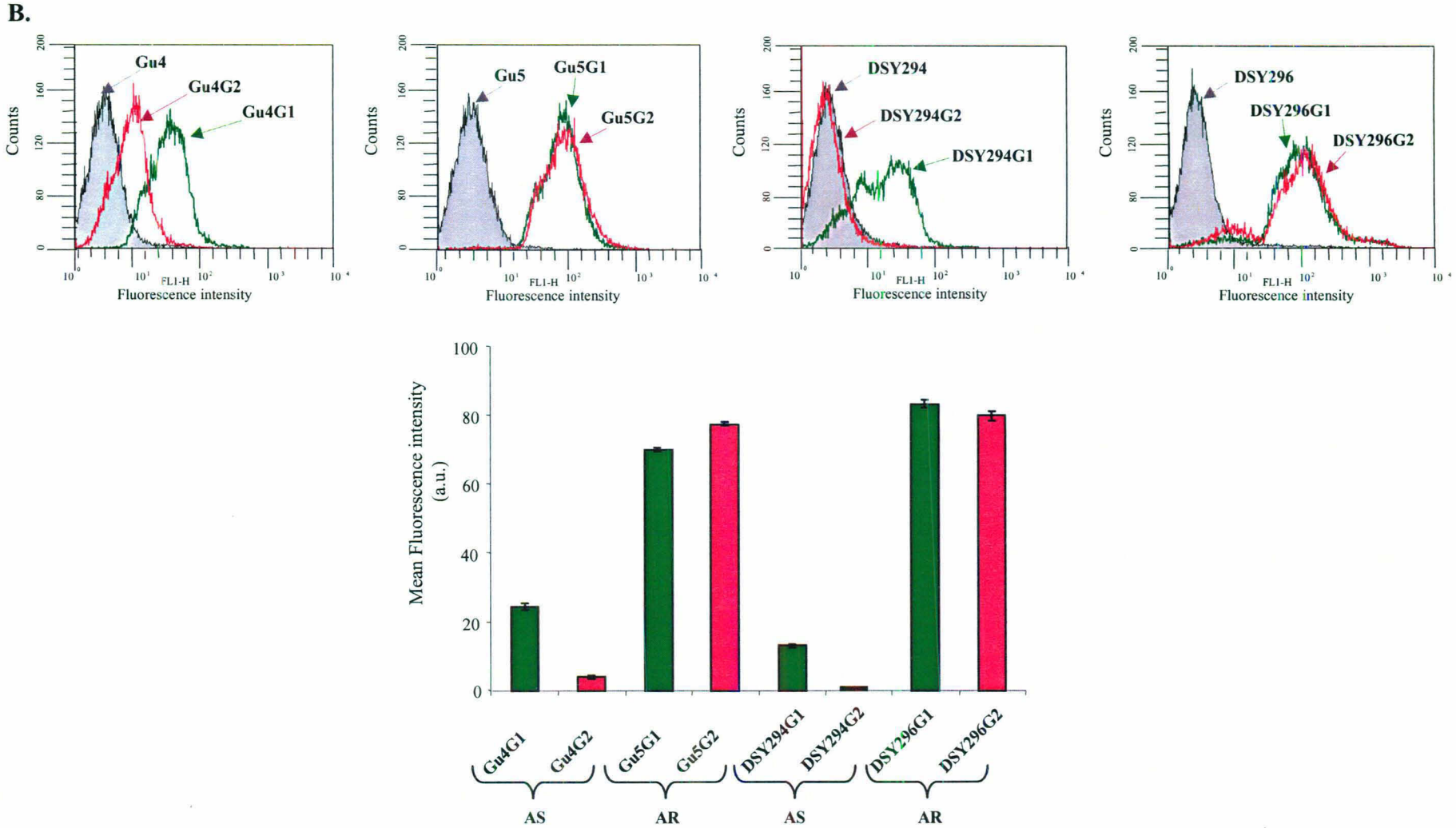


Figure 31. Expression of *GFP* reporter fusion integrants in AS and AR isolates.

(B) Cells of the reporter strains grown to exponential phase in YEPD medium were diluted to a density of 2×10^7 cells per ml in PBS (pH 7.0) and a total of twenty thousand events were analysed by flow cytometry. The parental strains of the transformants, which do not contain *GFP*, were used as a negative control. The mean fluorescence intensity is shown for each population of cells (bottom panel) after normalizing with their corresponding negative controls. Since the normalized mean fluorescence intensity of 'DSY294G2' was a negative value, we have taken it as '1.0' for calculating the fold change for this particular strain.

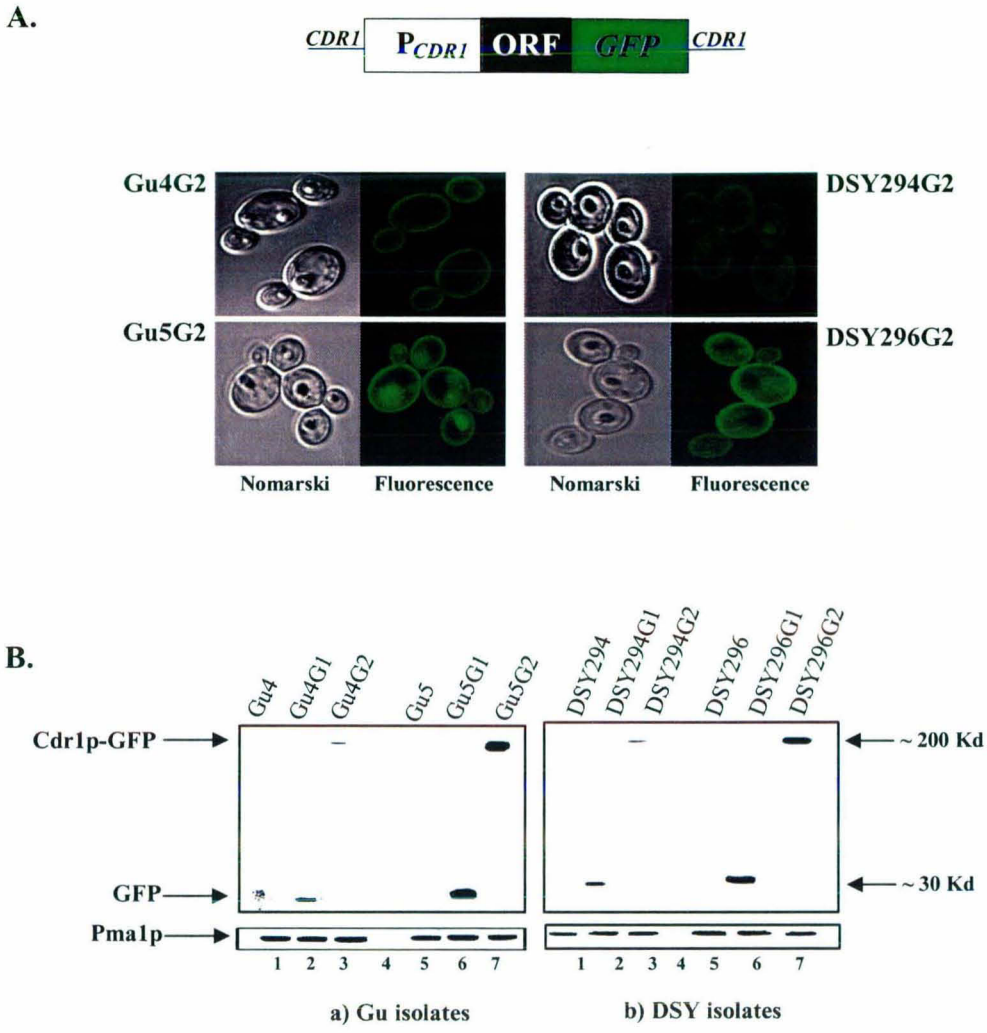


Figure 27. Localization of Cdr1p and Immunodetection of *GFP* in reporter fusion transformants.

(A) Nomarski (left) and corresponding confocal (right) pictures of the transformants harboring the chromosomally integrated P_{CDR1}-*CDR1-GFP* (translational fusion) reporter construct are shown which indicate the proper plasma membrane localization of chimeric Cdr1p in clinical *C. albicans* isolates. The cells were viewed directly on a glass slide with a 100× oil immersion objective. **(B)** The Western blot analyses were done with an anti-GFP monoclonal antibody on both the transcriptional and translational fusion integrants. Equal loading of protein was assessed by using an anti-Pma1p polyclonal antibody.

versus DSY294G1), whereas the two types of reporter fusions produced comparable fluorescence in AR isolates. Of note, SC5314-derived *CDR1* promoter showed sequence differences from the *CDR1* promoters in the matched AS or AR isolates (at nucleotide positions -21, -150, -171, -215, -238, -315, -368, -381, -418 and -455 relative to the TSP) which have been used in this study (*unpublished observation*). These differences, however, did not affect the ß-galactosidase reporter activity of integrated cassettes derived either from pCPL1 (Gaur *et al.*, 2005) which harbors the SC5314 *CDR1* promoter or from pCPL4 or pCPL5 which harbor Gu4- and Gu5-derived *CDR1* promoter, respectively (*unpublished observation*). Notably, confocal microscopy confirmed that the Cdr1p-GFP fusion protein was correctly localized to the cell membrane in all reporter strains expressing the translational fusion (Figure 27A). Immunoreactive bands of the expected sizes were observed in whole cell extracts and plasma membrane preparations of the P_{CDR1}-*GFP* and P_{CDR1}-*CDR1-GFP* reporter strains, respectively, after Western immunoblotting with an anti-GFP antibody (Figure 27B).

3.1.2.2 Expression of transcriptional and translational *lacZ* reporter fusions in AS and AR isolates.

To rule out that the reduced expression of the P_{CDR1}-*CDR1-GFP* translational fusion in AS isolates was an artifact intrinsic to the Cdr1p-GFP fusion protein, we used a codon-optimized *lacZ* (Uhl and Johnson, 2001) as an alternative reporter gene. As for *GFP*, transcriptional (P_{CDR1}-*lacZ*) and translational (P_{CDR1}-*CDR1-lacZ*) *lacZ* reporter fusions were generated and integrated at the native *CDR1* locus of the AS and AR isolates (*see Materials and Methods* and Figure 28A and B). The expression of Cdr1p in the P_{CDR1}-*CDR1-lacZ* construct was unaffected by its fusion to *lacZ* as tested by Western blotting with an anti-Cdr1p antibody (Figure 29). Two transformants of each parental strain containing a single copy of the reporter fusion were used for further analysis. The reporter strains were designated as Gu4L2 (P_{CDR1}-*lacZ*), Gu4L3 (P_{CDR1}-*CDR1-lacZ*); Gu5L2 (P_{CDR1}-*lacZ*), Gu5L3 (P_{CDR1}-*CDR1-lacZ*); DSY294L2 (P_{CDR1}-*lacZ*), DSY294L3 (P_{CDR1}-*CDR1-lacZ*); DSY296L2 (P_{CDR1}-*lacZ*) and DSY296L3 (P_{CDR1}-*CDR1-lacZ*).

Expression of the *lacZ* reporter gene in various strains was assessed by comparing the intensity of the blue color produced by cells grown on agar plates containing the indicator

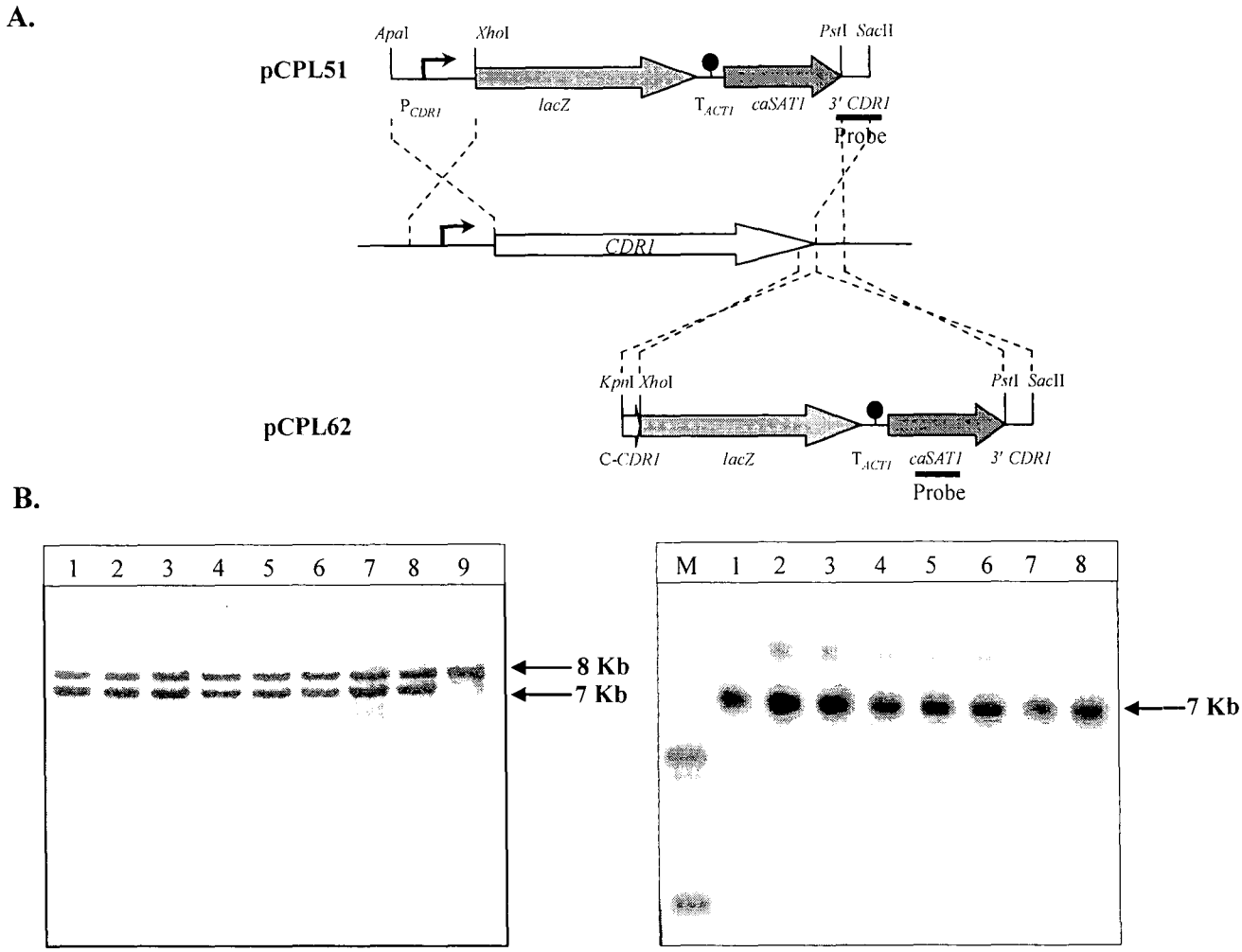


Figure 28. Schematic depiction of *lacZ* reporter fusion constructs and their integration in AS and AR isolates.

(A) Structure of the DNA cassettes which were used to integrate the transcriptional (P_{CDR1}-*lacZ*, *top*) and translational (P_{CDR1}-*CDR1-lacZ*, *bottom*) *lacZ* reporter fusions into the *CDR1* locus of the clinical *C. albicans* isolates (*middle*). The *CDR1* and *lacZ* coding regions are represented by *white and blue arrows* respectively, the *caSAT1* marker by the *grey arrow*, and the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the *filled circle*. *CDR1* upstream and downstream regions are represented by *solid lines*, the *CDR1* promoter (P_{CDR1}) is symbolized by the *bent arrow*. Only relevant restriction sites are shown. **(B)** Chromosomal DNA from *C. albicans* transformants was isolated as described under *Material and Methods*. Genomic DNA (~10 µg) of P_{CDR1}-*lacZ* (*left blot*: *Lanes* 1,2-Gu4L2; *Lanes* 3,4-Gu5L2; *Lanes* 5,6-DSY294L2; *Lanes* 7,8-DSY296L2; *Lane* 9-control SC5314 strain) and P_{CDR1}-*CDR1-lacZ* (*right blot*: *Lanes* 1,2-Gu4L3; *Lanes* 3,4-Gu5L3; *Lanes* 5,6-DSY294L3; *Lanes* 7,8-DSY296L3) reporter transformants were digested with *Bgl*II, separated on a 1% (*w/v*) agarose gel, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Probe labeling, hybridization, washing and signal detection were performed as described previously. Single copy integration of each construct at the desired locus was confirmed with gene specific probe indicated by a *thick line*. The size of the hybridizing fragments are indicated on right side of the each blot. *Lane M*, nucleotide size marker (*1 kb Marker*).

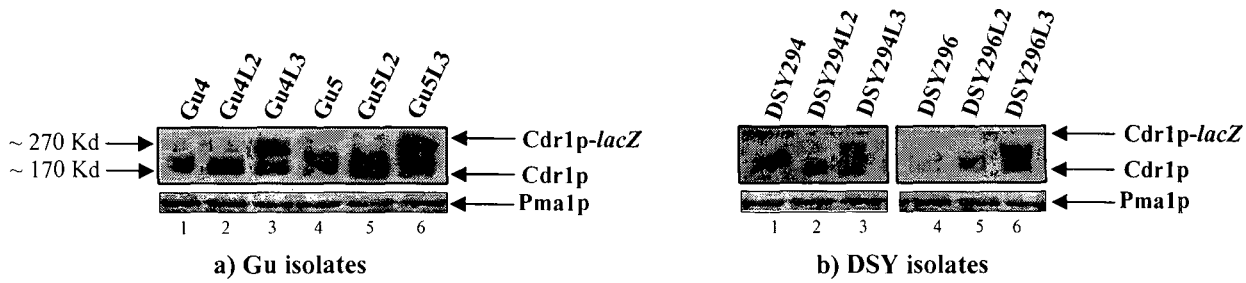


Figure 29. Immunodetection of Cdr1p in *lacZ* reporter fusion transformants.
Western blot analyses were done by anti-Cdr1p polyclonal antibody with crude protein extract of wild type, *lacZ* transcriptional (P_{CDR1}-*lacZ*) and translational (P_{CDR1}-*CDR1-lacZ*) fusion integrants in both AS and AR isolates. For AR isolates ~20 µg, while for AS isolates ~30 µg (because of relatively low expression of Cdr1p) of crude extract was loaded and separated by SDS-PAGE. Immunoreactive bands corresponding to Cdr1p and Cdr1p-*lacZ* correlates to wild type and translationally fused *CDR1* allele respectively. An equal loading and transfer of protein was assessed by deprobing and subsequently reprobing the immunoblot with anti-Pma1p polyclonal antibody.

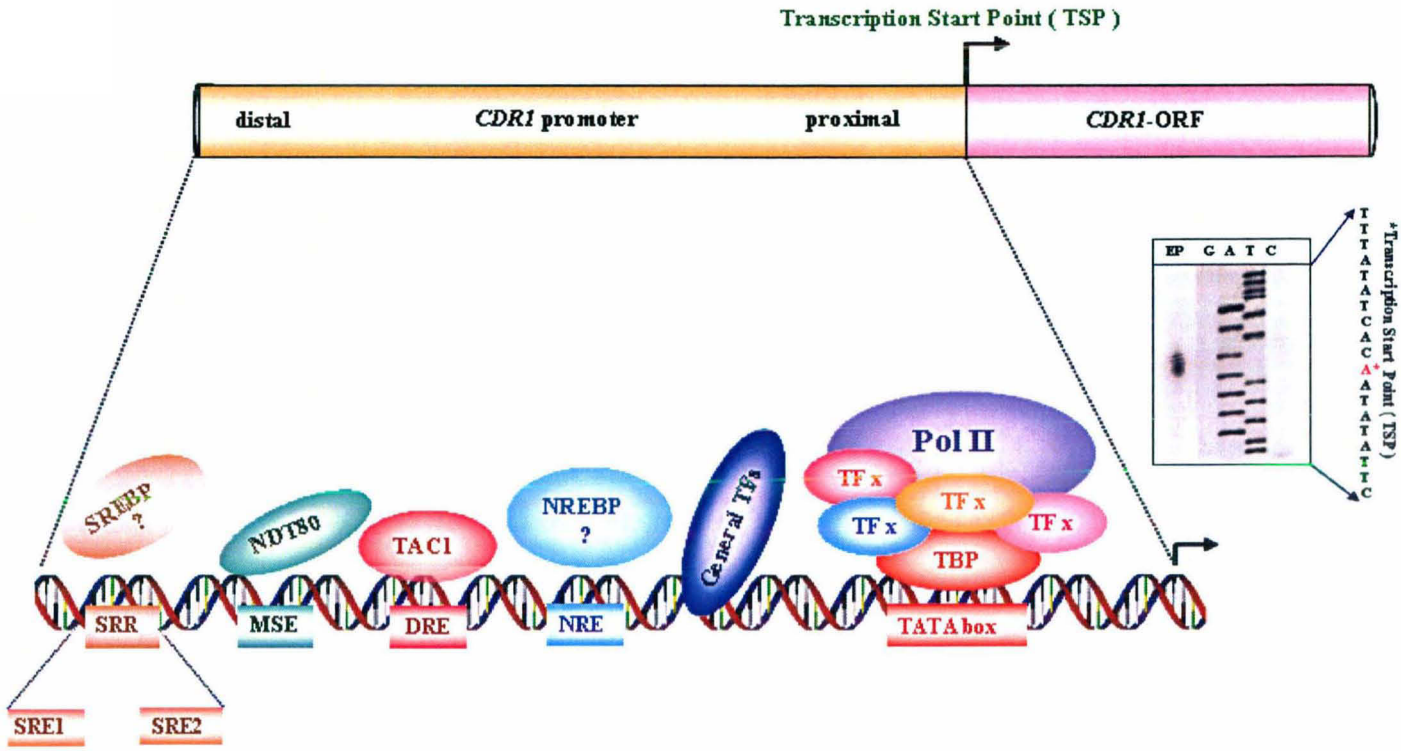


Figure 18. Untranslated 5'-regulatory region of the *CDR1* gene showing identified promoter elements and cognate binding factors.

Positions are relative to the TSP (Transcription Start Point), which is represented by the *bent arrow*.

Abbreviations:

NRE : Negative Regulatory Element; NREBP: Negative Regulatory Element Binding Protein

DRE : Drug Responsive Element; TAC1 : Transcription Activator of *CDR* genes

SRR : Steroid Responsive Region; SREBP : Steroid Responsive Element Binding Protein

SRE1 : Steroid Responsive Element 1; SRE2 : Steroid Responsive Element 2

MSE : Mid Sporulation Element; CaNDT80 : Transcription Activator of *CDR* genes

Inset blot shows the TSP that has been mapped by primer extension analysis as depicted by *asterisk*.

EP : Extension product; GATC : Sequencing marker (**Courtesy**: Puri *et al.*, 1999).

dye X-Gal (Figure 30A) and was quantified by determining ß-galactosidase activities in liquid assays (Figure 30B). The *lacZ* reporter study confirmed the results obtained with *GFP*. Higher *lacZ* expression levels were observed in transformants of the AR isolates than in transformants of the AS isolates (1.4-fold for Gu5L2 *versus* Gu4L2, 37-fold for Gu5L3 *versus* Gu4L3, 5.6-fold for DSY296L2 *versus* DSY294L2, and 13-fold for DSY296L3 *versus* DSY294L3). In addition, while the transcriptional and translational fusions yielded comparable activities in the AR isolates, expression of the translational fusion was much lower than that of the transcriptional fusion in the AS isolates (26-fold for Gu4L3 *versus* Gu4L2 and 2-fold for DSY294L3 *versus* DSY294L2). Of note, the integration of P_{CDR1}-*lacZ* reporter fusion constructs at the ectopic *ACT1* locus (Figure 31A and B). resulted in comparable ß-galactosidase activity as that of native *CDR1* locus integrants (Figure 32A and B). Additionally, the tagging of P_{CDR1} as well as P_{CDR1}-*CDR1* with *GFP/lacZ* did not alter the drug resistance profiling of AS and AR isolates which ruled out that the *GFP/lacZ* fusions caused any selective impact on Cdr1p functionality on either AS and AR isolates (Figure 33).

3.1.2.3 Growth phase *versus* ß-galactosidase reporter activity.

To investigate whether the observed differences in the expression of transcriptional and translational reporter gene fusions in AS and AR isolates depended on the growth phase, we quantitatively monitored ß-galactosidase activities in the *lacZ* reporter strains at various times during growth in batch cultures. As can be seen in Figure 34A and C, the lower reporter expression levels of the translational P_{CDR1}-*CDR1-lacZ* fusion as compared with the transcriptional P_{CDR1}-*lacZ* fusion in the AS isolates were observed at all growth stages In contrast, both types of reporter fusion produced comparable ß-galactosidase activities in the AR isolates throughout growth (Figure 34B and D).

Taken together, exploitation of reporter fusions and their expression analysis indicated that increase in *CDR1* expression levels in the AR isolates as compared to the corresponding AS isolates is contributed either by affecting *CDR1* promoter activity, mRNA stability, translational efficiency, or protein stability. Therefore, we performed further experiments on the native *CDR1* (endogenous gene) to get a real insight if

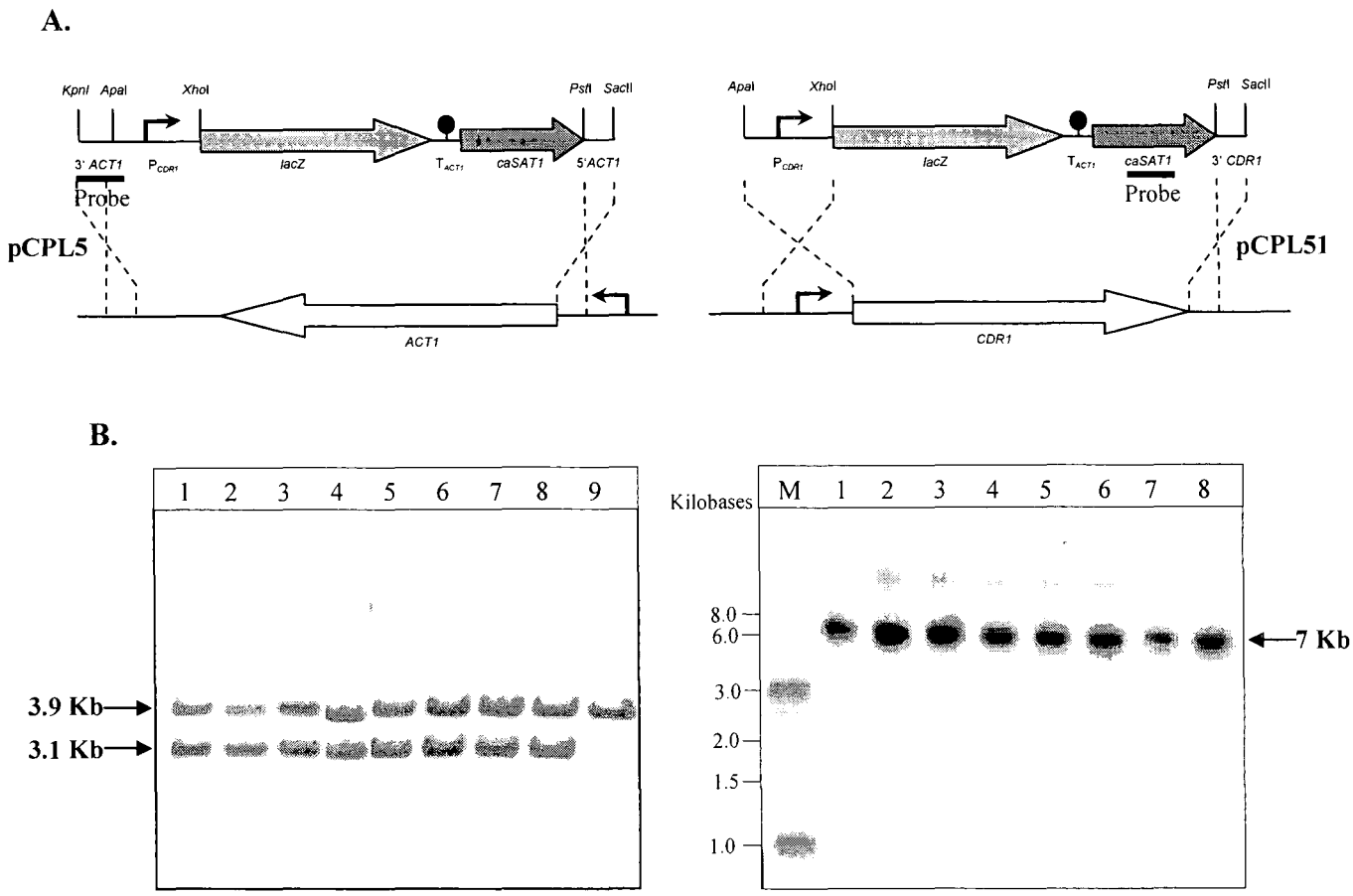
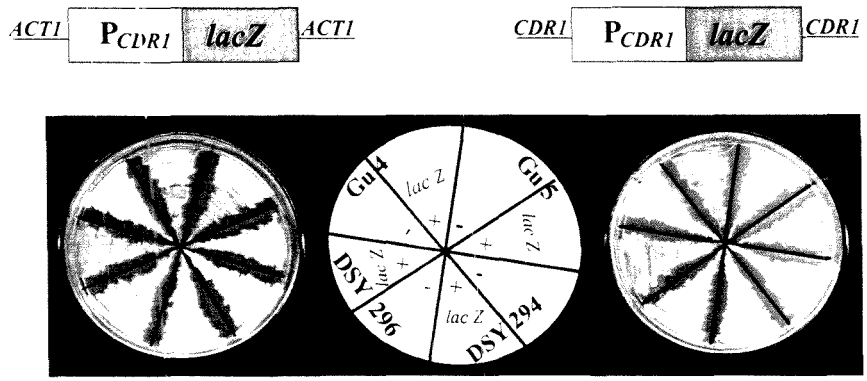


Figure 31. Schematic depiction of *CDR1* promoter-*lacZ* transcriptional reporter fusion constructs and their integration at *ACT1* and *CDR1* locus in AS and AR isolates.

(A) Structure of the DNA cassettes which were used to integrate *CDR1* promoter *lacZ* transcriptional reporter fusions (P_{CDR1}-*lacZ*) at *ACT1* (*left*) and *CDR1* locus (*right*) of the clinical *C. albicans* isolates. The *ACT1* and *CDR1* coding regions are represented by *white arrow*, while *lacZ* coding regions by *blue arrows* respectively, the *caSAT1* marker by the *grey arrow*, and the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the *filled circle*. *ACT1* and *CDR1* upstream and downstream regions are represented by *solid lines*, *ACT1* (P_{ACT1}) and *CDR1* (P_{CDR1}) promoter is symbolized by the *bent arrow*. Only relevant restriction sites are shown. **(B)** Chromosomal DNA from *C. albicans* transformants was isolated as described under *Material and Methods*. Genomic DNA (~10 µg) of P_{CDR1}-*lacZ* integrants at *ACT1* locus (*left blot*: *Lanes* 1,2-Gu4L1; *Lanes* 3,4-Gu5L1; *Lanes* 5,6-DSY294L1; *Lanes* 7,8-DSY296L1; *Lane* 9-control SC5314 strain) and at *CDR1* locus (*right blot*: *Lanes* 1,2-Gu4L2; *Lanes* 3,4-Gu5L2; *Lanes* 5,6-DSY294L2; *Lanes* 7,8-DSY296L2) reporter transformants were digested with *Bgl*II, separated on a 1% (*w/v*) agarose gel, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Probe labeling, hybridization, washing and signal detection were performed as described previously. Single copy integration of each construct at the desired locus was confirmed with gene specific probe indicated by a *thick line*. The size of the hybridizing fragments are indicated on left / right side of the each blot. *Lane M*, nucleotide size marker (*1 kb Marker*).

A.



B.

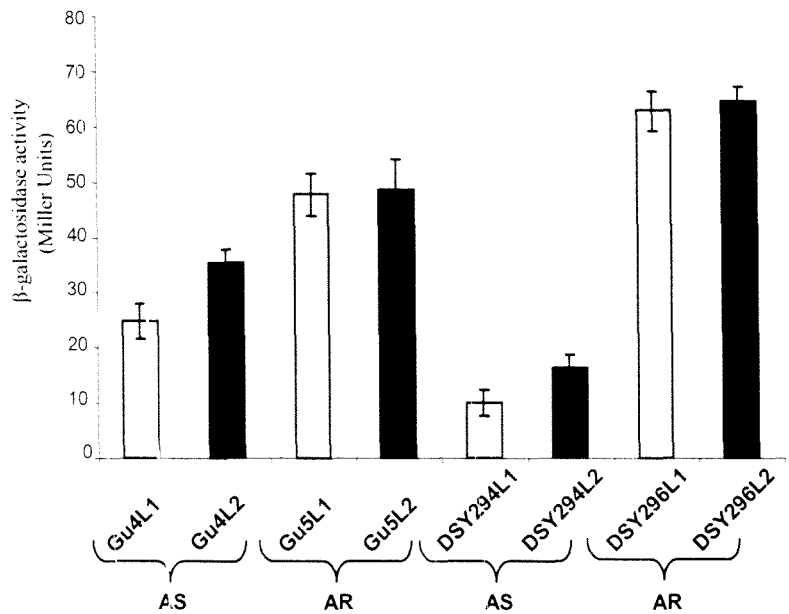
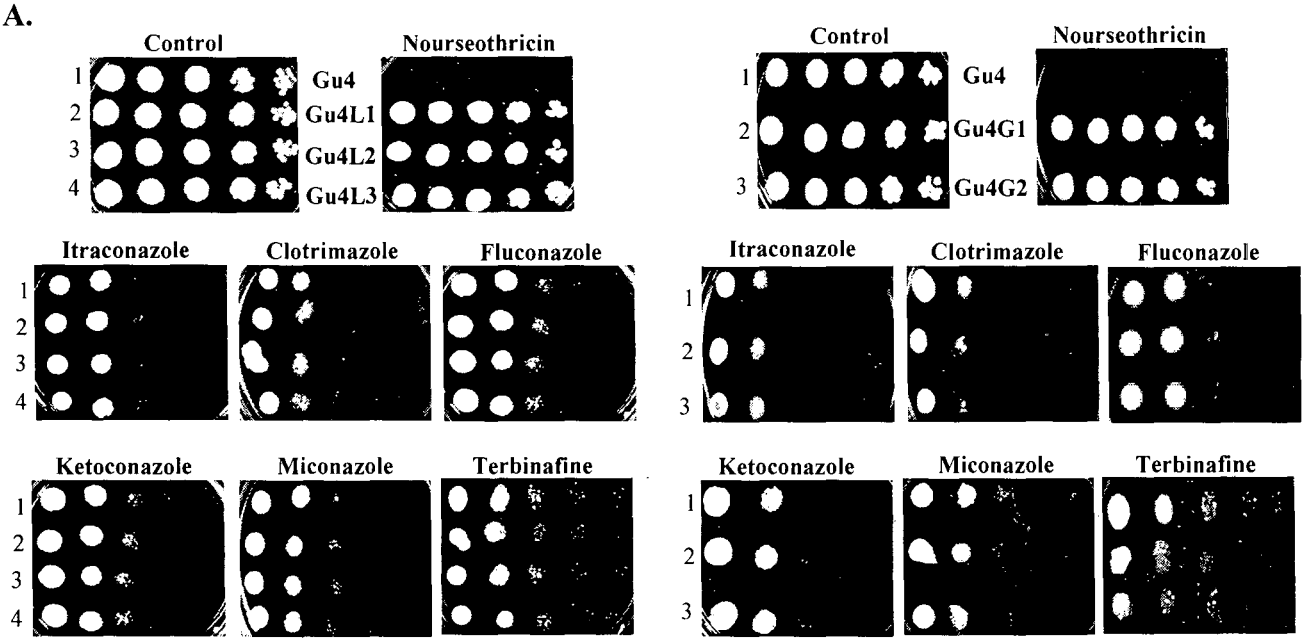


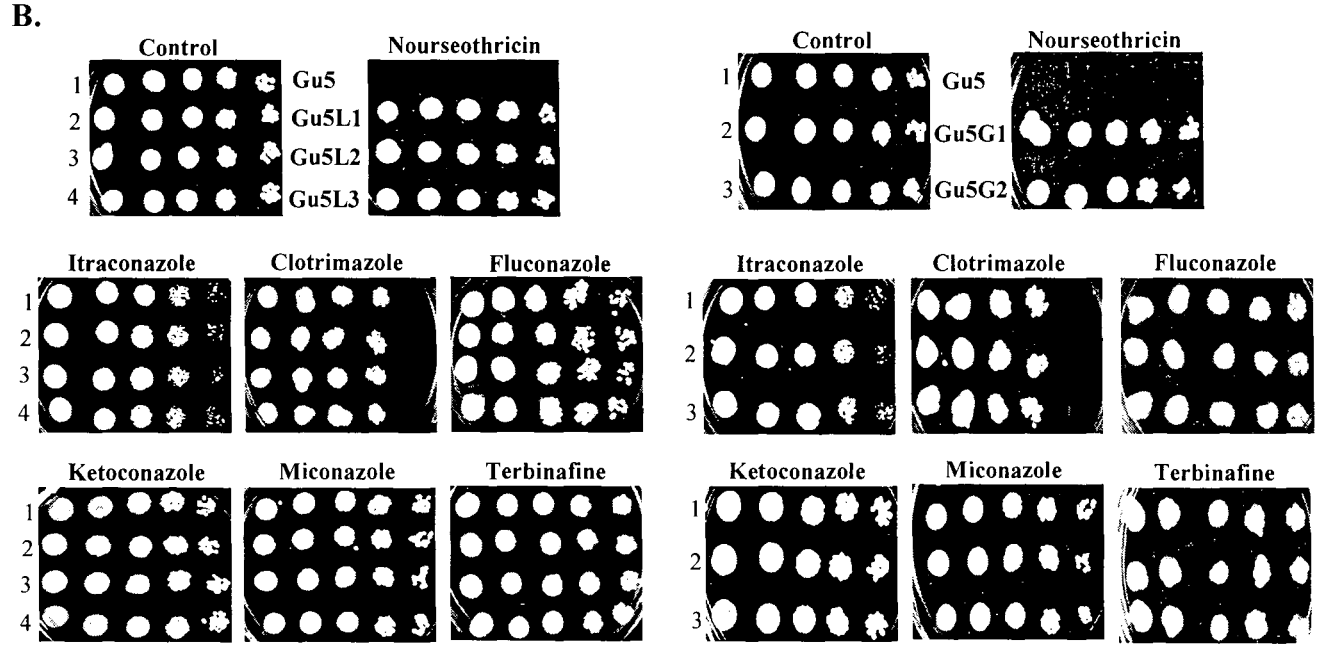
Figure 32. Qualitative and quantitative assay of β-galactosidase activity of *lacZ* reporter fusion integrants at *ACT1* or *CDR1* locus in AS and AR isolates.

(A) Transformants harboring the chromosomally integrated transcriptional fusion (P_{CDR1}-*lacZ*) at *ACT1* (left) and *CDR1* locus (right) and their corresponding parental strain (without *lacZ*) were streaked on minimal media plate containing X-gal and photographed after 3 days growth at 30°C. The positions of the individual strains on the plates are shown in the scheme (middle). **(B)** The transcriptional reporter fusion (P_{CDR1}-*lacZ*) transformants integrated at the *ACT1* and *CDR1* locus were designated as Gu4L1, Gu5L1, DSY294L1, DSY296L1 and Gu4L2, Gu5L2, DSY294L2, DSY296L2 respectively. β-galactosidase quantitative reporter activities of each transformants were determined as described in Materials and Methods. The values are means ± Standard Deviations (*indicated by the bars*) of three independent experiments with duplicate measurements of two independent clones. AS and AR stands for azole-susceptible and azole-resistant isolates. Empty and filled bars indicate *lacZ* transcriptional (P_{CDR1}-*lacZ*) fusion transformants at *ACT1* and *CDR1* locus respectively in both AS and AR backgrounds.

A.


Control
Nourseothricin
1
2
3
4
Gu4
Gu4L1
Gu4L2
Gu4L3
Control
Nourseothricin
1
2
3
Gu4
Gu4G1
Gu4G2
Itraconazole
Clotrimazole
Fluconazole
1
2
3
4
Itraconazole
Clotrimazole
Fluconazole
1
2
3
Ketoconazole
Miconazole
Terbinafine
1
2
3
4
Ketoconazole
Miconazole
Terbinafine
1
2
3

B.


Control
Nourseothricin
1
2
3
4
Gu5
Gu5L1
Gu5L2
Gu5L3
Control
Nourseothricin
1
2
3
Gu5
Gu5G1
Gu5G2
Itraconazole
Clotrimazole
Fluconazole
1
2
3
4
Itraconazole
Clotrimazole
Fluconazole
1
2
3
Ketoconazole
Miconazole
Terbinafine
1
2
3
4
Ketoconazole
Miconazole
Terbinafine
1
2
3

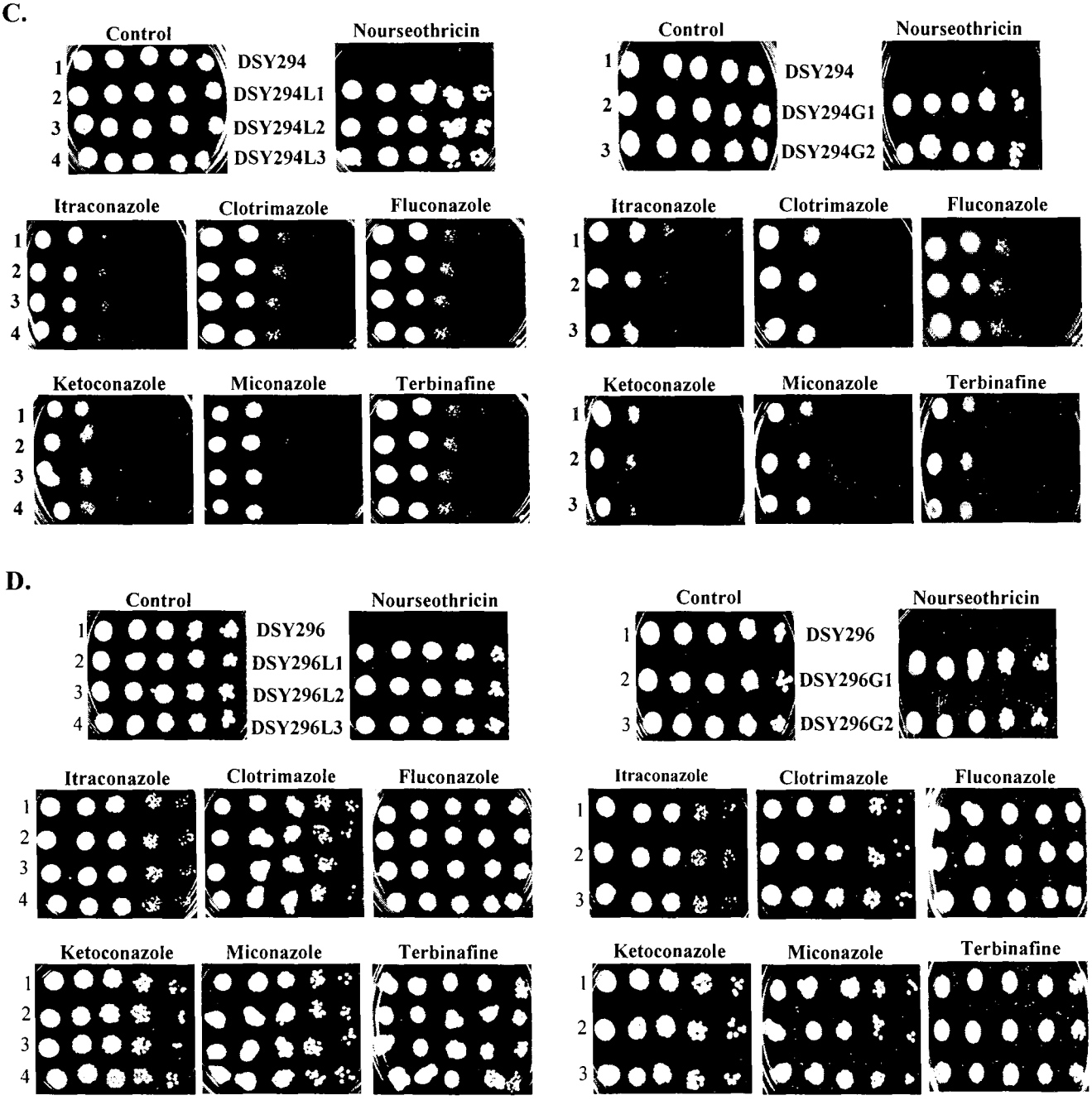


Figure 33. Drug resistance profiles of the *C. albicans* wild type, transcriptional and translational reporter (*GFP/lacZ*) fusion transformants in both AS and AR isolates.

Drug resistance profiling was determined by the spot assay which were done as described previously. In the spot assay, 5 µl of fivefold serial dilutions of each yeast culture (A_{600} = 0.1) was spotted onto YEPD plates in the absence (control) and the presence of the following drugs- Nourseothricin (200 µg/ml), Itraconazole (0.025 µg/ml), Clotrimazole (0.025 µg/ml), Fluconazole (2 µg/ml), Ketoconazole (0.025 µg/ml), Miconazole (0.05 µg/ml) and Terbinafine (0.1 µg/ml). Growth differences were evaluated by using drug-free controls following incubation of the plates for 48 h at 30°C. Growth was not affected by the presence of the solvents used for the drugs (data not shown). **(A)** Gu4 **(B)** Gu5 **(C)** DSY294 **(D)** DSY296 isolates and their transformant derivatives. Nourseothricin has been used as a transformants control. Itraconazole, Clotrimazole, Fluconazole, Ketoconazole, Miconazole and Terbinafine are well known substrates of *CDR1* (Kohli *et al.*, 2001 and 2002).

transcriptional/post-transcriptional control mechanisms are involved in the up-regulation of *CDR1* expression in AR isolates.

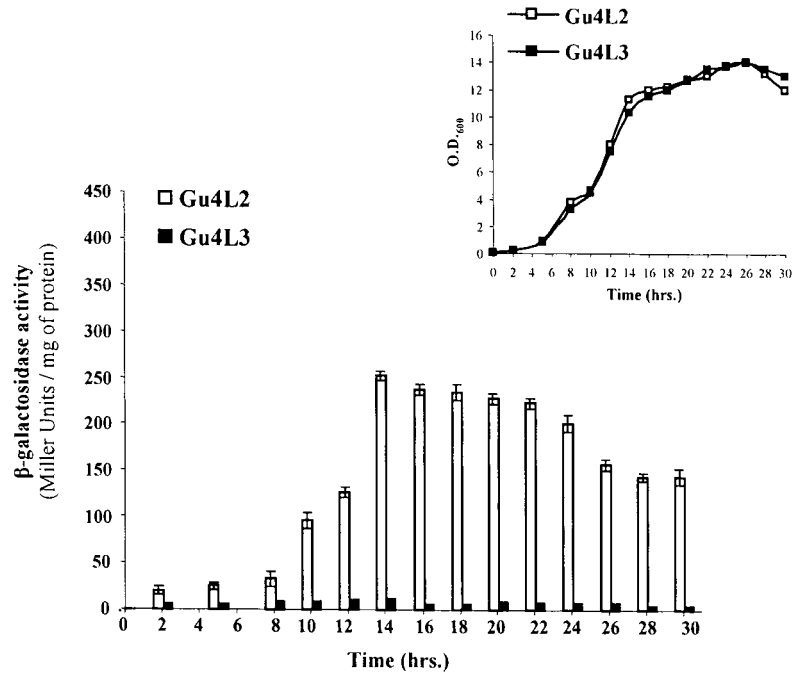
3.1.2.4 Transcriptional rate of *CDR1* is increased in AR isolates.

We first tested whether the transcription rate of *CDR1* was elevated in the AR isolates. For this purpose, transcription run-on assays were performed. Both AS and AR isolates were grown to an OD_{600} of ~1.0 and the cells were permeabilized with the detergent N-lauryl sarcosine sodium sulfate (sarkosyl) for the isolation of intact nuclei (Lyons and White, 2000; Garcia-Martinez *et al.*, 2004). The subsequent incubation of isolated nuclei with transcription buffer and radio-labeled [α-^{32}P]UTP reinitiated the transcription (*see Material and Methods*). The *in vivo* labeled nascent RNAs were then used as probes in reverse Northern hybridizations with dot blotted *CDR1* specific PCR amplified DNA. As controls, pACT1 plasmid DNA, containing the constitutively expressed *ACT1* gene, and the empty vector pBluescript were also dotted onto the membranes. As shown in Figures 35A and B, the AR isolates exhibited an increased transcription rate of *CDR1* as compared with the AS isolates (5-fold for Gu5 *versus* Gu4 and 7-fold for DSY296 *versus* DSY294).

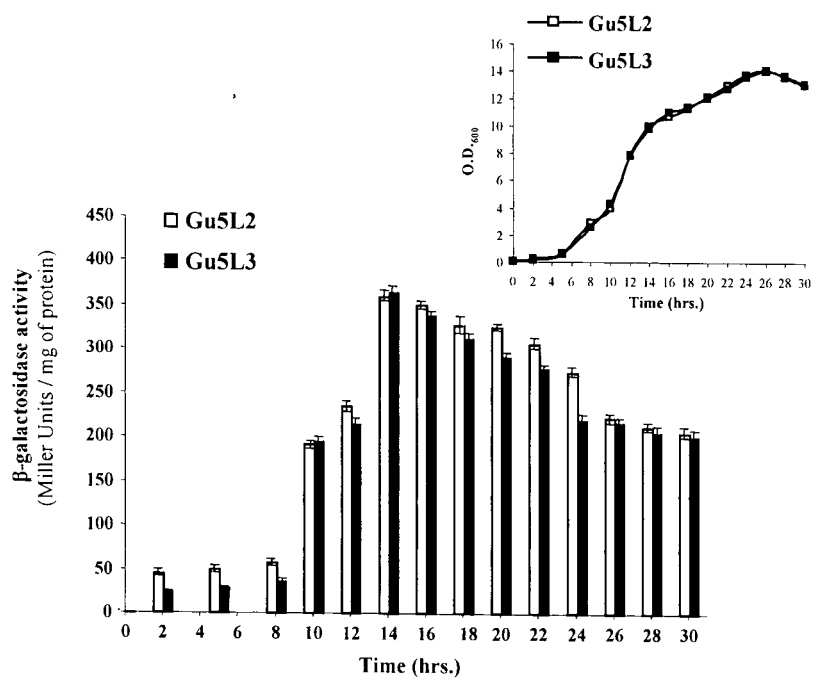
3.1.2.5 *CDR1* mRNA stability is increased in AR isolates.

To investigate if in addition to the increased transcription rates post-transcriptional events also contribute to the higher *CDR1* expression in drug resistant strains, we determined *CDR1* mRNA stability in the AS and AR isolates. To this end, we exploited an effective sulphur containing purine analogue, thiolutin, as a potent inhibitor of *de novo* transcription to determine mRNA stability in *C. albicans* (Kebaara *et al.*, 2006; Setiadi *et al.*, 2006). Thiolutin affected ^{3}H-Uridine incorporation into nascent RNAs, in a concentration dependent manner. About 40 µg/ml of thiolutin inhibited ~95% of the ^{3}H-Uridine incorporation in total RNA (Figure 36A). Methylene blue staining revealed no decline in cell viability of AS and AR isolates treated with 40 µg/ml thiolutin, although growth was inhibited to a certain extent (Figure 36B). This optimized thiolutin concentration was subsequently used for the mRNA chase assays. Total RNA was isolated at different time points after transcriptional inhibition with thiolutin and analysed

A.


Gu4L2
Gu4L3
β-galactosidase activity
(Miller Units / mg of protein)
Time (hrs.)
O.D600
Time (hrs.)

B.


Gu5L2
Gu5L3
β-galactosidase activity
(Miller Units / mg of protein)
Time (hrs.)
O.D600
Time (hrs.)

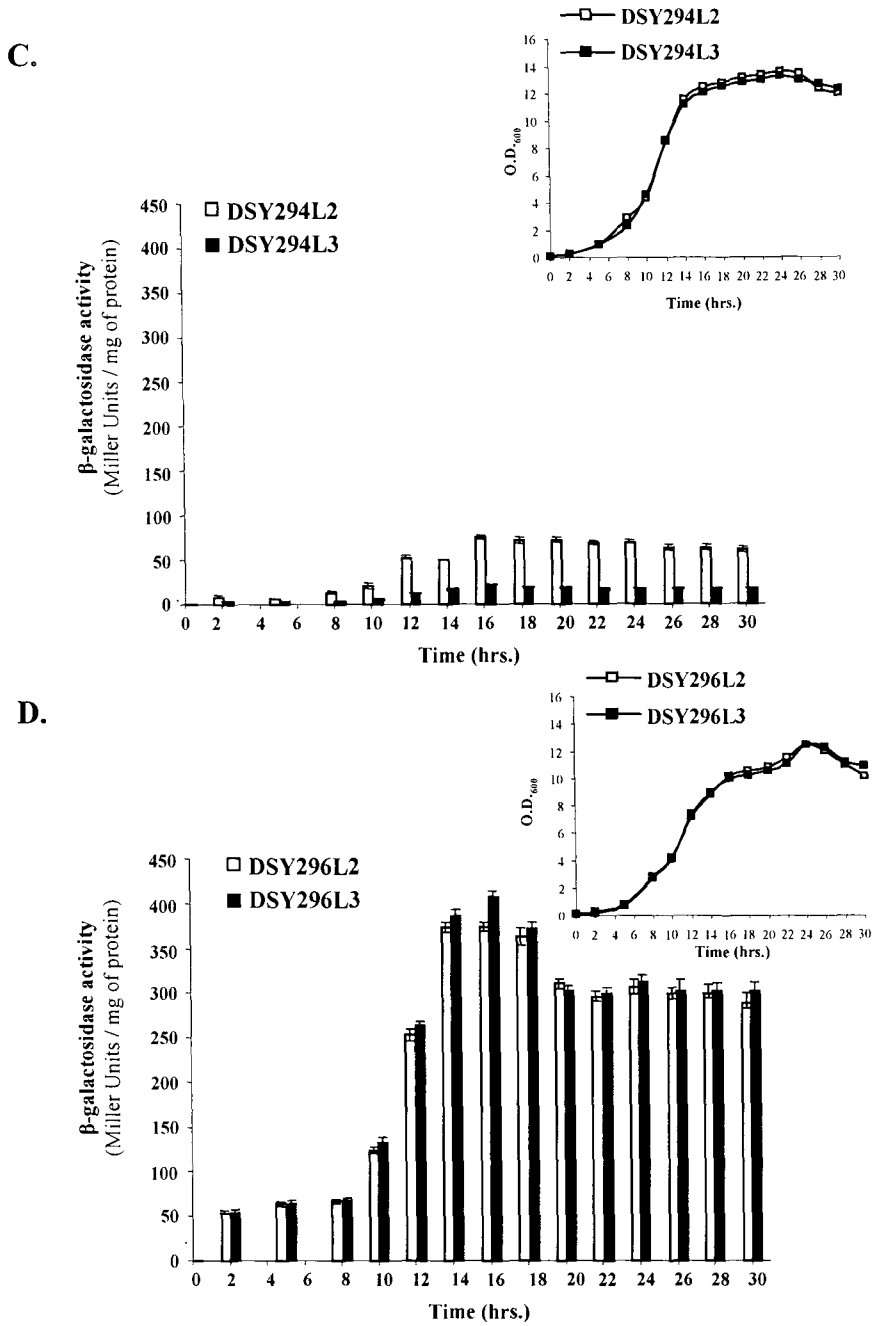
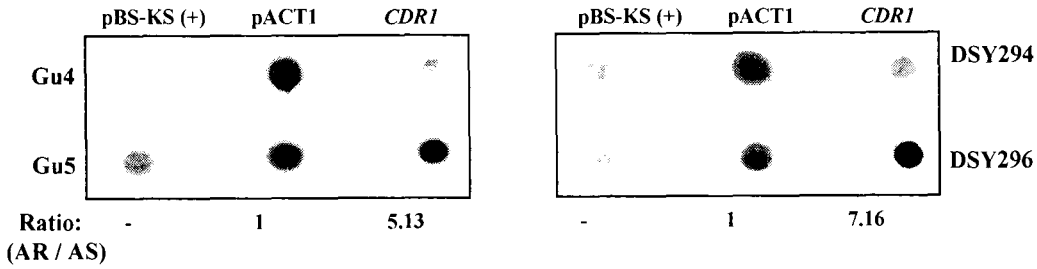


Figure 34. β-galactosidase reporter activity of *lacZ* reporter fusion integrants of AS and AR isolates during growth phase.

Transcriptional fusion (P_{CDR1}-*lacZ*) and translational fusion (P_{CDR1}-*CDR1-lacZ*) reporter transformants of each isolates were grown from an initial OD_{600} of 0.1 in YEPD broth and withdrawn at the indicated time points of growth for β-galactosidase reporter activity (A, B, C, D). The inset depicts growth curves of the P_{CDR1}-*lacZ* (—□—) and P_{CDR1}-*CDR1-lacZ* (—■—) reporter transformants in AS and AR isolates. Negative control parental strain (without *lacZ* fusion constructs) reporter activity value was always below 0.5 Miller Units and it was subtracted from the reporter activity of each corresponding transcriptional and translational fusion transformants. The values are means ± Standard Deviations (indicated by the bars) of three independent experiments with duplicate measurements of two independent clones. **(A)** Gu4 transformants **(B)** Gu5 transformants **(C)** DSY294 transformants **(D)** DSY296 transformants. Empty and filled bars indicate transcriptional (P_{CDR1}-*lacZ*) and translational fusion (P_{CDR1}-*CDR1-lacZ*) transformants in both AS and AR backgrounds.

A.



B.

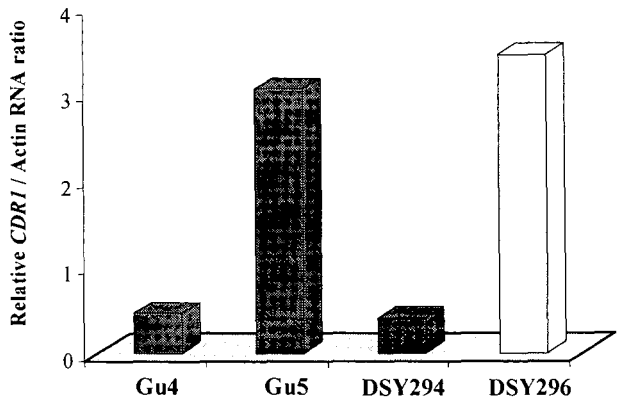


Figure 35. Transcription Run-On (TRO) analysis of AS and AR isolates.
(A) Approximately 2 μg each of *CDR1*, pACT1 and empty vector pBlueScript-KS (+) DNA was blotted and immobilized on charged nylon membranes (Hybond-N^+; Amersham Pharmacia Biotech) using a dot blot assembly apparatus. The blots were probed with total labeled nuclear run-on RNA as described in Materials and Methods. Hybridization signal intensities of nuclear RNA were quantified using densitometry scanning of phosphorimages. DNA from a pBlueScript-KS (+) plasmid was used as negative control for non-specific binding of nuclear RNA to a random DNA fragment. Signal intensities of each isolate was subtracted from the negative control and subsequently normalized to the intensity corresponding to their AS isolate. AR/AS ratio indicates the normalized nuclear RNA intensity between AR and AS isolates. **(B)** Relative intensity of *CDR1* with respect to actin RNA of each isolate is plotted.

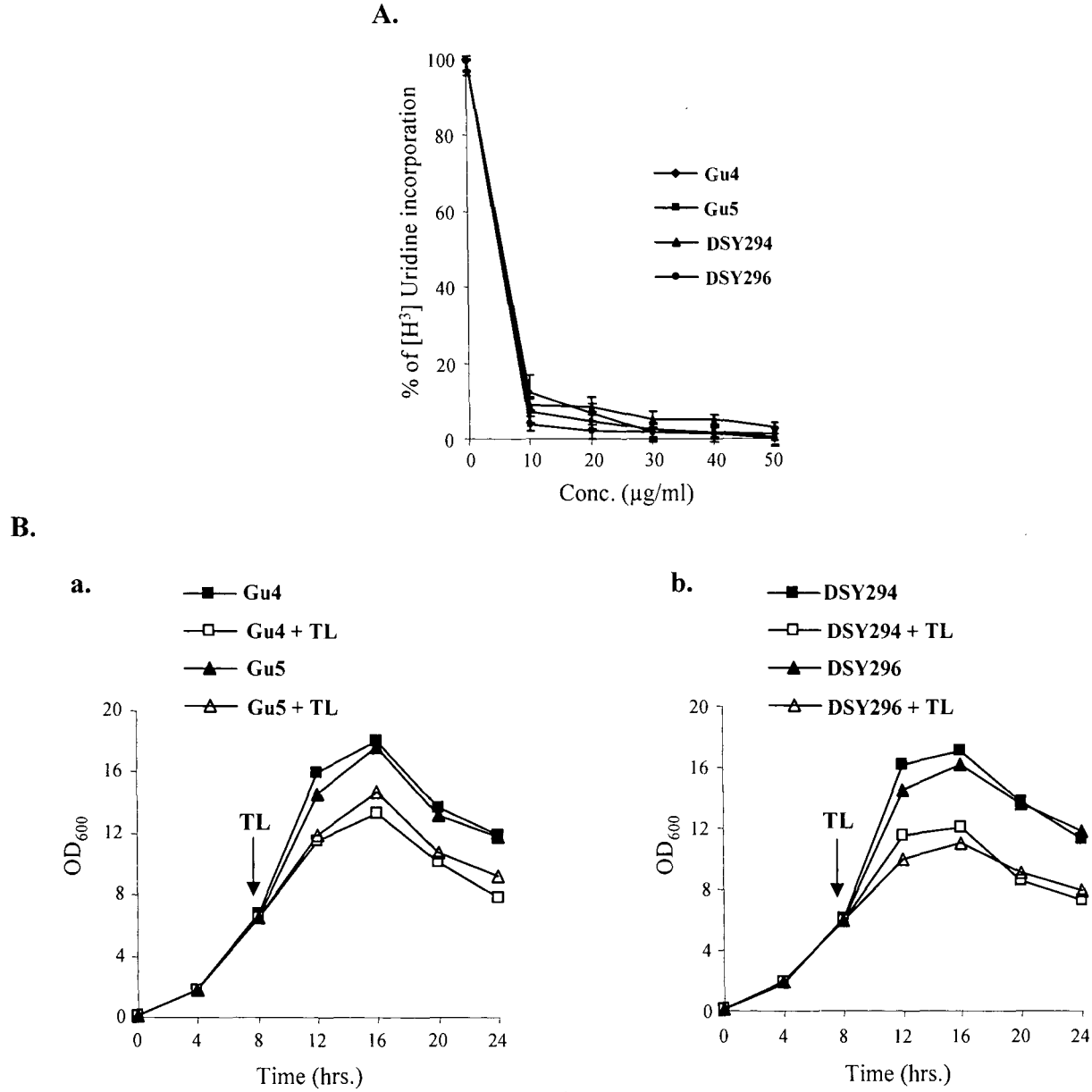


Figure 36. Effect of thiolutin on *in vivo* RNA synthesis and growth of clinical *C. albicans* isolates.

(A) Radio-labeled uridine was added to the cultures in mid-exponential growth phase as described under *Material and Methods*. Thiolutin was added at a linear concentration ranging from 0 to 50 µg/ml. Negative control includes no thilutin addition to cultures. RNA synthesis was followed by measuring *in vivo* incorporation of radiolabeled uridine by quantifying the TCA-precipitable counts in Liquid Scintillation analyzer, normalized with the corresponding untreated cells (*which was considered as 100%*), expressed as a percentage of maximum and plotted as a *line graph.* **(B)** Growth curves of cultures, which remained untreated (*closed square and triangle for AS and AR respectively*) or after addition of thiolutin (*open square and triangle for AS and AR respectively*) at the indicated time. The time points of thiolutin addition are marked by arrows. Growth was not affected by the presence of the solvents used for the inhibitors (*data not shown*). **(a)** Gu4/Gu5 **(b)** DSY294/DSY296.

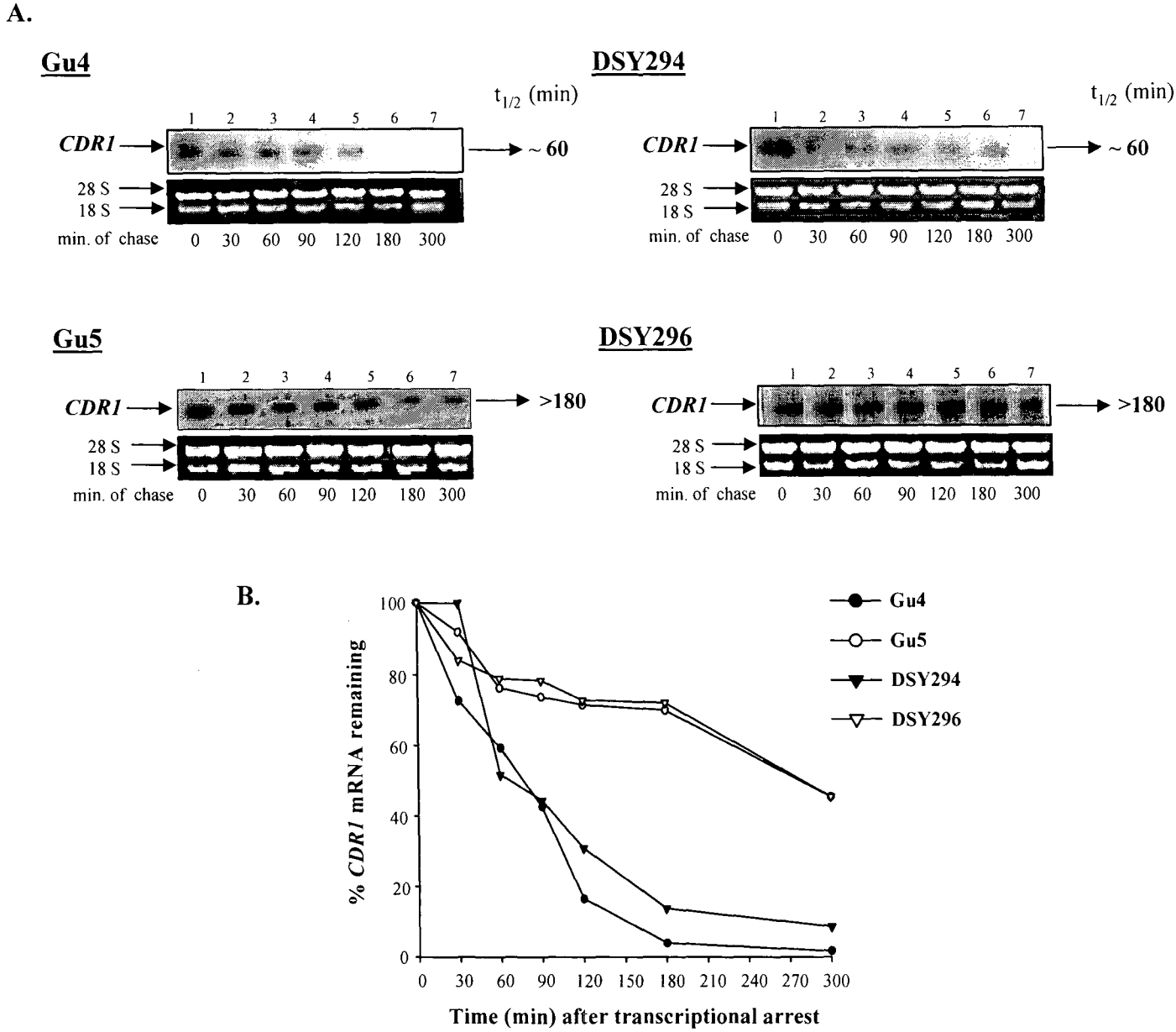


Figure 37. *CDR1* mRNA decay assay.

Exponentially growing cultures of *C. albicans* were incubated with the optimized thiolutin concentration (40 µg/ml) to inhibit ongoing *in vivo* transcription. Total RNA was isolated at the indicated times thereafter and fractionated on a 1% (*w/v*) agarose 2.2 M formaldehyde denaturing gel. **(A)** The gel was stained with ethidium bromide before blotting to monitor equal loading of the RNA and subsequently blotted onto a charged nylon membrane. The blot was hybridized with a *CDR1* specific probe. Time points in minutes are indicated below each phosphorimage. **(B)** The hybridization signals were quantified using densitometry scanning in a phosphorimager. Signal intensity at each time point was normalized to that of time T_0 (*expressed in percentage*) and plotted as described in *Materials and Methods*.

by RNA gel blots (Figure 37A). After probing the blots with a *CDR1* specific probe, hybridization signals were quantified by densitometry scanning in a phoshorimager. Figure 37B depicts a typical *CDR1* mRNA decay profile in the AS and AR isolates over a 300 min period from one of these experiments. *CDR1* mRNA could be detected in both AS isolates Gu4 and DSY294 at time T_0 and the signal intensity diminished progressively with time (mRNA half-life was approximately 60 minutes). The turnover of the *CDR1* transcript occurred much more slowly in the AR isolates Gu5 and DSY296, with a half-life of >180 minutes. These results demonstrated that the *CDR1* mRNA stability was increased in the AR isolates as compared to the AS isolates.

3.1.2.6 Cdr1 protein stability does not differ in AS and AR isolates.

To test whether increased protein stability might also contribute to the high Cdr1p levels in AR isolates, cycloheximide chase assays were performed. Cycloheximide is a translation elongation inhibitor in eukaryotes and a previous study reports that 25mM of cycloheximide is an efficient inhibitor and upto 0.4% of *de novo* total protein synthesis blockage has been reported in a clinical susceptible isolate IFM 40009 (ATCC48130) (Imanishi *et al.*, 2004). Therefore it was important to determine the effective cycloheximide concentration for *de novo* total protein synthesis inhibition in these isolates. For this purpose, we used 0 to 100 mM of cycloheximide concentration range and checked its effect on the inhibition of labeled amino acid incorporation. At 75 mM of cycloheximide concentration, there was an 85% and 80% inhibition in the incorporation of labeled amino acid into the nascent protein synthesized in AS and AR isolates, respectively (Figure 38). Of note, extent of inhibition was much more pronounced in AS isolates than AR isolates below the concentration of 75 mM. This observation in agreement with the fact that cycloheximide is a substrate for Cdr1p and its over-expression in AR isolates ensures its efficient efflux. Total crude protein extracts were isolated at different times after treatment of the cells with an optimized concentration (75 mM) of cycloheximide (*as described above*) and analysed by Western immunoblotting with a rabbit polyclonal anti-Cdr1p antibody. Figure 39A shows the Western blot of the decay experiment while Figure 39B shows the quantitative decay profile. The half-life of Cdr1p was similar in AS and AR isolates and was calculated to be approximately 90 min.

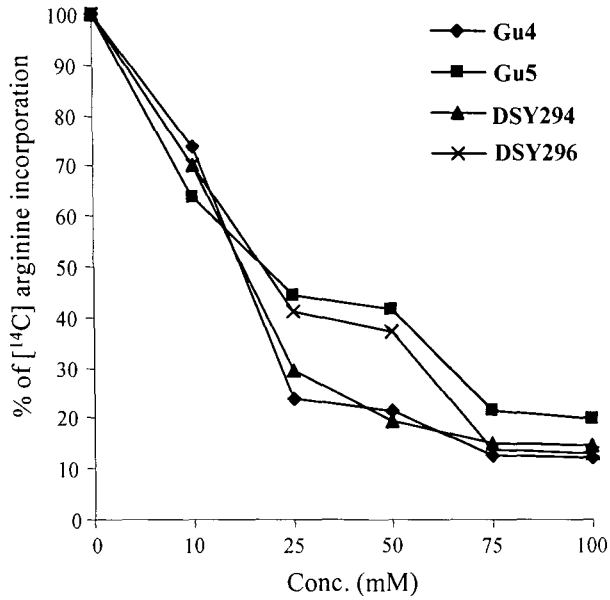
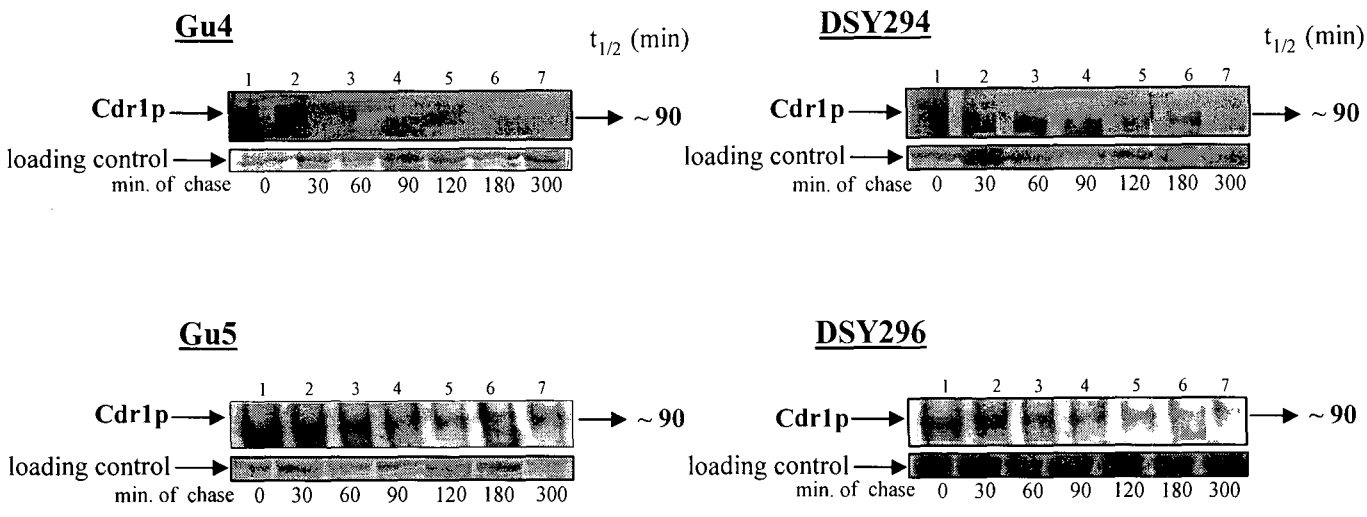


Figure 38. Cycloheximide effect on *in vivo* protein synthesis.
Radio-labeled arginine was added to the cultures in mid-exponential growth phase as described under *Material and Methods*. Cycloheximide was added at a linear concentration ranging from 0 to 100 mM. Negative control includes no cycloheximide addition to cultures. Aliquots of cells were collected after 1 h of treatment and protein synthesis inhibition was followed by measuring *in vivo* incorporation of radio-labeled arginine by quantifying the detergent lysed cell counts in Liquid Scintillation analyzer. Radioactive counts were plotted as a % inhibition of radio-labeled incorporation, normalized with the corresponding untreated cells (*assuming 100% incorporation*) expressed as a percentage of maximum and plotted as a *line graph.*

A.



B.

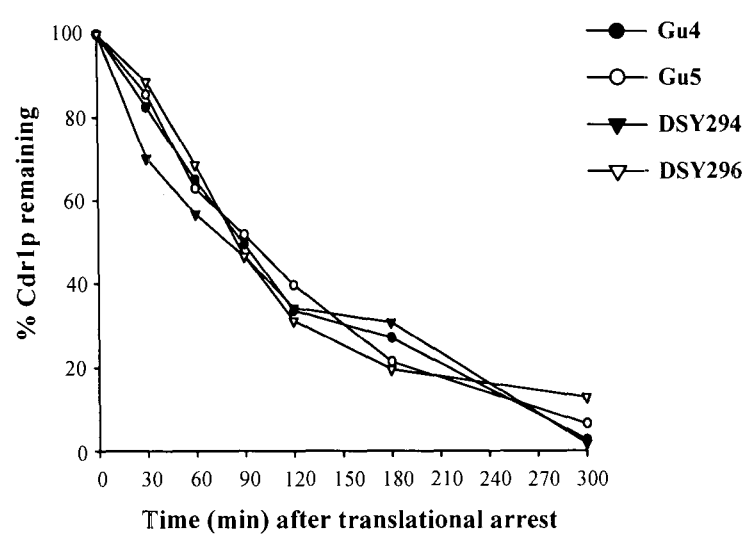


Figure 39. Cdr1p decay assay.

(A) Exponentially grown cultures of *C. albicans* were translationally halted at 30°C by addition of 75 mM of cycloheximide for 1h. Whole cell extracts were prepared at the indicated times after cycloheximide treatment. For AR isolates ~20 µg, while for AS isolates ~30 µg (because of relatively low expression of Cdr1p) of crude extract for each time was loaded and separated by SDS-PAGE. An equal loading of protein was assessed from coomassie stained gel. Cdr1p was detected using a polyclonal anti-Cdr1p antibody. The Cdr1p specific bands were subsequently quantified by densitometry scanning in a phosphorimager. **(B)** Band intensities (*represented as a percentage of the value at time* T_0) for each isolate were plotted against the chased time.

3.1.3 Discussion

In this study, we used two pairs of matched azole-susceptible (AS) and azole-resistant (AR) *C. albicans* clinical isolates to study the mechanisms of *CDR1* over-expression in AR isolates. Our results demonstrate that both increased transcriptional activation and enhanced mRNA stability contribute to the increased *CDR1* expression in these drug resistant isolates. Interestingly, we found that in the AS isolates reporter fusions with the *CDR1* coding region were expressed at lower levels than fusions in which the reporter genes were directly fused to the *CDR1* promoter, whereas in the AR isolates the two types of reporter fusions were expressed at comparable levels. This would mean that sequences in the *CDR1* coding region can also contribute to the increased *CDR1* expression in AR isolates.

It has been shown previously that *CDR1* over-expression in *C. albicans* is caused by an increased *CDR1* transcription rate in AR isolates as compared with AS isolates (24). Our transcription run-on experiments confirmed that the transcriptional initiation rate from the *CDR1* promoter was 5- to 7-fold higher in the AR isolates than in the AS isolates used in the present study (Figure 35). The *CDR1* upstream region contains many sequence elements which are involved in the regulation of *CDR1* expression (Puri *et al.*, 1999; de Micheli *et al.*, 2002; Karnani *et al.*, 2004; Gaur *et al.*, 2004 and 2005), however, no sequence differences were found in the *CDR1* upstream region of these matched pairs of AS and AR isolates (de Micheli *et al.*, 2002; Gaur *et al.*, 2005 and Figure 40). In line with this, it has recently been shown that a gain-of-function mutation in the transcription factor *TAC1*, which controls *CDR1* expression, causes *CDR1* up-regulation in the AR isolate DSY296 (Coste *et al.*, 2004). In order to evaluate if in addition to transcriptional activation of *CDR1*, differential mRNA and protein stability also contribute to the enhanced Cdr1p levels in AR isolates, we performed thiolutin and cycloheximide chase assays and observed that the up-regulation of *CDR1* mRNA in AR isolates was due to an increase in mRNA half-life (>180 min), which was approximately 3-fold higher than in AS isolates (Figure 37). In contrast, no difference in Cdr1p protein stability was observed between AS and AR isolates (Figure 39). There are examples in other organisms where over-expression of efflux pumps can be caused by increased mRNA stability. An increase in the mRNA half-life of *MDR1* (a *CDR1* homologue in humans) has been shown to

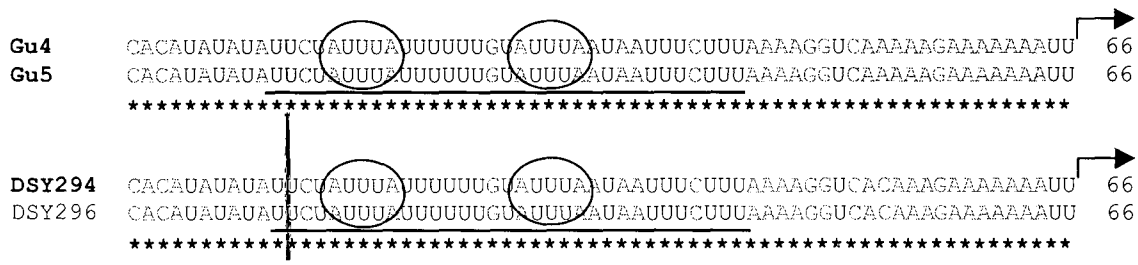


Figure 40. Pair-wise sequence alignment and analysis of *CDR1* 5′UTR.
Alignment of *CDR1* 5′UTR (66 bases) from the two matched pairs of clinical isolates was obtained using the *Clustal W* program (*version 1.83*). Conserved residues are indicated by *asterisks*. *Ellipses* denote the AU-rich elements (‘*AUUUA*’). *Continuous underlines* denote the polyuridine (*polyU*) rich tract respectively. Numbers at the right are relative to the ‘*AUG*’ translational start codon (*considered as position +1*).

contribute to doxorubicin and colchicine resistance in the myelogenous leukemic cell line K562 (Yague *et al.*, 2003). An enhanced mRNA stability of *bmr3* encoding a MultiDrug Transporter (MDT) also leads to an MDR phenotype in *Bacillus subtilis* (Ohki *et al.*, 2004). In addition, the reported MDR phenotype of *Entamoeba histolytica* trophozoites is also caused by transcriptional activation (Nieto *et al.*, 2005) as well as an increase in mRNA stability of the *EhPgp5* gene (Lopez-Camarillo *et al.*, 2003).

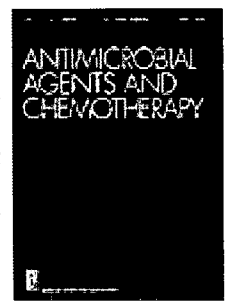
Notably though, *cis*-determinants located in the 3'UTR regulate the degradation of mRNA (Ross, 1996). Among these *cis* elements, AURE (Adenylate-Uridylate Rich Element) motifs of 3' UTR involved in destabilization of their corresponding mRNAs are of prime importance (Ross, 1996; Prokipcak *et al.*, 1999; Lopez-Camarillo *et al.*, 2003). Several reports have also suggested a relationship between the relative affinity of a given RNA for RNA-binding protein(s), and the stability of an mRNA containing these sequences (Ross, 1996; Prokipcak *et al.*, 1999). Our preliminary results reveal that *CDR1* 3'UTR is ~78% AU-rich, and also posseses several putative consensus binding sequences for regulatory RNA-binding protein(s). Therefore, any contribution of *CDR1* 3' UTR *cis* elements and of the mutation or alteration in *trans*-acting regulatory factor(s) corresponding to these conserved elements in determining mRNA stability between AS and AR isolates requires an in-depth analysis.

Our results with the reporter fusion transformants also suggest that sequences in the *CDR1* coding region could also be an important contributor for increased *CDR1* expression in AR isolates. In this context, it should be mentioned that Synonymous (S-SNPs) and Non-Synonymous Nucleotide Polymorphisms (NS-SNPs) have been observed in the *CDR1* coding region, but so far none of these has been linked to *CDR1* over-expression (Holmes *et al.*, 2006; Haque *et al.*, 2007). Our present study did not consider the role of these allelic differences in sustained over-expression of *CDR1* in AR isolates. However, a recent study has reported that a silent polymorphism does not influence the human *P-gp/MDR1* mRNA and protein expression, but affects post-translational events in terms of timing of co-translational folding and membrane insertion (Wang *et al.*, 2005; Kimchi-Sarfaty *et al.*, 2007).

In conclusion, our results demonstrate for the first time that *CDR1* is regulated by both transcriptional as well as post-transcriptional events. Our finding that the acquisition of

azole resistance involves transcriptional activation as well as decreased mRNA turnover opens up new possibilities for treatment regimes to circumvent MDR in *C. albicans*. In this context, it is worth mentioning that the intervention of over-expressing *MDR1* in multidrug-resistant (MDR) cell lines by verapamil (Muller *et al.*, 1995) and ecteinascidin-743 (E-743) (Scotto and Johnson, 2001) has been reported to be due to the transcriptional down-regulation of the gene.

✓ The work presented in this chapter has been published as **"Transcriptional activation and increased mRNA stability contribute to over-expression of *CDR1* in azole-resistant *Candida albicans*"** in *Antimicrobial Agents and Chemotherapy* (2008) **52**:1481-1492.



"However beautiful the strategy,
you should occasionally look at the results"

Section-2

Results and Discussion

3.2 Differential RNA-protein interaction and poly(A) tail length contributes to increased *CDR1* mRNA stability in azole resistant clinical isolates of *Candida albicans*

3.2.1 Review of literature

One of the major mechanisms of Multidrug resistance (MDR) phenotype in Azole-Resistant (AR) *C. albicans* clinical isolates is characterized by the over-expression of genes encoding ATP Binding Cassette (ABC) such as *CDR1/CDR2*), (Prasad *et al.*, 1995; Sanglard *et al.*, 1995 and 1997; White, 1997; White *et al.*, 1998; Franz *et al.*, 1998; Lopez-Ribot *et al.*, 1998; Sanglard and Odds, 2002; Akins, 2005; Manoharlal *et al.*, 2008) or Major Facilitators (MFS) Superfamily such as *CaMDR1* multidrug transporters (Wirsching *et al.*, 2000; Riggle and Kumamoto, 2006; Pasrija *et al.*, 2007). Once acquired, MDR is a stable phenotype that is maintained in azole resistant clinical isolates even in the absence of selection pressure by the drugs (White *et al.*, 1997 and 1997a). This implies that certain genetic alterations take place in the azole-susceptible (AS) isolates resulting in constitutive over-expression of the drug efflux pumps encoding genes in AR isolates. Thus what leads to and regulate over-expression of MDR genes in AR isolates is very critical in designing strategies for therapeutic interventions.

The regulation of *CDR1* has been extensively studied by several groups. It is shown that *CDR1* harbors various consensus (Sp1, AP-1, Y-box) as well as specific Basal (BRE), Negative (NRE) and Drug/Steroid Response Element (DRE and SRE) in the 5' flanking region (Puri *et al.*, 1999; de Micheli *et al.*, 2002; Karnani *et al.*, 2004; Gaur *et al.*, 2004 and 2005). Trans-acting factors regulating *CDR1* have also been identified. For example, Non DiTyrosine 80 (*CaNDT80*) a homolog to a meiosis specific transcription factor (TF) in *S. cerevisiae* has been identified as a potential activator of *CDR1* (Chen *et al.*, 2004). Coste *et al.* (2004) identified a TF, Transcriptional Activator of *CDR* genes (*TAC1*) that binds to the Drug Response Element (DRE) in both the *CDR1* and *CDR2* promoters. Interestingly, TF belonging to the zinc cluster family, Fluconazole Resistance 1 (*FCR1*), (Talibi and Raymond, 1999; Shen *et al.*, 2007) as well as the global repressor,

Thymidine uptake 1 (*Tup1*) acts as a negative regulator of *CDR1* expression (Murad *et al.*, 2001; Yang *et al.*, 2006). A recent genome wide location profiling (ChIP-chip) show that a TF of the zinc cluster family, Uptake control 2 (*Upc2*) which regulates *ERG* genes, also targets *CDR1* (Znaidi *et al.*, 2008). Efforts of several groups have also led to the identification of various *cis*-acting elements such as H_2O_2 Responsive Element (HRE) and Benomyl Responsive Element (BRE) in *CaMDR1* promoter region mediating its up-regulation in AS isolates and its constitutive activation in AR isolates (Hiller *et al.*, 2006; Rognon *et al.*, 2006). Recently a zinc cluster TF, designated as Multidrug Resistance Regulator 1 (*MRR1*) has been identified which is linked to the activation of *CaMDR1* expression (Morschhäuser *et al.*, 2007).

It is thus apparent that transcriptional regulation plays an important role in the mechanism underlying the over-expression of MDR genes, however, the relevance of the post-transcriptional events associated with it are poorly understood. By employing two pairs of matched *C. albicans* clinical isolates in which azole resistance is developed due to the over-expression of *CDR1* during prolonged azole therapy, we have recently shown that the high mRNA levels in AR isolates is predominantly contributed both by its enhanced transcription activation and mRNA stability (Manoharlal *et al.*, 2008). In this study, we further dissected the molecular basis of *CDR1* mRNA turnover. For this, we have identified and characterized the complete 3'-end of the *CDR1* mRNA by the 3'-Rapid Amplification of cDNA Ends (3' RACE)-PCR method and compared the sequence and structure motifs in these matched isolates. Our observations with RNA-Electrophoretic Mobility Shift Assay (R-EMSA) confirmed the existence of differential interaction of RNA-binding protein(s) to the 3'UTR of *CDR1* between AS and AR isolates. Interestingly, there is distinct increase in poly(A) tail in AR isolates which probably also contribute to enhanced mRNA stability (Manoharlal *et al.*, 2008), of *CDR1*. Notably, we also show that rapid and transient *in vitro* induced over-expression of *CDR1* is only contributed by transcriptional activation without affecting its mRNA stability and poly(A) tail length.

3.2.2 RESULTS

We have recently analyzed two pairs of fluconazole-resistant *C. albicans* strains isolated from recurrent episodes of oropharyngeal candidiasis (OPC) in two different human

immunodeficiency virus-positive (HIV^+) AIDS patients (Manoharlal *et al.*, 2008). The CARE-2 fingerprint patterns of these isolates demonstrated that in both the patients, stable azole resistance phenotype in AR isolates (Gu5 and DSY296) was developed from previously susceptible strains (Gu4 and DSY294) (Boerlin *et al.*, 1996; Franz *et al.*, 1999) (Figure 21). The AR in these isolates was predominantly due to the sustained over-expression of major efflux pump protein encoding gene *CDR1* (Figure 24). Our recent study on molecular characterization of *CDR1* and its products (mRNA/protein) in these clinical isolates suggested that the expression of the MDR phenotype is regulated by enhanced transcription activation as well as mRNA stability of *CDR1* transcript (Manoharlal *et al.*, 2008). In this study, we further explored the cause(s) of enhanced mRNA stability of *CDR1* in AR isolates.

3.2.2.1 3'UTR of *CDR1* mRNA display length heterogeneity.

As an initial step to study the post-transcriptional mechanisms involving enhanced *CDR1* mRNA stability, transcription termination sites of *CDR1* in AS and AR isolates were determined by the 3' RACE method. For this, RT-PCR products of *CDR1* of each isolate was generated (Figure 42A, step 1 and 2) using the *CDR1* specific forward primer, CT-*CDR1*-F and oligo$(dT)_{18}$ anchor primer (*see Table 8*), cloned directly into pGEM®T-Easy vector (Promega) and sequenced (Figure 42A, step 3). To further confirm the RT-PCR specificity, subsequent second round of Nested PCR of corresponding *CDR1* 3'UTR harboring clones (Figure 42A, step 3) was performed with 3'UTR specific primer for each isolate(Figure 42A, step 4) (*see Table 8*). 3'UTR products specific to *CDR1* for each isolate were amplified and further subcloned into pGEM®T-Easy vector (Figure 42A, step 5a). Direct sequencing (Figure 42A, step 5b) of multiples of 3'UTR specific clones of AS and AR isolates confirmed and revealed exact transcription termination sites of *CDR1* (Figure 42B). For the matched isolates-Gu4/Gu5 and DSY294/DSY296, 3'UTR length was mapped to 137/163 bases and 162/137 bases, respectively (*position relative to 'UAA' stop codon*).

3.2.2.2 *CDR1* 3'UTR sequences reveal polymorphism.

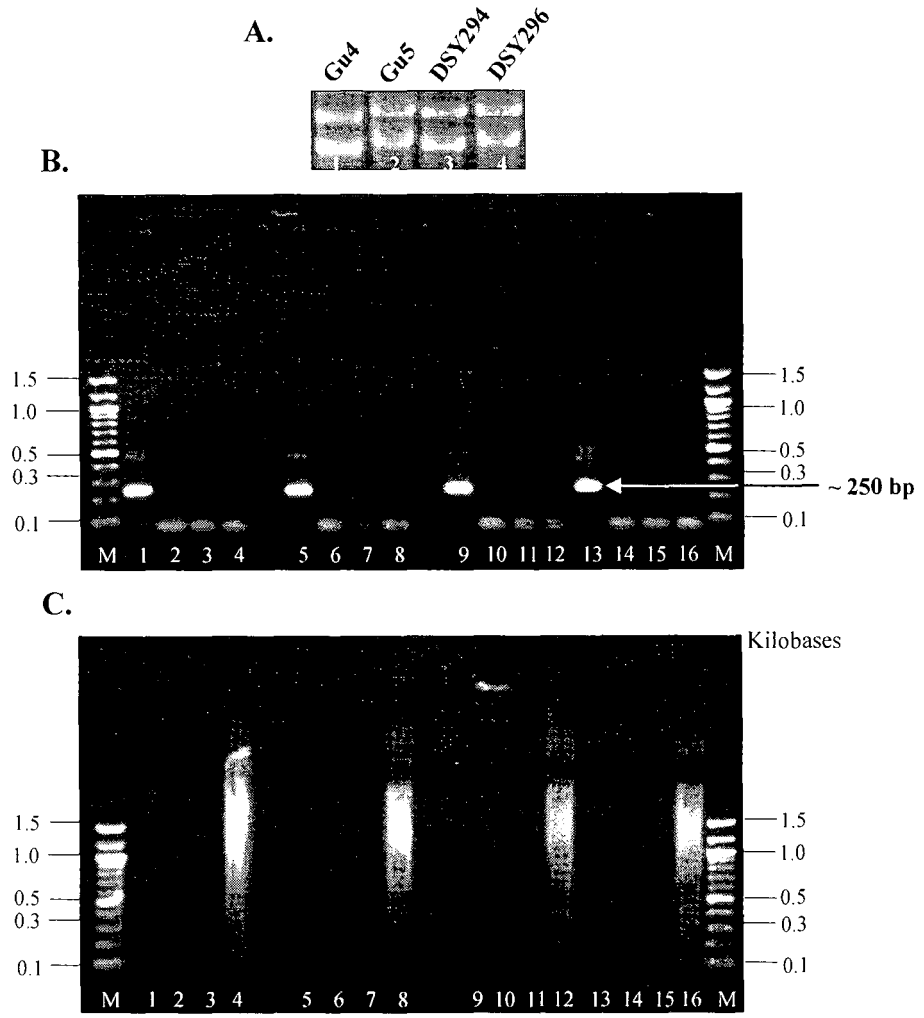
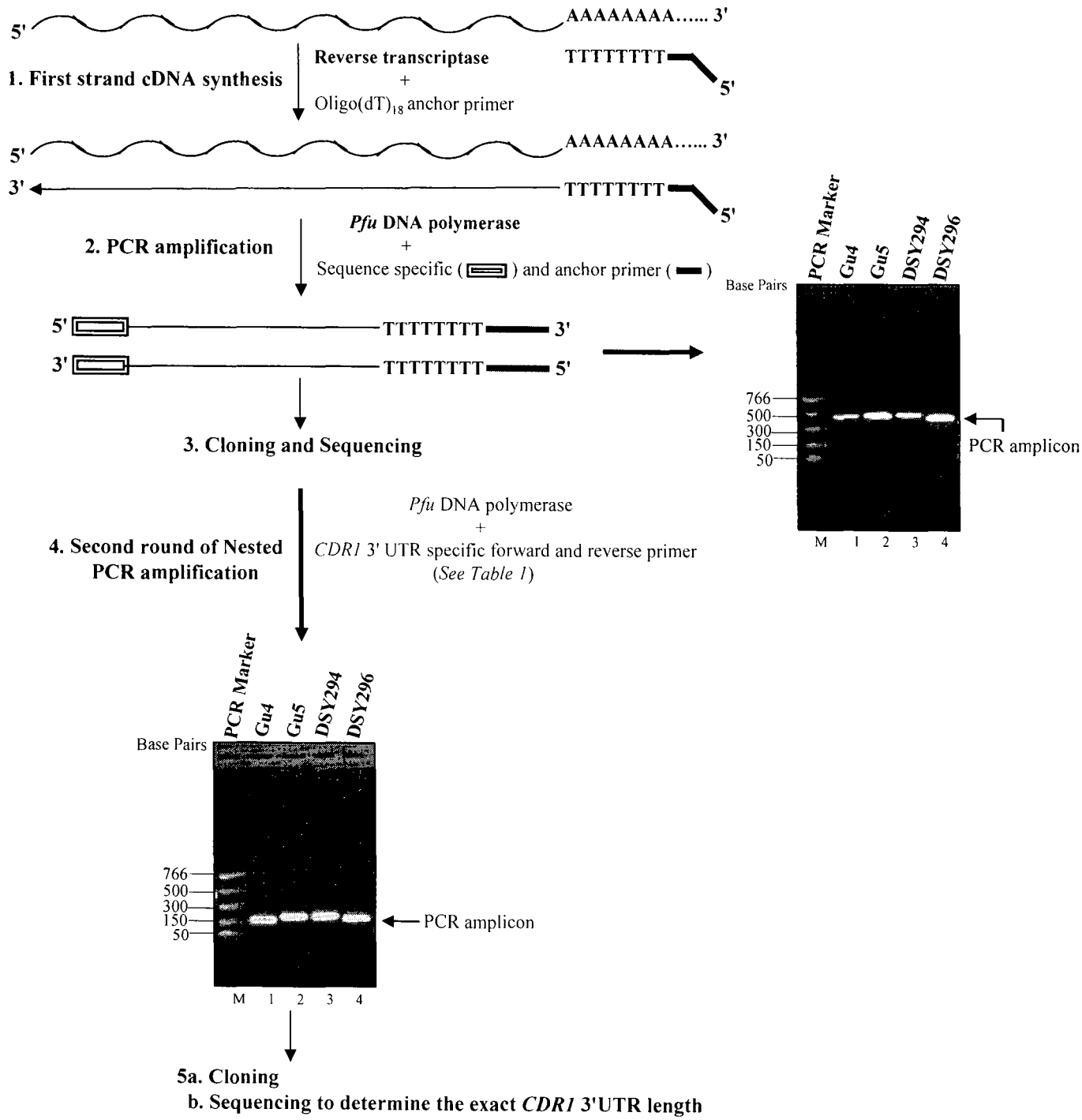
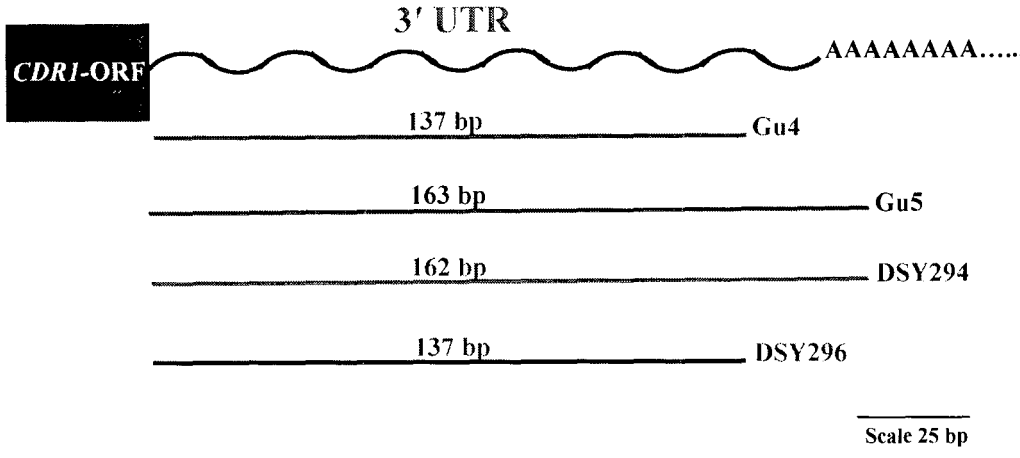


Figure 41. RT-PCR analysis of *CDR1* in clinical *C. albicans* isolates.
(A) Total RNA isolation. RNA isolated from was fractionated (~10 µg) on 1% (*w/v*) agarose, 2.2 M formaldehyde denaturing gels and stained with 0·5 µg/ml of ethidium bromide. **(B)** Assessment of genomic DNA contamination in poly-A^+ RNA preparations. Total RNA isolated was differentially enriched for poly-A^+ RNA as described under *Material and Methods*. Reverse transcription PCR reactions were performed with ~5 µg of poly-A^+ RNA. 15 µl of PCR reaction was electrophoresed on a 2% agarose gel and stained with 0·5 µg/ml of ethidium bromide. *Lanes*: M, 100 bp size marker; *Lanes* 1-4: Gu4; *Lanes* 5-8: Gu5; *Lanes* 9-12: DSY294 and *Lanes* 13-16: DSY296. *Lanes 1,5,9,13*: PCR reaction (with internal primers CDR32 and CDR31; see *table*) with genomic DNA (*positive control*); *Lanes 2,6,10,14*: without genomic DNA (*negative control*); *Lanes 3,7,11,15*: without Reverse Transcriptase (*no-RT, negative control*); *Lanes 4,8,12,16*: with RT. **(C)** cDNA Synthesis. cDNA (~10 µl of each reaction) prepared from 0.5 µg poly-A^+ RNA in the presence of oligo(dT)18 anchor-primer was separated on a 0·8% agarose gel and stained with 0·5 µg/ml ethidium bromide. *Lanes*: M, 100 bp size marker; *Lanes*: M, 100 bp size marker; *Lanes* 1-4: Gu4; *Lanes* 5-8: Gu5; *Lanes* 9-12: DSY294 and *Lanes* 13-16: DSY296. *Lanes 1,5,9,13*: RT-reaction without poly-A^+ RNA (*negative control*); *Lanes 2,6,10,14*: without oligo$(dT)_{18}$ anchor primer (*negative control*); *Lanes 3,7,11,15*: without Reverse Transcriptase (*no-RT, negative control*); *Lanes 4,8,12,16*: cDNA smear visualized with RT.

A.


5'
AAAAAAAA..... 3'
1. First strand cDNA synthesis
Reverse transcriptase
+
Oligo(dT)18 anchor primer
TTTTTTTT
5'
3'
2. PCR amplification
Pfu DNA polymerase
+
Sequence specific ( ) and anchor primer ( )
3. Cloning and Sequencing
4. Second round of Nested PCR amplification
Pfu DNA polymerase
+
CDR1 3' UTR specific forward and reverse primer
(See Table 1)
PCR Marker
Gu4
Gu5
DSY294
DSY296
Base Pairs
766
500
300
150
50
PCR amplicon
M 1 2 3 4
5a. Cloning
b. Sequencing to determine the exact CDR1 3'UTR length

B.



Strains	3′ UTR length (bases)
Gu4	137
Gu5	163
DSY294	162
DSY296	137

Figure 42. Determination of termination sites of *CDR1* mRNA by 3′RACE method.
(A) 3′RACE strategy employed for the detection of *CDR1* 3′UTR length is detailed under *Materials and Methods*. Briefly, the purified poly(A)$^+$-enriched mRNA samples from clinical *C. albicans* isolates were PCR amplified. Following electrophoresis, the amplified PCR products were visualized by staining with ethidium bromide (*shown by thick arrow*) and cloned. To confirm the RT-PCR specificity, another round of 3′ UTR specific Nested PCR amplification of each isolate was performed. The locations of DNA size markers (bp) are marked on the left side of the gel (*lane M*). *C. albicans* isolates are marked above the gels. **(B)** Schematic representations of the mapped 3′UTR of *CDR1*. The *open box* and *wavy lines* represent *CDR1* coding and poly(A) tail harboring 3′-end region respectively, while *thick lines* indicate the mapped 3′UTR length in AS and AR isolates. The numbers on top of thick lines indicate length of 3′ UTR in each isolates.

Since differences in sequence at the 3'UTR could be involved in polyadenylation site selection (Edwalds-Gilbert *et al.*, 1997), and mRNA stability (Russell *et al.*, 1998), we did a comparative *in silico* analysis of mapped 3'UTR fragments of *CDR1* of AS and AR isolates and analyzed its relevant sequence features. 3'UTR sequence alignment of *CDR1* revealed polymorphism at position -7U, U19C and C64U (Figure 43A, *open box,* where the base[s] before and after the number is the sequence from Gu4 and Gu5 isolates, respectively) and an additional extended 26 and 25 base 'AU' (Adenylate-Uridylate) rich stretch at 3'-end for only Gu5 (AR) and DSY294 (AS) isolates, respectively (Figure 43A). 3'UTR of *CDR1* is ~78% AU-rich, represents several putative consensus sequences for cytoplasmic RNA-binding protein(s) (Figure 43B) and has perfect AU-rich elements ('AURE') (Figure 43A, *ellipses box*). The 'AURE' motifs are characterized by the presence of the consensus sequence 'AUUUA' and have been reported to be involved in destabilization of their corresponding mRNAs (Trzaska and Dastych, 2005). The presence of one perfect (UAGU) and two degenerate (A/CAUAAA, AAUAAA/U) yeast polyadenylation signals (Figure 43A, *shadow box*) suggests that mRNA variants could influence the *CDR1* mRNA steady-state levels, as has been reported for other cells (Higgins, 1991; Edwalds-Gilbert *et al.*, 1997; Wickens *et al.*, 1997). A polyuridine (poly'U') rich and Cytoplasmic Polyadenylation Element ('CPE') tract were also predicted (Figure 43A, *Continuous and dis-continuous underlines*, respectively). All nucleotide positions are relative to 'UAA' translational stop codon (*considered as* +1). Notably, alignment of the full-length 3'UTR of *CDR1* with other 3'UTR sequences (163 bp's were randomly selected as the common length) of MDR genes of *C. albicans* (such as *CDR2, CaMDR1 and ERG11*) revealed a high degree of polymorphism (Figure 44).

3.2.2.3 3'UTR of *CDR1* mRNA forms altered secondary structures.

As noted above, the observed polymorphism may affect secondary structures of 3'UTR of *CDR1*. This possibility was explored by carrying out putative folding analyses (Figure 43C). Folding pattern prediction with nearest neighbor rules showed the co-existence of several possible Gibbs free energy of secondary structure conformations. The structure which displayed highest free energy value is expected to make the most stable arrangement. The free energy values for *CDR1* 3'UTR secondary structure were different

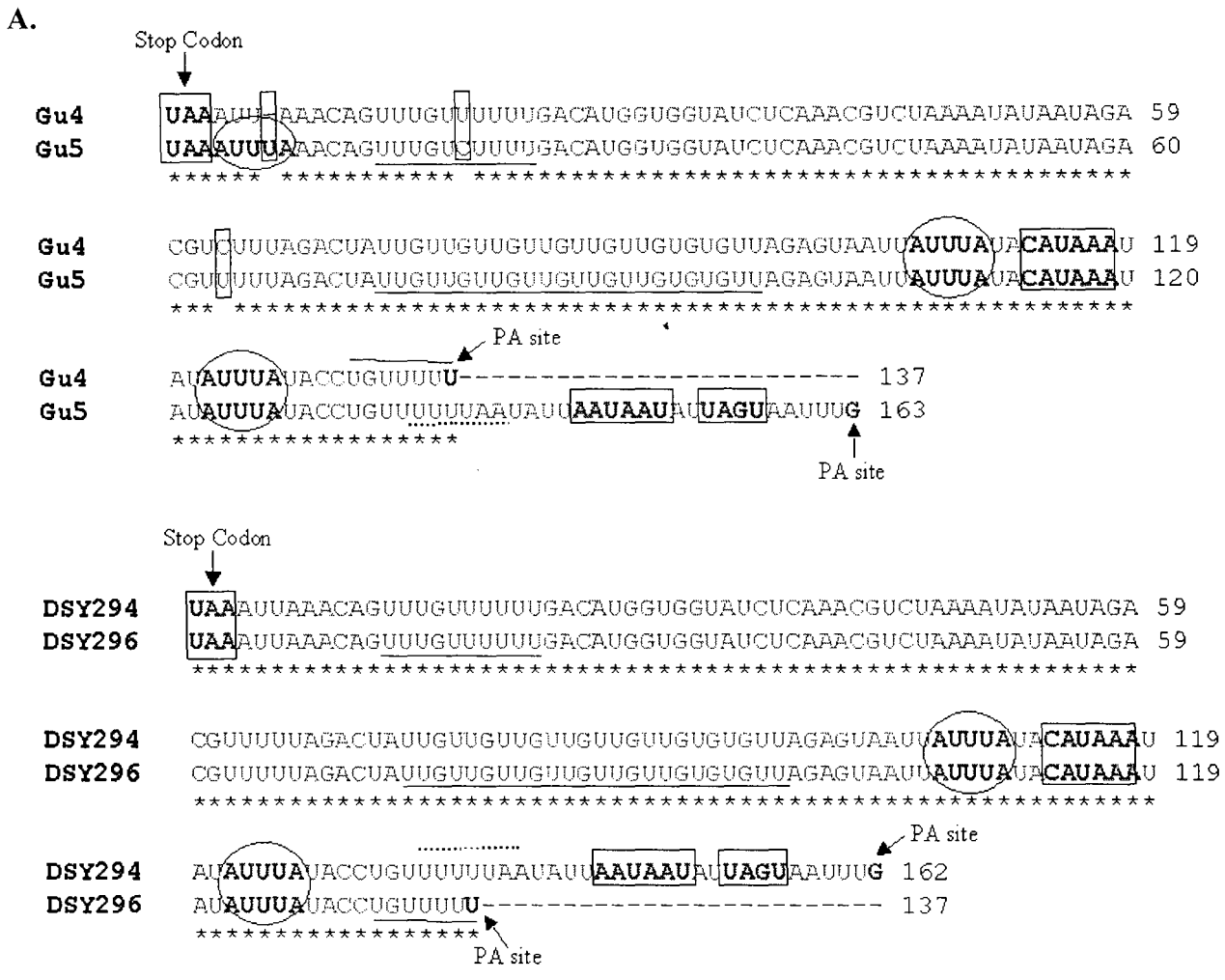


Figure 43. Sequence and Structure analysis of *CDR1* 3′UTR.

(A) Alignment of *CDR1* 3′UTR from the two matched pairs of clinical isolates obtained using the *ClustalW* program. Conserved residues are indicated by *asterisks*. Mismatched residues are represented by *open boxes*. Gaps (*marked with dashes*) have been introduced to maximize the alignments. *Continuous* and *dis-continuous underlines* denote the polypyrimidine (Py) and cytoplasmic polyadenylation element (CPE) tract respectively. Eukaryotic poly(A) signal is indicated by *shadowbox*. *Ellipses* denote the AU-rich elements ('AAUAAA'). *Numbers* at the *right* are relative to the 'UAA' translational stop codon (*considered as position +1*).

B.

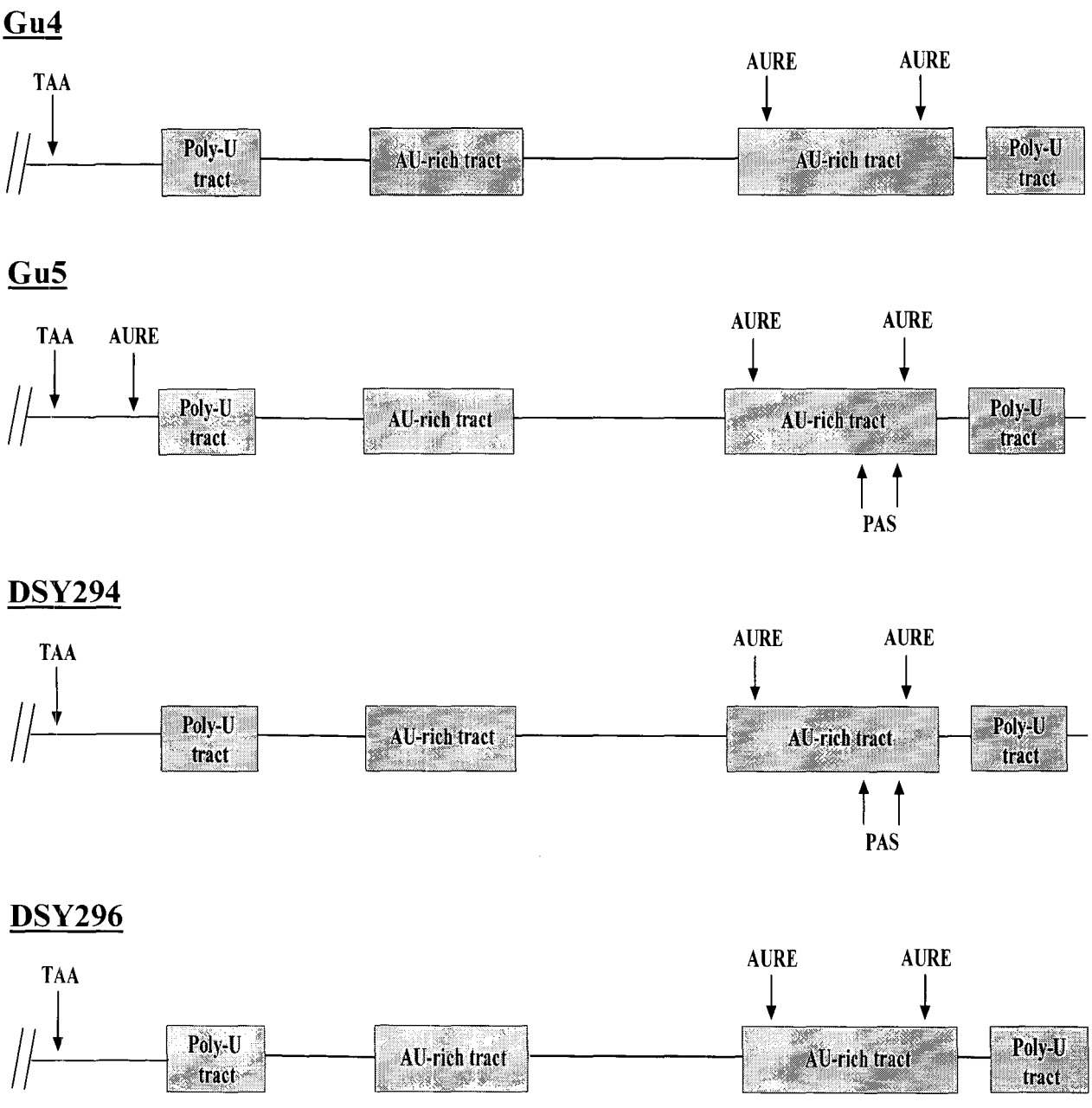
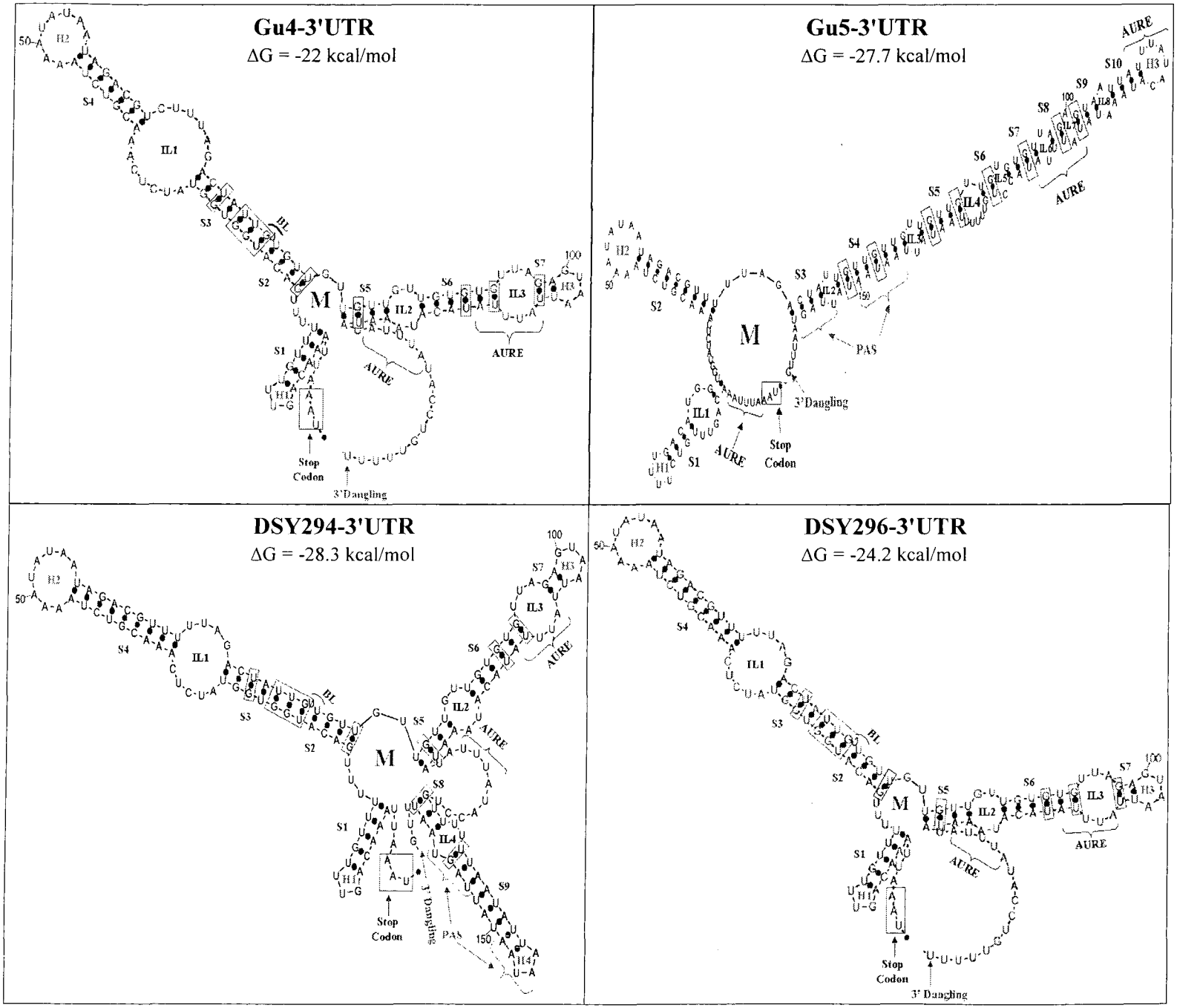


Figure 43. Sequence and Structure analysis of *CDR1* 3′UTR.
(B) Schematic representation of the predicted regulatory motifs in the 3′UTR are marked in *boxes*. **(C)** Predicted Secondary structure of *CDR1* 3′UTR. Only the top-scoring conformation displaying highest free-energy value for each isolate are shown. *Solid boxes* indicate non canonical G·U wobbles base pairing. *Oval boxes* mark polymorphic nucleotides. *Brackets* mark AU-rich elements ('AURE') and polyadenylation signal ('PAS'). *Patterned box* mark 'UAA' translational stop codon. 3′-*dangling* indicates poly(A)$_n$ addition site. *Abbreviations*: M-*Multibranch loop* (╬); H-*Hairpin loop* (Ω); IL-*Internal Loop* (Ω); BL-*Bulge Loop* (∩); S-*Stacks* (≡).

C.


Gu4-3'UTR
ΔG = -22 kcal/mol
Gu5-3'UTR
ΔG = -27.7 kcal/mol
DSY294-3'UTR
ΔG = -28.3 kcal/mol
DSY296-3'UTR
ΔG = -24.2 kcal/mol
Stop Codon
3'Dangling
PAS
AURE
M
IL1
IL2
IL3
IL4
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
H1
H2
H3
H4
BL

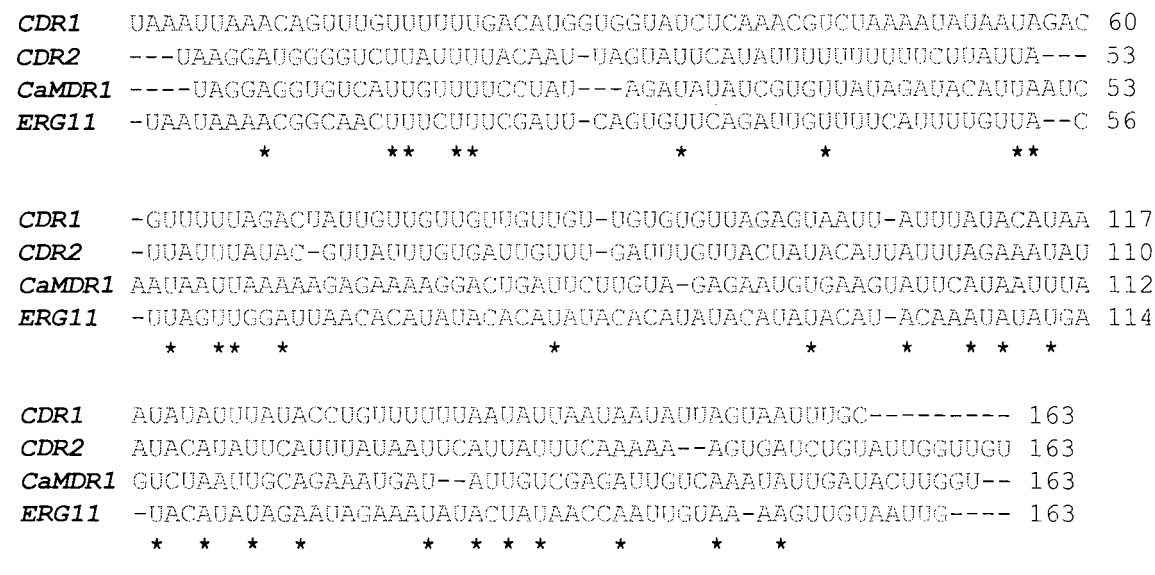


Figure 44. Multiple sequence alignment of mapped *CDR1* 3'UTR with other MDR genes of *C. albicans.*

Alignment of SC5314 *CDR1* 3'UTR with 163 bases of 3'-regions of *CDR2*, *MDR1* and *ERG11* was performed using the ClustalW (*version 1.83*) program. Conserved residues are indicated by *asterisks*. Gaps (*marked with dashes*) have been introduced to maximize the alignments. Numbers at the right are relative to the '*UAA*' translational stop codon (*considered as position +1*).

between AS and AR isolates (ΔG = -22 and -27.7 kcal/mol for Gu4/Gu5, while ΔG = -28.3 and -24.2 kcal/mol for DSY294/DSY296, respectively). Interestingly, 'AURE' motifs (AAUUUA) detected were located either in exposed hairpin or interior loop, while predicted polyadenylation signals resided in exposed sites of the molecule. Based on the predicted secondary structure, *CDR1*-3'UTR displayed differences between AS and AR isolates.

3.2.2.4 3'UTR swapping does not affect β-galactosidase reporter activity.

The sequence and structure analyses of 3'UTR of *CDR1* suggested that the polymorphism as well as differential secondary structure between AS and AR isolates might contribute to the observed enhanced mRNA stability. To obtain direct evidence, we tested whether the *CDR1* 3'UTR from AS isolates will have any destabilizing effect in AR isolates and *vice versa*. For this purpose, expression vectors harboring P_{CDR1}-*lacZ*-*CDR1* 3'UTR chimeric transcriptional reporter fusions were constructed (Figure 45A, *see Materials and Methods*). The linearised cassette harboring *CDR1* 3'UTR of each isolate was integrated into native *CDR1* genomic locus as well as into their corresponding matched isolates. Single copy integration of each construct was confirmed by Southern hybridization (Figure 45B). Two representative Nourseothricin resistant (Nou^R) transformants of each parental strain containing a single integrated copy of the reporter fusion were used for further analysis. The resulting reporter strains were designated as Gu4L2-CUN (P_{CDR1}-*lacZ*-3'UTR^N), Gu4L2-CUS (P_{CDR1}-*lacZ*-3'UTR^S); Gu5L2-CUN (P_{CDR1}-*lacZ*-3'UTR^N), Gu5L2-CUS (P_{CDR1}-*lacZ*-3'UTR^S); DSY294L2-CUN (P_{CDR1}-*lacZ*-3'UTR^N), DSY294L2-CUS (P_{CDR1}-*lacZ*-3'UTR^S); DSY296L2-CUN (P_{CDR1}-*lacZ*-3'UTR^N) and DSY296L2-CUS (P_{CDR1}-*lacZ*-3'UTR^S). The superscript 'N' and 'S' in designated constructs stand for native and swapped 3'UTR reporter constructs, respectively.

Expression of the *lacZ* reporter gene in the various strains was qualitatively assessed by comparing the intensity of the blue color produced by cells grown on agar plates containing the indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Figure 46A) and was quantitated by measuring the β-galactosidase reporter activity in liquid assays (Figure 46B). Two observations emerged from these results. Firstly, β-galactosidase activity observed in native as well as swapped 3'UTR transformants of each

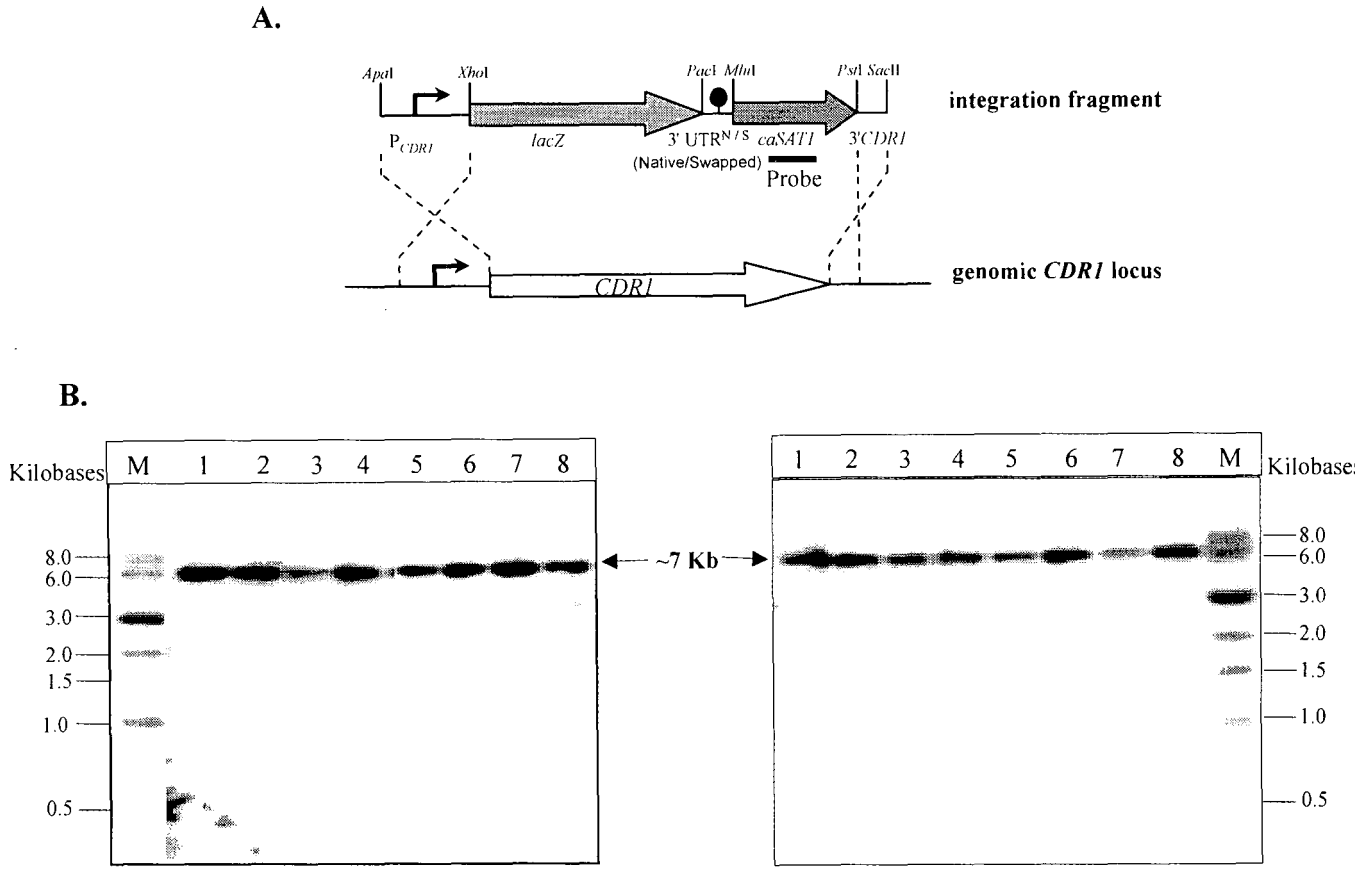


Figure 45. Schematic depiction of *lacZ* reporter fusion constructs and their integration in AS and AR isolates.

(A) Structure of the DNA cassettes that were used to integrate the P_{CDR1}-*lacZ*-3'UTR^N (Native) and P_{CDR1}-*lacZ*-3'UTR^S (Swapped) reporter fusions into the *CDR1* locus of the clinical *C. albicans* isolates (*middle*). The *CDR1* and *lacZ* coding regions are represented by *white* and *blue arrows* respectively, the *caSAT1* marker by *grey arrow*, and 3'UTR of *CDR1* by the *filled circle*. *CDR1* upstream and downstream regions are represented by *solid lines*, the *CDR1* promoter (P_{CDR1}) is symbolized by the *bent arrow*. The straight arrow indicates the direction of transcription. The probe used to verify the correct integration is indicated by a thick line. Only relevant restriction sites are shown. **(B)** Chromosomal DNA from *C. albicans* transformants was isolated as described previously. Genomic DNA (~10 µg) of native (*left blot*: *Lanes* 1,2-Gu4L2^N; *Lanes* 3,4-Gu5L2^N; *Lanes* 5,6-DSY294L2^N; *Lanes* 7,8-DSY296L2^N) and swapped (*right blot*: *Lanes* 1,2-Gu4L2^S; *Lanes* 3,4-Gu5L2^S; *Lanes* 5,6-DSY294L2^S; *Lanes* 7,8-DSY296L2^S) 3'UTR reporter transformants were digested with *Bgl*II, separated on a 1% (*w/v*) agarose gel, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Probe labeling, hybridization, washing and signal detection were performed as described previously. Single copy integration of each construct at the desired locus was confirmed with gene specific probe indicated by a thick line. The size of the hybridizing fragments and locations of DNA standards (kb) are indicated on centre and left / right side of the each blot respectively. *Lane M*, nucleotide size marker (*1 kb Marker*).

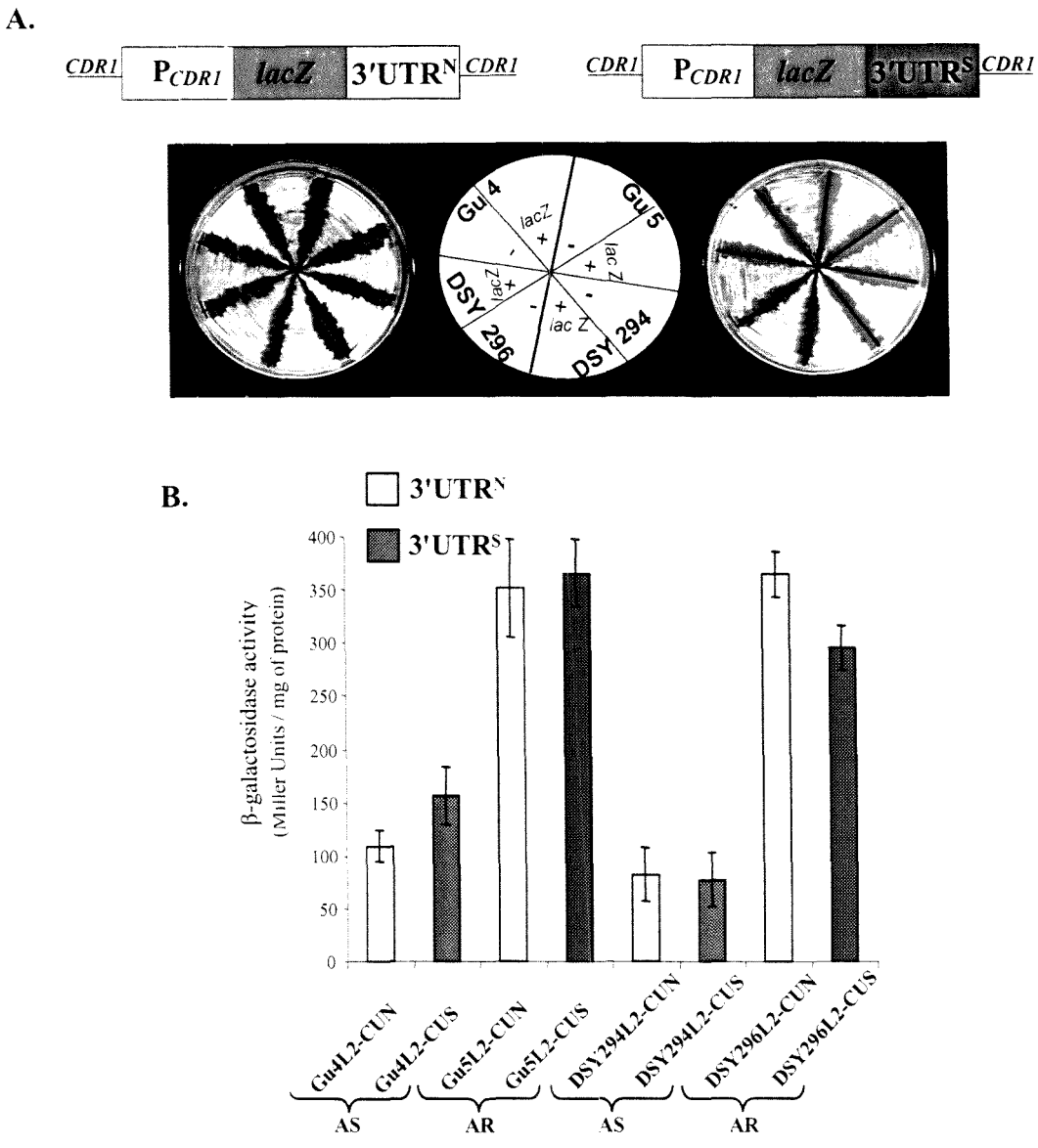


Figure 46. Qualitative and quantitative assay of β-galactosidase activity of *lacZ* reporter fusion integrants in AS and AR isolates.

(A) Transformants harboring the chromosomally integrated P_{CDR1}-*lacZ*-3′UTR^N (Native, *left*) and P_{CDR1}-*lacZ*-3′UTR^S (Swapped, *right*) and their corresponding parental strain (without *lacZ*) were streaked on minimal media plate containing X-gal and photographed after 3 days of growth at 30°C. The positions of the individual strains on the plates are shown in the scheme (*middle*). **(B)** β-galactosidase quantitative reporter activities of each transformants were determined as described under *Materials and Methods*. The reported quantities are mean values ± Standard Deviations (*indicated by error bars*) of three independent experiments with duplicate measurements of two independent clones. *Empty* and *filled bars* indicate P_{CDR1}-*lacZ*-3′UTR^N (Native) and P_{CDR1}-*lacZ*-3′UTR^S (Swapped) 3′UTR reporter fusion transformants in both AS and AR backgrounds.

Table 11. Table depicting the quantitative β-galactosidase activity of native and swapped 3'UTR-*lacZ* reporter fusion integrants in AS and AR isolates.

Strains	Average β-galactosidase activity (Miller Units/mg of protein)	Fold induction	
Native 3'UTR		**AR *v/s* AS**	**Native *v/s* Swapped**
Gu4L2-CUN	109 ± 14.2	Gu4L2-CUN / Gu5L2-CUN } 3.22	Gu4L2-CUN / Gu4L2-CUS } 0.69
Gu5L2-CUN	351.6 ± 45		
DSY294L2-CUN	81.9 ± 25.4	DSY294L2-CUN / DSY296L2-CUN } 4.44	Gu5L2-CUN / Gu5L2-CUS } 0.96
DSY296L2-CUN	364.6 ± 21.1		
Swapped 3'UTR			DSY294L2-CUN / DSY294L2-CUS } 1.07
Gu4L2-CUS	156.3 ± 26.1	Gu4L2-CUS / Gu5L2-CUS } 2.33	
Gu5L2-CUS	364.9 ± 31.1		DSY296L2-CUN / DSY296L2-CUS } 1.23
DSY294L2-CUS	76.5 ± 25.5	DSY294L2-CUS / DSY296L2-CUS } 3.86	
DSY296L2-CUS	295.6 ± 21.4		

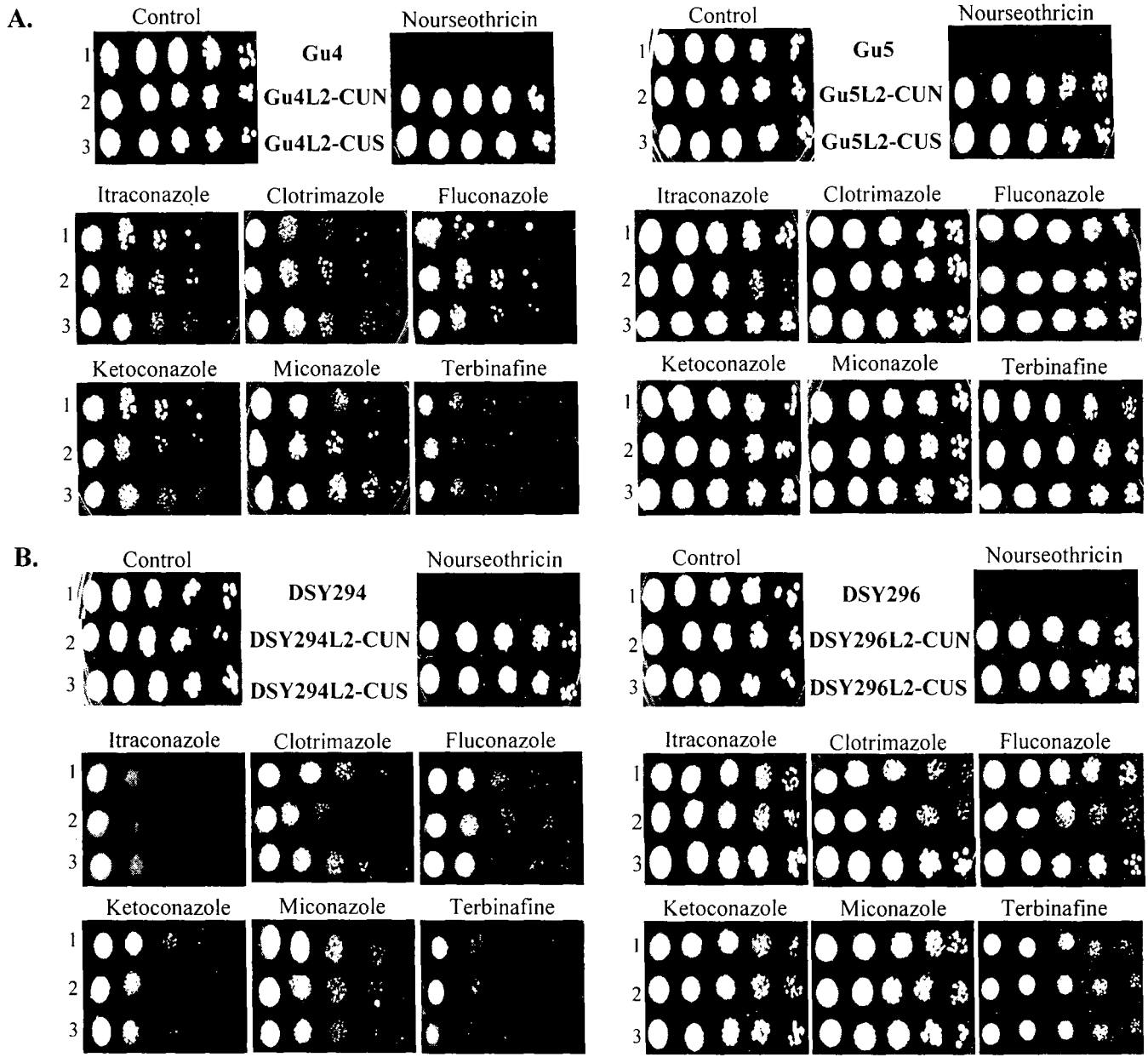


Figure 47. Drug resistance profiles of the wild type, native and swapped transcriptional reporter fusion transformants in AS and AR isolates.

Drug resistance profiling was determined by the spot assay as described previously. In the spot assay, 5 µl of fivefold serial dilutions of each yeast culture (A_{600}=0.1) was spotted onto YEPD plates in the absence (control) and the presence of the following drugs - nourseothricin (200 µg/ml), itraconazole (0.025 µg/ml), clotrimazole (0.025 µg/ml), fluconazole (2 µg/ml), ketoconazole (0.025 µg/ml), miconazole (0.05 µg/ml) and terbinafine (0.1 µg/ml). Growth differences were evaluated by using drug-free controls following incubation of the plates for 48 h at 30°C. Growth was not affected by the presence of the solvents used for the drugs (*data not shown*). **(A)** Gu4/Gu5 and **(B)** DSY294/DSY296 isolates and their transformant derivatives. Nourseothricin has been used as a transformants control. Itraconazole, clotrimazole, fluconazole, ketoconazole, miconazole and terbinafine are well known substrates of *CDR1* (Kohli *et al.*, 2001 and 2002).

AR isolate was always higher than their corresponding AS reporter transformants (*see* Table 12: 3.22 fold for Gu5L2-CUN *versus* Gu4L2-CUN, 2.33 fold for Gu5L2-CUS *versus* Gu4L2-CUS; 4.44 fold for DSY296L2-CUN *versus* DSY294L2-CUN and 3.86 fold for DSY296L2-CUS *versus* DSY294L2-CUS). Secondly, the reporter activity of the 3'UTR native transformants of each AS and AR isolates was comparable to their corresponding swapped reporter fusion transformants (Gu4L2-CUN *versus* Gu4L2-CUS, Gu5L2-CUN *versus* Gu5L2-CUS; DSY294L2-CUN *versus* DSY294L2-CUS and DSY296L2-CUN *versus* DSY294L2-CUS). Thus swapping of *CDR1* 3'UTR of AS and AR isolates does not affect the reporter activity. In another set of experiments, we tagged P_{CDR1}-*lacZ* with either native (*CDR1*) or heterologus (*ACT1*) 3'UTR (Manoharlal *et al.*, 2008), and observed comparable ß-galactosidase reporter activity (*See* Figure 30 *versus* Figure 46). Of note, none of the parent strains exhibited any reporter activity under similar experimental conditions (Figure 46A). Additionally, the tagging of P_{CDR1}-*lacZ* with *CDR1* 3'UTR and its swapping also did not alter the drug resistance profiling of AS and AR isolates (Figure 47). Taken together, these results suggests that 3'UTR sequence of AS and AR isolates do not contribute to transcriptional or post-transcriptional control of *CDR1*.

3.2.2.5 RNA-EMSA reveals reduced protein binding in AR isolates.

Relatively longer half-life of *CDR1* transcript in AR isolates (~3 folds) could be due to differential affinity of RNA binding protein(s) to the 3'UTR. To investigate the role of *CDR1* mRNA binding regulatory proteins, RNA-Electrophoretic Mobility Shift Assays (R-EMSA) was performed using the 3'UTR fragments as RNA probes. The results showed that 3'UTR of *CDR1* transcripts formed two RNA-protein complexes (C1 and C2) in both AS (Figure 48A, lanes-2, and 6) and AR isolates (Figure 48A, lanes-4 and 8). Band intensity of each complex for AR isolates was normalized to that of corresponding AS isolates and plotted as described under *Materials and Methods*. Figure 52B shows the intensities of RNA-protein complex C1 and C2 which were ~66% and 51% (for Gu5) and ~60% and 40% (for DSY296), lower than the corresponding complex intensity of AS isolates (Gu4 and DSY294 respectively). An equal amount of crude protein extract used

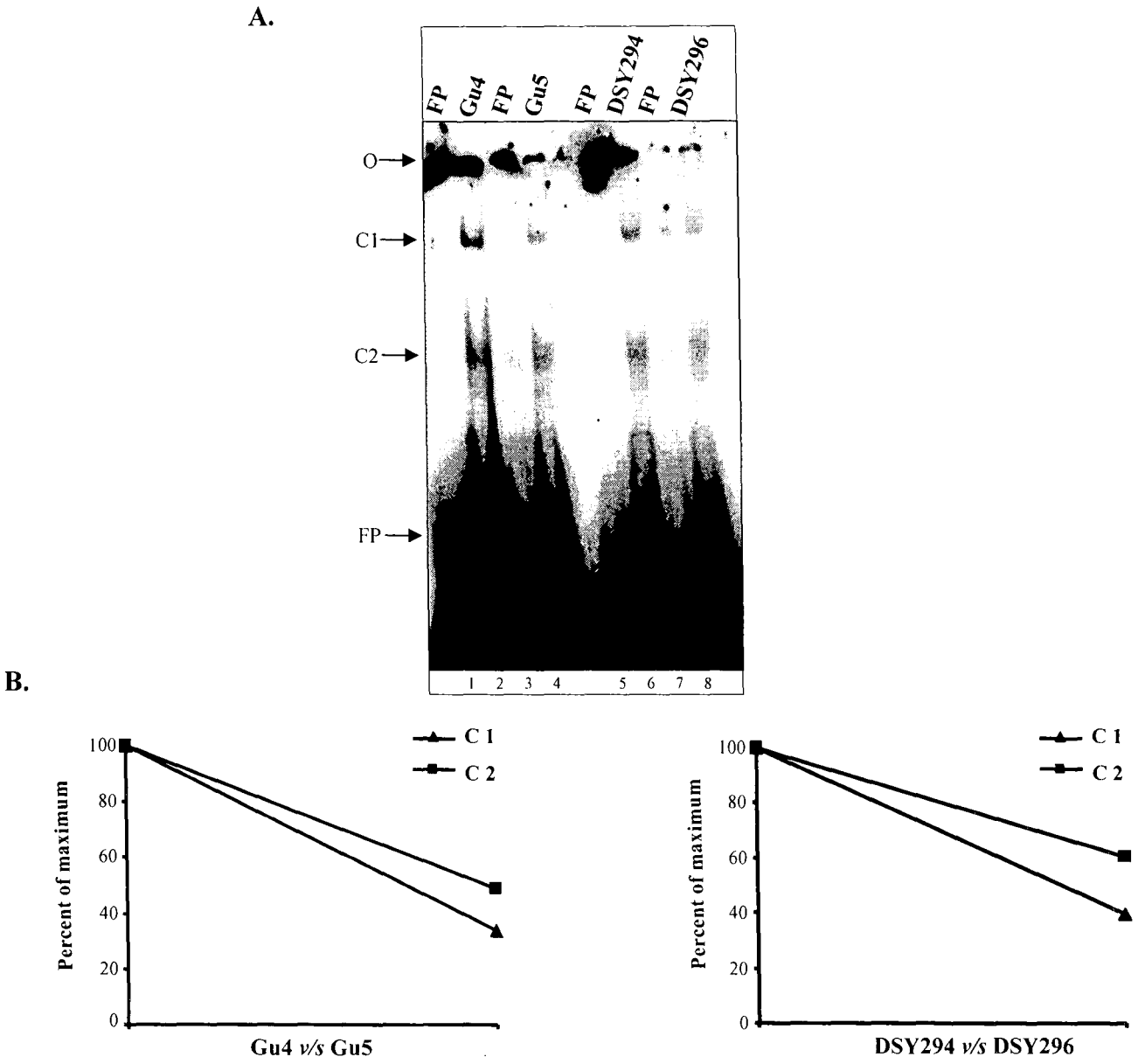


Figure 48. Differential RNA-protein complex(es) formation at 3'UTR mRNA of *CDR1*.
(A) [α-^{32}P] UTP labeled riboprobes of each isolate were incubated with the corresponding 30 μg of cellular extracts, as described under *Materials and Methods*. Lanes 1, 3, 5 and 7- free probe; lanes 2, 4, 6 and 8- radiolabeled riboprobe incubated with cellular extracts of Gu4, Gu5, DSY294 and DSY296 respectively. *Abbreviations*- FP: *Free Probe*; C1: *RNA-Protein Complex 1*, C2: *RNA-Protein Complex 2*; O: *Origin of gel.* **(B)** RNA-protein complex(es) signal intensity from a typical gel shift assay were quantitated using PhosphorImager scanner, normalized with the corresponding AS isolates (*considered as 100%*), expressed as a percentage of maximum and plotted as a line graph (Gu4 *v/s* Gu5 and DSY294 *v/s* DSY296).

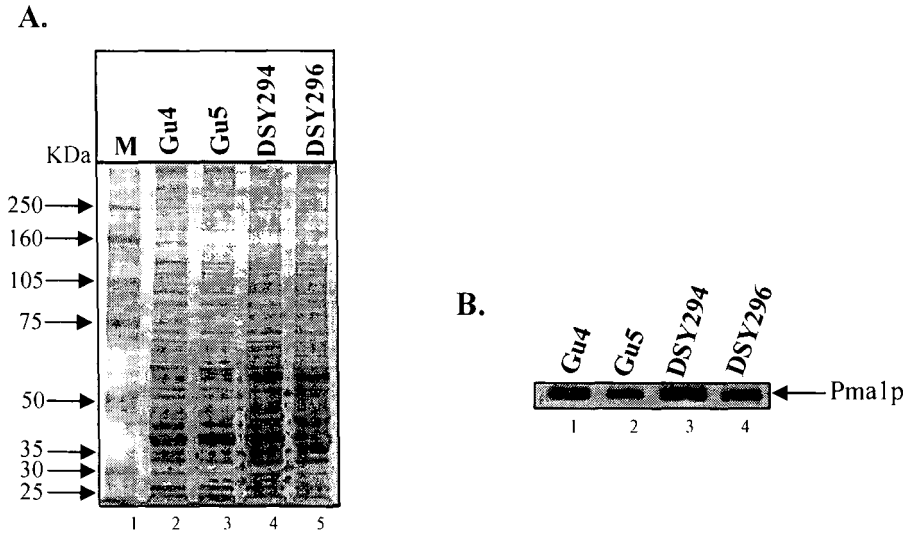


Figure 49. Assessing of integrity and equal loading of crude protein extract used for R-EMSA by (A) Silver staining of 8% SDS-PAGE as well as by **(B)** Western blot analyses using anti-Pma1p polyclonal antibody both AS and AR isolates respectively.

for R-EMSA was assessed by Western blot analyses using an anti-Pma1p polyclonal antibody for both AS and AR isolates (Figure 49).

3.2.2.6 *CDR1* mRNA poly(A) tail is longer in AR isolates.

As poly(A) tail removal is a critical step in the 3'-exonuclease mediated decay pathway (Ross, 1996; McCarthy, 1998), we investigated the polyadenylation state of *CDR1* mRNA between AS and AR isolates. For this, we employed PAT assay (Kusov *et al.*, 2001) to assess the polyadenylation state of mRNA between AS and AR isolates. We adopted a recently described PCR-based PAT assay where the 3'-end of the mRNA is polyguanylated using poly(A) polymerase (Figure 50A, step 1) as described under *Materials and Methods.* With this step, a poly(A)-oligo(G) junction is generated which serves as specific target for the amplification of the 3'-end of the transcriptome with the universal reverse oligo(dC_9T_6) anchor primer and a gene-specific forward primer (Figure 50A, step 2a & b). Considering the resolution of the gel and the intrinsic width of the band (Figure 50B), it was observed that *CDR1* PAT-PCR products from AS and AR isolates were of heterogeneous sizes, ranging between 300 and 525 bp. Since the amplified products derived from the region between 5' and the 3'-end of the transcript were of ~470 bp, it corresponded to poly(A) tail length of ~30 to ~50 A's .RT-PCR products were cloned and sequenced (Figure 50A, step 3) for the accurate measurement of the poly(A) tail length (Kusov *et al.*, 2001). Chromatogram analysis of cloned PAT-PCR products of each AS and AR isolate confirmed precisely that the poly(A) tail length was relatively shorter with ~24-26 adenylic residues in AS isolates, in contrast to ~33-35 residues in AR isolates (Figures 50C and D). It should be mentioned that a minimum of 10 random clones were subjected to sequencing to arrive at exact length of poly(A) tail. A maximum variation of 2 bases was observed between different clones.

3.2.2.7 Short term *in vitro* induced over-expression of *CDR1* display enhanced drug resistance.

In the following, we explored the regulatory mechanisms controlling the expression of *CDR1* mRNA when induced *in vitro* by short exposure of drug/steroids. To address this issue, the AS isolates were spotted on YEPD plates containing drugs-fluconazole,

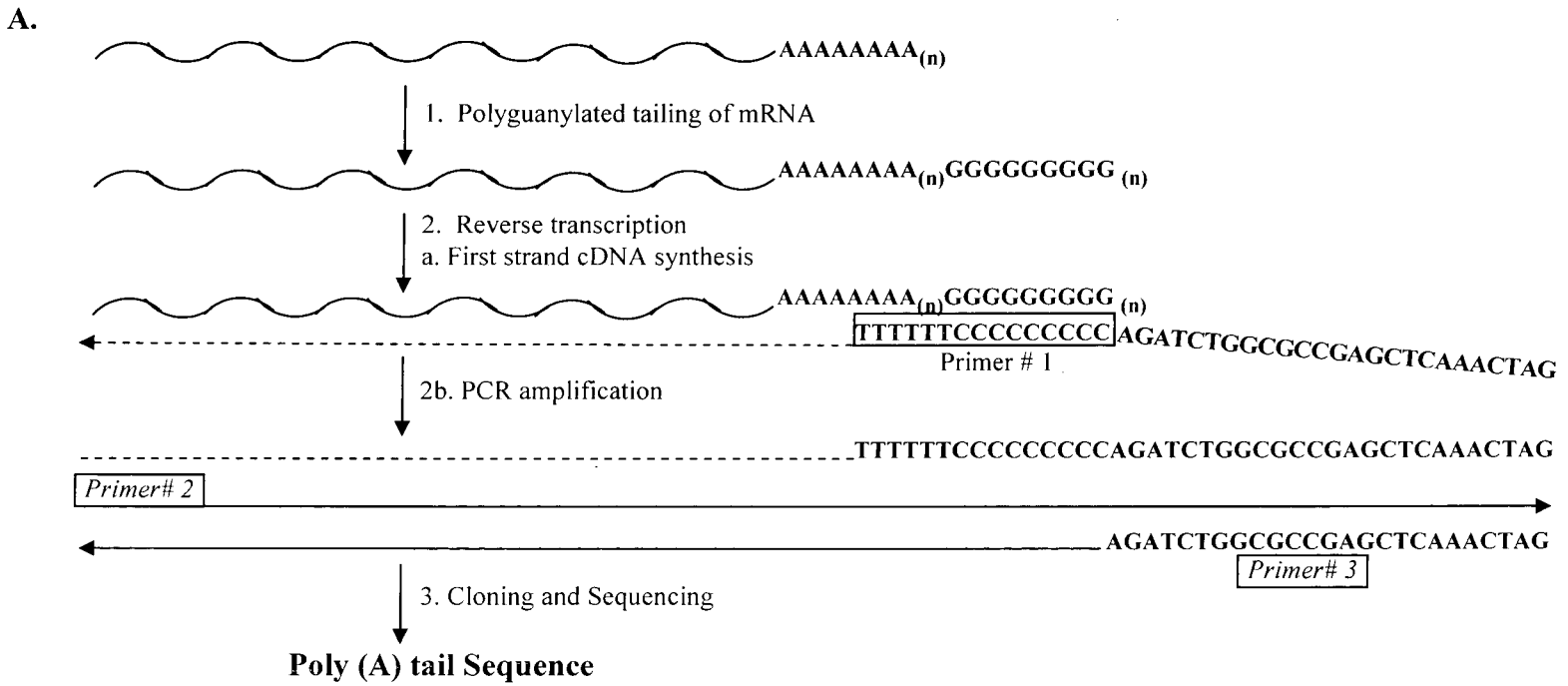
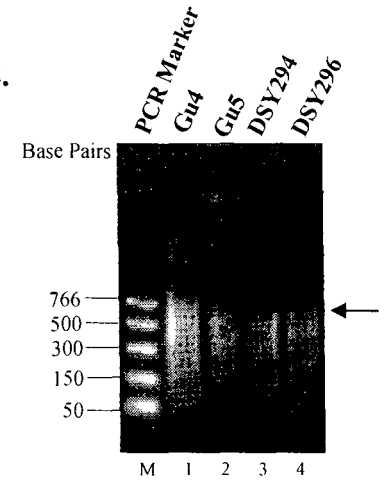


Figure 50. Polyadenylation test (PAT) of *CDR1* mRNA.

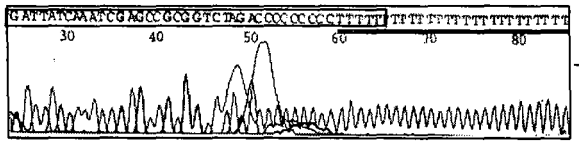
(A) Schematic representation of the modified PAT assay. In step 1, purified mRNA is polyguanylated using PAP and GTP. In step 2a, the guanylated mRNA is reverse transcribed using anti-sense primer, oligo(dC_9T_6)AP (*primer#1*). In step 2b, the RT product is amplified by PCR with *CDR1* specific sense primer, CT-*CDR1*-F-RML (*primer#2*) and anti-sense anchor primer, AP-RML (*primer#3*). **(B)** Specific amplification of polyguanylated mRNA. According to the scheme on *top*, poly(A)$^+$- enriched mRNA were subjected to the PAP reaction with GTP and subsequently to 3' RACE-RT-PCR specific amplification of *CDR1*. The RT-PCR product smear following electrophoresis through 1.2% agarose gel, were visualized by staining with ethidium bromide (*shown by an arrow*). **(C)** Representative chromatogram of cloned polyadenylated fragments obtained from each AS and AR isolate is shown. *Open box* sequence represents the oligo(C)-poly(T) junction. *Continuous underline* sequences denotes the poly'T' tract (*complementary to polyadenylated sequence*). **(D)** Table depicting the representative poly(A) tail length of *CDR1* in both AS and AR isolates.

B.



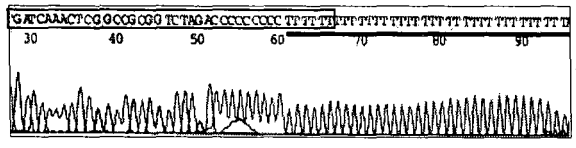
C.

Gu4



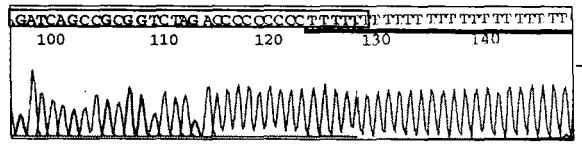
$\rightarrow \{A\}_{26\pm2}$

Gu5



$\rightarrow \{A\}_{35\pm2}$

DSY294



$\rightarrow \{A\}_{24\pm2}$

DSY296

$\rightarrow \{A\}_{36\pm2}$

D.

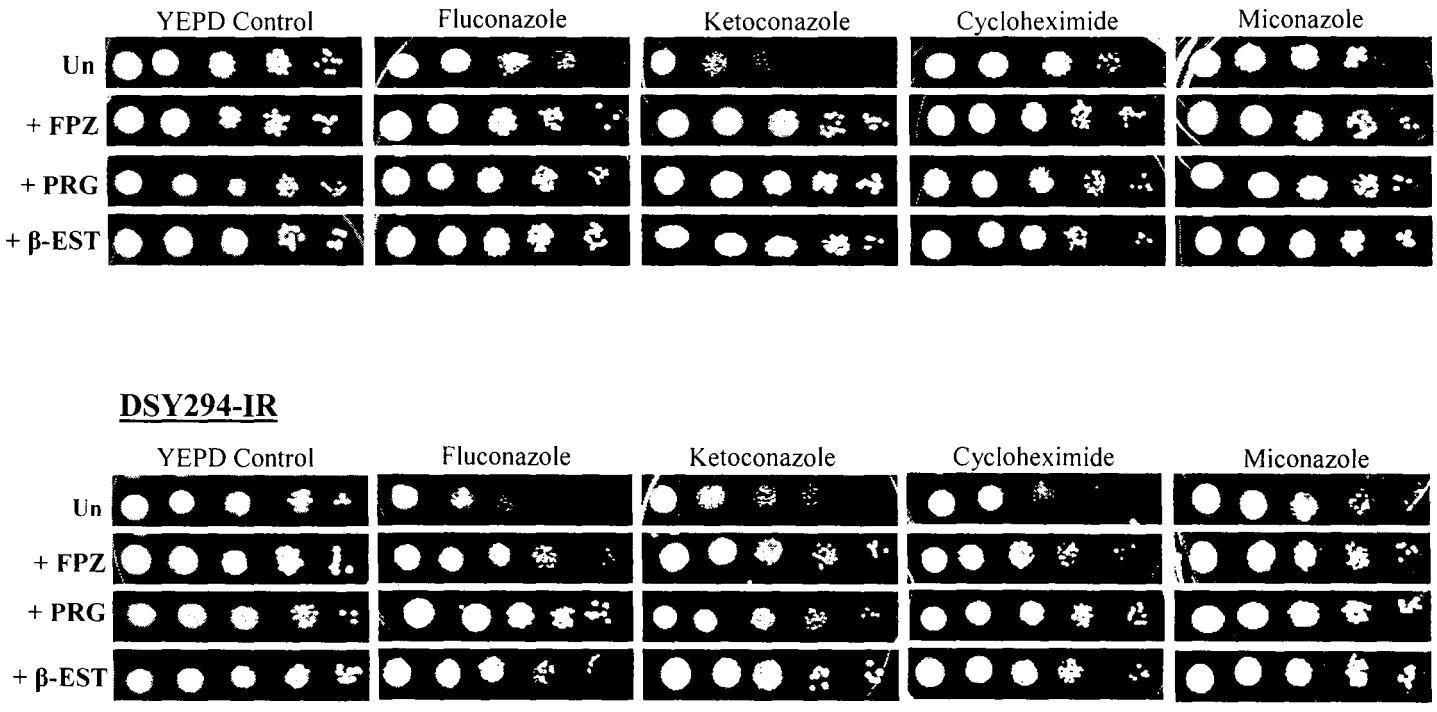
Strains	Poly (A) tail length
Gu4	26 ± 2 A's
Gu5	35 ± 2 A's
DSY294	24 ± 2 A's
DSY296	36 ± 2 A's

ketoconazole, miconazole and cycloheximide in the presence of *CDR1* inducers such as fluphenazine, progesterone and ß-estradiol (de Micheli *et al.*, 2002; Karababa *et al.*, 2004). A relatively increased drug resistance in Gu4-IR (for Gu4-induced resistance) and DSY294-IR (for DSY294-induced resistance) in the presence of all the tested inducers was observed after 48 hr of incubation at 30°C (Figure 51A). Notably, inducers alone did not have any effect on the growth of the AS isolates (Figure 51A). The increased efflux of a well known substrate of *CDR1*, Rhodamine-6G (R6G) (Shukla *et al.*, 2003), in induced AS cells corroborated the drug spot results (Figure 51B). Northern blot analysis and subsequent quantification of *CDR1* transcript confirmed its higher amount in both Gu4-IR and DSY294-IR isolates (Figure 51C). Increased Cdr1p level was also observed in plasma membrane preparations of Gu4-IR and DSY294-IR in contrast to uninduced AS isolates after Western immunoblotting with anti-*CDR1* polyclonal antibody (Figure 51D). Thus our results established an increase in steady state *CDR1* mRNA levels to be one of the reasons of the observed increases in drug resistance and enhanced efflux of R6G in Gu4-IR and DSY294-IR. Of note, no difference in drug resistance profiling and R6G efflux for both uninduced and induced AR isolates was observed, indicating no over and above increase of basal *CDR1* expression (Figures 52 A and B).

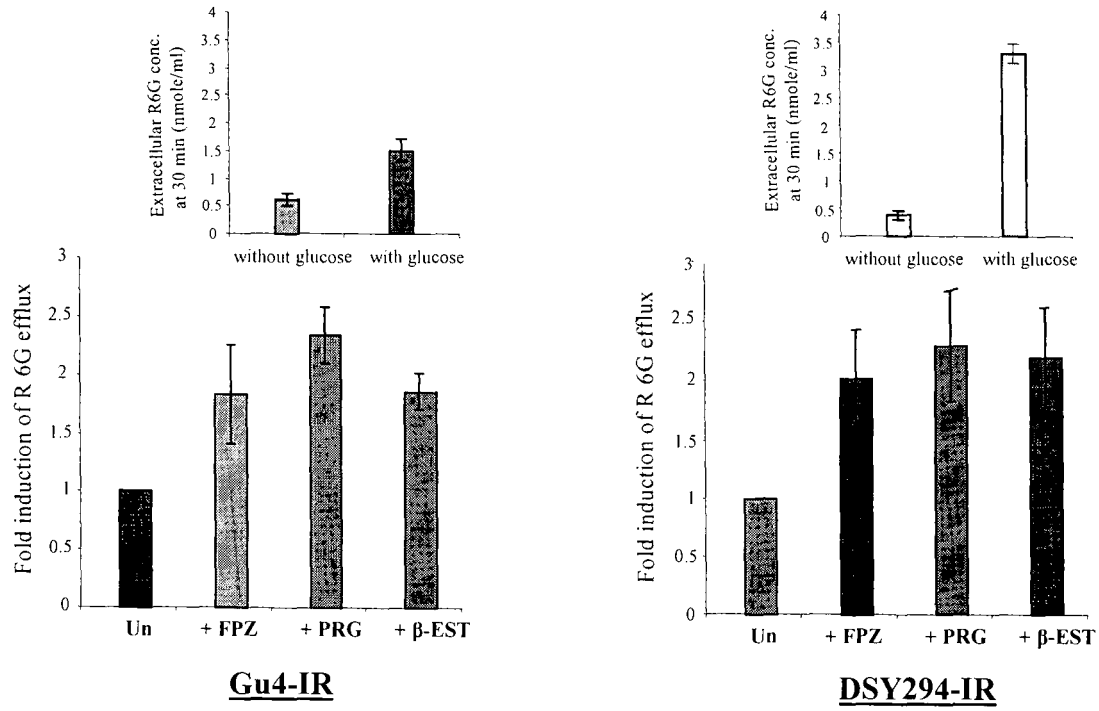
3.2.2.8 Induced up-regulation of *CDR1* is mediated by its enhanced transcriptional activation.

To determine whether induced *CDR1* mRNA up-regulation was due to transcriptional activation, we performed ß-galactosidase reporter assay in which a *CDR1* promoter fragment was placed upstream of the *lacZ* reporter gene (as described under *Materials and Methods*). Interestingly, ß-galactosidase activity observed in both induced AS isolates (Gu4-IR and DSY294-IR) was relatively higher than their corresponding untreated counterpart (4.5, 4.6 and 5.4 fold for Gu4 and 3.3, 7.6 and 6.6 fold for DSY294 in the presence of fluphenazine, progesterone and ß-estradiol respectively, Figure 53), indicating that *CDR1* inducers up regulate the activity of *CDR1* promoter by transcriptional activation. To validate this further, transcription run-on assays were performed as described under *Materials and Methods*. For controls, pACT1 plasmid DNA, containing the constitutively expressed *ACT1* gene, and the empty vector

A.


Gu4-IR
YEPD Control
Fluconazole
Ketoconazole
Cycloheximide
Miconazole
Un
+ FPZ
+ PRG
+ β-EST
DSY294-IR
YEPD Control
Fluconazole
Ketoconazole
Cycloheximide
Miconazole
Un
+ FPZ
+ PRG
+ β-EST

B.


Extracellular R6G conc. at 30 min (nmole/ml)
without glucose
with glucose
Fold induction of R 6G efflux
Un
+ FPZ
+ PRG
+ β-EST
Gu4-IR
Extracellular R6G conc. at 30 min (nmole/ml)
without glucose
with glucose
Fold induction of R 6G efflux
Un
+ FPZ
+ PRG
+ β-EST
DSY294-IR

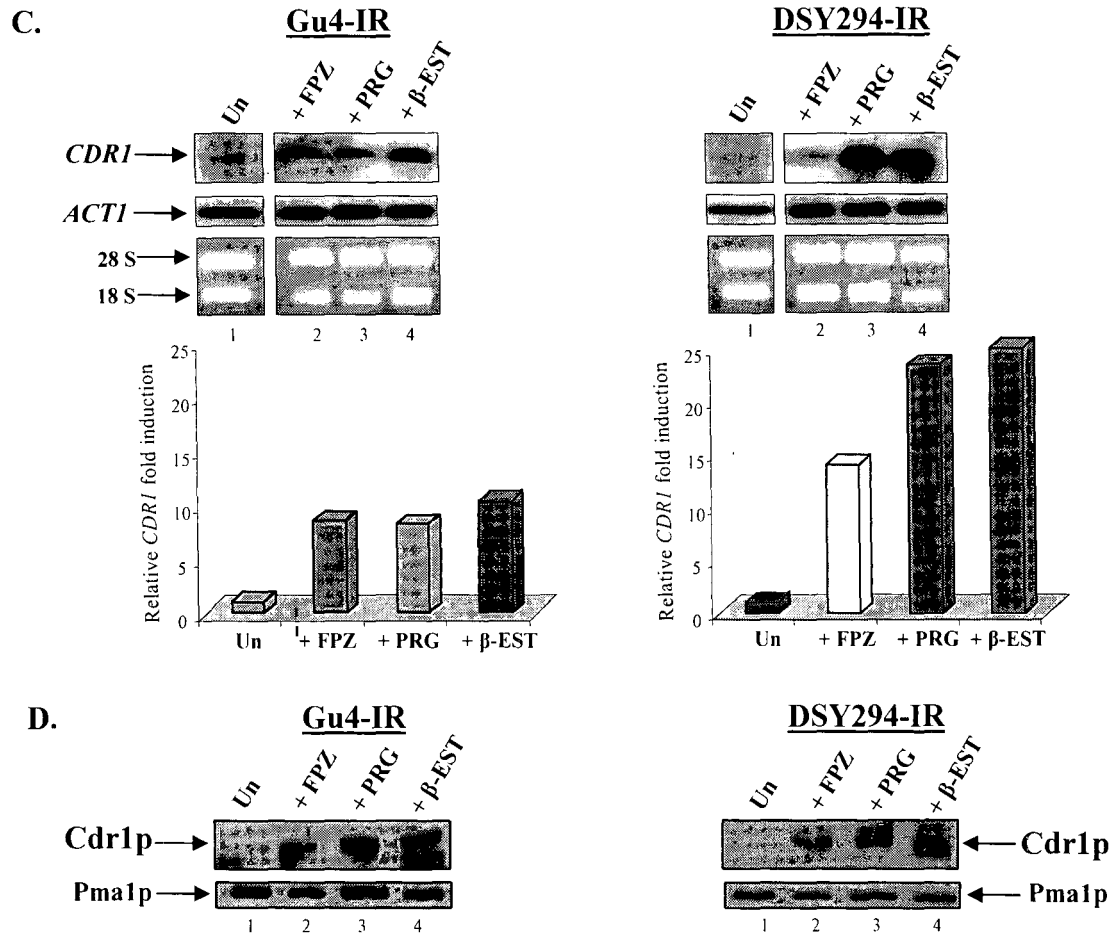


Figure 51. Inducibility of AS isolates.

(A) Drug susceptibility testing. Drug susceptibilities with the inducers (FPZ, PRG and β-EST) were measured by spot assays either alone or in the presence of the indicated drugs as described under *Material and Methods*. The final drug concentrations used (*the solvent used is given in parenthesis*) were FLC 2 μg/ml (*water*), KET 0.05 μg/ml (*methanol*), MIC 0.1 μg/ml (*methanol*) and CYC 300 μg/ml (*water*). Susceptibilities to various drugs were checked in YEPD. In the spot assay, 5 μl of fivefold serial dilutions of each yeast culture (A_{600}=0.1) was spotted onto YEPD plates in the absence (*control*) and the presence of the indicated drugs. **(B)** R6G efflux assay. The assay was performed essentially as described under *Materials and Methods*. The results are shown as relative fold induction of R6G efflux from induced AS isolates as compared to uninduced cells. The quantities reported are mean values ± Standard Deviations (*indicated by error bars*) of three independent experiments. The efflux of R6G is shown by open (for Gu4) and filled bars (for DSY294) respectively. The inset shows the extracellular concentration of R6G in the presence and absence of glucose. **(C)** Northern blot analysis. Northern blots were carried out employing standard protocols (Sambrook *et al.*, 1989). Equal loading of RNA was assessed by rRNA bands. The membranes were subsequently deprobed and rehybridized with the *ACT1* probe to monitor further equal RNA loading and transfer. **(D)** Immunodetection of Cdr1p in induced AS isolates. Exponentially grown cultures of both AS isolates (Gu4 and DSY294) were treated with *CDR1* inducers- fluphenazine, progesterone and β-estradiol for 30 min. Approximately 30 μg of whole cell extracts of both uninduced and induced AS isolates were separated on 10% SDS-PAGE. Cdr1p was detected using a polyclonal anti-Cdr1p antibody. An equal loading of protein was assessed from colloidal Coomassie blue G250 stained gel (*data not shown*) as well as by stripping and subsequent re-probing of the blot with anti-Pma1p polyclonal antibody (*lower panel*). The position of the immunoreactive bands corresponding to Cdr1p and Pma1p on the blot is marked by an arrow.

A.

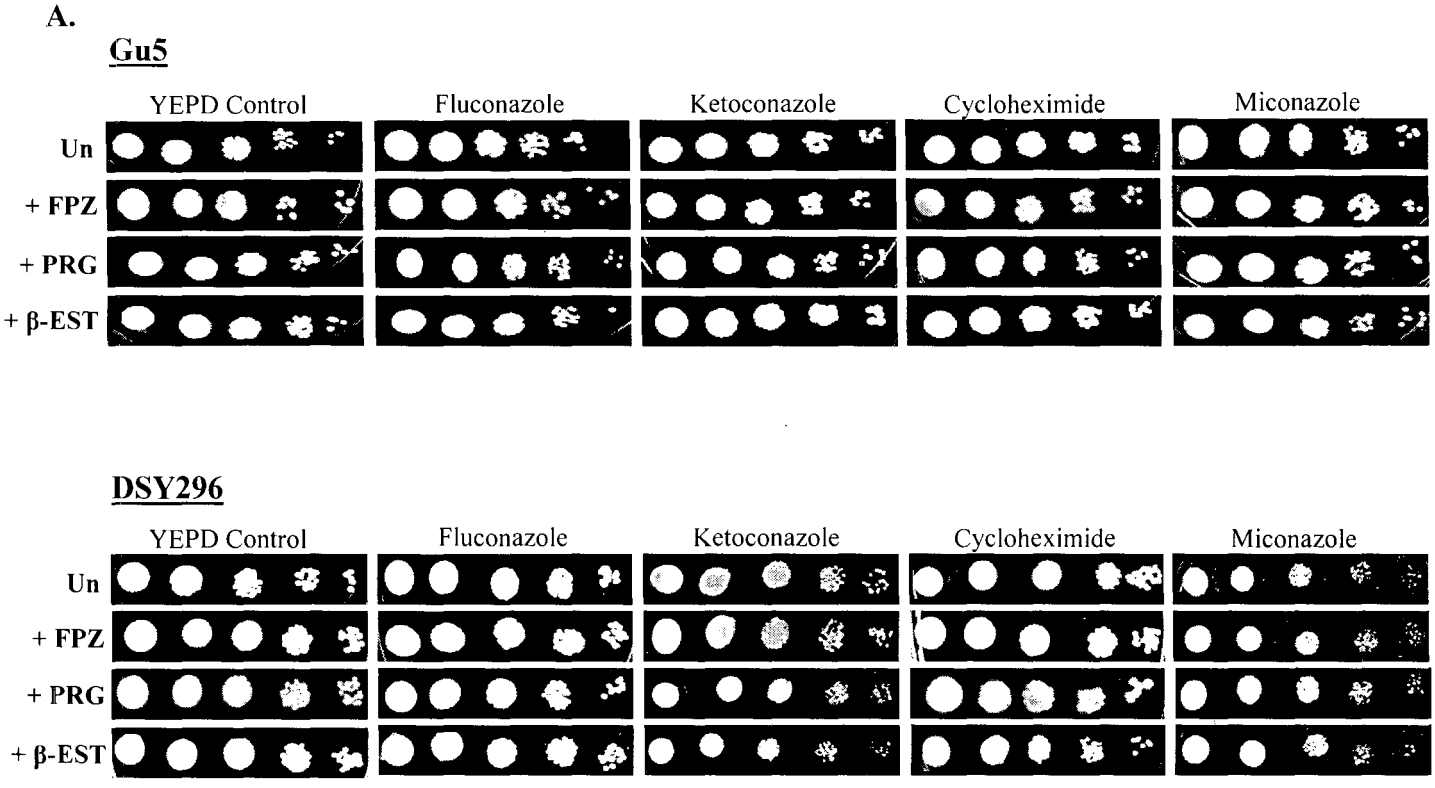


Figure 52. Non-inducibility of AR isolates.

(A) Drug susceptibility testing. Drug susceptibilities with the inducers (FPZ, PRG and β-EST) were measured by spot assays either alone or in the presence of the indicated drugs as described previously. The final drug concentrations used (the solvent used is given in parenthesis) were FLC 2 μg/ml (*water*), KET 0.05 μg/ml (*methanol*), MIC 0.1 μg/ml (*methanol*) and CYC 300 μg/ml (*water*). Susceptibilities to various drugs were checked in YEPD. In the spot assay, 5 μl of fivefold serial dilutions of each yeast culture (A_{600}=0.1) was spotted onto YEPD plates in the absence (control) and the presence of the indicated drugs.

B.

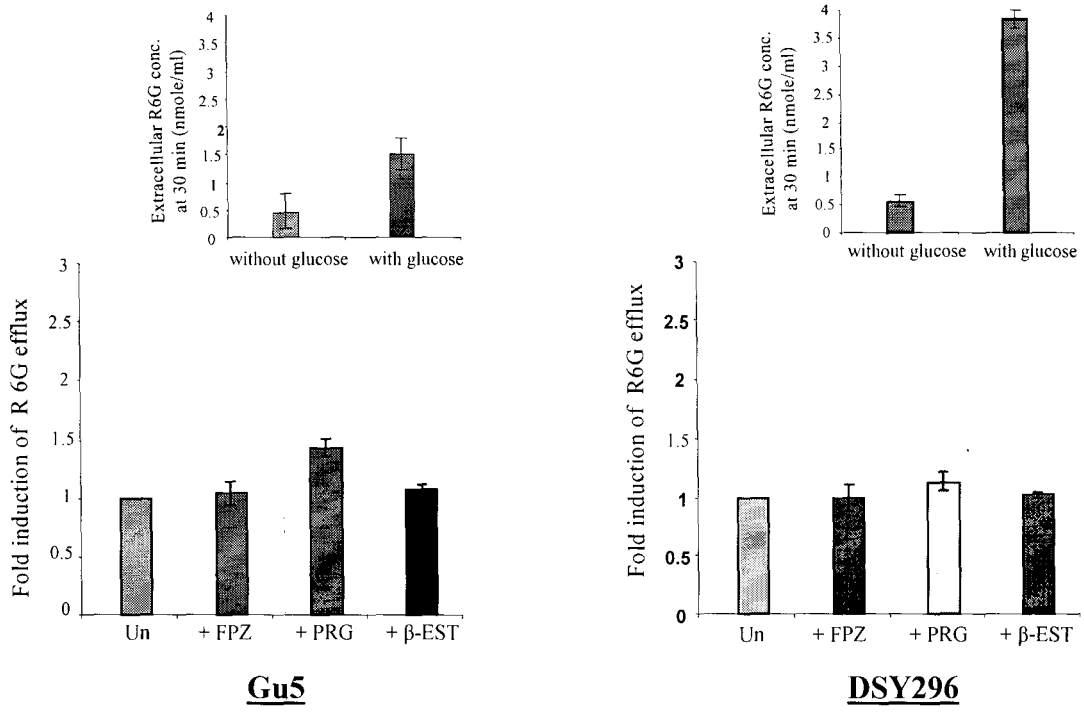


Figure 52. Non-inducibility of AR isolates.

(B) R6G efflux assay. The assay was performed essentially as described under *Materials and Methods*. The results are shown as relative fold induction of R6G efflux from induced AS isolates as compared to uninduced cells. The quantities reported are mean values ± Standard Deviations (*indicated by error bars*) of three independent experiments. The efflux of R6G is shown by open (for Gu5) and filled bars (for DSY296) respectively. The inset shows the extracellular concentration of R6G in the presence and absence of glucose.

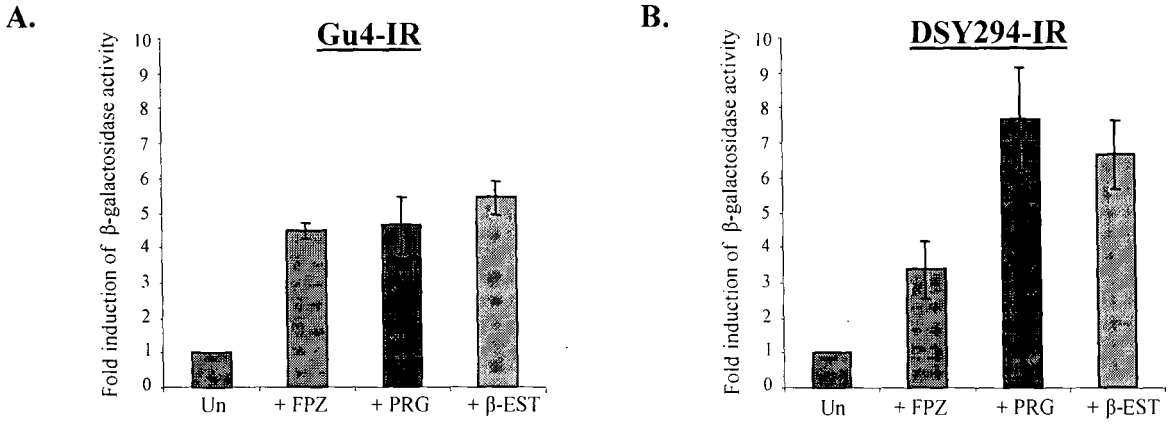


Figure 53. Analysis of *CDR1* promoter driven β-galactosidase reporter activity in induced AS isolates.

β-galactosidase reporter activity driven by the *CDR1* promoter was measured for control and induced AS isolates as described previously. Induced β-galactosidase reporter activity was normalized to that of corresponding untreated cells (*considered as '1'*), and represented as fold induction of reporter activity. Results are expressed as the Mean ± Standard Deviations (*indicated by error bars*) of three independent experiments. **(A)** Gu4 and **(B)** DSY294 isolates.

pBluescript, pBS-KS(+) were also dotted onto the membranes. As shown in Figures 54, the induced AS isolates exhibited an increased transcription rate of *CDR1* when compared with the untreated AS isolates (10, 12.3 and 12.2 fold for Gu4-IR and 24.2, 23.9 and 20.1 fold for DSY294-IR in the presence of fluphenazine, progesterone and ß-estradiol respectively).

3.2.2.9 Rapid acquired resistance in induced AS isolates is due to transiently enhanced *CDR1* transcription rate.

To compare the stability of the resistance phenotype between AR and induced AS isolates, both Gu4-IR and DSY294-IR isolates were subjected to Northern blot analysis after treatment with the inducers at indicated time points. Unlike in AR isolates (White *et al.*, 1997; White, 1997a; Manoharlal *et al.*, 2008), the *CDR1* mRNA levels in both the induced isolates reduced with time (Figure 55A). These findings also corresponded with the loss of *CDR1* promoter driven ß-galactosidase reporter activity indicating that enhanced *CDR1* transcription rate in AS isolates in response to inducers is time dependent (Figure 55B). As seen in Figure 55C too, each of the induced resistant isolate lost transcription activation with time, but the isolates did so at various rates for each inducer. Time dependent transcription run on assay further confirmed that transiently enhanced *CDR1* transcription rate is indeed at least one of the reason for rapid acquired resistance in both the induced AS.

3.2.2.10 Transient induction of *CDR1* is not mediated by an increase in its mRNA stability or poly(A) tail length.

We determined the *CDR1* mRNA half life and length of poly(A) tail in Gu4-IR and DSY294-IR isolates in order to analyze the role of these mechanisms, if any, in induced expression of *CDR1*. Total RNA was isolated at different time points after transcription inhibition by thiolutin in induced AS isolates and was analysed by RNA gel blots (Figure 56A). After probing the blots with a *CDR1* specific probe, hybridization signals were quantified by densitometry scanning in a phoshorimager. *CDR1* mRNA could be detected in both induced AS isolates (Gu4 and DSY294) at time T_0 (Figure 56B) and the signal intensity diminished progressively with time (mRNA half-life was approximately 60

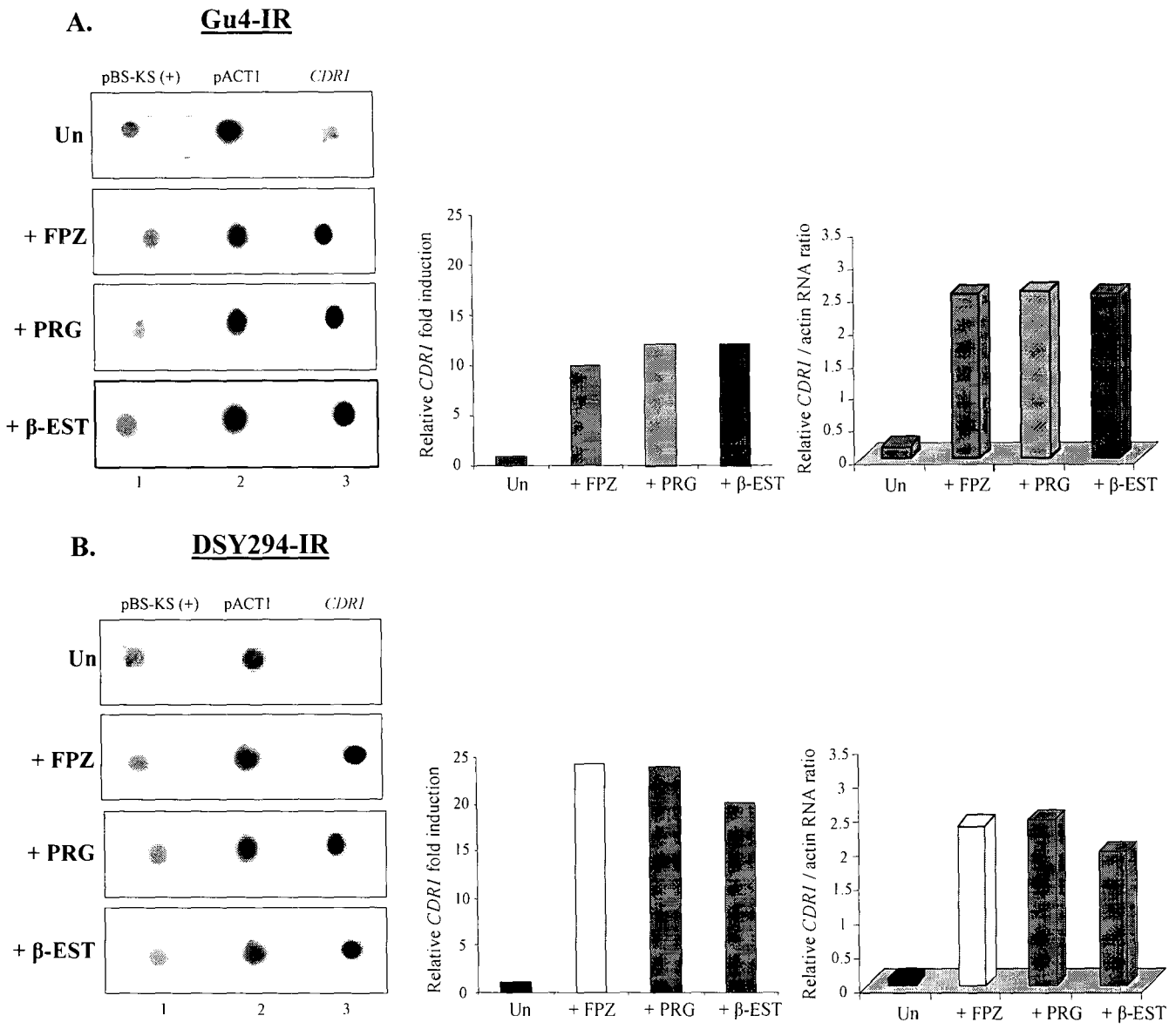


Figure 54. TRO analysis of induced AS isolates.

Approximately 2 μg (each) of *CDR1*, pACT1, and empty vector pBlueScript-KS(+) DNA was blotted and immobilized on charged nylon membranes (Hybond-N^+; Amersham Pharmacia Biotech) using a dot blot assembly apparatus. Nuclei were collected and nascent RNA was labeled with [α-^{32}P] UTP to elongate RNA transcripts. The blots were probed with total labeled nuclear run-on RNA as described under *Materials and Methods*. Hybridization signal intensities of nuclear RNA were quantified using densitometry scanning of phosphorimages. DNA from a Non-recombinant pBlueScript-KS(+) plasmid was used as a negative control to determine background and non-specific binding of labeled nuclear RNA to a random DNA fragment. Signal intensities for each isolate were subtracted from the negative control values and subsequently normalized to the intensity corresponding to their untreated counterpart (*considered as '1'*). Relative intensity of *CDR1* with respect to corresponding actin RNA for each isolate, is also plotted **(A)** Gu4 and **(B)** DSY294 isolates.

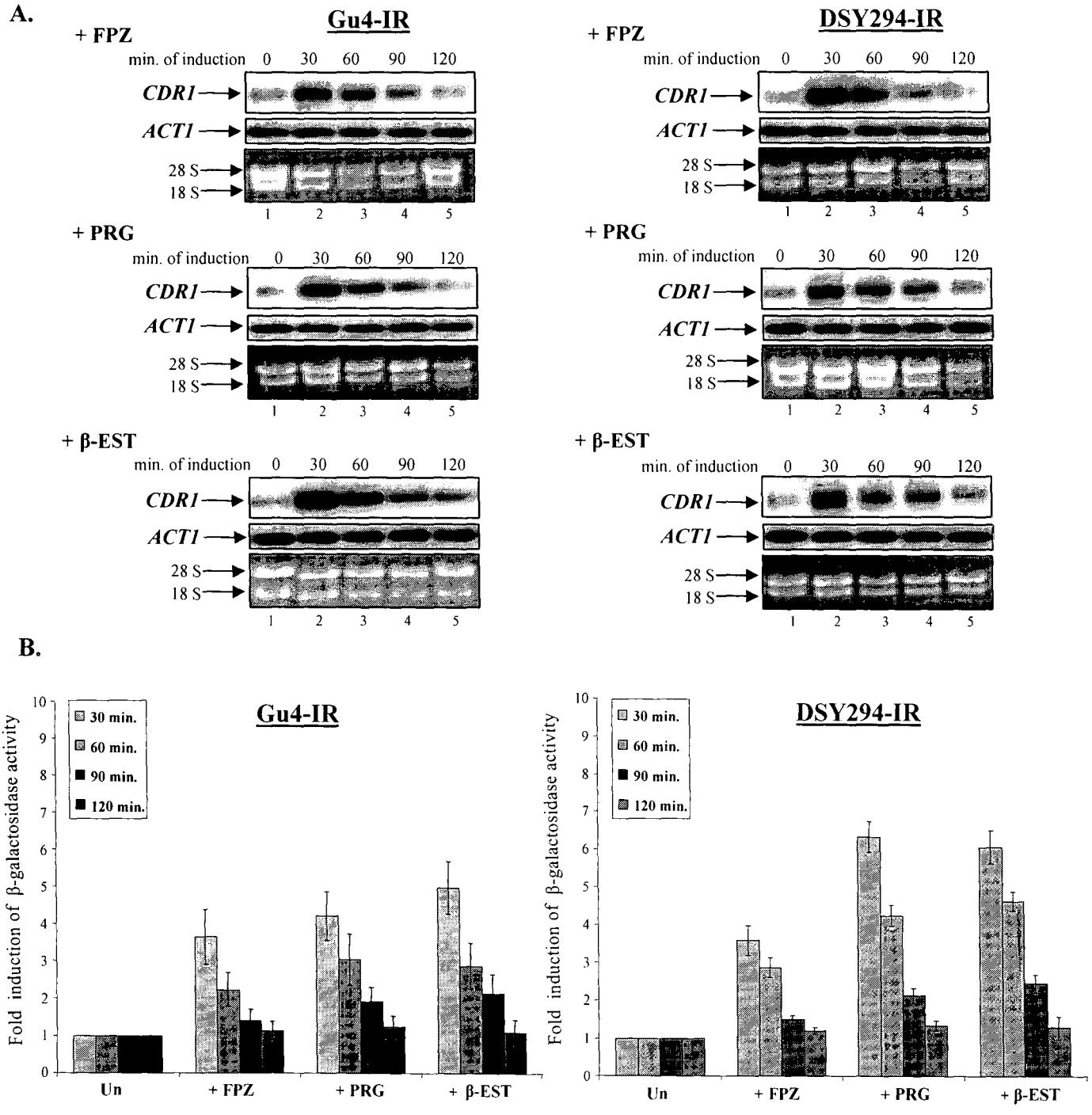
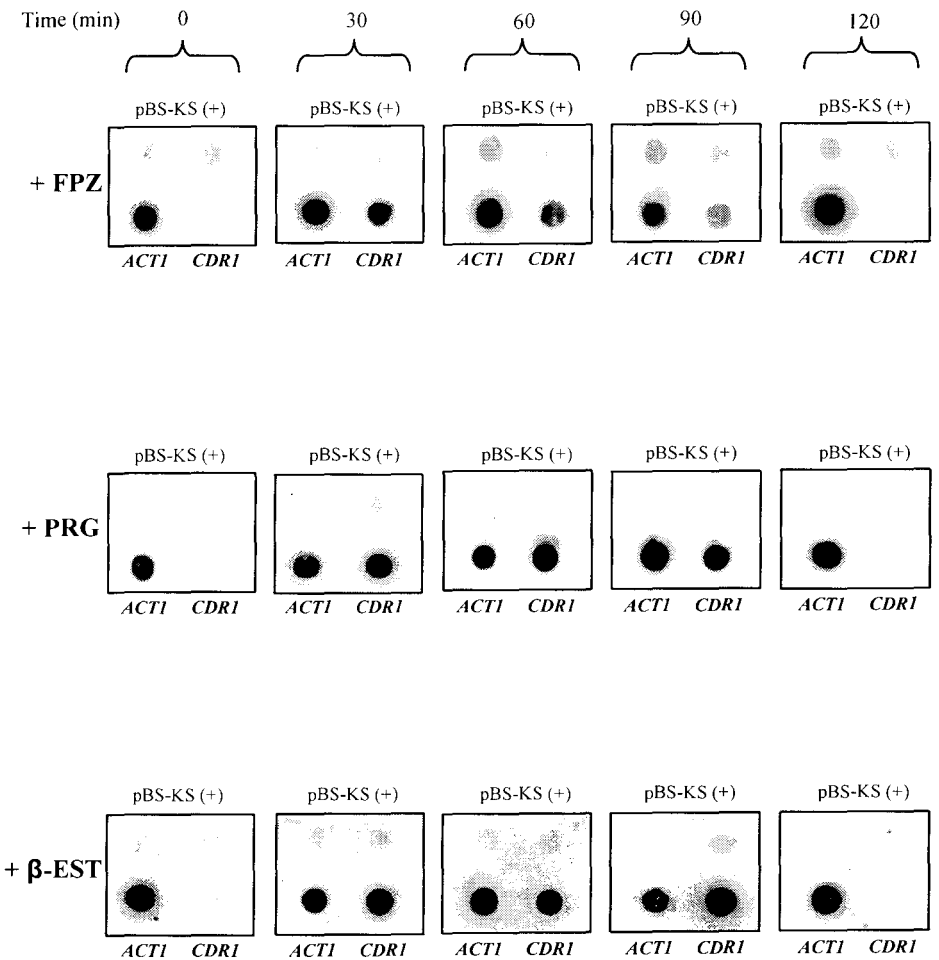
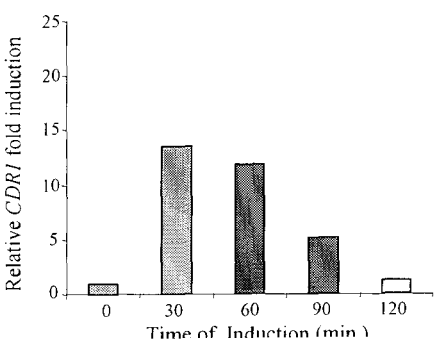


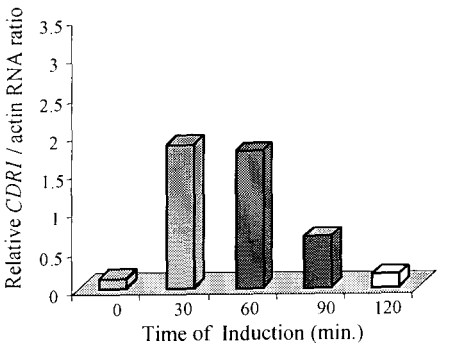
Figure 55. Time course of *CDR1* expression in AS isolates after exposure to inducers.
(A) AS isolates (Gu4 and DSY294) were exposed to inducers over a time interval of 0-120 min. Total RNA was extracted from AS isolates exposed to inducers at the indicated time points. Culture, treatment of cells, RNA extraction and Northern blot were performed as described under *Materials and Methods.* Equal RNA loading and transfer of RNA was assessed by rRNA bands as well as by subsequently deprobing and rehybridiztion of membranes with the *ACT1* probe. **(B)** β-galactosidase reporter analysis of AS isolates over a time interval of 0-120 min after exposure to inducers. Data represents the fold induction of *CDR1* promoter driven reporter activity as compared to untreated cells (*considered as '1'*) from three independent experiments.

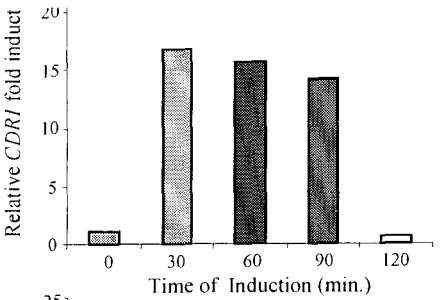
Gu4-IR

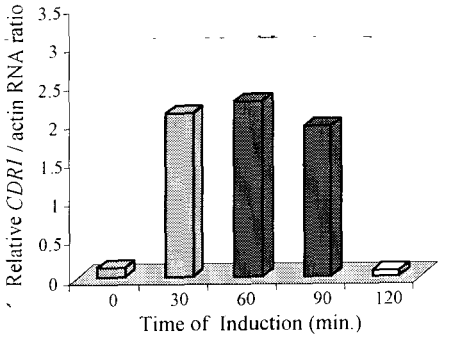
A.

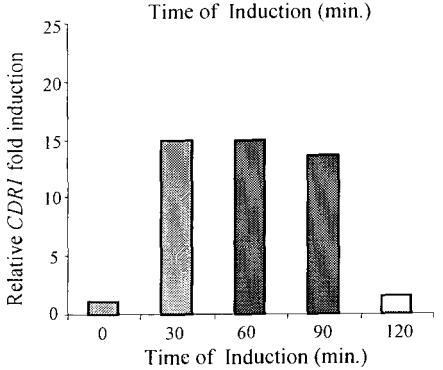

Time (min)
0
30
60
90
120
pBS-KS (+)
+ FPZ
+ PRG
+ β-EST
ACT1
CDR1

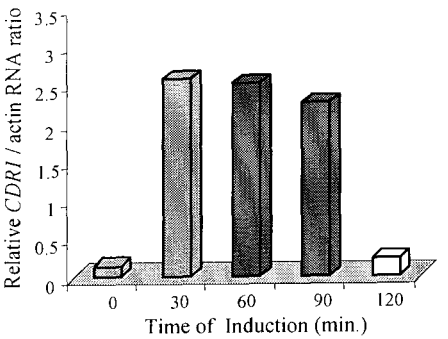

Relative CDR1 fold induction
0
5
10
15
20
25
0
30
60
90
120
Time of Induction (min.)


Relative CDR1 / actin RNA ratio
0
0.5
1
1.5
2
2.5
3
3.5
0
30
60
90
120
Time of Induction (min.)


Relative CDR1 fold induct
0
5
10
15
20
0
30
60
90
120
Time of Induction (min.)


Relative CDR1 / actin RNA ratio
0
0.5
1
1.5
2
2.5
3
3.5
0
30
60
90
120
Time of Induction (min.)


Relative CDR1 fold induction
0
5
10
15
20
25
0
30
60
90
120
Time of Induction (min.)


Relative CDR1 / actin RNA ratio
0
0.5
1
1.5
2
2.5
3
3.5
0
30
60
90
120
Time of Induction (min.)

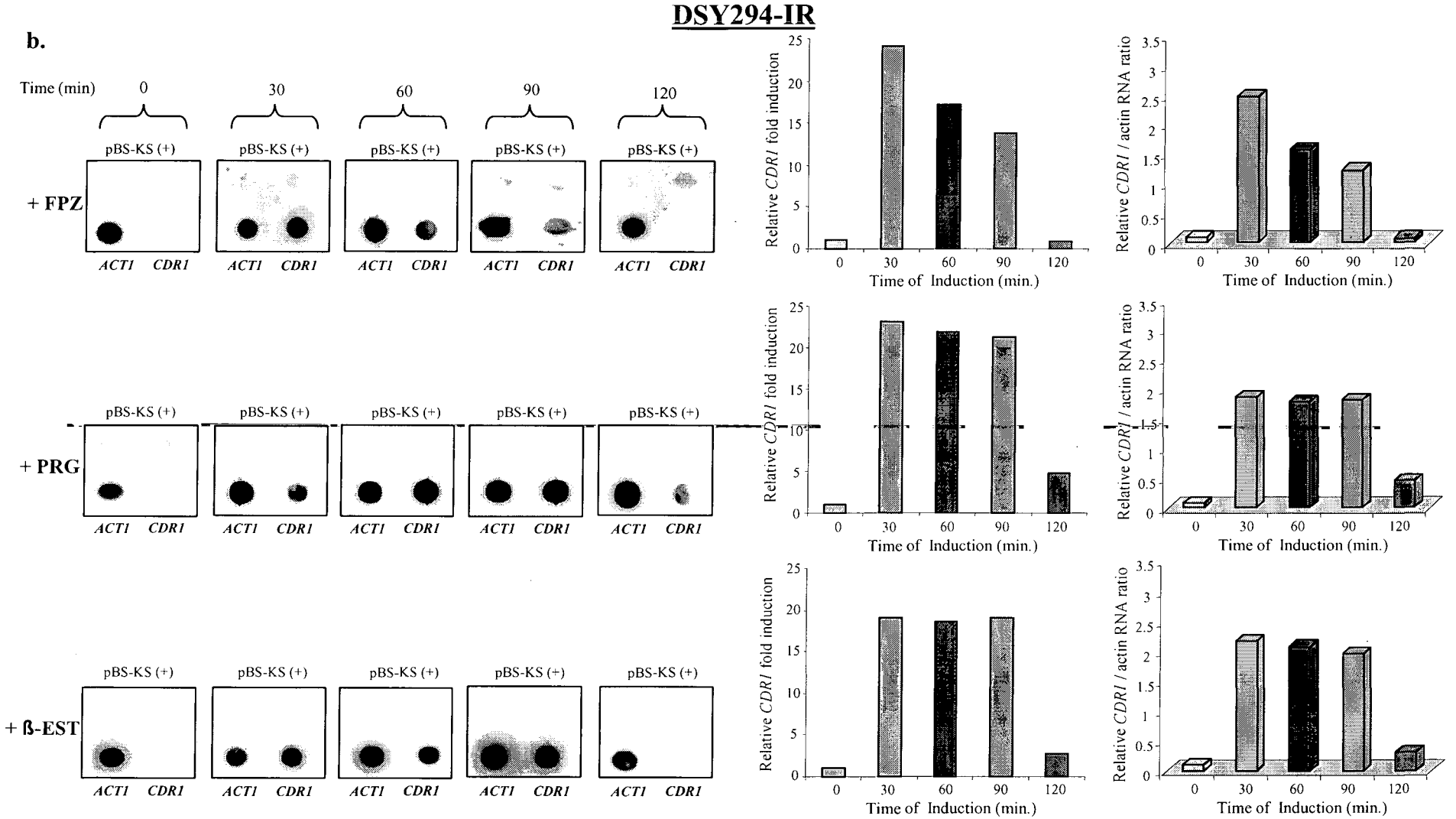


Figure 55. Time course of *CDR1* expression in AS isolates after exposure to inducers.

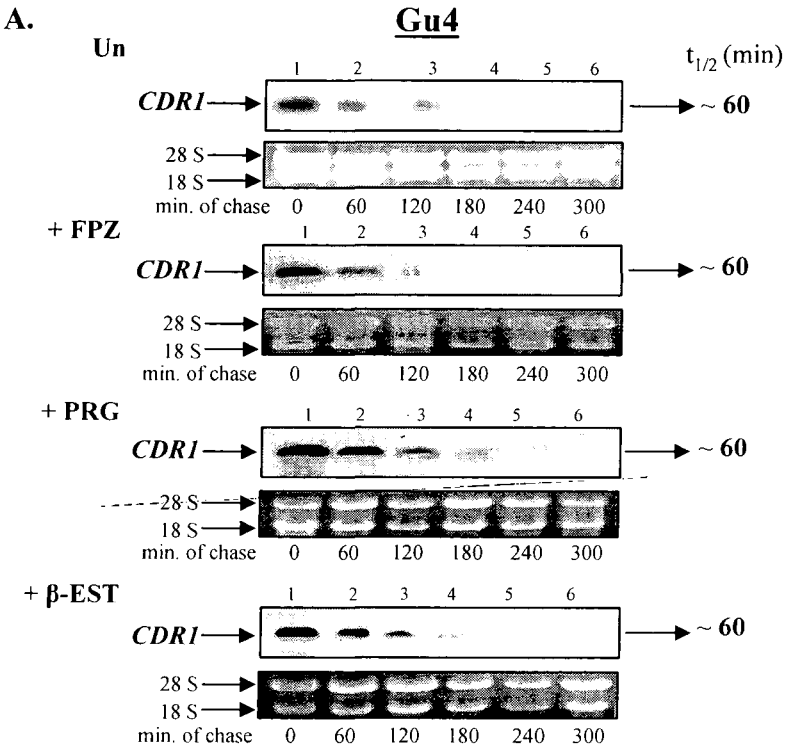
(C) Nuclear run-on transcription assays were done on nuclei isolated from both AS isolates at the indicated times after addition of inducers as described above. The labeled RNA from each indicated time point was hybridized to the pACT1 and *CDR1* specific PCR amplicon, which were immobilized by dot blotting on a nylon membrane. Signal intensities for each isolate were subtracted from the negative control values and subsequently normalized to the intensity corresponding to their untreated counterpart, which was considered as '1'. The relative intensity of *CDR1* with respect to corresponding actin RNA for each isolate at each time point was also plotted.
(a) Gu4-IR **(b)** DSY294-IR.

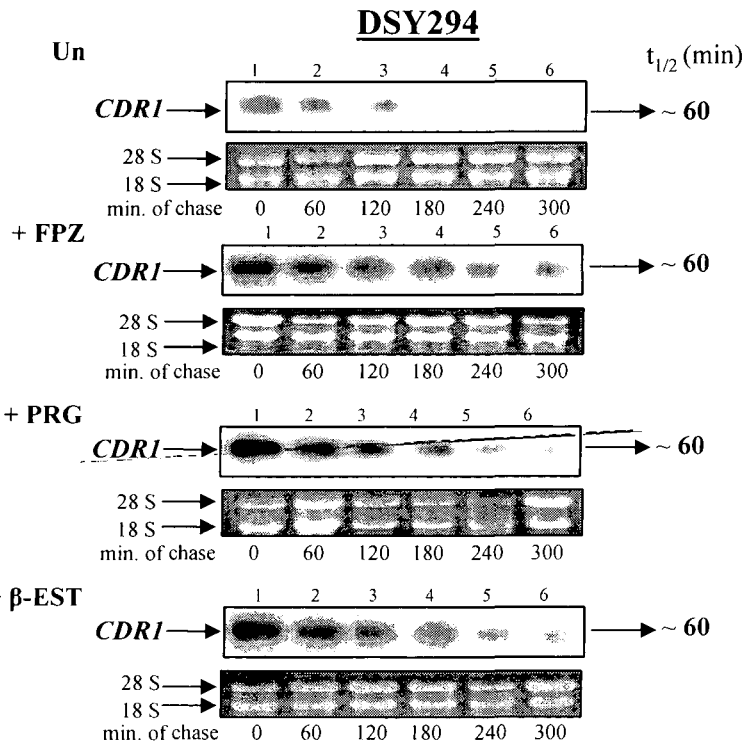
minutes). PAT analysis of both the induced AS isolates also did not show any change in poly(A) tail length as compared to untreated cells (Figure 57). Interestingly, Gu4-IR isolate restores the 3'UTR length similar to that of Gu5 isolates (Figure 58). However, clinical significance of this increased 3'UTR length in Gu4-IR isolate remains to be established.

3.2.3 Discussion

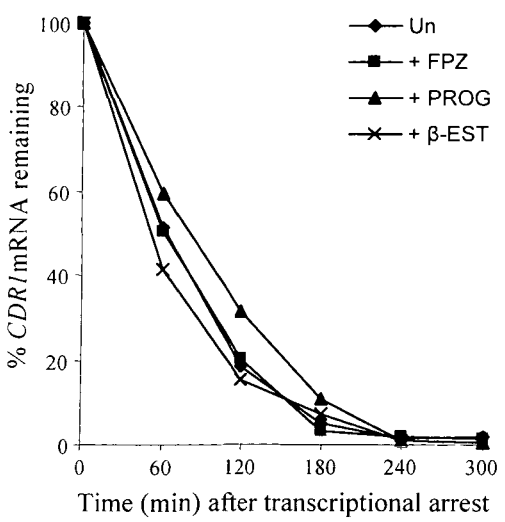
In many azole resistant clinical isolates of *C. albicans*, a positive correlation between resistance and *CDR1* over-expression has been reported by several independent research groups. (White, 1997a; Franz *et al.*, 1998; Lopez-Ribot *et al.*, 1998; White *et al.*, 1998; Franz *et al.*, 1999; de Micheli *et al.*, 2002; Chen *et al.*, 2004; Coste *et al.* 2004; Gaur *et al.*, 2005; Manoharlal *et al.*, 2008). Various other studies have dissected the *cis*-acting elements in promoter region (Puri *et al.*, 1999; de Micheli *et al.*, 2002; Karnani *et al.*, 2004; Gaur *et al.*, 2004 and 2005), and transcription factor(s) (Chen *et al.*, 2004; Coste *et al.* 2004; Znaidi *et al.*, 2008), that could contribute to differential *CDR1* mRNA expression between AS and AR isolates. We have recently observed that along with increased transcriptional activation, enhanced mRNA stability also contributes to the sustained *CDR1* over-expression in AR isolates (Manoharlal *et al.*, 2008). In the present study, we evaluate the cause of this increased mRNA stability of *CDR1* in AR isolates. By employing 3' RACE method (Figure 42A), we mapped and observed variation in the length of 3'UTR of *CDR1* (Figure 42B). Notably, heterogeneous products at 3'-ends observed were not specific to AS or AR isolates as each isolate has its own 3'UTR length irrespective of the level of *CDR1* expression (Figure 43A). Existence of this heterogeneity at 3'-end of *CDR1* can be explained by the alternative usage of several polyadenylation signals (Figure 43A and B), as has been well documented for other *MDR* genes (Hsu *et al.*, 1990; Edwalds-Gilbert *et al.*, 1997). For example, mouse *mdr1a* (Hsu *et al.*, 1990), and *EhPgp5* mRNA of *Entamoeba histolytica* trophozoites (Lopez-Camarillo *et al.*, 2003), show length variations at their 3'-ends which has been suggested to influence their mRNA half-lives. Notably, several other yeast genes also produce multiple transcripts with different 3'-ends as a result of carbon source regulated choice between alternative polyadenylation sites (Sparks *et al.*, 1998).

A.


Gu4
Un
t1/2 (min)
CDR1
~ 60
28 S
18 S
min. of chase 0 60 120 180 240 300
+ FPZ
+ PRG
+ β-EST


DSY294
Un
t1/2 (min)
CDR1
~ 60
28 S
18 S
min. of chase 0 60 120 180 240 300
+ FPZ
+ PRG
+ β-EST

B.


% CDR1 mRNA remaining
Time (min) after transcriptional arrest
Un
+ FPZ
+ PROG
+ β-EST

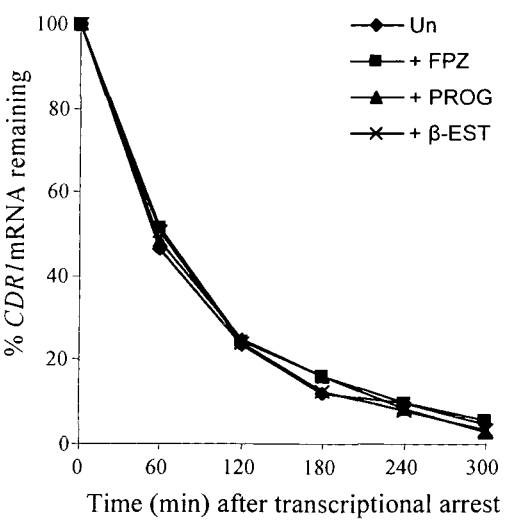
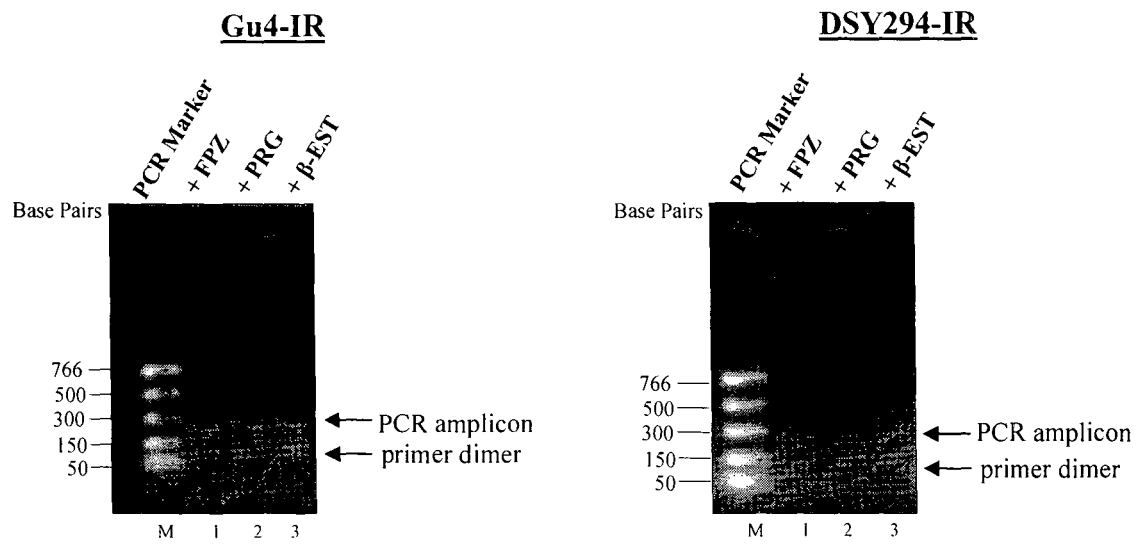
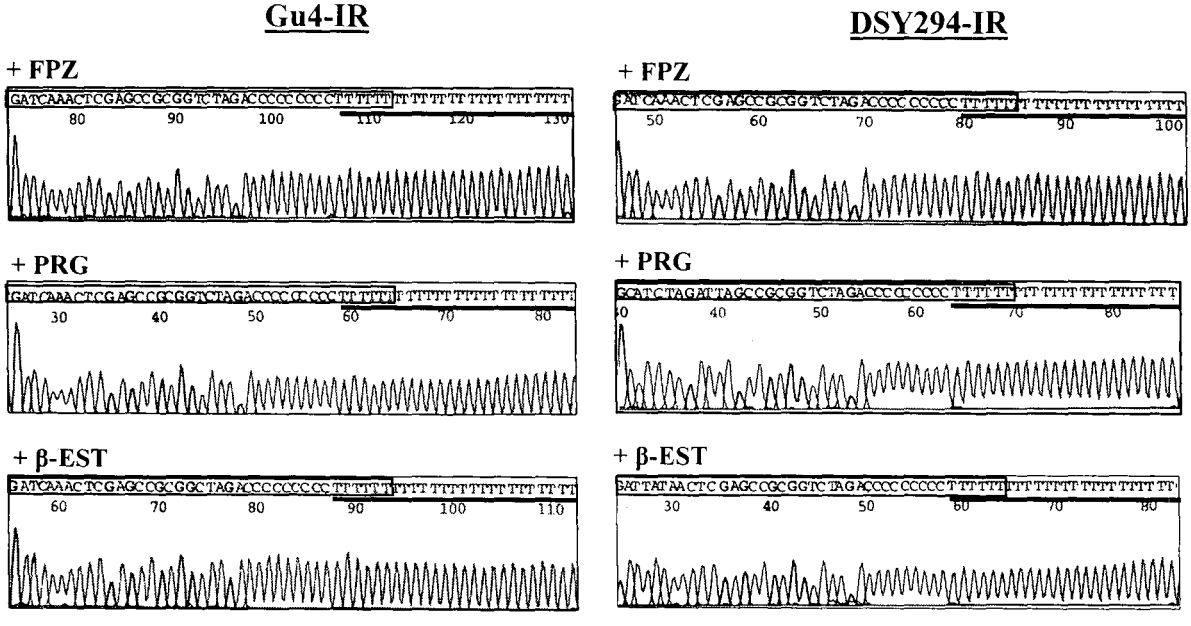

% CDR1 mRNA remaining
Time (min) after transcriptional arrest
Un
+ FPZ
+ PROG
+ β-EST

Figure 56. *CDR1* mRNA decay assay of induced AS isolates.
Exponentially growing cultures of *C. albicans* were treated with inducers (as mentioned under *Materials and Methods*) and subsequently incubated with the optimized thiolutin concentration (40 μg/ml) to inhibit ongoing *in vivo* transcription. Total RNA was isolated at the times indicated thereafter and fractionated on a 1% (*wt/vol*) agarose-2.2 M formaldehyde denaturing gel. **(A)** The gel was stained with ethidium bromide before blotting to monitor equal loading of the RNA and subsequently blotted onto a charged nylon membrane. The blot was hybridized with [α-^{32}P]dATP labeled *CDR1* specific probe. Time points in minutes are indicated below each phosphorimage. **(B)** The hybridization signals were quantified using densitometry scanning in a PhosphorImager scanner. The signal intensity at each time point was normalized to that of time T_0 (*expressed as a percentage*) and plotted as a line graph. $t_{1/2}$, half-life.

A.


Gu4-IR
DSY294-IR
PCR Marker
+ FPZ
+ PRG
+ β-EST
Base Pairs
766
500
300
150
50
PCR amplicon
primer dimer
M
1
2
3

B.


Gu4-IR
DSY294-IR
+ FPZ
+ PRG
+ β-EST

C.

Stains	Poly (A) tail length
Gu4-IR	
Fluphenazine	25 A's
Progesterone	25 A's
β-estradiol	25 A's
DSY294-IR	
Fluphenazine	22 A's
Progesterone	23 A's
β-estradiol	24 A's

Figure 57. Polyadenylation test (PAT) of *CDR1* mRNA in induced AS isolates.
(A) PAT assay was carried out as described earlier. Briefly, induced purified poly(A)$^+$-enriched mRNA of both AS isolates were polyguanylated using *PAP* and GTP and reverse transcribed using anti-sense primer, oligo(dC_9T_6)AP-RML. In subsequent step, the RT products were amplified by PCR with *CDR1* specific sense primer, CT-*CDR1*-F-RML and anti-sense anchor primer, AP-RML. The RT-PCR product following electrophoresis through 1.2% agarose gel, were visualized by staining with ethidium bromide (shown by an arrow). *C. albicans* isolates are given above the gel. *Lane M*, nucleotide size marker (*PCR Marker*). DNA standards are indicated on the left (*lane M*). **(B)** Sequences of cloned polyadenylated fragments obtained from each isolate. *Open box* sequence represent anti-sense primer, oligo(dC_9T_6)AP-RML used to generate them. *Continuous underline* sequences denotes the poly'T' tract (complementary to polyadenylated sequence). **(C)** Table depicting the representative poly(A) tail length of *CDR1* in both the induced AS isolates.

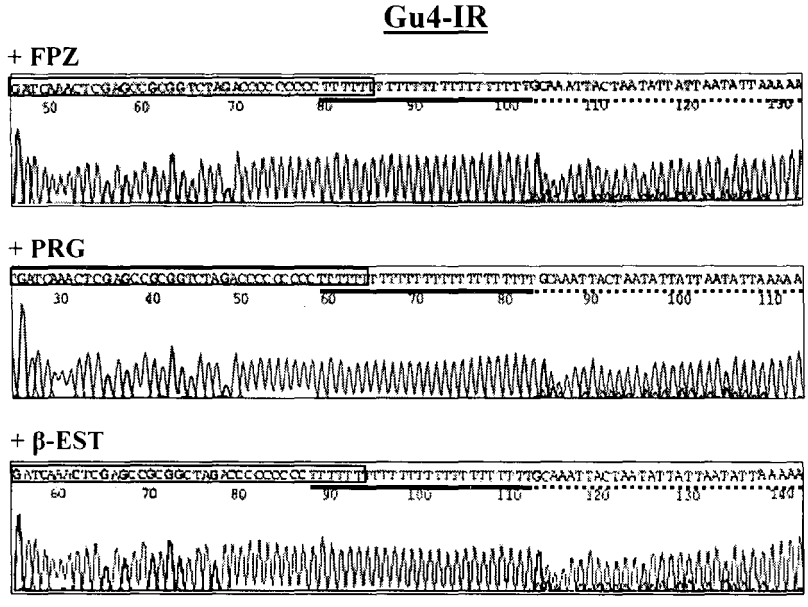


Figure 58. Increment in *CDR1* 3′UTR length of induced Gu4 isolate.
PAT assay was carried out as described in *Materials and Methods*. Sequences of cloned polyadenylated fragments obtained for each tested inducers. *Open box* sequence represent anti-sense primer, oligo(dC_9T_6)AP-RML used to generate them. *Continuous underline* sequences denotes the poly'T' tract (complementary to polyadenylated sequence). *Dotted underline* sequences represent the increased 3′UTR length of *CDR1* upon induction. Interestingly, induced Gu4 isolate restores the 3′UTR length similar to that of Gu5 isolates.

3'UTR of *CDR1* is ~78% AU-rich and showed sequence polymorphism between AS and AR isolates (Figure 43A). Changes in rate of turnover and RNA cleavage can be affected by these 3'UTR secondary structure motifs and their corresponding RNA-binding protein(s). *In silico* analysis and secondary structure prediction suggests the existence of exposed polypyrimidine (Py) tract, AURE and a CPE motif (Figure 43C). In other organisms, Py-tract and AURE binding proteins (AUBP) are known to be involved in mRNA stability, whereas CPE-motif interacting proteins target specific mRNAs to cytoplasmic polyadenylation, producing the translational activation of the transcripts (Czyzyk-Krzeska *et al.*, 1994; Irwin *et al.*, 1997; Hake *et al.*, 1998; Tillmar *et al.*, 2002). These signals in *CDR1* 3'UTR could also constitute a potential target site for RNA binding protein(s). It needs to be mentioned that the prediction of the stable secondary structures does not necessarily imply their involvement in the regulation of *CDR1* mRNA stability, which otherwise are known to play a key role as revealed in higher eukaryotes (Ross, 1996). However, these predictions do indicate differential RBP-RNA binding in AS and AR isolates which could be due to difference in the regulatory RNA motifs they recognize. To experimentally test the proposed mRNA destabilizing activity of the predicted *CDR1* 3'UTR regulatory motifs, expression vectors harboring heterologus and codon-optimized *Streptococcus thermophilus lacZ* reporter coding region (Uhl and Johnson, 2001), in transcriptional fusion with 3'UTR of either AS or AR isolates were constructed (Figure 45A). Interestingly, swapping of 3'UTR between AS or AR isolates had no effect on *lacZ* reporter activity (Figure 46A and B) indicating that *CDR1* 3'UTR is not sufficient to affect either transcription or mRNA stability. Our results are in agreement with mammalian *MDR1*-3'UTR (Prokipcak *et al.*, 1999), and *EhPgp5*-3'UTR (Lopez-Camarillo *et al.*, 2003), which also harbour putative regulatory RNA motifs, but do not behave as active destabilizing elements for their corresponding mRNA's. The regulatory RNA motifs residing in 3'UTRs control mRNA stability and in some cases mRNA nuclear export, cytoplasmic localization, translation efficacy, polyadenylation and translation control through 3'UTR sequence-binding proteins, *see* Figure 59 (De Maria and Brewer, 1996; Shalgi *et al.*, 2005).

A correlation between relative affinity of 3'UTR for RNA-binding protein(s) and mRNA stability has been documented in several studies (Wang *et al.*, 1995; Ross, 1996;

McCarthy, 1998). Existence of this correlation also suggests that rather than existence of 'all or none rule' phenomenon, interaction between 3'UTRs and proteins may be altered in either way, and subtle shifts in affinity may be associated with alteration in mRNA half-life.

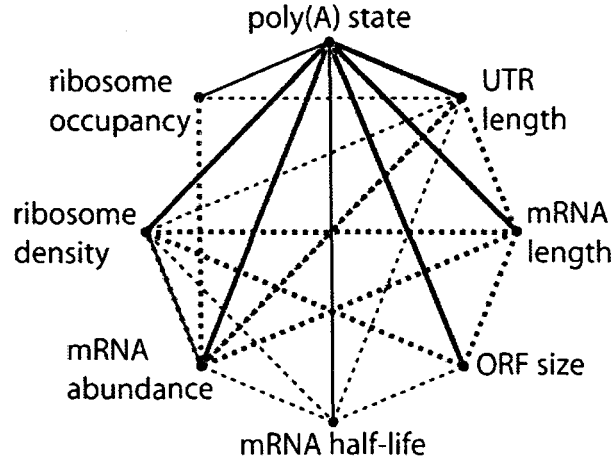


Figure 59. Correlation among various mRNA stability determinants.
Solid and dashed lines represent correlation among various determinants of mRNA stability. *Green lines* represent positive correlations; *red lines* represent negative correlations and *black lines* indicate no simple correlation. *See text for detail.*

Our R-EMSA results also supports this hypothesis as decreased affinity of 3'UTR of AR isolates (Figures 48A and B) for the *trans*-regulatory factor(s) is correlated with relative long half-life of *CDR1* mRNA. However, the identity, relative expression level, functional role and precise corresponding binding sites of *CDR1* 3'UTR mRNA-interacting protein(s) in both AS and AR isolates remains to be characterized.

The mRNA abundance is also affected by a concerted interplay of key factors such as the activities of poly(A) polymerase, deadenylases, and poly(A) binding proteins (Zhao *et al.*, 1999). The pre-mRNAs are polyadenylated in a reaction involving 3'-endonucleolytic cleavage followed by poly(A) tail synthesis (Zhao *et al.*, 1999). Poly(A) tail is also considered as a strong modulator of mRNA stability and its length is subjected to cellular control throughout the life span of the mRNA (Figure 60) (Wickens *et al.*, 1997; McCarthy, 1998). It is well known that longer poly(A) tails provide higher stability to mRNA and promote a more efficient translation (Ross, 1996).

Our PAT assay in the present study shows that *CDR1* mRNA has 30-35% longer poly(A) tail in AR isolates as compared to their respective AS isolates (Figure 50C and D),

suggesting polyadenylation and deadenylation events occurring at different rates could affect *CDR1*

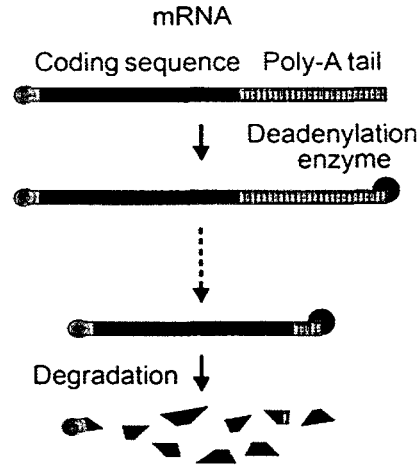


Figure 60. Shortening of poly(A) length targets the mRNA for its destruction.
Cartoon of mRNA senescence when the poly(A) tail is progressively shortened before degradation. *See text for detail.*

mRNA half-life. This finding is important since relatively longer poly(A) tail of *EhPgp5* mRNA is associated with its enhanced half-life in *E. histolytica* trophozoites grown in the presence of emetine (Lopez-Camarillo *et al.*, 2003). Interestingly, this increased poly(A) tail length has been associated to an induced nuclear poly(A) polymerase (*EhPAP*) expression (Garcia-Vivas *et al.*, 2005). *C. albicans PAP1* (*CaPAP1*) is located within the Mating Type-Like (MTL) locus, where *TAC1* and its linkage with the locus in determining its hyperactivity has been established (Coste *et al.*, 2006). It has been recently shown that a hyperactive allele of *TAC1* is responsible for *CDR1* as well as *CDR2* up-regulation in DSY296 (Coste *et al.*, 2006). However, it has not yet been explored if hyperactive *TAC1* allele contribute to the differential transcript stability and poly(A) tail length of *CDR1* in these isolates. The existence of a link between hyperactive *TAC1* allele, *CaPAP1* and MDR is thus an exciting possibility that needs to be explored. It would also be interesting to see if the increase in poly(A) tail length in AR isolates is specific to *CDR1* transcript or it is a general feature of mRNA population of MDR related genes.

MDR can also be acquired if *C. albicans* cells are exposed to the *CDR1* inducers for a short time (Karababa *et al.*, 2004; Larsen *et al.*, 2006). We observed that AS isolates, if

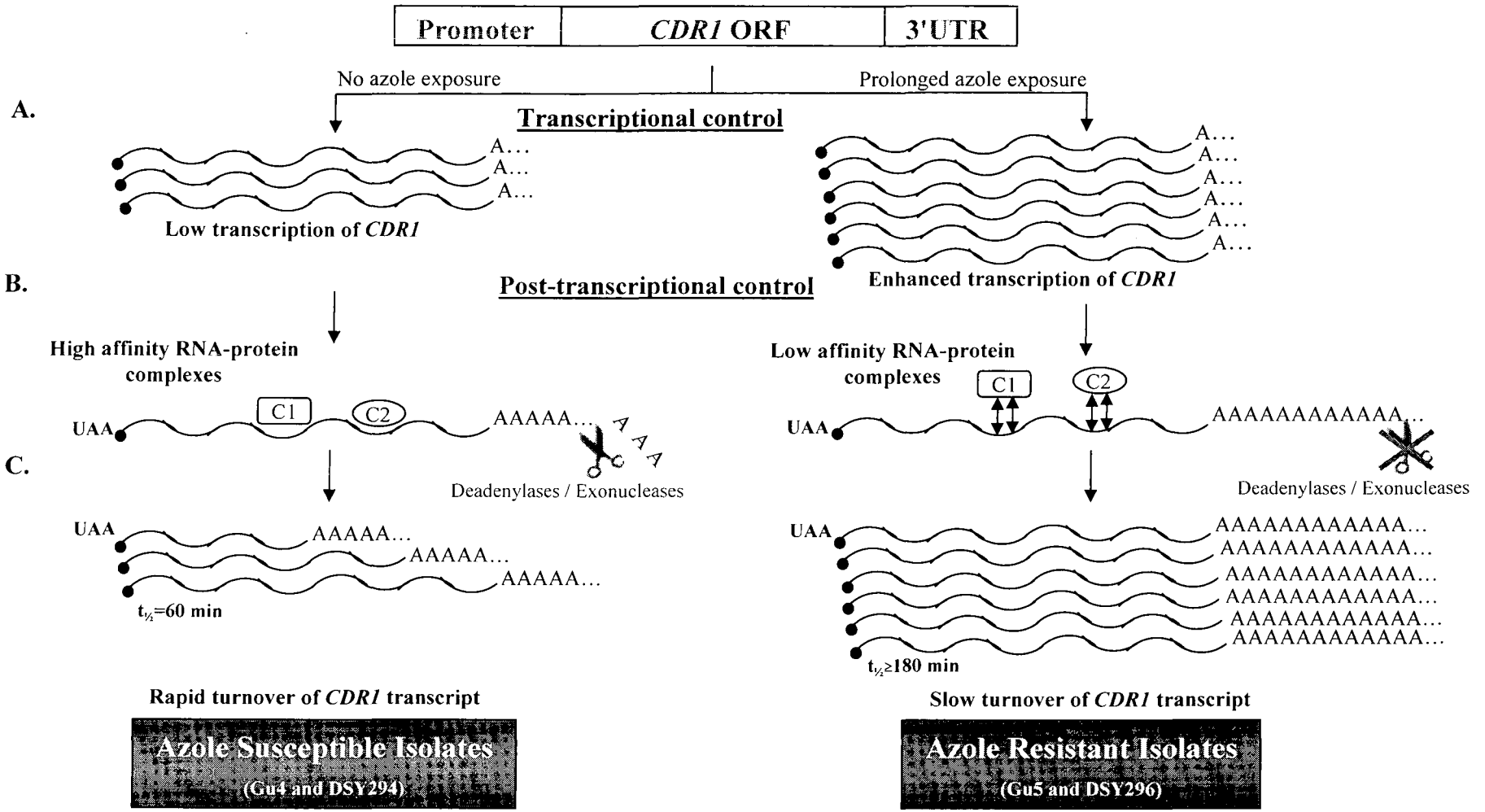


Figure 61. Hypothetical model of differential *CDR1* mRNA expression in AS and AR isolates.
(A) An enhanced transcription of *CDR1* gene occurs in both AR isolates (Gu5 and DSY296) due to prolonged azole exposure. **(B)** in nuclei and cytoplasm, mRNA processing and decay mechanisms influence the *CDR1* mRNA half-life. AR isolates (Gu5 and DSY296) have relatively low binding affinity for certain unidentified RNA binding protein(s) that might prevent rapid mRNA turnover of *CDR1* than their corresponding AS isolates (Gu4 and DSY294). **(C)** Additionally, AR isolates possess certain putative response factors and polyadenylation proteins, which could promote an efficient polymerization of poly(A) tails, resulting in a longer poly(A) tail and increased mRNA stability than corresponding AS isolates. *Wavy lines* denote the *CDR1* mRNA, and the *filled circle* represents the 'UAA' *translational stop codon.* ORF, *open reading frame*; C1: *RNA-Protein Complex 1*, C2: *RNA-Protein Complex 2.*

exposed to fluphenazine and steroids become resistant to antifungal drugs, with a concomitant increase in *CDR1* mRNA. This *in vitro* rapid acquired resistance phenotype is transient and temporal in nature. Our results demonstrate that *C. albicans* develops an unstable *in vitro* resistance to antifungals *via* transcription activation. In contrast, *in vivo* stable resistant developed in AR isolates is contributed by both enhanced transcription rate and mRNA stability of *CDR1*. Our working hypothesis thus assumes that in contrast to AS isolates, AR isolates obtained after prolonged fluconazole therapy have increase transcription of *CDR1* and maintain longer poly(A) tails, contributing to enhanced mRNA stability (Figure 61). The characterization of the 3' non-coding region of *CDR1* should lead to an understanding of the mechanism underlying MDR and should also be useful for the development of antisense oligonucleotides strategy that can be exploited as a mean to combat MDR in *C. albicans.*

✓ Part of this section was presented as an oral presentation **"Transcriptional and post-transcriptional regulation of *CDR1* mRNA in multidrug resistance of *Candida albicans*"** at the 9th American Society for Microbiology Conference on *Candida and Candidiasis* held on 24th-28th March, 2008 in New Jersey, USA.

"A summary is the place where you get tired of thinking"

Summary

Summary

Oral infections of the pathogenic yeast *Candida albicans* are one of the most frequent and serious life-threatening problems in human immunodeficiency virus-infected patients (Graybill, 1988; Vanden Bossche *et al.*, 1994). Oral candidiasis has been controlled largely by use of the systemic azole drug fluconazole and topical azole and polyene drugs (Vanden Bossche *et al.*, 1989; Williams and Wilkins, 2004). However, the use and occasional over-use of fluconazole has resulted in the emergence of azole-resistant strains of *C. albicans* (Sanglard *et al.*, 1995; White *et al.*, 1998; Franz *et al.*, 1999). Factors contributing to the development of clinical resistance in patients are numerous and include the extent of immuno-suppression, the level of exposure to azole drugs, and intrinsic resistant of the fungus to antifungal drugs (White *et al.*, 1998; Akins, 2005). To date, four molecular resistance mechanisms have been identified in the development of azole resistance in clinical *C. albicans* isolates: (i) increased cellular content of the azole target encoded by *ERG11*; (ii) the decreased affinity of Erg11p to azoles by mutation(s) in *ERG11*; (iii) alteration in ergosterol biosynthetic pathway and (iv) failure of azoles to accumulate inside the cells (Sanglard *et al.*, 1998). Among these mechanisms, the last is the most commonly observed and involves the consistent up-regulation of MDR genes responsible for their drug-resistant phenotype: *CDR1* and *CDR2* (encoding homologous transporters of the ATP binding cassette family functioning as multidrug efflux pumps and phospholipid flippases) (Prasad *et al.*, 1995; Sanglard *et al.*, 1995 and 1997), *MDR1* (encoding a multidrug transporter of the major facilitator super-family) (Sanglard *et al.*, 1995; Franz *et al.*, 1998), *ERG11* (Oliver *et al.*, 2007; Hoot *et al.*, 2008) and *PDR16* (encoding a phospholipid transfer protein) (Saidane *et al.*, 2006; Znaidi *et al.*, 2007).

Constitutive and stable over-expression of these drug resistance genes (*CDR1*, *CDR2*, *MDR1*, *ERG11* and *PDR16*) in clinical *C. albicans* isolates have been shown to be regulated at the level of transcription (Lyons and White, 2000; Coste *et al.* 2004; Hiller *et al.*, 2006; Rognon *et al.*, 2006; Znaidi *et al.*, 2007; Oliver *et al.*, 2007; Morschhäuser *et al.*, 2007; Hoot *et al.*, 2008). Understanding the transcriptional control of these genes by both *cis*- and *trans*-acting effectors, is therefore important for determining how azole resistance mechanisms are regulated. So far, regulatory aspects of *CDR1* has been studied extensively by several research groups. It has been shown that basal as well as induced

transcription activation of *CDR1* is controlled largely by various *cis*-acting consensus (Sp1, AP-1, Y-box) and specific [Basal (BRE) (de Micheli *et al.* 2002), Negative (NRE) (Gaur *et al.*, 2004 and 2005) and Drug/Steroid Response Element (DRE and SRE) (De Micheli *et al.* 2002; Karnani *et al.*, 2004) elements as well as by *trans*-acting factors [*CaNDT80* (Chen *et al.*, 2004), *TAC1* (Coste *et al.*, 2004), *FCR1* (Talibi and Raymond, 1999; Shen *et al.*, 2007), *Upc2* (Znaidi *et al.*, 2008) and *Tup1* (Murad *et al.*, 2001)] in their 5' flanking region. Transcriptional regulatory *cis*-acting elements H_2O_2 (HRE)/Benomyl (BRE) Responsive Element (Rognon *et al.*, 2006) and more recently a zinc cluster TF, designated as *MRR1* (Multidrug Resistance Regulator 1) has also been identified for *CaMDR1* promoter region (Morschhäuser *et al.*, 2007). Recent studies have also shown the *CaUPC2* dependent Azole-Responsive Enhancer element (ARE), and a perfect Sterol Response Element (SRE: TCGTATA) residing in *ERG11* promoter are crucial for its transcription activation (Oliver *et al.*, 2007). *CaUPC2* itself is also reported o be transcriptionally induced in response to antifungal drugs and anaerobicity through Upc2p-dependent and -independent mechanisms (Hoot *et al.*, 2008). It is thus apparent that transcriptional regulation plays an important role in the mechanism underlying the over-expression of MDR genes. However, the relevance of the post-transcriptional events associated with it is relatively poorly understood.

In this study, we evaluate the molecular mechanisms that contribute to the maintenance of constitutively high *CDR1* transcript levels in two matched pairs of azole-susceptible (AS) and azole-resistant (AR) clinical isolates of *C. albicans*.

- Expression analysis of reporter constructs of *GFP* and *lacZ* fused either to the *CDR1* promoter (P_{CDR1}-*GFP/lacZ*; transcriptional fusion) or to the *CDR1* ORF (P_{CDR1}-*CDR1-GFP/lacZ;* translational fusion) integrated at the native *CDR1* locus indicates that expression of the two reporter genes as transcriptional fusion in the AR isolates is higher than in matched AS isolates. However, the fold difference in the reporter activity between the AS and the AR isolates is even higher for the translational fusions, indicating that the sequences within the *CDR1* coding region also contribute to its increased expression in AR isolates.

- Further analysis of these observations by TRO assays demonstrated a ~5-7 fold difference in the transcription initiation rates in the AR isolates as compared to their respective matched AS isolates (5-fold for Gu5 *versus* Gu4 and 7-fold for DSY296 *versus* DSY294).

- Measurement of mRNA stability using thiolutin as a transcription inhibitor showed that the half-life of *CDR1* mRNA in the AR isolates was 3-fold higher than in the corresponding AS isolates. The turnover of the *CDR1* transcript occurred much more slowly in the AR isolates (half-life >180 minutes) in contrast to AS isolates (half-life ~ 60 minutes).

- Cycloheximide chase assays reveals Cdr1p stability does not differ in AS and AR isolates. The half-life of Cdr1p was similar in AS and AR isolates and was calculated to be approximately 90 min.

Taken together, our results demonstrate that both increased transcription and enhanced mRNA stability contribute to the over-expression of CDR1 in azole-resistant C. albicans isolates.

- It became apparent now that the regulatory elements are mostly embedded in the non-coding part of the genomes. Among non-coding regions, the 5'- and 3'-UnTranslated Regions (5'-UTR and 3'-UTR) of eukaryotic mRNAs contain sequence motifs crucial for many aspects of transcriptional and post-transcriptional gene regulation respectively. To identify the critical control regions involved in *CDR1* transcription control, 5' upstream proximal stretch of the *CDR1* promoter was dissected. Sequence analysis of 5'-UTR of *CDR1* promoter reveals identical promoter sequence rules out the *cis*-mutation in 5'-UTR, thereby suggesting the possible role of mutation(s) in *trans*-regulatory factor(s) in matched set of AS and AR isolates. Our hypothesis is also in agreement with the existence of hyperactive allele of *TAC1* that has been shown to be responsible for *CDR1* as well as *CDR2* up-regulation in AR isolate (DSY296).

- To evaluate the post-transcriptional control point involving *CDR1* mRNA stability, the complete 3'-end of the *CDR1* mRNA was mapped by 3'-Rapid Amplification of cDNA Ends (3' RACE)-PCR method. 3'UTR of *CDR1* mRNA display length heterogeneity. For the matched isolates-Gu4/Gu5 and DSY294/DSY296, 3'UTR length was mapped to 137/163 bases and 162/137 bases, respectively (*position relative to 'UAA' stop codon*).

- Subsequent characterization of mapped 3'UTR of *CDR1* reveals that it was ~78% AU-rich, possessed perfect destabilizing AU-rich elements ('AURE'), heterogeneous polyadenylation sites, and had several putative consensus sequences for cytoplasmic RNA-binding proteins(s).

- Comparison of the sequence and structure motifs of 3'UTR of *CDR1* in reveal polymorphism at position -7U, U19C and C64U (where the base[s] before and after the number is the sequence from Gu4 and Gu5 isolates, respectively) and an additional extended 26 and 25 base 'AU' (Adenylate-Uridylate) rich stretch at 3'-end for only Gu5 (AR) and DSY294 (AS) isolates, respectively.

- Swapping of heterologous and chimeric *lacZ-CDR1* 3'UTR transcriptional reporter fusion did not alter the ß-galactosidase reporter activity in AS and AR isolates, indicating that *cis*-acting sequences within the *CDR1* 3'UTR itself are not sufficient to confer the observed differential mRNA decay.

- RNA-Electrophoretic Mobility Shift Assay showed reduced binding of *trans*-regulatory factor(s) in AR isolates. Signal intensities of two RNA-protein complex '*C1*' and '*C2*' (~66% and 51% for Gu5 and ~60% and 40% for DSY296) was lower than the corresponding complex intensity of AS isolates (Gu4 and DSY294 respectively).

- PCR-based PAT (Polyadenylation Test) assay establish relatively longer poly (A) tail length of *CDR1* mRNA in AR isolates. Poly(A) tail length of the *CDR1* mRNA of AR isolates was ~35% to 50% hyperadenylated compared with AS isolates.

Taken together, our results suggested that enhanced CDR1 mRNA stability in AR isolates results mainly from the reduced interaction of RNA binding trans-regulatory factor(s) to corresponding 3'UTR and hyper-adenylation of its transcript.

- Short term *in vitro* induced over-expression of *CDR1* display enhanced efflux of R6G and drug resistance in AS isolates and this rapid acquired resistance in induced AS isolates is due to transiently enhanced transcription rate of *CDR1*.

- Transient drug resistance in AS isolates induced by drug and steroids is not mediated by an increase in *CDR1* mRNA stability or its 3'UTR polyadenylation. Thus, AR and transiently induced AS adopt different strategies for maintaining high expression level of *CDR1*.

Future perspectives

The designing of anti-sense oligonuclotides, that will elicit specific effects by promoting the rapid destabilization of the CDR1 mRNA can be used either in those AR isolates which have mRNA stability as their primary mechanism of CDR1 over-expression, or as a combinational treatment alongside other conventional anti-MDR therapies. Thus, our observations acquire greater clinical significance as transcriptional and post-transcriptional regulatory pathway of CDR1 in pathogenic yeasts C. albicans can serve as an attractive antifungal drug target in circumvention of multidrug resistance. Of note, due to similar eukaryotic structural organization of fungal and mammalian cells, future prospects of these potential antifungal drug directed against C. albicans will depend on elucidating their highly specificity towards C. albicans MDR phenomenon only.

"When you achieve the goal, let's remember the former's role"

References

References

- **Akache B. and Turcotte B.** (2002). New regulators of drug sensitivity in the family of yeast zinc cluster proteins. *J. Biol. Chem.* **277**: 21254-21260.

- **Akache B., MacPherson S., Slyvain M.A. and Turcotte B.** (2004). Complex interplay among regulators of drug resistance genes in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279**: 27855-27860.

- **Akins R.A.** (2005). An update on antifungal targets and mechanisms of resistance in *Candida albicans*. *Med. Mycol.* **43**: 285-318.

- **Alarco A.M. and Raymond M.** (1999). The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. *J. bact.* **181**: 700-708.

- **Alarco A.M., Balan I., Talibi D., Mainville N. and Raymond M.** (1997). *AP1*-mediated multidrug resistance in *Saccharomyces cerevisiae* requires *FLR1* encoding a transporter of the major facilitator superfamily. *J. Biol. Chem.* **272**: 19304-19313.

- **Albertson G.D., Niimi M., Cannon R.D. and Jenkinson H.F.** (1996). Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. *Antimicrob. Agents Chemother.* **40**: 2835-2841.

- **Ambudkar S.V. and Gottesman M.M.** (1998). ABC Transporters: Biochemical, cellular and molecular aspects. Academic Press, NewYork.

- **Angel P., Imagawa M., Chiu R., Stein B., Imbra R.J., Rahmsdork H.J., Jonat C., Herrlich P. and Karin M.** (1987). Phorbol ester-inducible genes contain a common *cis*- element recognized by a TPA-modulated *trans*-acting factor. *Cell.* **49**: 729-739.

- **Arceci R.J., Baas F., Raponi R, Horwitz S.B. Housman D. and Croop J.M.** (1990). Multidrug resistance gene expression is controlled by steroid hormones in the secretory epithelium of the uterus. *Mol. Rep. Dev.* **25**: 101-109.

- **Arceci R.J., Croop J.M., Horwitz S.B. and Housman D.** (1988).The gene encoding multidrug resistance is induced and expressed at high levels during pregnancy in the secretory epithelium of the uterus. *Proc. Natl. Acad. Sci. USA.* **85**: 4350-4354.

- **Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A. and Struhl K.** (1987). Current protocols in Molecular Biology. John Wiley, New York.

- **Balan I., Alarco A.M. and Raymond M.** (1997). The *Candida albicans CDR3* gene codes for an opaque-phase ABC transporter. *J. Bacteriol* .**179**: 7210-7218.

- **Balzi E. and Goffeau A.** (1994). Genetics and biochemistry of yeast multidrug resistance. *Biochim. Biophys. Acta*.**1187**: 152-162.

- **Balzi E. and Goffeau A.** (1995). Yeast multidrug resistance: The PDR network. *J. Bioenerget. Biomemb.* **27**: 71-76.

- **Barrett-Bee K. and Dixon G.** (1995). Ergosterol biosynthesis inhibition: a target for antifungal agents. *Acta Biochem. Polonica.* **42**: 465-480.

- **Ben-Yaacov R., Knoller S., Caldwell G.A., Becker J.M. and Koltin Y.** (1994). *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. *Antimicrob. Agents Chemother.* **38**: 648-652.

- **Biswas K. and Morschhäuser J.** (2005). The Mep2p ammonium permease controls nitrogen starvation-induced filamentous growth in *Candida albicans*. *Mol. Microbiol.* **56**: 649-669.

- **Blaxall B.C., Pellett A.C., Wu S.C., Pende A. and Port J.D.** (2000). Purification and characterization of ß-adrenergic receptor mRNA-binding proteins. *J. Biol. Chem.* **274**: 4290-4297.

- **Bloch K.D.** (1999). Regulation of endothelial NO synthase mRNA stability: RNA-binding proteins crowd on the 3'-untranslated region. *Circulation Res.* **85**: 653-655.

- **Blum H., Bier H. and Gross H.J.** (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide Gel. *Electrophoresis.* **8**: 93-99.

- **Bobrowicz P., Wysocki R., Owsianik G., Goffeau A. and Ulaszewski S.** (1997). Isolation of three contiguous genes, *ACR1*, *ACR2* and *ACR3*, involved in resistance to arsenic compounds in the yeast *Saccharomyces cerevisiae*. *Yeast.* **13**: 819-828.

- **Boerlin P., Boerlin-Petzold F., Goudet J., Durussel C., Pagani J.L. and Chave J.P.** (1996). Typing *Candida albicans* oral isolates from human immunodeficiency virus-infected patients by multilocus enzyme electrophoresis and DNA fingerprinting. *J. Clinical Microbiol.* **34**: 1235-1248.

- **Bossche H.V.** (1994a). Biochemical targets for antifungal azole derivatives: hypothesis on the mode of action, in *Current Topics in Med. Mycol.* 313-351.

- **Bossche H.V., Willemsens G. and Marichal P.** (1987). Anti-*Candida* drugs-the biochemical basis for their activity. *Crit. Rev. Microbiol.* **15**: 57-72.

- **Bossier P., Fernandes L., Rocha D. and Rodrigues-Pousada C.** (1993). Over expression of *YAP2*, coding for a new yAP protein and yAP1 in *Saccharomyces cerevisiae* alleviates growth inhibition caused by 1,10-phenanthroline. *J. Biol. Chem.* **268**: 23640-23645.

- **Bradford M.M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.

- **Bradley G., Sharma R., Rajalakshmi S. and Ling V.** (1992). P-glycoprotein expression during tumor progression in the rat liver. *Cancer Res.* **52**: 5154-5161.

- **Brôco N., Tenreiro S., Viegas C.A. and Sá-Correia I.** (1999). *FLR1* gene (ORF YBR008c) is required for benomyl and methotrexate resistance in *Saccharomyces cerevisiae* and its benomyl-induced expression is dependent on pdr3 transcriptional regulator. *Yeast.* **15**: 1595-1608.

- **Burgener-Kairuz P., Zuber J.P., Jaunin P., Buchman T.G., Bille J. and Rossier M.** (1994). Rapid detection and identification of *Candida albicans* and *Torulopsis* (*Candida*) *glabrata* in clinical specimens by species-specific nested PCR amplification of a cytochrome P-450 lanosterol-alpha-demethylase (L1A1) gene fragment. *J. Clin. Microbiol.* **32:** 1902-1907.

- **Burt R.K. and Thorgeirsson S.S.** (1988). Coinduction of *MDR1* multidrug resistance and cytochrome P-450 genes in rat liver by xenobiotics. *J. Nat. Cancer Inst.* **80**: 1383- 1386.

- **Capieaux E., Ulaszewski S., Balzi E. and Goffeau A.** (1991). Physical, transcriptional and genetical mapping of a 24 kb DNA fragment located between the *PMA1* and *ATE1* loci on chromosome VII from *Saccharomyces cerevisiae. Yeast.* **7**: 275-280.

- **Carvajal E., Van Den Hazel H.B., Cybularz-Kolaczkowska A., Balzi E. and Goffeau A.** (1997) Molecular and phenotypic characterisation of yeast *PDR1* mutants that show hyperactive transcription of various ABC multidrug transporter genes. *Mol. Gen. Genet.* **256**: 406-415.

- **Chaudhary P.M. and Roninson I.B.** (1992). Activation of *MDR1* (P-glycoprotein) gene expression in human cells by protein kinase C agonists. *Oncol. Res.* **4**: 281-290.

- **Chaudhary P.M. and Roninson I.B.** (1993). Induction of mutlidrug resistance in human cells by transient exposure to different chemotherapeutic drugs. *J. Nat. Cancer Inst.* **85**: 632-639.

- **Chen C.G., Shih H.I., Yang Y.L. and Lo H.J.** (2003). Identification of *trans*-regulatory factors of the efflux pumps involved in drug resistance in *Candida albicans*. XXIth YGM conference. Göteborg, Sweden, July 7-12th, 2003. Abstract 4-4.

- **Chen C.G., Yang Y.L., Shih, H.I., Su C. L. and Lo H.J.** (2004). *CaNdt80* is involved in drug resistance in *Candida albicans* by regulating *CDR1*. *Antimicrob. Agents Chemother.* **48**: 4505-4512.

- **Chen C.-Y. and Shyu A.B.** (1994). Selective degradarion of early-response-gene mRNAs: functionaly analyses of sequence features of the AU-rich elements. *Mol. Cell. Biol.* **14**: 8471-8482.

- **Childs S. and Ling V.** (1994). The MDR superfamily of genes and its biological implications. *Import. Adv. Oncol.* 21-36.

- **Chin K.V., Chauhan S.S., Abraham I., Sampson K.E., Krolczyk A.J., Wong M., Schimmer B., Pastan I. and Gottesman M.M.** (1992). Reduced mRNA levels for the multidrug resistance genes in cAMP-dependent protein kinase mutant cell lines. *J. Cell. Physiol.* **152**: 87-94.

- **Chin K.V., Chauhan S.S., Pastan I. and Gottesman M.M.** (1990b). Regulation of *mdr* RNA levels in response to cytotoxic drugs in rodent cells. *Cell Growth and Differ.* **1**: 361-365.

- **Chin K.V., Tanaka S., Darlington G., Pastan I. and Gottesman M.M.** (1990a). Heat shock and arsenite increase expression of the multidrug resistance (*MDR1*) gene in human renal carcinoma cells. *J. Biol. Chem.* **265**: 221-226.

- **Chin K.V., Ueda K., Pastan I. and Gottesman M.M.** (1992). Modulation of activity of the promoter of the human *MDR1* gene by Ras and p53. *Science.* **255**: 459-462.

- **Choi K., Chen C.-J., Krieger M. and Roninson I.B.** (1988). An altered pattern of cross-resistance in multidrug resistant human cells results from spontaneous mutations in the *MDR1* (P-Glycoprotein) gene. *Cell.* **53**: 519-529.

- **Cohen D., Piekarz R.L., Hsu S.I., DePinho R.A., Carrasco N. and Horwitz S.B.** (1991). Structural and functional analysis of the mouse *mdr1b* gene promoter. *J. Biol. Chem.* **266**: 2239-2244.

- **Copping V.M., Barelle C.J., Hube B., Gow N.A., Brown A.J. and Odds F.C.** (2005). Exposure of *Candida albicans* to antifungal agents affects expression of *SAP2* and *SAP9* secreted proteinase genes. *J. Antimicrob. Chemother.* **55**: 645-654.

- **Cornwell M.M.** (1990). The human multidrug resistance gene: sequences upstream and downstream of the initiation site influence transcription. *Cell Growth Differ.* **1**: 607-615.

- **Cornwell M.M. and Smith D.E.** (1993a). SP1 activates the *MDR1* promoter through one of two distinct G-rich regions that modulate promoter activity. *J. Biol. Chem.* **268**:19505-19511.

- **Cornwell M.M. and Smith D.E.** (1993b). A signal transduction pathway for activation of the *mdr1* promoter involves the proto-oncogene *c-raf* kinase. *J. Biol. Chem.* **268**: 15347-15350.

- **Coste A., Turner V., Ischer F., Morschhäuser J., Forche A., Selmecki A., Berman J., Bille J. and Sanglard D.** (2006). A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. *Genetics.* **172**: 2139-2156.

- **Coste A.T., Karababa M., Ischer F., Bille J. and Sanglard D.** (2004). *TAC1*, transcriptional activator of *CDR* genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters *CDR1* and *CDR2*. *Eukaryot. Cell.* **3**: 1639-1652.

- **Crowley J.H., Leak Jr. F.W, Shianna K.V., Tove S. and Parks L.W.** (1998). A mutation in a purported regulatory gene affects control of sterol uptake in *Saccharomyces cerevisiae*. *J. Bacteriol.* **180**: 4177-4183.

- **Cui Z., Shiraki T., Hirata D. and Miyakawa T.** (1998). Yeast gene *YRR1*, which is required for resistance to 4-nitroquinoline N-oxide, mediates transcriptional activation of the multidrug resistance transporter gene *SNQ2*. *Mol. Microbiol.* **29**:1307-1315.

- **Cusack S.** (1999). RNA-protein complexes. *Curr. Opin. Struct. Biol.* **9**: 66-73.

- **Czyzyk-Krzeska M.F., Dominski Z., Kole R. and Millhorn D.E.** (1994). Hypoxia stimulates binding of a cytoplasmic protein to a pyrimidine-rich sequence in the 3'-untranslated region of rat tyrosine hydroxylase mRNA. *J. Biol. Chem.* **269**: 9940-9945.

- **Dassa E. and Bouige P.** (2001). The ABC of ABCS: a phylogenetic and functional classification of ABC systems in living organisms. *Res. Microbiol.* **152**: 211-229.

- **De Backer M.D., Ilyina T., Ma X.J., Vandoninck S., Luyten W.H. and Vanden Bossche H.** (2001). Genomic profiling of the response of *Candida*

albicans to itraconazole treatment using a DNA microarray. *Antimicrob. Agents Chemother.* **45**: 1660-1670.

- **de Micheli M., Bille J., Schueller C. and Sanglard D.** (2002). A common drug-responsive element mediates the up-regulation of the *Candida albicans* ABC transporters *CDR1* and *CDR2*, two genes involved in antifungal drug resistance. *Mol. Microbiol.* **43**: 1197-1214.

- **Decottignies A., Lambert L., Catty P., Degand H., Epping E.A., Moye-Rowley W.S., Balzi E. and Goffeau A.** (1995). Identification and characterization of SNQ2, a new multidrug ATP binding cassette transporter of the yeast plasma membrane. *J. Biol Chem.* **270**: 18150-18157.

- **Del Sorbo G., Schoonbeek H.-J. and De Waard M.A.** (2000) Fungal transporters involved in efflux of natural toxic compounds and fungicides. *Fung. Genet. Biol.* **30**: 1-15.

- **Delahodde A., Delaveau T. and Jacq C.** (1995). Positive autoregulation of the yeast transcription factor Pdr3p, which is involved in control of drug resistance. *Mol. Cell. Biol.* **15**: 4043-4051.

- **DeMaria C.T. and Brewer G.** (1996). AUF1 binding affinity to A+U-rich elements correlates with rapid mRNA degradation. *J. Biol. Chem.* **271**: 12179-12184.

- **DeRisi J., van den Hazel B., Marc P., Balzi E., Brown P., Jacq C. and Goffeau A.** (2000). Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. *FEBS Lett.* **470**: 156-160.

- **Devaux F., Marc P., Bouchoux C., Delaveau T., Hikkel I., Potier M.C. and Jacq C.** (2001). An artificial transcription activator mimics the genome-wide properties of the yeast *Pdr1* transcription factor. *EMBO Rep.* **2**: 493-498.

- **Dexter D., Moye-Rowley W.S., Wu A.L. and Golin J.** (1994). Mutations in the yeast *PDR3, PDR4, PDR7 and PDR9* pleiotropic (multiple) drug resistance loci affect the transcript level of an ATP binding cassette transporter encoding gene, *PDR5 .Genetics.* **136**: 505-515.

- **Douglas C.M.** (2001). Fungal β-(1,3)-D-glucan synthesis. *Med. Mycol.* **39**: 55-66.

- **Douglas C.M., D'ipplolito J.A., Shei G.J., Meinz M., Onishi J., Marrinan J.A., Li W., Abruzzo G.K., Flattery A., Bartizal K., Mitchell A. and Kurtz M.B.** (1997). Identification of the *FKS1* gene of *Candida albicans* as the essential target of 1, 3-ß-D-glucansynthase inhibitors. *Antimicrob. Agents Chemother.* **41**: 2471-2479.

- **Dunkel N., Liu T.T.,. Barker K.S., Homayouni R., Morschhäuser J. and Rogers P.D.** (2008). A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical *Candida albicans* isolate. *Eukaryot. Cell.* **7**: 1180-1190.

- **Edwalds-Gilbert G., Veraldi K.L. and Milcarek C.** (1997). Alternative poly (A) site selection in complex transcription units: means to an end? *Nucleic Acids Res.* **25**: 2547-2561.

- **Espinell-Ingroff A., Barchiesi F., Hazen K. C., Martinez-Suarez J. V. and Scalise G.** (1998). Standardization of antifungal susceptibility testing and clinical relevance. *Med Mycol.* **36**: 68-78.

- **Fairchild C.R., Ivy S.P., Rushmore T., Lee G., Koo P., Goldsmith M.E., Myers C.E., Farber E. and Cowan K.H.** (1987). Carcinogen-induced *mdr* overexpression is associated with xenobiotic resistance in rat preneoplastic liver nodules and hepatocellular carcinomas *Proc. Natl. Acad. Sci. USA.* **84**: 7701-7705.

- **Fardel O., Lecureur V. and Guillouzo A.** (1996). The P-glycoprotein multidrug transporter. *Gen. Pharmacol.* **27**: 1283-1291.

- **Fardel O., Lecureur V., Corlu A. and Guillouzo A.** (1996). P-glycoprotein induction in rat liver epithelial cells in response to acute 3-methylcholanthrene treatment. *Biochem. Pharm.* **51**: 1427-1436.

- **Favre B., Didmon M. and Ryder N.S.** (1999). Multiple amino acid substitutions in lanosterol 14α-demethylase contribute to azole resistance in *Candida albicans*. *Microbiology.* **145**: 2715-2725.

- **Fernandes L., Rodrigues-Pousada C. and Struhl K.** (1997). Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. *Mol. Cell. Biol.* **17**: 6982-6993.

- **Fling M.E., Kopf J., Tamarkin A., Gorman J.A., Smith H.A. and Koltin Y.** (1991). Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. *Mol. Gen. Genet.* **227**: 318-329.

- **Fojo A.T., Ueda K., Slamon D.I., Poplack D.G., Gottesman M.M. and Pastan I.** (1987). Expression of a multidrug-resistance gene in human tumors and tissues. *Proc. Natl. Acad. Sci. USA.* **84**: 265-269.

- **Franz R., Kelly S.L., Lamb D.C. Kelly D.E., Ruhnke M. and Morschhäuser J.** (1998a). Multiple molecular mechanisms contribute to a stepwise development

of fluconazole resistance in clinical *Candida albicans* strains. *Antimicrob. Agents Chemother.* **42**: 3065-3072.

- **Franz R., Michel S. and Morschhäuser J.** (1998b). A fourth gene from the *Candida albicans CDR* family of ABC transporters. *Gene.* **220**: 91-98.

- **Franz R., Ruhnke M. and Morschhäuser J.** (1999). Molecular aspects of fluconazole resistance development in *Candida albicans*. *Mycoses.* **42**: 453-458.

- **Garcia-Martinez J., Aranda A. and Perez-Ortin J.E.** (2004). Genomic run-on evaluates transcription rates for all yeast genes and identifies gene regulatory mechanisms. *Mol. Cell.* **15**: 303-313.

- **Garcia-Vivas J., Lopez-Camarillo C., Zuara-Liceaga E., Orozco E. and Marchat L.A.** (2005). *Entamoeba histolytica*: cloning and expression of the poly (A) polymerase *EhPAP*. *Exp. Parasitol.* **110**: 226-232.

- **Gardner R.G., Swarbrick G.M., Bays N.W., Cronin S.R., Wilhovsky S., Seelig L., Kim C. and Hampton R.Y.** (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling: Transmembrane control of Hrd1p by Hrd3p. *J. Cell Biol.* **151**: 69-82.

- **Gasch A.P., Spellman P.T., Kao C.M., Carmel-Harel O., Eisen M.B., Storz G., Botstein D. and Brown P.O.** (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell.* **11**: 4241-4257.

- **Gaur N.A., Manoharlal R., Saini P., Prasad T., Mukhopadhyay G., Hoefer M., Morschhäuser J. and Prasad, R.** (2005). Expression of the *CDR1* efflux pump in clinical *Candida albicans* isolates is controlled by a negative regulatory element. *Biochem. Biophys. Res. Commun.* **332**: 206-214.

- **Gaur N.A., Puri N., Karnani N., Mukhopadhyay G., Goswami S.K. and Prasad R.** (2004). Identification of a negative regulatory element which regulates basal transcription of a multidrug resistance gene *CDR1* of *Candida albicans*. *FEMS Yeast Res.* **4**: 389-399.

- **Geber A., Hitchcock C.A., Swartz J.E., Pullen F.S., Marsden K.E., Kwon-Chung K.J. and Bennett J.E.** (1995). Deletion of the *Candida glabrata ERG3* and *ERG11* genes: effect on cell viability, cell growth, sterol composition, and antifungal susceptibility. *Antimicrob. Agents Chemother.* **39**: 2708-2717.

- **Georgopapadakou N.K. and Walsh T.J.** (1994) Human mycoses: drugs and targets for emerging pathogens. *Science* **264**: 371-373.

- **Gerads M. and Ernst J.F.** (1998). Overlapping coding regions and trancriptional units of two essential chromosomal genes (*CCT8*, *TRP1*) in the fungal pathogen *Candida albicans*. *Nucleic Acids Res.* **26**: 5061-5066.

- **Ghannoum M. and Rice L.B.** (1999a). Antifungal agents: mode of action, mechanism of resistance and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.***12**: 501-512.

- **Goffeau A.** (2008). Drug resistance: the fight against fungi. *Nature.* **452**: 541-542.

- **Goldsmith, M.E., Madden M.J., Morrow C.S. and Cowan K.H.** (1993). A Y-box consensus sequence is required for basal expression of the human multidrug resistance (*MDR1*) gene. *J. Biol. Chem.* **268**: 5856-5860.

- **Görlich D., Dabrowski M., Bischoff F.R., Kutay U., Bork P., Hartmann E., Prehn S. and Izaurralde E.** (1997). A novel class of Ran GTP binding proteins. *J. Cell Biol.* **138**: 65-80.

- **Gottesman M.M., Hrycyna C.A., Schoenlein P.V., Germann U.A. and Pastan I.** (1995). Genetic analysis of the multidrug transporter. *Ann. Rev. Genet.* **29**: 607-649.

- **Gottesman M.M., Pastan I. and Ambudkar S.V.** (1996). P-glycoprotein and multidrug resistance. *Curr. Opin. Genet. Dev.* **6**: 610-617.

- **Graybill J.R.** (1988). The long and the short of antifungal therapy. *Infect. Dis. Clin. North Am.* **2**: 805-825.

- **Haase E., Servos J. and Brendel M.** (1992). Isolation and characterization of additional genes influencing resistance to various mutagens in the yeast *Saccharomyces cerevisiae. Curr. Genet.* **21**: 319-324.

- **Hake L.E., Mendez R. and Richter J.D.** (1998). Specificity of RNA binding by CPEB: requirement for RNA recognition motifs and a novel zinc finger. *Mol. Cell Biol.* **18**: 685-693.

- **Haque A., Rai V., Bahal B.S., Shukla S., Lattif A.A., Mukhopadhyay G. and Prasad R.** (2007). Allelic variants of ABC drug transporter Cdr1p in clinical isolates of *Candida albicans. Biochem. Biophys. Res. Commun.* **352**: 491-497.

- **Harry J.B., Song J.L., Lyons C.N. and White T.C.** (2002).Transcription initiation of genes associated with azole resistance in *Candida albicans. Med Mycol.* **40**: 73-81.

- **Harshman K.D., Moye-Rowley W.S. and Parker C.S.** (1988). Transcriptional activation by the SV40 AP-1 recognition element in yeast is mediated by a factor similar to AP-1 that is distinct from GCN4. *Cell.* **53**: 321-330.

- **Hellauer K., Akache B., MacPherson S., Sirard E. and Turcotte B.** (2002). Zinc cluster protein Rdr1p is a transcriptional repressor of the *PDR5* gene encoding a multidrug transporter. *J Biol. Chem.* **277**: 17671-17676.

- **Henry K.W., Nickels J.T. and Edlind T.D.** (2000). Upregulation of *ERG* genes in *Candida* species by azoles and other sterol biosynthesis inhibitors. *Antimicrob. Agents Chemother.* **44**: 2693-2700.

- **Herker E., Jungwirth H., Lehmann K.A., Maldener C., Fröhlich K.U., Wissing S., Büttner S., Fehr M., Sigrist S. and Madeo F.** (2004). Chronological aging leads to apoptosis in yeast. *J. Cell Biol.* **164**: 501-507.

- **Hertle K., Haase E. and Brendel M.** (1991). The *SNQ2* gene of *Saccharomyces cerevisiae* confers hyper-resistance to several functionally unrelated chemicals. *Curr. Genet.* **19**: 429-433.

- **Higgins C.F.** (1991). Stability and degradation of mRNA. *Curr. Opin. Cell Biol.* **3**: 1013-1018.

- **Higgins C.F.** (1992). ABC transporters: from microorganisms to man. *Ann. Rev. Cell Biol.* **8**: 67-113.

- **Higgins C.F.** (1993). Introduction: the ABC transporter channel superfamily- an overview. *Sem. Cell Biol.* **4**: 1-5.

- **Hikkel I., Lucau-Danila A., Delaveau T., Marc P., Devaux F. and Jacq C.** (2003). A general strategy to uncover transcription factor properties identifies a new regulator of drug resistance in yeast. *J. Biol. Chem.* **278**: 11427-11432.

- **Hiller D., Sanglard D. and Morschhäuser J.** (2006). Overexpression of the *MDR1* gene is sufficient to confer increased resistance to toxic compounds in *Candida albicans*. *Antimicrob. Agents Chemother.* **50**: 1365-1371.

- **Hiller D., Stahl S. and Morschhäuser J.** (2006). Multiple *cis*-acting sequences mediate upregulation of the *MDR1* efflux pump in a fluconazole-resistant clinical *Candida albicans* isolate. *Antimicrob. Agents Chemother.* **50**: 2300-2308.

- **Hirata D., Yano K. and Miyakawa T.** (1994). Stress induced transcriptional activation mediated by *YAP1* and *YAP2* genes that encode the JUN family of transcriptional activators in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **242**: 250-256.

- **Hitchcock C.A., Russel N.J. and Barrett-Bee K.J.** (1987). Sterols in *Candida albicans* mutants resistant to polyene or azole antifungals, and of a double mutant *C. albicans* 6.4. *Crit. Rev. Microbiol.* **15**: 111-115.

- **Hoffman C.S. and Winston F.** (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene.* **57**: 267-272.

- **Holland I.B. and Blight M.A.** (1999). ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *J. Mol. Biol.* **293**: 381-399.

- **Holmes A.R., Tsao S., Ong S.W., Lamping E., Niimi K., Monk B.C., Niimi M., Kaneko A., Holland B.R., Schmid J. and Cannon R.D.** (2006). Heterozygosity and functional allelic variation in the *Candida albicans* efflux pump genes *CDR1* and *CDR2*. *Mol. Microbiol.* **62**: 170-186.

- **Holzer K.P. and Hammes G.G.** (1989). Cloning and expression of the yeast plasma membrane ATPase in *Escherichia coli*. *J. Biol. Chem.* **264**: 14380-14395.

- **Hoot S.J., Oliver B.G. and White T.C.** (2008). *Candida albicans UPC2* is transcriptionally induced in response to antifungal drugs and anaerobicity through Upc2p-dependent and -independent mechanisms. *Microbiology.* **154**: 2748-2756.

- **Hsu S.I., Cohen D., Kirschner L.S., Lothstein L., Hartstein M. and Horwitz S.B.** (1990). Structural analysis of the mouse *mdr1a* (P-glycoprotein) promoter reveals the basis for differential transcript heterogeneity in multidrug resistant J774.2 cells. *Mol. Cell. Biol.* **10**: 3596-3606.

- **Hu Z., Jin, S. and Scotto K.W.** (2000). Transcriptional activation of the *MDR1* gene by UV irradiation. *J. Biol. Chem* **275**: 2979-2895.

- **Hussain M. and Lenard J.** (1991). Characterization of *PDR4*, a *Saccharomyces cerevisiae* gene that confers pleiotropic drug resistance in high-copy number: identity with YAP1, encoding a transcriptional activator. *Gene.* **101**: 149-152.

- **Ikeguchi M., Teeter L.D., Eckersberg T., Ganapathi R. and Kuo M.T.** (1991). Structural and functional analyses of the promoter of the murine multidrug resistance gene *mdr3/mdr1a* reveal a negative element containing the AP-1 binding site. *DNA Cell Biol.* **10**: 639-649.

- **Imanishi Y., Yokoyama K. and Nishimura K.** (2004). Inductions of germ tube and hyphal formations are controlled by mRNA synthesis inhibitor in *Candida albicans*. *Nippon Ishinkin Gakkai Zasshi.* **45**: 113-119.

- **Irwin N., Baekelandt V., Goritchenko L. and Benowitz, L.I.** (1997). Identification of two proteins that bind to a pyrimidine-rich sequence in the 3'-untranslated region of GAP-43 mRNA. *Nucleic Acids Res.* **25**: 1281-1288.

- **Jin S.** (1998). Transcriptional Regulation of the *MDR1* gene by histone acetyltransferase and deacetylase is mediated by NF-Y. *Mol. Cell. Biol.* **18**: 4377-4384.

- **Joseph-Horne T. and Hollomon D.W.** (1997). Molecular mechanisms of azole resistance in fungi. *FEMS Microbiol. Lett.***149**: 1141-149.

- **Juliano R.L. and Ling V.** (1976). A surface glycoprotein modulating drug permeability in chinese hamster ovary cell mutants. *Biochim Biophys Acta.* **455**: 152-162.

- **Jungwirth H. and Kuchler K.** (2006).Yeast ABC transporters- a tale of sex, stress, drugs and aging.*FEBS Lett.* 580:1131-1138.

- **Kakeya H., Miyazaki Y., Miyazaki H., Nyswaner K., Grimberg B. and Bennett J.E.** (2000). Genetic analysis of azole resistance in the Darlington strain of *Candida albicans*. *Antimicrob. Agents Chemother.* **44**: 2985-2990.

- **Karababa M., Coste A.T., Rognon B., Bille J. and Sanglard D.** (2004). Comparison of gene expression profiles of *Candida albicans* azole-resistant clinical isolates and laboratory strains exposed to drugs inducing multidrug transporters. *Antimicrob. Agents Chemother.* **48**: 3064-3079.

- **Karnani N., Gaur N.A., Jha S., Puri N., Krishnamurthy S., Goswami S.K., Mukhopadhyay G. and Prasad R.** (2004). SRE1 and SRE2 are two specific steroid responsive modules of *Candida* drug resistance gene 1 (*CDR1*) promoter. *Yeast.* **21**: 219-239.

- **Kebaara B.W., Nielsen L.E., Nickerson K.W. and Atkin A.L.** (2006). Determination of mRNA half-lives in *Candida albicans* using thiolutin as a transcription inhibitor. *Genome.* **49**: 894-899.

- **Kelly R., Card D., Register E., Mazur P., Kelly T., Tanaka K.-I., Onishi J., Williamson J.M., Fan H., Satoh T. and Kurtz M.** (2000). Geranylgeranyltransferase I of *Candida albicans*: null mutants or enzyme inhibitors produce unexpected phenotypes. *J. Bacteriol.* **182**: 704-713.

- **Kelly S.L., Lamb D.C. and Kelly D.E.** (1999a). Y132H substitution in *Candida albicans* sterol 14alpha-demethylase confers fluconazole resistance by preventing binding to haem. *FEMS Microbiol. Lett.* **180**: 171-175.

- **Kelly S.L., Lamb D.C., Kelly D.E., Manning N.J., Loeffler J., Hebart H., Schumacher U. and Einsele H.** (1997). Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol $A^{5,6}$- desaturation. *FEBS Lett.* **400**: 80-82.

- **Kelly S.L., Lamb D.C., Loeffler J., Einsele H. and Kelly D.E.** (1999b). The G464S amino acid substitution in *Candida albicans* sterol 14alpha-demethylase causes fluconazole resistance in the clinic through reduced affinity. *Biochem. Biophys. Res. Commun.* **262**: 174-179.

- **Kelly S.L., Lamb D.C., Taylor M., Corran A.J., Baldwin B.C. and Powderly W.G.** (1994). Resistance to amphotericin B associated with defective sterol Δ8-7 isomerase in a *Cryptococcus neoformans* strain from an AIDS patient. *FEMS Microbiol. Lett.* **122**: 39-42.

- **Kerridge D.** (1985). The protoplast membrane and antifungal drugs, in *Fungal Protoplasts: Applications in Biochemistry and Genetics* 135-169, Marcel Deker, NewYork.

- **Kerridge D.** (1986) Mode of action of clinically important antifungal drugs. *Advan. in Microbial Physiol.* **27**: 1-72.

- **Kerridge D. and Whelan W.L.** (1984). The polyene macrolide antibiotics and 5-fluorocytosine: molecular actions and interactions, in *Mode of action of antifungal agents* 343-375, Cambridge Univerisity Press, Cambridge.

- **Kimchi-Sarfaty, C., Oh J.M., Kim I.W., Sauna Z.E., Calcagno A.M., Ambudkar S.V. and Gottesman M.M.** (2007). A "silent" polymorphism in the *MDR1* gene changes substrate specificity. *Science.* **315**: 525-528.

- **Klausner R.D., Rouault T.A. and Harford J.B.** (1993). Regulating the fate of mRNA : the control of cellular iron metabolism. *Cell.* **72**: 19-28.

- **Klepser M.E., Ernst E.J. and Pfaller M.A.** (1997). Update on antifungal resistance. *Trends Microbiol.* **5**: 372-375.

- **Kohli A., Gupta V., Krishnamurthy S., Hasnain S.E. and Prasad R.** (2001). Specificity of drug transport mediated by *CaMDR1*: a major facilitator of *Candida albicans*. *J. Biosci.* **26**: 101-107.

- **Kohli A., Smriti, Mukhopadhyay K., Rattan A. and Prasad, R.** (2002). *In vitro* low-level resistance to azoles in *Candida albicans* is associated with changes in membrane lipid fluidity and asymmetry. *Antimicrob. Agents Chemother.* **46**: 1046-1052.

- **Kolaczkowska A. and Goffeau A.** (1999). Regulation of pleiotropic drug resistance in yeast. *Drug Resist. Updates.* **2**: 403-414.

- **Kolaczkowski M. and Goffeau A.** (1997). Active efflux by multidrug transporters as one of the strategies to evade chemotherapy and novel practical implications of yeast pleiotropic drug resistance. *Pharmacol. Ther.* **76**: 219-242.

- **Kozovská Z. and Subik J.** (2003). Screening for effectors that modify multidrug resistance in yeast. *Int. J. Antimicrob. Agents.* **22**: 284-290.

- **Kren A., Mamnun Y.M., Bauer B.E., Schüller C., Wolfger H., Hatzixanthis K., Mollapour M., Gregori C., Piper P. and Kuchler K.** (2003). Warlp, a novel transcription factor controlling weak acid stress response in yeast. *Mol. Cell. Biol.* **23**: 1775-1785.

- **Krishnamurthy S., Gupta V., Prasad R., Panwar S.L. and Prasad R.** (1998). Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: *in vitro* transcriptional activation by heat shock, drugs and human steroid hormones. *FEMS Microbiol. Lett.* **160**: 191-197.

- **Kurtz M.B., Abruzzo G., Flattery A., Bartizal K., Marrinan J.A., Li W., Milligan J., Nollstadt K. and Douglas C.M.** (1996). Characterisation of echinocandin-resistant mutants of *Candida albicans*: Genetic, biochemical and virulence studies. *Infec. Immun.* **64**: 3244-3251.

- **Kusov Y.Y., Shatirishvili G., Dzagurov G. and Gauss-Muller V.** (2001). A new G-tailing method for the determination of the poly (A) tail length applied to hepatitis A virus RNA. *Nucleic Acids Res.* **29**: E57.

- **Laemmli U.K.** (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature.* **227**: 680-685.

- **Lamb D.C., Kelly D.E., White T.C. and Kelly S.L.** (2000). The R467K amino acid substitution in *Candida albicans* sterol 14 alpha-demethylase causes drug resistance through reduced affinity. *Antimicrob. Agents Chemother.* **44**: 63-67.

- **Lamping E., Monk B.C., Niimi K., Holmes A.R., Tsao S., Tanabe K., Niimi M., Uehara Y. and Cannon R.D.** (2007). Characterization of three classes of membrane proteins involved in fungal azole resistance by functional hyperexpression in *Saccharomyces cerevisiae. Eukaryot. Cell.* **6**: 1150-1165.

- **Larsen B., Anderson S., Brockman A., Essmann M. and Schmidt M.** (2006). Key physiological differences in *Candida albicans CDR1* induction by steroid hormones and antifungal drugs. *Yeast.* **23**: 795-802.

- **Law D., Moore C.B., Wardle H.M., Ganguli L.A., Keaney M.G. and Denning D.W.** (1994). High prevalence of antifungal resistance in *Candida* spp. from patients with AIDS. *J. Antimicrob. Chemother.* **34**: 659-668.

- **Le Crom S., Devaux P.F., Marc P., Zhang X., Moye-Rowley W.S. and Jacq C.** (2002). New insights into the pleiotropic drug resistance network from genome-wide characterization of the *YRR1* transcription factor regulation system. *Mol. Cell Biol.* **22**: 2642-2649.

- **Lee C.H., Bradley G. and Ling V.** (1998). Increased P-glycoprotein messenger RNA stability in rat liver tumors in *vivo*. *J. Cell. Physiol.* **177**: 1-2.

- **Lee C.H., Bradley G., Zhang J.-T. and Ling V.** (1993). Differential expression of P-glvcoprotein genes in primary rat hepatocyte culture. *J. Cell. Physiol.* **157**: 392-402.

- **Li D., Gurkovska V., Sheridan M., Calderone R. and Chauhan N.** (2004). Studies on the regulation of the two-component histidine kinase gene *CHK1* in *Candida albicans* using the heterologous *lacZ* reporter gene. *Microbiology.* **150**: 3305-3313.

- **Liu I., Shen X., Nguyen V., Kunos G. and Gao B.** (2000). α_1 adrenergic agonist induction of $p21^{waf1/cip1}$ mRNA stability in transfected HepG2 cells correlates with the increased binding of an AU-rich element binding factor. *J. Biol. Chem.* **275**: 11846-11851.

- **Liu T.T., Lee R.E., Barker K.S., Lee R.E., Wei L., Homayouni R. and Rogers P.D.** (2005). Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. *Antimicrob. Agents Chemother.* **49**: 2226-2236.

- **Looi C.Y., D' Silva E.C., Seow H.F., Rosli R., Ng K.P. and Chong P.P.** (2005). Increased expression and hotspot mutations of the multidrug efflux transporter, *CDR1* in azole-resistant *Candida albicans* isolates from vaginitis patients. *FEMS Microbiol Lett.* **249**: 283-289.

- **Lopez-Camarillo C., Luna-Arias J.P., Marchat L.A. and Orozco E.** (2003). *EhPgp5* mRNA stability is a regulatory event in the *Entamoeba histolytica* multidrug resistance phenotype. *J. Biol. Chem.* **278**: 11273-11280.

- **Lopez-Ribot J. ., McAtee R.K., Lee L.N., Kirkpatrick W.R., White T.C., Sanglard D. and Patterson T.F.** (1998). Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. *Antimicrob. Agents Chemother.* **42**: 2932-2937.

- **Lucau-Danila A., Delaveau T., Lelandais G., Devaux F. and Jacq C.** (2003). Competitive promoter occupancy by two yeast paralogous transcription factors controlling the multidrug resistance phenomenon. *J. Biol. Chem.* **278**: 52641-52650.

- **Lum B.L. and Gosland M.P.** (1995). MDR expression in normal tissues. Pharmacologic implications for the clinical use of P-glycoprotein inhibitors. *Hematol. Oncol. Clin. North Am.* **9**: 319-336.

- **Lyons, C. N. and T. C. White.** 2000. Transcriptional analyses of antifungal drug resistance in *Candida albicans*. *Antimicrob. Agents Chemother.* **44**: 2296-2303.

- **MacPherson S., Akache B., Weber S., DeDeken X., Raymond M. and Turcotte B.** (2005). *Candida albicans* zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. *Antimicrob. Agents Chemother.* **49**: 1745-1752.

- **Madden M.J., Morrow C.S., Nakagawa M., Goldsmith M.E., Fairchild C.R. and Cowan K.H.** (1993). Identification of 5' and 3' sequences involved in the regulation of transcription of the human mdr1 gene *in vivo*. *J. Biol. Chem.* **268**: 8290-8297.
- **Mahé Y., Parle-McDermott A., Nourani A., Delahodde A., Lamprecht A. and Kuchler K.** (1996). The ATP-binding cassette multidrug transporter *Snq2* of *Saccharomyces cerevisiae*: a novel target for the transcription factors Pdr1 and Pdr3. *Mol. Microbiol.* **20**: 109-117.

- **Mamnun Y.M., Pandjaitan R., Mahe Y., Delahodde A. and Kuchler K.** (2002). The yeast zinc finger regulator Pdr1p and Pdr3p control pleiotropic drug resistance (PDR) as homo-and heterodimers *in vivo*. *Mol. Microbiol.* **46**: 1429-1440.

- **Mamnun Y.M., Schüller C. and Kuchler K.** (2004). Expression regulation of the yeast *PDR5* ATP-binding cassette (ABC) transporter suggests a role in cellular detoxification during the exponential growth phase. *FEBS Lett.* **559**: 111-117.

- **Maniatis T., Goodbom S. and Fischer I.A.** (1987). Regulation of inducible and tissue specific gene expression. *Science.* **236**: 1237-1245.

- **Manoharlal R., Gaur N.A., Panwar S.L., Morschhäuser J. and Prasad R.** (2008). Transcriptional activation and increased mRNA stability contribute to over-expression of *CDR1* in azole-resistant *Candida albicans*. *Antimicrob. Agents Chemother.* **52**: 1481-1492.

- **Marger M.D. and Saier M.H. Jr.** (1993). A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends Biochem. Sci.* **18**: 13-20.

- **Marichal P.** (1999a). Mechanisms of resistance to azole antifungal compounds. *Curr. Opin. Anti-infective Investig. Drugs.* **1**: 318-333.

- **Marichal P., Koymans L., Willemsens S., Bellens D., Verhasselt P., Luyten W., Borgers M., Ramaekers F.C.S., Odds F.C. and Vanden Bossche H.** (1999). Contributions of mutations in the cytochrome P450 14α-demethylase

(Erg11p, Cyp51p) to azole resistance in *Candida albicans*. *Microbiology*. 145: 2701-2713.

- **Marichal P., Vanden Bossche H., Odds F.C., Nobels G., Warnock D.W., Timmerman V., Van Broeckhoven C., Fay S. and Mose-Larsen P.** (1997). Molecular biological characterization of an azole-resistant *Candida glabrata* isolate. *Antimicrob. Agents Chemother.* **41**: 2229-2237.

- **Marino P.A., Gottesman M.M. and Pastan I.** (1990). Regulation of the multidrug resistance gene in regenerating rat liver. *Cell Growh and Differ.* **1**: 57-62.

- **Martens J.A., Genereaux J., Saleh A. and Brandl C.J.** (1996).Transcriptional activation by yeast *PDR1* is inhibited by its association with Ngg1p/Ada3p. *J. Biol. Chem.* **271**: 15884-15890.

- **Marthinet E., Divita G., Bernaud J., Rigal D. and Baggetto L.G.** (2000). Modulation of the typical multidrug resistance phenotype by targeting the MED-1 region of human *MDR1* promoter. *Gene Ther.* **7**: 1224-1233.

- **Martinez-Pastor M.T., Marchler G., Schuller C., Marchler-Bauer A., Ruis H. and Estruch F.** (1996). The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *EMBO J.* **15**: 2227-2235.

- **Martins M.D. and Rex J.H.** (1997). Antifungal drug resistance: a focus on *Candida*. *Clin. Updates Fung. Infect.* **1**: 1-6.

- **Maurer F., Tierney M. and Medcalf R.L.** (1999). An AU-rich sequence in the 3'-UTR of plasminogen activator inhibitor type 2 (PAI-2) mRNA promotes PAI-2 mRNA decay and provides a binding site for nuclear HuR. *Nucleic Acids Res.* **27**: 1664-1673.

- **McCarthy J.E.** (1998). Post-transcriptional control of gene expression in yeast. *Microbiol. Mol. Biol. Rev.* **62**: 1492-1553.

- **Meyers M.B., Spengler B.A., Chang T.D., Melera P.W. and Biedler J.L.** (1985). Gene amplification-associated cytogenetic aberrations and protein changes in vincristine resistant chinese hamster, mouse, and human cells. *J. Biol. Chem.* **100**: 588-597.

- **Mickley L.A., Bates S.E., Richert N.D., Currier S., Tanaka S., Fos F., Rosen N. and Fojo A.T.** (1989). Modulation of the expression of a multidrug resistance gene (*mdr-1*/ P-glycoprotein) by differentiating agents. *J. Biol. Chem.* **264:** 18031-18040.

- **Miyahara K., Hirata D. and Miyakawa T.** (1996). yAP-1- and yAP-2-mediated, heat shock-induced transcriptional activation of the multidrug resistance ABC transporter genes in *Saccharomyces cerevisiae*. *Curr. Genet.* **29**: 103-105.

- **Morita T. and Nozawa Y.** (1985). Effects of antifungal agents on ergosterol biosynthesis in *Candida albicans* and *Trichophyton mentagrophytes*: differential inhibitory sites of naphthiomate and miconazole. *J. Invest. Dermatol.* **85**: 434-437.

- **Morschhäuser J., Barker K.S., Liu T.T., BlaB-Warmuth J., Homayouni R. and Rogers P.D.** (2007). The transcription factor Mrr1p controls expression of the *MDR1* efflux pump and mediates multidrug resistance in *Candida albicans*. *PLoS Pathog.* **3**: e164.

- **Morschhäuser J., Michel S. and Hacker J.** (1998). Expression of a chromosomally integrated, single-copy *GFP* gene in *Candida albicans*, and its use as a reporter of gene regulation. *Mol. Gen. Genet.* **257**: 412-420.

- **Moye-Rowley W.S.** (2002). Transcription factors regulating the response to oxidative stress in yeast. *Antioxid. Redox Signal.* **4**: 123-140.

- **Moye-Rowley W.S.** (2003). Regulation of the transcriptional response to oxidative stress in fungi: similarities and differences. *Eukaryot. Cell.* **2**: 381-389.

- **Moye-Rowley W.S., Harshman K.D. and Parker C.S.** (1989).Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. *Genes Dev.* **3**: 283-292.

- **Mukhopadhyay K., Kohli A. and Prasad R.** (2002). Drug susceptibilities of yeast cells are affected by membrane lipid composition. *Antimicrob. Agents Chemother.* **46**: 3695-3705.

- **Muller C., Goubin F., Ferrandis E., Comil-Schamtz I., Bailly J.D., Bordier C., Benard J., Sikic B.I. and Laurent G.** (1995). Evidence for transcriptional control of human *mdr1* gene expression by verapamil in multidrug-resistant leukemic cells. *Mol. Pharm.* **47**: 51-56.

- **Murad A.M., d'Enfert C., Gaillardin C., Tournu H., Tekaia F., Talibi D., Marechal D., Marchais V., Cottin J. and Brown A.J.** (2001). Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors *CaTup1*, *CaMig1* and *CaNrg1*. *Mol. Microbiol.* **42**: 981-993.

- **Nakatsukasa H., Evarts R.P., Burt R.K., Nagy P. and Thorgeirsson S.S.** (1992). Cellular pattern of multidrug resistance gene expression during chemical hepatocarcinogenesis in the rat. *Mol. Carcinogenesis.* **6**: 190-198.

- **Nieto A., Guillermo Perez D., Orozco E., Paz F. and Gomez C.** (2005). *Entamoeba histolytica EhPgp5* transcriptional activation depends on putative emetine response elements. *Exp. Parasitol.* **110**: 233-237.

- **Nourani A., Papajova D., Delahodde A., Jacq C. and Subik J.** (1997). Clustered amino acid substitutions in the yeast transcription regulator Pdr3p increase pleiotropic drug resistance and identify a new central regulatory domain. *Mol. Gen. Genet.* **256**: 397-405.

- **Odds F.C.** (1988). *Candida and Candidiasis*: A review and bibliography. Ballière Tindall, London.

- **Ohki R. and Tateno K.** (2004). Increased stability of *bmr3* mRNA results in a multidrug-resistant phenotype in *Bacillus subtilis*. *J. Bacteriol.* **186**: 7450-7455.

- **Oliver B.G., Song J.L., Choiniere J.H. and White T.C.** (2007). *Cis*-acting elements within the *Candida albicans ERG11* Promoter mediate the azole response through transcription factor Upc2p. *Eukaryot. Cell.* **6**: 2231-2239.

- **Parissenti A.M., Gannon B.R., Villeneuve D.J., Kirwan-Rhude A.F. and Chadderton A.** (1999). Lack of modulation of *MDR1* gene expression by dominant inhibition of cAMP-dependent protein kinase in doxorubicin-resistant MCR-7 breast cancer cells. *Int. J. Cancer* **82**: 893-900.

- **Park K.S., Kwon J. and Choi S.Y.** (1998). Cloning, characterization, and expression of the *CIP2* gene induced under cadmium stress in *Candida* sp. *FEMS Microbiol. Lett.* **162**: 325-330.

- **Pasrija R., Banerjee D. and Prasad R.** (2007). Structure and function analysis of CaMdr1p, a MFS antifungal efflux transporter protein of *Candida albicans*: identification of amino acid residues critical for drug/ H^+ transport. *Eukaryot. Cell.* **6**: 443-453.

- **Paulsen I.T., Brown M.H. and Skurray R.A.** (1996). Proton-dependent multidrug efflux systems. *Microbiol. Rev.* **60**: 575-608.

- **Pennisi E.** (1998). The nucleus's revolving door. *Science.* 279: 1129-1131.

- **Perea S., Lopez-Ribot J.L., Kirkpatrick W.R., McAtee R.K., Santillan R.A., Martinez M., Calabrese D., Sanglard D. and Patterson T.F.** (2001). Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob.Agents Chemother.* **45**: 2676-2684.

- **Pesole G. and Liuni S.** (1999). Internet resources for the functional analysis of 5' and 3' untranslated regions of eukaryotic mRNAs. *Trends Genet.* **15**: 378.

- **Pesole G., Liuni S., Grillo G., Licciulli F., Mignone F., Gissi C. and Saccone C.** (2002). UTRdb and UTRsite: specialized databases of sequences and functional elements of 5' and 3' untranslated regions of eukaryotic mRNAs. Update 2002. *Nucleic Acids Res.* **30**: 335-340.

- **Peters W.** (1996). History and current status of drug resistance, *Book chapter (16) of chemotherapy of malaria*: 423-443.

- **Prasad R.** (1991). *Candida albicans*: Cellular and Molecular Biology, (Springer-Verlang, Berlin).

- **Prasad R., Panwar S.L and Puri N.** (2000b). Sex and drug ATPases in fungi. (A chapter in the book *"Biophysical Processes In Living Systems"*).

- **Prasad R., Panwar S.L. and Krishnamurthy S.** (2000a). Drug resistance mechanisms of human pathogenic fungi-*in the Book by Marcel Dekker-"Fungal Pathogenesis: Principles and Clinical Applications"*.

- **Prasad R., Panwar S.L. and Smriti** (2002). Drug resistance in yeasts- an emerging scenario, in *Advances in Microbial Physiol.* (Poole RK ed) 155-201, Academic Press, London.

- **Prasad R., Worgifosse P. D., Goffeau A. and Balzi E.** (1995). Molecular cloning and characterisation of a novel gene of *C. albicans*, *CDR1*, Conferring Multiple Resistance to Drugs and Antifungals. *Curr. Genet.* **27**: 320-329.

- **Prokipcak R.D., Raouf A. and Lee C.** (1999). The AU-rich 3' untranslated region of human *MDR1* mRNA is an inefficient mRNA destabilizer. *Biochem. Biophys. Res. Commun.* **261**: 627-634.

- **Pujol C., Reynes J., Renaud F., Raymond M., Tibayrenc M., Ayala F.J., Janbon F., Malli'e M. and Bastide J.M.** (1993). The yeast *Candida albicans* has a clonal mode of reproduction in a population of infected human immunodeficiency virus-positive patients. *Proc. Natl. Acad. Sci. USA.* **90**: 9456-9459.

- **Puri N., Krishnamurthy S., Habib S., Hasnain S.E., Goswami S.K. and Prasad R.** (1999). *CDR1,* a multidrug resistance gene from *Candida albicans,* contains multiple regulatory domains in its promoter and the distal AP-1 element mediates its induction by miconazole. *FEMS Microbiol. Lett.* **180**: 213-219.

- **Rajagopalan L.E. and Malter J.S.** (1997). Regulation of eukaryotic messenger RNA turnover. *Prog. Nucl. Acids Res. Mol. Biol.* **56**: 257-283.

- **Raviv Y., Pollard H.B., Bruggemann E.P., Pastan I. and Gottesman M.M.** (1990). Photosensitised labeling of a functional multidrug transporter in living drug resistant tumour cells. *J. Biol. Chem.* **265**: 3975-3980.

- **Raymond M., Rose E., Housman D.E. and Gros P.** (1990). Physical mapping, amplification and overexpression of the mouse *mdr* gene family in multidrug-resistant cells. *Mol. Cell. Biol.* **10**: 1642-1651.
- **Reuss O., Vik A., Kolter R. and Morschhäuser J.** (2004). The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene.* **341**: 119-127.

- **Rex J.H., Pfaller M.A., Galgiani J.N., Bartlett M.S., Espinel-Ingroff A., Ghannoum M.A., Lancaster M., Odds F.C., Rinaldi M.G., Walsh T.J. and Barry A.L.** (1997). Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of *in vitro-in vivo* corrrelation data for fluconazole, itraconazole and *Candida* infections. *Clin. Infect. Dis.* **24**: 235-247.

- **Rex J.H., Rinaldi M.G. and Pfaller M.A.** (1995). Resistance of *Candida* species to fluconazole. *Antimicrob. Agents Chemother.* **39**: 1-8.

- **Reynolds T.B. and Fink G.R.** (2001). Baker's yeast, a model for fungal biofilm formation. *Science.* **291**: 878-881.

- **Riggle P.J. and Kumamoto C.A.** (2006). Transcriptional regulation of *MDR1*, encoding a drug efflux determinant, in fluconazole-resistant *Candida albicans* strains through an Mcm1p binding site. *Eukaryot. Cell.* **5**: 1957-1968.

- **Riordan J.R., Deuchars K., Kartner N., Alon N., Trent J. and Ling V.** (1985). Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature.* **316**: 817-819.

- **Rodrigues-Pousada C.A., Nevitt T., Menezes R., Azevedo D., Pereira J. and Amaral C.** (2004). Yeast activator proteins and stress response: an overview. *FEBS Lett.* **567**: 80-85.

- **Rognon B., Kozovska Z., Coste A.T., Pardini G. and Sanglard D.** (2006). Identification of promoter elements responsible for the regulation of *MDR1* from *Candida albicans*, a major facilitator transporter involved in azole resistance. *Microbiology.* **152**: 3701-3722.

- **Ross J.** (1996). Control of messenger RNA stability in higher eukaryotes. *Trends Genet.* **12**: 171-175.

- **Rothenberg M.L., Mickley L.A., Cole D.E., Balis F.M., Tsuruo T., Poplack D.G. and Fojo A.T.** (1989). Expression of the mdr-1/P-170 gene in patients with acute lymphoblastic leukemia. *Blood.* **74**: 1388-1395.

- **Russell J.E., Morales J., Makeyev A.V. and Liebhaber S.A.** (1998). Sequence divergence in the 3' untranslated regions of human ?- and α-globin mRNAs mediates a difference in their stabilities and contributes to efficient α- to -? gene development switching. *Mol. Cell Biol.* **18**: 2173-2183.

- **Rustad T.R., Stevens D.A., Pfaller M. and White T.C.** (2002). Homozygosity at the *Candida albicans* MTL locus associated with azole resistance. *Microbiology.* **148**: 1061-1072.

- **Saidane S., Weber S., De Deken X., Germain G.S. and Raymond M.** (2006). *PDR16*-mediated azole resistance in *Candida albicans*. *Mol. Microbiol.* **60**: 1546-1562.

- **Saier M.H. Jr. and Reizer J.** (1991). Families and super familes of transport proteins common to prokaryotes and eukaryotes. *Curr. Opin. Struct. Biol.* **1**: 362-368.

- **Saleh A., Lang V., Cook R. and Brandl C.J.** (1997). Identification of native complexes containing the yeast coactivator/repressor proteins Ngg1p/Ada3p and Ada2p. *J. Biol. Chem.* **272**: 5571-5578.

- **Sambrook J., Fritsch E.F. and Maniatis T.** (1989). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

- **Sanglard D. and Odds F.C.** (2002). Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect. Dis.* **2**: 73-85.

- **Sanglard D., Ischer F., Calabrese D., de Micheli M. and Bille J.** (1998a). Multiple resistance mechanism to azole antifungals in yeast clinical isolates. *Drug Resist Updates* **1**: 255-265.

- **Sanglard D., Ischer F., Koymans L. and Bille J.** (1998b). Amino acid substitutions in the cytochrome P-450 lanosterol 14 alpha-demethylase (CYP51A1) from azole resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob. Agents Chemother.* **42**: 241-253.

- **Sanglard D., Ischer F., Monod M. and Bille J.** (1996). Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. *Antimicrob. Agents Chemother.* **40**: 2300-2305.

- **Sanglard D., Ischer F., Monod M. and Bille J.** (1997). Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterisation of *CDR2*, a new multidrug ABC transporter gene. *Microbiology.* **143**: 405-416.

- **Sanglard D., Ischer F., Monod M., Dogra S., Prasad R. and Bille J.** (1999). Analysis of the ATP-binding cassette (ABC)-transporter gene of *CDR4* from *Candida albicans*. ASM conference on *Candida* and Candidiasis. C27.

- **Sanglard D., Isher I., Calabrese D., De Micheli M. and Bille, J.** (1998). Multiple mechanisms to azole antifungals in yeast. *Drug Resist. Updates.* **1**: 255-265.

- **Sanglard D., Kuchler K., Ischer F., Pagani J.-L., Monod M. and Bille J.** (1995). Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob. Agents Chemother.* **39**: 2378-2386.

- **Schnell N., Krems B. and Entian, K.D.** (1992). The *PAR1* (*YAP1/SNQ3*) gene of *Saccharomyces cerevisiae*, a *c-jun* homologue, is involved in oxygen metabolism. *Curr. Genet.* **21**: 269-273.

- **Schuller C., Mamnun Y.M., Mollapour M., Krapf G., Schuster M., Bauer B.E., Piper P.W. and Kuchler K.** (2004). Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae. Mol. Biol. Cell.* **15**: 706-720.

- **Scotto K.W. and Egan D.** (1998). Transcriptional regulation of *MDR* genes. *Cytotechnology.* **27**: 257-269.

- **Scotto K.W. and Johnson R.A.** (2001). Transcription of the multidrug resistance gene *MDR1*: a therapeutic target. *Mol. Interv.* **1**: 117-125.

- **Scotto K.W., Biedler J.L and Melera P.W.** (1988). Amplification and expression of genes associated with rnultidrug resistance in mammalian cells. *Science.* **232**: 751-755.

- **Seet B.T. and Pawson T.** (2004). MAPK signaling: Sho business. *Curr. Biol.* **14**: R708-R710.

- **Servos J., Haase E. and Brendel M.** (1993). Gene *SNQ2* of *Saccharomyces cerevisiae,* which confers resistance to 4-nitroquinoline-N-oxide and other chemicals, encodes a 169 kDa protein homologous to ATP-dependent permeases. *Mol Gen Genet.* **236**: 214-218.

- **Sessions A. and Horwitz A.F.** (1983). Differentiation-related differences in the plasma membrane phospholipid asymmetry of mycogenic and fibrogenic cells. *Biochim. Biophys. Acta.* **728**: 103-111.

- **Setiadi E.R., Doedt T., Cottier F., Noffz C. and Ernst J.F.** (2006). Transcriptional response of *Candida albicans* to hypoxia: linkage of oxygen sensing and Efg1p-regulatory networks. *J Mol. Biol.* **361**: 399-411.

- **Shalgi R., Lapidot M., Shamir R. and Pilpel Y.** (2005). A catalog of stability-associated sequence elements in 3' UTRs of yeast mRNAs. *Genome Biol.* **6**: R86.
- **Sharom F.J.** (1997). The P-glycoprotein efflux pump: how does it transport drugs? *J. Memb. Biol.* **160**: 161-175.

- **Shen D.W., Fojo A., Chin J.E., Roninson I.B., Eüchert N., Pastan I. and Gottesman M.M.** (1986). Human multidrug-resistant cell lines: increased *mdr1* expression can precede gene amplification. *Science.* **232**: 643-645.

- **Shen H., An M.M., Wang D.J., Xu Z., Zhang J.D., Gao P.H., Cao Y.Y., Cao Y.B. and Jiang Y.Y.** (2007). Fcr1p inhibits development of fluconazole resistance in *Candida albicans* by abolishing *CDR1* induction. *Biol. Pharm. Bull.* **30**: 68-73.

- **Shukla S., Saini P., Smriti, Jha S., Ambudkar S.V. and Prasad R.** (2003). Functional characterization of *Candida albicans* ABC transporter Cdr1p. *Eukaryot. Cell.* **2**: 1361-1375.

- **Silver P.M., Oliver B.G. and White T.C.** (2004). Role of *Candida albicans* transcription factor Upc2p in drug resistance and sterol metabolism. *Eukaryot. Cell.* **3**: 1391-1397.

- **Silverman J.A. and Schrenk D.** (1997). Hepatic canalicular membrane 4: expression of the multidrug resistance genes in the liver. *FASEB J.* **11**: 308-313.

- **Siomi H. and Dreyfuss G.** (1997). RNA-binding proteins as regulators of gene expression. *Curr. Opin. Genet. Devel.* **7**: 345-353.

- **Skovsgaard T., Nielsen D., Maare C. and Wasserman K.** (1994). Cellular resistance to cancer chemotherapy. *Int. Rev. Cytol.* **156**: 77-157.

- **Song J.L., Harry J.B., Eastman R.T., Oliver B.G. and White T.C.** (2004). The *Candida albicans* lanosterol 14-alpha-demethylase (*ERG11*).gene promoter is maximally induced after prolonged growth with antifungal drugs. *Antimicrob. Agents Chemother.* **48**: 1136-1144.

- **Sparks K.A. and Dieckmann C.L.** (1998). Regulation of poly(A) site choice of several yeast mRNAs. *Nucleic Acids Res.* **26**: 4676-4687.

- **Sternberg S.** (1994). The emerging fungal threat. *Science.* **266**: 1632-1634.

- **Subik J., Ulaszewski S. and Goffeau A.** (1986). Genetic mapping of nuclear mucidin resistance mutations in *Saccharomyces cerevisiae. Curr. Genet.* **10**: 665-670.

- **Sundseth R., MacDonald G., Ting J. and King A.C.** (1997). DNA elements recognizing NF-Y and Sp1 regulate the human multidrug-resistance gene promoter. *Mol. Pharmacol.* **51**: 963-971.

- **Talibi D. and Raymond M.** (1999). Isolation of a putative *Candida albicans* transcriptional regulator involved in pleiotropic drug resistance by functional complementation of a *pdr1 pdr3* mutation in *Saccharomyces cerevisiae. J. Bacteriol.* **181**: 231-240.

- **Thakur J.K., Arthanari H., Yang F., Pan S.J., Fan X., Breger J., Frueh D.P., Gulshan K., Li D.K., Mylonakis E., Struhl K., Moye-Rowley W.S., Cormack B.P., Wagner G. and Näär A.M.** (2008). Nuclear receptor-like pathway regulating multidrug resistance in fungi. *Nature.* **452**: 604-609.

- **Thayer S., Ince T., Wright A. and Scotto K.W.** (1996). An upstream protein binding element is required for efficient expression of the *MDR1* promoter in human neuroblastoma cells. In *Proceedings of the American association of cancer research.*

- **Thayer S.P.** (1999) Transcriptional activation of the *MDR1* gene in retinoic acid stimulated neuroblastoma cells involves derepression and activation by multiple SP family members (Ph.D. thesis, Cornell Medical College).

- **Thompson J.D., Higgins D.G. and Gibson, T.J.** (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673-4680.

- **Thomson A.M., Rogers, J.T., Walker C.E., Staton J.M. and Leedman P.J.** (1999). Optimized RNA gel-shift and UV cross-linking assays for characterization of cytoplasmic RNA-protein interactions. *BioTechniques.* **27**: 1032-9, 1042.

- **Thorgeirsson S.S., B.E. Huber, Sorrel S., Fojo A., Pastan I. and Gottesman M.M.** (1987). Expression of the multidrug-resistant gene in hepatocarcinogenesis and regenerating rat liver. *Science.* 236: 1120-1122.

- **Tian D., Huang D., Brown C. and Jungmann R.A.** (1998). Protein kinase A stimulates binding of multiple proteins to a U-rich domain in the 3'untranslated

region of lactate dehydrogenase A mRNA that is required for the regulation of mRNA stability. *J. Biol. Chem.* **273**: 28454-28460.

- **Tillmar L., Carlsson C. and Welsh N.** (2002). Control of insulin mRNA stability in rat pancreatic islets. Regulatory role of a 3'-untranslated region pyrimidine-rich sequence. *J. Biol. Chem.* **277**: 1099-1106.

- **Trzaska D. and Dastych J.** (2005). Role of AURE sequences in the regulation of mRNA stability. *Postepy Biochem.* **51**: 28-35.

- **Turi T.G. and Loper J.C.** (1992). Multiple regulatory elements control expression of the gene encoding the *Saccharomyces cerevisiae* cytochrome P450, lanosterol 14 alpha-demethylase (*ERG11*). *J. Biol. Chem.* **267**: 2046-2056.

- **Uchiumi, T., K. Kohno, H. Tanimura, K. Matsuo, S. Sato, Y. Uchida and M. Kuwano** (1993). Enhanced expression of the human multidrug resistance 1 gene in response to UV light irradiation. *Cell Growh and Differ.* **4:** 147-157.

- **Ueda K., Clark D.P., Chen C.J., Roninson I.B., Gottesman M.M. and Pastan I.** (1987a). The human rnuitidrug resistance (*mdr1*) gene. cDNA cloning and transcription initiation *J. Biol. Chem.* **262**: 505-508.

- **Ueda K., Pastan I. and Gottesman M.M.** (1987b). Isolation and sequence of the promoter region of the human multidrug resistance (P-glycoprotein) gene. *J. Biol. Chem.* **262**: 17432-27436.

- **Uhl M.A. and Johnson A.D.** (2001). Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans*. *Microbiology.* **147**: 1189-1195.

- **Van denbroucke-Grauls C.M.** (1993). The threat of multidrug resistant microorganisms. *Eur. J. Clin. Microbiol. Infect. Dis.* **1**: 27-30.

- **Van Groenigen M., Valentijn L.J. and Baas F.** (1993). Identification of a functional initiator sequence in the human *MDR1* promoter. *Biochim. Biophys. Acta.* **1172**:138-146.

- **Vanden Bossche H. and Koymans L.** (1998). Cytochromes P450 in fungi. *Mycoses.* **41**: 32-38.

- **Vanden Bossche H., Dromer F., Improvisi L., Lozano-Chiu M., Rex J.H. and Sanglard D.** (1998). Antifungal drug resistance in pathogenic fungi. *Med. Mycol.* **36**: 119-128.

- **Vanden Bossche H., Marichal P. and Odds F.C.** (1994). Molecular mechanisms of drug resistance in fungi. *Trends Microbiol.* **2**: 393-400.

- **Vanden Bossche H., Marichal P., Gorrens J., Coene M.C., Willemsens G., Bellens D., Roels I., Moereels H. and Janssen P.A.** (1989). Biochemical approaches to selective antifungal activity. Focus on azole antifungals. *Mycoses.* **32**: 35-52.

- **Vanden Bossche H., Warnock D.G., Dupont B., Kerridge D., Sengupta S., Improvisi L., Marichal P., Odds F.C., Provost F. and Ronin O.** (1994b). Mechanisms and clinical impact of antifungal drug Resistance. *J. Med. Vet. Mycol.* **32**: 189-202.

- **Vik A. and Rine J.** (2001). Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae. Mol. Cell Biol.* **21**: 6395-6405.

- **Wang D., Johnson A.D., Papp A.C., Kroetz D.L. and Sadee W.** (2005). Multidrug resistance polypeptide 1 (*MDR1, ABCB1*) variant 3435C>T affects mRNA stability. *Pharmacogenet. Genomics.* **15**: 693-704.

- **Wang X., Kiledjian M., Weiss I.M. and Liebhaber S.A.** (1995). Detection and characterization of a 3' untranslated region ribonucleoprotein complex associated with human α-globin mRNA stability. *Mol. Cell Biol.* **15**: 1769-1777.

- **Wemmie J.A., Szczypka M.S., Thiele D.J. and Moye-Rowley W.S.** (1994). Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter- encoding gene, *YCF1. J. Biol. Chem.* **269**: 32592-32597.

- **Wendler F., Bergler H., Prutej K., Jungwirth H., Zisser G., Kuchler K. and Hogenauer G.** (1997). Diazoborine resistance in the yeast *Saccharomyces cerevisiae* reveals a link between *YAP1* and the pleitropic drug resistance genes *PDR1* and *PDR3. J. Biol. Chem.* **272**: 27091-27098.

- **Whelan W.L. and Soll D.R.** (1982). Mitotic recombination in *Candida albicans*: recessive lethal alleles linked to a gene required for methionine biosynthesis. *Mol. Gen. Genet.* **187**: 477-485.

- **White T.C.** (1997a). Increased mRNA levels of *ERG16, CDR,* and *MDR1* correlate with increased azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. *Antimicrob. Agents Chemother.* **41**: 1482-1487.

- **White T.C.** (1997b). The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14 alpha-demethylase in *Candida albicans. Antimicrob. Agents Chemother.* **41**: 1488-1494.

- **White T.C. and Silver. P.M.** (2005). Regulation of sterol metabolism in *Candida albicans* by the *UPC2* gene. *Biochem. Soc. Trans.* **33**: 1215-1218.

- **White T.C., Marr K.A. and Bowden R.A.** (1998). Clinical, cellular and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* **11**: 382-402.

- **White T.C., Pfaller M.A. Rinaldi M.G., Smith J. and Redding S.W.** (1997). Stable azole drug resistance associated with a substrain of *Candida albicans* from an HIV-infected patient. *Oral Diseases.* **3**: 102-109.

- **Wickens M., Anderson P. and Jackson R.J.** (1997). Life and death in the cytoplasm: messages from the 3' end. *Curr. Opin. Genet. Dev.* **7**: 220-232.

- **Wilcox L.J., Balderes D.A., Wharton B., Tinkelenberg A.H., Rao G. and Sturley S.L.** (2002). Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. *J. Biol. Chem.* **277**: 32466-32472.

- **Williams D.L., Sensel M., McTigue M. and Binder R.** (1993). Hormonal and developmental regulation of mRNA turnover, in *Control* of *Messenger RNA stability* (J. Belasco and G. Brawerman, eds.). Academic Press, Inc, San Diego, 161-197.

- **Williams, L. and Wilkins.** (2004). Clinical drug therapy: rationale for nursing practices. 7th edition by Anne Collins Abrams.

- **Wirsching S., Michel S., Kohler G. and Morschhäuser J.** (2000). Activation of the multidrug resistance gene *MDR1* in fluconazole resistant, clinical *Candida albicans* strains is caused by mutations in a *trans*-regulatory factor. *J. Bacteriol.* **182**: 400-404.

- **Wolfger H., Mahe Y., Parle-McDermott A., Delahodde A. and Kuchler K.** (1997). The yeast ATP binding cassette (ABC) protein genes *PDR10* and *PDR15* are novel targets for the *Pdr1* and *Pdr3* transcriptional regulators. *FEBS Letters.* **418**: 269-274.

- **Wolfger H., Mamnun Y.M. and Kuchler K.** (2004). The yeast Pdr15p ATP-binding cassette (ABC) protein is a general stress response factor implicated in cellular detoxification. *J. Biol. Chem.* **279**: 11593-11599.

- **Wu A., Wemmie J.A., Edgington N.P., Goebl M., Guevara J.L. and Moye-Rowley W.S.** (1993). Yeast bZip proteins mediate pleiotropic drug and metal resistance. *J. Biol. Chem.* **268**: 18850-18858.

- **Wysocki R., Fortier P.K., Maciaszczyk E., Thorsen M., Leduc A., Odhagen A., Owsianik G., Ulaszewski S., Ramotar D. and Tamas M.J.** (2004). Transcriptional activation of metalloid tolerance genes in *Saccharomyces*

cerevisiae requires the AP-1 like proteins Yap1p and Yap8p. *Mol. Biol. Cell.* **15**: 2049-2060.

- **Yague E., Armesilla A.L., Harrison G., Elliott J., Sardini A., Higgins C.F. and Raguz S.** (2003). P-glycoprotein (*MDR1*) expression in leukemic cells is regulated at two distinct steps, mRNA stabilization and translational initiation. *J. Biol. Chem.* **278**: 10344-10352.

- **Yang Y.L., Lin Y.H., Tsao M.Y., Chen C.G., Shih H.I., Fan J.C., Wang J.S. and Lo H.J.** (2006). Serum repressing efflux pump *CDR1* in *Candida albicans*. *BMC. Mol. Biol.* **7**: 22.

- **Zhang X., Cui Z., Miyakawa T. and Moye-Rowley W.S.** (2001). Cross-talk between transcriptional regulators of multidrug resistance in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **276**: 8812-8819.

- **Zhao J., Hyman L. and Moore C.** (1999). Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. *Microbiol. Mol. Biol. Rev.* **63**: 405-445.

- **Zhou G. and Kuo T.** (1998). Wild-type p53-mediated induction of rat *mdr1b* expression by the anticancer drug daunorubicin. *J. Biol. Chem.* **273**: 15387-15394.

- **Znaidi S., De Deken X., Weber S., Rigby T., Nantel A. and Raymond M.** (2007). The zinc cluster transcription factor Tac1p regulates *PDR16* expression in *Candida albicans*. *Mol. Microbiol.* **66**: 440-452.

- **Znaidi S., Weber S., Zin Al-Abdin O., Bomme P., Saidane S., Drouin S., Lemieux S., De D.X., Robert F. and Raymond M.** (2008). Genome-wide location analysis of *Candida albicans* Upc2p, a regulator of sterol metabolism and azole drug resistance. *Eukaryot. Cell.* **7**: 836-847.

- **Zuker M., Mathews D. and Turner D.** (1999). Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide in RNA biochemistry and biotechnology. In: Clark B.F.C. Barciszewski, Editor, NATO ASI Series, Kluwer Academic Publishers, New York. p.11.

Appendices

Appendices

APPENDIX I: Culture Media

1. YEPD (Yeast Extract Peptone Dextrose)

Component	Concentration (g/100ml)
Yeast extract	1
Bacto peptone	2
D-glucose	2
Bacto agar (when required)	2.5

2. YNB (Yeast Nitrogen Base)

Component	Concentration (g/100ml)
Yeast Nitrogen Base (w/o a.a.)	0.67
D-glucose	2
Bacto agar (when required)	2.5
Supplements:	
Uracil	30 μg/ml

3. LB (Luria Bertini Broth): Bacterial culture media

Component	Concentration (g/100ml)
Tryptone	1
Yeast extract	0.5
Sodium chloride	1
Bacto agar (when required)	1.5

4. SD Drop out mix

Component	Concentration (g/100ml)
Amino acid	*Amount (in gm)*
Adenine	2.0
Trytophan	2.0
Histidine	1.0
Arginine	1.0
Methionine	1.0
Tyrosine	1.5
Isoleucine	1.5
Valine	7.5
Lysine	1.5
Phenylalanine	2.5
Glutamic acid	5.0
Aspartic acid	1.8
Threonine	10.0
Serine	1.8
Leucine	3.0
Uracil	1.0

5. SD-URA$^-$ media

Component	Concentration (g/100ml)
Yeast Nitrogen Base (w/o aa)	0.67
Glucose	2
Ura$^-$ amino acid dropout	0.2
Bacto agar (when required)	2.5

APPENDIX II: Gel Electrophoresis solutions

1. 50X TAE (pH 8.0)

Component	*g/ litre*
Tris-base	242 g
Acetic acid	57.1 ml
0.5M EDTA (pH 8.0)	100 ml

2. 10X TBE (pH 8.0)

Component	*g/ litre*
Tris-base	108
Boric Acid	55
0.5M EDTA (pH 8.0)	40 ml

3. 1% Agarose

Components	*Volume for 50 ml*
1% agarose	0.5 g
1X TAE	50 ml

4. 5 % Polyacrylamide Gel (PAGE) (for EMSA)

Components	*Volume for 50 ml*
30 % Polyacrylamide	8.25 ml
10X TBE	2.5 ml
10 % APS	500 µl
TEMED	50 µl
M.Q. water	38.25 ml

6. 8 % Polyacrylamide Gel (PAGE)

Components	*Volume for 50 ml*
30 % Polyacrylamide	13.3 ml
1.5 M Tris (pH 8.8)	12.5 ml
10 % SDS	500 µl
10 % APS	500 µl
TEMED	30 µl
M.Q. water	23.2 ml

7. 10 % Polyacrylamide Gel (PAGE)

Components	*Volume for 50 ml*
30 % Polyacrylamide	16.7 ml
1.5 M Tris (pH 8.8)	12.5 ml
10 % SDS	500 µl
10 % APS	500 µl
TEMED	20 µl
M.Q. water	19.8 ml

APPENDIX III: Yeast Lysis Buffer (TENTS Buffer)

Components	Volume for 100 ml
1 M Tris (pH 7.5)	1 ml
0.5 M EDTA (pH 8.0)	200 µl
5 M NaCl	2 ml
Triton X-100	2 ml
10% SDS	10 ml
M.Q. water	up to 100 ml

APPENDIX IV: Hybridization solutions

1. Southern Hybridization

Component	Amount
Pre-Hyb/ Hybridization solution	
SSC	6X SSC
Denhardt's solution	5X
SDS	0.5 %
Salmon Sperm DNA	100 µg/ml
100X Denhardt's Solution	
BSA	2 % (*w/v*)
Ficoll	2 % (*w/v*)
Polyvinyl pyrollidone	2 % (*w/v*)

2. Northern Hybridization

Component	For 10 ml
Pre-Hyb/ Hybridization solution	
0.1M Sodium phosphate buffer (pH 7.0)	3 ml
10% SDS	7 ml
0.5M EDTA (pH 8.0)	200 µl
Salmon Sperm DNA	100 µg/ml

3. Western Hybridization

Component	*Amount*
Transfer Buffer *(For 1000 ml)*	
Tris	3.03 g (25 mM)
Glycine	14.4 g (198 mM)
Methanol	200 ml (20%)
Blocking Buffer *(For 10 ml)*	
Skimmed milk	500 mg (5 %)
Phosphate buffer saline-Tween80 (PBS-T)	10 ml
Hybridization solution *(For 10 ml)*	
Phosphate buffer saline-Tween80 (PBS-T)	10 ml
Monoclonal Anti-GFP antibody	1:5000
Polyclonal Anti-Cdr1p antibody	1:500
Polyclonal Anti-Pma1p antibody	1:1000

APPENDIX V:

Solvents for Drugs

Drugs	Solvent used
α-amanitin	Methanol
1,10-Phenanthroline	Methanol
Actinomycin D	Methanol
Clotrimazole	Dimethyl formamide (DMF)
Cycloheximide	Water
Fluconazole	Water
Fluphenazine	Water
Itraconazole	Methanol
Ketoconazole	Methanol
Miconazole	Methanol
Nourseothricin	Water
Progesterone	Ethanol
Rhodamine6G (R6G)	DMSO
Terbinafine	DMSO
Thiolutin	DMSO
ß-estradiol	Ethanol

APPENDIX VI: Molecular Biology solutions / Buffers

I. Crude nuclear extract preparation

1. Zymolyase Buffer

Components	For 50 ml
1M 50mM Tris-Cl (pH 7.5)	2.5 ml
1M $MgCl_2$	0.5 ml
1M DTT	1.5 ml
2M Sorbitol	25 ml
M.Q. water	20 ml

2. Lysis Buffer

Components	For 20 ml
1M Tris-Cl (pH 7.5)	1 ml
1M $MgSO_4$	200 µl
3M Potassium acetate	60 µl
0.5M EDTA (pH 8.0)	40 µl
100mM PMSF	20 µl
1M DTT	20 µl
M.Q. water	18.66 ml

3. Nuclear Lysis Buffer

Components	For 5 ml
1M HEPES (pH 8.0)	100 µl
100% Glycerol	1 ml
5M NaCl	400 µl
1M $MgCl_2$	7.5 µl
0.5M EDTA	10 µl
1M DTT	50 µl
100mM PMSF	25 µl
M.Q. water	3.4 ml

4. Dialysis Buffer

Components	*For 1 litre*
1M HEPES (pH 8.0)	20 ml
100% Glycerol	200 ml
5M NaCl	4 ml
2M DTT	500 µl
100mM PMSF	5 ml
M.Q. water	770.5 ml

II. *DNA-protein Binding Buffer:*

1. for *DNase I* Footprinting

Components	*Volume*
5X Binding Buffer *(For 1 ml)*	
100 mM Tris (pH 7.6)	500 µl
5M NaCl	50 µl
100 mM DTT	50 µl
10 mM EDTA	50 µl
100% glycerol	250 µl
200 mM $MgCl_2$	~63 µl
100 mM $CaCl_2$	25 µl
M.Q. water	~12 µl
2.5X *DNA Mix Buffer *(For 200 µl)*	
5X Binding Buffer	100 µl
*DNA (~30,000 cpm/µl)	50 µl
Poly dI-dC (Stock 1µg/µl)	10 µl
BSA (Stock 10 mg/ml)	30 µl (30 µg/reaction)
M.Q. water	10 µl
DNase I digestion reaction (50 µl/reaction)	
2.5X *DNA Mix Buffer	20 µl (final 1X)

Nuclear extracts	10-25 μl (40-80 μg)
M.Q. water	upto 45 μl
DNase I (0.001 U)	5 μl (0.005 U/ reaction)

2. for DNA-EMSA

Components	*Volume For 30 μl*
500 mM Tris (pH 7.6)	0.6 μl
500 mM NaCl	3 μl
10 mM DTT	3 μl
10 mM EDTA	0.3 μl
50% glycerol	3 μl
100 mM $MgCl_2$	0.75 μl
Poly dI-dC (Stock 1μg/μl)	1 μl
Nuclear extracts	~8-10 μl (30 μg)
M.Q. water	upto 30 μl

3. for RNA-EMSA

Components	*Volume For 30 μl*
1 M HEPES-KOH (pH 7.5)	0.75 μl
1 M KCl	1.5 μl
10 mM DTT	3 μl
10 mM EDTA	3 μl
50% glycerol	3 μl
E. coli tRNA (Stock 10 μg/μl)	1 μl
RNase inhibitor (40 U/μl)	1 μl
Crude cellular extracts	8-10 μl (30 μg)
DEPC treated M.Q. water	upto 30 μl

III. Reporter assay buffer: for β-galactosidase activity

1. *Z*-Buffer (pH 7.0)

Components	*For 1 litre*
$Na_2HPO_4.7H_2O$	16.1 g
$NaH_2PO_4.H_2O$	5.5 g
KCl	0.75 g
$MgSO_4.7H2O$	0.246 g
β-mercaptoethanol	2.7 ml
M.Q. water	upto 1000 ml

2. O-Nitrophenyl β-D-galactopyronoside (ONPG) Buffer

Components	*For 10 ml*
ONPG	40 mg (4 mg/ml)
0.1M Potassium phosphate buffer (pH 7.0)	10 ml

Publications

This thesis is based on the following original published / unpublished research article.

1. Thiolutin as a potent transcription inhibitor of pathogenic yeast *Candida albicans.*

2. Transcriptional activation and increased mRNA stability contribute to over-expression of *CDR1* in azole-resistant *Candida albicans*

3. Differential RNA-Protein interaction and poly(A) tail length contributes to increased *CDR1* mRNA stability in azole resistant clinical isolates of *Candida albicans*

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 332 (2005) 206–214
BBRC
www.elsevier.com/locate/ybbrc

Expression of the *CDR1* efflux pump in clinical *Candida albicans* isolates is controlled by a negative regulatory element

Naseem Akhtar Gaur [a], Raman Manoharlal [a], Preeti Saini [a], Tulika Prasad [a], Gauranga Mukhopadhyay [b], Milan Hoefer [c], Joachim Morschhäuser [d], Rajendra Prasad [a,*]

[a] *Membrane Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India*
[b] *Special Center for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India*
[c] *University of Bonn, Bonn, Germany*
[d] *University of Würzburg, Würzburg, Germany*

Received 2 April 2005
Available online 28 April 2005

Abstract

Resistance to azole antifungal drugs in clinical isolates of the human fungal pathogen *Candida albicans* is often caused by constitutive overexpression of the *CDR1* gene, which encodes a multidrug efflux pump of the ABC transporter superfamily. To understand the relevance of a recently identified negative regulatory element (NRE) in the *CDR1* promoter for the control of *CDR1* expression in the clinical scenario, we investigated the effect of mutation or deletion of the NRE on *CDR1* expression in two matched pairs of azole-sensitive and resistant clinical isolates of *C. albicans*. Expression of *GFP* or *lacZ* reporter genes from the wild type *CDR1* promoter was much higher in the azole-resistant *C. albicans* isolates than in the azole-susceptible isolates, reflecting the known differences in *CDR1* expression in these strains. Deletion or mutation of the NRE resulted in enhanced reporter gene expression in azole-sensitive strains, but did not further increase the already high *CDR1* promoter activity in the azole-resistant strains. In agreement with these findings, electrophoretic mobility shift assays showed a reduced binding to the NRE of nuclear extracts from the resistant *C. albicans* isolates as compared with extracts from the sensitive isolates. These results demonstrate that the NRE is involved in maintaining *CDR1* expression at basal levels and that this repression is overcome in azole-resistant clinical *C. albicans* isolates, resulting in constitutive *CDR1* overexpression and concomitant drug resistance.

Keywords: *Candida albicans*; *CDR1*; Multidrug resistance; Promoter; Regulation

Azole-resistant clinical *Candida albicans* isolates frequently exhibit reduced intracellular drug accumulation that correlates with enhanced expression of certain multiple drug resistance genes, the ATP-binding cassette (ABC) transporters, *CDR1* and *CDR2*, and the major facilitator, *CaMDR1* [1–5]. Fluconazole resistance is usually a stable phenotype that is maintained in the absence of selection pressure by the drug. This implies that certain genetic alterations have occurred in the resistant isolates that result in a constitutive overexpression of genes that encode the drug efflux pump proteins. Earlier studies suggested that *CDR1* is a highly regulatable gene, which is activated by several stresses like drugs, higher temperatures, and human steroids [6]. In silico analysis of the *CDR1* promoter region revealed the presence of several putative stress inducible *cis*-regulatory elements (HSE, MDR-NF1/YB-1, AP-1, etc.) [7]. Promoter deletion analyses and electrophoretic mobility shift assays (EMSAs) with *CDR1* promoter fragments confirmed the presence of a steroid responsive region (SRR) in the distal part of the promoter [8]. Deletion analysis within the SRR further delimited these steroid responsive sequences to two distinct elements, viz.,

* Corresponding author. Fax: +91 11 2671 7081, +91 11 2616 5886.
E-mail address: rp47@hotmail.com (R. Prasad).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.04.113

SRE1 and SRE2. Comparison of SRE1/2 with the promoter sequence of MDR (*CDR2* and *PDR5*) and non-MDR (*HSP90*) steroid responsive genes revealed a similarity with respect to conservation of three 5 bp stretches (AAGAA, CCGAA, and ATTGG) [8]. In addition, a common drug/steroid responsive element has also been identified in the *CDR1* and *CDR2* promoters of *C. albicans* [9].

The transcriptional activation of *CDR1* in the development of azole resistance is well known [4,6]. However, the mechanisms by which *CDR1* levels are altered in clinical resistant isolates are only partially understood [4]. *CDR1* expression in azole-resistant clinical isolates of *C. albicans* could be increased due to mutations in the promoter region of the gene, alterations in *trans*-regulatory factors controlling its expression, or molecular changes taking place during mRNA processing [10–13]. We had earlier identified a negative regulatory element (NRE) at positions −272 to −265 upstream of the transcriptional start point of *CDR1* [14]. Mutation or deletion of this sequence, 5′-CTGATTGA-3′, resulted in an activation of the *CDR1* promoter. A purified 55 kDa nuclear protein (NREBP) was shown to specifically bind to the NRE [14]. Considering the importance of the NRE in controlling expression of *CDR1*, in this study, we explored its clinical relevance. For this purpose, we investigated the role of the NRE in two matched pairs of azole-susceptible/-resistant clinical *C. albicans* isolates in which drug resistance correlated with the stable *CDR1* overexpression [4,15].

Materials and methods

Strains and media. All plasmids were maintained in *Escherichia coli* DH-5α. *E. coli* was cultured in LB (Luria–Bertani) broth or on LB plates, supplemented with ampicillin (0.1 mg/ml). *C. albicans* strains used in this study (Table 1) were maintained on YEPD (yeast extract 1%, bactopeptone 2%, and glucose 2%). When grown on solid media, 2.5% agar was added to the liquid media.

Plasmid construction and yeast transformation. Plasmids pCPL1, pCPLM, pCPLD, pCPG1, pCPGM, and pCPGD contain *lacZ* or *GFP* reporter genes under control of the wild type *CDR1* promoter or derivatives in which the NRE was mutated or deleted (see Figs. 1A and B). The flanking *ACT1* sequences in these plasmids serve for genomic integration of the reporter fusions into the *ACT1* locus of clinical *C. albicans* isolates and the dominant *caSAT1* marker was used to select nourseothricin-resistant transformants. For construction of pCPL1, an *Xho*I–*Pst*I fragment from plasmid pMEP2LACZ2 [16] containing the *Streptococcus thermophilus lacZ* gene [17], the transcription termination sequence of the *C. albicans ACT1* gene, and the *URA3* selection marker was first ligated between flanking *ACT1* sequences of plasmid pMPG2 (S. Stahl and J. Morschhäuser, unpublished) to obtain pLACZ6. An *Xho*I–*Pst*I fragment containing the *caSAT1* selection marker [18] was then substituted for the *Sal*I–*Pst*I fragment containing the *URA3* marker to generate pLACZ7. A *Kpn*I–*Apa*I fragment containing 0.5 kb of the *ACT1* coding region was PCR amplified from genomic DNA of *C. albicans* strain SC5314 with the primers ACT23

Table 1
C. albicans strains used in this study

Strain	Description	Source
Gu4	Fluconazole-susceptible clinical isolate	Franz et al. [15]
NGY4G	*CDR1*p-*GFP* (wild type) integrated in Gu4 at *ACT1* locus	This study
NGY4MG	*CDR1*p-*GFP* (NREmut) integrated in Gu4 at *ACT1* locus	This study
NGY4DG	*CDR1*p-*GFP* (NREdel) integrated in Gu4 at *ACT1* locus	This study
NGY4L	*CDR1*p-*lacZ* (wild type) integrated in Gu4 at *ACT1* locus	This study
NGY4ML	*CDR1*p-*lacZ* (NREmut) integrated in Gu4 at *ACT1* locus	This study
NGY4DL	*CDR1*p-*lacZ* (NREdel) integrated in Gu4 at *ACT1* locus	This study
Gu5	Fluconazole-resistant clinical isolate	Franz et al. [15]
NGY5G	*CDR1*p-*GFP* (wild type) integrated in Gu5 at *ACT1* locus	This study
NGY5MG	*CDR1*p-*GFP* (NREmut) integrated in Gu5 at *ACT1* locus	This study
NGY5DG	*CDR1*p-*GFP* (NREdel) integrated in Gu5 at *ACT1* locus	This study
NGY5L	*CDR1*p-*lacZ* (wild type) integrated in Gu5 at *ACT1* locus	This study
NGY5ML	*CDR1*p-*lacZ* (NREmut) integrated in Gu5 at *ACT1* locus	This study
NGY5DL	*CDR1*p-*lacZ* (NREdel) integrated in Gu5 at *ACT1* locus	This study
DSY294	Fluconazole-susceptible clinical isolate	Sanglard et al. [4]
NGY294G	*CDR1*p-*GFP* (wild type) integrated in DSY294 at *ACT1* locus	This study
NGY294MG	*CDR1*p-*GFP* (NREmut) integrated in DSY294 at *ACT1* locus	This study
NGY294DG	*CDR1*p-*GFP* (NREdele) integrated in DSY294 at *ACT1* locus	This study
NGY294L	*CDR1*p-*lacZ* (wild type) integrated in DSY294 at *ACT1* locus	This study
NGY294ML	*CDR1*p-*lacZ* (NREmut) integrated in DSY294 at *ACT1* locus	This study
NGY294DL	*CDR1*p-*lacZ* (NREdel) integrated in DSY294 at *ACT1* locus	This study
DSY296	Fluconazole-resistant clinical isolate	Sanglard et al. [4]
NGY296G	*CDR1*p-*GFP* (wild type) integrated in DSY296 at *ACT1* locus	This study
NGY296MG	*CDR1*p-*GFP* (NREmut) integrated in DSY296 at *ACT1* locus	This study
NGY296DG	*CDR1*p-*GFP* (NREdel) integrated in DSY296 at *ACT1* locus	This study
NGY296L	*CDR1*p-*lacZ* (wild type) integrated in DSY296 at *ACT1* locus	This study
NGY296ML	*CDR1*p-*lacZ* (NREmut) integrated in DSY296 at *ACT1* locus	This study
NGY296DL	*CDR1*p-*lacZ* (NREdel) integrated in DSY296 at *ACT1* locus	This study

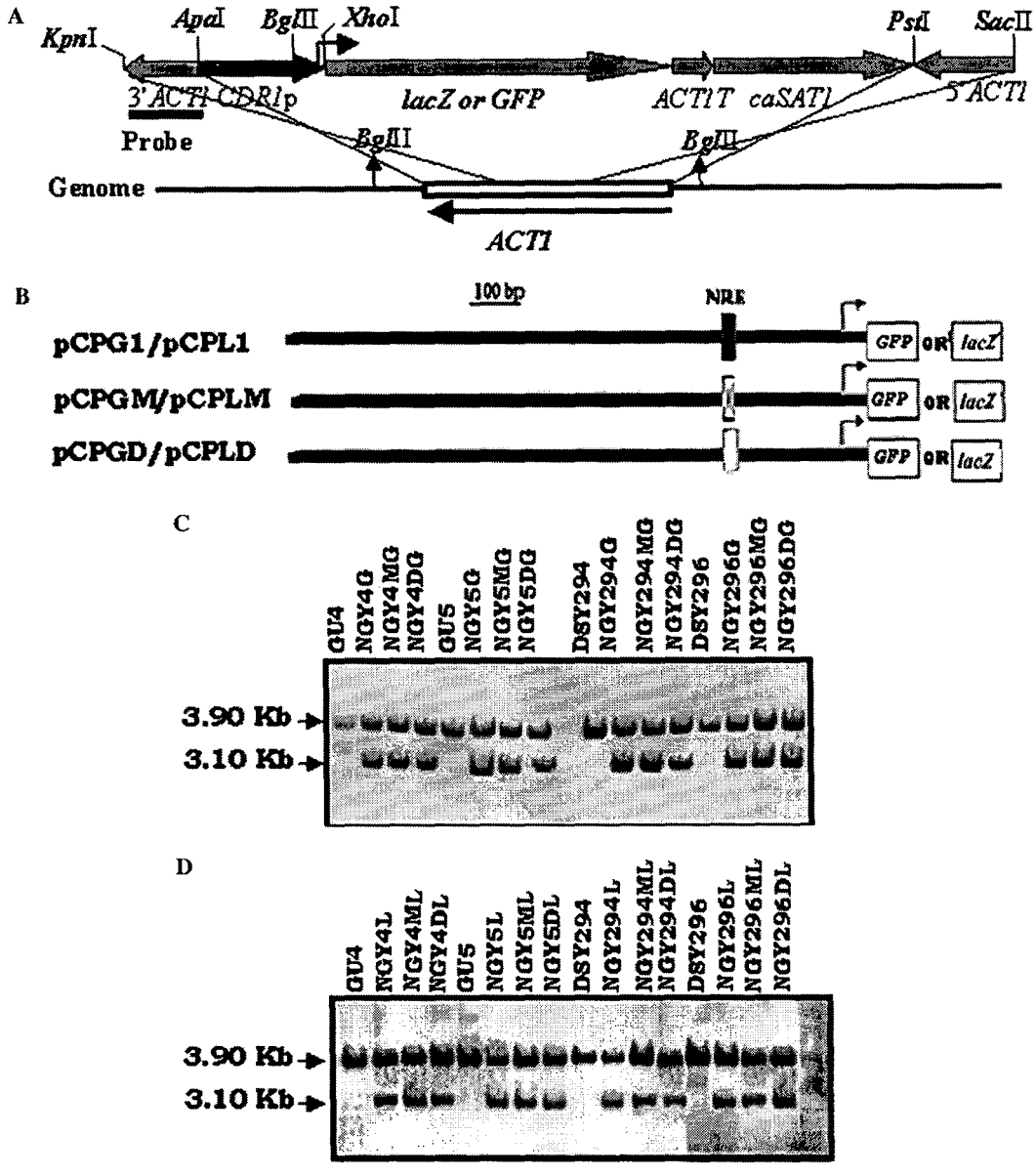


Fig. 1. Integration of *CDR1*p-*GFP* and *CDR1*p-*lacZ* fusions into the genome of clinical *C. albicans* isolates. (A) Structure of the DNA fragments used for integration of *CDR1*p-*GFP* or *CDR1*p-*lacZ* reporter fusions at the *ACT1* locus of the clinical *C. albicans* isolates. The genomic *ACT1* locus is represented by an open bar, the straight arrow indicating the direction of transcription. The dark arrow represents the *CDR1* promoter. The probe used to verify the correct integration is indicated by the thick line. Relevant restriction sites are shown. (B) Schematic representation of the *CDR1* promoter–reporter gene constructs. The crossed and open boxes indicate the mutation and deletion, respectively, of the NRE in the *CDR1* promoter. The names of the plasmids harboring the reporter fusions are shown on the left. (C,D) Southern hybridization of *Bgl*II-digested genomic DNA of the parental clinical *C. albicans* isolates and transformants containing the various *CDR1*p-*GFP* (C) and *CDR1*p-*lacZ* (D) reporter fusions with the *ACT1*-specific probe shown in (A). The sizes of the hybridizing fragments are given on the left side of the blots. The appearance of a 3.1 kb *Bgl*II fragment in the transformants in addition to the 3.9 kb wild type *ACT1* fragment confirms correct integration of the reporter gene fusions in one of the *ACT1* alleles.

(5′-GACATAACAATGGTACCGTATAATTC-3′) and ACT34 (5′-CGGACGATATGGGCCCAATCTGGCATCAC-3′) (the introduced *Kpn*I and *Apa*I restriction sites are underlined). In addition, an *Apa*I–*Xho*I fragment containing 1196 bp of *CDR1* upstream sequences was amplified from SC5314 genomic DNA with the primers CDR1F (5′-GATCGGGCCCTCGTTACTCAATAAGTAT-3′) and CDR1R (5′-AGCTCTCGAGTTCTTTTTGACCTTTTAAAG-3′). The *ACT1* fragment and the *CDR1* promoter fragment were ligated together into the *Kpn*I/*Xho*I-digested vector pLACZ7 to generate pCPL1, which contains the *lacZ* reporter gene under control of the wild type *CDR1* promoter. Substitution of a *C. albicans*-adapted *GFP* gene [19] for the *lacZ* gene generated pCPG1. A mutation (CTGATTGA to CCCCGGGG) or deletion of the NRE was introduced in pCPL1 and pCPG1 by site directed mutagenesis using the primer pairs FMF/FMR and FDLF/FDLR for NRE mutation and deletion, respectively [14]. The resulting plasmids were named pCPLM (NRE mutated), pCPLD (NRE deleted), pCPGM (NRE mutated), and pCPGD (NRE deleted). All constructs were confirmed by sequencing. The gel-purified *Kpn*I–*Sac*II fragments from pCPL1, pCPG1, pCPLM, pCPLD, pCPGM, and pCPGD, containing the *CDR1*p-*lacZ* and *CDR1*p-*GFP* fusions

(see Fig. 1A), were used to transform the fluconazole-susceptible clinical *C. albicans* isolates Gu4 and DSY294 as well as the corresponding fluconazole-resistant matched isolates Gu5 and DSY296. Transformations were performed as described earlier [14,18] and transformants were selected on YPD agar plates containing 200 μg/ml of nourseothricin after 24 h of incubation at 30 °C.

Microscopy. The strains were grown overnight in YPD liquid medium and aliquots were spotted on microscopic slides. Fluorescence was detected with a Zeiss Axiolab microscope equipped for epifluorescence microscopy with a 5-W mercury high-pressure bulb and the Zeiss fluorescein-specific filter set 09 [8,11].

β-Galactosidase assays. β-Galactosidase assays were performed as described by Uhl and Johnson [17]. Visual screening was carried out by streaking the *C. albicans* strains onto minimal media plates containing X-Gal. For plates, 1.70 g yeast nitrogen base without amino acids or ammonium sulfate, 20 g glucose, 5 g ammonium sulfate, and 20 g agar were dissolved in 930 ml H_2O. After autoclaving, 70 ml of 1 M potassium phosphate, pH 7.0, and 2.0 ml of a 20 mg/ml X-Gal solution were added [17].

For liquid assays, 1 ml of cell culture was pelleted and the cells were resuspended in an equal volume of *lacZ* buffer and placed on ice. The OD_{600} was determined for each sample [17]. Then 100 μl of cells was added to the *lacZ* buffer to a final volume of 1.0 ml and the cells were permeabilized with 15 μl of 0.1% SDS and 30 μl chloroform. Cells were equilibrated at 37 °C for 15 min and then 0.2 ml of ONPG (4 mg/ml) was added, and the cells were mixed and incubated at 37 °C. Reactions were stopped by addition of 0.5 ml of 1 M Na_2CO_3 and centrifuged for 5 min at 10,000*g* and the OD of the samples was measured at 420 and 550 nm. Units of activity were determined by the standard equation given by Ausubel [17].

Electrophoretic mobility shift assay. The EMSA was performed essentially as reported earlier [14]. The double stranded oligonucleotide harboring the NRE, generated by annealing of complementary strands (NRE1: 5′-GATCTGCCAACTGATTGAGGTTGA-3′, NRE2: 5′-GATCTCAACCTCAATCAGTTGGCA-3′), was end labeled with [γ-^{32}P]ATP and incubated with 30 μg of nuclear extracts isolated from each susceptible and resistant isolate for 20 min at room temperature [14]. The protein content of nuclear extracts for each isolate was estimated by Bradsford method and also checked on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [14].

Results

Expression of CDR1p-GFP reporter fusions in matched azole-susceptible and resistant clinical C. albicans isolates

In order to explore the significance of the NRE in the regulation of *CDR1* expression in drug-resistant clinical isolates of *C. albicans*, we monitored the activity of the wild type *CDR1* promoter and derivatives in which the NRE was either mutated or deleted in two matched azole-susceptible and resistant clinical *C. albicans* isolate pairs using *GFP* as a reporter gene. The wild type *CDR1* promoter was amplified from the commonly used azole-susceptible *C. albicans* model strain SC5314 (*CDR1*p-*GFP*, wild type) and an NRE mutation (*CDR1*p-*GFP*, NREmut) or deletion (*CDR1*p-*GFP*, NREdel) was introduced by site directed mutagenesis (see Materials and methods). To avoid recombination between the cloned *CDR1* promoters and the resident *CDR1* promoter of the host strains, the reporter fusions were integrated at the *ACT1* locus (see Fig. 1A). Nourseothricin-resistant transformants of the azole-susceptible *C. albicans* isolates Gu4 and DSY294, and of the corresponding matched, azole-resistant isolates Gu5 and DSY296, respectively [4,15], were selected using the recently developed dominant selection marker *caSAT1* [18]. Single copy integration was confirmed by Southern hybridization (Fig. 1C) and the resulting reporter strains were designated as NGY4G (*CDR1*p-*GFP*, wild type), NGY4MG (*CDR1*p-*GFP*, NREmut), NGY4DG (*CDR1*p-*GFP*, NREdel), NGY5G (*CDR1*p-*GFP*, wild type), NGY5MG (*CDR1*p-*GFP*, NREmut), NGY5DG (*CDR1*p-*GFP* NREdel), NGY294G (*CDR1*p-*GFP*, wild type), NGY294MG (*CDR1*p-*GFP*, NREmut), NGY294DG (*CDR1*p-*GFP*, NREdel), NGY296G (*CDR1*p-*GFP*, wild type), NGY296MG (*CDR1*p-*GFP*, NREmut), and NGY296DG (*CDR1*p-*GFP*, NREdel) (see Table 1 for details). No growth differences were observed between transformants and their respective parental strains in the absence of nourseothricin (data not shown).

The expression of the *CDR1*p-*GFP* fusions was analyzed by epifluorescence microscopy of cells grown to mid-log phase ($OD_{600} = 1.0$) in YPD liquid medium. Two transformants of each strain were analyzed. As shown in Fig. 2, all transformants of susceptible (Gu4 and DSY294) and resistant (Gu5 and DSY296) isolates (NGY4G, NGY294G, NGY5G, and NGY296G) containing the wild type *CDR1*p-*GFP* fusion showed a green fluorescent phenotype, but the fluorescence intensity of the cells was much higher in azole-resistant strains (NGY5G and NGY296G) as compared with their matched sensitive counterparts (NGY4G and NGY294G). This observation is in agreement with the previously described increased *CDR1* transcript levels in the resistant isolates [4,15]. The fact that the *CDR1* promoter from the azole-susceptible *C. albicans* strain SC5314 displayed stronger activity in resistant isolates than in the matched sensitive isolates demonstrated that in both resistant isolates *CDR1* overexpression was caused by alterations in *trans*-regulatory factors [20,21]. Interestingly, in the azole-susceptible strains expressing *GFP* from *CDR1* promoters in which the NRE was mutated or deleted (NGY4MG, NGY4DG and NGY294MG, NGY294DG), the intensity of fluorescence was found to be much stronger than in the corresponding strains that expressed *GFP* from the wild type *CDR1* promoter (NGY4G and NGY294G). These results matched well with our previous *CDR1* promoter deletion analysis in which NRE deletion/mutation led to derepression of *Renilla* luciferase reporter activity in a *C. albicans* laboratory strain [14]. In contrast, mutation or deletion of the NRE did not result in a further increase in the fluorescence intensity of azole-resistant strains expressing the *CDR1*p-*GFP* fusions (Fig. 2).

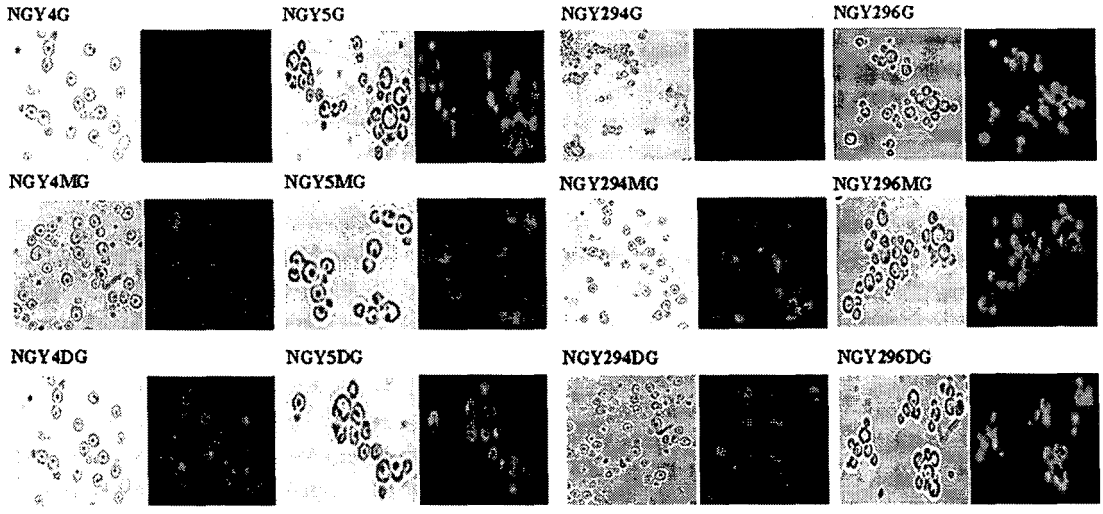


Fig. 2. Expression of the *GFP* reporter gene from wild type as well as NRE mutated and deleted *CDR1* promoters in azole-susceptible and resistant *C. albicans* isolates. Phase contrast (left) and corresponding fluorescence (right) micrographs of representative transformants harboring the chromosomally integrated *CDR1*p-*GFP* fusions.

Azole-resistant C. albicans isolates overcome the negative effect of the NRE on CDR1 expression

The activity of the various *CDR1* promoter derivatives in the clinical *C. albicans* isolates was confirmed using *lacZ* instead of *GFP* as a reporter gene in plate and liquid assays [17]. For this purpose, the *lacZ* gene from *S. thermophilus* [17] was fused to the wild type *CDR1* promoter (*CDR1*p-*lacZ*, wild type) and the derivatives in which the NRE was mutated (*CDR1*p-*lacZ*, NREmut) or deleted (*CDR1*p-*lacZ*, NREdel) (see Materials and methods). The *CDR1*p-*lacZ* reporter constructs were integrated at the *ACT1* locus of the two pairs of matched azole-susceptible and resistant isolates in the same way as the *CDR1*p-*GFP* fusions (see Figs. 1A and B). Single copy integration was confirmed by Southern hybridization (Fig. 1D) and the resulting reporter strains were designated as NGY4L (*CDR1*p-*lacZ*, wild type), NGY4ML (*CDR1*p-*lacZ*, NREmut), NGY4DL (*CDR1*p-*lacZ*, NREdel), NGY5L (*CDR1*p-*lacZ*, wild type), NGY5ML (*CDR1*p-*lacZ*, NREmut), NGY5DL (*CDR1*p-*lacZ*, NREdel), NGY294L (*CDR1*p-*lacZ*, wild type), NGY294ML (*CDR1*p-*lacZ*, NREmut), NGY294DL (*CDR1*p-*lacZ*, NREdel), NGY296L (*CDR1*p-*lacZ*, wild type), NGY296ML (*CDR1*p-*lacZ*, NREmut), and NGY296DL (*CDR1*p-*lacZ*, NREdel), respectively (see Table 1 for details).

Expression of the *CDR1*p-*lacZ* reporter fusions was first qualitatively observed by streaking two transformants of each strain and the parental control (without *lacZ*) on X-Gal plates. All transformants of resistant strains exhibited a dark colored phenotype after 48 h incubation at 30 °C, demonstrating high *lacZ* expression, whereas transformants of the sensitive isolates showed a less intense staining, indicative of a comparably lower *lacZ* expression (Fig. 3A, left panel). However, the sensitive strains exhibited an increased staining when the NRE was mutated or deleted in the *CDR1*p-*lacZ* reporter fusion (Fig. 3A, middle and right panels).

For better comparison of *CDR1* promoter activities, expression of the *CDR1*p-*lacZ* fusions was quantified in liquid β-galactosidase assays. As depicted in Fig. 3B, *lacZ* expression from the wild type *CDR1* promoter was about fivefold higher in transformants of the resistant isolate Gu5 (NGY5L) than in transformants of the matched susceptible isolate Gu4 (NGY4L) and approximately 21-fold higher in transformants of the resistant isolate DSY296 (NGY296L) than in transformants of the corresponding susceptible isolate DSY294 (NGY294L), which exhibited the lowest basal *CDR1* expression levels. In agreement with the results obtained with the *GFP* reporter gene, mutation as well as deletion of the NRE from the *CDR1* promoter resulted in higher *CDR1*p-*lacZ* expression levels in transformants of the susceptible isolates, but not in transformants of the resistant isolates. The β-galactosidase activity in the Gu4 transformants NGY4ML (NREmut) and NGY4DL (NREdel) was about threefold higher than in NGY4L (wild type), and the DSY294 transformants NGY294ML (NREmut) and NGY294DL (NREdel) exhibited approximately ninefold higher *lacZ* expression levels than NGY294L (wild type). Altogether, these results demonstrate that the NRE is involved in maintaining *CDR1* expression at basal levels in azole-susceptible *C. albicans* isolates and that this inhibitory function is overcome in some way in azole-resistant isolates, resulting in constitutive *CDR1* overexpression.

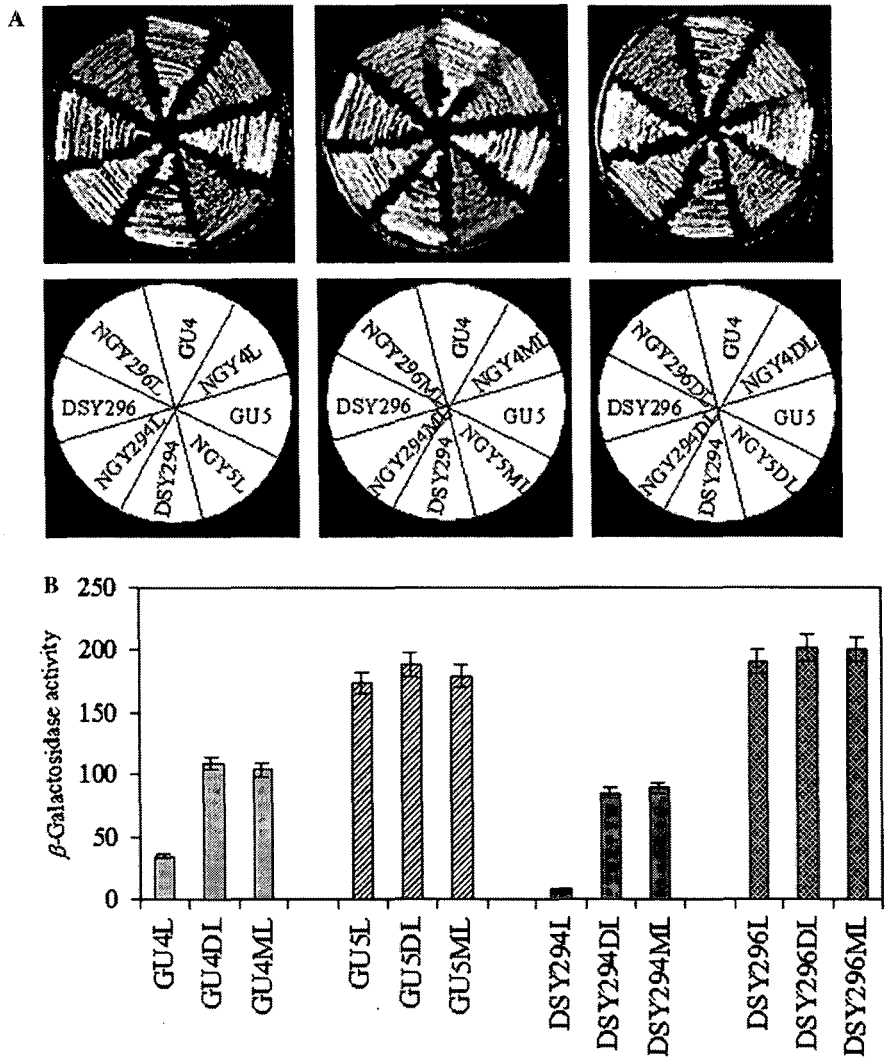


Fig. 3. Effect of mutation or deletion of the NRE in the *CDR1* promoter on β-galactosidase activity in clinical isolates of *C. albicans*. (A) Transformants harboring the chromosomally integrated *CDR1*p-*lacZ* fusions and their parental strains (without *lacZ*) were streaked on minimal media plates containing X-gal and photographed after 48 h of growth at 30 °C (upper panels). The positions of the individual strains on the plates are shown in the scheme below. (B) β-Galactosidase activities of strains harboring the various *CDR1*p-*lacZ* reporter fusions were determined as described in Materials and methods. The values are means ± SD (indicated by the bars) of three independent experiments with duplicate measurements with two independent clones. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Azole-resistant C. albicans isolates display reduced binding of NRE-binding protein (NREBP) to the CDR1 promoter

Since mutation as well as deletion of the NRE resulted in enhanced *CDR1* promoter activity only in azole-susceptible isolates, we hypothesized that the NRE-binding protein keeps *CDR1* expression down-regulated in susceptible isolates but may fail to do so in azole-resistant isolates, resulting in derepression of the *CDR1* promoter. Therefore, we performed electrophoretic mobility shift assays (EMSAs) with a radioactively labeled synthetic double stranded oligonucleotide harboring the NRE and equal amounts of nuclear extract proteins isolated from the fluconazole-susceptible (Gu4 and DSY294) and resistant (Gu5 and DSY296) *C. albicans* isolates. As shown in Fig. 4, a single, specific DNA–protein complex was formed with nuclear extracts from sensitive and resistant isolates. However, the signal intensity of the shifted DNA fragment was strongly reduced when the nuclear extracts from the azole-resistant isolates Gu5 and DSY296 were used in the binding reaction as compared with the signal intensity of the corresponding complex formed with nuclear extracts from the azole-susceptible isolates Gu4 and DSY294.

Discussion

In this study, we have explored the clinical relevance of a negative regulatory element (NRE) which we had

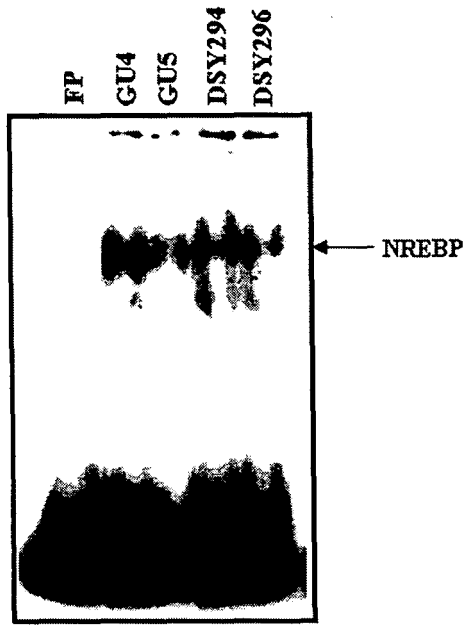


Fig. 4. Differential interaction of NREBP with NRE of *CDR1* in clinical isolates. EMSAs were performed using a synthetic double stranded oligonucleotide (annealed) harboring the NRE as [γ-^{32}P]ATP labeled probe and 30 μg of nuclear extract isolated from each drug-susceptible (Gu4 and DSY294) and resistant (Gu5 and DSY296) isolates. FP: free probe (without protein).

earlier identified in the *CDR1* promoter region [14]. For this purpose, two different reporter genes, *GFP* and *lacZ*, were placed under the control of the *CDR1* promoter from an azole-susceptible *C. albicans* strain and integrated at an ectopic locus in the genome of two pairs of matched clinical *C. albicans* isolates in which stable *CDR1* overexpression coincided with azole resistance. We observed that the reporter genes were expressed at higher levels in the azole-resistant strains as compared with the corresponding sensitive strains, although the *CDR1* promoter fragment controlling their expression was the same in all cases. This result demonstrated that in both resistant isolates the *CDR1* promoter was upregulated by alterations in *trans*-regulatory factors. In agreement with this, sequence analysis of the *CDR1* upstream region (1196 bp from ATG) did not reveal any differences in isolates Gu4 and Gu5 (data not shown), and no mutations in the regulatory region of *CDR1* had been detected by others in the second pair of isolates used in our present study, DSY294/DSY296 [9]. Coste et al. recently reported that a mutated allele of the transcriptional activator of *CDR* genes (*TAC1*) is responsible for *CDR1* and *CDR2* overexpression in the azole-resistant isolate DSY296 [22].

Mutation and deletion of the NRE gave further insight into its role in the control of *CDR1* expression in the clinical *C. albicans* isolates. Deletion or mutation of the NRE resulted in activation of the *CDR1* promoter in the azole-susceptible isolates, but did not further enhance the constitutively elevated *CDR1* promoter activity in the azole-resistant isolates. From these results we conclude that the NRE is involved in maintaining *CDR1* expression at basal levels in the absence of inducing conditions and that the NRE-mediated repression is overcome in azole-resistant clinical *C. albicans* isolates, resulting in constitutive *CDR1* overexpression. A ~55 kDa protein from nuclear extracts was previously found to specifically bind to and protect the NRE from DNaseI digestion in in vitro experiments. In the present study, we observed reduced binding to a *CDR1* promoter fragment comprising the NRE of nuclear extracts from the azole-resistant isolates as compared with extracts from the azole-sensitive isolates. Furthermore, DNaseI footprinting revealed no protection of the NRE by nuclear extracts from resistant isolates (data not shown). Taken together, these results suggest that in azole-sensitive *C. albicans* strains the NREBP is bound to the NRE and functions as a transcriptional repressor to keep *CDR1* expression at basal levels. In azole-resistant isolates binding of the NREBP to the *CDR1* promoter is impaired, resulting in activation of *CDR1* expression. Whether the reduced binding of the NREBP to the NRE is caused by decreased NREBP levels, altered cellular localization, or protein modification remains to be established. However, the recent findings by Coste et al. [22] imply that at least in the clinical isolate DSY296 the transcriptional activator Tac1p may be involved in overcoming the NRE-mediated repression.

In silico analysis revealed that the NRE (5′-CTGA TTGA-3′) of *CDR1* harbors ATTGA and shows significant homology with an inverted CCAAT element (ICE), also known as the Y-box (ATTGA), which is present in the promoter region of several genes including human *MDR1* and topoisomerase IIα (topo IIα) that have been shown to be involved in the development of drug resistance in human cancer cells [23,24]. Interestingly, point mutation in the ICE of the topo IIα promoter resulted in a significant increase in its transcriptional activity in drug-sensitive CEM cells (a human leukemia cell line) which was not the case in drug-resistant cells [23]. In addition, in EMSAs the element was recognized by a protein complex that was present at reduced levels in drug-resistant cell lines, suggesting a negative regulatory role of the ICE in transcriptional activation of human topo IIα [23], similar to the proposed role of the NRE in the *C. albicans CDR1* promoter.

Of note, the transcriptional activation of *PDR5* of *S. cerevisiae*, a close homologue of Cdr1p, is simultaneously controlled by both transcriptional activators (*PDR1* and *PDR3*) and a repressor (*RDR1*) [25–28]. *PDR1* and *PDR3* are considered as the master regulators in *S. cerevesiae*, because mutations in their activation domain generated hyperactive alleles [29,30]. Interestingly, the activators (*PDR1* and *PDR3*) and repressor (*RDR1*) in *S. cerevesiae* recognize a common

cis-regulatory element (PDRE) and contribute to the expression of *PDR5* [25,31]. Transcriptional regulation of *CDR1* also appears to be the result of a combinatorial effect of positive and negative regulatory factors. Coste et al. [22] also suggested that in addition to *TAC1* other regulators of *CDR1* may exist. One such regulator, *CaNDT80*, was recently identified by Chen et al. [32]. Inactivation of *CaNDT80* did not completely abolish the drug-induced upregulation of *CDR1*, implying the involvement of other regulators which may contribute to the overexpression of *CDR1*. The *CDR1* regulatory region contains multiple upstream activating (UAS) and repressing sequences (URS), which may interact with *trans*-regulatory factors and control *CDR1* expression [7,8]. That *CDR1* expression is also under negative control is supported by transcription profiling studies with strains deleted for the global repressors *TUP1*, *MIG1*, and *NRG1*, in which *CDR1* expression was increased [33]. Our present study suggests that the NREBP is a transcriptional repressor that is involved in the control of *CDR1* expression.

Acknowledgments

Strains DSY294 and DSY296 were a kind gift from Dominique Sanglard. The work presented in this paper has been supported by grants from the Department of Biotechnology, India, to R.P. (BT/PR3825/MED/14/488(a)/ 2003), (BT/PR4862/BRB/10/360/2004), and by a grant from the European Commission, Brussels, to M.H., J.M., and R.P. (QLK-CT-2001-02377). R.P. and M.H. acknowledge financial assistance from DST-DAAD Grant (INT/DAAD/ p-79/2003) to support N.A.G. to visit Germany.

References

[1] R. Franz, S.L. Kelly, D.C. Lamb, D.E. Kelly, M. Ruhnke, J. Morschhauser, Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains, Antimicrob. Agents Chemother. 42 (1998) 3065–3072.

[2] J.L. Lopez-Ribot, R.K. McAtee, L.N. Lee, W.R. Kirkpatrick, T.C. White, D. Sanglard, T.F. Patterson, Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal Candidiasis, Antimicrob. Agents Chemother. 42 (1998) 2932–2937.

[3] R. Prasad, P.D. Worgifosse, A. Goffeau, E. Balzi, Molecular cloning and characterisation of a novel gene of *C. albicans*, *CDR1*, conferring multiple resistance to drugs and antifungals, Curr. Genet. 27 (1995) 320–329.

[4] D. Sanglard, K. Kuchler, F. Ischer, J.-L. Pagani, M. Monod, J. Bille, Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters, Antimicrob. Agents Chemother. 39 (1995) 2378–2386.

[5] T.C. White, Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlate with increased azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus, Antimicrob. Agents Chemother. 41 (1997) 1482–1487.

[6] S. Krishnamurthy, V. Gupta, R. Prasad, S.L. Panwar, R. Prasad, Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: in vitro transcriptional activation by heat shock, drugs and human steroid hormones, FEMS Microbiol. Lett. 160 (1998) 191–197.

[7] N. Puri, S. Krishnamurthy, S. Habib, S.E. Hasnain, S.K. Goswami, R. Prasad, *CDR1*, a multidrug resistance gene from *Candida albicans*, contains multiple regulatory domains in its promoter and the distal AP-1 element mediates its induction by miconazole, FEMS Microbiol. Lett. 180 (1999) 213–219.

[8] N. Karnani, N.A. Gaur, S. Jha, N. Puri, S. Krishnamurthy, S.K. Goswami, G. Mukhopadhyay, R. Prasad, SRE1 and SRE2 are two specific steroid-responsive modules of *Candida* drug resistance gene 1 (*CDR1*) promoter, Yeast 21 (2004) 219–239.

[9] M. de Micheli, J. Bille, C. Schueller, D. Sanglard, A common drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters *CDR1* and *CDR2*, two genes involved in antifungal drug resistance, Mol. Microbiol. 43 (2002) 1197–1214.

[10] D. De, X.M. Raymond, Constitutive activation of the *PDR16* promoter in a *Candida albicans* azole-resistant clinical isolate overexpressing *CDR1* and *CDR2*, Antimicrob. Agents Chemother. 48 (2004) 2700–2703.

[11] S. Wirsching, S. Michel, G. Kohler, J. Morschhauser, Activation of the multidrug resistance gene *MDR1* in fluconazole resistant, clinical *Candida albicans* strains is caused by mutations in a *trans*-regulatory factor, J. Bacteriol. 182 (2000) 400–404.

[12] M. Goto, S. Masuda, H. Saito, K. Inui, Decreased expression of P-glycoprotein during differentiation in the human intestinal cell line Caco-2, Biochem. Pharmacol. 66 (2003) 163–170.

[13] C.M. Laurencot, G.L. Scheffer, R.J. Scheper, R.H. Shoemaker, Increased LRP mRNA expression is associated with the MDR phenotype in intrinsically resistant human cancer cell lines, Int. J. Cancer 72 (1997) 1021–1026.

[14] N.A. Gaur, N. Puri, N. Karnani, G. Mukhopadhyay, S.K. Goswami, R. Prasad, Identification of a negative regulatory element which regulates basal transcription of a multidrug resistance gene *CDR1* of *Candida albicans*, FEMS Yeast Res. 4 (2004) 389–399.

[15] R. Franz, M. Ruhnke, J. Morschhauser, Molecular aspects of fluconazole resistance development in *Candida albicans*, Mycoses 42 (1999) 453–458.

[16] K. Biswas, J. Morschhauser, The Mep2p ammonium permiase controles nitrogen starvation-induced filamentous growth in *Candida albicans*, Mol. Microbiol. 56 (2005) 649–669.

[17] M.A. Uhl, A.D. Johnson, Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans*, Microbiology 147 (2001) 1189–1195.

[18] O. Reuss, A. Vik, R. Kolter, J. Morschhauser, The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*, Gene 27 (2004) 119–127.

[19] J. Morschhauser, S. Michel, J. Hacker, Expression of a chromosomally integrated, single-copy *GFP* gene in *Candida albicans*, and its use as a reporter of gene regulation, Mol. Gen. Genet. 257 (1998) 420.

[20] M. Karababa, A.T. Coste, B. Rognon, J. Bille, D. Sanglard, Comparison of gene expression profiles of *Candida albicans* azole-resistant clinical isolates and laboratory strains exposed to drugs inducing multidrug transporters, Antimicrob. Agents Chemother. 48 (2004) 3064–3079.

[21] J. Morschhauser, The genetic basis of fluconazole resistance development in *Candida albicans*, Biochem. Biophys. Acta 1587 (2002) 240–248.

[22] A.T. Coste, M. Karababa, F. Ischer, J. Bille, D. Sanglard, *TAC1*, transcriptional activator of *CDR* genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters *CDR1* and *CDR2*, Eukaryot. Cell 3 (2004) 1639–1652.

[23] S.E. Morgan, W.T. Beck, Role of an inverted CCAAT element in human topoisomerase IIalpha gene expression in ICRF-187-sensitive and -resistant CEM leukemic cells, Mol. Pharmacol. 59 (2001) 203–211.

[24] K. Asakuno, K. Kohno, T. Uchiumi, T. Kubo, S. Sato, M. Isono, M. Kuwano, Involvement of DNA binding protein, MDR-NF-1/YB-1, in human *MDR1* gene expression by actinomycin D, Biochem. Biophys. Res. Commun. 199 (1994) 1428–1435.

[25] K. Hellauer, B. Akache, S. MacPherson, E. Sirard, B. Turcotte, Zinc cluster protein Rdr1p is a transcriptional repressor of the *PDR5* gene encoding a multidrug transporter, J. Biol. Chem. 277 (2002) 17671–17676.

[26] T.C. Hallstrom, W.S. Moye-Rowley, Divergent transcriptional control of multidrug resistance genes in *Saccharomyces cerevisiae*, J. Biol. Chem. 273 (1998) 2098–2104.

[27] D.J. Katzmann, T.C. Hallstrom, Y. Mahe, W.S. Moye-Rowley, Multiple Pdr1p/Pdr3p binding sites are essential for normal expression of the ATP binding cassette transporter protein-encoding gene *PDR5*, J. Biol. Chem. 271 (1996) 23049–23054.

[28] A. Kolaczkowska, A. Goffeau, Regulation of pleiotropic drug resistance in yeast, Drug Resist. Updates 2 (1999) 403–414.

[29] A. Nawrocki, S.J. Fey, A. Goffeau, P. Roepstorff, P.M. Larsen, The effects of transcription regulating genes *PDR1*, *pdr1-3* and *PDR3* in pleiotropic drug resistance, Eur. J. Mass Spectrom. 7 (2001) 195–205.

[30] E. Carvajal, H.B. Van Den Hazel, A. Cybularz-Kolaczkowska, E. Balzi, A. Goffeau, Molecular and phenotypic characterisation of yeast *PDR1* mutants that show hyperactive transcription of various ABC multidrug transporter genes, Mol. Gen. Genet. 256 (1997) 406–415.

[31] B. Akache, B. Turcotte, New regulators of drug sensitivity in the family of yeast zinc cluster proteins, J. Biol. Chem. 277 (2002) 21254–21260.

[32] C.G. Chen, Y.L. Yang, H.I. Shih, C.L. Su, H.J. Lo, CaNdt80 is involved in drug resistance in *Candida albicans* by regulating *CDR1*, Antimicrob. Agents Chemother. 48 (2004) 4505–4512.

[33] A. Munir, A. Murad, C.D. Enfert, C. Gaillardin, H. Tournu, F. Tekaia, D. Talibi, D. Marechal, V. Marchais, J. Cottin, A.J.P. Brown, Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors CaTup1, CaMig1 and CaNrg1, Mol. Microbiol. 42 (2001) 981–993.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2008, p. 1481–1492 Vol. 52, No. 4
0066-4804/08/$08.00+0 doi:10.1128/AAC.01106-07

Transcriptional Activation and Increased mRNA Stability Contribute to Overexpression of *CDR1* in Azole-Resistant *Candida albicans*▽

Raman Manoharlal,[1] Naseem Akhtar Gaur,[1]† Sneh Lata Panwar,[2] Joachim Morschhäuser,[3] and Rajendra Prasad[1]*

Membrane Biology[1] and Yeast Molecular Genetics[2] Laboratories, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India, and Institut für Molekulare Infektionsbiologie, Universität Würzburg, Röntgenring 11, D-97070 Würzburg, Germany[3]

Received 22 August 2007/Returned for modification 23 September 2007/Accepted 2 February 2008

Many azole-resistant (AR) clinical isolates of *Candida albicans* display increased expression of the drug transporters *CDR1* and *CDR2*. In this study, we evaluate the molecular mechanisms that contribute to the maintenance of constitutively high *CDR1* transcript levels in two matched pairs of azole-susceptible (AS) and AR clinical isolates of *C. albicans*. To address this, we use reporter constructs of *GFP* and *lacZ* fused either to the *CDR1* promoter (P_{CDR1}-*GFP*/*lacZ*; transcriptional fusion) or to the *CDR1* open reading frame (P_{CDR1}-*CDR1*-*GFP*/*lacZ*; translational fusion) integrated at the native *CDR1* locus. It is observed that expression of the two reporter genes as a transcriptional fusion in the AR isolates is higher than that in matched AS isolates. However, the difference in the reporter activity between the AS and AR isolates is even greater for the translational fusions, indicating that the sequences within the *CDR1* coding region also contribute to its increased expression in AR isolates. Further analysis of these observations by transcription run-on assays demonstrated a ~5- to 7-fold difference in the transcription initiation rates for the AR isolates from those for their respective matched AS isolates. Measurement of mRNA stability showed that the half-life of *CDR1* mRNA in the AR isolates was threefold higher than that in the corresponding AS isolates. Our results demonstrate that both increased *CDR1* transcription and enhanced *CDR1* mRNA stability contribute to the overexpression of *CDR1* in AR *C. albicans* isolates.

Resistance of the human fungal pathogen *Candida albicans* to azole antifungals is often caused by increased expression of genes encoding multidrug efflux pumps, the ATP-binding cassette transporter genes *CDR1* and *CDR2* and/or the major facilitator gene Ca*MDR1* (1, 7, 23, 29, 34, 36, 37, 38, 44, 45, 46). However, the molecular mechanisms leading to the constitutive overexpression of efflux pump-encoding genes in drug-resistant, clinical *C. albicans* isolates are only beginning to be understood. In particular, the regulation of *CDR1* expression has been studied by many groups (2, 3, 4, 5, 10, 11, 17, 20, 32). Various unrelated stresses, including elevated temperature or the presence of drugs and steroids, induce a transient transcriptional activation of *CDR1* in drug-susceptible *C. albicans* strains (20). Several *cis* elements in the *CDR1* upstream region that affect basal as well as induced *CDR1* expression have been identified. Puri et al. (32) identified four upstream activating sequences and four upstream repressing sequences in the 5′ noncoding region of *CDR1*. A basal regulatory element and a negative regulatory element, in the proximal region of the promoter, have also been characterized and implicated in the basal expression of *CDR1* (10, 11). A specific steroid-responsive region in the distal part of the *CDR1* promoter, consisting of two progesterone-responsive sequences and one β-estradiol-responsive sequence, has been shown to contribute exclusively to steroid responsiveness of *CDR1* (17). Another basal expression element in the *CDR1* upstream region and a *d*rug *r*esponse *e*lement (DRE), which is present in the upstream region of *CDR1* and *CDR2*, have been identified by de Micheli et al. (5). The DRE was found to mediate both the transient upregulation of *CDR1* and *CDR2* by steroid hormones and drugs and their constitutive overexpression in a resistant strain (5).

Coste et al. identified a transcription factor, *TAC1* (*t*ranscriptional *a*ctivator of *CDR* genes), that binds to the DRE in the *CDR1* and *CDR2* promoters (3). Inactivation of *TAC1* resulted in the loss of fluphenazine-induced upregulation of *CDR1* and *CDR2*, with little impact on basal expression levels, and also abrogated the constitutive overexpression of these efflux pumps in a drug-resistant strain (3, 4, 5). CaNdt80p, a homolog of the meiosis-specific transcription factor Ndt80p of *Saccharomyces cerevisiae*, is another positive regulator of *CDR1*. Deletion of Ca*NDT80* abolished the induced expression of *CDR1* and increased the sensitivity of *C. albicans* to antifungals (2). Interestingly, the global repressor CaTup1p acts as a negative regulator of *CDR1* expression (26, 48).

Although transcriptional regulation is considered to be the key step accounting for complex basal and induced patterns of *CDR1* expression, the possibility of posttranscriptional control of *CDR1* expression, which could also affect drug resistance, still remains to be explored. The large amounts of Cdr1p, which correlate with high *CDR1* mRNA levels, in azole-resistant (AR) *C. albicans* strains not only may be due to increased

* Corresponding author. Mailing address: Jawaharlal Nehru University, School of Life Sciences, JNU Campus. New Mehrauli Road, New Delhi 110067, India. Phone: 91-11-26704509. Fax: 011-91-11-26187338. E-mail: rp47jnu@gmail.com or rp47@mail.jnu.ac.in.

† Present address: Laboratory of Gene Regulation and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892.

▽ Published ahead of print on 11 February 2008.

CDR1 transcription but could also be caused by increased stability of its mRNA and protein. It is therefore of interest to compare the following: (i) *CDR1* transcription initiation rates, (ii) *CDR1* mRNA stability, and (iii) Cdr1 protein stability in drug-susceptible and *CDR1*-overexpressing, drug-resistant *C. albicans* strains. In this study, we have addressed these issues by exploiting two pairs of matched azole-susceptible (AS) and *CDR1*-overexpressing, AR isolates. By using transcriptional and translational reporter gene fusions, transcriptional run-on (TRO), thiolutin, and cycloheximide chase assays, we demonstrate that *CDR1* overexpression in *C. albicans* is caused by an increase in its transcriptional initiation rate and by increased mRNA stability.

MATERIALS AND METHODS

Materials. Medium chemicals were obtained from HiMedia (Mumbai, India). Restriction endonuclease, DNA-modifying enzymes, ultra-pure deoxyribonucleotides (dATP, dGTP, dCTP, and dTTP) for PCR, and ribonucleotides (ATP, CTP, GTP, and UTP) for TRO were purchased from New England Biolabs (NEB, Inc.). Thiolutin (CP-4092) was a generous gift from Pfizer, Inc., Groton, CT. Radiolabeled [5,6-^{3}H]uridine, [α-^{32}P]dATP, and [α-^{32}P]UTP were obtained from Amersham Biotech and Bhabha Atomic Research Center, India. Polyclonal anti-Cdr1p antibody was custom synthesized from Covance Research Products, Inc., PA. Oligonucleotides used were commercially synthesized from Sigma-Aldrich. All molecular biology-grade chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO), and routinely used chemicals (Tris, sodium chloride, glycine, potassium acetate, sodium carbonate, magnesium chloride, sodium hydroxide pellets, methanol, glacial acetic acid, urea, sodium dodecyl sulfate [SDS], formamide, and ethanol) were obtained from Qualigens and Merck, Mumbai, India.

Bacterial and yeast strains and growth media. *Escherichia coli* DH5α was used as a host for plasmid constructions and propagation. *C. albicans* strains used in this study are listed in Table 1. *C. albicans* strains were maintained on yeast extract-peptone-dextrose (YEPD) medium. All strains were stored as frozen stocks with 15% glycerol at −80°C. Before each experiment, cells were freshly revived on YEPD plates from this stock.

Plasmid construction. All primers and plasmids used in this study are listed in Table 2. Plasmids pCPL51 and pCPG3, harboring the P_{CDR1}-*lacZ* and P_{CDR1}-*GFP* transcriptional fusions, were constructed as follows. A *CDR1* promoter fragment was first amplified from genomic DNA of isolate Gu5 with the primers CDR1F and CDR1R, digested at the introduced ApaI and XhoI sites, and substituted for the *CDR1* promoter fragment in the previously described plasmid pCPL1 (11) to generate pCPL5. A *CDR1* downstream fragment was then amplified from genomic DNA of strain SC5314 with the primers CDR29 and CDR30. The PCR product was digested at the introduced PstI and SacII sites and ligated with the PstI/SacII-digested plasmid vectors pCPL5 and pCPG1 (11) to generate pCPL51 and pCPG3, respectively. Notably, plasmid pCPG1 harbors the *CDR1* promoter isolated from SC5314 genomic DNA, which has been used in our previous studies (11). The SC5314-derived *CDR1* promoter showed sequence differences from the *CDR1* promoters in the matched AS or AR isolates (at nucleotide positions −21, −150, −171, −215, −238, −315, −368, −381, −418, and −455 relative to the transcription start point) which have been used in this study (unpublished observation). These differences, however, did not affect the β-galactosidase reporter activity of integrated cassettes derived either from pCPL1 (11), which harbors the SC5314 *CDR1* promoter, or from pCPL4 or pCPL5, which harbor the Gu4- or Gu5-derived *CDR1* promoter, respectively (data not shown). Plasmid pCPG2, which contains the *GFP* reporter gene fused in frame with the last codon of the *CDR1* open reading frame (ORF), was constructed as follows. The C-terminal region of *CDR1* was amplified from SC5314 genomic DNA with the primers CDR32 and CDR31, digested at the introduced KpnI and BamHI sites, and ligated together with a BamHI-PstI fragment from pADH1G3 (14) containing *GFP*, the *ACT1* transcription termination sequence, and the Ca*SAT1* marker into the KpnI/PstI-digested pCPL51. To generate pCPL52, in which the *lacZ* reporter gene instead of *GFP* is fused in frame to the *CDR1* ORF, the C-terminal part of *CDR1* was PCR amplified from SC5314 genomic DNA with the primers CDR32 and CT-CDR1-R-RML, digested at the introduced KpnI and XhoI sites, and ligated into the KpnI/XhoI-digested pCPL51. To ensure in-frame translational fusion of the *CDR1* ORF with *lacZ*, pCPL62 was generated, in which an additional "A" in pCPL52 before the *lacZ* ATG was removed by site-directed mutagenesis employing the A-del pCPL52-F and A-del pCPL52-R primers. All constructs were confirmed by appropriate restriction digestion analysis and by sequencing. The flanking *CDR1* sequences in all of these plasmids served for genomic integration of the reporter fusions at the native *CDR1* locus of clinical *C. albicans* isolates, and the dominant Ca*SAT1* marker (33) was used to select nourseothricin-resistant (Nou^r) transformants. Briefly, for the electroporation, 5 μl (approximately 1 μg) of the linearized DNA fragments were mixed with 40 μl of electrocompetent cells (33) and electroporated using a Bio-Rad Genepulser XL system (0.2-cm cuvette, 1.5 kV). After electroporation, *C. albicans* transformants were washed with 1 ml of 1 M sorbitol, resuspended in 1 ml YEPD medium, incubated for 3 to 4 h with shaking at 30°C prior to plating on YEPD plates containing 200 μg/ml of nourseothricin, and grown at 30°C (33). Nou^r transformants were picked up after 24 h of growth, and single-copy integration of each construct at the desired locus was confirmed by Southern hybridization with gene-specific probes (data not shown).

TABLE 1. *C. albicans* strains used

Strain	Description	Reference
Gu4	Fluconazole-susceptible clinical isolate	Franz et al. (6)
Gu4G1	P_{CDR1}-*GFP* integrated at *CDR1* locus	This study
Gu4G2	P_{CDR1}-*CDR1-GFP* integrated at *CDR1* locus	This study
Gu4L2	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
Gu4L3	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study
Gu5	Fluconazole-resistant clinical isolate	Franz et al. (6)
Gu5G1	P_{CDR1}-*GFP* integrated at *CDR1* locus	This study
Gu5G2	P_{CDR1}-*CDR1-GFP* integrated at *CDR1* locus	This study
Gu5L2	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
Gu5L3	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study
DSY294	Fluconazole-susceptible clinical isolate	Sanglard et al. (38)
DSY294G1	P_{CDR1}-*GFP* integrated at *CDR1* locus	This study
DSY294G2	P_{CDR1}-*CDR1-GFP* integrated at *CDR1* locus	This study
DSY294L2	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
DSY294L3	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study
DSY296	Fluconazole-resistant clinical isolate	Sanglard et al. (38)
DSY296G1	P_{CDR1}-*GFP* integrated at *CDR1* locus	This study
DSY296G2	P_{CDR1}-*CDR1-GFP* integrated at *CDR1* locus	This study
DSY296L2	P_{CDR1}-*lacZ* integrated at *CDR1* locus	This study
DSY296L3	P_{CDR1}-*CDR1-lacZ* integrated at *CDR1* locus	This study

Fluorescence microscopy and flow cytometry of the cells. The strains were grown overnight in YEPD liquid medium, and aliquots were spotted on microscope slides. Fluorescence microscopy was performed with a Zeiss Axiolab microscope equipped for epifluorescence microscopy with a 50-W mercury high-pressure bulb and the Zeiss fluorescein-specific filter set 09. Imaging scans were acquired with an Argon laser of 488-nm wavelength and corresponding filter settings for green fluorescent protein (GFP) and parallel transmission images. For confocal microscopy, the cells were placed on glass slides and directly viewed with a Bio-Rad confocal microscope (Radiance 2100, AGR, 3Q/BLD; Bio-Rad, United Kingdom) under a 100× oil immersion objective (41). Fluorescence-

TABLE 2. Plasmids and oligonucleotides used

Name	Description	Source (reference) or sequence[a]
Plasmids		
pBS-KS(+)	Plasmid vector used for cloning purpose	Stratagene
pACT1	Plasmid harboring *ACT1* gene	Gift from Anand Bachhawat
pSAT1	Plasmid harboring *caSAT1* marker	Reuss et al. (33)
pLACZ6	Plasmid harboring *lacZ* reporter gene	Gaur et al. (11)
pADH1G3	Plasmid harboring *GFP* reporter gene and *caSAT1* marker	Hiller et al. (14)
pCPG1	Plasmid harboring P_{CDR1}-*GFP* fusion for *ACT1* locus integration	Gaur et al. (11)
pCPG3	Plasmid harboring P_{CDR1}-*GFP* fusion for *CDR1* locus integration	This study
pCPG2	Plasmid harboring *CDR1-GFP* fusion for *CDR1* locus integration	Prasad et al. (30)
pCPL1	Plasmid harboring P_{CDR1}-*lacZ* fusion for *ACT1* locus integration	Gaur et al. (11)
pCPL51	Plasmid harboring P_{CDR1}-*lacZ* fusion for *CDR1* locus integration	This study
pCPL52	Plasmid harboring *CDR1-lacZ* fusion for *CDR1* locus integration	This study
pCPL62	Plasmid harboring in frame *CDR1-lacZ* fusion for *CDR1* locus integration	This study
Oligonucleotides		
CDR1F	Forward primer for *CDR1* promoter amplification	5′-*GATC*GGGCCCTCGTTACTCAATAAGTAT-3′
CDR1R	Reverse primer for *CDR1* promoter amplification	5′-*AGCT*CTCGAGTTCTTTTTGACCTTTTAAAG-3′
CDR1-F-RML	Forward primer for *CDR1* promoter amplification	5′-GCTTCGCCTCAACTTCTTATAAAGTTTTGAAAG-3′
CDR1-R-RML	Reverse primer for *CDR1* promoter amplification	5′-CGTGGTATTCATTAATGGAATGGTTTTCTGAAG-3′
CDR29	Forward primer for amplification of *CDR1* downstream region	5′-*AATT*CTGCAGTTTGTTTTTTGACATGGTGGTATC-3′
CDR30	Reverse primer for amplification of *CDR1* downstream region	5′-*TCGTG*CCGCGGAACACTTTTTCTTTCTAATTATAA-3′
CDR32	Forward primer for amplification of *CDR1* C-terminal region	5′-*ATTT*GGTACCATACATTAAATTTGCTGGTGGG-3′
CDR31	Reverse primer for amplification of *CDR1* C-terminal region	5′-*GTTT*GGATCCTTTCTTATTTTTTTTCTCTCTGTTACC C-3′
CT-CDR1-R-RML	Reverse primer for amplification of *CDR1* C-terminal region	5′-*GTTT*CTCGAGTTTCTTATTTTTTTTCTCTCTGTTACC C-3′
A-del pCPL52-F	Forward primer for in-frame translational fusion of CDR1-ORF with *lacZ*	5′-GAGAGAAAAAAAATAAGAAACTCGAGATGAACAT GACTGAAAAAATTCAAAC-3′
A-del pCPL52-R	Reverse primer for in-frame translational fusion of *CDR1*-ORF with *lacZ*	5′-GTTTGAATTTTTTCAGTCATGTTCATCTCGAGTTTCT TATTTTTTTTCTCTC-3′
KM1	Forward primer for amplification of *CDR1* N-terminal region	5′-CTTTTCCACTGGTAACTACT-3′
KM2	Reverse primer for amplification of *CDR1* N-terminal region	5′-CAATAAACCTGCTGACGAG-3′

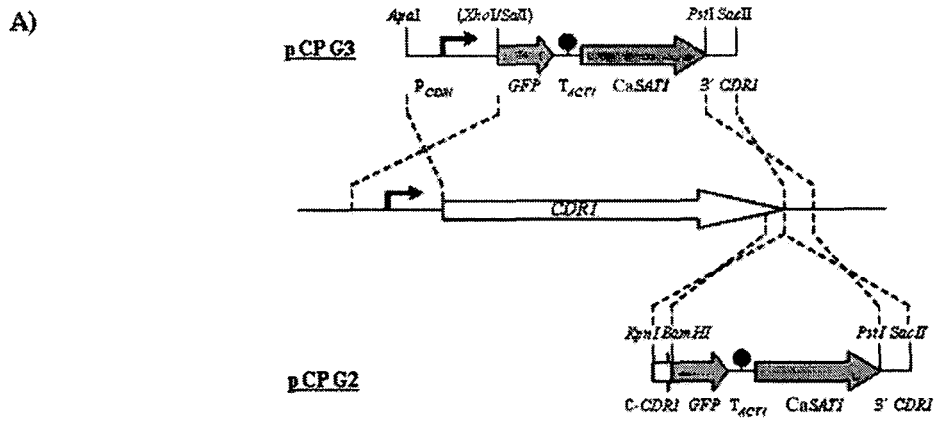
[a] Restriction sites introduced into the primers are underlined, while the flanking bases are written in italics.

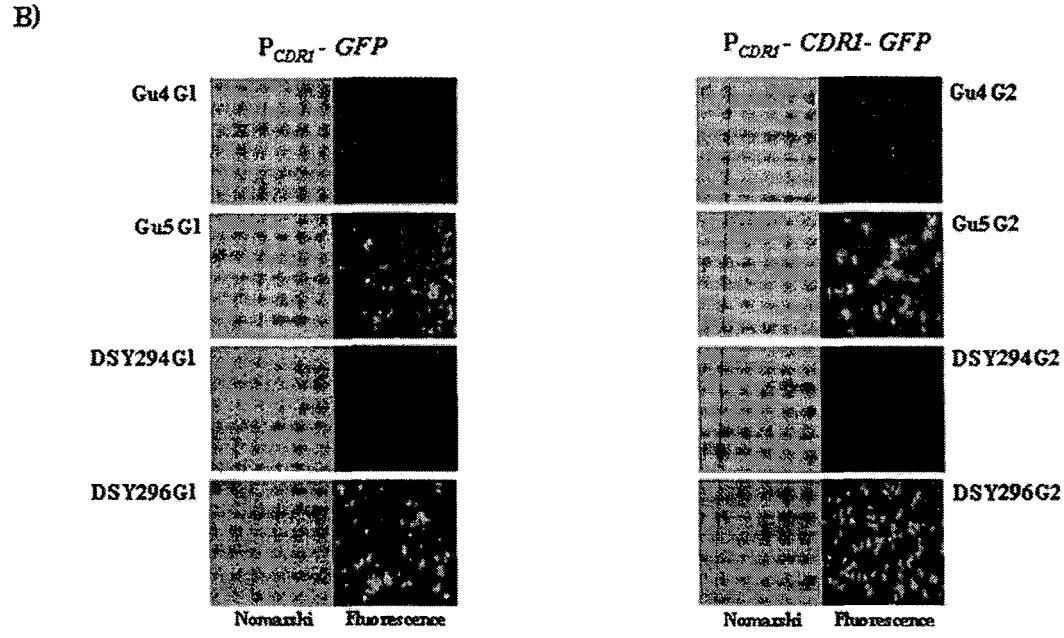
activated cell sorter (FACS) analysis was performed using a FACSCalibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA) equipped with an argon laser emitting at 488 nm. Fluorescence was measured on the FL1 fluorescence channel equipped with a 530-nm bandpass filter. A total of 20,000 events were counted at the low flow rate. Fluorescence data were collected using logarithmic amplifiers. Data analysis was performed using CellQuest software (Becton Dickinson Immunocytometry Systems). The mean fluorescence intensity was calculated using the histogram statistics program.

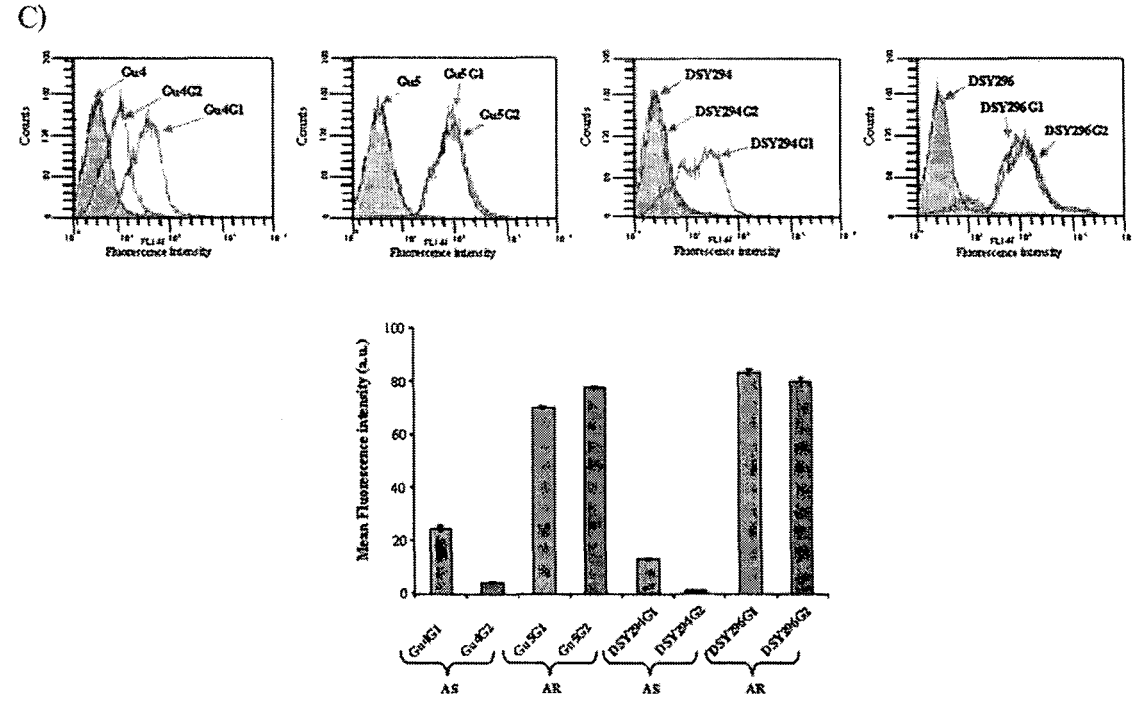
Immunodetection of GFP in AS and AR reporter strains. Purified plasma membrane (PM) and crude-extract fractions of *GFP* reporter strains were prepared as described previously (13, 41). For Western blots, membranes were incubated with a 1:5,000 dilution of monoclonal anti-GFP antibody (JL-8) (BD Biosciences, Clontech) or a 1:1,000 dilution of polyclonal anti-Pma1p (plasma membrane ATPase) antibody. Immunoreactivity was detected using horseradish peroxidase-labeled secondary antibodies at a dilution of 1:5,000 (goat antimouse antibody for GFP and goat antirabbit antibody for Pma1p) using the enhanced chemiluminescence assay system (ECL kit; Amersham Biosciences, Arlington Heights, IL).

β-Galactosidase assays. β-Galactosidase assays were performed using duplicate samples of cells from three independent experiments as described previously (11, 21, 42). β-Galactosidase activity was determined by the standard equation (11, 21, 42) and is expressed in Miller units per mg of protein (Miller units are arbitrary units): β-galactosidase activity (Miller units) = $[OD_{420} \times 1{,}000]/[OD_{600} \times t \times v]$, where t is time of reaction expressed in min, v is volume of culture used in the assay, expressed in ml, and OD_{420} and OD_{600} are optical densities at 420 and 600 nm, respectively.

TRO analysis. TRO experiments were performed as described previously (8, 24) with the following modifications. Cells were grown at 30°C in YEPD with agitation until the culture reached an OD_{600} of 1.0. An aliquot of yeast cells (6×10^8 per ml) was used to perform TRO. The cells were centrifuged for 5 min at 4,000 × *g* and resuspended in 5 ml of cold TMN (10 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$; pH 7.4). The cells were again centrifuged for 5 min at 4,000 × *g*, and the cell pellet was resuspended in 900 μl of sterile cold diethyl pyrocarbonate (DEPC)-treated water. Next, the cell suspension was transferred to a fresh microcentrifuge tube containing 50 μl of 10% *N*-lauryl sarcosine sodium sulfate (sarkosyl) and was incubated for 20 min on ice. After the permeabilization step, cells were recovered by low-speed centrifugation at 6,000 rpm for 2 min at 4°C and the supernatant was removed. In vivo transcription was reinitiated by resuspending the permeabilized cell fraction in 120 μl of 2.5× transcription buffer (50 mM Tris-HCl [pH 7.7], 500 mM KCl, 80 mM $MgCl_2$), 16 μl of AGC mix (10 mM







each of ATP, GTP, and CTP), 6 μl of dithiothreitol (0.1 M), 1 U of RNase inhibitor per μl, 10 mM phosphocreatine, 1.2 μg of creatine phosphokinase per μl, and 15 μl of [α-^{32}P]UTP (3,000 Ci/mmol, 10 μCi/μl). Cells were maintained on ice at all times. The final volume was adjusted to 300 μl with DEPC-treated water, and the mix was incubated for 15 min at 30°C to allow transcription elongation. The reaction was stopped by adding 1 ml of ice-cold DEPC-treated water to the mix. Cells were recovered by centrifugation to remove nonincorporated radioactive nucleotides. Total RNA was isolated using the Trizole reagent (Sigma) as per the manufacturer's specifications except that 200 μl of ice-cold acid-washed 0.4- to 0.6-mm-diameter glass beads (Sigma, St. Louis, MO) were also used for efficient and complete lysis of permeabilized cells. Isolated total labeled RNA was again precipitated by adding 2.5 M NH_4 acetate and an equal volume of isopropanol. The mixture was stored overnight at −20°C. To pellet the RNA, tubes were centrifuged at 14,000 rpm for 15 min in the microcentrifuge. The isopropanol was removed, and the labeled RNA pellet was washed twice with 70% ethanol, dried, and resuspended in 100 μl of DEPC-treated water. This double precipitation of RNA was used to minimize DNA contamination. Total extracted RNA was spectrophotometrically quantified. An aliquot was used for specific radioactivity determination in a Tri-CARB 2900 TR liquid scintillation analyzer (Packard instrument Co., Inc.). All of the in vivo-labeled RNA of each isolate (~2×10^6 to 2.5×10^6 cpm) was subsequently used for reverse Northern hybridization with a dot blotted Nylon membrane (Hybond-N$^+$; Amersham Pharmacia Biotech) containing PCR-amplified gene-specific N-terminal *CDR1* sequences (nucleotides −242 to +256 from the transcription start point), plasmid pACT1 (positive control), and pBlueScript-KS(+) (negative control) as probes, as per the manufacturer's recommendation. Northern blots were scanned with a phosphorimager scanner (FLA-5000 Fuji phosphorimager). Signal intensities of hybridized nuclear RNA were quantified and subsequently normalized to the actin intensities using densitometry scanning.

Thiolutin chase assay. In order to measure the *CDR1* mRNA half-life, a potent in vivo transcriptional inhibitor of *C. albicans*, thiolutin, was used (18, 40). AS and AR isolates of *C. albicans* were incubated with an optimized concentration (40 μg/ml) of thiolutin (data not shown). For this purpose, cultures were treated with 150 μM of the copper chelator bathocuprioinedisulphonic acid and incubated at 30°C for an additional 10 min at 200 rpm. Transcription was subsequently shut off by the addition of 150 nM of $CuSO_4$ and 40 μg/ml of thiolutin. Addition of bathocuprioinedisulphonic acid and $CuSO_4$ was found to enhance the response of the cells to thiolutin (40). Briefly, 100 ml of cells were grown to an OD_{600} of 1.0 at 30°C. Aliquots of cells were taken at the indicated times after transcriptional shutoff. Total RNA was isolated using Ambion's RiboPure-Yeast RNA isolation kit (catalog no. 1926) as per the manufacturer's instructions. For Northern blots, approximately 20 μg of total RNA from the above samples was hybridized with probes derived from gene-specific sequences of the *CDR1* gene. Hybridization signal intensity was quantified with a phosphorimager and was normalized to the band intensity at time T_0 and plotted as a line graph.

Cycloheximide chase assay. Cycloheximide chase assays were performed as described earlier (9) with certain modifications that included the use of an optimized concentration of cycloheximide (16) (data not shown) and the alkaline extraction procedure used for the preparation of crude protein extract (13). Briefly, aliquots of mid-log-phase-grown cells were withdrawn for analysis after translational shutoff at the indicated times and lysed in solution containing 1.85 M NaOH and 7.5% β-mercaptoethanol. Crude proteins isolated were precipitated with 50% trichloroacetic acid and sedimented. The sediment was resuspended in loading buffer (40 mM Tris-HCl [pH 6.8], 8 M urea, 5% SDS, 0.1 M EDTA, 1% β-mercaptoethanol, and 0.1 mg/ml bromophenol blue) and incubated at 37°C for 10 min. Nonsolubilized material was cleared by a centrifugation step, and solubilized proteins (approximately 20 and 30 μg for AR and AS isolates, respectively) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane for Western blotting. Immunodetected Cdr1p signal intensity was quantified with a phosphorimager and was normalized to the band intensity at time T_0 and plotted as a line graph.

RESULTS

Expression of transcriptional and translational *GFP* reporter fusions in AS and AR isolates. To analyze the molecular basis of *CDR1* upregulation in azole-resistant, clinical *C. albicans* isolates in more detail, we employed two matched pairs of AS and AR isolates. The resistant isolates Gu5 and DSY296, which were obtained after fluconazole therapy for oropharyngeal candidiasis in two different human immunodeficiency virus-positive patients, were shown by DNA fingerprinting to be highly related to the susceptible isolates Gu4 and DSY294, respectively, which were recovered from earlier infection episodes in the same patients (6, 38). It was recently shown that a mutation in the transcription factor *TAC1* is responsible for *CDR1* and *CDR2* upregulation in DSY296 (4). However, it has not yet been explored if other mechanisms contribute to the overexpression of the efflux pumps in these isolates.

We compared the expression of two different *GFP* reporter fusions in these isolates, one in which *GFP* was expressed from the *CDR1* promoter (P_{CDR1}-*GFP*) and another where *GFP* was fused in frame to the last codon of the *CDR1* ORF and expressed from the *CDR1* promoter (P_{CDR1}-*CDR1-GFP*) (see Materials and Methods) (Fig. 1A). The reporter fusions were integrated at the native *CDR1* locus, and two transformants of each of the four parental strains were used for further analysis. The reporter strains were designated Gu4G1 (P_{CDR1}-*GFP*) and Gu4G2 (P_{CDR1}-*CDR1-GFP*); Gu5G1 (P_{CDR1}-*GFP*) and Gu5G2 (P_{CDR1}-*CDR1-GFP*); DSY294G1 (P_{CDR1}-*GFP*) and DSY294G2 (P_{CDR1}-*CDR1-GFP*); and DSY296G1 (P_{CDR1}-*GFP*) and DSY296G2 (P_{CDR1}-*CDR1-GFP*).

Expression of the P_{CDR1}-*GFP* transcriptional and P_{CDR1}-*CDR1-GFP* translational fusions in cells grown to mid-exponential phase (OD_{600} of ~1.0) was detected by epifluorescence microscopy (Fig. 1B) and quantified by FACS analysis (Fig. 1C). As expected, the fluorescence intensities of the AR reporter fusion strains were higher than those of the corresponding AS strains (2.5-fold for Gu5G1 versus Gu4G1, 19-fold for Gu5G2 versus Gu4G2; 6-fold for DSY296G1 versus DSY294G1; and 80-fold for DSY296G2 versus DSY294G2), confirming the previously reported increased *CDR1* transcript and Cdr1p protein levels in the AR isolates (4, 6, 38). Inter-

FIG. 1. Schematic depiction of *GFP* reporter fusion integrants and their expression in AS and AR isolates. (A) Structure of the DNA cassettes which were used to integrate the transcriptional (P_{CDR1}-*GFP*, top) and translational (P_{CDR1}-*CDR1-GFP*, bottom) *GFP* reporter fusions into the *CDR1* locus of the clinical *C. albicans* isolates (middle). The *CDR1* and *GFP* coding regions are represented by white and green arrows, respectively, the *CaSAT1* marker by the gray arrow, and the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the filled circle. *CDR1* upstream and downstream regions are represented by solid lines, and the *CDR1* promoter (P_{CDR1}) is symbolized by the bent arrow. Only relevant restriction sites are shown. (B) Nomarski and corresponding fluorescence micrographs of transformants containing the chromosomally integrated P_{CDR1}-*GFP* (left) or P_{CDR1}-*CDR1-GFP* (right) reporter fusion. (C) Cells of the reporter strains grown to exponential phase in YEPD medium were diluted to a density of 2×10^7 cells per ml in phosphate-buffered saline (pH 7.0), and a total of 20,000 events were analyzed by flow cytometry. The parental strains of the transformants, which do not contain GFP, were used as a negative control. The mean fluorescence intensity is shown for each population of cells (bottom panel) after normalization with values for their corresponding negative controls. Since the normalized mean fluorescence intensity of DSY294G2 was a negative value, we designated it "1.0'" for calculating the degree of change for this particular strain. a.u., arbitrary units.

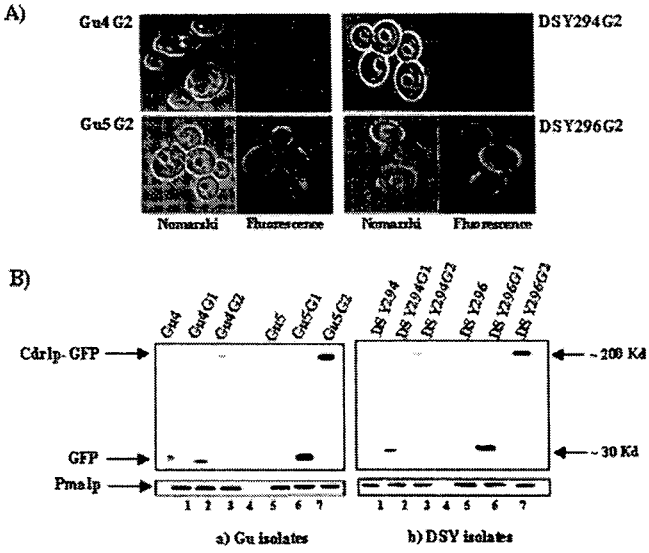


FIG. 2. Localization of Cdr1p and immunodetection of GFP in reporter fusion transformants. (A) Nomarski (left) and corresponding confocal (right) pictures of the transformants harboring the chromosomally integrated P_{CDR1}-*CDR1-GFP* (translational fusion) reporter construct are shown which indicate the proper plasma membrane localization of chimeric Cdr1p in clinical *C. albicans* isolates. The cells were viewed directly on a glass slide with a 100× oil immersion objective. (B) The Western blot analyses were done with an anti-GFP monoclonal antibody on both the transcriptional and translational fusion integrants. Equal loading of protein was assessed by using an anti-Pma1p polyclonal antibody.

estingly, however, expression of the P_{CDR1}-*CDR1-GFP* translational fusion resulted in much lower fluorescence than expression of the P_{CDR1}-*GFP* transcriptional fusion in AS isolates (6-fold for Gu4G2 versus Gu4G1 and 13-fold for DSY294G2 versus DSY294G1), whereas the two types of reporter fusions produced comparable fluorescence in AR isolates. Notably, confocal microscopy confirmed that the Cdr1p-GFP fusion protein was correctly localized to the cell membrane in all reporter strains expressing the translational fusion (Fig. 2A). Immunoreactive bands of the expected sizes were observed in whole-cell extracts and plasma membrane preparations of the P_{CDR1}-*GFP* and P_{CDR1}-*CDR1-GFP* reporter strains, respectively, after Western immunoblotting with an anti-GFP antibody (Fig. 2B). Additionally, the tagging of P_{CDR1} and P_{CDR1}-*CDR1* with *GFP* did not alter the drug resistance profiles of AS and AR isolates, which ruled out that the *GFP* fusions caused any selective impact on Cdr1p functionality for either AS or AR isolates (data not shown).

Expression of transcriptional and translational *lacZ* reporter fusions in AS and AR isolates. To rule out that the reduced expression of the P_{CDR1}-*CDR1-GFP* translational fusion in AS isolates was an artifact intrinsic to the Cdr1p-GFP fusion protein, we used codon-optimized *lacZ* (42) as an alternative reporter gene. As for *GFP*, transcriptional (P_{CDR1}-*lacZ*) and translational (P_{CDR1}-*CDR1-lacZ*) *lacZ* reporter fusions were generated and integrated at the native *CDR1* locus of the AS and AR isolates (see Materials and Methods) (Fig. 3A). The expression of Cdr1p in the P_{CDR1}-*CDR1-lacZ* construct was unaffected by its fusion to *lacZ* as tested by Western blotting with an anti-Cdr1p antibody (data not shown). Two

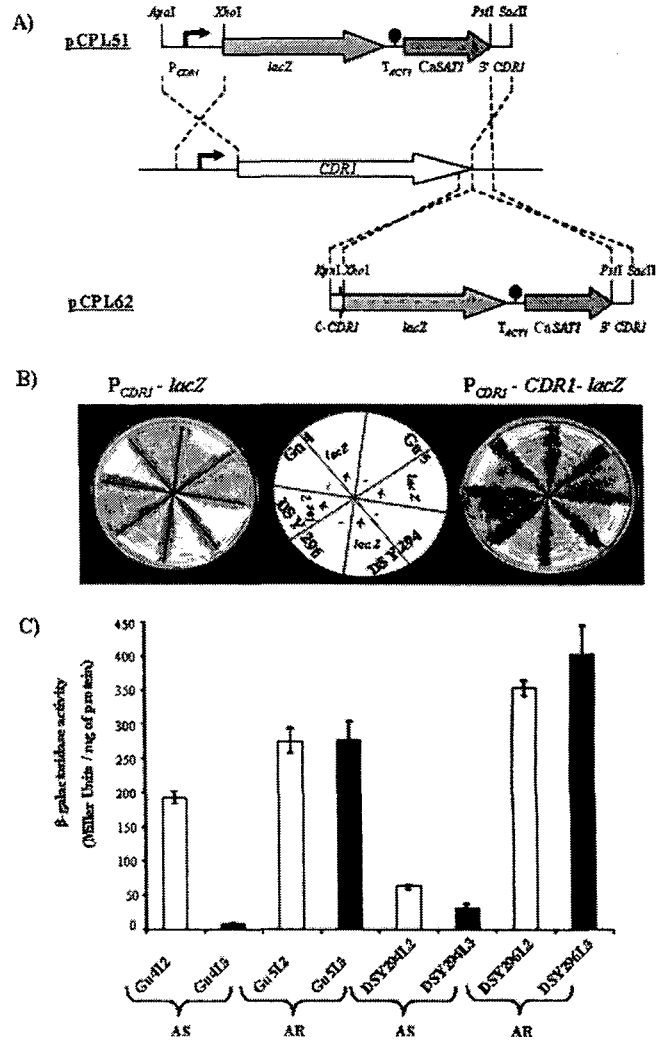


FIG. 3. Schematic depiction of *lacZ* reporter fusion integrants and qualitative and quantitative assay of β-galactosidase activity in AS and AR isolates. (A) Structure of the DNA cassettes which were used to integrate the transcriptional (P_{CDR1}-*lacZ*, top) and translational (P_{CDR1}-CDR1-*lacZ*, bottom) *lacZ* reporter fusions into the *CDR1* locus of the clinical *C. albicans* isolates (middle). The *CDR1* and *lacZ* coding regions are represented by white and blue arrows, respectively, the Ca*SAT1* marker by the gray arrow, and the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the filled circle. *CDR1* upstream and downstream regions are represented by solid lines, and the *CDR1* promoter (P_{CDR1}) is symbolized by the bent arrow. Only relevant restriction sites are shown. (B) Transformants harboring chromosomally integrated P_{CDR1}-*lacZ* (transcriptional fusion, left) and P_{CDR1}-*CDR1-lacZ* (translational fusion, right) and their corresponding parental strain (without *lacZ*) were streaked on minimal medium plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and photographed after 3 days' growth at 30°C. The positions of the individual strains on the plates are shown in the scheme (middle). (C) β-Galactosidase quantitative reporter activities of each transformant were determined as described previously (11, 21, 42). The values are means ± standard deviations (indicated by the bars) of three independent experiments with duplicate measurements of two independent clones. Empty and filled bars indicate transcriptional (P_{CDR1}-*lacZ*) and translational fusion (P_{CDR1}-*CDR1-lacZ*) transformants in both AS and AR backgrounds.

transformants of each parental strain containing a single copy of the reporter fusion were used for further analysis. The reporter strains were designated Gu4L2 (P_{CDR1}-*lacZ*) and Gu4L3 (P_{CDR1}-*CDR1-lacZ*); Gu5L2 (P_{CDR1}-*lacZ*) and Gu5L3

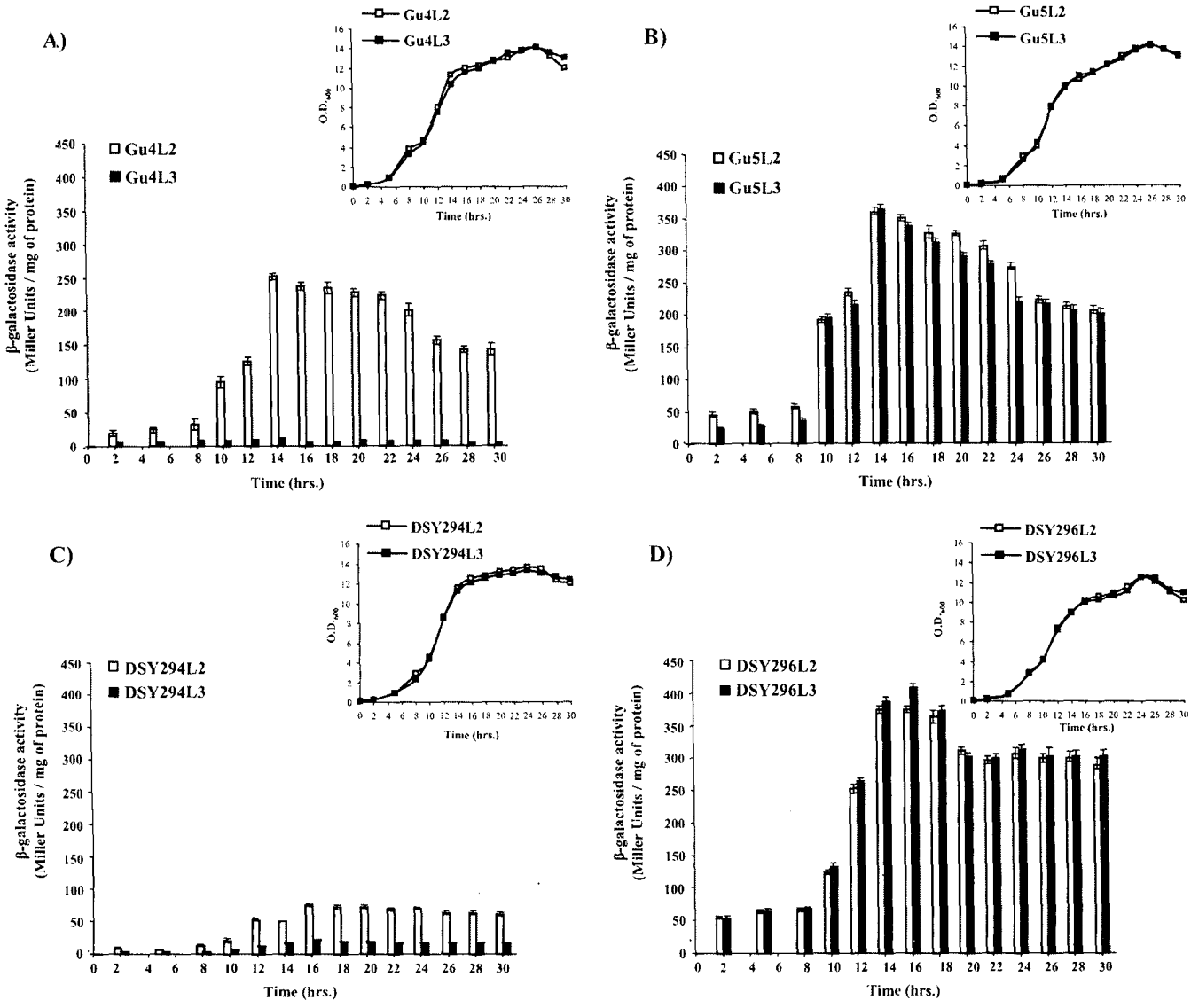


FIG. 4. β-Galactosidase reporter activity of *lacZ* reporter fusion integrants of AS and AR isolates during growth phase. Transcriptional fusion (P_{CDR1}-*lacZ*) and translational fusion (P_{CDR1}-*CDR1-lacZ*) reporter transformants of each isolates were grown from an initial OD_{600} of 0.1 in YEPD broth and withdrawn at the indicated time points of growth for β-galactosidase reporter activity (Fig. 4A, B, C, and D). The inset depicts growth curves of the P_{CDR1}-*lacZ* (□) and P_{CDR1}-*CDR1-lacZ* (■) reporter transformants in AS and AR isolates. The negative-control parental strain (without *lacZ* fusion constructs) reporter activity value was always below 0.5 Miller units, and it was subtracted from the reporter activity of each corresponding transcriptional and translational fusion transformant. The values are means ± standard deviations (indicated by the bars) for three independent experiments with duplicate measurements of two independent clones. Gu4 transformants (A), Gu5 transformants (B), DSY294 transformants (C), and DSY296 transformants (D) were analyzed. Empty and filled bars indicate transcriptional (P_{CDR1}-*lacZ*) and translational fusion (P_{CDR1}-*CDR1-lacZ*) transformants in both AS and AR backgrounds.

(P_{CDR1}-*CDR1-lacZ*); DSY294L2 (P_{CDR1}-*lacZ*) and DSY294L3 (P_{CDR1}-*CDR1-lacZ*); and DSY296L2 (P_{CDR1}-*lacZ*) and DSY296L3 (P_{CDR1}-*CDR1-lacZ*).

Expression of the *lacZ* reporter gene in various strains was assessed by comparing the intensity of the blue color produced by cells grown on agar plates containing the indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Fig. 3B) and was quantified by determining β-galactosidase activities in liquid assays (Fig. 3C). The *lacZ* reporter study confirmed the results obtained with *GFP*. Higher *lacZ* expression levels were observed in transformants of the AR isolates than in transformants of the AS isolates (1.4-fold for Gu5L2 versus Gu4L2, 37-fold for Gu5L3 versus Gu4L3, 5.6-fold for DSY296L2 versus DSY294L2, and 13-fold for DSY296L3 versus DSY294L3). In addition, while the transcriptional and translational fusions yielded comparable activities in the AR isolates, expression of the translational fusion was much lower than that of the transcriptional fusion in the AS isolates (26-fold for Gu4L3 versus Gu4L2 and 2-fold for DSY294L3 versus DSY294L2). Of note, the integration of P_{CDR1}-*lacZ* reporter fusion constructs at the ectopic *ACT1* locus resulted in β-galactosidase activity comparable to that of native *CDR1* locus integrants (data not shown).

Growth phase versus β-galactosidase reporter activity. To investigate whether the observed differences in the expression

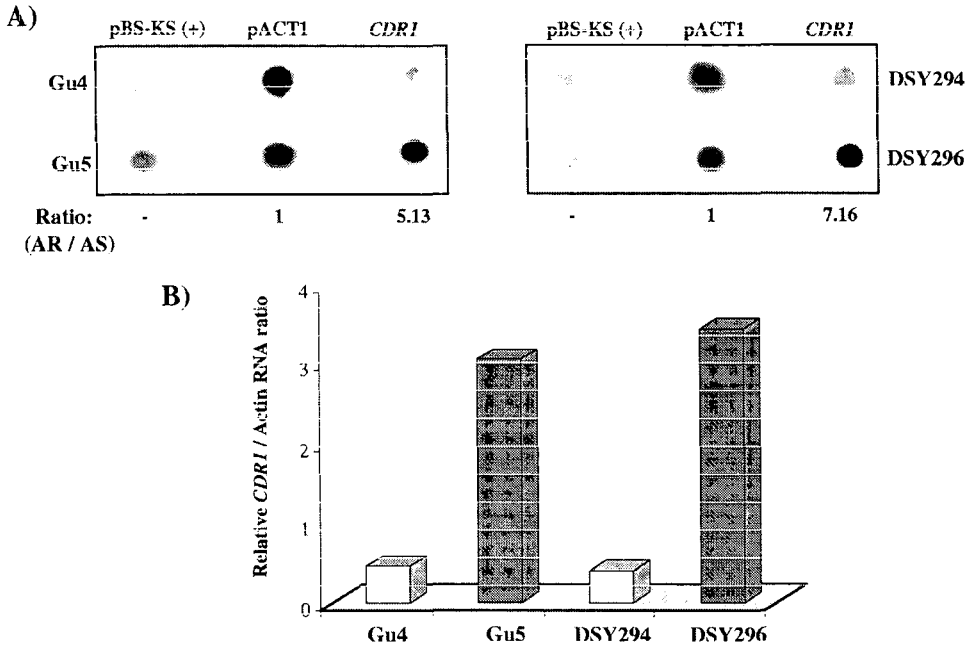


FIG. 5. TRO analysis of AS and AR isolates. (A) Approximately 2 μg (each) of *CDR1*, pACT1, and empty vector pBlueScript-KS(+) DNA was blotted and immobilized on charged nylon membranes (Hybond-N$^+$; Amersham Pharmacia Biotech) using a dot blot assembly apparatus. The blots were probed with total labeled nuclear run-on RNA as described in Materials and Methods. Hybridization signal intensities of nuclear RNA were quantified using densitometry scanning of phosphorimages. DNA from a pBlueScript-KS(+) plasmid was used as a negative control for nonspecific binding of nuclear RNA to a random DNA fragment. Signal intensities for each isolate were subtracted from the negative control values and subsequently normalized to the intensity corresponding to their AS isolate. The AR/AS ratio is the normalized nuclear RNA intensity between AR and AS isolates. (B) The relative intensity of *CDR1* with respect to actin RNA of each isolate is plotted.

of transcriptional and translational reporter gene fusions in AS and AR isolates depended on the growth phase, we quantitatively monitored β-galactosidase activities in the *lacZ* reporter strains at various times during growth in batch cultures. As can be seen in Fig. 4, the low reporter expression levels of the translational P_{CDR1}-*CDR1-lacZ* fusion compared with those of the transcriptional P_{CDR1}-*lacZ* fusion in the AS isolates were observed at all growth stages (Fig. 4A and C). In contrast, both types of reporter fusion produced comparable β-galactosidase activities in the AR isolates throughout growth (Fig. 4B and D).

Taken together, exploitation of reporter fusions and their expression analysis indicated that an increase in *CDR1* expression levels in the AR isolates compared to those in the corresponding AS isolates is contributed by affecting either *CDR1* promoter activity, mRNA stability, translational efficiency, or protein stability. Therefore, we performed further experiments on native *CDR1* (endogenous gene) to get a real insight into whether transcriptional/posttranscriptional control mechanisms are involved in the upregulation of *CDR1* expression in AR isolates.

Transcriptional rate for *CDR1* is increased in AR isolates. We first tested whether the transcription rate for *CDR1* was elevated in the AR isolates. For this purpose, TRO assays were performed. Both AS and AR isolates were grown to an OD_{600} of ~1.0, and the cells were permeabilized with the detergent *N*-lauryl sarcosine sodium sulfate (sarkosyl) for the isolation of intact nuclei (8, 24). The subsequent incubation of isolated nuclei with transcription buffer and radiolabeled [α-^{32}P]UTP reinitiated the transcription (see Materials and Methods). The in vivo-labeled nascent RNAs were then used as probes in reverse Northern hybridizations with dot blotted *CDR1*-specific PCR-amplified DNA. As controls, pACT1 plasmid DNA, containing the constitutively expressed *ACT1* gene, and the empty vector pBluescript were also dotted on the membranes. As shown in Fig. 5A and B, the AR isolates exhibited an increased rate of transcription of *CDR1* compared with that for the AS isolates (fivefold for Gu5 versus Gu4 and sevenfold for DSY296 versus DSY294).

***CDR1* mRNA stability is increased in AR isolates.** To investigate if in addition to the increased transcription rates posttranscriptional events also contribute to the higher level of *CDR1* expression in drug-resistant strains, we determined *CDR1* mRNA stability in the AS and AR isolates. To this end, we exploited an effective sulfur-containing purine analogue, thiolutin, as a potent inhibitor of de novo transcription to determine mRNA stability in *C. albicans* (18, 40). Thiolutin affected [^{3}H]uridine incorporation into nascent RNAs in a concentration-dependent manner. About 40 μg/ml of thiolutin inhibited ~95% of the [^{3}H]uridine incorporation in total RNA (data not shown). Methylene blue staining revealed no decline in cell viability of AS and AR isolates treated with 40 μg/ml thiolutin, although growth was inhibited to a certain extent (data not shown). This optimized thiolutin concentration was subsequently used for the mRNA chase assays. Total RNA was isolated at different time points after transcriptional inhibition with thiolutin and analyzed by RNA gel blots (Fig. 6A). After probing the blots with a *CDR1*-specific probe, hybridization signals were quantified by densitometry scanning in a phosphorimager. Figure 6B depicts a typical *CDR1* mRNA decay profile

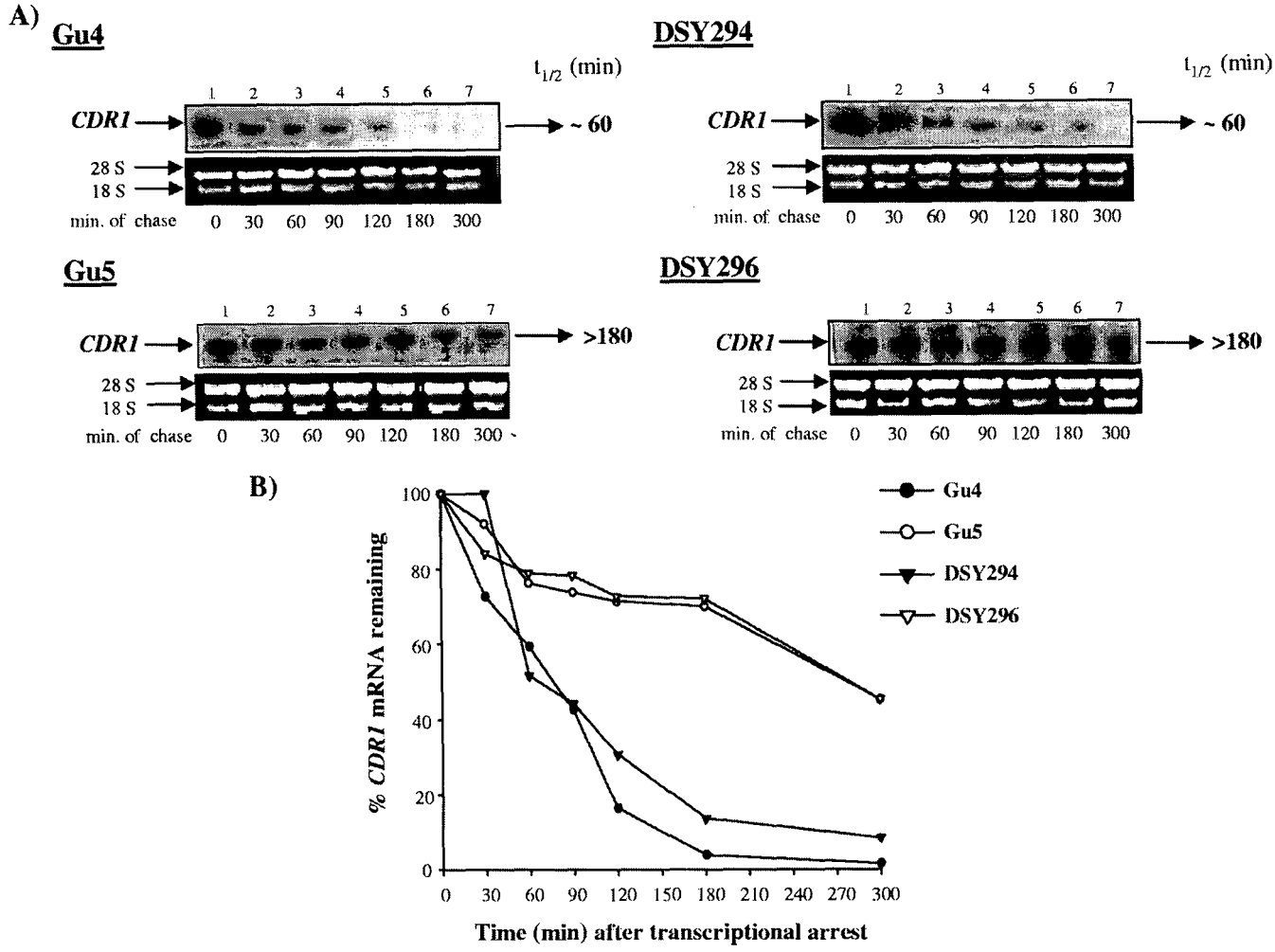


FIG. 6. *CDR1* mRNA decay assay. Exponentially growing cultures of *C. albicans* were incubated with the optimized thiolutin concentration (40 μg/ml) to inhibit ongoing in vivo transcription. Total RNA was isolated at the indicated times thereafter and fractionated on a 1% (wt/vol) agarose–2.2 M formaldehyde denaturing gel. (A) The gel was stained with ethidium bromide before blotting to monitor equal loading of the RNA and subsequently blotted onto a charged nylon membrane. The blot was hybridized with a *CDR1*-specific probe. Time points in minutes are indicated below each phosphorimage. (B) The hybridization signals were quantified using densitometry scanning in a phosphorimager. The signal intensity at each time point was normalized to that of time T_0 (expressed as a percentage) and plotted as described in Materials and Methods. $t_{1/2}$, half-life.

in the AS and AR isolates over a 300-min period from one of these experiments. *CDR1* mRNA could be detected in both AS isolates Gu4 and DSY294 at time T_0, and the signal intensity diminished progressively with time (mRNA half-life was approximately 60 min). The turnover of the *CDR1* transcript occurred much more slowly in the AR isolates Gu5 and DSY296, with a half-life of >180 min. These results demonstrated that *CDR1* mRNA stability was increased in the AR isolates over that in the AS isolates.

Cdr1 protein stability does not differ in AS and AR isolates. To test whether increased protein stability might also contribute to the high Cdr1p levels in AR isolates, cycloheximide chase assays were performed. Total crude protein extracts were isolated at different times after treatment of the cells with an optimized concentration (75 mM) of cycloheximide (16) and analyzed by Western immunoblotting with a rabbit polyclonal anti-Cdr1p antibody. Figure 7A shows the Western blot of the decay experiment, while Fig. 7B shows the quantitative decay profile. The half-life of Cdr1p was similar in AS and AR isolates and was calculated to be approximately 90 min.

DISCUSSION

In this study, we used two pairs of matched AS and AR *C. albicans* clinical isolates to study the mechanisms of *CDR1* overexpression in AR isolates. Our results demonstrate that both increased transcriptional activation and enhanced mRNA stability contribute to increased *CDR1* expression in these drug-resistant isolates. Interestingly, we found that in the AS isolates reporter fusions with the *CDR1* coding region were expressed at lower levels than fusions in which the reporter genes were directly fused to the *CDR1* promoter, whereas in the AR isolates the two types of reporter fusions were expressed at comparable levels. This would mean that sequences in the *CDR1* coding region can also contribute to the increased *CDR1* expression in AR isolates.

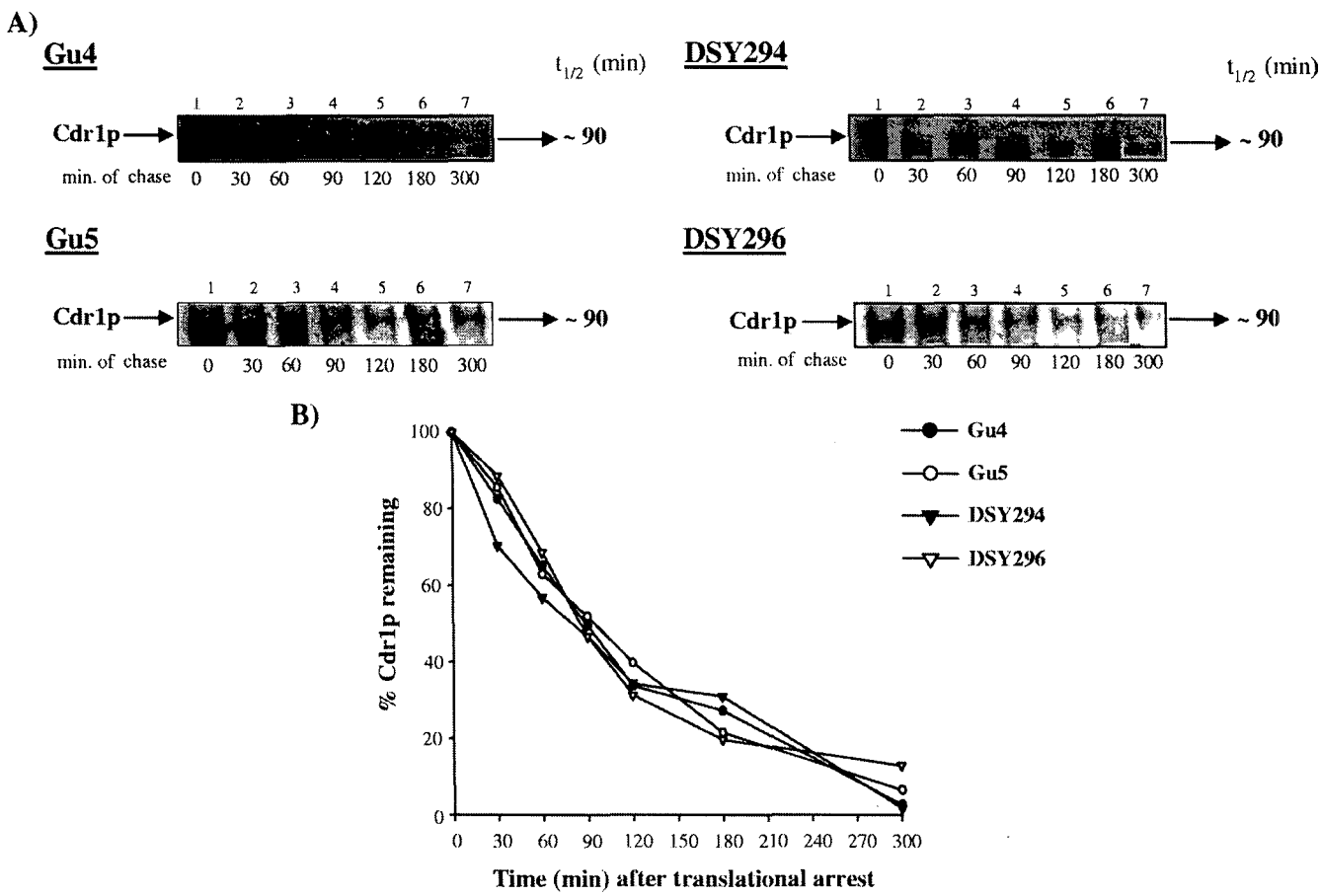


FIG. 7. Cdr1p decay assay. (A) Exponentially grown cultures of *C. albicans* were translationally halted at 30°C by addition of 75 mM of cycloheximide for 1 h. Whole-cell extracts were prepared at the indicated times after cycloheximide treatment. For AR isolates, ~20 μg, and for AS isolates, ~30 μg (because of relatively low expression of Cdr1p) of crude extract for each time was loaded and separated by SDS-polyacrylamide gel electrophoresis. Equal loading of protein was assessed using a Coomassie-stained gel (data not shown). Cdr1p was detected using a polyclonal anti-Cdr1p antibody. The Cdr1p-specific bands were subsequently quantified by densitometry scanning in a phosphorimager. (B) Band intensities (represented as percentages of the value at T_0) for each isolate were plotted against the chased time. $t_{1/2}$, half-life.

It has been shown previously that *CDR1* overexpression in *C. albicans* is caused by an increased *CDR1* transcription rate in AR isolates compared with that in AS isolates (24). Our TRO experiments confirmed that the transcriptional initiation rate from the *CDR1* promoter was five- to sevenfold higher in the AR isolates than in the AS isolates used in the present study (Fig. 5). The *CDR1* upstream region contains many sequence elements which are involved in the regulation of *CDR1* expression (5, 10, 11, 17, 32); however, no sequence differences were found in the *CDR1* upstream region of these matched pairs of AS and AR isolates (5, 11; also unpublished observations). In line with this, it has recently been shown that a gain-of-function mutation in the transcription factor *TAC1*, which controls *CDR1* expression, causes *CDR1* upregulation in the AR isolate DSY296 (4).

In order to evaluate if, in addition to transcriptional activation of *CDR1*, differential mRNA and protein stability also contribute to the enhanced Cdr1p levels in AR isolates, we performed thiolutin and cycloheximide chase assays and observed that the up-regulation of *CDR1* mRNA in AR isolates was due to an increase in the mRNA half-life (>180 min), which was approximately threefold greater than that in AS isolates (Fig. 6). In contrast, no difference in Cdr1p protein stability was observed between AS and AR isolates (Fig. 7). There are examples in other organisms where overexpression of efflux pumps can be caused by increased mRNA stability. An increase in the mRNA half-life of *MDR1* (a *CDR1* homologue in humans) has been shown to contribute to doxorubicin and colchicine resistance in the myelogenous leukemic cell line K562 (47). An enhanced mRNA stability of *bmr3*, encoding a multidrug transporter, also leads to a multidrug-resistant (MDR) phenotype in *Bacillus subtilis* (28). In addition, the reported MDR phenotype of *Entamoeba histolytica* trophozoites is also caused by transcriptional activation (27), as well as an increase in mRNA stability of the Eh*Pgp5* gene (22).

Notably, though, *cis* determinants located in the 3′ untranslated region (UTR) regulate the degradation of mRNA (35). Among these *cis* elements, adenylate-uridylate-rich-element motifs of the 3′ UTR involved in destabilization of their corresponding mRNAs are of prime importance (22, 31, 35). Several reports have also suggested a relationship between the relative affinity of a given RNA for RNA-binding protein(s) and the stability of an mRNA containing these sequences (31, 35). Our preliminary results reveal that the *CDR1* 3′ UTR is

~78% AU rich and also possesses several putative consensus binding sequences for a regulatory RNA-binding protein(s). Therefore, any contribution of *CDR1* 3′ UTR *cis* elements and of the mutation or alteration in *trans*-acting regulatory factor(s) corresponding to these conserved elements in determining mRNA stability between AS and AR isolates requires an in-depth analysis.

Our results with the reporter fusion transformants also suggest that sequences in the *CDR1* coding region could also be an important contributor for increased *CDR1* expression in AR isolates. In this context, it should be mentioned that synonymous and nonsynonymous nucleotide polymorphisms have been observed in the *CDR1* coding region, but so far none of these has been linked to *CDR1* overexpression (12, 15). Our present study did not consider the role of these allelic differences in sustained overexpression of *CDR1* in AR isolates. However, a recent study has reported that a silent polymorphism does not influence human *P-gp/MDR1* mRNA and protein expression but affects posttranslational events in terms of timing of cotranslational folding and membrane insertion (19, 43).

In conclusion, our results demonstrate for the first time that *CDR1* is regulated by both transcriptional and posttranscriptional events. Our finding that the acquisition of azole resistance involves transcriptional activation as well as decreased mRNA turnover opens up new possibilities for treatment regimes to circumvent MDR in *C. albicans*. In this context, it is worth mentioning that the intervention of overexpressing *MDR1* in MDR cell lines by verapamil (25) and ecteinascidin 743 (39) has been reported to be due to the transcriptional down-regulation of the gene.

ACKNOWLEDGMENTS

We thank Dominique Sanglard for providing the *C. albicans* isolates DSY294 and DSY296. Thiolutin (CP-4092) was a generous gift from Pfizer, Inc. (Groton, CT). We also acknowledge R. Serrano for the PM-ATPase antibody. We especially thank K. Natarajan for his valuable suggestions in improving the manuscript.

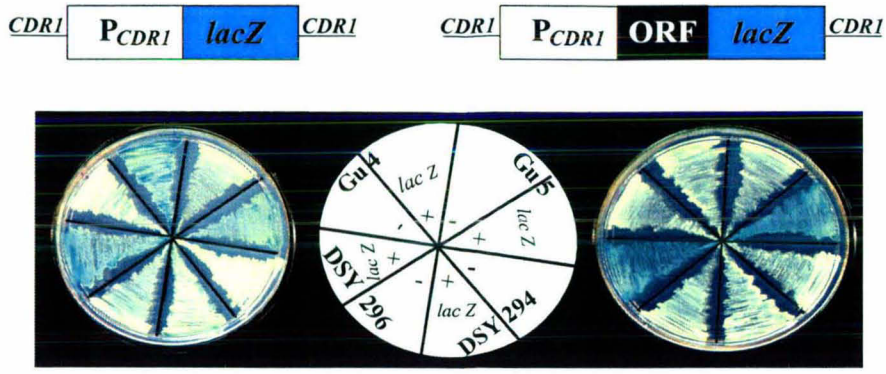
The work presented in this paper has been supported in part by grants to R.P. from the Department of Biotechnology, India [BT/PR3825/MED/14/488(a)/2003 and BT/PR4862/BRB/10/360/2004], the Council of Scientific and Industrial Research [38(1122)/06/EMR-II], Department of Science Technology (SR/SO/BB-12/2004), Indo-French (IFC/A/3403-2/2006). Work in J.M.'s lab was supported by the Deutsche Forschungsgemeinschaft (SFB 630). S.L.P acknowledges a grant from the Department of Science and Technology (SR/FT/L-26/2006), India. R.M. thanks the Council of Scientific and Industrial Research (C.S.I.R.) for the award of junior and senior research fellowships.

REFERENCES

1. **Akins, R. A.** 2005. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. Med. Mycol. **43**:285–318.
2. **Chen, C. G., Y. L. Yang, H. I. Shih, C. L. Su, and H. J. Lo.** 2004. CaNdt80 is involved in drug resistance in *Candida albicans* by regulating *CDR1*. Antimicrob. Agents Chemother. **48**:4505–4512.
3. **Coste, A. T., M. Karababa, F. Ischer, J. Bille, and D. Sanglard.** 2004. *TAC1*, transcriptional activator of *CDR* genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters *CDR1* and *CDR2*. Eukaryot. Cell **3**:1639–1652.
4. **Coste, A., V. Turner, F. Ischer, J. Morschhäuser, A. Forche, A. Selmecki, J. Berman, J. Bille, and D. Sanglard.** 2006. A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. Genetics **172**:2139–2156.
5. **de Micheli, M., J. Bille, C. Schueller, and D. Sanglard.** 2002. A common drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters *CDR1* and *CDR2*, two genes involved in antifungal drug resistance. Mol. Microbiol. **43**:1197–1214.
6. **Franz, R., M. Ruhnke, and J. Morschhäuser.** 1999. Molecular aspects of fluconazole resistance development in *Candida albicans*. Mycoses **42**:453–458.
7. **Franz, R., S. L. Kelly, D. C. Lamb, D. E. Kelly, M. Ruhnke, and J. Morschhäuser.** 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrob. Agents Chemother. **42**:3065–3072.
8. **Garcia-Martinez, J., A. Aranda, and J. E. Perez-Ortin.** 2004. Genomic run-on evaluates transcription rates for all yeast genes and identifies gene regulatory mechanisms. Mol. Cell **15**:303–313.
9. **Gardner, R. G., G. M. Swarbrick, N. W. Bays, S. R. Cronin, S. Wilhovsky, L. Seelig, C. Kim, and R. Y. Hampton.** 2000. Endoplasmic reticulum degradation requires lumen to cytosol signaling: transmembrane control of Hrd1p by Hrd3p. J. Cell Biol. **151**:69–82.
10. **Gaur, N. A., N. Puri, N. Karnani, G. Mukhopadhyay, S. K. Goswami, and R. Prasad.** 2004. Identification of a negative regulatory element which regulates basal transcription of a multidrug resistance gene *CDR1* of *Candida albicans*. FEMS Yeast Res. **4**:389–399.
11. **Gaur, N. A., R. Manoharlal, P. Saini, T. Prasad, G. Mukhopadhyay, M. Hoefer, J. Morschhäuser, and R. Prasad.** 2005. Expression of the *CDR1* efflux pump in clinical *Candida albicans* isolates is controlled by a negative regulatory element. Biochem. Biophys. Res. Commun. **332**:206–214.
12. **Haque, A., V. Rai, B. S. Bahal, S. Shukla, A. A. Lattif, G. Mukhopadhyay, and R. Prasad.** 2007. Allelic variants of ABC drug transporter Cdr1p in clinical isolates of Candida albicans. Biochem. Biophys. Res. Commun. **352**:491–497.
13. **Hiller, D., D. Sanglard, and J. Morschhäuser.** 2006. Overexpression of the *MDR1* gene is sufficient to confer increased resistance to toxic compounds in *Candida albicans*. Antimicrob. Agents Chemother. **50**:1365–1371.
14. **Hiller, D., S. Stahl, and J. Morschhäuser.** 2006. Multiple *cis*-acting sequences mediate upregulation of the *MDR1* efflux pump in a fluconazole-resistant clinical *Candida albicans* isolate. Antimicrob. Agents Chemother. **50**:2300–2308.
15. **Holmes, A. R., S. Tsao, S. W. Ong, E. Lamping, K. Niimi, B. C. Monk, M. Niimi, A. Kaneko, B. R. Holland, J. Schmid, and R. D. Cannon.** 2006. Heterozygosity and functional allelic variation in the *Candida albicans* efflux pump genes *CDR1* and *CDR2*. Mol. Microbiol. **62**:170–186.
16. **Imanishi, Y., K. Yokoyama, and K. Nishimura.** 2004. Inductions of germ tube and hyphal formations are controlled by mRNA synthesis inhibitor in *Candida albicans*. Nippon Ishinkin Gakkai Zasshi **45**:113–119.
17. **Karnani, N., N. A. Gaur, S. Jha, N. Puri, S. Krishnamurthy, S. K. Goswami, G. Mukhopadhyay, and R. Prasad.** 2004. SRE1 and SRE2 are two specific steroid-responsive modules of *Candida* drug resistance gene 1 (*CDR1*) promoter. Yeast **21**:219–239.
18. **Kebaara, B. W., L. E. Nielsen, K. W. Nickerson, and A. L. Atkin.** 2006. Determination of mRNA half-lives in *Candida albicans* using thiolutin as a transcription inhibitor. Genome **49**:894–899.
19. **Kimchi-Sarfaty, C., J. M. Oh, I. W. Kim, Z. E. Sauna, A. M. Calcagno, S. V. Ambudkar, and M. M. Gottesman.** 2007. A "silent" polymorphism in the *MDR1* gene changes substrate specificity. Science **315**:525–528.
20. **Krishnamurthy, S., V. Gupta, R. Prasad, S. L. Panwar, and R. Prasad.** 1998. Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: in vitro transcriptional activation by heat shock, drugs and human steroid hormones. FEMS Microbiol. Lett. **160**:191–197.
21. **Li, D., V. Gurkovska, M. Sheridan, R. Calderone, and N. Chauhan.** 2004. Studies on the regulation of the two-component histidine kinase gene *CHK1* in *Candida albicans* using the heterologous *lacZ* reporter gene. Microbiology **150**:3305–3313.
22. **Lopez-Camarillo, C., J. P. Luna-Arias, L. A. Marchat, and E. Orozco.** 2003. EhPgp5 mRNA stability is a regulatory event in the *Entamoeba histolytica* multidrug resistance phenotype. J. Biol. Chem. **278**:11273–11280.
23. **Lopez-Ribot, J. L., R. K. McAtee, L. N. Lee, W. R. Kirkpatrick, T. C. White, D. Sanglard, and T. F. Patterson.** 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob. Agents Chemother. **42**:2932–2937.
24. **Lyons, C. N., and T. C. White.** 2000. Transcriptional analyses of antifungal drug resistance in *Candida albicans*. Antimicrob Agents Chemother. **44**:2296–2303.
25. **Muller, C., F. Goubin, E. Ferrandis, I. Cornil-Scharwtz, J. D. Bailly, C. Bordier, J. Benard, B. I. Sikic, and G. Laurent.** 1995. Evidence for transcriptional control of human mdr1 gene expression by verapamil in multidrug-resistant leukemic cells. Mol. Pharmacol. **47**:51–56.
26. **Murad, A. M., C. d'Enfert, C. Gaillardin, H. Tournu, F. Tekaia, D. Talibi, D. Marechal, V. Marchais, J. Cottin, and A. J. Brown.** 2001. Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors *CaTup1*, *CaMig1* and *CaNrg1*. Mol. Microbiol. **42**:981–993.
27. **Nieto, A., D. Guillermo Perez, E. Orozco, F. Paz, and C. Gomez.** 2005. *Entamoeba histolytica EhPgp5* transcriptional activation depends on putative emetine response elements. Exp. Parasitol. **110**:233–237.

28. **Ohki, R., and K. Tateno.** 2004. Increased stability of *bmr3* mRNA results in a multidrug-resistant phenotype in *Bacillus subtilis*. J. Bacteriol. **186**:7450–7455.
29. **Prasad, R., P. D. Worgifosse, A. Goffeau, and E. Balzi.** 1995. Molecular cloning and characterization of a novel gene of *Candida albicans CDR1*, conferring multiple resistance to drugs and antifungals. Curr. Genet. **27**:320–329.
30. **Prasad, T., P. Saini, N. A. Gaur, R. A. Vishwakarma, L. A. Khan, Q. M. Haq, and R. Prasad.** 2005. Functional analysis of *CaIPT1*, a sphingolipid biosynthetic gene involved in multidrug resistance and morphogenesis of *Candida albicans*. Antimicrob Agents Chemother. **49**:3442–3452.
31. **Prokipcak, R. D., A. Raouf, and C. Lee.** 1999. The AU-rich 3′ untranslated region of human *MDR1* mRNA is an inefficient mRNA destabilizer. Biochem. Biophys. Res. Commun. **261**:627–634.
32. **Puri, N., S. Krishnamurthy, S. Habib, S. E. Hasnain, S. K. Goswami, and R. Prasad.** 1999. *CDR1*, a multidrug resistance gene from *Candida albicans*, contains multiple regulatory domains in its promoter and the distal AP-1 element mediates its induction by miconazole. FEMS Microbiol. Lett. **180**: 213–219.
33. **Reuss, O., A. Vik, R. Kolter, and J. Morschhäuser.** 2004. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. Gene **341**:119–127.
34. **Riggle, P. J., and C. A. Kumamoto.** 2006. Transcriptional regulation of *MDR1*, encoding a drug efflux determinant, in fluconazole-resistant *Candida albicans* strains through an Mcm1p binding site. Eukaryot. Cell **5**:1957–1968.
35. **Ross, J.** 1995. mRNA stability in mammalian cells. Microbiol. Rev. **59**:423–450.
36. **Sanglard, D., and F. C. Odds.** 2002. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect. Dis. **2**:73–85.
37. **Sanglard, D., F. Ischer, M. Monod, and J. Bille.** 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of *CDR2*, a new multidrug ABC transporter gene. Microbiology **143**: 405–416.
38. **Sanglard, D., K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille.** 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agents Chemother. **39**:2378–2386.
39. **Scotto, K. W., and R. A. Johnson.** 2001. Transcription of the multidrug resistance gene *MDR1*: a therapeutic target. Mol. Interv. **1**:117–125.
40. **Setiadi, E. R., T. Doedt, F. Cottier, C. Noffz, and J. F. Ernst.** 2006. Transcriptional response of *Candida albicans* to hypoxia: linkage of oxygen sensing and Efg1p-regulatory networks. J. Mol. Biol. **361**:399–411.
41. **Shukla, S., P. Saini, Smriti, S. Jha, S. V. Ambudkar, and R. Prasad.** 2003. Functional characterization of *Candida albicans* ABC transporter Cdr1p. Eukaryot. Cell **2**:1361–1375.
42. **Uhl, M. A., and A. D. Johnson.** 2001. Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans*. Microbiology **147**: 1189–1195.
43. **Wang, D., A. D. Johnson, A. C. Papp, D. L. Kroetz, and W. Sadee.** 2005. Multidrug resistance polypeptide 1 (MDR1, ABCB1) variant 3435C>T affects mRNA stability. Pharmacogenet. Genomics **15**:693–704.
44. **White, T. C.** 1997. Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlate with increased azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother. **41**:1482–1487.
45. **White, T. C., K. A. Marr, and R. A. Bowden.** 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin. Microbiol. Rev. **11**:382–402.
46. **Wirsching, S., S. Michel, G. Kohler, and J. Morschhäuser.** 2000. Activation of the multidrug resistance gene *MDR1* in fluconazole-resistant, clinical *Candida albicans* strains is caused by mutations in a *trans*-regulatory factor. J. Bacteriol. **182**:400–404.
47. **Yague, E., A. L. Armesilla, G. Harrison, J. Elliott, A. Sardini, C. F. Higgins, and S. Raguz.** 2003. P-glycoprotein (*MDR1*) expression in leukemic cells is regulated at two distinct steps, mRNA stabilization and translational initiation. J. Biol. Chem. **278**:10344–10352.
48. **Yang, Y. L., Y. H. Lin, M. Y. Tsao, C. G. Chen, H. I. Shih, J. C. Fan, J. S. Wang, and H. L. Lo.** 2006. Serum repressing efflux pump *CDR1* in *Candida albicans*. BMC Mol. Biol. **7**:22.

A.



B.

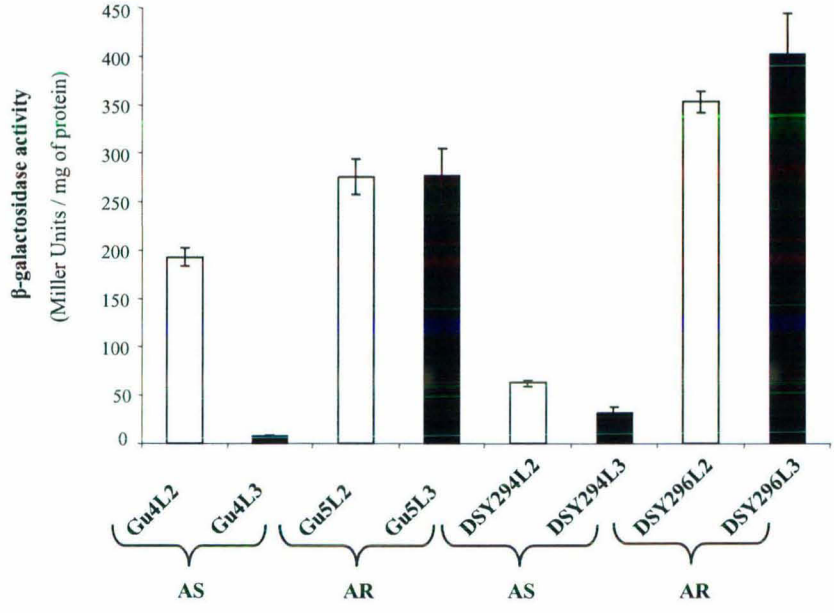


Figure 30. Qualitative and quantitative assay of β-galactosidase activity of *lacZ* reporter fusion integrants in AS and AR isolates.

(A) Transformants harboring the chromosomally integrated P_{CDR1}-*lacZ* (transcriptional fusion, left) and P_{CDR1}-*CDR1-lacZ* (translational fusion, right) and their corresponding parental strain (without *lacZ*) were streaked on minimal media plate containing X-gal and photographed after 3 days growth at 30°C. The positions of the individual strains on the plates are shown in the scheme (*middle*). **(B)** β-galactosidase quantitative reporter activities of each transformants were determined as described under *Materials and Methods*. The values are means ± Standard Deviations (*indicated by the bars*) of three independent experiments with duplicate measurements of two independent clones. AS and AR stands for azole-susceptible and azole-resistant isolates. Empty and filled bars indicate transcriptional (P_{CDR1}-*lacZ*) and translational fusion (P_{CDR1}-*CDR1-lacZ*) transformants in both AS and AR backgrounds.