Expression of *HXK1*, N-acetylglucosamine Kinase, in *Candida albicans*

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CERTIFICATE

The research work embodied in this thesis entitled **"Expression of *HXK1*, N-acetylglucosamine Kinase, in *Candida albicans*"** has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi -110067, India. The work is original and has not been submitted so far, in part or in full for any degree or diploma of any other university.

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1. INTRODUCTION

Candida albicans, an opportunistic yeast pathogen of humans, normally exists as commensal and turns pathogenic when the host is immunocompromised. It can cause variety of infections, frequently in the gastrointestinal, respiratory and genital tracts. *Candida* present in the lesions and sites of infections exhibits a variety of morphological forms like yeast (blastospores) true hyphae and intermediate forms like germ tube or pseudo hyphae (fig.1.0.). The ability of *Candida* to switch its mode of growth i.e. from yeast to filamentous form in response to various environmental signals is important for its establishment as a pathogen.

Yeast cells are round to ovoid in shape and separate readily from each other. Pseudo hyphae resemble elongated, ellipsoid yeast cells that remain attached to one another at the constricted septation site and usually grow in a branching pattern that is thought to facilitate foraging for nutrients away from the parental cell and colony. True hyphal cell are long and highly polarized with parallel sides and no obvious constrictions between cells. Germ tubes are formed during the transition between yeast and hypha.

1.1. IMPORTANCE OF DIMORPHISM

The ability to switch between the morphological forms (hyphal and yeast form) is important for *C.albicans* virulence. Hyphal form is often found at sites of tissue invasion, and cells that do not readily form hyphae often have reduced virulence (Odds, 1988). Importantly, *Candida* that do not readily form true hyphae are much less frequently isolated from the human host, indicating that they are less virulent. But strains that are unable to grow in the yeast form are also less virulent (Lo et al, 1997 ; Laprade et al., 2002.). It is generally thought that hyphal cells expressing cell-wall proteins that facilitate adhesion to human tissue are important for tissue invasion, as well as escape from phagocytosis meditated by neutrophils or macrophages. By contrast, the yeast form is thought to be important for dissemination of the pathogen through the blood stream.

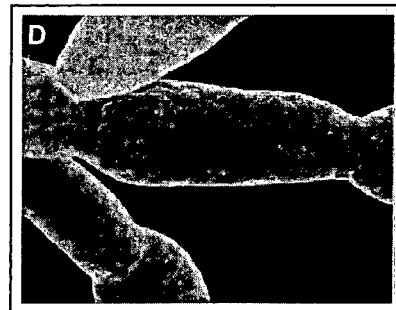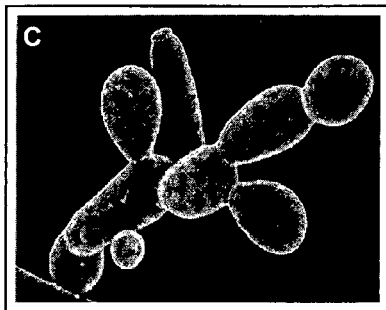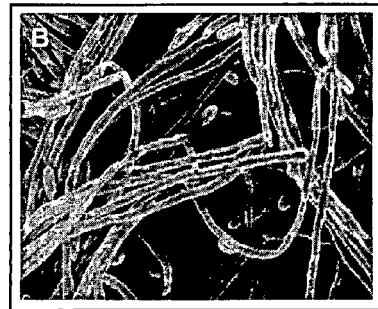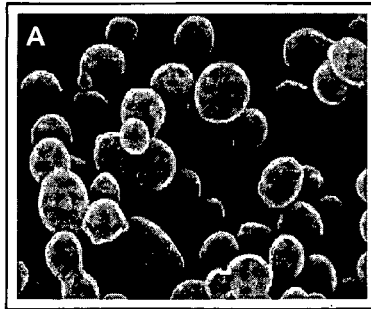**Fig. 1.0. Different morphological forms of *C. albicans***
Scanning Electron Micrograph of various forms of *C.albicans*. A.
Blastospores B. True hyphae C. Pseudohyphae with constrictions at septal
junctions. D. Enlarged photograph of pseudohyphae

However, the proof that this is the case is still lacking, and the issue remains controversial among the Candida research community.

1.2. *Candida albicans* GENOME ORGANISATION

There is hope that recently developed techniques of manipulating *C. albicans* and the sequencing of its genome will lead to a thorough understanding of the virulence and biology of this fungal pathogen, thus offering the possibility of a knowledge-based approach to novel antifungal agents.

1.2.1. Genome Sequencing

1. *Candida albicans* is one of the first eukaryotic pathogens selected for genome sequencing. Its genome was sequenced by the Stanford Genome Technology centre, and draft of the assembled sequence can be down loaded and searched at their web site. *C.albicans* has a diploid genome that is split between 8 pairs of chromosomes. Haploid genome of ~16 Mb is slightly greater than that of *S.cerevisiae*. The reference haploid genome contains 7,677 ORFs of 100 amino acid or greater, including incomplete ORFs at the ends of supercontigs. A reduced set of 6,419 ORFs was derived by eliminating the smaller pair of ORFs that overlap by >50%. It contains several large families of genes that encode proteases, lipases and cell wall proteins that are not present in such large families in *S.cerevisiae*. The genes of these two yeasts usually lack introns. But relatively small fraction of *C.albicans* genes contains introns, as in *S.cerevisiae*. Unlike some other fungal species *C.albicans* does not have extensively spliced genes. The *C.albicans* structure is similar to that of *Saccharomyces*. *C.albicans* and its close relatives translate codon CUG as serine rather than usual leucine in nuclear genes. Approximately two thirds of the ORFs make use of this unusual codon. Although centromeric sequences have remained elusive, several ORFs that encode conserved centromeric proteins are present in the genome sequence. Telomere sequences and telomere homologues have also been identified (Ted et al, 2004).

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2. An interesting, but poorly understood, property of *C.albicans* clinical isolates is their variable karyotype (Merz et al, 1988). As has been observed in *S.cerevisiae*, the length of chromosome that carries the ribosomal (r) DNA is highly variable, owing to changes in the number of rDNA repeats (Wickes et al., 1991). A set of nested repetitive sequences-multiple repeat sequences (MRSs) – seem to be the main site of the translocations that are found in the clinical isolates. Karyotype changes are caused by expansion and contraction of repeat sequences in the MRS repeats. MRSs are found in one or two copies on all chromosomes except chromosome 3 (Chindamporn et al., 1998).

3. Although unintended, genome rearrangements occur in *S.cerevisiae* strains that go through rounds of transformation, non-disjunction apparently occur with a higher frequency in *C.albicans*, perhaps as a mechanism to adapt stressful conditions. For example the loss of chromosome 5 occurs frequently in strains that are forced to grow on sorbose as the sole carbon source, presumably because a repressor of sorbose use resides on chromosome 5(Janbon et al, 1998). Similarly, strains that are resistant to the anti-fungal fluconazole have an increased frequency of chromosome 4 loss or chromosome 5 gain (Perepnikhatka et al, 1999). The mechanism by which these events affect fluconazole resistance is not clear.

4. Genome sequencing uncovered many *C.albicans* ORFs that have obvious S.cervisiae homologues. Among them are many of the putative homologues of S.cervisiae genes that are required for sexual differentiation and meiosis (Tzung et al, 2001). *C.albicans* also contains many genes that are most similar to genes from other fungi, but others that encode novel gene products(Scherer, 2002) . Because most *S.cerevisiae* strains do not adhere to, or invade human tissues, gene products that have no homologues in *S.cerevisiae* are considered by some to be good candidates for broad spectrum anti-fungal targets. *C.albicans* genes that lack human homologues are considered especially promising in this respect, because they are less likely to cause the negative side effects that are associated with most anti- fungal therapies.

The S. cerevisiae genome is thought to have undergone duplication ~100 million years ago. The C. albicans contains fewer sets of duplicated genes with related or redundant

functions indicating that it might not have undergone duplication. For example, the B-cyclin genes of *S.cerevisiae* correspond to only two obvious B-cyclin homologues in *C.albicans*. Even if gene sequences indicate related functions, their roles might be different. For example, *spt3* mutants in *S.cerevisiae* are defective in filamentous growth, where as *spt3* mutant in *C.albicans* are hyperfilamentous. Further more, genes that are essential for viability in *S.cerevisiae* might not be essential in C. albicans, and vise versa. Accordingly, *S.cerevisiae* has two RAS homologues and together they are essential for viability, but the single obvious RAS homologue of *C.albicans* is not essential, indicating that a path way controlled by RAS/cAMP in *S.cerevisiae* is either not controlled by RAS in *C. albicans* or not important for *C.albicans* viability. So, knowing role of gene product in S. cerevisiae is not sufficient to infer its properties in *C.albicans*

1.2.2. DNA array analysis

The availability of the *C.albicans* genome sequence facilitated the development of DNA arrays for gene-expression analysis. Incyte, Inc. generated microarrays of 6000 *C.albicans* open reading frames (ORFs) that had been determined on the basis of genomic and proprietary cDNA sequences. So far, these arrays have been used to analyse the expression patterns of cells exposed to Itroconazole, a broad spectrum anti-fungal drug (De Backer et al, 2001). Groups led by AI Brown and Haoping Liu have used partial genome microarrays to analyse the regulatory pathways that orchestrate gene expression during the yeast to hyphal transition. Several groups are now constructing and using whole-genome microarrays. The first whole-genome arrays for *C.albicans* (6,334) to be published came from Whiteway and co-workers, who used them to analyse the evolution of resistant to anti-fungal and yeast-to-hyphal transition (Nantel et al, 2002).

1.3. Virulence Determinants of C. albicans

C. albicans needs to use several attributes which are potential pathogenicity parameters, to establish pathogenesis in the host. In an effort to understand this process, various physiological and biochemical activities of *C. albicans* have been studied over the years. These include factors related to species and strains, adherence, dimorphism, toxin and

enzyme production, and cell surface composition. *C. albicans* virulence is a function of a multiplicity of factors working jointly to overcome the host defenses. A lack or debility in any of these parameters will reflect negatively on its infectivity and make it difficult for *Candida* to establish itself, particularly in a healthy individual (Ghannoum and Abu-Elteen, 1986).

1.3.1. Adherence

C. albicans maintains a commensal relationship with human hosts probably by adhering to mucosal tissue in a variety of physiological conditions. The adherence of yeasts to oral mucous cells is one of the main characteristics of pathogenicity. Strains of *C. albicans* isolated from the buccal mucosa of HIV-infected patients in the initial stages of AIDS, adhered more to oral mucous cells than the isolated strains from subjects without HIV infection. An interesting breakthrough came with the discovery of ALA1 gene in C. albicans. Adherence due to ALA1 gene in *C. albicans* comprises of two sequential steps. Initially *C. albicans* attaches itself to extracellular matrix (ECM) protein coated magnetic beads in small numbers (the attachment phase). This is followed by a relatively slower step in which cell to cell interactions predominate (the aggregation phase). Neither of these phases is observed in *Saccharomyces cerevisiae,* but expression of ALA1 gene from a high copy vector results in both attachment and aggregation. The adherence of *C. albicans* and *S.cerevisiae*, overexpressing ALA1, to a number of protein ligands, occurs over a broad pH range, is resistant to shear forces generated by vortexing, and is unaffected by the presence of sugars, high salt levels, free ligands, or detergents. Adherence is, however, inhibited by agents that disrupt hydrogen bonds. The similarities in the adherence and aggregation properties of *C. albicans* and *S. cerevisiae* overexpressing ALA1 suggest a role in adherence and aggregation for ALA1-like genes in *C. albicans* (Gaur et al., 1999).

In a landmark finding, Gale et al., in 1998 reported a single gene INT1 linked to adhesion, filamentous growth, and virulence in *C.albicans*. Int1p is a *C. albicans* surface protein with limited similarity to vertebrate integrin. INT1 expression in *S.cerevisiae* was sufficient to direct the adhesion of this normally nonadherent yeast to human epithelial cells.

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Furthermore, disruption of INT1 in *C. albicans* suppressed hyphal growth, adhesion to epithelial cells, and virulence in mice. INT1 links adhesion, filamentous growth, and pathogenicity in *C.albicans*, and Int1p may be an attractive target for the development of antifungal therapies.

1.3.2. Phospholipase

Microbial pathogens like bacteria, parasite, and pathogenic fungi, use secretion of enzymes such as phospholipase, as a genetic strategy to invade the host and cause infection. Phospholipases are important pathogenicity determinants in *C.albicans*. They play a significant role in damaging cell membranes and invading host cells. High phospholipase production is correlated with an increased ability of adherence and a higher mortality rate in animal models (Mayser et al., 1996). Extracellular (secreted) phospholipase activities that have been reported from *C. albicans* include phospholipases A, B, and C, and D. Phospholipase A and lysophospholipase activities have been found in the cell wall of yeast cells and hyphae. Enzyme activity was more in the walls of older yeast cells than of younger cells and was more prominent at the tip of growing hyphae. When yeast cells and germ tubes were grown in the same medium but at a different pH, the specific activity of extracellular phospholipase A was similar for yeast cells and germ tubes, while that of lysophospholipase was higher for the yeast form (Goyal and Khuller, 1992).

The virulence of strains deleted for the *C. albicans* phospholipase B gene Caplb1, for hematogenously disseminated candidiasis, was significantly attenuated, compared with the isogenic wild-type parental strain. Although deletion of CaPLB1 did not produce any detectable effects on candidal adherence to human endothelial or epithelial cells, the ability of the *Caplb1* null mutants to penetrate host cells was dramatically reduced. Thus, phospholipase B may well contribute to the pathogenicity of *C. albicans* by abetting the fungus in damaging and traversing host cell membranes, processes which likely increase the rapidity of disseminated infection (Leidich et al., 1998). Another evidence procured by Ghannoum, 1998, claims that a phospholipase-producing strain caused more fatality in mice, while the phospholipase-deficient null mutant strain was avirulent. These data prove

that phospholipase B is essential for *Candida* virulence, and paves the way for studies directed at determining the mechanism(s) through which phospholipase modulate virulence in this organism.

1.3.3. Acid Proteinase

Aspartyl proteinases are secreted by pathogenic species of *C. albicans in vivo* during infection. (Staib et al., 2000). This enzyme is also secreted *in vitro* when the organism is cultured in presence of exogenous protein (usually BSA) as nitrogen source. The *C. albicans* isolates which adhered most strongly to buccal epithelial cells had the highest relative proteinase activities and were most pathogenic (Ghannoum and Abu-Elteen, 1986). It was found that the fungal isolates from HIV-infected symptomatic patients secreted, on average, up to eight fold more proteinase, than the isolates from uninfected or HIV-infected, but asymptomatic, subjects. This differential property was stably expressed by the strains even after years of maintenance in stock cultures. Moreover, representative high-proteinase isolates were significantly more pathogenic for mice than low-proteinase isolates of *C. albicans* (De Bernardis et al., 1996).

The secreted aspartyl proteinases of *C. albicans* are thought to contribute to virulence through their effects on *Candida* adherence, invasion, and pathogenicity. The role of *Candida* secreted aspartyl proteinase referred to as Sap (SAP gene and Sap protein), has been studied by a number of laboratories as a potential virulence factor of *C.albicans*. As a protease, the enzyme may have a spectrum of substrates, depending upon the host organ, e.g., skin or blood that is colonized or infected. Virulence genes like SAP are differentially activated during infection. *C. albicans* can colonize or infect virtually all body sites because of its high adaptability to different host niches, which involves the activation of appropriate sets of genes in response to complex environmental signals. An *in vivo* expression technology was used that is based on genetic recombination as a reporter of gene expression to monitor the differential activation of individual members of a gene family encoding secreted aspartic proteinases (Saps) at various stages of the infection process. It is shown that SAP expression depends on the type of infection, with different

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SAP isogenes being activated during systemic disease as compared with mucosal infection. In addition, the activation of individual SAP genes depends on the progress of the infection, some members of the gene family being induced immediately after contact with the host, whereas others are expressed only after dissemination into deep organs. In the latter case, the number of invading organisms determines whether induction of a virulence gene is necessary for successful infection.

So far, nine distinct SAP genes (SAP1 to SAP9) have been identified. The levels of the Sap1, Sap2, and Sap3 isoenzymes were monitored under a variety of growth conditions for several *C. albicans* strains, including strain *WO-1*, which alternates between two switch phenotypes, white (W) and opaque (O) (Soll, 1992). These studies revealed that the specific Sap isoenzyme produced is determined by the cell type (strain) whereas the level of Sap production is affected by environmental factors, and they showed that both the yeast-to-mycelium transition and phenotypic switching can determine which of the Sap isoenzymes is produced. SAP1 and SAP3 levels were regulated during the phenotypic transition between W and O forms. SAP2 was the dominant transcript in the yeast form, and its expression was autoinduced by peptide products of its own enzymatic activity and repressed by amino acids (Hube et al., 1997). SAP4 and SAP6 expression was observed only at neutral pH during morphogenetic conversion from yeast to hypha induced by serum. Expression of SAP7 was not detected under any of the experimental conditions used throughout the study. SAP8 is the third gene of the family to be expressed in the opaque phenotype (Soll, 1992).

Implication of Sap proteins in virulence has also come from recent studies by Hube, Sanglard and colleagues. The authors constructed strains harboring disruptions in a number of SAP genes, including SAP1, SAP2, and SAP3 (Hube et al., 1997) and a triple-knockout of SAP4, SAP5, and SAP6 (Sanglard et al., 1997). In all cases, mutants showed decreased virulence in an animal model of disseminated candidiasis. Interestingly, Sap4, Sap5, and Sap6, are produced by *C. albicans* cells after phagocytosis by macrophages. A sap4, sap5, sap6 null mutant was killed more effectively by 53% after contact with macrophages, than the wild-type strain. (Von Zepelin et al., 1998). However, expression of Sap2p as a sole putative virulence factor did not cause *S. cerevisiae* to become virulent and constitutive

overexpression of SAP2 did not augment virulence of *C. albicans* in experimental oral or systemic infection (Dubois et al., 1998).

1.3.4. pH AND PATHOGENICITY

C. albicans has to survive at host environment of diverse pH range. The environmental pH acts as a manipulator for many physiological functions including morphogenesis. It has been shown that pH can alter the expression of the pathogenic trait also. *C. albicans* PHR1 gene which is expressed at ambient pH at 5.5 or higher (neutral to alkaline pHs), and PHR2, expressed at an ambient pH below 5.5, play a role in morphogenesis (Saporito-Irwin et al., 1995). The virulence of the organism also is affected in this pattern, when either or both of the genes are disrupted. Deletion of PHR1, results in pH-conditional defects in growth, morphogenesis, and virulence, evident at neutral to alkaline pH, but absent at acidic pH. Conversely, a *phr2* null mutant exhibited pH-conditional defects in growth and morphogenesis analogous to those of phr1 mutants, but manifests at acid rather than alkaline pH values. Engineered expression of PHR1 at acid pH in a phr2 mutant strain and PHR2 at alkaline pH in a phr1 mutant strain complemented the defects in the opposing mutant. Deletion of both PHR1 and PHR2 resulted in a strain with pH-independent, constitutive growth and morphological defects (Ghannoum et al., 1995; Muhlschlegel and Fonzi, 1997). When these strains were tested for pathogenicity in various niches of the host with different pH (systemic pH is near neutrality and vaginal pH is around 4.5), the virulence phenotype paralleled the pH dependence of the *in vitro* phenotypes. The phr1 null mutant was avirulent in a mouse model of systemic infection, but uncompromised in its ability to cause vaginal infection in rats. The virulence phenotype of a *phr2* null mutant was the inverse. The mutant was virulent in a systemic infection model, but avirulent in a vaginal infection model. Heterozygous mutants exhibited partial reductions in their pathogenic potential, suggesting a gene dosage effect (De Bernardis et al., 1998). Another pH regulatory gene of *C. albicans* whose maximal expression occurs at neutral pH, with no expression detected below pH 6.0, has been cloned (Sentandreu et al., 1998). This gene was designated as PRA1, for pH regulated antigen. The protein predicted from nucleotide

sequence was 299 amino acids long, with motif characteristics of secreted glycoproteins. The predicted surface localization and N- glycosylation of the protein were demonstrated directly by cell fractionation and immunoblot analysis. The PRA1 protein was homologous to surface antigens of *Aspergillus* species, which react with serum from aspergillosis patients, suggesting that the PRA1 protein may have a role in the host-parasite interaction during candidal infection.

1.3.5. Integrins

The existence of integrin-like proteins in *C. albicans* has been postulated because monoclonal antibodies to the leukocyte integrins, bind to blastospores and germ tubes, recognize a candidal surface protein, and inhibit candidal adhesion to human epithelium. The gene α INT1 has motifs common to human integrins, and α-Int1p is surface localized in *C.albicans*. Expression of αINT1 led to the production of germ tubes in haploid *S. cerevisiae* and in the corresponding ste12 mutant. Studies of αInt1p reveal a role for integrin-like proteins in adhesion and in STE12-independent morphogenesis. Disruption of INT1 in *C. albicans* suppressed hyphal growth, adhesion to epithelial cells, and virulence in mice. INT1 links adhesion, filamentous growth, and pathogenicity in *C. albicans* (Gale et al., 1996).

1.3.6. High-affinity Iron Permease: An Essential Virulent Factor of *C. albicans*

Two high-affinity iron permease genes, CaFTR1 and CaFTR2, have been isolated. CaFTR1 expression was induced under iron-limited conditions and repressed when iron supply was sufficient, whereas the expression of CaFTR2 was regulated in a reversed manner. Mutants lacking CaFTR1, but not CaFTR2, exhibited a severe growth defect in iron-deficient medium, and were unable to establish systemic infection in mice. Thus, CaFTR1-mediated iron-uptake mechanism constitutes a virulence factor of *C. albicans*. It could also be a target for the development of anti-candida therapies (Ramanan and Wang, 2000).

1.4. SIGNAL TRANSDUCTION PATHWAYS

Several environmental factors can induce yeast cells to form hyphae and pseudohyphae through several signal transduction pathways (fig.1.1.). This probably reflects the variety of microenvironments in which this opportunist must survive *in vivo*. As in *S.cerevisiae*, the cAMP and the mating pheromone response- MAP kinase pathways target transcription factors that promote morphogenesis. Inactivation of cAMP pathway (by deleting EFG1) blocks filamentation in most conditions, whereas inactivation of MAP-kinase pathway (deletion of CPH1) blocks filament formation only in response to limited set of conditions. So, it seems that cAMP pathway has a more prominent role in *C.albicans* morphogenesis than in *S.cerevisiae*. A cph1 efg1 mutant, in which both the MAP-kinase and cAMP pathways are disabled, failed to form filaments in most *in vitro* conditions, and is avirulent in a systemic mouse model of candidiasis. This observation is often sited as evidence that the ability to form hyphae or pseudo hyphae is an essential virulence factor. But other two important caveats to this interpretation are, first, these mutations block the expression of hyphal specific genes, many of which are also required for virulence and second, cph1 efg1 mutants are able to produce filaments under some *in vivo* and *in vitro* conditions. This might be due to action of other pathways of hyphal growth induction, such as the Rim101 pathway, which is activated by alkaline pH and the Czf1 pathway which is activated by growth in solid matrix.

Recent advances indicate that *C.albicans* uses a common set of conserved pathways to regulate dimorphism, mating and phenotypic switching. Major pathways known to regulate dimorphism include a MAP Kinase pathway via Cph1, the cAMP-dependent protein kinase pathway through Efg1 and Tup1-mediated repression through Rfg1 and Nrg1 (Fig. 1.1.). All these developmental pathways regulate the expression of hyphal specific and phase specific genes. A high proportion of these genes contribute directly or indirectly to pathogenesis and virulence of *C.albicans*, therefore, virulence genes are co-regulated with cell morphogenesis (Liu, 2002).
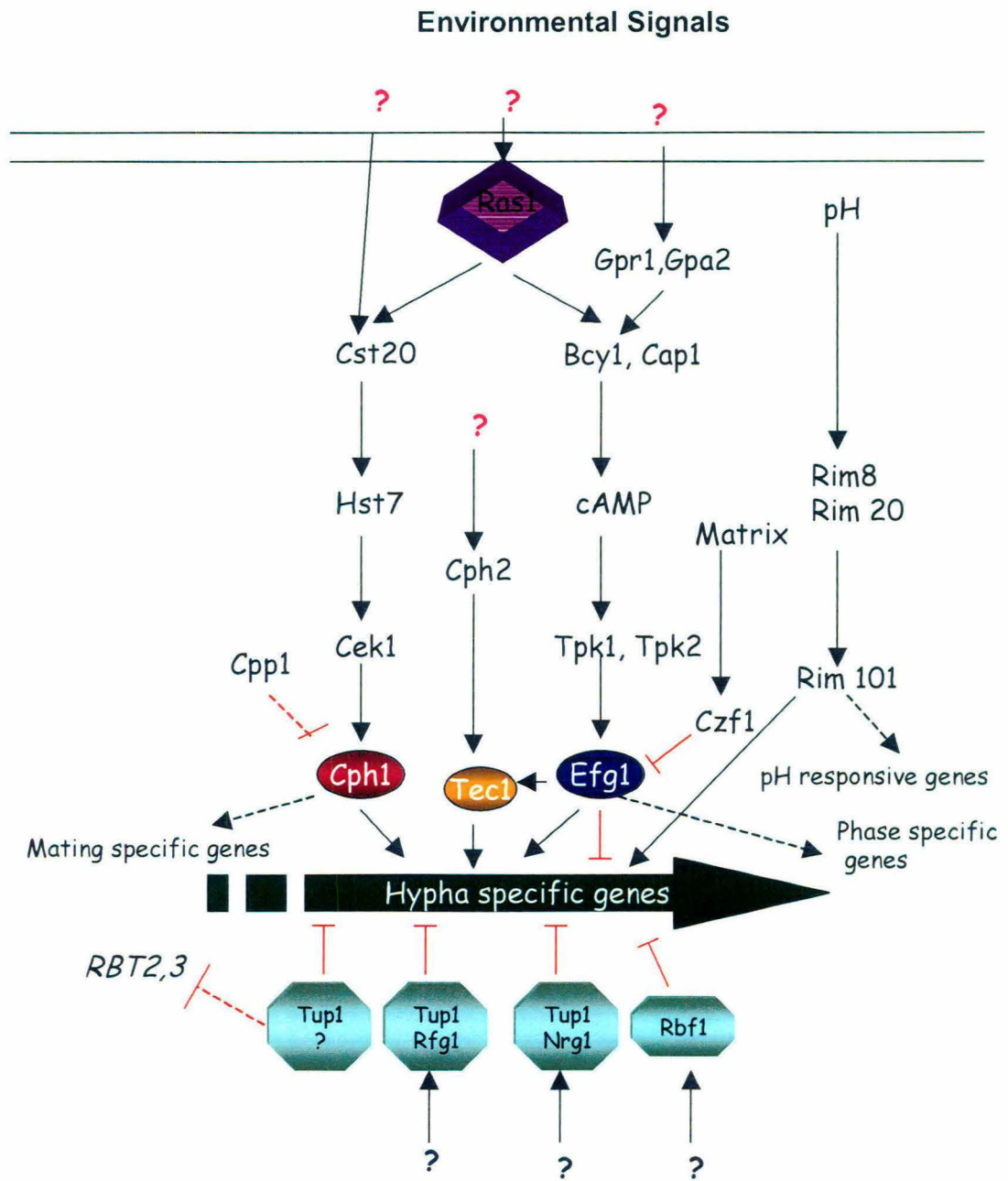
Fig. 1.1. Regulation of dimorphism in *C. albicans* by multiple signaling pathways.

1.4.1. MAP Kinase Pathway

Like *S.cerevisiae*, a mitogen-activated protein kinase (MAPK) pathway is involved in filamentation in *C. albicans*. The cascade consists of the kinases Cst20 (homologous to the p21-activated kinase [PAK] kinase Ste20), CaSte7/Hst7 (homologous to the MAP kinase kinase Ste7), and Cek1 (homologous to the Fus3 and Kss1 MAP kinases) (Clark et al., 1995; Kohler and Fink, 1996; Leberer et al., 1996; Singh et al., 1997; Whiteway et al., 1992). A transcription factor, Acpr/Cph1, which is homologous to the Ste12 that regulates mating and pseudohyphal growth in *S. cerevisiae*, has been identified (Malathi et al., 1994; Liu et al., 1994). Null mutations in any of the genes in the MAP kinase cascade (Cst20, Hst7, or Cek1) or the transcription factor Cph1 confer a hyphal defect on solid medium in response to many inducing conditions; however, all of these mutants filament normally in response to serum (Csank et al., 1998; Leberer et al., 1996). Interestingly, although a cek1 MAP kinase mutant strain forms morphologically normal filaments in response to serum, it has a minor growth defect on serum-containing medium (Csank et al., 1998). The *cek1* mutant strain also has a virulence defect that may be attributable to this growth defect (Csank et al., 1998, Guhad et al., 1998b). This indicates that the Cek1 MAP kinase may function in more than one pathway or that deletion of the gene causes aberrant cross talk between distinct MAP kinase cascades, similar to the altered signaling that occurs in a *fus3* mutant of *S.cerevisiae*. The other elements of the pathway have small but varied effects on virulence. *cst20* mutant strains have a modest virulence defect in a mouse model of systemic candidiasis (Leberer et al., 1996). However, *hst7* and *cph1* mutant strains are able to cause lethal infection in mice at rates comparable to wild-type strains (Leberer et al., 1996; Lo et al., 1997). In addition to these components, a MAP kinase phosphatase, Cpp1, has been identified which regulates filamentous growth in *C. albicans* (Csank et al., 1997). Disruption of both alleles of the CPP1 gene derepresses hyphal production and results in a hyperfilamentous phenotype. This hyperfilamentation is suppressed by deletion of the MAP kinase Cek1 (Csank et al., 1997). *cpp1* mutant strains are also reduced for virulence in both systemic and localized models of candidiasis (Csank et al., 1997; Guhad et al., 1998a). Recently a G-protein α subunit homologue has been identified from *C.albicans*. Deletion of both the alleles of GPA2 causes *in vitro* defects in filamentation formation in

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Spider and SLAD media and in embedded conditions but not in serum containing media. These defects cannot be reversed by exogenous addition of cyclic AMP. However over expression of HST7 can complement the filamentation defect showing that GPA2 is required for MAP kinase signaling pathway (Sanchez-Martinez and Perez-Martin, 2002b).

1.4.2. cAMP-PKA Pathway

The cAMP-dependent protein kinase A (PKA) pathway plays a very important role in filamentation in S. cerevisiae, C. albicans and other fungi (Sonneborn et al., 2000). In C.albicans, an increase in cAMP level accompanies the yeast to hyphal transition, and inhibition of the cAMP phosphodiesterase induces this transition (Sabie and Gadd, 1992). Moreover, two cell-permeating PKA inhibitors, myristoylated protein kinase inhibitor (myrPKI) amide and the small-molecule PKA inhibitor H-89, both block hyphal growth induced by N-acetylglucosamine, but not in response to serum (Castilla et al., 1998).

Genes similar to GPA2, encoding G-protein, and GPR1, coding for G-protein receptor have been identified in the C. albicans genome-sequencing project, Stanford University. GPA2 has been reported to act upstream to MAP kinase pathway (Sanchez-Martinez and Perez-Martin, 2002b). C. albicans has a single gene homologous to the S. cerevisiae adenylate cyclase gene (CDC35/CYR1). The cyclase is not essential for growth in C. albicans, but is required for hyphal development (Rocha et al., 2001). Recently, the adenylate cyclase associated protein (CAP1) has been identified and disrupted (Bahn and Sundstrom, 2001). An increase in cytoplasmic cAMP is seen to precede germ tube emergence in wild type strains, but not in cap1/cap1 mutants. The cap1/cap1 mutant is defective in germ tube formation and hyphal development, the defects being suppressible by exogenous cAMP or dibutyryl cAMP. cap1/cap1 strains are avirulent in a mouse model for systemic candidosis.

A single Ras homolog, Ras1, has been identified in C.albicans, which is not essential for survival (Feng et al., 1999). The ras1 mutant strains have a severe defect in hyphal growth in response to serum and other conditions (Feng et al., 1999). In addition, while a dominant negative Ras1 mutation (Ras1A16) caused a defect in filamentation, a dominant active

Ras1 mutation (Ras1V13) enhanced the formation of hyphae (Feng et al., 1999). The *in vitro* defects in morphological transition were reversed by either supplementing the growth media with cAMP or over expressing components of the filament inducing MAP Kinase cascade, demonstrating that it functions upstream of both cyclic AMP as well as MAP Kinase pathway (Leberer et al., 2001).

The Ras1 protein is likely to activate two protein kinase A (PKA) isoforms Tpk1p and Tpk2p (Bockmühl et al., 2001; Cloutier et al., 2003). *tpk1* mutants are defective in hyphal formation on solid media but not so much in liquid (Sonneborn et al., 2000). Hyphal formation in *tpk2* mutants, on the other hand, is partially affected on solid media but is blocked in liquid. A strain mutated for TPK2 containing a single allele of TPK1 under a regulatable promoter is unable to grow properly at low expression levels.

Efg1, a basic helix-loop-helix (bHLH) protein, functions downstream of the PKAs. It is similar to Sok2 in *S. cerevisiae* (Pan and Heitman, 2000; Shenhar and Kassir, 2001). *efg1/efg1* null mutant strains are defective in filamentation under most inducing conditions, and are also defective in the induction of hypha-specific genes (Lo et al., 1997; Stoldt et al., 1997). Overexpression of TPK2 is unable to suppress the mutant phenotype of *efg1/efg1*, whereas overexpression of EFG1 can nullify the filamentation defect in *tpk2/tpk2* (Sonneborn et al., 2000). The suppression activity of EFG1 depends on a threonine residue at the position 206, a potential phosphorylation site for a PKA (Bockmühl and Ernst, 2001). The presumed bHLH motif in Efg1p indicates an E box binding site on promoter regions of target genes (5'CANNTG3') (Ernst, 2000). Recent studies have shown the involvement of EFG1 in normal biofilm formation (Ramage et al., 2002).

Studies by Sohn et al., 2003, revealed that this transcription factor is a major regulator of cell wall dynamics in *C.albicans*. Microarray studies showed that Efg1p was essential for the transcription of both hyphae specific genes such as HWP1 and HWP2 as well as yeast form specific genes such as YWP1. During hyphal induction, EFG1 transcript levels decline to low levels; downregulation is affected at the level of transcriptional initiation as shown by an EFG1 promoter-LAC4 fusion. Under standard induction conditions, using

serum or GlcNAc as inducers in liquid or on solid media, there is a complete block of hyphal formation in *efg1/efg1* null mutants. Contrastingly, under microaerophilic or embedded conditions hyphal formation is not defective at all in homozygous efg1 mutants, but rather appears stimulated (Giusani et al., 2002). These results indicate that depending on environmental cues (and depending on the genetic background), Efg1p has a dual role as a transcriptional activator and repressor, whose balanced activity is essential for yeast, pseudohyphal and hyphal morphogenesis of *C. albicans*.

The ras1 mutant strains exhibit a filamentation defect similar to that of *efg1 cph1* mutant strains, and evidence from *S. cerevisiae* indicates that Ras2 lies upstream of both the cAMP-PKA and MAP kinase pathways regulating pseudohyphal growth (Lorenz and Heitman, 1997; Mösch and Fink, 1997; Pan and Heitman, 1999; Roberts et al., 1997). These findings are consistent with a model in which Ras1 lies upstream of the related pathways in *C. albicans* (Leberer et al., 2001).

1.4.3. Other Signaling pathways and Regulators involved in Dimorphism

a) Tec1

Tec1 has recently been shown to regulate hyphal development and virulence in *C. albicans* (Schweizer et al., 2000). In *S. cerevisiae*, TEC1 transcription is regulated by Ste12, and cooperation between Tec1 and Ste12 is important for pseudohyphal growth (Madhani and Fink, 1997). In *C.albicans*, however, TEC1 transcription is not regulated by CPH1 (Lane et al., 2001a). *tec1/tec1* mutants exhibit suppressed filamentation in liquid serum-containing media. EFG1 overexpression does not suppress the morphological defect of the *tec1/tec1* mutant, whereas TEC1 overexpression has a partial phenotype in the *efg1/efg1* mutant (Schweizer et al., 2000). These results, coupled with the fact that *efg1/efg1* strains have a more severe defect in hyphal development than do *tec1/tec1* strains, suggest that TEC1 is one of the downstream effectors of Efg1.

b) Cph2

Cph2, a myc family bHLH protein, has been found to regulate hyphal development in *C. albicans* (Lane et al., 2001b). *cph2/cph2* mutant strains are impaired in hyphal development and in the induction of hypha-specific genes in liquid Lee's media, and Cph2 is necessary for the transcriptional induction of TEC1. Cph2 binds directly to two sterol-regulatory-element-1-like elements upstream of TEC1. Furthermore, the ectopic expression of TEC1 expression suppresses the defect of *cph2/cph2* in hyphal development. The function of Cph2 in hyphal transcription is therefore mediated, in part, through Tec1.

c) Bmh1

Recently a 14-3-3 gene, BMH1 has been isolated from *C.albicans*. In *S.cerevisiae*, there are two such genes BMH1 and BMH2, which are essential for normal pseudohyphal induction and normal bud cell development. In *C.albicans*, however, there is just one copy of this gene and is essential for survival gene. The heterozygous mutant is defective in growth and morphogenesis (Cognetti et al., 2002).

d) Czf1

An Efg1p-independent pathway of filamentation operates under microaerophilic/embedded conditions (Sonneborn et al., 1999a; Riggle et al., 1999; Giusani et al., 2002). The putative transcription factor Czf1 is probably an important element of the alternative pathway of filamentation in *C. albicans* (Brown et al., 1999). Homozygous *czf1* null mutants filament normally under standard induction conditions, but they are defective in hyphal development when embedded in agar. Hyperfilamentation of *efg1* and *efg1 cph1* double mutants suggests that Efg1p is a negative modulator of the Czf1p pathway under microaerophilic/embedded conditions (Giusani et al., 2002).

e) Cdc5

CaCDC5, a cell cycle regulator polo-like kinase in *C.albicans* has been identified. Cells lacking this gene are blocked early in nuclear division. The cell cycle defects are accompanied by the formation of hyphal like filaments under yeast growth conditions. The filaments resembled serum induced hyphae. Filament formation is not dependent on Cph1 or Efg1 but requires Cdc35 (Bachewich et al., 2003).

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f) Fkh2

In *S.cerevisiae*, two forkhead transcription factors, ScFKH1 and ScFKH2 regulate the expression of B-cyclin genes. They also influence morphogenesis. *C.albicans* has only one homologue, CaFKH2. Cells lacking this gene formed constitutive pseudohyphae under all yeast and hyphal growth conditions tested. Under hyphal growth conditions levels of hyphae specific mRNA were reduced, and under yeast growth conditions levels of several genes encoding proteins likely to be important for cell wall separation were reduced. Together these results imply that Fkh2p is required for morphogenesis of true hyphae as well as yeast cells. Cph1 and Efg1 were not required for pseudohyphal morphology of *fkh2* mutants, implying that it acts in pathways downstream or parallel to them. Cells lacking Fkh2p were unable to damage human epithelial and endothelial cells *in vitro*, suggesting that Fkh2p contributing to *C.albicans* virulence (Bensen et al., 2002).

g) Mcm1

C.albicans homologue of MCM1 was identified while screening for genes which could activate FLO11::lacZ expression in *S.cerevisiae*. Both over expression and repression of this gene led to the induction of hyphae. A hyphal specific gene HWP1 was induced by repression of CaMCM1. But the change in expression was not dependent on NRG1 or TUP1 (Rottmann et al., 2003). Thus CaMCM1 is a component of a hitherto unknown regulatory mechanism of hyphal growth.

h) Cdc42 and Cdc24

The function of different polarity establishment proteins in dimorphism was studied. Ectopic expression of a Rho G-protein, CaCDC42 (Ushinsky et al., 2002; Hazan and Liu, 2002) or it's exchange factor Cdc24 were unable to form invasive hyphal filaments and germ tubes in response to serum or elevated temperature and yet could grow normally as a budding yeast. Further these mutants were avirulent in a mouse model for systemic infection. These results suggest that these proteins are required for invasive hyphal growth and pathogenicity of *C.albicans* (Bassilana et al. 2003).

i) Farnesoic acid

Studies on the morphological transition from a filamentous to a budding yeast form in *C.albicans* have shown that this organism excretes an autoregulatory substance into the culture media. This was identified as farnesoic acid. This substance inhibited filamentous growth and might be involved in developmental signaling (Oh et al., 2001).

j) Hog1

Apart from these, Hog1 MAP Kinase is reported to be essential in oxidative stress response and chlamydospore formation in *C.albicans* (Alonso-Monge et al., 2003). The mutants are hyper invasive (Alonso-Monge et al., 1999).

1.5. REPRESSION OF FILAMENTATION

a) Tup1

The Tup1 transcription factor may be involved in constituting the hypha-repressed state in the presence of glucose and other non-inducing conditions. In *S.cerevisiae*, the Tup1 protein regulates about 60 genes involved in glucose regulation, oxygen stress response and DNA damage. A *C. albicans* homologue of Tup1p was identified that is 67% identical to *S. cerevisiae* Tup1p (Braun and Johnson, 1997). Tup1p contains seven conserved WD40 repeats at the C terminus, which could anchor it to some of its DNA-binding proteins, and an N-terminal domain that could interact with a homologue of Ssn6p, as in *S.cerevisiae* (Keleher et al., 1992; Komachi and Johnson, 1997). A homozygous *C. albicans tup1* mutant grew in filamentous form in all media tested; filaments on most media had the characteristics of pseudohyphae, but in some media had the appearance of true hyphae. Pseudohyphae of a *tup1* mutant, unlike pseudohyphae produced by EFG1 overexpression (Stoldt et al., 1997) could not be induced to form germ tubes or true hyphae by the addition of serum (Braun and Johnson, 1997). Tup1p had activities besides repression of filamentation, because *tup1* mutants failed to grow at 42°C grew faster on glycerol and had misshapen cell walls compared to the wild-type. In epistasis experiments most, but not all

of the filamentation phenotype induced by the tup1 mutation, was abolished by the presence of an efg1 mutation, while a cph1 mutation had very little effect. A comparison of a *tup1 efg1* mutant with a *tup1 efg1 cph1* mutant showed a slight influence of the cph1 mutation on hyphal morphogenesis (Braun and Johnson, 2000). An analysis of transcript levels of hypha-specific genes including HYR1, ALS1, HWP1 and ECE1 showed no differences between *tup1* and *tup1 cph1* mutants, only the HWP1 transcript was lowered slightly in the *tup1 efg1 cph1* mutant compared to the *tup1 efg1* mutant. These results indicate that Efg1p is the main, and Cph1p a minor contributor to the tup1 hyphal phenotype. Genes repressed by Tup1p have been identified recently, of which some are expressed in a filament-specific manner (Braun and Johnson, 2000).

b) Nrg1

A DNA binding protein, Nrg1 that represses filamentous growth in *C.albicans* has been identified. It contains a Zinc finger domain that is conserved in transcriptional regulators from fungi to human. It is most closely related to ScNrg1 which represses transcription in Tup1- dependent fashion. The *nrg1* mutant cells are predominantly filamentous under non-filament inducing conditions. They also show attenuated virulence. Nrg1 represses several filament specific genes such as ECE1 and HWP1. Most of these genes contain Nrg1 response element (NRE) in their promoter. These genes constitute a subset of those under Tup1 control, providing further evidence that Nrg1 acts by recruiting Tup1 to target genes (Braun et al., 2001; Murad et al., 2001a and b).

c) Rfg1

Rfg1 is a HMG protein which was cloned in an attempt to characterize the *C.albicans* homologue of *S.cerevisiae* Rox1, a repressor of hypoxic genes. When a homologous deletion was generated, the cells became constitutively filamentous (Khalaf and Zitomer, 2001; Kadosh and Johnson, 2001).

d) Rap1

In *S.cerevisiae*, the Rap1 protein acts as both a transcriptional silencer and a structural protein at telomeres by binding to a sequence designated the RPG box (Drazinic et al., 1996). A *C.albicans* homologue of Rap1 has been identified which is not essential for

survival. The *rap1* mutants formed budding as well as pseudohyphal cells under conditions that promote budding yeast growth in wild type strain. The phenotype was reverted upon reintroduction of a functional copy. Thus this gene is required to repress pseudohyphae formation under conditions favouring growth as budding yeast (Biswas et al., 2003).

e) Rbf1

A *C. albicans* protein, Rbf1p, was identified which is not homologous to Rap1p, but binds to the RPG box of *S. cerevisiae* (Ishii et al., 1997). Rbf1p contains two glutamine-rich regions embedding a region with weak similarity to bHLH domains, which binds to RPG sequences. Homozygous *rbf1* null mutants grew in filamentous form in all media tested; the filaments formed had the characteristics of pseudohyphae rather than true hyphae (Ishii et al., 1997; N. Ishii, M. Watanabe and Y. Aoki, unpublished). Thus, Rbf1p seems to be involved exclusively in pseudohyphal, but not true hyphal growth. Interestingly, the authors reported that three alleles of RBF1 were present in the standard disruption strain CAI4. Aneuploidy or triploidy had been previously demonstrated in other *C.albicans* strains, such as strain SGY-243 (Gow et al., 1994), but not in strain CAI4. Besides derepression of filamentous growth, the *rbf1* knockout strain showed significantly slower growth and increased sensitivities to high temperature, high osmolarity and hydrogen peroxide compared to the wild-type strain. Virulence of the *rbf1* mutant in the mouse model of systemic infection was significantly attenuated. Recently, by screening for sequences that mediate Rbf1p-dependent transcriptional regulation, target genes were identified in the heterologous host *S. cerevisiae*. Among the genes identified as Rbf1p targets was the WH11 gene, which in phenotypic switching between a white and an opaque phenotype is specifically expressed in the white phase (Soll, 1997); the level of WH11 transcripts is reduced in homozygous *rbf1* mutants compared to wild-type cells.

f) Sir2

The Sir2 protein represses hyphal formation, which is consistent with the role of Sir2p as a repressor in *S.cerevisiae* (although it is unrelated to pseudohyphal growth in this species) (Perez-Martin et al., 1999).

g) Rad6

Another repressing factor is the Rad6 protein, which besides contributing to UV protection, represses hyphal growth under inducing conditions by an unknown pathway; its deficiency under non-inducing conditions generates a pseudohyphal phenotype (Leng et al., 2000).

h) Ssn6

C.albicans Ssn6 encodes a putative global transcriptional co-repressor. It is highly homologous to the *S.cerevisiae* Ssn6. Its expression level declines significantly in response to strong hyphal inducer such as serum. The mutant lacking Ssn6 displayed a stubby pseudohyphal growth pattern, derepressed filament specific genes in response to increased temperature and failure to develop true hyphae. Such morphological defects were not rescued by over expression of Tup1, Cph1 and efg1. Over expression of Ssn6 led to increased filamentation and decreased virulence (Hwang et al., 2003).

1.6.ROLE OF CELL CYCLE REGULATORY ELEMENTS IN MORPHOGENESIS

Cell differentiation is the process by which cells undergo stable alteration in form and function that enables them to be distinguished from precursor cells. It has been established in number of systems that pathways regulating the cell proliferation influence the cell differentiation and that cells undergoing differentiative processes utilize cell cycle regulatory elements in distinct ways. Specific signal transduction pathways mediate communication between environmental stimuli and cellular components that coordinate complex cellular changes (metabolism, gene expression, cell division, morphology etc.) and controls cell growth and differentiation.

Diploid yeast cells have two mutually exclusive choices upon starvation: pseudo hyphae growth or sporulation. Nitrogen starvation triggers pseudohyphal growth in presence of abundant and fermentable carbon source, where as starvation for nitrogen (or other nutrients) in the absence of a fermentable carbon source induces sporulation. In the wild, after spore germination, haploid yeast cells mate immediately to form diploid zygote that in presence of nutrients can reenter a vegetative growth phase. During vegetative

growth, diploid and haploid cells divide by budding. Therefore, depending on nutrient availability and ploidy, yeast cells have atleast four mutually exclusively differentiative fates: vegetative growth, mating, pseudohyphal growth or sporulation. Each of pathways includes a clearly recognized effect on the cell cycle regulatory apparatus.

The budding yeast provides an excellent experimental system for addressing questions regarding the role of cell cycle-regulatory apparatus in cell differentiation and development. Still the extent to which cell regulation influence the process of differentiation is not uniformly not understood. In $S.cerevisiae$, morphogenesis is regulated during cell cycle by the association of cyclins with the Cdc28 cyclin dependent kinase (CDK). Association of the CDK with G1 cyclins (Cln1, Cln2) promote polarized growth; its association with B-cyclins promotes ISOTROPIC growth. The $C.albicans$ Cln1 G1 cyclin is not required for filamentous growth, although it seems to promote the maintenance of filamentous growth in wild-type cell.

Many lines of evidence indicate that pseudohyphal growth in $S.cerevisiae$ might involve regulation of the Clb2 Cdc28 kinase, but the mechanisms by which this comes is still unclear. In $S.cerevisiae$, transcription of CLB2,the main mitotic B-cyclin, regulated by the fork head transcription factor family members Fkh1 and Fkh2. Cells that lack these transcription factors have reduced periodicity of CLB2 transcription and grow constitutively as pseudohyphae. Only one homologue of Fkh1/2, Fkh2, is present in $C.albicans$, and its deletion results in constitutive pseudohyphal phenotype under both yeast and hyphal growth conditions. Cells that lack Fkh2 in $C.albicans$ have increased levels of hyphal cell wall proteins and of enzymes that dissolve the connections between mother and daughter yeast cells. These observations have led to a model in which Fkh2 regulates the cell cycle processes that are necessary for the morphogenesis of true hyphal and yeast cells(reviewed from Curt W and Robert L V, 2003).

2. HEXOKINASE

2.1. INTRODUCTION

Fungal diseases have become a major health threat in the last few years and are likely to increase in severity. Most fungal pathogens are opportunists, and their emerging importance is due to the success of modern medical practices. These diseases thrive in debilitated patients who are treated with procedures which diminish their immune responses. The major fungal pathogen in such patients is *Candida albicans*, which can grow in a variety of forms, ranging from budding yeast to thread-like hyphae. Colonization of *C.albicans* can lead to systemic infection with or without tissue invasion or to disseminated disease where single or multiple organs are sites of infection.

C.albicans can undergo morphological transition in response to a variety of environmental cues in infection sites. The ability to switch between the morphological forms is important for *C.albicans* virulence. Both hyphal and yeast forms are important for virulence *C.albicans*. So morphological transitions or morphogenesis has gained significance for understanding the pathogenesis.

2.2. SIGNAL TRANSDUCTION PATHWAYS

Several environmental factors can induce the yeast cells to form hyphae and pseudohyphae through several signal transduction pathways (Fig.1.1.). This probably reflects the variety of microenvironments in which this opportunist must survive (Brown, 2002). As in *S.cerevisiae*, the cAMP and the mating pheromone response-MAP kinase pathways target transcription factors that promote the morphogenesis. Inactivation of the cAMP pathway (by deleting EFG1) blocks filamentation in most conditions, where as inactivation of the MAP-kinase pathway (by deleting CPH1) blocks filament formation only in response to limited set of conditions. So, it seems that the cAMP pathway has a more prominent role in *C.albicans* morphogenesis than in *S.cerevisiae*. An *efg1 cph1* mutant, in which both cAMP pathway and MAP-kinase pathways are disabled, fails to form filaments in most in vitro conditions, and is avirulent in systemic mouse model of

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candidiasis. This observation is often cited as evidence that the ability to form hyphae or pseudohyphae is an essential virulence factor. But there are two important caveats to this interpretation. First, these mutations block the expression of hyphal specific genes, many of which are also required for virulence. Second, *cph1 efg1* mutants are able to produce filaments under some in vivo and *in vitro* conditions. This might be due to the action of other pathways of hyphal growth induction, such as the Rim101pathway, which is activated by alkaline pH and the Czf1 pathway, which is activated by growth in a solid matrix

RAS1, homologous to *S.cerevisiae RAS1*, has been identified in this organism (Feng et al., 1999). Homozygous *ras1* mutants are unable to form true hyphae in the presence of serum. Similar to *S.cerevisiae*, Ras1p acts upstream to both MAP Kinase pathway, as well as cAMP dependent PKA pathway. Recently, the *TPK1* and *TPK2* genes encoding the catalytic subunit isoforms of a protein kinase A (PKA) have been characterized which are homologous to *S. cerevisiae TPK1* and *TPK2* (Sonneborn et al., 2000; Bockmuhl et. al., 2001). They function downstream to Ras1p and are regulated by cAMP levels. Hyphal morphogenesis of a homozygous *tpk2* mutant is blocked on liquid media whereas *tpk1* mutant is severely compromised for hyphal formation in solid inducing media. It has been strongly suggested that Tpk1p and Tpk2p are members of a signaling pathway that operates in parallel to the MAP kinase pathway in *C. albicans* (Sonneborn et al., 2000; Bockmuhl et. al., 2001).

EFG1, a transcriptional activator is involved not only in yeast to hyphal inter conversions (Stoldt et al., 1997; Lo et al., 1997), but also regulates phenotypic switching and chlamydospore formation of this pathogen (Sonneborn et al., 1999 a and b). It is the *Candida* homolog of the *S. cerevisiae PHD1* gene and is a direct downstream target of *TPK1/TPK2*. In contrast to *cph1* mutant, the *efg1* mutant cells are strongly attenuated in hyphal formation in response to serum and also shows reduced filamentous growth on most, but not all, solid media (Lo et al., 1997).

ACPR/CPH1 and *EFG1* regulate the transcriptional activation of the two major filamentation pathways, MAP Kinase and PKA respectively. The *cph1 efg1* double mutant fails to form filaments under any condition tested, whereas neither of the single mutation can block morphogenesis completely (Lo et al., 1997). Several negative regulators of hyphal formation have been identified and characterized. Possibly as in *S.cerevisiae*, repression of pseudohyphal development in *C.albicans* is also brought about by a repressor complex consisting of *TUP1*, a global repressor (Braun and Johnson, 1997), *SSN6* (Hwang et al., 2003), *NRG1* (Braun et al., 2001; Murad et al., 2001b) and *RFG1* (Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001). It has been reported that deletion of *TUP1* causes constitutive filamentous growth on all media tested (Braun and Johnson, 1997). Tup1p and Ssn6p do not have any DNA binding activity. They are recruited to specific promoters through the interaction with distinct DNA binding proteins such as Nrg1p (Fig.2.0). Recently *CaMCM1* has been identified which causes suppression of hyphal formation in a *TUP1/NRG1* independent pathway (Rottmann et al., 2003).

2.3. GlcNAc Catabolic Pathway

The mucous membrane at the sites of infection of this organism is rich in the aminosugar N-acetylglucosamine (GlcNAc). The pathogenic strains can utilize GlcNAc as sole carbon source (Sigh and Datta, 1979a); and this has led investigations to try and delineate the role of this molecule in pathogenicity. Investigations about GlcNAc catabolic pathway had begun by the study on the induction and regulation N-acetylglucosamine kinase (Bhattacharya et al; 1974) and N-acetyl glucosamine -6- phosphate deacetylase (Rai and Datta, 1982) in *C.albicans*.

There are three enzymes, which are sequentially involved in the catabolic path way of GlcNA are 1) GlcNAc Kinase, HXK1 (phosphorylation of GlcNAc), 2) DAC1 (deacetylation of GlcNAc-6-phosphate) and 3) NAG1 (deamination of GlcN-6-phosphate). The final product, Fructose-6-phosphate is metabolized through Embden-Meyerhoff pathway (Fig.2.1., shown: Nag cluster & GlcNAc catabolic pathway).
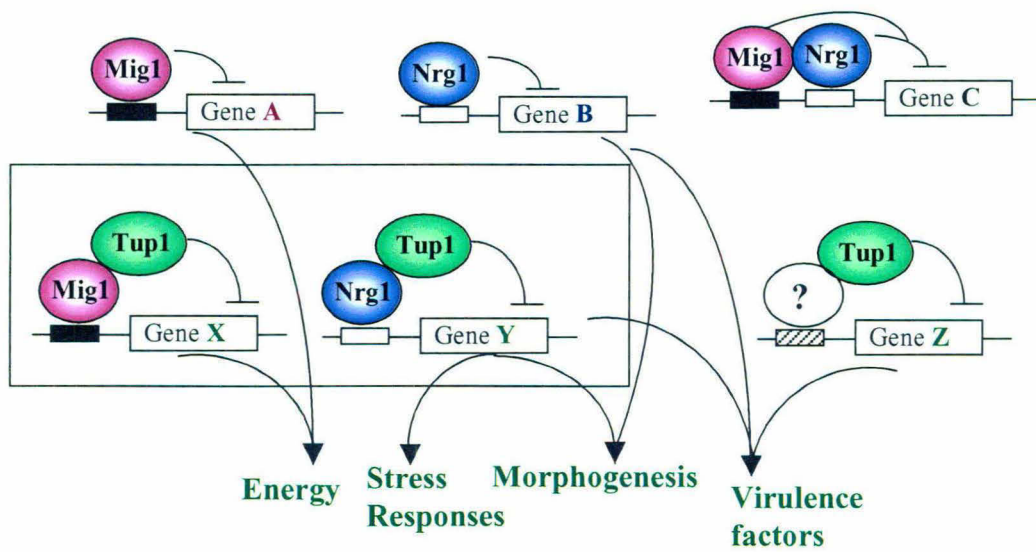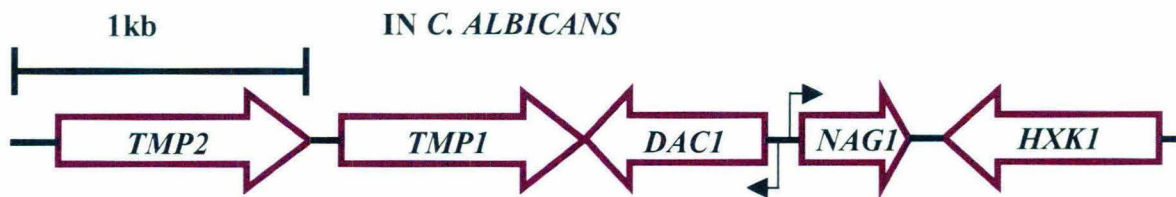
Fig. 2.0. Model summarizing the transcriptional repression mediated by CaTup1p, CaNrg1p and CaMig1p in *C. albicans*.

In *C. albicans*, GlcNAc is transported into cells by GlcNAc permease and then by the sequential action of GlcNAc kinase (*HXK1*), GlcNAc-6-phosphate deacetylase (*DAC1*) and glucosamine-6-phosphate deaminase (*NAG1*) (Fig. 2.1), it is converted to fructose 6-phosphate which is metabolized through the Embden-Meyerhoff pathway. The terminal enzyme of this pathway, *NAG1*, was cloned and characterized previously (Natarajan and Datta, 1993; Kumar et al., 2000).

To study the role of GlcNAc utilization pathway in virulence of the organism, *NAG1* gene was disrupted in wild type *C.albicans* (Singh et. al., 2001). Presence of *HXK1* gene in the same cluster was accidentally discovered while reintroducing a functional copy of *NAG1* into the double disruptant. The reintroduced strain behaved like the homozygous mutant and could not grow in GlcNAc containing medium. Southern blot analysis confirmed the correct integration of the gene, the only possibility was, the reintegration of the *NAG1* disrupted an adjacent gene responsible for GlcNAc catabolism. Sequence analysis of upstream and downstream regions of *NAG1* revealed the presence of *DAC1* and *HXK1*. In an attempt to disrupt *NAG1*, the entire catabolic pathway or the "nag regulon" had been disrupted. The *nag1/dac1/hxk1* mutant generated was unable to utilize GlcNAc as the sole carbon source and demonstrated attenuated virulence in a murine model of systemic candidiasis. Not only that, the mutant was hyper filamentous in various inducing media such as Spider and SLAD (Singh et al., 2001). While reintroducing *NAG1* in the triple mutant, functional copies of both *DAC1* and *NAG1* were integrated generating an *hxk1/hxk1* mutant. The mutant so generated mimicked the phenotypes of the nag regulon mutant (Ghosh et al., communicated). So we speculated that the unique hyper filamentous phenotype of the nag mutant was due to the lack of HXK1 gene. An *hxk1/hxk1* mutant was generated which showed hyperfilamentation phenotype. Several nag mutants were generated in our lab in background of various filamentation pathway mutants such as *efg1/efg1; tpk2/tpk2; cph1/cph1 and ras1/ras1* to determine the role of HXK1 in dimorphism. Except for *efg1/hxk1* double mutant, all the other mutants showed hyperfilamentation phenotype suggesting that HXK1 is involved in this process and works in concert with efg1 morphogenetic regulator (Ghosh et al., communicated).

(A) CHROMOSOMAL ORGANIZATION OF *NAG* GENE CLUSTER IN *C. ALBICANS*

1kb

TMP2   TMP1   DAC1   NAG1   HXK1

(B) GlcNAc CATABOLISM IN *C. ALBICANS*

GlcNAc

GlcNAc
inducible genes

GlcNAc

Permease

ManNac
GlcN

Epimerase

GlcNAc

Kinase(*HXK1*)

ManNac

GlcNAc-6-phosphate

General
permease

GlcN

Deacetylase(*DAC1*)

GlcN-6-phosphate

Deaminase(*NAG1*)

Fructose-6-phosphate

GLYCOLYSIS
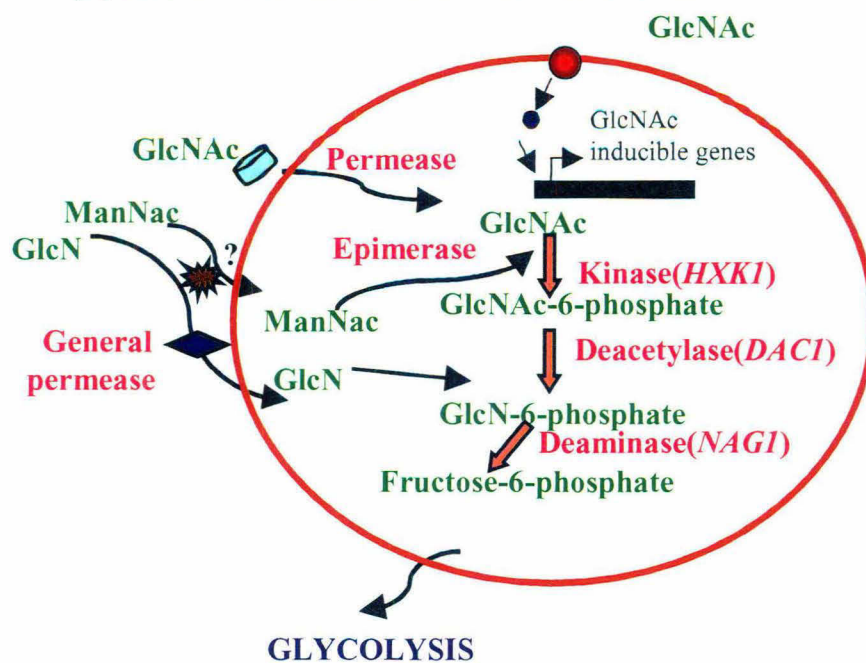
Fig. 2. 1. (A) Chromosomal organization of the Nag gene cluster in *C. albicans* The organization of the cluster genes on chromosome 6 along with their lengths and the direction of the transcription are illustrated. *NAG1* and *DAC1* are transcribed in opposite directions from a bidirectional promoter. **(B) Catabolism of GlcNAc in *C. albicans*.** GlcNAc kinase, GlcNAc-6-phosphate deacetylase and GlcN-6-phosphate deaminase act sequentially on GlcNAc, to generate Fructose-6-phosphate that is fed into the glycolytic pathway. N-acetylmannosamine epimerase converts the N-acetylmannosamine, which is imported in the cell by an unknown permeases (?), into N-acetylglucosamine. Glucosamine can directly enter the cell via a general sugar permease and gets converted to Glucosamine-6-phosphate by the action of a GlcN-kinase.

HXK1 was cloned and disrupted in *C.albicans*. The double disruptant was hyperfilamentous in hyphal inducing media and mimicked the Nag mutant. We tried to decipher the role of HXK1 in GlcNAc catabolism and its contribution to morphological transition.

2.4. Hyphal Specific Gene Expression

The conditions that induce hyphal growth (box) also induce the expression of hyphal-specific genes (HSGs). Identifying the HSGs is complicated by the fact that the conditions that induce morphogenesis will also induce other cell responses are not necessarily connected with the morphogenesis but are required for physiological adaptation to the new environment. For the most part, induction of pseudohyphae requires combination of two environmental conditions such as high temperature and serum or high temperature and neutral pH. So, a gene is only considered to be an HSG when it is induced during hyphal development, but not when only one of these conditions applies (Brown, 2002). Many of the HSGs that have been isolated so far encode known or putative virulence factors. These include genes that encode several secreted aspartylproteases (SAP4, 5, 6), cell-wall proteins (HWP1), adhesions, ALS3 and ALS8 and proteins that are required for invasive growth (RBT1).

None of the HSGs that have been isolated so far are actually required for hyphal or pseudohyphal morphogenesis. Rather they are coordinately induced by the signals that also induce morphogenesis. Their expression is blocked in an *efg1efg1* mutant and is induced in *tup1 tup1, nrg1 nrg1* or *rfg1 rfg1* mutants, indicating that they might be among the targets of the morphogenesis signaling pathway.

2.5. Hexokinase

It has been reported that in plants, *Hexokinase* has a multifarious role. It not only catalyzes the ATP-dependent phosphorylation of glucose, but also senses glucose level and its phosphorylation status, transmitting this information to the nucleus through a signal transduction pathway (Fig.2.2.). The catalytic and the glucose sensing properties of this

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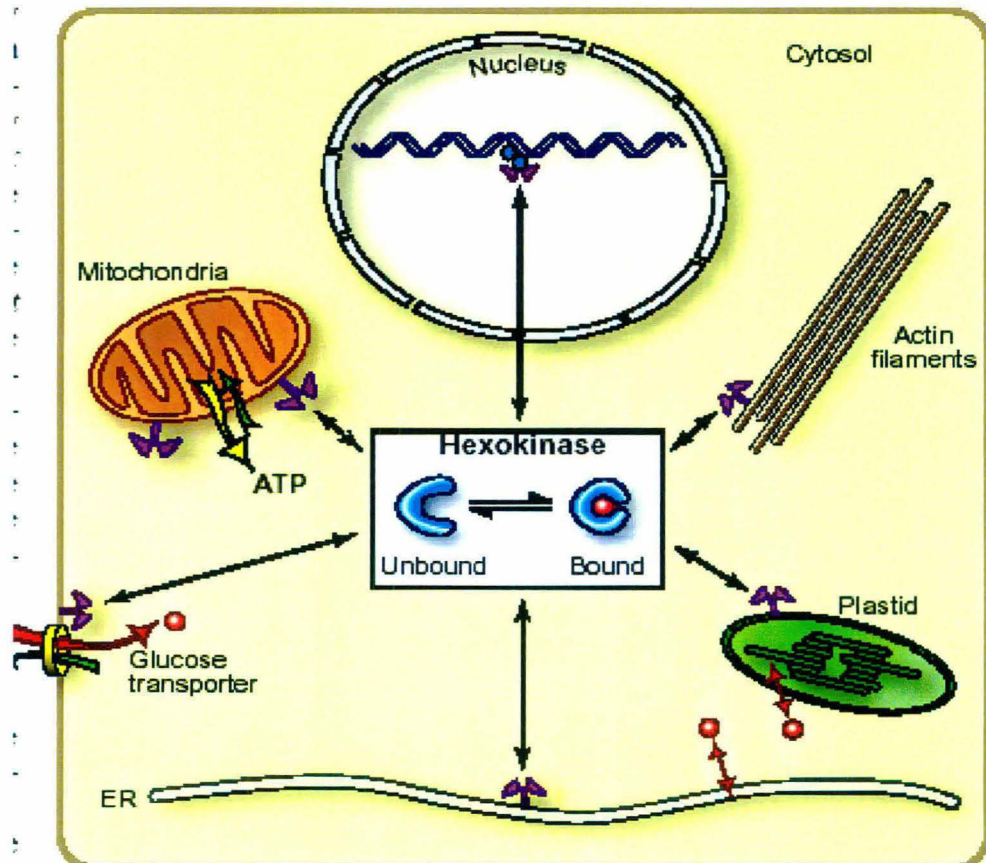
Fig.2.2.Sticking to membranes. Hexokinases associate with various cellular membranes, and this association affects their activity. These enzymes are not only involved in glucose sensing and metabolism but also in signal transduction. This duality is achieved by switching between a bound and unbound form that interacts with different proteins, such as regulatory DNA-protein complexes in the nucleus. Receptors for hexokinases (purple) must be present to enable differential targeting of these enzymes to different subcellular locations. Hexokinases associate with membranes of subcellular compartments, such as the endoplasmic reticulum (ER) and mitochondria.

enzyme could be separated in *Arabidopsis* (Moore et al., 2003). Catalytically inactive *HXK1* mutants were constructed by replacement of conserved amino acids in the ATP binding site. The mutants so generated had lost ATP binding property, but still retained the sugar binding ability. It was shown that though the mutants could not catabolize glucose, they could exhibit similar glucose signaling function as the wild type *HXK1* and able to retain morphology.

In *E.coli*, yeast, mammals, and plants, Hexokinase (HXKs) and other sugar kinases are most ancient and evolutionarily conserved sugar sensors. Most organisms make multiple forms of Hexokinase. Unicellular yeast produce hexokinases P1 and P2 and glucokinase. Mammalian genomes encode two classes of hexokinases: the first comprises of low affinity glucokinase; the second contains three high affinity hexokinases I, II and III that form dimers. Isoforms of this hexokinase interact not only with each other to form dimers, but also with other proteins and with cellular membranes. This enzyme has various sub cellular localizations (Frommer et al., 2003). In addition to their cytosolic localization, hexokinase isoforms are found to be associated with membranes of the endoplasmic reticulum and plasma membrane (Travis et al., 1999) and nucleus. Some hexokinase isoforms are also found bound to two different binding sites on mitochondria, where they may be coupled to ATP production. In plants, hexokinases are associated with the chloroplast outer envelope, where they might help in glucose export by phosphorylating glucose before it enters the cytosol (Wiese et al., 1999). Mammalian glucokinase is associated with the actin cytoskeleton, but depending on physiological conditions, it can move to the nucleus and alter gene expression (Murata et al., 1997; Herrero et al., 1998). The marked difference in the sub cellular localization of hexokinase, its ability to form dimers and interact with other proteins and cellular membranes reflect its diverse activities.

The regulation of glucose metabolism and homeostasis is essential for survival; for example, defects in human hexokinases can result in diabetes, regulation of blood sugar levels in animals, optimal nutrition for yeast, and coordination of photosynthesis and

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storage activity in plants are just a few of the processes requiring constant glucose sensing and regulation of glucose metabolism. Thus, knowledge of signaling pathways and of the proteins that sense glucose and its metabolites will be essential for understanding homeostasis. The findings of Moore *et al.* has shed new light on how even simple enzymes can serve to integrate a variety of essential environmental signals.

```
S.c1    ----MVHLGPKKPQARKGSMADVPKELMDEIHQLEDMFT-VDSETLRKVVKHFIDELNKG
S.c2    ----MVHLGPKKPQARKGSMADVPKELMQQIENFEKIFT-VPTETLQAVTKHFISELEKG
C.a     MTETSISGLRGPKSMYFMEIVDVSSQESSVLSSIVESFTSAVSASNLGVYSDEVLCDIKS
             :          .     .:.**..:  . : .: . ** . : :   * .. :    *.


S.c1    LTKKGGNIPMIPGWVMEFPTGKESGNYLAIDLGGTNLRVVLVKLSGNHTFDTTQSKYKLP
S.c2    LSKKGGNIPMIPGWVMDFPTGKESGDFLAIDLGGTNLRVVLVKLGGDRTFDTTQSKYRLP
C.a     SLKENSPITMLPNYNVS-PTGDEHGQYLVIDLGGSTLRIAVVDISKPHPNLSRSERITIV
         *:.. *.*:*.: :. ***.* *::*.*****:.**::*.:.  :.  : ..:  :


                    PHOSPHATE 1 BINDING DOMAIN


S.c1    HDMRTTK------HQEELWSFIADSLKDFMVEQELLNTKDTLPLGFTFSYPASQNKINEG
S.c2    DAMRTTQ------NPDELWEFIADSLKAFIDEQFPQGISEPIPLGFTFSFPASQNKINEG
C.a     VEKSWIIGNDFKRIDGEFFKYIGSKINEILMGQNVIDVKSVINTGITWSFPLETTDYNRG
           *::.:*...:: ::  *   . ...:  *:*:*:*  . ... *.*


                    SUGAR BINDING DOMAIN


S.c1    ILQRWTKGFDIPNVEGHDVVPLLQNEISKRE--LPIEIVALINDTVGTLIASYYTDPETK
S.c2    ILQRWTKGFDIPNIENHDVVPMLQKQITKRN--IPIEVVALINDTTGTLVASYYTDPETK
C.a     KIKHVSKGYTVGEDIYDKDLKMVLEDTLRQEYGLTLDVQSILNDSLAVYSAGCFIDSKMK
         :::  :**:  : :   .. : :: ::  ::: :..:: :::**:  .. *. : *.: *


S.c1    MGVIFGTGVNGAFYDVVSDIEKLEGKLADDIPSNSPMAINCEYGSFDNEHLVLPRTKYDV
S.c2    MGVIFGTGVNGAYYDVCSDIEKLQGKLSDDIPPSAPMAINCEYGSFDNEHVVLPRTKYDI
C.a     LAMVLGTGINMCCSLKRSSDIHPSKMLADAT------LFNCELSLFGQNLCKDFATKYDI
         :.::::***:* .   *.  : . *:*       :*** . *.::    ****:


                    PHOSPHATE 2 BINDING DOMAIN


S.c1    AVDEQSP---------------RPGQQAFEKMTSGYYLGELLRLVLLELNEKGLMLKDQ
S.c2    TIDEESP---------------RPGQQTFEKMSSGYYLGEILRLALMDMYKQGFIFKNQ
C.a     IIDKRFAGFSHHFKTFMEPDPITKTLFQPHELMTSGRYLPELTRLVVVDLIEAGEIFQNV
         :*:. .               :.  *..* *:** **  *: **.:::: : *  ::::


S.c1    DLSKLKQPYIMDTSYPARIEDDPFENLEDTDDIFQKDFGVKTT-LPERKLIRRLCELIGT
S.c2    DLSKFDKPFVMDTSYPARIEEDPFENLEDTDDLFQNEFGINTT-VQERKLIRRLSELIGA
C.a     DHQQMYQEYGGFSGELMCFVHE-NDDYDDIHDKLCKAYGWTTVGLSDIVCLKEVVSCIIK
         *  .:: : :  :.     :.   : .: :: :* .* : : :* .*. : :  ::.: . *


S.c1    RAARLAVCGIAAICQKRG-YKTGHIAADGSVYNKYPGFKEAAAKGLRDIYGWTGDASKD-
S.c2    RAARLSVCGIAAICQKRG-YKTGHIAADGSVYNRYPGFKEKAANALKDIYGWTQTSLDDY
C.a     RAAFIVANAIIAFFKLLGSDELGGDVTIGYVGSVLNYFHKYRRLIVEYVN--NAEEAKGI
         *** : . .* *: : * : * .: * * .  *::    :. :  .  ..


                    ADENOSIN INTERACTION DOMAIN


S.c1    PITIVPAEDGSGAGAAVIAALSEKRIAEGKSLGIIGA
S.c2    PIKIVPAEDGSGAGAAVIAALAQKRIAEGKSVGIIGA
C.a     KVDLKLIENSSIIGAAIGAAYHK-------------
         : :   *:.*  ***; ** :
```

Fig. 2.3. Clustal W analysis of HXK1 of *Candida albicans*

A comparison of predicted amino acid sequences of *Candida albicans* GlcNAc kinase, *HXK1* (C.a), *Saccharomyces cerevisiae HXK1* (S.c1) and *HXK2* (S.c2). The ATP binding site and sugar binding site have been indicated with bold letters. The conserved phosphate binding sites and adenosine interaction region have been indicated. Identical and similar residues have been marked with * and : respectively.

3. MATERIALS AND METHODS

3.1.Strains and Plasmids used

The strains and plasmids used in this study have been listed in Table

3.1. A. Strains used in this study

SC5314 (*nag+/nag+*), N216 (*nag/nag*), HLC67(*efg1/efg1*), HLC 67-16-19 (*efg1/efg1 nag/nag*), CAN52 (*ras1/ras1*), HR 1-4-2 (*ras1/ras1 hxk1/hxk1*), A11-1-1 (*cph1/cph1*), AN 8-1-16 (*cph1/cph1 nag1/nag1*), AS1 (*tpk2/tpk2*) and AS1-3-18 (*tpk2/tpk2 nag/nag*).

3.1.B. Plasmids used in this study

Plasmids	Description	Source or reference
pGEM-T Easy	T-vector for cloning TAQ amplified PCR products	STRATAGENE
pHHiBX	*HXK1* cloned in pBS/*Eco*RV	This study
pGT-NRG1	821bp partial clone of *NRG1* in pGEM-T Easy	This study
pGT-ECE1	1026bp partial clone of *ECE1* in pGEM-T Easy	This study
pGT-TUP1	1015bp partial clone of *TUP1* in pGEM-T Easy	This study

3.1.C. Media for growth of *Candida albicans*

1. YPD: 1% Yeast extract, 2% Peptone, 2% Dextrose

2. YPD Agar: YPD with 2% agar

3. SD: 0.67% Yeast nitrogen base without amino-acids (YNB w/o aa), 2% dextrose supplemented with Uridine at the concentration of 25 μg/ml, when required.

4. SD Agar: SD with 2.0% agar.

5. Spider (pH 7.2): 1% Nutrient Broth, 1% Mannitol, 0.2% K_2HPO_4 , 1.35% Bactoagar for plates. Adopted from Liu et al., 1994.

6. 2.5mM GlcNAc in salt base: 0.45% NaCl and 0.335% YNB w/o aa. Adopted from Delbruck and Ernst, 1993.

7. 20% Serum: 20% Bovine Calf Serum in YPD.

3.1.D. Growth and Maintenance of strains

Candida albicans strain SC5314 was routinely cultured in YPD or SD media at 30°C at 200rpm shaking. For growing the *ura3* auxotroph, such as CAF3-1 and other *ura* cured transformants of *C.albicans*, SD medium was supplemented with Uridine at the concentration of 25μg/ml.

E.coli strains were cultured in Luria-Bertani broth or agar plates containing 50μg/ml and 75μg/ml ampicillin respectively.

3.1.E. Storage of *C.albicans* and *E.coli* strains

Bacterial and *C.albicans* strains were stored as glycerol stocks at minus 80°C. To the overnight grown cultures, sterile glycerol was added to the final concentration of 15%.

3.2. Media and Solutions

All % shown are on a W/V basis unless mentioned otherwise. All solutions and media were made in Milli-Q and Milli-RO water respectively. All solutions and media were sterilized by autoclaving at 15 lb/ sq inch for 15 minutes or filter sterilized by passing through a 0.22μM Millipore filter. Media used were from Invitrogen or Difco. Chemicals used were of analytical grade (Qualigens or Merck) or molecular biology grade mostly from Sigma, Invitrogen or USB. Restriction enzymes and DNA modifying enzymes were obtained from NEB, MBI-Fermentas, Roche, Promega, Amersham or Perkin Elmer. Buffers provided with the enzymes were used.

3.2.1.Reagents and Buffers used for Agarose Gel Electrophoresis

1. 50X TAE: 242g Tris base, 57.1ml Glacial acetic acid and 100ml 0.5M EDTA, pH 8.0 for 1 liter.

2. Ethidium Bromide:10mg/ml in water.

3. EndoR (6X):   30% Ficoll 400, 60mM EDTA pH 8.0, 0.6% SDS and 0.06% bromophenol blue.

3.2.2. Reagents and Buffers used for RNA isolation and Northern Blotting:

Loading buffe:50% glycerol, 1mM EDTA, 0.002% bromophenol blue and 0.002% xylene cyanol

10X MOPS: 0.2M MOPS (pH 7.0), 20mM sodium acetate, 10mM EDTA. Dissolve 41.8g of (3-[N-morpholino] propanesulfonic acid) in 700ml of sterile DEPC-treated water; adjust pH to 7.0 with 2N NaOH. Add 20ml of DEPC-treated 1M sodium acetate and 20ml of DEPC-treated 0.5M EDTA (pH 8.0). Adjust the volume of the solution to 1 liter with DEPC-treated water. Sterilize the solution by filtration through a 0.45-μm Millipore filter, and store it at room temperature protected from light. The buffer yellows with age if it is exposed to light or is autoclaved.

Methylene blue solution: 0.02% methylene blue in 0.3M Sodium Acetate.

3.2.3. Buffers used for Northern Blot analysis and Hybridization

20X SSC: 175.3 g NaCl and 88.2 g trisodium citrate were dissolved in 800 ml MQ water. The pH was adjusted to 7.0 by adding HCl; volume was made up to 1 liter and sterilized by autoclaving.

3.3. Hyphal induction, RNA isolation and Northern blot analysis

3.3.1. Hyphal Induction

Overnight grown preculture of mutant strains (Table) were diluted 1:100 times in fresh YPD and grown to mid log phase. Cells were harvested, washed twice with water, and starved for 10 hours. The cells were then resuspended in equal volumes of fresh YPD, GlcNAc (2.5mM), 20% Serum, and Spider medium. The cultures were grown with continuous shaking at 200 rpm, under the conditions as below mentioned in the table. Cells were harvested   and stored at minus 80°C for further use. Total RNA was isolated from the samples and northern blot was performed. The blot was probed with specific probes as described in section **3.3.3.2.** Equal quantity of RNA was loaded from each sample in a 1.5% agarose gel and stained with ethidium bromide to ensure equal loading.

Medium	Temperature($^{\text{O}}$C)	Incubation time(hrs)
YPD	30	2
GlcNAc 2.5Mm	37	4
SPIDER	37	2
SERUM 20%	37	2

3.3.2.Isolation of total RNA from *C.albicans* cells

TriPure Method of RNA Isolation:

TriPure isolation reagent was added to the polypropylene tubes containing cell pellet at room temperature. 1 ml reagent was used for each $\sim10^7$ cells.

Equal volumes of 0.45 mm chilled glass beads were added and homogenized for 3 minutes on cyclomixer.

This was incubated at room temperature for 5 minutes to ensure complete dissociation of the nucleoprotein complexes. For phase separation, chloroform was added, 0.2 ml for each 1 ml of TriPure reagent used. The tubes were capped, vortexed vigorously for 15 seconds and incubated at room temperature for 10 minutes.

The tubes were centrifuged at 12,000 g for 15 minutes at 4°C, to separate the solution into three phases.

The colourless upper aqueous phase was collected in a new tube and RNA was precipitated by adding isopropanol (0.5 ml for each 1 ml TriPure reagent used) and incubated at room temperature for 10 minutes.

RNA was pelleted down by centrifuging the tubes at 12,000 g for 10 minutes at 4°C; supernatant was discarded and the pellet was given 75% ethanol wash.

RNA pellet was then air-dried and dissolved in DEPC-treated RNase free water incubating at 55°C for 10 minutes.

3.3.3. Northern Blot Analysis

3.3.3.1. Agarose gel electrophoresis of RNA

Formaldehyde-denatured RNA gel was prepared as described by Sambrook *et al.* (1989), formaldehyde (Glaxo) pH 3-3.5, was used. Formamide was deionised, stored in small aliquots at $-20°C$.

RNA samples (30μg)   10 μl

10xMOPS   3 μl

Formamide   10 µl

Formaldehyde   6 µl

The above components were mixed and incubated at 60°C for 15 minutes and then chilled on ice. 2 µl loading buffer (50% glycerol, 1mM EDTA, 0.002% bromophenol blue and 0.002% xylene cyanol) was added to the above mix.

1.5% (W/V) agarose gel containing 1xMOPS and 2.2.M formaldehyde was used.

The gel was placed in electrophoresis tank with 1 x MOPS buffer for 15 minutes prior to loading.

The denatured RNA was loaded in the wells and was left for 15 minutes.

Gel was run at 80V till the bromophenol blue ran 2/3 distance (3-4 hours).

After electrophoresis, the gel was thrice rinsed in five times the gel volume of DEPC treated water for 30 min, followed by soaking in 20X SSC for 30mins.

RNA was transferred to gene screen membrane using 10XSSC as described (gene screen Plus protocols, NEN), and transferred RNA was UV crosslinked to membrane.

Equal loading was verified by Methylene blue staining (Sambrook *et. al.*, 1989) of parallel lanes.

3.3.3.2. Probe preparation

The plasmids mentioned in the section 3.1.B were digested with *EcoR V / Not I / Eco R I*, gel eluted , purified and used for probe preparation in Northern hybridization studies.

A. Random Priming Reaction:

Labeling was done according to Feinberg and Vogelstein (1983).

1. 100 ng template DNA of fragment was taken in a volume of 15.5 μl sterile MQ-water.

2. Denatured in boiling water bath for 5 minutes and quickly placed in ice for 5 minutes.

3. Centrifuged briefly in the cold.

4. The following reagents were added to the DNA in the order listed:

   2.5 μl 10X Labeling Buffer which includes Random Octadeoxyribonucleotides (NEB Kit)

   3 μl dNTP mixture (1μl of dATP, dTTP, and dGTP)

   2 μl α ^{32}P dCTP (3,000 ci/mmol, 50 μCi) (Amersham, USA)

   1 μl DNA Polymerase I-Klenow fragment (5 units)

   Total reaction volume was 25 μl.

5. Incubated at 37°C for 4 hours or 30°C for 16 hours.

6. The reaction was terminated by adding 5 μl of 0.2 M EDTA and the probe was stored at -20°C till further use.

B. Purification of Labeled Probes:

Probes synthesized were separated from unincorporated nucleotides by filtration through Sephadex® G-50 spin columns. The reaction volume was increased to 100 μl with 1X TE (pH 8.0) before purification. 5μl of purified probe was used to detect for incorporation of radioactive nucleotide using liquid scintillation counter.

C. Preparation of Probe Solution for Hybridization

Salmon sperm DNA (100μg/ml) was added to probe solution was denatured by heating for 5 minutes in a boiling water bath and chilled on ice for 5 minutes before adding to the prehybridization buffer.

3.3.3.3. Hybridization

Hybridization was carried out in hybridization bottles, at 42°C, for 16 hours in a hybridization incubator (Robbins Scientific) with continuous mixing.

A. Post-hybridization Washing

1. The membrane was washed with 2X SSC at room temperature for 10 minutes.

2. The second washing was with 2X SSC/1% SDS at 42°C for 20 minutes. The washing was repeated as per requirement.

3. The membrane was then washed with 0.2X SSC/1% SDS at 42°C for 20 minutes. As per requirement the washing was repeated.

4. During 2nd wash the background count was monitored frequently with a Handheld Monitor (Grieger Muller counter) to avoid washing off specifically bound signal. Only when background signals are high, 3rd wash was performed.

3.3.3.4. Exposing and Developing of Film

1. The membrane was kept securely in a poly bag in wet condition, and radioactive markers were used at different positions to align the blot on the film later.

2. The blot was exposed to Kodak X-Omat™ film in a film cassette, and incubated at -- 80°C for 16-20 hours.

3. The film was developed, aligned with the blot and tracing, to mark the positions of the bands.

4. RESULTS AND DISCUSSION

4.1. Expression of *HXK1* gene in various filamentous inducing media

As *HXK1* or *Nag* regulon mutated strains of *C.albicans* showed hyper filamentation in filament inducing media like 2.5Mm GlcNAc, Spider and Serum (20%). So, we wanted to study the expression of *HXK1* gene under the same conditions. The wild type SC5314 strain was induced with YPD, 2.5Mm GlcNAc, Spider and Serum (20%) (as described in section 3.3.1.). Total RNA was isolated and Northern blot analysis was done. The blot was probed with *HXK1* specific probe. The Northern analysis results showed the differential expression of *HXK1* in filamentous inducing and non-inducing media; basal expression in YPD and higher expression in2.5Mm GlcNAc, Spider and Serum (20%) (Fig.4.1.). Reasonably this expression pattern is supporting our morphological observations which indicate that *HXK1* is getting induced more to suppress or fine-tune the filamentation (morphogenesis).

4.2. Expression of *HXK1* gene in various filamentation pathway mutants

Various signaling pathways are involved in dimorphic transition of *C.albicans*. Ras1p, acts upstream of MAP Kinase and cAMP dependent PKA pathways. It controls hyphal formation in both solid and liquid media as CAN52; *ras1/ras1* strain is severely compromised in all liquid as well as solid inducing media checked (Feng et al., 1999). Tpk2p, one of the two isoforms of PKA catalytic subunit is important for morphogenesis in liquid inducing media as *tpk2/tpk2* strain, AS1 exhibits reduced filamentation in serum and Spider (Sonneborn et al., 2000). The transcription factor Efg1p is responsible for hyphae formation in most inducing conditions (Stoldt et al., 1997; Lo et al., 1997; Leng et al., 2009). The *efg1/efg1* mutant HLC67 is unable to form hyphae in liquid serum and displays pseudohyphal chains on solid plates (Lo. et al., 1997). A significant block in filamentation is observed on Spider plates (Stoldt et al., 1997).

Since *hxk1/hxk1* strain is hyper filamentous in most inducing media, we wanted to check if there is any difference in the expression of *HXK1* gene in filamentation pathway mutants.

The strains used were SC5314 (wild type), AS1 (*tpk2/tpk2*), HLC67 (*efg1/efg1*), CAN52 (*ras1/ras1*), A11-1 -1 (*cph1/cph1*) and N2-1-6 (*nag/nag*). The results showed that *HXK1* is expressed differentially in all the strains except for N2-1-6(*nag/nag*), when grown in YPD, GlcNAc, Spider and Serum media. HLC67 (*efg1/efg1*), CAN52 *(ras1/ras1)* showed higher expression of *HXK1*, under filamentation inducing conditions (Fig.4.2., 4.3.).As similar to that of SC5314 strain (Fig.4.1.) but AS1 *(tpk2/tpk2)* showed higher expression of *HXK1* in Spider medium (Fig.4.4.). A11-1-1 *(cph1/cph1)* showed lower expression of *HXK1* in serum (Fig.4.5.).Further studies on these expressions will elucidate these results.

4.3. Gene Expression studies in Nag mutant strains

4.3.1. Expression of hyphal specific genes in Nag regulon mutant

Several hyphal specific genes of *C.albicans* have been identified, such as *HWP1* which encodes for a protein of unknown function that is homologous to GPI anchor protein (Staab and Sundstrom, 1998). It is present in filamentous condition and is downstream of *EFG1* (Sharkey et al., 1999). The other hyphal specific gene identified is *ECE1* (Birse et al., 1993). It is expressed during cell elongation (Sharkey et al., 1999). Adherence is an important parameter for pathogenicity. In 1996, Gale et al., reported *INT1* gene linked to adhesion, filamentous growth and virulence in *C.albicans*. It is a surface protein with similarity to vertebrate integrins. Disruption of this gene causes reduced adhesion, hyphal growth and virulence in mice (Gale et al., 1998). We were interested to study the expression pattern of these genes in *nag/nag* mutant strain under different inducing conditions.

The expression of hyphal specific genes *ECE1* (Fig.4.6. to 4.10.) was studied in SC5314 (*nag+/nag+*), N216 (*nag/nag*), HLC67(*efg1/efg1*), HLC 67-16-19 (*efg1/efg1 nag/nag*), CAN52 (*ras1/ras1*), HR 1-4-2 (*ras1/ras1 hxk1/hxk1*), A11-1-1 (*cph1/cph1*), AN 8-1-16 (*cph1/cph1 nag1/nag1*), AS1 (*tpk2/tpk2*) and AS1-3-18 (*tpk2/tpk2 nag/nag*). *ECE1* was expressed in all filamentation inducing conditions except for GlcNAc induction in *nag* mutants but it was not expressed in YPD. It is overexpressed in hyper filamentous condition. These results are in correlation with hyper filamentous morphology of

C.albicans. and *INT1* was studied in the *hxk1* mutant. *ECE1* was expressed in all filament inducing conditions but it was not expressed in YPD. It is overexpressed in hyperfilamentous conditions. These results are in correlation with hyperfilamentous morphology.

4.3.2. Expression of hyphal repressor genes in Nag regulon mutants

Filamentation is a complex event resulting from transcription and repression of many genes. The *C.albicans TUP1* plays a significant role in filament repression. A homozygous mutant of *TUP1* is constitutively filamentous (Braun and Johnson, 1997). Similarly *NRG1* (Braun et al., 2001; Murad et al., 2001b) and *RBF1* (Ishii et al., 1997) are supposed to form a repressor complex along with *TUP1*. Since N2-1-6 (*nag/nag*), HR1-4-2 (*ras/ras hxk1/hxk1*), AN8-1-16 (*cph1/cph1 nag/nag*) and AS1 3-18 (*tpk2/tpk2 nag/nag*) gave hyper filamentous phenotype, we were interested to check the expression of few repressor genes. Both *TUP1* and *NRG1* transcripts could be identified in all conditions tested (Fig.4.11 to 4.16.) *TUP1* expression was observed less in GlcNAc induction. Further work has to be done to determine exact mechanism of regulation.

4.4. CONCLUSION

In plants, *HXK1* has a multifarious role. It not only catalyzes the ATP-dependent phosphorylation of glucose, but also senses glucose level and its phosphorylation status, transmitting this information to the nucleus through a signal transduction pathway. The catalytic and the glucose sensing properties of this enzyme could be separated in an *Arabidopsis* mutant that lacked hexokinase property (Moore et al., 2003). Catalytically inactive *HXK1* mutants were constructed by replacement of conserved amino acids in the ATP binding site. The mutants so generated had lost ATP binding property, but still retained the sugar binding ability. It was shown that though the mutants could not catabolize glucose, they could exhibit similar glucose signaling function as the wild type *HXK1*.

Studies of glucose kinase in other systems have shown that this enzyme has various sub cellular localizations (Frommer et al., 2003). In addition to their cytosolic localization, hexokinase isoforms are found to be associated with membranes of the endoplasmic reticulum and plasma membrane (Travis et al., 1999). Some hexokinase isoforms are also found bound to two different binding sites on mitochondria, where they may be coupled to ATP production. In plants, hexokinases are associated with the chloroplast outer envelope, where they might help in glucose export by phosphorylating glucose before it enters the cytosol (Wiese et al., 1999). Mammalian glucokinase is associated with the actin cytoskeleton, but depending on physiological conditions, it can move to the nucleus and alter gene expression (Murata et al., 1997; Herrero et al., 1998). The marked difference in the sub cellular localization of hexokinase, its ability to form dimers and interact with other proteins and cellular membranes reflect its versatile activity.

The GlcNAc kinase gene *HXK1* too has a multifarious role in *C.albicans* physiology. It not only phosphorylates GlcNAc, but negatively regulates the filament formation in inducing media. The genetic inactivation of Hxk1p resulted in the stimulation of filamentous growth in several inducing solid media such as Spider agar and SLAD. The hyper filamentous phenotype is more evident in a nonfilamentous strain CAN52, where both the alleles of *RAS1* gene have been mutated. In this strain, the double disruption of *HXK1* gene causes hyper filamentous phenotype in both liquid as well as solid media. This suggests that Hxk1p participates in the general pathway of yeast to filament transition.

Since earlier reports have suggested *EFG1* to be the main regulator of filamentation under most of the inducing conditions. On the basis of our expression studies the hyper filamentous morphology of *hxk1/hxk1* mutant is supposedly independent of the *EFG1* regulation. But at the same time this does not rule out the possibility of *HXK1* interaction with *EFG1* at the DNA level.

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The mechanism of repression could be defined to some extent as we were able to detect comparatively higher *HXK1* transcript levels in filamentous inducing media than non inducing media. It can be argue that the hyperfilamentous phenotype of H 8 1 108 (*hxk1/hxk1*) or N216 (*nag/nag*), HR1-4-2 (*ras1/ras1 hxk1/hxk1*), AN 8-1-16 (*cph1/cph1 nag/nag*) and AS1 3 18 (*tpk2/tpk2 nag/nag*) is due to lose of repressor role of *HXK1*. Interestingly in *hxk1/hxk1* mutant strain, the expression of one hyphal specific gene *ECE1* is comparatively higher in filament inducing conditions, indication that *HXK1* maintain a balance on some hyphal specific genes and the regulation of metabolism.

Our results suggest that, like glucose kinases in other organisms, GlcNAc kinase to has catalytic as well as regulatory roles. It is not unlikely that it might possess a separate catalytic domain for phosphorylation of GlcNAc and one or more regulatory domains for sensing various environmental cues and regulating filament formation. It may be possible that *HXK1* interacts with repressor complex for DNA binding and helps in the binding of the repressor complex to filament specific genes. Anyhow, further work has to be done to delineate the exact role of *HXK1*.
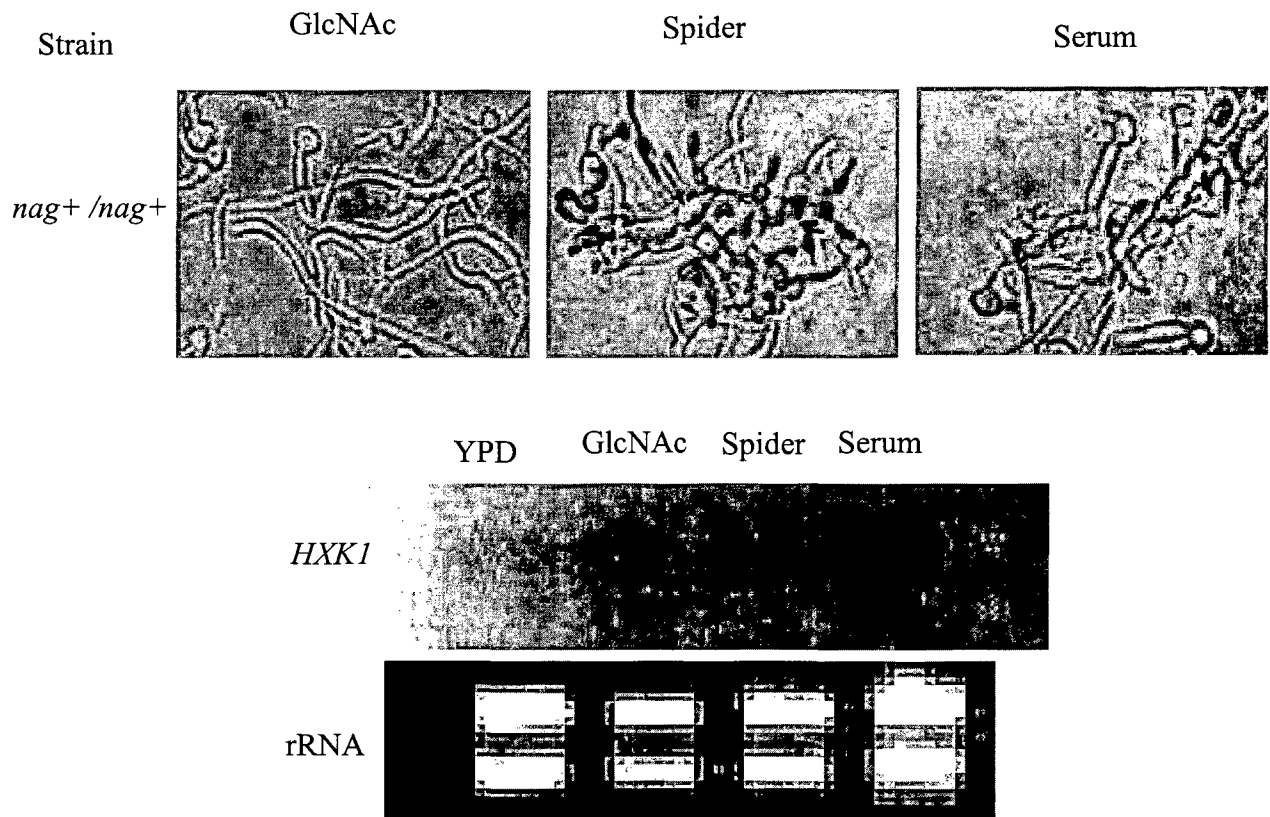
Fig.4.1. **Expression of** *HXK1* **gene in differentation inducing media** Northern blot analysis to show the expression of *HXK1* in various filamentation inducing media. Total RNA was isolated from SC5314 strain induced in YPD , GlcNAc, Spider and 20% Serum. 20μg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, EtBr staining of rRNA was used to ensure equal loading of RNA
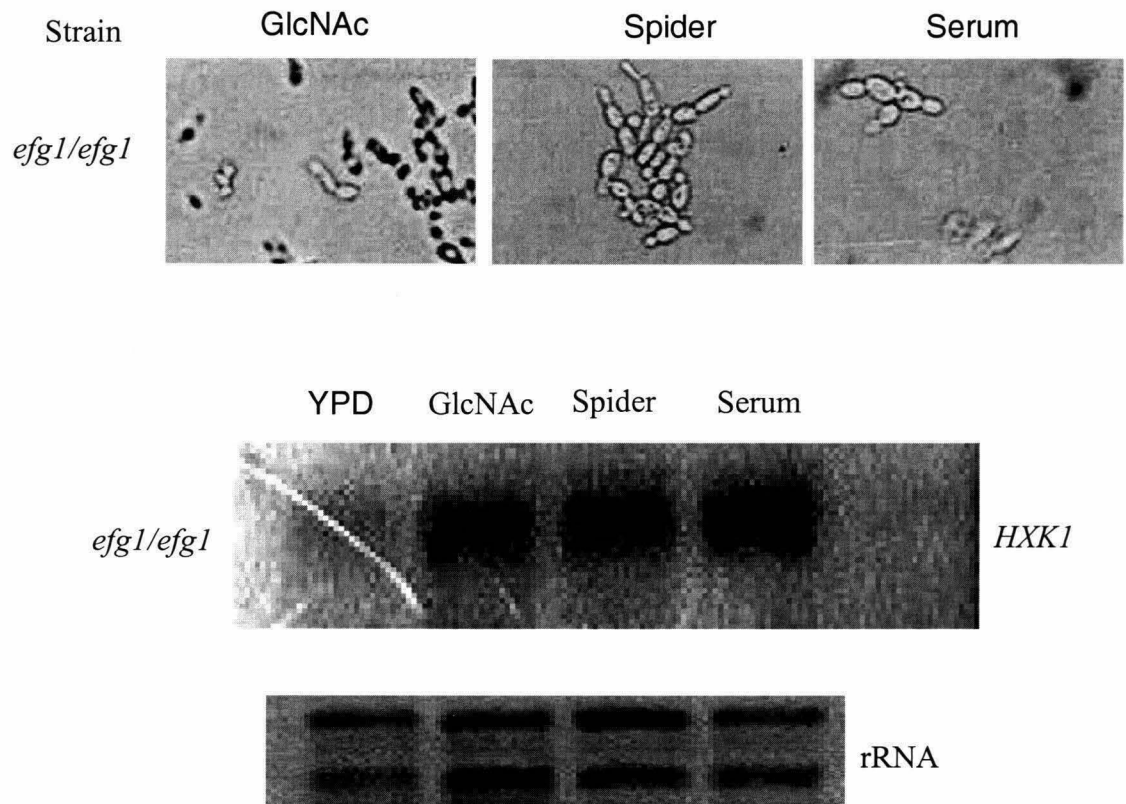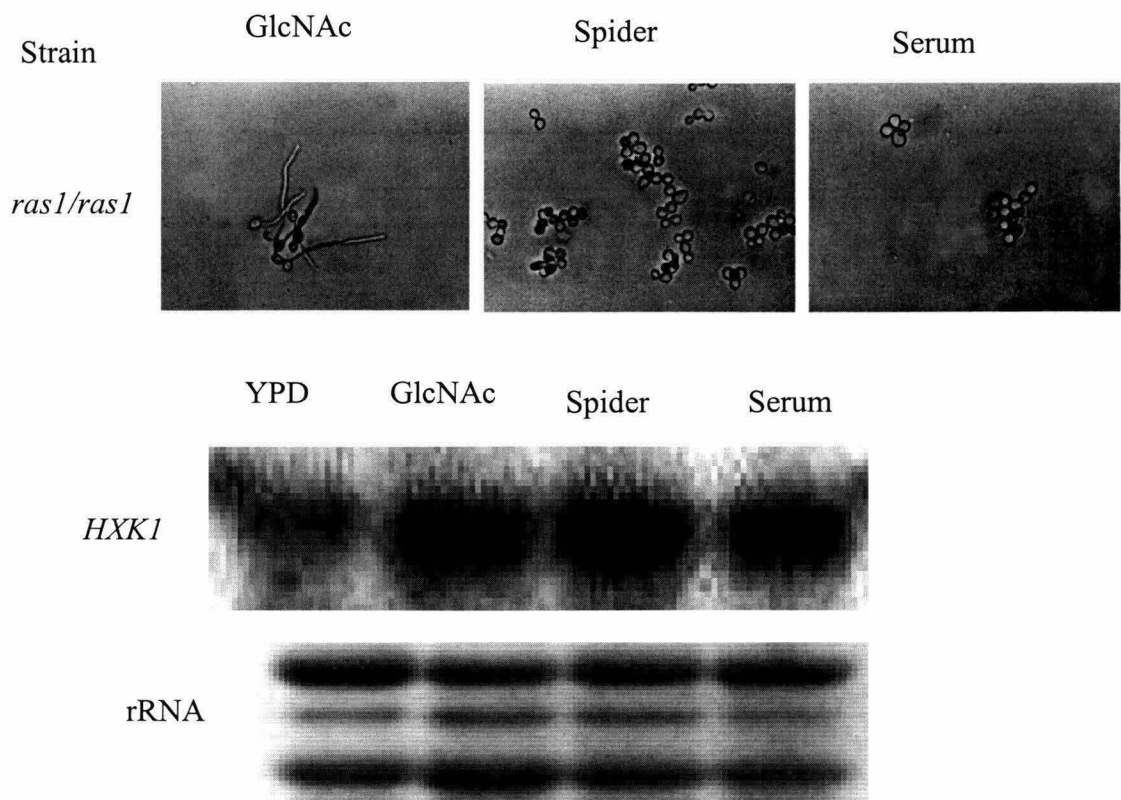
Strain	GlcNAc	Spider	Serum
efg1/efg1			

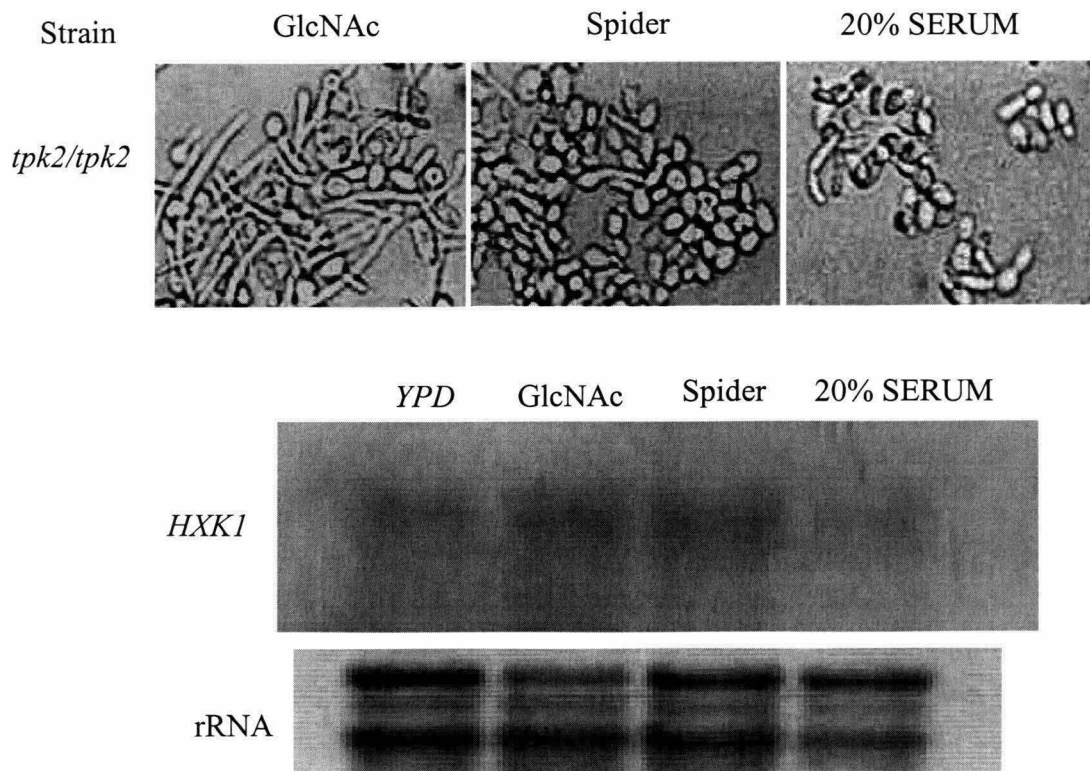|	YPD	GlcNAc	Spider	Serum	
efg1/efg1					*HXK1*
					rRNA

Fig.4.2. Expression of *HXK1* gene in differentation inducing media Northern blot analysis to show the expression of *HXK1* in various filamentation inducing media. Total RNA was isolated from HLC67 (efg1/efg1) strain induced in YPD , GlcNAc, Spider and 20% Serum. 20µg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA
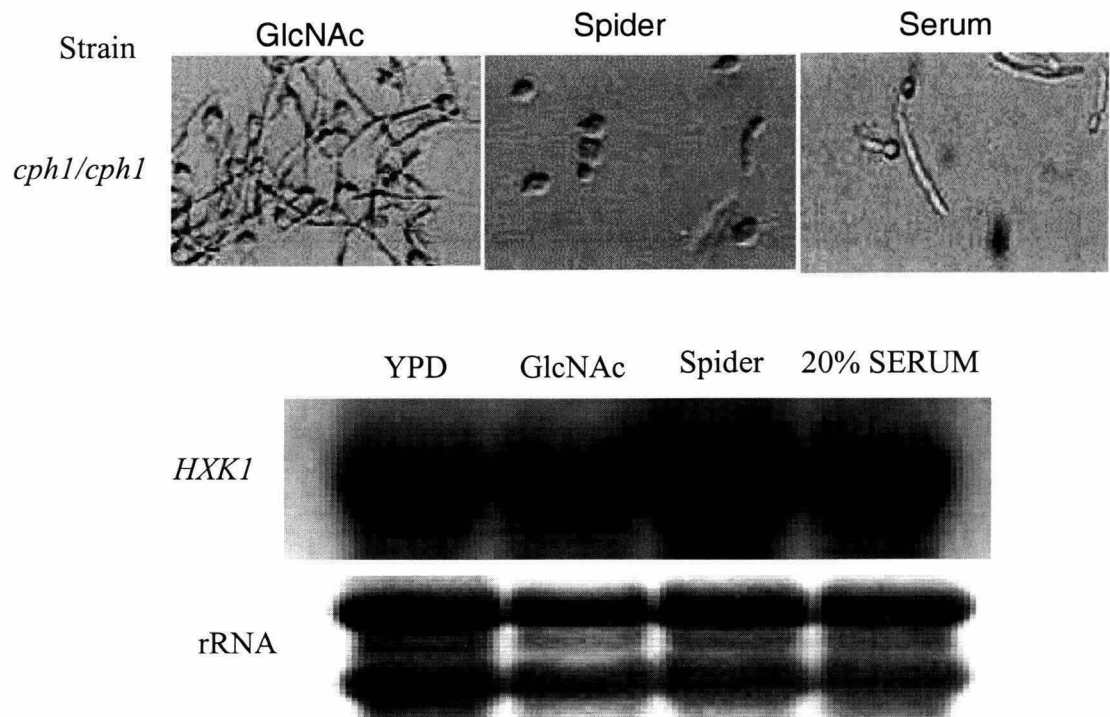
Strain   GlcNAc   Spider   Serum

ras1/ras1

YPD   GlcNAc   Spider   Serum

HXK1

rRNA

Fig.4.3. Expression of *HXK1* gene in differentation inducing media. Northern blot analysis to show the expression of *HXK1* in various filamentation inducing media. Total RNA was isolated from CAN52 (ras/ras) strain induced in YPD , GlcNAc, Spider and 20% Serum. 20μg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA

Strain   GlcNAc   Spider   20% SERUM

tpk2/tpk2

         YPD   GlcNAc   Spider   20% SERUM

HXK1

rRNA

Fig.4.4. Expression of *HXK1* gene in differentation inducing media Northern blot analysis to show the expression of *HXK1* in various filamentation inducing media. Total RNA was isolated from AS1 3 18 (tpk2/tpk2) strain induced in YPD , GlcNAc, Spider and 20% Serum. 20µg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA

Strain	GlcNAc	Spider	Serum
cph1/cph1			

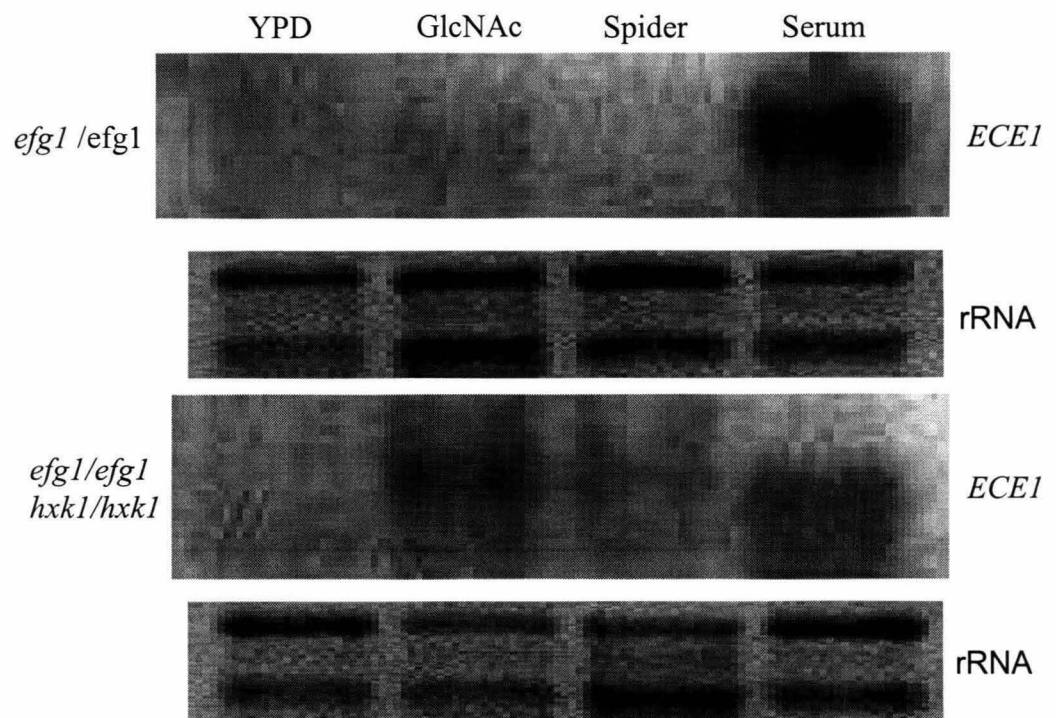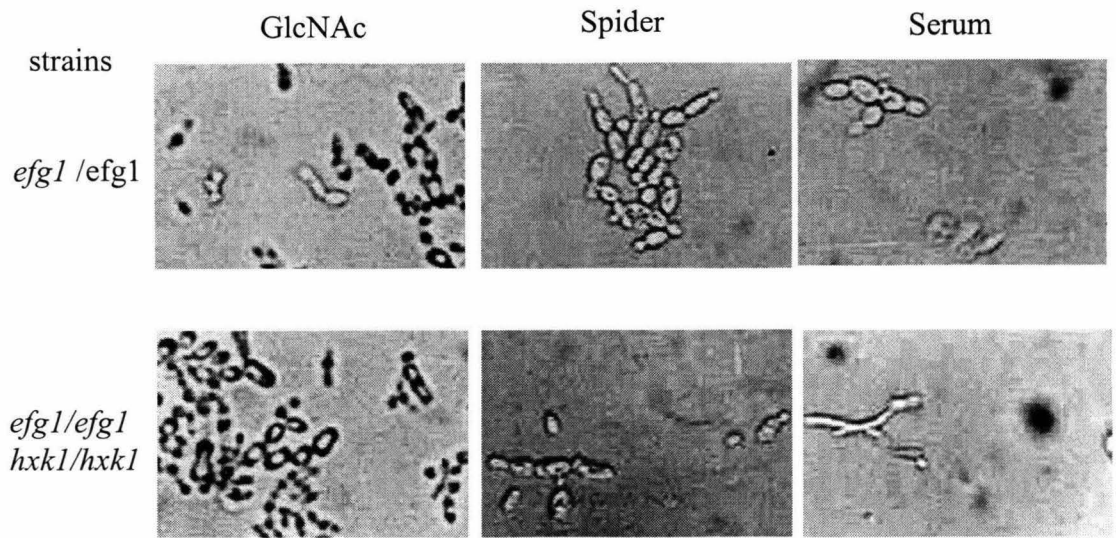|	YPD	GlcNAc	Spider	20% SERUM
HXK1				
rRNA				

Fig.4.5. Expression of *HXK1* gene in differentation inducing media. Northern blot analysis to show the expression of *HXK1* in various filamentation inducing media. Total RNA was isolated from A11 1 1 (cph1/cph1) strain induced in YPD , GlcNAc, Spider and 20% Serum. 20µg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA
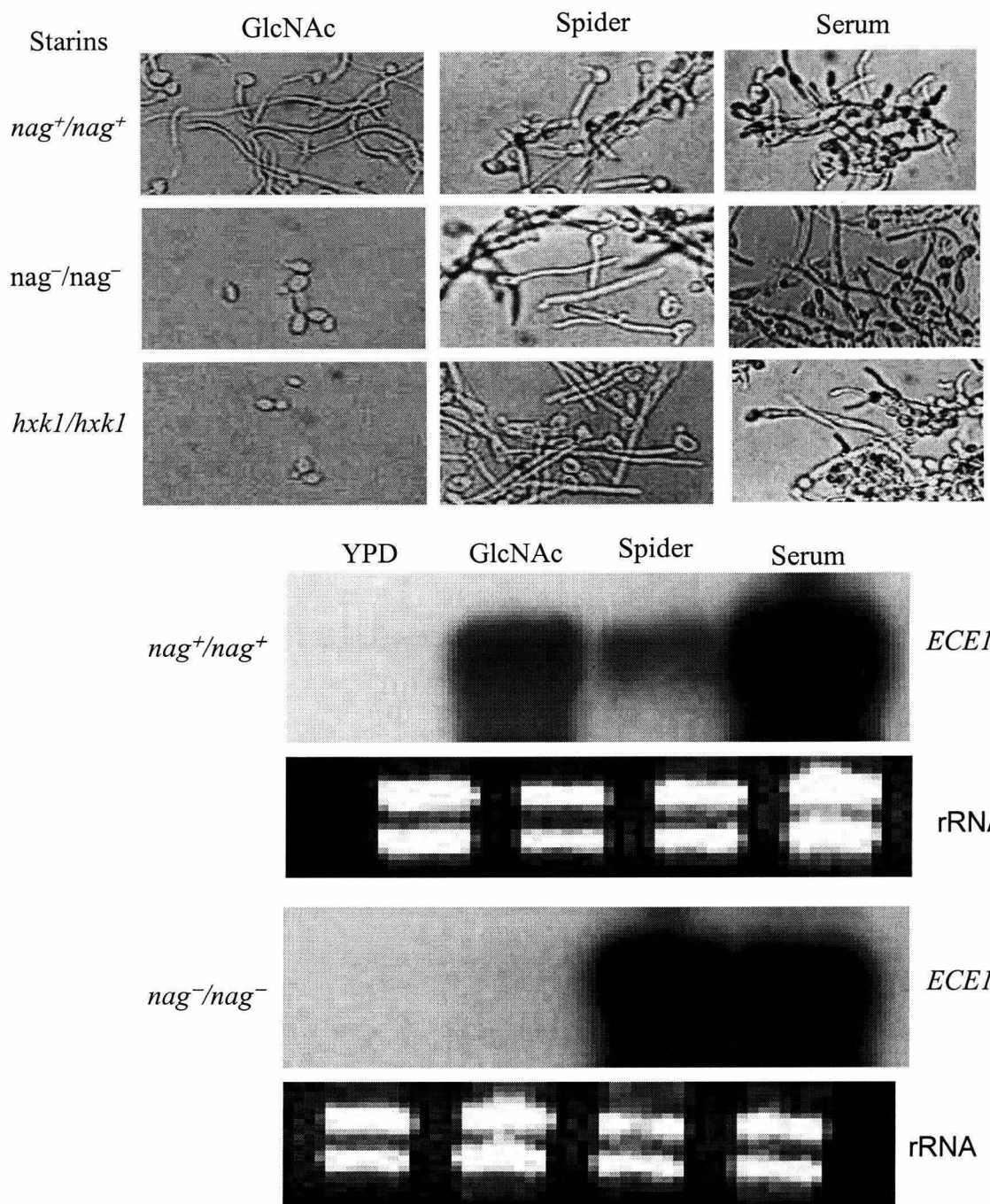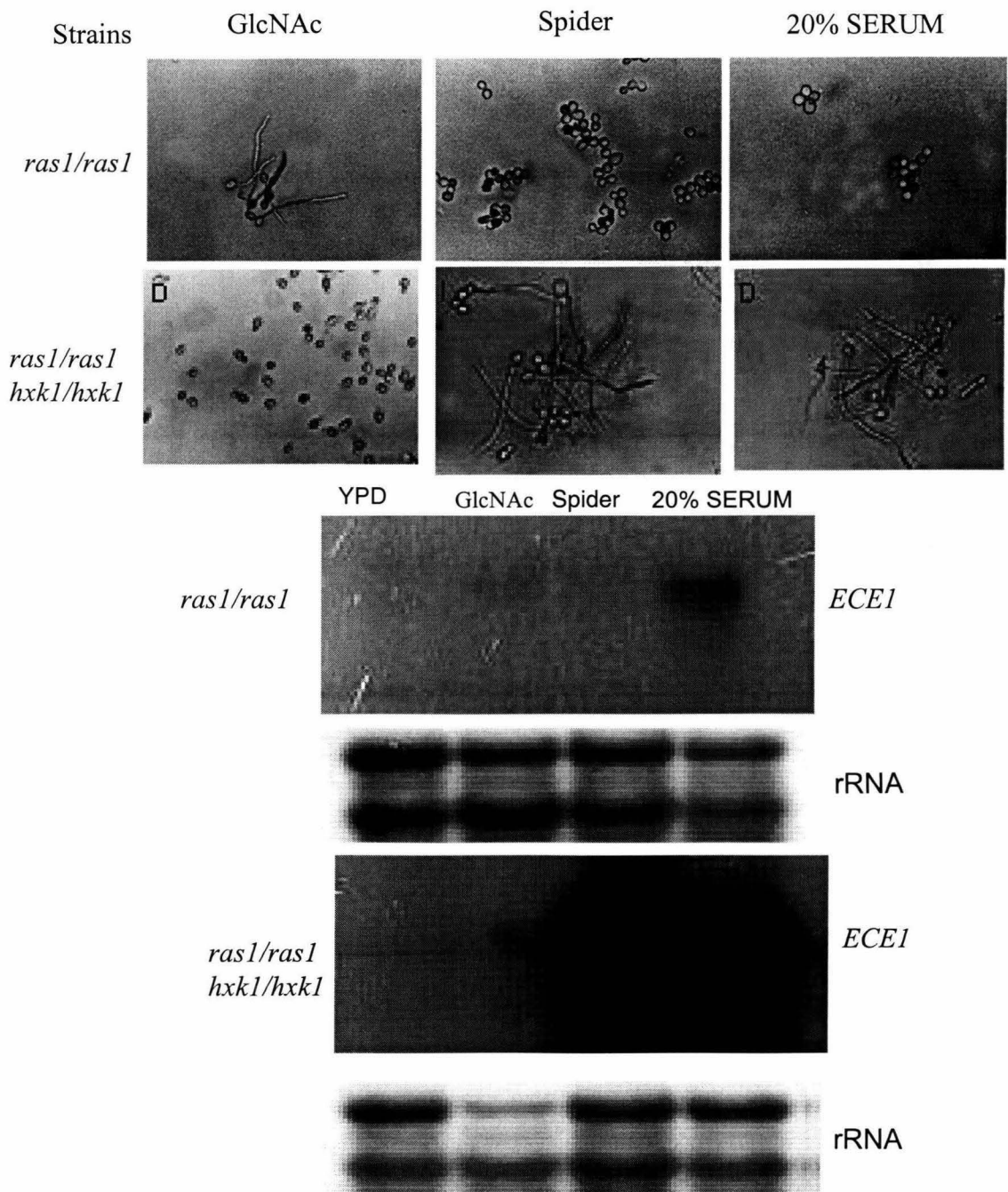
Fig. 4.6. Expression of *ECE1* gene in differentation inducing media. Northern blot analysis to show the expression of *ECE1* in various filamentation inducing media. Total RNA was isolated from SC5314 (*nag+/nag+)* and N216 (*nag-/nag-*) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20µg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, EtBr staining of rRNA was used to ensure equal loading of RNA

Fig.4.7. Expression of *ECE1* gene in differentation inducing media Northern blot analysis to show the expression of *ECE1* in various filamentation inducing media. Total RNA was isolated from HLC67 (*efg1/efg1*) and HLC67-16-19 (*efg1/efg1,hxk1/hxk1*) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20µg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA

Strains	GlcNAc	Spider	20% SERUM
ras1/ras1			
ras1/ras1 hxk1/hxk1			

	YPD	GlcNAc	Spider	20% SERUM	
ras1/ras1					ECE1
					rRNA
ras1/ras1 hxk1/hxk1					ECE1
					rRNA

Fig.4.8. Expression of *ECE1* gene in differentation inducing media Northern blot analysis to show the expression of *ECE1* in various filamentation inducing media. Total RNA was isolated from CAN52 (*ras/ras*) and HR 1 4 2 (*ras/ras, hxk1/hxk1*) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20µg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA
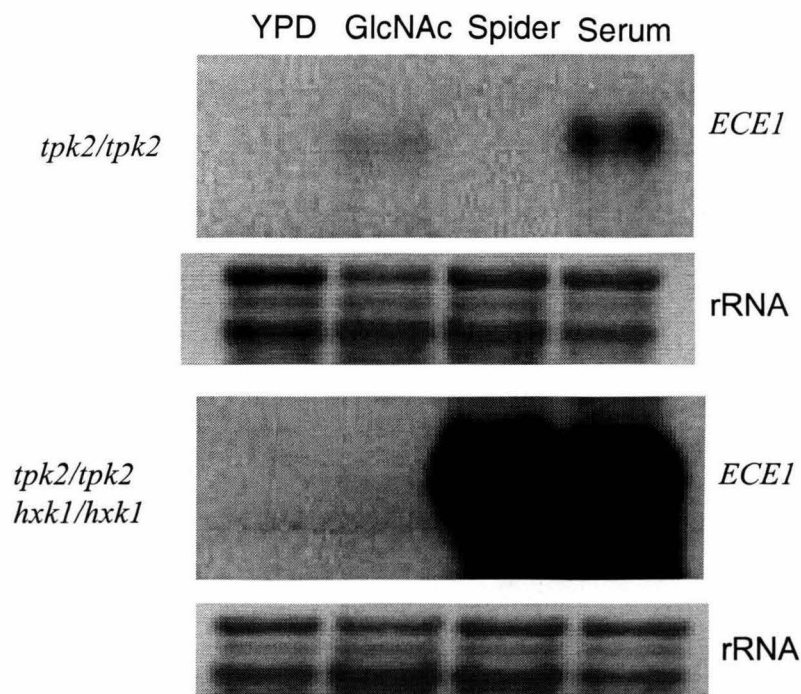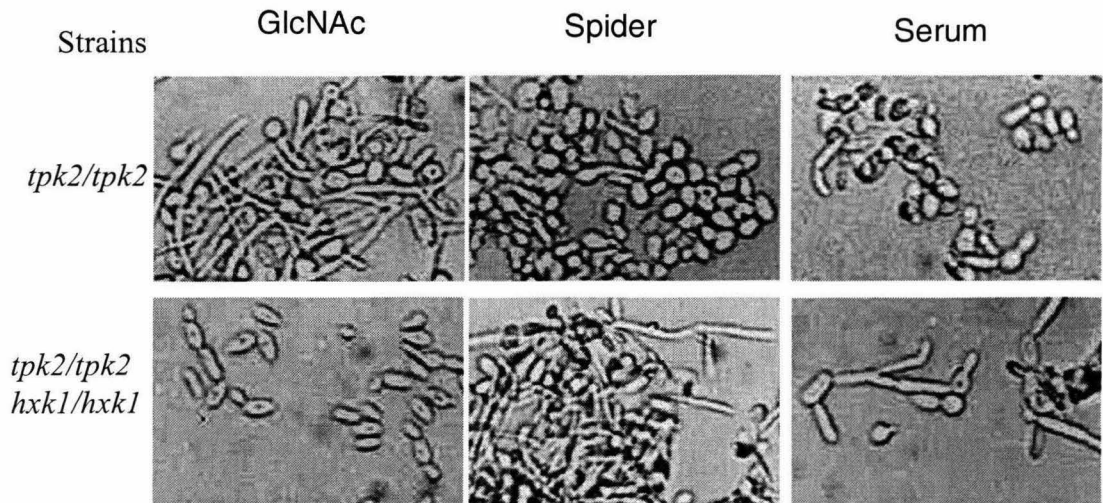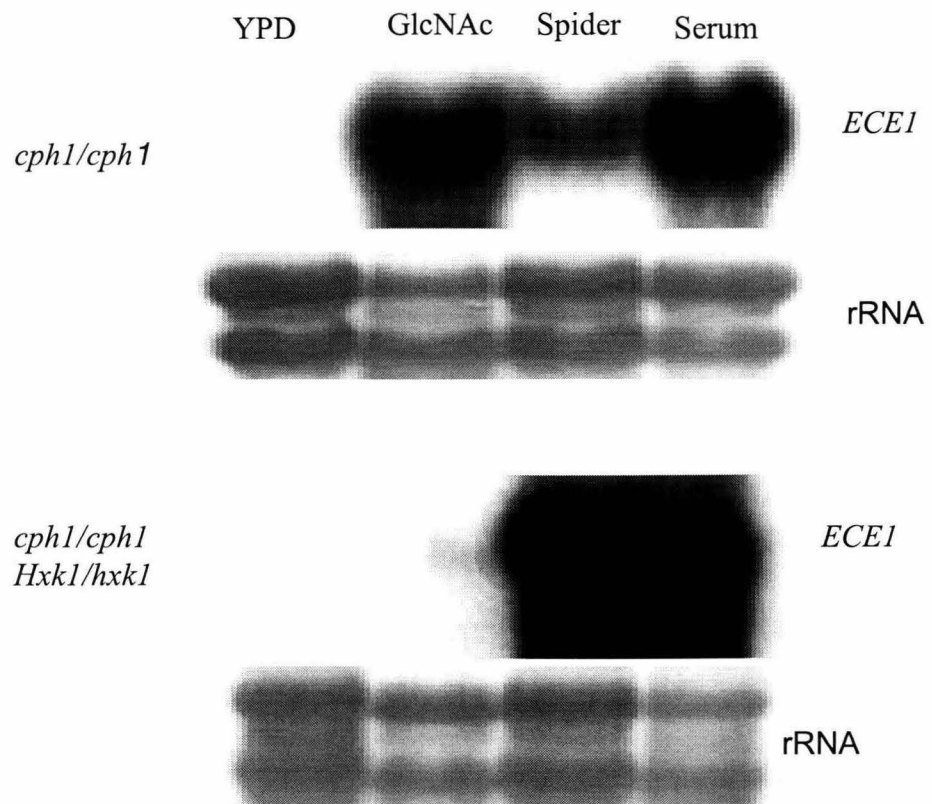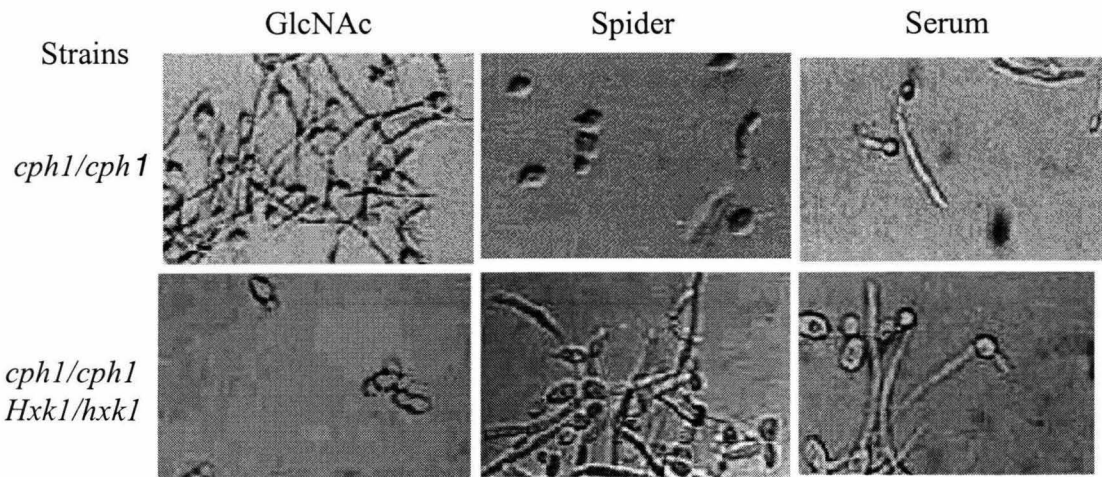
Fig. 4.9. Expression of *ECE1* gene in filamentation inducing media Northern blot analysis to show the expression of *ECE1* in various filamentation inducing media. Total RNA was isolated from AS1 (*tpk2/tpk2*) and AS1 3 18 (*tpk2/tpk2, hxk1/hxk1*) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20µg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA

Fig. 4.10.Expression of *ECE1* gene in differentation inducing media Northern blot analysis to show the expression of *ECE1* in various filamentation inducing media. Total RNA was isolated from A11 1 1 (*cph1/cph1*) and AN8 1 16 (*cph1/cph1 hxk1/hxk1*) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20µg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA
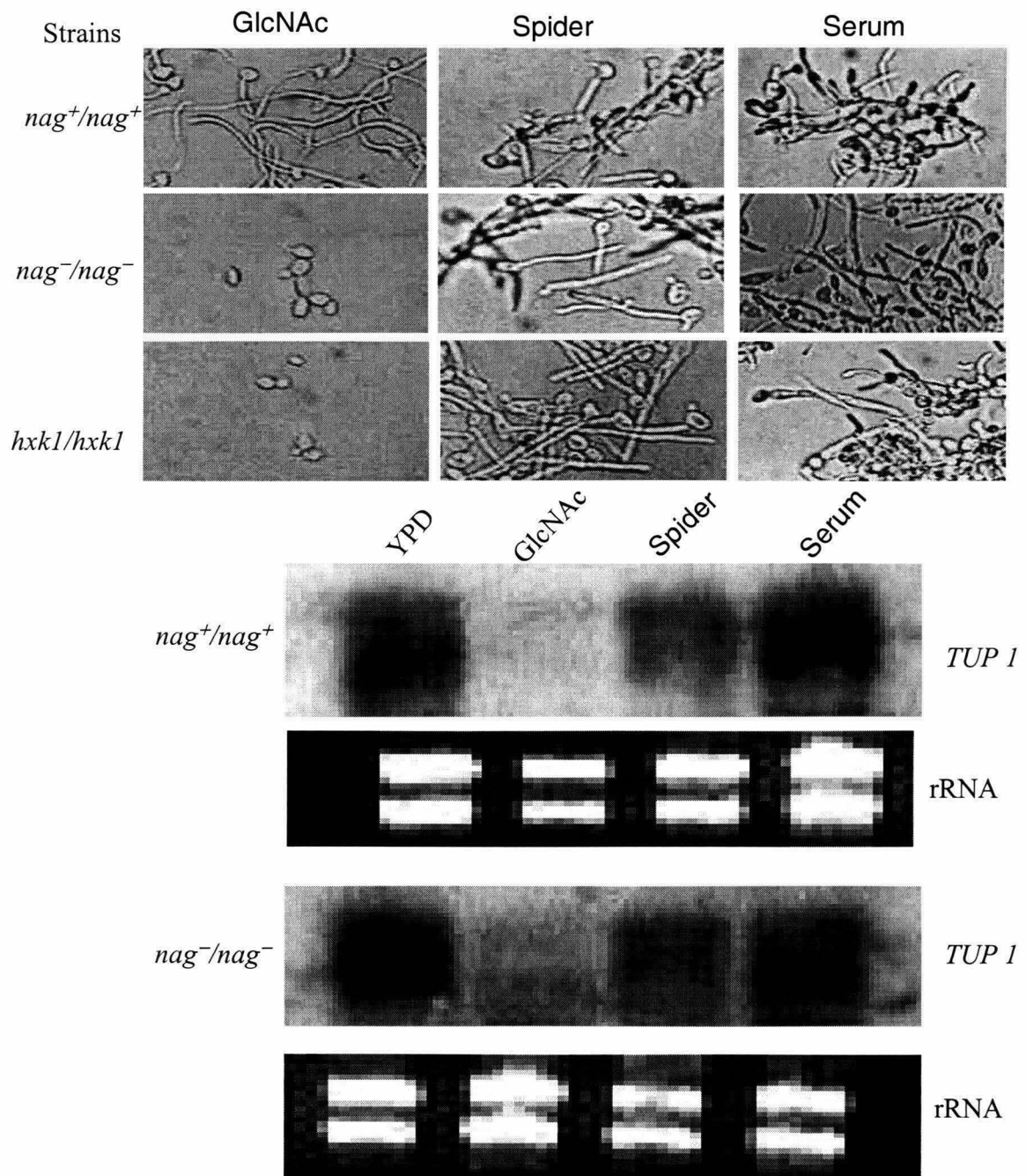
Fig.4.11. Expression of *TUP1* gene in filamentation inducing media. Northern Blot analysis to show the expression of *TUP1* in various filamentation inducing media. Total RNA was isolated from SC5314 (nag+/nag+) and N216 (nag-/nag-) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20μg of RNA was loaded in each lane and Northern Blotting was performed. As an internal control, EtBr staining of rRNA was used to ensure equal loading of RNA
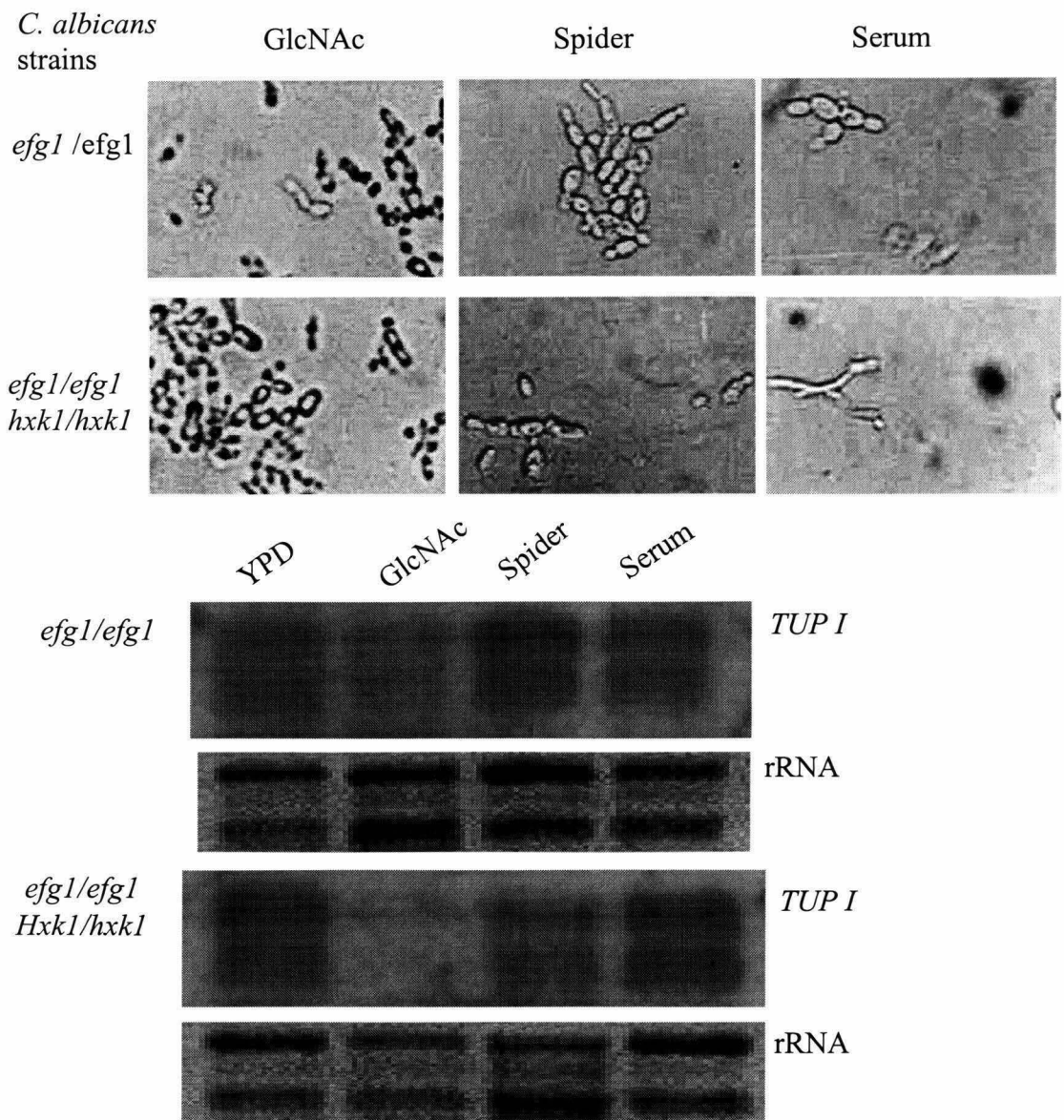
C. albicans strains	GlcNAc	Spider	Serum
efg1 /efg1			
efg1/efg1 hxk1/hxk1			

	YPD	GlcNAc	Spider	Serum	
efg1/efg1					TUP I
					rRNA
efg1/efg1 Hxk1/hxk1					TUP I
					rRNA

Fig.4.12. Expression of *TUP1* gene in filamentation inducing media. Northern blot analysis to show the expression of *TUP1* in various filamentation inducing media. Total RNA was isolated from HLC67 (*efg1/efg1*) and HLC67-16-19 (*efg1/efg1,hxk1/hxk1*) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20μg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA
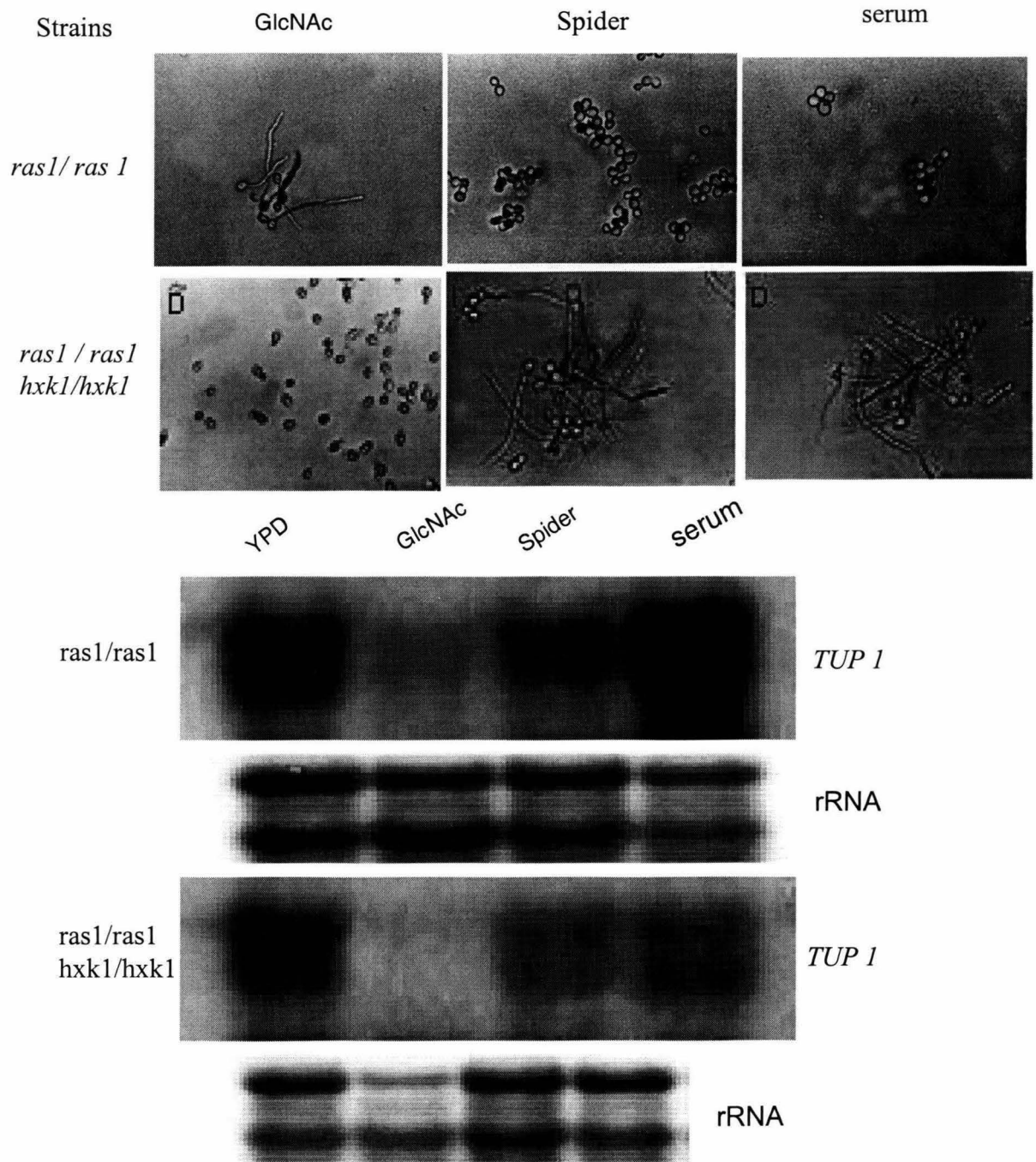
Fig.4.13. Expression of *TUP1* gene in filamentation inducing media. Northern blot analysis to show the expression of *TUP1* in various filamentation inducing media. Total RNA was isolated from CAN52 (*ras1/ras1*) and HR 1 4 2 (*ras1/ras1, hxk1/hxk1*) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20μg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA
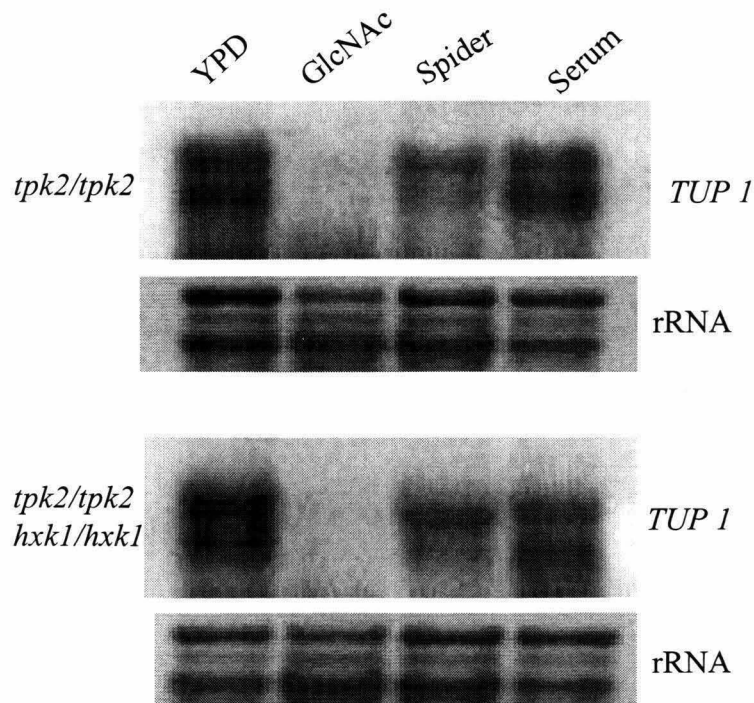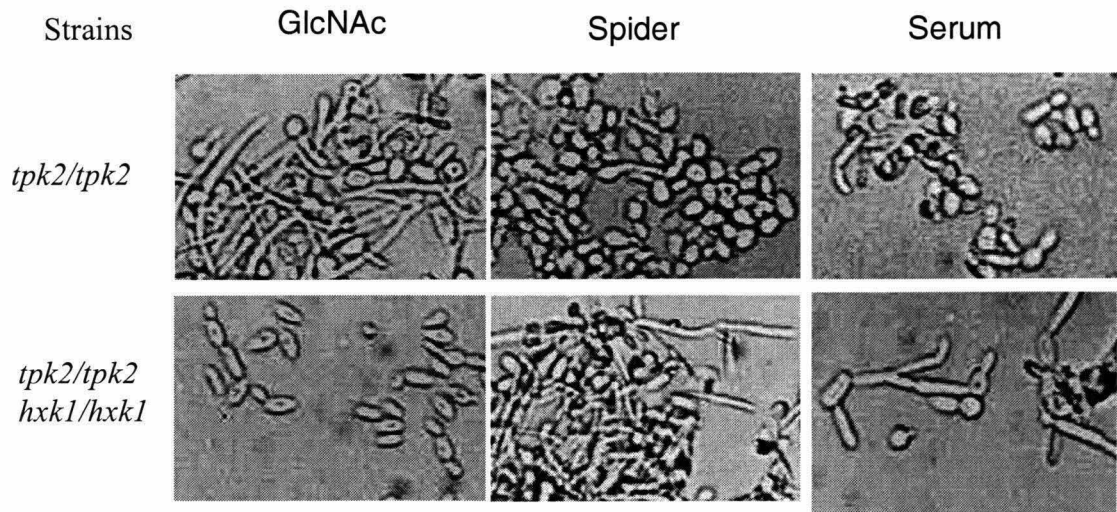
Strains	GlcNAc	Spider	Serum
tpk2/tpk2			
tpk2/tpk2 hxk1/hxk1			

Fig.4.14. Expression of *TUP1* gene in filamentation inducing media. Northern blot analysis to show the expression of *TUP1* in various filamentation inducing media. Total RNA was isolated from AS1 (*tpk2/tpk2*) and AS1 3 18 (*tpk2/tpk2, hxk1/hxk1*) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20μg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA
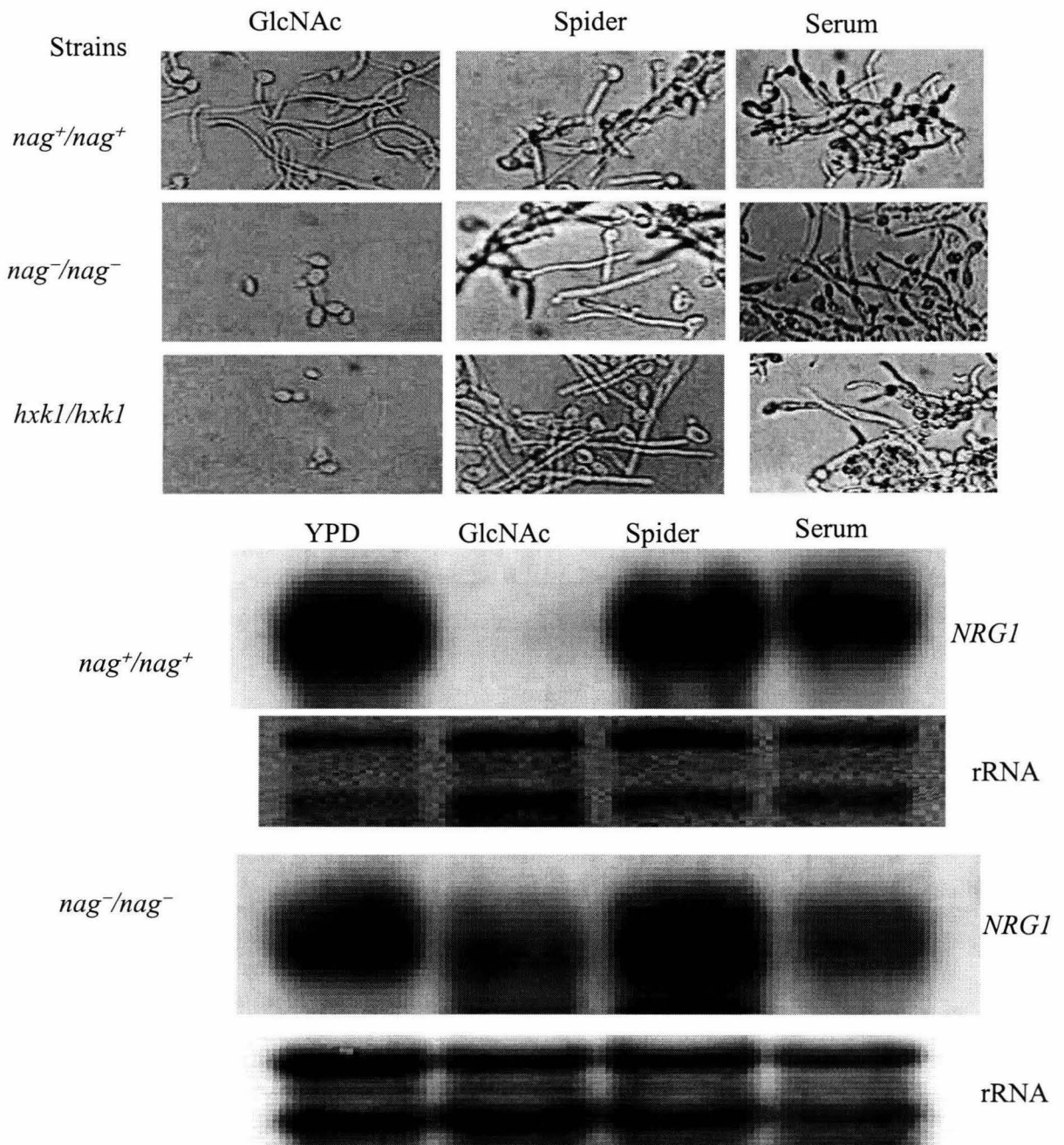
	GlcNAc	Spider	Serum
Strains			
nag⁺/nag⁺			
nag⁻/nag⁻			
hxk1/hxk1			

	YPD	GlcNAc	Spider	Serum	
nag⁺/nag⁺					*NRG1*
					rRNA
nag⁻/nag⁻					*NRG1*
					rRNA

Fig.4.15. Expression of *NRG1* gene in filamentation inducing media. Northern blot analysis to show the expression of *NRG1* in various filamentation inducing media. Total RNA was isolated from SC5314 (*nag+/nag+)* and N216 (*nag ⁻/nag⁻*) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20µg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, EtBr staining of rRNA was used to ensure equal loading of RNA
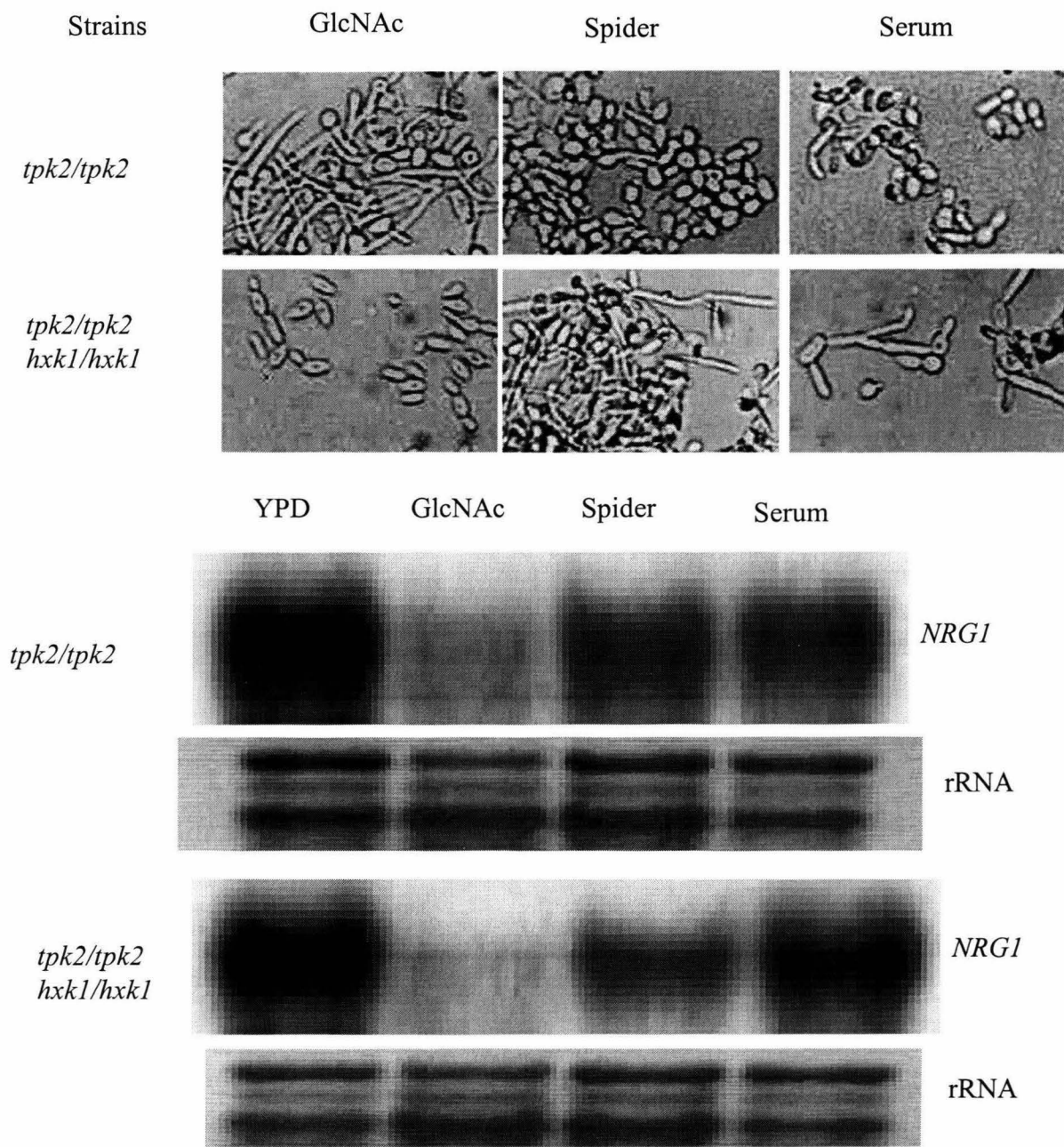
Fig.4.16. Expression of *NRG1* gene in filamentation inducing media. Northern blot analysis to show the expression of *NRG1* in various filamentation inducing media. Total RNA was isolated from AS1 (*tpk2/tpk2*) and AS1 3 18 (*tpk2/tpk2, hxk1/hxk1*) strains induced in YPD , GlcNAc, Spider and 20% Serum. 20µg of RNA was loaded in each lane and a Northern blotting was performed. As an internal control, methylene blue staining of rRNA was used to ensure equal loading of RNA

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