# Molecular Characterization of Cysteine Proteases of *P. falciparum* and *P. berghei* using RNA Interference

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of
**Doctor of Philosophy**

by

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# Declaration

I hereby declare that the research work embodied in this thesis entitled **"Molecular Characterization of Cysteine proteases of *P. falciparum* and *P. berghei* using RNA interference"** has been carried out by me under the supervision of Dr. Pawan Malhotra at the Malaria Research Group, International Centre for Genetic Engineering and Biotechnology, New Delhi.

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# *Certificate*

This is to certify that the research work embodied in this thesis entitled "Molecular characterization of Cysteine proteases of *P. falciparum* and *P. berghei* using RNA interference" has been carried out in the Malaria Research Group, International Centre for Genetic Engineering and Biotechnology, New Delhi. This work is original and no part of this thesis has been submitted for the award of any other degree or diploma to any other university.

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To my parents and brothers

# List of Abbreviations

| | |
|---|---|
| % | Percent |
| aa | Amino acid |
| Ab | Antibody |
| ADCC | Antibody dependent cell cytotoxicity |
| ADCI | Antibody dependent cell inhibition |
| Amp | Ampicillin |
| APS | Ammonium Per Sulfate |
| ATCC | American Type Culture Collection |
| bp | Base pair (s) |
| BCIP | 5-Bromo-4-chloro-3-indolyl phosphate |
| BGH | Bovine growth hormone |
| BSA | Bovine serum albumin |
| cDNA | Complementary DNA |
| C-terminal | Carboxy terminal |
| cpm | Counts per minute |
| DAPI | 6-Diamidino-2-phenylindole |
| DAB | Diamino benzidine |
| ddNTP | Di-deoxyribose nucleotide phosphate |
| DEPC | Diethyl pyrocarbonate |
| DsRNA | Double stranded RNA |
| DNA | Deoxyribose nucleic acid |
| dNTPs | Deoxynucleoside triphosphates |
| DTT | Dithiothreitol |
| *E.coli* | *Escherichia coli* |
| EDTA | Ethylenediamine tetracetic acid |
| E-64 | L-transepoxysuccinyl-leucylamido-[4-guanido] |
| butane | |
| g | Gram |
| h | Hour/Hours |
| 6X-His | hexa-histidine tag |
| HRPO | Horse radish peroxidase |
| HS | Human serum |
| IEM | Immuno electron microscopy |
| IFA | Immunofluorescence assay |

| | |
|---|---|
| IPTG | Isopropyl β-D-thio-galactopyranoside |
| i.v. | Intravenous |
| Kan | Kanamycin |
| kDa | Kilo dalton |
| kb | Kilo base pairs |
| LB | Luria broth |
| LMP | Low melting point |
| LMW | Low molecular weight |
| M | Molarity |
| mAb | Monoclonal antibody |
| min | Minutes |
| mg | Milligram |
| ml | Milliliter |
| mM | Millimolar |
| mRNA | Messenger RNA |
| N-terminal | Amino terminal |
| ng | Nanogram |
| nm | Nanometer |
| O/N | Overnight |
| °C | Degree Centigrade |
| OD | Optical Density |
| OPD | o-phenylene diamine dihydrochloride |
| ORF | Open reading frame |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline with Tween-20 |
| PCR | Polymerase chain reaction |
| pg | Pico gram |
| pmol | Pico mole |
| PMSF | Phenylmethylsulfonyl fluoride |
| PTGS | Post Transcriptional Gene Silencing |
| PVM | Parasitophorous vacuolar membrane |
| TVM | Tubulo vesicular membrane |

| | |
|---|---|
| RBCs | Red blood cells |
| RNA | Ribo nucleic acid |
| RNAi | RNA interference |
| rpm | Revolutions per minute |
| siRNA | Small interfering RNA |
| RdRP | RNA dependent RNA Polymerase |
| SDS | Sodium dodecyl sulphate |
| sec | Seconds |
| SI | Stimulation index |
| SDS-PAGE | Sodium Dodecyl sulphate-polyacrylamide gel electrophoresis |
| Sp. | Species |
| TE | Tris-EDTA |
| TEMED | N, N, N', N'-Tetramethylethylenediamine |
| US$ | US dollar |
| WHO | World Health Organization |
| X-gal | 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside |
| βME | Beta mercaptoethanol |
| µg | Microgram |
| µl | Microliter |
| µM | Micromolar |
| vs | Versus |

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# Chapter 1: Introduction

Malaria remains a public health problem of enormous magnitude, affecting over 500 millions people every year. In the fight against malaria, there is an urgent need to develop new antimalarials and effective malaria vaccine because of wide spread resistance to common antimalarials. The progress in the development of effective antimalarials/ vaccine has been slow due to the lack of understanding of the functions of different parasite proteins. Reverse genetics is one of the ways to assess the function of parasite genes. Gene function elucidation by antisense approach, ribozyme technology and gene disruption technology has met with limited success. Even though transfection technology to knock out different genes has been developed for different malaria parasites, they are not being applied routinely due to extensive selection procedures. With the availability of malaria genome sequence, there is an urgent need to develop an effective molecular tool to carry out functional genomic studies in malaria.

Recently, RNAi has emerged as a powerful and quick tool to study the gene function in a wide variety of organisms. RNA silencing is a sequence specific RNA degradation process mediated by its own double stranded RNA. It differs from other methods of reverse genetics in terms of specificity and simplicity. Extensive genetic and biochemical studies have revealed a three-step mechanism for RNAi induced gene silencing. The first step involves degradation of dsRNA to small interfering RNA (siRNA) of 21-25nt long. An RNAse III like enzyme called DICER has been shown to be involved in this step. In the second step, RISC (**R**NA **i**nduced **s**ilencing **c**omplex) is activated by forming a complex with siRNA. The third step involves recognition of corresponding mRNA by siRNA-RISC complex and degradation of that mRNA by nucleases present in the complex. Currently RNAi is being used extensively in *C.elegans*, *Drosophila*, in cultured insect and mammalian cell lines to elucidate the function of the genes. In *C.elegans* RNAi has been successfully used to carry out large-scale functional genomic studies.

In this study, we developed and applied RNAi to know the function of two cysteines protease genes of *P.falciparum* and *P.berghei.* Since, RNAi was being used for the first time in malaria parasite, we attempted to silence the expression of cysteines protease genes because the effects of inhibitors of cysteines protease on parasite morphology and biochemistry was well documented. Moreover, both these

falcipain1&2 had been produced by recombinant means and their role in hemoglobin degradation was well established. However, direct evidence using gene disruptions as well as other gene silencing techniques were lacking to show the involvement of this protein in other parasite functions. In spite of extensive research on cysteine proteases, their precise role in parasite development and maturation has remained largely speculative.

Keeping this in mind, we made an attempt to understand the exact role of these proteases in *Plasmodium* using RNA interference. The experimental approaches applied were as follows:

- To develop methodology to carryout RNAi in *Plasmodium*
- DsRNA mediated gene silencing of falcipain 1&2 in *P.falciparum*
- Use siRNA of Falcipain1&2 to carryout RNAi in *P.falciparum*
- *In vivo gene silencing in P.berghei*- a mouse malaria model.

# Chapter 2: Review of Literature

## 2.1. History of Malaria

Malaria has been a major cause of human suffering for thousands of years and despite considerable advances in our understanding of the disease, it continues to be one of the main causes of serious illness and death in the world. It is caused by a protozoan parasite belonging to the genus *Plasmodium*. Human malaria is as old as mankind. Hippocrates in 400 B.C. gave first clinical descriptions of malarial fever. In the middle of $17^{th}$ century, malaria was treated by giving the extract made from the bark of Peruvian tree, now known as chinchona tree. In the year 1820, Pelletier and Cavention, isolated active ingredient from the bark of chinchona and named it as quinine, which was found to have an anti-malarial effect. In 1880, Lavern, a French army surgeon in Algeria, first observed and described malaria parasites in the red blood cells of man. Machiafava and Celli (1884) studied the morphological aspects of blood schizogony of the parasite. Sir Ronald Ross, in 1887 described sporogony of *Plasmodium relictum* in anopheles mosquitoes and also showed that the *P. falciparum* was transmitted to humans through the bite of mosquitoes.

To date, four species of *Plasmodium* are known to infect humans, namely, *P. vivax, P. falciparum, P. ovale and P. malarie*. The four species differ morphologically, immunologically, in geographical distribution, relapse pattern and drug response. Of the four human malaria species, *P. falciparum* causes hemorrhagic malaria, which is fatal. It is highly abundant in Africa and responsible for the death of many children. In India, *P. vivax* and *P. falciparum* are widely prevalent. WHO launched malaria eradication programme during 1955-56 in many countries, which achieved unprecedented success. However, in some areas the disease re-emerged mainly due to two factors, 1) Multiple drug resistance of the parasite and 2) resistance of mosquito vector against many insecticides. Therefore, new tools and improved use of the control measures are essential for an effective malaria control.

## 2.2 Life Cycle of Malaria Parasite

*Plasmodium*, the causative agent of malaria completes its life cycle in two hosts: Human and mosquito. In human it undergoes asexual cycle whereas in mosquito it goes through sexual cycle called sporogony.

### 2.2a Life Cycle in Humans

Malaria infection is initiated when an infected female Anopheles mosquito injects sporozoites into the vertebrate host while taking a blood meal. The sporozoites, which disappear from the blood in less than an hour, invade host hepatocytes. The mechanism of hepatocyte invasion of sporozoites is poorly understood, although some of the reports have shown involvement of the kuffer cells in hepatocyte invasion (Yoshida *et al.,* 1980). Two major proteins, circumsporozoite protein (CSP) and thrombospondin related adhesive protein (TRAP) on the surface of the sporozoite were shown to be responsible for the invasion process. Once in the hepatocyte, sporozoites develop over a period of 2-10 days (for *P. falciparum*) to mature liver-stage schizonts, which rupture releasing upto, 40,000 merozoites. These merozoites then escape into the sinusoids of the liver and invade erythrocytes.

### 2.2b Erythrocytic Cycle

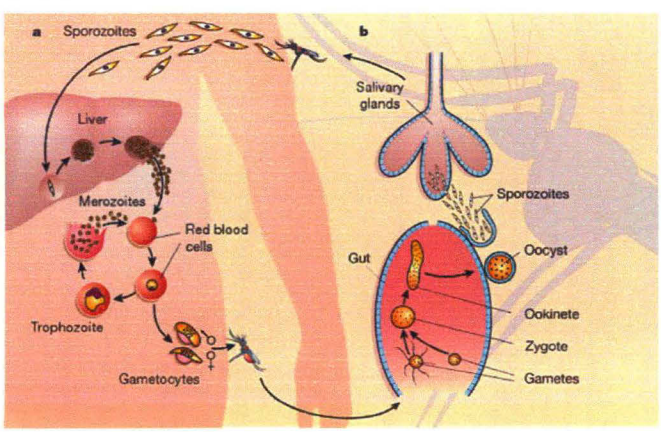
Merozoites released at the end of exo-erythrocytic cycle are ovoid or elongated structures and vary in size. Merozoites proceed to invade the erythrocytes (Mitchell and Bannister, 1988) and this process has been studied extensively through electron microscopy (Hadley *et al.*, 1986). Erythrocyte invasion by merozoite involves an initial attachment at any point on the erythrocyte and subsequently reorient in a way that the apical end of the parasite comes in contact with RBC membrane. This contact induces the release of microneme proteins, which subsequently leads to the formation of "Tight Junction" (Aikawa *et al.*, 1981). Meanwhile, the contents of the apical organelles are released around the erythrocyte membrane and vacuole is formed (Bannister *et al.*, 1977). As the invasion proceeds, the vacuole inside the merozoite leads to a 'ring' stage.

The vacuole surrounding the parasite is called parasitophorous vacuole. The parasite ingests hemoglobin, which is digested inside numerous phagosomes, and heme is polymerised into hemozoin, the typical malaria pigment. At this stage, the parasite is termed as trophozoite. At this time infected erythrocytes become distorted and nuclear division starts. The cytoplasm surrounds each nucleus and a schizont containing several merozoites is formed. The mature schizont ruptures releasing 10-30 merozoites, each of which can reinvade another erythrocyte. Alternatively, the merozoites can develop within the erythrocyte to give the sexual forms called macro gametocyte (female gametes) and micro gametocyte (male gametes).

### 2.2c Sporogony in the Mosquito

When a mosquito ingests erythrocytes containing gametocytes, the gametocytes transform into gametes. The gametes are released from the erythrocytes in the midgut of the insect, followed by fertilization leading to 'zygote' formation within 18 hours of the blood meal. The immobile zygote elongates into motile ookinite, which enters into the intestinal epithelium and comes to rest beneath the basal lamina forming an oocyte 24-72 h after blood meal. Oocyte matures in 7-15 days after the blood meal, giving rise to as many as 10,000 sporozoites, which escape through pores in the oocyte membrane and travel into haemocytic fluid to accumulate in the acinal cells of the salivary gland. Here the sporozoites mature into infective sporozoites that are inoculated into the vertebrate host as the mosquito takes its blood meal.

**Fig. 1 shows the complete life cycle of *Plasmodium falciparum* in mosquito and human**



## 2.3 Current Situation of Malaria

Malaria still remains as a major parasitic disease inspite of more than a century of efforts to eradicate it. About 300-500 millions of people come in contact with malaria every year and around 1.5 million people scum due to the disease. Thus malaria is one of the major infectious diseases and it exerts an enormous toll on developing countries through out the tropics. In particular, cerebral malaria is an over whelming problem in sub-saharan A frica, where most malarial morbidity and mortality occurs. Taking these into consideration international agencies has recently initiated new programs that will provide increased funds for malaria control and research. The roll back malaria initiative was formed in 1998 to expedite and coordinate worldwide malaria control efforts. The principle focus of these programs is to address the pressing needs of those, who are at risk of severe malaria in particular children and pregnant woman in Africa and other endemic regions. In addition, two important focused programs were established in 1999; Malaria vaccine initiative and the medicine for malaria venture. Importantly, these programs are much better funded than existing national and international programs for malaria control. Inspite of huge funding and major advancements in the field of v accine a nd d rug, m alaria s till r emains a s a major h ealth p roblem i n t he developing countries. This drawback is because of the poor understanding of the biology of the parasite.

An international effort was launched in 1996 to sequence the *P. falciparum* genome with the expectation that the genome sequence would open new avenues for research. The complete genome sequence of *P. falciparum* was published in the year 2002 (Gardner *et al.*, 2002). The annotation of completed *P. falciparum* sequence revealed that of the 5,268 predicted proteins; about 60 % are hypothetical, which means that these proteins did not have sufficient similarity to proteins in other organism to justify provision of functional assignments. Hence, it is very much essential to develop tools to study the function of these genes whose functions are not

assigned. In recent years transfection technology has been developed in *P. falciparum, P. berghei, P. knowlesi and P. yeolii* to study function of genes.

## 2.4. Transfection in *Plasmodium*

Transfection of microorganisms was first achieved in the late 1920. Later, it was achieved in yeast, Trypanosomatid parasites, *Leishmania, Toxoplasma gondii* and *Entameoba histolytica*. Transfection of malaria parasite was first reported in *Plasmodium gallinaceum* (Goonewardena *et al.*, 1993) and later in *Plasmodium berghei* (van Djik *et al.*, 1995) and *Plasmodium falciparum* (Wu *et al.*, 1995). This technology has been used to investigate many aspects of *Plasmodium* biology such as genes functions, drug resistance, vaccine development, cell biology, genome organization, and inducible gene expression. Two kinds of transfection methods have been developed for *Plasmodium*: 1) transient transfection and 2) stable transfection.

### 2.4a Transient Transfection

In transient transfection, the vector used doesnot integrate into the genome of the parasite but expresses the gene episomally. Transient transfection has been used to provide insight into the regulation of gene expression. Many vectors have been developed for transient expression both in *Plasmodium falciparum* as well as *P. berghei*. The gene to be expressed transiently in *Plasmodium* should be flanked by 5′UTR and 3′UTR sequences. Vectors carrying luciferase as well as CAT (chloramphemicol acetyl transferase has been used successfully in *P. berghei* (de Koning-Ward *et al.*, 2000) as well as in *P. falciparum* (Crabb *et al.*, 1997) to study the promoter elements. Recently, vectors containing GFP fusions have been developed to study the localization of proteins inside *Plasmodium* (Waller *et al.*, 2000; Wickham *et al.*, 2001).

### 2.4b Stable Transfection

In stable transfection unlike transient transfection, the transgene gets integrated in to the genome. The development of stable transfections has provided the opportunity to express transgenes in *Plasmodium*, as well as

elucidate the function of proteins by disrupting, modifying, or replacing the genes encoding them. The vectors designed for stable transfections require selectable marker flanked by 5´UTR and 3´UTR and gene of interest also flanked by 5´UTR and 3´UTR. The most important aspect of stable transfection is the orientation of the two expression cassettes. It has been shown that higher level of expression of the selectable marker and transgene have been obtained when the two cassettes are arranged in a head to head orientation, presumably due to co-operation between elements in each promoter (Andy Waters, 2002). Based on the type of integration, two types of plasmids have been developed 1) insertional plasmids, 2) replacement plasmids.

2.4b(i) Insertional Plasmid

They contain a single continuous targeting sequence cloned next to the selectable marker. Insertion takes place mainly by single cross over and it is very well demonstrated in *P. falciparum*. Few of the important genes involved in invasion as well as the metabolic pathways of the parasite have been knocked out in *P. falciparum* by using insertional plasmids (Cowman *et al.*, 2003). The selectable marker used for insertional plasmids are PbDHFR or hDHFR. The drugs used for the selection of these markers pyrimethamine and WR99210 respectively. The only disadvantage in using insertional plasmids is the reversion of knockout parasites to wild types through a reversal of the integration mechanism leading to the excision of the insertion vector (Transfection workshop booklet, 2002).

2.4b(ii) Replacement Vectors

In replacement vectors, two regions of targeting sequences were cloned at either end of the selectable marker. As little as 250-300 bp of targeting sequence appears sufficient to force integration into the genome. The replacement constructs integrate into the genome via double cross over between pairs of homologous sequences, leading to the replacement of the target gene. These vectors have been commonly used for knockouts in *P. berghei*. Recently, Alan Cowman and his group developed a replacement vector to carry out transfection in *P. falciparum*. This vector carries thymidine

kinase gene to negatively select against its maintenance episomally (Duraisingh *et al.*, 2002). This is an important advancement because it allows better-defined deletions and mutations in *P. falciparum* genome and it also facilitates the production of double mutations and knockouts into the genome. The major disadvantage of replacement vectors over integration vectors is the difficulty in constructing the vector and the intensive selection procedures for the double cross over event to take place.

In spite of major advances in the field of transfection technology, its usage is limited because of the difficulties listed below:

1. Laborious and time consuming.
2. Intensive selection procedures
3. Limitations in the usage of selectable markers and promoter elements.
4. Knockout of multiple genes.
5. Essential genes cannot be knocked out by using transfection.
6. Redundancy among different genes/proteins.

Taking these points into consideration and moreover with the completion of *P. falciparum* genome sequencing, there is a need and necessity to develop fast track tools which can make the functional genomics of *Plasmodium* an easier job. In parallel to gene knockout by transfection, antisense technology by using single stranded oligonucleotides and ribozyme approach were also developed in *P. falciparum* to silence the genes (Mc Robert *et al.*, 2002). Recently dsRNA/siRNA mediated gene silencing (RNA interference) has emerged as an important tool to study the gene function in a variety of organism (Agrawal *et al.*, 2003).

## 2.5 RNA Interference (RNAi) or Double-Stranded RNA Mediated Gene Silencing

RNA interference is a process by which expression of a gene is inhibited by its dsRNA, which elicits the degradation of cognate mRNA. The RNAi phenotypes are either identical to the genetic null mutants or resembles an allelic series of mutants. Jorgensen's group first time reported PTGS (Post transcriptional gene silencing) in plants. In an attempt to deepen the purple colour of *Petunia* flower, Jorgensen's group introduced extra copies of

Chalcone synthase gene, which is responsible for the production of anthocyanin pigment in to *Petunia*. Surprisingly, some of the transgenic petunia plants harboring the chsA coding region under the control of a 3 5S promoter lost both endogene and transgene chalcone synthase activity, and as a result many of the flowers were variegated or developed white sectors (Napoli *et al.*, 1990). This manifestation of gene silencing was termed as co-suppression, because of the apparent communication between the unlinked but homologous loci. Nuclear run on experiments showed that silencing is at posttranscriptional level rather than transcriptional, thus it was renamed as posttranscriptional gene silencing (PTGS) (Van Blokland *et al.*, 1994). Co-suppression or PTGS is not only induced by related genes but can also be initiated by viruses, termed as virus induced gene silencing (VIGS). Later on, transgene silencing was also shown in *Neurospora crassa*, a process referred to as quelling (Romano *et al.*, 1992; Cogoni *et al.*, 1997). Fire and his group did a systematic and careful study to describe homology dependent silencing of the genes. They introduced double stranded RNA in *C. elegans* and showed that the double stranded RNA was able to silence its cognate RNA (Fire *et al.*, 1998). RNAi appears to be effective in number of invertebrate species, including insects, protozoan, planaria, and hydra, with limited efficiency in zebra fish as well as vertebrates.

**Table 1: List of organisms where RNAi has been successfully applied** (Adapted from the Agrawal *et al.*, 2003).

| Kingdom | Species | Stage tested | Delivery method |
|---|---|---|---|
| *Protozoan* | *Trypanosome brucei* | Procyclic forms | Transfection |
| | *Plasmodium falciparum* | Blood stage | Electroporation and soaking |
| | *Toxoplasma gondii* | Mature forms in fibroblast | Transfection |
| | *Paramacieum* | Mature form | Transfection and feeding |
| | *Leishmania* | | Unsuccessful |
| *nvertebrates* | | | |

| | | | |
|---|---|---|---|
| | *C. elegans* | Larval stage and adult stage | Transfection, Feeding bacteria expressing dsRNA, soaking |
| | *C. briggsae* | Adult | Injection |
| | *Brugia malayi (Filarial worm)* | Adult worm | Soaking |
| | *Schistosoma mansoni* | sporocyts | Soaking |
| | *Hydra* | Adult | Delivered using micropipette |
| | *Planaria* | Adult | Soaking |
| | *Lymnea stagnalis(Snail)* | Adult | Injection |
| | *Drosophila* | Cell lines, Adult, embryonic | Injection for adult and embryonic stages, Soaking and transfection for cell lines |
| | *Cyclorrphan fly* | Early embryonic stages | Injection |
| | *Milkweed bug* | Early embryonic stages | Injection |
| | *Beetle* | Early embryonic stages | Injection |
| | *Cockroach* | Larval stage | Injection |
| | *Spodoptera* | Adult and cell line | Injection and soaking |
| *Vertebrate* | *Zebra fish** | Embryo | Micro injection |
| | *Xenopus* | Embryo | Injection |
| | Mice | Prenatal,embryonic stages and Adult | Injection |
| | Humans | Human cell lines | Transfection |
| *Plants* | Monocots/Dicots | Plant | Particle bombardment using siRNA/ transgenics |
| *Fungi* | *Neurospora* | Filamentous fungi | Transfection |
| | Fission yeast | Filamentous fungi | Transgene |
| | *Dictyostelium* | | Transgene |

| | | | |
|---|---|---|---|
| *Algae* | *Chlamydomonas* | | Transfection |

RNAi, Quelling and PTGS have been shown to work through a common mechanism, which involves degradation of dsRNA into small RNA species known as small Interfering RNA (siRNA). These siRNA with the help of protein complex binds and degrades mRNA (Tijsterman *et al.*, 2002). RNAi has attracted considerable attention because it results in knocking out the activity of a specific gene within short time and with a less labor. With a huge amount of genomic data accumulating, RNAi acts as a genetic magic wand to study the function of the unknown genes in the genome (Bosher *et al.*, 2000).

## 2.6 Components of RNAi

To identify genes/proteins responsible for RNAi, many genetic and biochemical approaches have been undertaken. Many mutants have been created either by transposon mediated or chemical mutagenesis to identify proteins involved in RNAi mechanism. These proteins are either involved in an initiation or in an effector and amplification steps. The components involved in RNAi mechanism are described below.

### 2.6a Dicer

In an attempt to isolate genes responsible for RNAi, Bernstein *et al.* identified RNAseIII like enzyme from the extract of *Drosophila*, which could cut dsRNA into 22nt fragments (Bernstein *et al.*, 2001). They also reported that this enzyme is involved in the initiation step and because of its ability to digest dsRNA into uniformly sized, small RNA, it was named as Dicer. Chemical sequencing studies revealed that siRNA generated from dsRNA by cutting of dicer has 3′ overhang of 2 nucleotides, 5′ phosphate and 3′ OH group, a characteristic feature of RNAseIII enzyme (Elbashir *et al.*, 2001). *Drosophila* dicer has four distinct domains, two RNAseIII motifs, a dsRNA binding domain, a ATP dependent RNA helicase domain and a PAZ domain, which is present in RDE1/QDE1/Argonaute family of proteins that has been

genetically linked to RNAi (Catalanotto *et al.*, 2000; Tabara *et al.*, 1999). A functional ortholog of *Drosophila* dicer protein was predicted from the genome of *C. elegans*, K12H4.8 and was referred as DCR-1 (Hammond *et al.*, 2000). The mutants of DCR-1 in *C. elegans* showed defects in RNAi of germline expressed genes but no effect on the RNAi response of somatic genes (Knight and Bass, 2001). CAF-1 a homolog of dicer has been identified in *Arabidopsis* but its role in PTGS is yet to be demonstrated. Recently, a functional homolog of dicer has been isolated and characterized from humans. Unlike other dicer proteins, human dicer is not dependent on ATP for its activity (Provost *et al.*, 2002).

### 2.6b RNA-Induced Silencing Complex (RISC)

RNA induced silencing complex, is a sequence specific multi component nuclease that degrades messenger RNA, homologous to the siRNA present in the complex. Hammond *et al.* showed that the cultured *Drosophila* cells when transfected with specific dsRNA, show loss of function phenotype (Hammond *et al.*, 2000). RISC has been purified from cultured *Drosophila* cells. The active fraction contains a multi component protein complex with nuclease activity of approx 500 kDa. One constituent of this complex is a member of argonaute family of proteins indicated as AGO2. Argonaute2 (AGO2) is 130 kDa protein with polyglutamine residues; PAZ/PIWI domains are characteristic of argonaute family of protein. AGO2 is homologous to RDE-1, protein essential for RNAi in *C. elegans*. Co-immunoprecipitation studies with AGO2 showed that Dicer and Ago2 interact with each other. Current model suggest that interaction of AGO2 and Dicer helps in incorporation of siRNA into RISC complexes (Hammond *et al.*, 2001).

Recently, two putative RNA binding proteins (L5 and L11), the *Drosophila* homolog of human fragile X syndrome protein (FMRP), FMR1 and VIG (Vasa intronic gene), have been shown to be associated with RISC (Caudy *et al.*, 2002). FMRP, a product of Human fragile X syndrome gene, regulates expression of numerous genes through unknown mechanism. Its counter part in *Drosophila*, Fmr1 binds and represses the translation of mRNA encoding microtubule-associated protein, Futsch (Zhang *et al.*, 2001). Two ribosomal binding proteins L5 and L11 along with 5S rRNA have been

isolated from Fmr-1 associated complex. The Fmr-1 also contains AGO2 and *Drosophila* homolog of p68 RNA helicase (Dmp68). Involvement of Fmr-1 with essential components of RNAi machinery raises the possibility that defects in RNAi related machinery might cause human diseases (Ischizuka *et al.*, 2002).

### 2.6c Helicases Involved in Silencing

A mutant strain (mut-7) of *C. elegans* with high transposition frequency was shown to be RNAi defective, temperature sensitive for fertility and produce excess of male offspring. The mut-7 gene was cloned and found to encode a protein with domains homologous to RnaseD and Werner syndrome helicase (Ketting *et al.*, 1999). To strengthen the link of helicases to RNAi, another gene called qde-3 (**q**uelling **de**fective-**3**) was isolated from *Neurospora*, which exhibits quelling, a process similar to PTGS in plants. QDE-3 protein has homology with several polypeptides belonging to the class of REC Q DNA helicases. QDE-3 protein was also shown to be involved in DNA-DNA interaction, which suggests its role in transcriptional part of the gene silencing (TGS) (Cogoni *et al.*, 1997).

In unicellular green algae *Chlamydomonas reinhardii*, loss of DEAH-box RNA helicases, *Mut6*, not only results in loss of PTGS but also leads to enhanced transposition of retero-element TOC1 and DNA mediated transposon (Gulliver). By blocking *de novo* RNA synthesis, mature endogenous transcripts are degraded normally where as TOC1 transcripts become stable, suggesting the role of Mut-6 in degradation of these mRNA. Interestingly, plasmid insertion into Mut-6 locus, causing the defect, resulted in improper processing of transcripts with defects in splicing and poly-adenylation. These aberrant RNAs disappeared when Mut-6 mutant was complemented by the introduction of a single copy of the wild type Mut-6 allele. This could mean that aberrant RNA was specifically degraded by Mut-6 dependent process, whereas correctly processed mRNA was left untouched (Wu-Scharf *et al.*, 2000).

Based on the observation that both RNAi and nonsense mediated decay involve degradation of RNA, Domeier *et al.* examined whether the proteins involved in nonsense mediated decay also functioned during RNAi. Seven

smg genes were identified, each of which was involved in nonsense mediated decay of RNA (Domeier *et al.*, 2000). Of all the seven smg mutants, smg-2, smg-5 and smg-6 subsequently recovered from the effects of RNAi and ultimately expressed nearly WT (untreated) levels of RNA. Unexpectedly, smg-1 mutants failed to recover from RNAi phenotype showing that it has no role in RNAi mediated degradation process. But it plays a critical role in nonsense-mediated decay of RNA. Mutant smg-3 and smg-4 animals gave a weak variable response, suggesting that smg-3 and smg-4 may not be required for the persistence of RNAi. These authors have also shown that there was no correlation between strength of RNAi phenotype associated with particular allele and the effect of that allele on nonsense mediated decay. SMG proteins involved in RNAi facilitate amplification of the signal essential for silencing. On the basis of homology of these proteins with Upf1, which encodes an adenosine triphosphatase with RNA binding and helicase activities (Leeds *et al.*, 1992), the SMG proteins could unwind dsRNA to provide template for RNA–directed RNA polymerase.

### 2.6d RNA-Dependent RNA Polymerase

One of the provocative aspects of RNAi includes ability to spread through the organism, even when triggered by low quantities of dsRNA or siRNA. It was found that in addition to siRNA that could be attributed to the input dsRNA, siRNA was also detected from sequence other than the targeted region. This could mean that siRNA are also produced from targeted mRNA transcript, as secondary product of mRNA degradation. Such siRNA are called as secondary siRNA and are found to be functional (Sijen *et al.*, 2001). These secondary siRNA can be generated by *de novo* RNA synthesis by RdRp like enzymes. Initially, an enzyme homologous to RdRp was isolated from tomato. This enzyme was shown to possess polymerase activity, and biochemical studies will be required to establish its involvement in PTGS. In *Arabidopsis,* SDE1/SGS2 is required for transgene silencing, but not for viral induced gene silencing (VIGS) (Dalmay *et al.*, 2000). This suggests that SDE-1/SGS2 may act as an RdRp, as viral replicase substitute for this function in VIGS. In *Neurospora*, QDE-1, a homolog of RdRp is required for efficient quelling effect (Catalanotto *et al.*, 2002). EGO-1 a homolog of RdRp was isolated from

*C. elegans* and demonstrated to be responsible for RNAi in germ line cells. However, for RNAi in somatic cells, a putative RdRp, RRF-1/ RDE-9 was isolated and shown to be functional. In *C. elegans* another gene belonging to the family of RdRp, RRF-3 was isolated. Mutations in this gene made *C. elegans* hypersensitive to RNAi. This phenotype can be explained by considering the possibility of RRF-3 strongly interacting with EGO-1 and RRF-1 proteins (Sijen *et al.*, 2001).

**Table 2: Proteins/genes from different organisms and their role in RNAi/PTGS/Quelling** (Adapted from *Agrawal et al., 2003*)

| Phenomenon | Organism | Silencing defective mutant | Gene Function | Developmental defect |
|---|---|---|---|---|
| Post Transcriptional Gene Silencing (PTGS) | Plants (*Arabidopsis thaliana*) | Sgs2/Sde1<br>Sgs3<br>Sde3<br>Ago1<br><br>CAF 1 | RdRP<br>Unknown Function<br>RecQ helicase<br>Translation Initiation factor<br>RNA helicase & RNase III | None<br>None<br><br>Pleiotropic effects on development & fertility |
| Quelling | Fungus (*Neurospora crassa*) | Qde-1<br>Qde-2<br><br>Qde-3 | RdRP<br>Translation Initiation factor<br>RecQ DNA helicase | None<br>None |

| | | | | |
|---|---|---|---|---|
| RNA Interference | Worm (*Caenorhabditis elegans*) | Ego-1 | RdRP | Gametogenetic defect & sterility |
| | | Rde-1 | Translation Initiation factor | None |
| | | Rde-2, Rde-3, Rde-4 & Mut-2 | Unknown Function<br>Unknown Function<br>Dicer homologue | None<br>Sterility |
| | | K12H4.8 (dcr-1) | RNA helicase & RNase III | |
| | | Mut-7 | Helicase & RNaseD DEAD box RNA helicase | None |
| | | Mut-14 | Upf1p helicase | |
| | | Smg-2 | Unknown Function | |
| | | Smg-5 | Unknown Function | |
| | | Smg-6 | Transmembrane protein | |
| | | Sid-1 | | |
| RNA Interference | Alga (*Chlamydomonas reinhardtii*) | Mut-6 | DEAH-box RNA helicase | |

Independently, Nellen and co-workers showed in *Dictyostelium discoideum* that hairpin constructs could silence expression of homologous transgenes and endogenous genes. Here too, silencing requires the action of an enzyme with presumed RdRp action. Three RdRp homologs (rrpA, rrpB and rrpC) were identified in the genome of *Dictyostelium*, out of which only rrpA is required to induce RNA silencing (Martens *et al.*, 2002). Although the homologs of RdRp described above are not found in *Drosophila* and human genome by blast searches, *in vitro* experiments by using *Drosophila* extract suggests that siRNA might prime RdRp action on complementary RNA targets. Role of RdRp in gene silencing in humans was convincingly disproved using siRNA modified at 3' end with FITC moiety, which blocks the RdRp action, but still is a potent inducer of RNAi (Lipardi *et al.*, 2001).

## 2.7 Mechanism of RNAi

Based on various genetic and biochemical studies especially in *C. elegans* and *Drosophila*, a three-step mechanism has been proposed for RNAi.

1. Initiation or cleavage of dsRNA
2. Silencing complex formation and activation
3. mRNA degradation

### 2.7. i Initiation Step

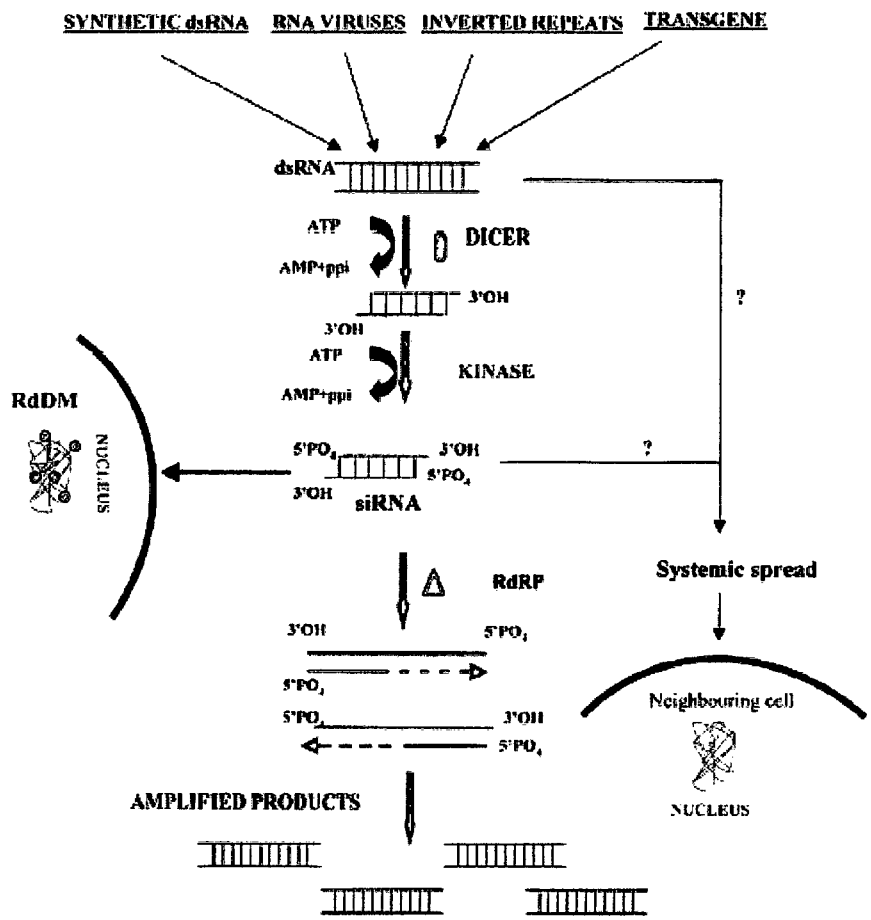



**Fig.2 Schematic representation shows the degradation of dsRNA to siRNA and amplification of siRNA into multiple copies of siRNA by RdRP.** This figure also shows the role of siRNA in DNA methylation and systemic silencing.

The first step in the RNAi reaction is the initiation step that involves cleavage of dsRNA into small RNA molecules of size 21-25nt, known as siRNA. The first evidence regarding the presence of small RNA species (25

nt) was shown in plants, both in transgene induced-PTGS and virus induced PTGS (Hamilton *et al.,* 1999). Hammond *et al.* for the first time partially purified nuclease complex carrying 25 nt species from the cell extract of dsRNA transfected *Drosophila* cells (Hammond *et al.,* 2000). They showed that this species confer specificity to the enzyme through homology to substrate mRNA. Subsequently, Sharp and coworkers developed *in vitro* cell extract model and demonstrated the formation of 25 nt species by incubating radiolabeled dsRNA in the cell extract (Tuschl *et al.,* 1999). Based on the binding and cleavage properties of *E.coli* RNaseIII enzyme, Bass for the first time predicted involvement of RNaseIII type endonucleases in the degradation of dsRNA to siRNA of 21-25 nt lengths (Bass *et al.,* 2000). Elegant experiments by Elbashir *et al.* showed direct involvement of RNaseIII like enzyme in RNAi process (Elbashir *et al.,* 2001). Bernstein *et al.* isolated bidentate ribonuclease (Dicer) from *Drosophila* extract and showed its involvement in the initiation step of RNAi pathway. Among RNaseIII enzymes, Dicer is unique in terms of cleavage of dsRNA as it requires ATP for its cleavage (Bernstein *et al.,* 2001) (Fig.2). This finding has been attributed to the presence of ATP–dependent RNA helicase domain. This domain may serve to unwind dsRNA locally, allowing its subsequent cleavage. But it seems unlikely, as other RNaseIII enzymes require no helicase function. More likely, helicase domain may be an RNA translocase, using ATP energy to drag Dicer down the dsRNA. Recently, Dicer was shown to be associated with R2D2, a protein homologous to *C. elegans* RNAi protein RDE-4, in *Drosophila.* This protein forms a bridge between initiator and effector function by facilitating the passage of siRNA from Dicer to RISC (Liu *et al.,* 2003).

### 2.7. ii RISC Complex Formation and Activation

The second step in the RNAi pathway is the association of the siRNA with RISC complex. Zamore and his group first reported the association of siRNA with a protein complex. They incubated $^{32}P$ labeled 501 nt *Pr-luc* dsRNA in a standard RNAi reaction in absence of target to permit its cleavage into siRNA. After two hours of incubation, this reaction was chromatographed on a Sephadex-200 gel filtration column. SiRNA formed were predominantly

associated with 360-kDa protein. They also showed that ATP was not required for the loading of siRNA onto the complex (Zamore *et al.*, 2000). A protein belonging to PAZ family of proteins, AGO2 has been suggested to be responsible for transferring of the siRNA from dicer onto the RISC (Hammond *et al.*, 2001). The next step after siRNA-protein complex formation is the activation of the complex. Activation involves unwinding of siRNA duplex, which is an ATP dependent process. This complex is termed as RISC*(Fig.3).

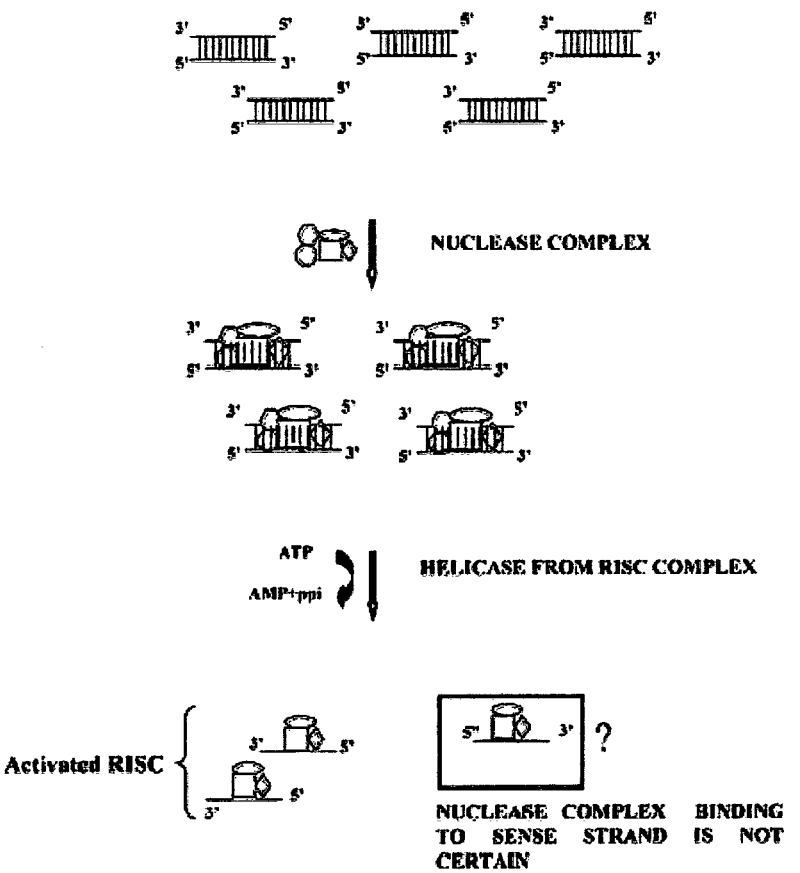



**Fig.3 Schematic representation shows RISC-siRNA complex formation and the activation of RISC.** This step involves separation of double stranded siRNA to single stranded siRNA by helicase activation.

Recently, it was shown that unwinding and retention of one of the strands of siRNA depends upon the internal energy at the 5′ end of the siRNA. It was also shown that helicase in the RISC complex binds to the 5′ end of that

strand where the internal energy is lesser when compared to the 5′ end of the other strand and unwinds the siRNA (Anastasia *et al.*, 2003 and Schwarz *et al.*, 2003). The activated RISC brings about degradation of mRNA homologus to siRNA (Nykanen *et al.*, 2001). Recently, two putative RNA binding proteins (FMR1 and VIG), two ribosomal proteins, L5 and L11 along with 5S RNA were shown to be present in the RISC complex, but the exact role in RNAi function is not known (Caudy *et al.*, 2002). A recent report also indicated the association of micrococcal nuclease with RISC, which is involved in the degradation of mRNA (Caudy *et al.*, 2003).

### 2.7. iii mRNA Degradation

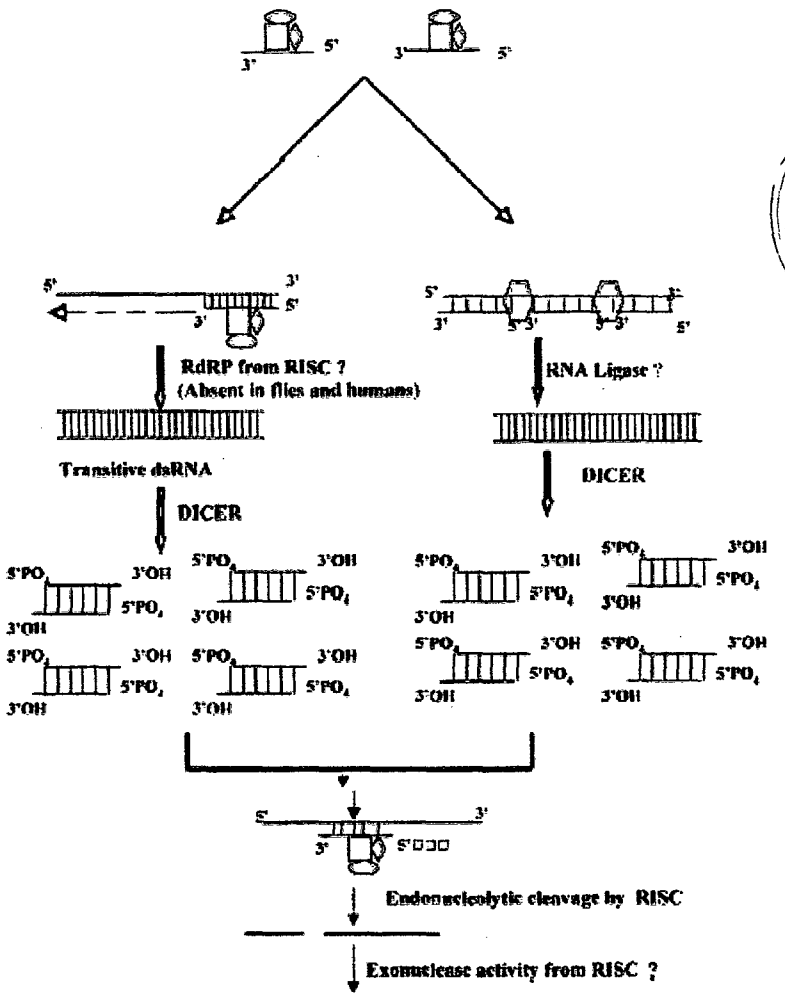


**Fig.4 Schematic representation shows degradation of mRNA by activated RISC.** In *C. elegans,* siRNA acts as a template for RdRP and brings about amplification of its mRNA, forming transitive dsRNA, which is further cleaved by DICER to siRNA. In the case of flies and humans multiple siRNA binds to different positions on the mRNA. siRNAs bound to the mRNA are joined by RNA ligase forming transitive dsRNA, which is cleaved by DICER to siRNA. These siRNAs join the RISC complex and binds to the mRNA. mRNA degradation is brought about by the initial endonucleolytic cleavage followed by exonucleolytic cleavage by the RISC.

The third step in an RNAi pathway involves degradation of target mRNA by siRNA–protein complex. The antisense strand on the RISC complex acts as a guiding strand. The degradation of target mRNA was mediated by ribonuclease, which was distinct from Dicer. This enzyme was named as slicer, which unlike Dicer does not require ATP for its cleavage activity (Gyorgy *et al.,* 2002). Experiments with siRNA revealed the requirement for a 5′ phosphate on the siRNA strand complimentary to the target RNA for efficient cleavage (Elbashir *et al.,* 2001). Tuschl and co-workers have proposed that the site of cleavage of target RNA is measured from the 5′ end of the complimentary siRNA strand. The 5′ phosphate of the siRNA may therefore serve as a reference point, from which the cleavage site is measured. A kinase in the *Drosophila* embryo lysate was shown to be responsible for the 5′ phosphorylation of synthetic siRNA. This kinase can differentiate between 5′ ribo and deoxyribo siRNA. The cleavage site on target RNA has been mapped to 11 or 12 nucleotides downstream of 5′ end of the guide siRNA. Three models have been proposed for the cleavage of mRNA mediated by siRNA in the RISC complex. According to the first model, the degraded fragments of target RNA are converted to duplex forms by RdRP like activity. These forms might have siRNA like functions and eventually enter the pool of amplification reactions (Sijen *et al.,* 2001; Lipardi *et al.,* 2001). The major drawback of this model is the absence of RdRP in *Drosophila* and humans. But the essentiality of RdRP in plants supports the validity of this model in plants. In second model, it has been proposed that siRNA do not act as a primer, but instead assemble along the length of target RNA and then ligate together by RNA ligase to generate cRNA (complementary RNA). DCR proteins cleaved the cRNA and target RNA

hybrid and the siRNA generated enters the RNAi pathway. In the third model, the siRNA in the RISC guide the RISC to its cognate mRNA, which subsequently cleaves the target mRNA in a step or a series of steps requiring no nucleotide triphosphates (Schwarz *et al.,* 2002). Of these three models, the third model seems to be more valid.

## 2.8 Functional Genomics Using RNAi

With the completion of genome sequencing for number of organisms such as human and *P. falciparum*, there is a need to develop new reverse genetic approaches to carry out functional genomic analysis. Of these different reverse genetic approaches, even though gene knockout technique by homologous recombination has been successfully used to carryout functional genomic analysis in *P. falciparum*, it is still cumbersome and time consuming. Other two reverse genetic approaches such as antisense and ribozyme have also worked with some limitations. The discovery of RNAi in *C. elegans* and many organisms including mammals had opened a new way to study the function of genes more effectively and in a rapid way. The demonstration of RNAi in mammals opened doors for wide scale screening of human genome.

### 2.8a RNAi and Functional Genomics in Lower Eukaryotes (*Drosophila and C. elegans)*

Fire and Mello successfully demonstrated RNAi for the first time in *C. elegans* by injecting dsRNA. Later different methods like soaking the worm in dsRNA solution or by feeding the worm with bacteria engineered to express dsRNA were developed to induce RNAi in *C. elegans*. The facile nature of this technique has made it a favored method for large-scale functional genomic analysis of different genes. *In C. elegans,* two groups used RNAi successfully for the large-scale functional genomic studies. Hyman group analyzed all the genes on the third chromosome of the worm by micro injecting over 2000 *in vitro* synthesized double-stranded RNAs. They mainly looked for defects in early embryonic cell divisions using time-lapse video and identified 133 genes with distinct embryonic phenotypes. Another group led by Julie Arhinger, examined the function of the genes of the chromosome I in

*C. elegans*, by feeding the worm with bacteria carrying dsRNA expressing library for 90 % of the predicted gene on chromosome I (Fraser *et al.,* 2000). The Arhinger group used this library to screen for gross phenotypes, and later re-examined embryonic phenotype of 147 essential genes in more detail (Zipperlen *et al.,* 2001). The same group later used bacterial library for inactivation of 16,757 genes that corresponds to 86 % of total predicted genes of the worm genome. They identified mutant phenotypes for 1,722 genes, about 2/3 of which were not previously associated with any phenotype (Kamath *et al.,* 2003). *Drosophila* is another popular model organism where RNAi has been successfully used to study function of individual genes and group of genes. Unlike *C. elegans* and Plants, RNAi in *Drosophila* is not systematic, meaning that RNAi doesn't spread into other cells or tissues, which allows us to do cell specific RNAi in *Drosophila* cells. Recently genome wide analysis of nuclear mRNA export pathways using RNAi in *Drosophila* was carried out and mRNA was grouped based on the protein, which exports them outside the nucleus (Herold *et al.,* 2002).

### 2.8b RNAi and Functional Genomics in Mammals

The machinery of RNAi in eukaryotic cell offers a protection mechanism against the foreign nucleic acids. Transcriptional regulation using dsRNA technology provides an easy means to identify the cellular characteristics in response to both internal and external cues. However, application of RNAi in mammals was hindered because of the fact that long dsRNA have been shown to nonspecifically suppress gene expression in mammalian cells. Long dsRNA activates dsRNA dependent protein kinase (PKR) and 2′-5′-oligoA synthase leading to nonspecific translational inhibition and RNA degradation, respectively (Gil *et al.,* 1999). This pathway does not exist in embryonic stage, allowing specific RNAi using dsRNA in mouse oocytes and embryos (Wianny *et al.,* 2000). RNAi was initially thought to be not feasible in mammalian cells, but Thomas Tuschl's group and others by using synthetic siRNA showed that duplexes (21nt) are too short to induce non-specific inhibitory affect (Elbashir *et al.,* 2001; Caplen *et al.,* 2001). The application of RNAi in mammalian cells opened doors for wide scale

screening of human genome. This would be much more painstaking than screening in *C. elegans* because number of human genes is almost twice that of *C. elegans*. An efficient screening system is necessary to carryout RNAi in humans. However, efforts are underway, to develop libraries of siRNAs and automatic screening system to achieve the long-standing goal of genome wide functional studies in humans. siRNA have been now used to carryout functional genomics in a variety of organisms.

One of the critical factors, which determine the efficiency of RNAi, is the mode of delivery of siRNA/dsRNA. Table 1 describes different delivery methods of siRNA/dsRNAs employed by various researchers. In case of *C. elegans*, the most commonly used method for the delivery of dsRNA/siRNA has been the soaking method, feeding of dsRNA expressing bacteria and transfection. In *Drosophila*, the frequently used method for the delivery of siRNA/dsRNA is transfection using different chemical reagents. However in mammalian cell lines, two different approaches have been used to deliver siRNAs.

1) Transfection of synthetic siRNA.
2) DNA based expression of small hairpin (sh) RNA.

2.8b(i) Transfection of Synthetic siRNA

The method developed by Tuschl's group and others involves transfection of synthetic siRNA into cultured cells. Because of the simplicity, siRNA transfection is now being widely used. Synthetic siRNA are available from Dharmacon RNA Technologies (USA), Qiagen (Germany) and Ambion (USA). There are several factors that may influence the efficiency of RNAi in mammalian system (McManus *et al.,* 2002). First, the choice of the target site is important. Originally, it was suggested that the best target site is around the first 100 nt down stream of the translational start site. However, it is still not clear yet which region on mRNA is most vulnerable to RNAi. A computer programme has been developed by Lin *et al.* and is available on ambion website, which helps in selecting a potential siRNA sequences. This programme also helps in performing BLAST analysis to avoid a chance of designed siRNA to be complementary to other unrelated mRNA. Second, the transfection method makes a difference in the outcome. SiRNA can also be

efficiently transfected in to the cells when lipophillic agents such as oligofectamineTM and transit-TKOTM are used. OligofectamineTM is most frequently used because of its low toxicity when used on HeLa and HEK 293 cells, virtually 100 % transfection efficiency was seen. Primary cells and T cell lines that are usually difficult to transfect have also been transfected with siRNA with relatively high efficiency using these reagents. Apart from transfection using lipophilic agents, electroporation have been successfully used for certain cell types such as T cells (McManus *et al.,* 2002) and human hepatoma cell lines (Wilson *et al.,* 2003). Massive cell death during electroporation should be taken into consideration during a particular application.

Third, the turnover rate of the protein should be taken into account because RNAi only aims at the mRNA and not the protein. siRNA mediated RNAi lasts only for 3-5 cell doubling times, probably due to gradual dilution of siRNA through cell division. Therefore multiple transfections are necessary in cases where the protein is unusually stable. Fourth, in case of two siRNA being used simultaneously, there is a chance of these siRNAs competing with each other suggesting that the RNAi machinery may be limiting in human cells. Thus, careful control of siRNA concentration is necessary for simultaneous knockdown of multiple genes.

Recently, an alternative method for siRNA preparation has been developed. Long dsRNA that are transcribed *in vitro* using RNA polymerase were incubated with recombinant Dicer to generate esiRNA (Myers *et al.,* 2003). The resulting diced products contained a mixture of siRNA that can bind to multiple sites on target mRNA, eliminating the need to design and test multiple individual siRNA.

2.8b(ii) DNA Based Expression of Small Hairpin (sh) RNA

In spite of efficient knock down capabilities, the siRNA transfection method has drawbacks such as transient effects and difficulties in transfection depending on cell types. Stable transgene silencing had been developed based on the expression of siRNA from DNA templates. The first type makes use of RNA polymerase III promoter such as U6 promoter (Paddison *et al.,* 2002, Paul *et al.*, 2002, Lee *et al.,* 2002, Yu *et al.,* 2002, Miyagishi *et al.,* 2002). The

advantage of using polIII system is that the transcription terminates at a stretch of four thymidines, making it possible to produce short RNA with 1-4 uridines at the 3′ ends. To construct shRNA expression cassette, gene specific targeting sequence of about 19-29 nt sequence from the target transcript separated by a short spacer sequence from the reverse complement sequences is inserted between polIII promoter and terminator. Some argue that longer stem (upto 29 nt) is more efficient than shorter ones (Paddison *et al.,* 2003). However, there are no clear guidelines to make the best hairpin at the moment. This partly is because of insufficient understanding of small RNA processing. Therefore, studies on small RNA processing would be important for the development of RNAi technique.

A similar approach to the shRNA methods is to transcribe 21 nt sense and anti sense RNA separately from PolIII promoters (Lee *et al.,* 2002, Yu *et al.,* 2002, Miyaghishi *et al.,* 2002). shRNA can also be generated from PolII promoters such as human cytomegalovirus (CMV), an immediate early promoter (Zeng *et al.,* 2002). Using PolII promoter would be advantageous in terms of regulated expression of siRNA. A variety of inducible/repressible promoters are available for the specific expression. This type of shRNA expression system has not been widely used yet and further experiments are needed to prove its efficiency. However, plasmid based shRNA expression has limitations in cases where transfection efficiency is low. To overcome this problem, viral vectors have been employed to deliver shRNA expression cassette. Retero viral vectors are most commonly used vectors for *in vitro* gene transfer and for *in vivo* gene therapy. Among retero viral vectors, lenti based and murine-based vectors have been efficiently used to deliver shRNA expression cassette (Abbas-terki *et al.*, 2002; Barton *et al.*, 2002). Adenoviral vectors are highly effective but allow only transient expression of siRNA which, might be advantageous in some applications such as cancer therapy where persisted expression is not necessary (Shen *et al.,* 2002).

## 2.9 RNAi and Therapy

In addition to their use in functional genomic studies, siRNA have recently been shown to represent a new avenue for gene therapy. The expression of mRNA coding for mutated proteins, which give rise to dominant genetic disorder and neoplastic growth, might be decreased or blocked by specific siRNA. Bitko and Barik for the first time successfully used siRNA to silence gene expressed from respiratory syncytial virus (RSV) (Bitko *et al.*, 2001). They also demonstrated that siRNAs donot induce interferon–mediated responses, by showing the absence of phosphorylation of the translational factor eIF-2A. Lee *et al.* (2002), reported effective siRNA-mediated degradation of HIV-1 *rev* transcripts in a cell assay by co-transfecting of proviral DNA and siRNA expression vectors. Thus raising the possibility that siRNA may become useful to treat HIV infection.

In leukemia and lymphomas, the most frequent cancers in childhood, oncogene activation frequently occurs through reciprocal translocations. Recently, siRNA have been targeted against BCR-ABL positive leukemia and this approach was compared to that of ST1571, a protein kinase inhibitor, mediated cell killing of cancerous cells. This leukemia was caused by the translocation of the long arms of chromosome 9 and 22, which results in the fusion of *BCR* gene from chromosome 22 and ABL gene from chromosome 9, creating an oncogenic *BCR-ABL* hybrid gene. The siRNA treatment of these cells readily reduced the expression of *BCR-ABL* mRNA, followed by a reduction of BCR-ABL oncoprotein, leading to apoptosis in leukemic cells. The extraordinary sequence specificity of the RNAi mechanism may also allow for the targeting of point mutated transcripts of transforming oncogenes such as Ras (Tuschl *et al.*, 2002). Over expressed oncogene and apoptosis inhibitors are another kind of potential targets for siRNA technology based tumour gene therapy (Cioca *et al.*, 2003). These and number of other studies thus provided advances to show that in addition to their use in functional genomic studies, siRNA also holds great potential for the treatment of infectious diseases as well as cancers.

## 2.10 RNAi *in vivo*

RNAi has also been successfully triggered *in vivo* in the whole organism. Mc Caffrey, first reported that siRNA from an exogenously administered plasmid could silence a concomitantly administered transgene expression in adult mouse liver cells (McCaffrey *et al.,* 2002). Song *et al.* demonstrated that the intravenous injection of Fas siRNA specifically reduced Fas mRNA levels and expression of FAS protein in mouse hepatocytes (Song *et al.,* 2003). Recently many groups reported the generation of transgenic knockdown mice from embryonic stem cells expressing siRNA in the form of shRNA, either from plasmid, retrovirus or lentivirus based vectors (Caplen, 2003). This technology helps in producing animals showing spatial and temporal specific inhibition of gene expression by expresing siRNA from tissue specific or inducible promoters.

In spite of considerable advances in this field, still many hurdles have to be overcome for the successful application of RNAi *in vivo*. One of the major issues to be taken care of is the delivery of siRNA to the specific tissue, while ensuring appropriate level of efficacy with minimum toxicity.

## 2.11 RNAi in Parasites

RNAi has also been shown to have potential to study the function of the genes at genome wide level. Table 1A lists some of parasitic organisms where RNAi has been successfully used. This technology particularly favours researchers if they are dealing with specific genes or gene families, which are difficult to knockout by using existing techniques. Among different parasites, RNAi has been extensively studied in *Trypanosomes.*

### 2.11a RNAi in Trypanosomes

Among the different known parasites, RNAi was first demonstrated in *Trypanosoma bruceii.* Genetic manipulation has been difficult in *T. brucei* because it is haploid through out the life cycle, lacks an easily manipulated sexual life cycle and has many genes in multiple copies. These barriers are largely overcome by the application of RNAi, which has proven extremely useful for studies of *Trypanosome* biology and of the interference process

itself. Initial study of RNAi in *Trypanosome* involved electroporating synthetic dsRNA of tubilin gene into *T. brucei* thereby silencing α-tubilin gene (Ngo *et al.,* 1998). Since then, RNAi has been used to study numerous aspects of *Trypanosome* biology, including flagellum ontogeny (Bastin *et al.,* 2000), a mitochondrial RNA polymerase (Grams *et al.,* 2002) and enzyme compartmentation in glycosomes (Guerra-Giraldez *et al.,* 2002). Euglund and his group have constructed *Trypanosome* genomic libraries in RNAi vector, allowing forward genetic applications of this technique (Morris *et al.,* 2002). RNAi has also been used to study gene families, such as *Tb PDE2C*, a cAMP–specific phosphodiesterase and the *TbPDE2* family to examine the effects of inhibiting expression of individual family members as well as family members simultaneously (Zoraghi *et al.,* 2002).

### 2.11b RNAi in *Plasmodium*

*Plasmodium falciparum*, a causative agent of cerebral malaria in humans is the most common and deadly protozoan parasite. Just like *Trypanosome, Plasmodium* has complex life cycle and has many genes in multiple copies. In contrast to *Trypanosomes*, application of RNAi in *Plasmodium* is at early stages. First report of RNAi in *Plasmodium* came from Mc Conkey and his group, who had electroporated *DHODH* dsRNA in to *P. falciparum* and showed silencing of dihydro orotate dehydrogenase gene, which is essential for purine biosynthesis pathway. They also showed reduction of *DHODH* mRNA levels and growth inhibition (Mc Robert *et al.,* 2002). Later another group showed silencing of pyrophosphotase-I (*PfPPI*) gene by electroporating *PfPPI* siRNA into *P. falciparum*. They for the first time demonstrated that siRNA could be successfully used in *P. falciparum* for silencing. We initiated RNAi studies to understand the function of the enzymes involved in the hemoglobin degradation pathway of *P. falciparum*.

## 2.12 Hemoglobin Degradation Pathway

Malaria disease is responsible for nearly two million deaths each year particularly in developing countries. With the increasing prevalence of drug resistant strains, there is an urgent need to identify new drug targets. It is

important to look for pathways that are unique to *Plasmodium* and develop inhibitors against these pathways. Hemoglobin degradation pathway is one such pathway, which is essential for the survival of the parasite. This pathway has many essential enzymes, which are good candidates for chemotherapeutic development. Intra erythrocytic stages of *Plasmodium* are capable of very limited *de novo* amino acid synthesis. To obtain amino acids for its own protein synthesis, the malaria parasite must degrade host cell hemoglobin as well as import extra cellular nutrients. In about 12 h, trophozoite stage parasite degrades 75 % of host cell hemoglobin (Loria *et al.,* 1999). For the normal growth, intra-erythrocytic *Plasmodium* requires amino acids like, methionine, glutamine, glutamic acid, cysteine and isoleucine, which are either limiting or absent in the hemoglobin. Jensen and his group observed significant growth retardation of parasites, when isoleucine was omitted from the medium (Divo *et al.,* 1995). Cultures grown in a medium containing only these four amino acids are sensitive to hemoglobin degradation inhibitors than cultures grown in complete medium thereby suggesting that amino acids obtained from hemoglobin degradation is essential for the protein synthesis. In contrary to the above studies, several groups have shown that only 7 % of the total red cell hemoglobin support normal parasite development (Rangachari *et al.,* 1987). It has also been shown that all the amino acids, even those present in the hemoglobin are taken up by the parasite from the culture medium and incorporated into the parasite proteins (McCormick *et al.,* 1970). Ginsburg and his group have shown that excess of amino acids produced from the hemoglobin degradation are diffused freely into the erythrocyte cytosol (Ginsburg *et al.,* 1986). This has raised the possibility that hemoglobin degradation occurs in order to reduce host cell volume and create space for the development of merozoites to schizonts (Desai *et al.,* 1993). Whatever might be the reason, hemoglobin degradation is an essential step for the survival of the parasite.

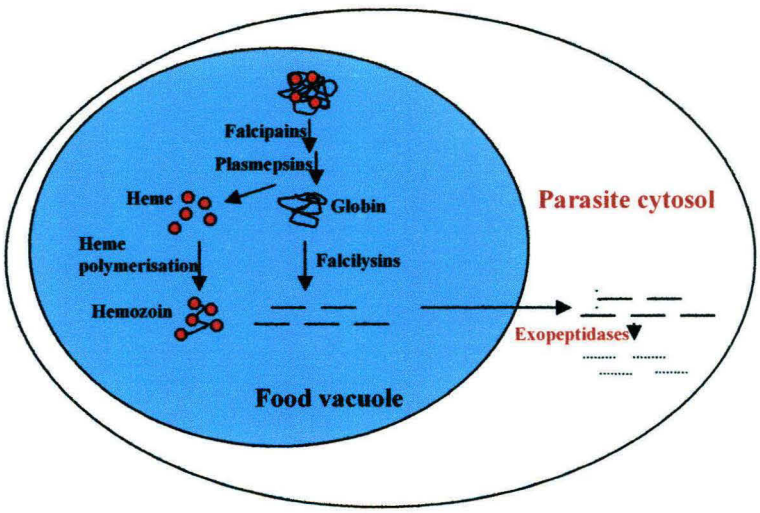



**Fig.4. Represents the hemoglobin degradation pathway in *Plasmodium* species**

### 2.12a Ingestion of Hemoglobin

The first step before the hemoglobin degradation is the ingestion of hemoglobin by the intraerythrocytic parasite. Hemoglobin ingestion and digestion vary with intraerythrocytic stages. In very early rings, parasites attaches to the erythrocyte membrane and may obtain nutrients directly through a passage way or metabolic window in the apposed membrane. Although most of the host cell cytosol is taken up by micro pinocytosis, only minimal amount of hemoglobin digestion and hemozoin production was observed (Slomianny *et al.,* 1990). In the more metabolically active trophozoite stage, hemoglobin catabolism is a complex and efficient process. Hemoglobin is taken through the cytostome, a pear shaped structure that is formed by the invagination of the parasitophorous vacuolar membrane and the parasite plasma membrane (Rosenthal, 2002). It was also shown that along with the hemoglobin other proteins present in the erythrocyte cytosol were also taken up by the parasite (Cowman *et al.*, 2001). In *Plasmodium falciparum,* double membrane transport vesicle bud off from cytostome and migrate towards and fuse with the large central food vacuole, where most of the hemoglobin degradation takes place (Slomianny *et al.,* 1990; Goldberg *et al.,* 1993; Akiwa *et al.,* 1971).

### 2.12b Food Vacuole

Mature trophozoites develop a large food vacuole, perhaps as a result of fusion of small vesicles. In *P. falciparum,* the food vacuole appears to be a large lysosome with an acidic pH, essential equipped with hemoglobinases to degrade hemoglobin. Food vacuole in *Plasmodium* is highly specialized and doesn't contain non-proteolytic hydrolases such as β-galactosidase, β-glucorinidase and acid phosphatase, which are present in lysosome (Goldberg *et al.,* 1990). This organelle is devoted only for hemoglobin degradation and is not probably involved in general protein degradation. Some of the erythrocyte proteins like superoxide dismutase and catalase, which are accumulated in the food vacuole, were not digested by the proteases present in the food vacuole. This shows that these proteases are specific for hemoglobin degradation.

### 2.12c Proteases in Food Vacuole

Hemoglobin degrading proteases have been categorized into three classes, aspartic proteases (Plasmepsins I-X) (Banerjee *et al.,* 2002), cysteine proteases (Falcipain 1, 2 and 3) (Shenai *et al.,* 2000; Salas *et al.,* 1995; Sijwali *et al.,* 2001) and metallo proteases (Falcilysins). These proteases have been proposed to act in a semi-orderd fashion (Goldberg *et al.,* 1993) in which aspartic proteases act first on hemoglobin (Goldberg at al., 1991; Gluzman *et al.,* 1994), while cysteine and metallo proteases act later in the degradation pathway (Eggleson *et al.,* 1999; Rosenthal and Nelson, 1992; Francis *et al.,* 1996).

#### 2.12c(i) Aspartic proteases

Among different aspartic proteases identified, Plasmepsin I and II were the first to be isolated. It was shown that under non-reducing conditions, Plasmepsin I was found to readily cleave native hemoglobin and Plasmepsin II prefers denatured hemoglobin, thereby suggests that the degradation as an ordered process initiated by Plasmepsin I (Goldberg *et al.,* 1990). Recently, Golberg and coworkers found 10 aspartic prorteases, out of which only Plasmepsin I, II, IV and histo-aspartic protease (HAP) are involved in

hemoglobin degradation (Banerjee *et al.,* 2002). However, further studies predicted reducing environment in the food vacuole because of the influx of reduced glutathione, which facilitates cleavage of hemoglobin by Falcipains (Francis *et al.,* 1996). This was further supported by E-64 and other cysteine protease inhibitor studies on *P. falciparum* culture, which showed accumulation of undegraded hemoglobin in food vacuole of treated parasites (Rosenthal *et al.,* 1996). Inspite of extensive studies, the role of proteases in the initial degradation of hemoglobin still remains unclear.

2.12c(ii) Falcipains

Falcipains are another class of proteases that have been shown to play an important role in hemoglobin degradation. They belong to the papain class of cysteine proteases. Rosenthal and his group for the first time showed cysteine protease activity in the extracts of trophozoites (Rosenthal *et al.,* 1998, 1987). Morphological observation of cysteine protease inhibitor-treated parasites revealed abnormally swollen, dark stained food vacuoles, and biochemical evaluation indicating that the abnormality was caused by the accumulation of undigested hemoglobin in food vacuole (Rosenthal *et al.,* 1989; Bailly *et al.,* 1992). These results suggested a central role for a cysteine protease in early steps in hemoglobin degradation by *P. falciparum*. The first identified cysteine protease that was attributed for hemoglobinase activity in food vacuole was a single copy *falcipain* gene, renamed now as *falciapin 1*.This gene was isolated, cloned and expressed in baculovirus. It was synthesized as a 67 kDa proform, which was cleaved into 26 kDa mature form, which has 37 % identity with cathepsin L (Rosenthal *et al.,* 1992). This recombinant protein showed hemoglobinase activity. Northern blot analysis of different stages of parasite showed that falcipain1 mRNA was mostly expressed at the ring stage. Recently, Greenbaum *et al.* (2002) showed the presence of falcipain1 protein at the apical end of the merozoite during the schizont stage rather than food vacuole, and helps in the invasion process. Although, its role in the invasion process has been clearly shown, the authors failed to explain the reason for the presence of this protein in vesicular membrane network during the ring as well as the trophozoite stages. Rosenthal's group later identified two more cysteine proteases and named them as falcipain 2 and 3 (Shenai *et al.,*

2000; Sijwali *et al.,* 2001). Both the falcipains were cloned and expressed and were shown to have hemoglobin degradation properties. Falcipain 2&3 have a large prodomain, which is cleaved to form a mature 27 kDa protein. Falcipain 2 has 33 % identity at aminoacid level with falcipain 1 and 80 % identity with falcipain 3. The expression profile of all the three proteins varies considerably. Falcipain 2 is expressed more at the trophozoite stage whereas Falcipain 3 is expressed only at schizont stage. Although Falcipain 3 is very similar to Falcipain 2, the enzymes have important difference, which suggest differences in their biological roles. The stability and activity of Falcipain 3 is greater at acid pH and less at neutral pH than that of *Falcipain 2* (Sijwali *et al.,* 2001). Both the falcipains lack a typical signal sequence, but contain a 20 amino acid stretch of hydrophobic domain, which might act as a transmembrane domain. Several studies have shown the involvement of cysteine proteases in the release of merozoites from the mature schizonts (Wickham *et al.,* 2003). Treatment of schizonts with 8 μM conc of E-64 inhibited the release of merozoites, supporting the role of cysteine proteases in the release process. Hanspal and colleagues have shown that the recombinant Falcipain 2 can degrade ankyrin *in vitro.* Later the same group showed that the peptides designed from the ankyrin cleavage site of Falcipain 2 inhibited the cleavage activity of recombinant Falcipain 2 and when added to the culture blocked the parasites at schizont stage. These results clearly indicate the involvement of Falcipain 2 in the release of parasites (Dhawan *et al.,* 2003). Recently, Rosenthal *et al.,* have constructed knockouts of Falcipain 2 and showed their role in stage specific degradation of hemoglobin (Woodshole meeting abstract).

2.12c(iii) Falcilysins

Falcilysins, zinc metallo proteases are the third class of proteases that have been shown to be involved in hemoglobin degradation. It was for the first time identified in 1999 when analysis of degraded globin fragments from the food vacuole revealed several peptides with cleavage sites that could not be attributed to the known proteases (Kolakovich *et al.,* 1997). Later, the native enzyme was purified from the food vacuole and was shown to be monomeric in nature with molecular weight of 130 kDa, the largest hemoglobin catabolic

protease. Falcilysins are characterized by an inverted active site motif, HXXEH, where the histidines coordinate a catalytic zinc ion. This enzyme unlike other food vacuole proteases, doesnot has a propiece. It is targeted and functional without processing (Eggleson *et al.,* 1992). The peptides of length 8-10 amino acids generated in the food vacuole are exported outside the food vacuole for terminal degradation by cytoplasmic exopeptidases (Kolakovich *et al.,* 1997). The other proteins that are present in the food vacuole include ATPases (Karcz *et al.,* 1994), PGH-1 (Cowman *et al.,* 1991) and oxidant defense enzymes (Francis *et al.,* 1996).

In conclusion, hemoglobin degradation process in the parasite thus involves interplay between three classes of the enzymes. However, the order in which these enzymes act is still not clearly known. There are two schools of thought regarding the involvement of different proteases in hemoglobin degradation. Goldberg and co-workers proposed that aspartic proteases act during the intial step of hemoglobin degradation separating heme and globin. Further degradation of globin moiety in the hemoglobin degradation pathway occurs by cysteine proteases along with aspartic proteases. Based on cysteine protease inhibitor studies, Rosenthal's group proposed that both cysteine proteases as well as aspartic proteases act simultaneously on the hemoglobin and separate globin from heme, which is further degraded by aspartic proteases and Falcilysins. In our present study, we made an attempt to resolve the ongoing controversy regarding the role of proteases in the initial degradation process of hemoglobin by silencing both the cysteine protease genes using RNAi.

# Chapter 3: Materials and Methods

## 3.1 Materials

### 3.1.1 *E. coli strains*

| Strain | Genotype | Application | Source |
|---|---|---|---|
| DH5α | $F^-$ (φ80d *lac* ZM15) Δ(*lac*ZYA - *arg*F) U169 *deo*R *ace*A1 *end* A1 *hsd* R17 (r-m+) *sup*E44λ-*thi*-1 *gyr* A96 *rel*A1 | Plating and growth of plasmids, permits α-complementation with amino terminus of β-galactosidase encoded in pUC vectors | GIBCO BRL, USA |

### 3.1.2 Plasmodium strains

| Strain | Source |
|---|---|
| *Plasmodium falciparum* | MR4 (USA) |
| *Plasmodium berghei* | MR4 (USA) |

### 3.1.3 Standard markers

| Marker | Source |
|---|---|
| 1 Kb DNA ladder | Invitrogen life technologies (Carlsbad, USA) |
| Protein marker | MBI Fermentas |
| Pre-stained protein marker | MBI Fermentas |

### 3.1.4 Restriction Endonucleases and DNA Modifying Enzymes

Restriction endonucleases, Calf intestinal alkaline phosphatase, T4 kinase, T4 DNA Polymerase, Klenow (DNA polymerase I large fragment) and T4 DNA Ligase used in all routine cloning and transformation experiments were procured from New England Biolabs Inc. MA, USA (NEB). Taq polymerase for PCR amplification was an in-house preparation. Analytical and molecular biology products including

antibiotics, enzymes like proteinase K and lysozyme were purchased from Sigma, USA.

### 3.1.5 Standard Media

All the media were made in milliQ water and sterilized by autoclaving for 20 min at 15 lb/sq. in. pressure unless otherwise indicated.

#### 3.1.5.1 LB Medium (Luria Bertani Medium)

| Per Liter: | | |
|---|---|---|
| | Bacto-tryptone | 10 g |
| | Bacto-yeast extract | 5 g |
| | NaCl | 5 g |
| | MilliQ water | 950 ml |

The media was dissolved; the pH adjusted to 7.5 with 5 N NaOH and the volume made up to 1000 ml with MilliQ water. Agar at a concentration of 1.5 % was added whenever required.

#### 3.1.5.2 RPMI Medium

| Per Liter: | | |
|---|---|---|
| | RPMI | 1 pack |
| | HEPES | 5.96 g |
| | $NaHCO_3$ | 2.00 g |
| | Triple distilled milliQ | 980 ml |

The medium was filtered through 0.25 μm filter and 10 % FCS was added at the time of use to make complete RPMI.

#### 3.1.5.3 CPD

| Per Liter: | | |
|---|---|---|
| | Sodium Citrate | 100 mM |
| | Citric Acid | 15 mM |
| | Sodium Phosphate | 16 mM |
| | Dextrose | 32.44 g |

### 3.1.6 Molecular Biology Kits

Commercially available kits for preparation of plasmid DNA, Endofree Plasmid Giga Kit, QIA filter Plasmid Maxi kit, QIA filter Plasmid midi kit, QIA prep spin miniprep kit, QIA quick PCR Purification Kit, QIA quick Gel Extraction Kit, were obtained from Qiagen GmbH, Hilden, Germany.

### 3.1.7 Immuno-chemicals and Other Consumables

Nitrocellulose membrane for western blotting was procured from Amersham Pharmacia Biotech (Uppasala, Sweden). Alkaline phosphatase as well as HRP conjugated anti-mouse IgG was from Sigma chemical company St. Louis, USA. X-ray (ready-pack) films for autoradiography were procured from Sigma (St. Louis, USA). Flourescein conjugated anti-mouse IgG was obtained from Sigma St. Louis, USA).

### 3.1.8 Primers

Primers for PCR amplification of the various genes from the genomic DNA and sequencing of the constructs were procured from Integrated DNA Technologies, Inc. (Coralville, USA).

Table 1: Sequences of the primers synthesized for the amplification.

| PRIMER | SEQUENCE |
|---|---|
| T7 primer | 5′-GTA ATA CGA CTC ACT ATA GGG -3′ |
| SP6 primer | 5'-ATA AGA TAT CAC AGT GGA TTT A-3′ |
| Fal1 forward | 5′-ATG GTT GCC ATA AAA GAA ATG -3′ |
| Fal1 reverse | 5′-TTA CAA GAT AGG ATA GAA GAC -3′ |
| Fal2 forward | 5′-ATG GAT TAC AAC ATG GAT ATA -3′ |
| Fal2 reverse | 5′-TTA TTC AAT TAA TGG AAT GAA -3′ |

### 3.1.9 Chemicals, Media Components, Kits and Other Consumables

Chemicals used in the present investigation and their sources are listed in Table 2-2.

*Table 2-2-List of chemicals and bio-chemicals used in the present study*

| Chemical | Source |
|---|---|

| | |
|---|---|
| Acrylamide | Sigma Chemical Co., St. Louis, USA |
| Ammonia E | Merck (India) Ltd., Mumbai |
| Ammonium sulfate E | Merck (India) Ltd., Mumbai |
| Ammonium per sulfate | Sigma Chemical Co., St. Louis, USA |
| Agarose DNA grade | Sigma Chemical Co., St. Louis, USA |
| Agarose DNA grade low melting point | GIBCO BRL |
| Ampicillin | Sigma Chemical Co., St. Louis, USA |
| Bacto Agar | Difco, Becton, Dickinson Company, Maryland, USA |
| Bacto Peptone | Difco, Becton, Dickinson Company, Maryland, USA |
| BCIP/NBT | Promega Life Science, Madison,WI, USA |
| Boric acid | Sigma Chemical Co., St. Louis, USA |
| Bovine serum albumin | Sigma Chemical Co., St. Louis, USA |
| Bromophenol blue | Sigma Chemical Co., St. Louis, USA |
| Calcium chloride | Sigma Chemical Co., St. Louis, USA |
| Citric acid anhydrous | Sigma Chemical Co., St. Louis, USA |
| Dextrose E | Sigma Chemical Co., St. Louis, USA |
| Dulbecco's modified eagle medium | GIBCO Invitrogen Corporation, Grand Island, New York |
| Dimethylsulfoxide | Sigma Chemical Co., St. Louis, USA |
| Dithiothreitol | Sigma Chemical Co., St. Louis, USA |
| EDTA | Sigma Chemical Co., St. Louis, USA |
| Ethidium bromide | GIBCO Invitrogen Corporation, Grand Island, New York |
| Ethanol | Bengal Chemicals and Pharmaceuticals Ltd., Calcutta, India |
| Fetal Calf serum | GIBCO Invitrogen Corporation, Grand Island, New York |
| Formaldehyde | Merck (India) Ltd. Mumbai |

| | |
|---|---|
| Formamide E | Sigma Chemical Co., St. Louis, USA |
| Kanamycin | Sigma Chemical Co., St. Louis, USA |
| Glycerol | Sigma Chemical Co., St. Louis, USA |
| Glycine | Sigma Chemical Co., St. Louis, USA |
| Imidazole | Sigma Chemical Co., St. Louis, USA |
| Lipofectamine reagent | Invitrogen Corporation |
| Luria Bertani medium | Difco, Becton, Dickinson Company, Maryland, USA |
| β-Mercaptoethanol | Sigma Chemical Co., St. Louis, USA |
| Methanol E | Merck (India) Ltd. , Mumbai |
| PMSF | Sigma Chemical Co., St. Louis, USA |
| di-Potassium hydrogen phosphate | Sigma Chemical Co., St. Louis, USA |
| Potassium Dihydrogen Orthophosphate | Sigma Chemical Co., St. Louis, USA |
| Propan-2-ol | Merck (India) Ltd. , Mumbai |
| Sodium bicarbonate | Sigma Chemical Co., St. Louis, USA |
| Sodium chloride | Sigma Chemical Co., St. Louis, USA |
| Sodium hydroxide | Sigma Chemical Co., St. Louis, USA |
| Sodium phosphate | Sigma Chemical Co., St. Louis, USA |
| di-Sodium hydrogen phosphate | Sigma Chemical Co., St. Louis, USA |
| SDS | Sigma Chemical Co., St. Louis, USA |
| Sulfuric acid E | Merck (India) Ltd. Mumbai |
| TEMED | Sigma Chemical Co., St. Louis, USA |
| Trypsin EDTA | Invitrogen Corporation |
| Tris-HCl | Sigma Chemical Co., St. Louis, USA |
| Triton X-100 | Sigma Chemical Co., St. Louis, USA |
| Tween-20 | Sigma Chemical Co., St. Louis, USA |
| T4 DNA ligase | (GIBCO BRL) |

### 3.1.10 Antibiotics and Substrates

| Reagent | Stock solution | Final concentration in use |
|---|---|---|
| Ampicillin | 100 mg/ml | 100 µg/ml |
| Kanamycin | 25 mg/ml | 25 µg/ml |
| IPTG | 1M | 1 mM |
| X-gal | 40 mg/ml | 40 µg/ml |

### 3.1.11 Reagents and Buffers

All reagents and buffers for DNA and protein work were prepared in MilliQ grade water and sterilized by autoclaving for 15 min at 15 lb/sq. in. pressure unless otherwise mentioned.

#### 3.1.11.1 Commonly Used Buffers

*1. Phosphate buffered saline (PBS)*

| | | |
|---|---|---|
| Per Liter: | NaCl | 5.8 g |
| | $Na_2HPO_4$ | 5.3 g |
| | $NaH_2PO_4$ | 1.63 g |
| | pH | 7.4 |

| | | |
|---|---|---|
| *2. 1X TE Buffer* | Tris (pH 8.0) | 10 mM |
| | EDTA (0.5 M) | 1 mM |

#### 3.1.11.2 Buffers for Isolation of Genomic DNA

| | | |
|---|---|---|
| *1. Lysis buffer:* | Tris (pH 8.0) | 10 mM |
| | EDTA | 1 mM |
| | SDS | 0.5 % |
| | NaCl | 100 mM |
| | Proteinase K | 200 µg/ml |

*2. Proteinase K* 20 mg/ml in MilliQ water

*3. Phenol: Chloroform: Isoamylalcohol*

Solution contains 25 parts of buffered phenol, 24 parts of chloroform mixed with 1 part of isoamyl alcohol. The solution is stored in a glass container at 4 °C.

### 3.1.11.3 Solutions for Preparation of Chemically Competent Cells

| | | |
|---|---|---|
| *1. Solution I* | $CaCl_2$ | 50 mM |
| *2. Solution II* | $CaCl_2$ | 50 mM |
| | Glycerol | 20 % |

### 3.1.11.4 Buffers for Electrophoresis

*1. Tris Acetate EDTA (TAE) Buffer:*

| | | |
|---|---|---|
| Per Liter: | Tris base | 242 g |
| | EDTA (0.5 M) | 100 ml |
| | Glacial acetic acid | 57.1 ml |
| | Milli Q water | to 1000 ml |
| | pH | 8.3 |

*2. 10X TBE Buffer*

| | | |
|---|---|---|
| Per Liter: | Tris base | 108 g |
| | Boric Acid | 55 g |
| | EDTA (0.5 M) | 40 ml |
| | MilliQ water | to 1000 ml |

*3. 10X Tris-Glycine buffer*

| | | |
|---|---|---|
| Per Liter | Tris base | 30.3 g |
| | Glycine | 144.1 g |
| | SDS | 10 g |

### 3.1.11.5 Buffers for Plasmid Isolation

| | | |
|---|---|---|
| *1. Solution I:* | Glucose | 50 mM |
| | Tris-HCl [pH 8.0] | 25 mM |
| | EDTA | 10 mM |
| | RNAse | 10 µg/ml |
| *2. Solution II* | NaOH | 0.2 M |
| | SDS | 1 % |

| | | |
|---|---|---|
| *3. Solution III* | Sodium Acetate | 3 M |

### 3.1.11.6 Buffers for Gel Loading

*1. Laemmli 4X Sample Buffer (180)*

| | | |
|---|---|---|
| Per 50 ml | Glycerol | 22.4 ml |
| | βME | 10 ml |
| | Tris (1 M), pH 6.8 | 1.4 ml |
| | SDS | 6.0 g |
| | Bromophenol blue | 0.02 g |

*6X dye for agarose gel electrophoresis*

| | | |
|---|---|---|
| | Bromophenol blue | 0.25 % |
| | Sucrose | 40 % |

Dye is prepared in 6X TE buffer

### 3.1.11.7 Reagents for SDS-PAGE

| | | |
|---|---|---|
| *1. Acrylamide 30%* | Acrylamide | 29.2 g |
| | N,N'-methylenebisacrylamide | 0.8 g |
| | MilliQ water | 50 ml |

The solution was stirred to dissolve the acrylamide. The volume was made up to 100 ml and the solution was filtered through Whatman filter paper no. 1 before use.

| | | |
|---|---|---|
| *2. Ammonium per sulfate (APS)* | | 10 % |
| *3. SDS* | | 20 % |
| *4. Buffer for resolving gel* | Tris-HCl (pH 8.8) | 1.5 M |
| *5. Buffer for stacking gel* | Tris-HCl (pH 6.8) | 1 M |
| *6. Coomassie Blue staining solution* | Brilliant blue (R250) | 0.1 % |
| | Acetic acid | 10 % |
| | Methanol | 40 % |

The solution was filtered through Whatmann filter paper no. 1 before use

| | | |
|---|---|---|
| *7. Destaining Solution* | Methanol | 40 % |
| | Acetic acid | 10 % |

### 3.1.11.8 Reagents for Western Blot analysis and ELISA

| | | |
|---|---|---|
| *1. Transfer Buffer* | Tris | 0.25 M |
| | Glycine | 0.019 M |
| | Methanol | 20 % |
| | SDS | 0.1 % |
| *2. Blocking Buffer* | 2 % milk in PBS | |
| *3. Wash Buffer* | 0.05 % Tween-20 in PBS | |
| *4. Developing buffer (HRP)* | DAB | 10 mg |
| | PBS | 10 ml |
| | $H_2O_2$ | 10 µl |
| *5. Coating buffer* | $Na_2CO_3$ | 0.015 M |
| | $NaHCO_3$ | 0.035 M |
| | pH 9.6 | |

### 3.1.11.9 Buffers for Protein Purification using $Ni^{2+}$-NTA

| | | |
|---|---|---|
| *1. Sonication buffer* | Tris-HCl | 50 mM |
| | NaCl | 300 mM |
| | pH 7.4 | |
| *2. Lysis buffer* | Urea | 8 M |
| | Tris-Hcl pH 8.0 | 20 mM |
| | NaCl | 300 mM |
| 3. *Buffers for refolding* | | |
| i. Urea Buffer | Urea | 8 M |
| | Tris-HCl pH 8.0 | 20 mM |
| ii. TB | Tris-HCl pH 8.0 | 20 mM |
| *4. Elution Buffer* | Imidazole | 10 mM-1M |
| | Tris-HCl, pH 8.0 | 20 mM |

## 3.2 Methods

### 3.2.1 Complete Media Preparation

RPMI powder sealed in a packet was opened and dissolved in 1000 ml of glass distilled water to make 1litre of RPMI medium. For 100ml of complete media, RPMI-HEPES was supplemented with 10 % of heat inactivated $O^+$ve human serum, 5.8 ml of 3.6 % Sodium bicarbonate and 20 µg/ml of gentamicin. In case of *P. berghei* cultures fetal calf serum was used instead of human serum.

**Incomplete media=Complete media – Human serum.**

### 3.2.2 *P. falciparum* Strains used for the Experiments

Parasite strains used for the present study were 3D7, 3D7-GFP +His. 3D7 was obtained from MR4 and 3D7-GFP +His is a kind gift from Dr.Alan Cowman, Walter Eliza Institute, Melbourne, Australia.

### 3.2.3 Thawing of *P. falciparum* Culture Frozen Stock

Frozen vial of parasite culture was thawed at 37 °C in circulating water bath for 1 min and immediately transferred to sterile tube. An equal volume of thawing solution was added drop by drop with gentle mixing of sample to the sterile tube. The mixture was centrifuged at 1200 rpm for 2 min at room temperature. The supernatant was discarded and 1 ml of thawing solution was added to the pellet by gently mixing the sample. The mixture was again centrifuged at same speed and time as indicated above. This step of centrifugation and addition of thawing solution was continued till there was no lysis of RBC. Fresh complete media of 5 ml with 2 % hematocrit and 10 % human serum was added to the pellet and mixed thoroughly. The parasite culture was transferred to 6 well microtitre plate and kept in a humid chamber, which was flushed with gas mixture (90 % N2, 5 % CO2 and 5 % O2) (SMS Multitech, India). The chamber was incubated at 37 °C for the growth of the parasite.

### 3.2.3a Maintenance of *P. falciparum* Culture (Trager and Jensen, 1976)

The media was changed after every 48 h of incubation. Fresh prewarmed complete media was added to the culture plate and mixed thoroughly. A small

quantity of sample was taken out for making thick and thin smears to determine parasite growth after staining with Geimsa stain. The plate was re-incubated at 37 °C in a humid chamber.

### 3.2.3b Subculture and Dilution of Parasite Culture

The parasite culture should be diluted or subcultured, once the culture reached 5-10 % parasitemia. Before dilution, the culture in the plate was thoroughly mixed to make sure that no RBC is stuck to the bottom of the plate. After wards, the culture was diluted by removing appropriate amount of culture media and the same amount of complete media with 4 % hematocrit was added. In case of transgenic lines i.e., 3D7-GFP+His, an appropriate amount of WR99210 was added to the culture before incubation.

### 3.2.3c Synchronization of *Plasmodium falciparum* by Sorbitol Treatment (Lambros *et al.*, 1979)

The culture was harvested at about 10 % parasiteamia with majority at ring stage by centrifuging at 1600 rpm for 5 min at RT. To the cell pellet 5 vol of 5 % sorbitol solution was added and mixed gently. This solution was incubated at 37 °C for 5 min and centrifuged at 1600 rpm for 5 min. The supernatant was carefully discarded with out disturbing the pellet. Prewarmed complete media was added to the pellet. The culture was mixed thoroughly and incubated at 37 °C for the growth of parasite. Repeat the sorbitol treatment once again after one cycle of growth to ensure synchronization.

### 3.2.3d Isolation of Purified Parasite from Infected Blood by Saponin Lysis

The culture for saponin lysis was harvested by centrifuging at 1600 rpm for 5 min at RT. To the pellet, 1.5 volumes of 0.15 % saponin was added and mixed gently. The solution was incubated in ice for 10 min and centrifuged at 2000 rpm for 10 min. The supernatant was discarded without disturbing the pellet. The pellet was washed with ice-cold 1XPBS till the red colour of the supernatant disappeared. Parasite pellet was stored at –70 °C till further use.

### 3.2.3e Preparation of Frozen Stocks of Parasite Culture

Once the parasitemia reaches 5 % ring stage, it was harvested by centrifuging at 1500 rpm for 5 min. To the pellet, 1.5 volumes of freezing mixture was added and dispensed in freezing vial (1ml per vial) and transferred immediately to liquid nitrogen.

### 3.2.4 Maintenance of *Plasmodium berghei*

The most frequently used strains of *P. berghei* are: K173, ANKA, NK65, SP11 and LUKA. In our present studies, we have used ANKA strain of *P. berghei* and this strain can be maintained 1. ***in vitro*** and 2.**rodent host**.

1. **Maintenance of *P. berghei in vitro*:** Blood was collected from the infected mice in to heparinized syringe or the frozen stock was thawed to set up initial culture. The blood which was collected from the mice should be passed through the CF11 column to remove white blood cells. The initial culture was established at 8-12 % hematocrit and parasitemia of 0.5-2 %. Culture was maintained at 37 °C under constant flow of 5 % CO2 in 10 % O2 and 85 % N2 in T75 flask. The culture was constantly stirred at 50 rpm and once every 24 h was stirred at 400 rpm to assist in the rupture of the accumulated schizonts. The medium was changed daily twice by stopping the magnetic stirrer, allowing the RBC to sediment, so that the old medium was removed and fresh medium was added.

2. **Maintenance of *P. berghei* in mice:** BALB/c mice were used for the maintenance of *P. berghei.* To infect mice, frozen stabilate or heparinised blood was used. Routinely, 100 µl of blood $10^5$ parasitized red blood cells was injected intraperitoneally into mice, so that the parasitemia will reach 10 % within 7 days of injection.

### 3.2.4a Monitoring Parasitemia

In case of *in vitro* cultures, smears were made from the T75 flasks just before changing of the medium. But in the case of infected rodents, the tail was held tight and a small incision was made at the tip and a small drop of blood was collected on the glass slide. Smear was made with the help of other slide, dried in the stream of warm air, fixed in methanol for 30 s and stained in Geimsa for 10-15 min. The slide

was observed under oil immersion and the parasitemia was estimated by counting number of infected and uninfected RBC.

### 3.2.4b Cryopreservation of *P. berghei* Parasite Culture

Blood from *P. berghei* infected mice was collected into heparinised syringe (final heparin concentration-30 µg/ml), transferred in to 15 ml falcon and was placed immediately on ice. Equal volumes of 20 % DMSO (Dimethyl sulfoxide) made in blood stage culture medium was added in drop wise over a period not less than 10 min to the heparinized blood. Numerous aliquots (0.1 ml) of the final mixture were made in cryotubes. The samples were frozen in a controlled manner to –80 °C. Subsequently, the cryotubes were transferred to the liquid nitrogen and can be stored indefinitely.

### 3.2.5 Cloning in pGEMT

Gene of interest was PCR amplified from the genomic DNA using gene specific primers. The PCR reaction was set up using following reagents

Chromosomal DNA : 100 ng

10X PCR buffer : 5 µl

dNTPs (1.25mM) : 8 µl

Forward primer : 50 pMoles

Backward pri : 50 pMoles

Taq enzyme : 1 unit

The reaction volume was set to 50 µl using MQ water.

The PCR reaction was run on 1 % agarose gel and the amplified product was gel extracted by using gel extraction kit (Novagen) as per manufacturer's instructions. The eluted fragment was cloned in pGEMT vector (Promega). The ligation in pGEMT was setup as follows.

pGEMT Vector : 1 µl

Insert : X µg

2X buffer : 5 µl

Ligase : 1 µl

The reaction volume was set to 10 µl using MQ water.

The ligation mixture was incubated at 4 °C O/N.

### 3.2.6 Preparation of Chemically Competent Cells

An overnight seed culture of *E. coli* was prepared in LB by incubating at 37 °C under shaking conditions. The seed culture was diluted fifty fold using fresh LB medium and allowed to grow at 37 °C with shaking till OD reached to 0.5-0.8 at 600 nm. The cells were pellet down by centrifugation at 4000 rpm for 30 min at 4 °C. The media was discarded and the cells were washed with chilled 50 mM calcium chloride. The cells were suspended in 50 mM Calcium chloride and incubated on ice for 1hr to make them competent. The competent cells were pelleted and resuspended in chilled $1/12.5^{th}$ volume of freshly prepared and sterile 50 mM calcium chloride containing 20 % glycerol. These competent cells were stored at –70 °C for several months.

### 3.2.7 Transformation into Chemically Competent *E. coli* Cells

Ligation mixture was mixed with Chemical competent *E. coli* cells (JM109) and incubated for 30 min on ice. Heat shock was given at 42 °C for 90 sec and immediately transferred onto the ice for 2 min. Appropriate amount of LB broth was added to the transformants and incubated at 37 °C with shaking for 1 h. The cells were plated on LB plate containing appropriate antibiotic for the selection of the transformants. When cloning in pGEMT, the plate was spread with 40 µl of 40 % X-gal and 40 µl of 0.1 mM IPTG for the selection of blue- white colonies.

### 3.2.8 Isolation of Plasmid from *E. coli*

Plasmid was isolated by alkali-lysis method. Culture for plasmid was inoculated in LB broth containing appropriate antibiotic and incubated O/N at 37 °C under shaking conditions. The cells were harvested by centrifugation at 6000 rpm for 10 min and suspended in 250 µl of P1 buffer. To the same tube 250 µl of P2 buffer was added, mixed gently and incubated for 5 min at room temperature. Later, P3 was added and incubated on ice for 15 min. The precipitate was pellet down by centrifugation at 13 K for 15 min at RT. Supernatant was carefully separated into another fresh eppendorf and phenol extraction was done to remove protein contamination. The DNA was precipitated by adding 0.7 volumes of isopropanol and pellet was recovered by centrifugation at maximum speed for 15 min. The plasmid DNA pellet was washed with 70 % ethanol, air-dried and dissolved in TE buffer.

### 3.2.9 Polyacrylamide Gel Electrophoresis of Proteins

PAGE was performed according to the protocol of Laemmli (1970). Gels were prepared and run in the presence of 0.1 % SDS (denaturing). Protein samples were prepared by mixing with equal volume of 2 X sample buffer (100 mM Tris-HCl pH 6.8, 4 % SDS, 20 % glycerol, 4 % β-mercaptoethanol, 0.01 % bromophenol blue). Samples were kept in boiling water for 5 min and loaded on the gel. Gels were run at 50 V till the proteins was stacked properly and thereafter gels were run at a constant voltage of 100 V. Gels were either electro blotted onto the nitrocellulose membrane (Hybond-C, Amersham, England) or stained with Coomassie blue R-250, 50 % methanol and 10 % acetic acid as described by Laemmli (1970).

### 3.2.10 Western Blotting

Western blotting was done according to Towbin *et al.* (1979). Mini Trans-blot Electrophoretic Cell (Bio-Rad) was used to transfer the proteins from gel onto nitrocellulose membrane. The apparatus for electroblotting was assembled according to the manufacturer's instructions. Electroblotting was performed in the presence of 39 mM glycine, 48 mM Tris base, 0.037 % SDS and 20 % methanol at a constant voltage of 50 V for 2 h at 4 °C. Transfer of proteins onto the membrane was checked by staining with Ponceau S stain (0.2 % w/v Ponceau-S, 3 % w/v tri-chloroacetic acid, 3 % (w/v) sulfosalicylic acid). The membrane was rinsed briefly in TBST (10 mM Tris, 150 mM NaCl, pH 7.5 and 0.05 % Tween-20) and then incubated in blocking solution (3 % BSA in TBST) for 1 h with gentle shaking at 37 °C. The blocking solution was replaced with primary antibody solution (1:2000 dilution of Fal2 and HRPII in TBST containing 1 % BSA) and incubation was continued at 37 °C for another 1 h with gentle shaking. Thereafter, the blots were washed thrice with TBST for 5 min each. After washing, blots were incubated with HRP conjugated secondary antibody solution (1:30,000 dilution in TBST containing 1 % BSA) at 37 °C for 1 h. The blots were washed as described above. The colour of protein-antibody complex was developed by using $H_2O_2$ and DAB. Alternatively, the blots were also developed by using ECL kit (Amersham Pharmacia).

### 3.2.11 dsRNA Preparation

Individual DNA fragments coding for *falcipain 1*, *falcipain 2* and *Aminopeptidae-N* (*Spodoptera litura)* are PCR amplified from pGEMT by using T7 and SP6 primers. The amplified products were PCR purified using PCR purification kit (Qiagen) as per manufacturer's instructions. The purified DNA fragments were used to generate sense as well as antisense RNA using T7 and SP6 polymerase.

**Reaction mixture is as follows**:

DNA : 5 µg

5Xtranscription buffer: 20 µl

rNTPs (2.5mM) : 8 µl

T7/SP6 : 3 µl each

RNase IN : 1 µl

The reaction was set to 100 µl using MilliQ water and incubated for 2 h at 37°C.

The tubes in which the reaction was setup were kept at –20 °C for 15 min to the polymerase activity. DNase I treatment was given by incubating at 37 °C to remove the DNA template contamination. To make dsRNA, the sense and antisense strands were mixed, heated to 65 °C and annealed by cooling slowly to 25 °C over several hours. Individual ssRNA and dsRNA were analyzed on 1 % agarose gel.

### 3.2.12 Preparation α-[$^{32}$P] Labeled dsRNA

The labeled dsRNA was prepared in the same way as discussed above. But instead of normal rUTP, α [$^{32}$P rUTP] was used in the rNTP mix. The internally labeled sense and anti sense RNA was annealed together to make labeled dsRNA. RNaseT1 digestion was done to eliminate ssRNA contamination.

### 3.2.13 Treatment of *P. falciparum* Culture with dsRNA

The parasites were synchronized as described in section 3.2.3c. For the analysis of the effects of dsRNAs, the synchronized cultures were adjusted to 5 % hematocrit with 1 % infected red blood cells, and 1ml of these cultures was treated in 24 well culture plates in triplicates in serum free medium for 30 min with intermittent mixing.

Subsequently human serum was added to these cultures to a final concentration of 10 % and parasites were maintained for 48 h. For microscopic analysis, smears were made from each well, fixed in methanol and stained with Geimsa.

### 3.2.14 Hypoxanthine Uptake Assay

To assess the effects of various dsRNA/inhibitors on parasite metabolic activity, a $^{3}$[H]- hypoxanthine uptake assay was performed as described by Rosenthal *et al.* (1996). Parasite cultures (1 % parasitemia) of 100 µl volume at early ring stage were taken in 96 well microtitre plate in triplicates. DsRNA was added to each of these wells and incubated for 24 h. Once the parasite reaches trophozoite stage, $^{3}$[H]-hypoxanthine (Dupont-NEN) was added (1.2 µCi), and cultures were maintained for additional 24 h. Cultures of mature parasite were frozen and thawed to lyse the infected RBCs. Lysed cultures were harvested on glass fibre filters, which were subsequently, washed with water, dried with ethanol and counted on a scintillation counter.

### 3.2.15 Treatment of *P. falciparum* cultures with Labeled dsRNA

The parasites were treated in the same way as described in the section 3.2.13. But instead of maintaining culture for 48 h, they were removed after 24 h and analyses for low molecular weight RNA (LMW) formation.

### 3.2.16 Isolation of RNA from Parasite and Fractionation on Agarose Gel

Culture from which RNA to be isolated was pellet down by centrifuging at 1500 rpm for 5 min. The pellet was taken into DEPC treated eppendorfs and saponin lysed as described in the section 3.2.3d. The parasites obtained after saponin lysis were washed with DEPC treated 1X PBS. Total RNA was isolated from the parasites using RNA isolation kit (Qiagen) as per manufacturer's instructions. Total RNA obtained was mixed with 6X RNA loading dye, heated at 65 °C for 10 min and quenched in ice for 5 min. The agarose gel for running of total RNA was made by dissolving 1.2 gms of agarose in 1X TBE and boiled. Once the temperature comes below 50 °C, 0.5 ml of 1 M GITC was added to 1X TBE containing agarose and poured on to the casting tray. After solidification, comb was carefully removed and

the gel along with the tray was transferred in to the buffer tank containing 1X TBE. The gel was run at 60-70 volts till the dye reaches 3/4$^{th}$ of total gel size.

### 3.2.17 Northern Blotting

Once the total RNA was properly resolved, the gel was washed thrice each for 15 min in denaturation solution (7.5 mM NaOH). The transfer was done over night in denaturation solution by using capillary transfer method. Briefly in this method, the gel was stacked between six pieces of 3 mm Whatmann sheet on either side with nylon membrane (Amersham N+ hybond) present above the gel. Filters papers were cut as per the exact size of the gel and placed above the Whatmann sheets. For proper transfer to take place, a heavy weight generally a thick book or glass bottle with uniform bottom was placed on the top of the filter sheets. After the transfer, position of the wells on the membrane was properly marked and the membrane was allowed to dry. RNA transferred on to the membrane was cross-linked using UV cross linker (Startagene) and washed in 6X SSC for 5 min. The membrane was dried properly, wrapped in saran wrap and stored at 4 °C till further use.

### 3.2.18 Preparation of Random Labeled Probe

The DNA probe for Northern hybridization was made by using random labeling kit (Gibco BRL). The DNA to be labeled was either gel-extracted fragment or PCR purified product, which was diluted in 20 µl of water in such a way that the amount of DNA was around 50-100 ng. This DNA was boiled, quenched in ice and was used in random labeling reaction. The reaction for random labeling was set as follows,

Random buffer: 15 µl
dATP : 2 µl
dTTP : 2 µl
dGTP : 2 µl
Taq Klenow : 1 µl
SDW : 7 µl

To the 28 µl reaction, 20 µl of diluted DNA along with 2 µl of α-[$^{32}$P] CTP was added and incubated at RT for 1 h. Later, the probe was purified by using nucleotide removal column (Qiagen) as per manufacturer's instructions.

### 3.2.19 Northern Hybridization

The blot for Northern hybridization was incubated in prehybridization solution for 2-3 h under constant rotation at 52 °C. Afterwards; the random labeled probe was added to the prehybridization solution and incubated for 12 h at 52 °C under constant rotation. Next, the blot was washed once in wash buffer I (3X SSC, 0.5 % SDS) and wash buffer II (2X SSC, 0.5 % SDS) at 52 °C for 15 min each. The last wash was given in 2X SSC to remove SDS and then the blot was dried and exposed to X-ray film (Kodak). The cassette was stored at –70 °C.

### 3.2.20 Analysis of Globin Hydrolysis in *P. falciparum* Parasites

To determine the effects of *falcipain* dsRNA or E-64 on hemoglobin degradation in malaria parasite, *P. falciparum* cultures were treated with this inhibitor/dsRNA for 24 h. After treatment, parasite infected erythrocytes were collected by centrifugation and lysed with saponin as described in the section 3.2.3d. The lysed samples were washed three times with ice cold PBS to remove erythrocyte cytoplasmic contents. The washed parasite pellet were solubilized in SDS sample buffer containing β-mercaptoethanol, boiled and fractionated on 15 % SDS-PAGE. The gels were stained with coomasie blue.

### 3.2.21 Extraction of Low Molecular Weight (LMW) RNA (25nt)

The dsRNA/siRNA treated cultures were lysed with saponin and the parasites obtained were washed with cold 1X PBS two to three times to remove erythrocyte cytosol contamination. Total RNA was obtained by using trizol based RNA extraction method. Initially, parasites were dissolved in 500 µl of trizol and were frozen at –70 °C. Later for extraction of RNA, tubes were thawed at 37 °C, 200 µl of chloroform was added and centrifuged at 13 K for 20 min at RT. The aqueous layer was carefully recovered and to this equal volume of Phenol/Chloroform was added and centrifuged at high speed for 10min to remove protein and lipid contamination. Total RNA was precipitated by using 3 volumes of absolute ethanol and 0.1 vol of sodium acetate. The pellet was washed once with 70 % ethanol and after drying was dissolved in DEPC treated water. The solution was heated to 65 °C to disrupt any association of the 25 nt RNA with larger RNA and DNA molecules and this treatment also quickens

the dissolving of the pellet. The tube was placed on the ice and PEG (MW 8000) to a final concentration of 5 % and NaCl (final conc. 0.5 M) was added to the solution and incubated on ice for further 30 min. The precipitate, which is high molecular weight nucleic acid, was pellet down by centrifugation at 13 K for 10 min. Three volumes of absolute ethanol was added to the supernatant of PEG precipitate and incubated at –20 °C for at least 2 h. The pellet was obtained by centrifugation at 13 K for 10 min. This pellet comprises mainly tRNA, small rRNA and 25 nt RNA.

### 3.2.22 Northern Blotting for LMW RNA

The sample was dissolved in formamide by heating at 65 °C for 5min and placed on ice immediately. To the sample 1/3 volume of 4X loading dye (2X TBE 40 % sucrose and 0.1 % bromophenol blue) was added. To run LMW RNA sample, 15 % polyacrylamide denaturation gel (15 % polyacrylamide 19:1, 8 M urea and 0.5X TBE) was used. The gel was run in 0.5X TBE at 100-500 V until BPB was almost at the bottom. The gel was carefully separated, placed on a prewetted (0.5X TBE) piece of nylon membrane (N+ hybond Amersham) and electroblotted for 45 min at about 100 V in 0.5X TBE. After blotting, membrane was placed on several layers of filter paper soaked on 20X SSC to equilibrate the membrane. After about 30 min, the membrane was fixed by UV cross linker (Stratagene). Membrane was stored in dark until further use.

If α-[$^{32}$P] UTP labeled dsRNA was used instead of normal dsRNA, after running of LMW RNA, the gel was dried on vacuum dryer and exposed to X-ray film for the presence of 25 nt small RNA species.

### 3.2.23 Preparation of Probe for Northern hybridization of LMW RNA

The probe was prepared by *in vitro* transcribing the DNA in the presence of $^{32}$P labeled rUTP. The single stranded labeled RNA was hydrolyzed using 0.2 M sodium carbonate (80 mM $NaHCO_3$, 120 mM $Na_2CO_3$). For hydrolysis, to 20 µl of *in vitro* transcribed RNA, 300 µl of carbonate buffer was added and incubated at 60 °C for as long as it takes to reduce the ssRNA to an average size of 50 nt. Time was calculated by using the below indicated formula.

$T= (L_i-L_f)/ (k.L_i.L_f)$

Where

T= time

$L_i$=Initial length of the probe in Kb

$L_f$ = final length of the probe in Kb

K= rate constant =0.11 Kb/min.

### 3.2.24 Northern Hybridization

The membrane carrying LMW RNA was incubated in prehybridization solution (50 % formamide, 7 % SDS, 50 mM $NaHPO_4/NaH2PO_4$ (pH 7.0), 0.3 M NaCl, 5X Denhardt solutions, 100 µg/ml sheared denatured salmon sperm DNA) for 5-6 h at 40 °C. For hybridization, the probe prepared as described in the section 3.2.23, was added to the prehybridization solution and incubated overnight at 40 °C under constant rotation. After O/N incubation, the blot was washed at 50 °C in 2 X SSC/0.2 % SDS, dried and exposed to X-ray film (Kodak).

### 3.2.25 *Plasmodium falciparum* Extract Preparation and Degradation of mRNA *in vitro*

The intact parasites treated with dsRNA as well as controls were collected by saponin lysis and washed thrice with ice cold PBS to remove erythrocyte cytoplasmic components. The washed parasite pellets were lysed by sonication in 200 µl of lysis buffer (100 mM Phosphate acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate and 5 mM dithiothreitol, pH 7.4). The lysates were centrifuged at 13000 rpm for 30 min at 4 °C, and supernatants were flash frozen and stored at –70 °C. The supernatants were used for *in vitro* assay. Typically, the reaction mixtures (50 µl) contained 25 µl of parasite extract and 50000 cpm of synthetic $^{32}P$ labeled mRNA as a substrate in *in vitro* assay buffer (20 mM HEPES, pH 7.3, 110 mM KoAc, 1 mM $Mg(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1mM DTT and 1mM ATP. The reaction mixtures were incubated for different time periods at 37 °C. Reactions were quenched by addition of 10 volumes 2X PK buffer (200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 300 mM NaCl and 2 % w/v SDS). Protein K was added to a final concentration of 465 µg/ml. reactions were analyzed by electrophoresis in a 0.8 % agarose gel containing GITC. Radiolabeled mRNA degradation was detected by exposing the agraose gel dried under vacuum to X-ray film (Kodak).

### 3.2.26 siRNA Design and Synthesis

Different siRNAs, fal 2, fal 1, BP 1, BP 2, AP-N, and GFP were designed for *falcipain 2*, *falcipain 1*, *bergheipain 1*, *bergheipain 2*, *aminopeptidase-N* and *green fluorescence protein* genes respectively, by using software developed by Elbhashir *et al.* (2002). All siRNA were obtained in annealed and lyophilized form from Dharmocon research (Colorado). These siRNAs were suspended in RNase free water at concentration of 5 µg/µl.

### 3.2.27 Treatment of *P. falciparum* Cultures with siRNAs

Synchronized *P. falciparum* cultures were adjusted to 4 % hematocrit with 1 % infected erythrocytes, and 0.5 ml of these cultures were treated with siRNAs in 24 well microtire plates in triplicates in serum free medium for 30 min with intermittent mixing. Subsequently, human serum was added to these cultures at a final concentration of 10 % and parasites were maintained for 48 h. Microscopic analysis and hypoxanthine assay was done as described in the previous section.

### 3.2.28 Indirect Immunofluorescence Assay

Indirect immunofluorescence assays were performed on 3D7 parasite lines. Thin smears of *P. falciparum* infected erythrocytes were made and fixed with mixture of methanol/ acetone. Slides were blocked in blocking buffer (1X PBS, 10 % FCS) for 2 h at 37 °C. After blocking, slides were incubated with primary antibody of appropriate dilution (mice anti Falcipain2, 1:200; rabbit anti $MSP_{42}$, 1:200; rabbit anti PfEMP1, 1;100) made in blocking buffer for 1 h. Slides were washed with 1X PBS for 1 h and incubated with appropriate secondary antibody conjugated to fluorescence dye (dilution 1:100) for 1 h. Later, the slides were stained with DAPI (4′, 6′-diamino-2-phenylindole) for 30 min at 37 °C at final concentration of 2 µg/ml and then washed twice in 1X PBS–Tween 0.05 %, once in 1X PBS and mounted on a cover slip in the presence of anti fade. The slides were viewed in confocal microscopy (Nikon).

### 3.2.29 Fractionation of Infected Erythrocytes and Western Blotting

Infected erythrocytes were fractionated by permeabilisation with streptolysin O (SLO) and saponin. Hemolytic activity of bacterially expressed recombinant streptolysin O was checked by incubating the RBCs with different concentration of

enzyme. Fraction showing considerable amount hemolytic activity was used for further experiments. Parasites (2X $10^7$) were incubated in RPMI containing 3-4 hemolytic units of SLO for 10min at 37 °C. Supernatant obtained after spinning was suspended in Laemmli sample buffer, pellet was washed and subsequently resuspended in Laemmli sample buffer. For saponin lysis, $2X10^7$ parasites were incubated in 1.5 volumes of 0.15 % saponin for 10 min on ice, centrifuged and the supernatant was suspended in protein loading dye. The pellet was washed in 1X PBS and subsequently resuspended in the protein loading dye. The samples were boiled and separated on SDS-PAGE, transferred to nitrocellulose membrane and visualized by ECL using mouse anti Falcipain anti serum (1:2000).

### 3.2.30 Fluorescence Microscopy

*P. falciparum* culture carrying GFP constructs were synchronized by sorbitol treatments consecutively twice with 4 h gap between each treatment. These synchronized parasites were treated with siRNA at ring stage and allowed to grow until early stage schizonts. Samples were taken hourly during the process of schizogony and merozoite release. SiRNA treated control and treated parasites were cultured in DAPI for 30 min at 37 °C at final concentration (2 µg/ml) prior to imaging. Fluorescence from DAPI and GFP was observed and captured from live cells within 30 min of mounting the sample in culture medium under coverslip on a glass slide using confocal microscopy (Nikon, Japan).

### 3.2.31 Immuno Electron Microscopy

*P. falciparum* infected erythrocytes were fixed in 1 % gluteraldehyde, in 0.1 M phosphate buffer, pH 7.4 for 30 min at RT, followed by washing with 1X PBS and suspended in 1X PBS. Samples were dehydrated in 70 % ethanol and embedded in L.R. white resin and polymerized at 37 °C for 2 days. Thin sections were incubated in Falcipain 2 specific antibodies (1:100 diuluted in 1X PBS, 1 % BSA), washed thoroughly and incubated with gold labeled secondary antibodies. Sections were stained with 2.5 % uranyl acetate and visualized under electron microscope (Morgagni Transmission Electron Microscope, FEI Germany).

### 3.2.32 Injection of siRNA into *P.berghei* Infected Mice

Female BALB/c, of 4-6 weeks age and weighing 20-25 gms were used. Mice were infected by intravenous injection of $10^5$ parasitized RBC. After 8-10 h, desired amount of siRNA were suspended in 200 µl of PBS and injected intravenously into infected mice in three replicates. The injections were repeated after 24 h and 48 h. Control mice were injected with GFP siRNA or PBS. Blood was collected from mice 24 h after the last injection. Blood smears stained with Geimsa stain were analyzed for parasite morphology and total parasitemia was counted.

### 3.2.33 Isolation of Parasites and Analysis of Globin Hydrolysis

Blood collected from siRNA treated and control nice was passed through CF11 column to remove white blood cells. Erythrocytes were lysed by 0.1 % saponin at 37 °C for 15 min. The parasite pellets were collected after centrifugation and washed three times with PBS to remove erythrocyte cytoplasmic content. The parasite pellets were supended in SDS sample buffer with β-mercaptoethanol, boiled and separated on 15 % SDS-PAGE. The gel was stained with Coomassie blue R-250.

### 3.2.34 Isolation and Detection of Low Molecular Weight RNA

For the siRNA uptake assay and to detect the formation of low molecular weight RNA, mice were first infected with $10^5$ parasitised erythrocytes. After parasitemia has reached 2 %, $^{33}P$ labeled or unlabeled siRNAs were injected intravenously into these mice. After 12-14 h, blood was collected and processed for the extraction of total RNA. Low molecular weight RNA was extracted as described in the section 3.2.21.

# Chapter 4: Results

*Plasmodium falciparum*, the causative agent of cerebral malaria, is responsible for millions of deaths every year. Annotation of *P. falciparum* complete genome sequence revealed a number of genes with unknown functions. There is a need for a rapid and an efficient tool to study the function of these genes. Recently, RNAi has emerged as an efficient tool to study the function of the genes in number of organisms. We thought of developing and applying RNAi in malaria, an intracellular parasite. To carryout RNAi in malaria parasite, we chose to silence the expression of cysteine protease genes, *falcipain 1&2*. These genes were selected as the morphological effects on parasites due to cysteine proteases inhibitors have been well described and moreover, cysteine proteases have been shown to play an important role in the hemoglobin degradation pathway, an essential step in parasite life cycle. We used both dsRNA as well as siRNA to carryout RNAi in *P. falciparum*.

## 4.1 DsRNA mediated gene silencing in *P. falciparum*

### 4.1a Synthesis of dsRNA corresponding to *falcipain1&2* and their uptake by *P. falciparum*

To prepare dsRNAs corresponding to *falcipain 1&2* we followed the protocol by Clemens *et al.* (2001). Briefly, the cysteine protease genes, *falcipain1* (1.6 Kb) and *Falcipain 2* (1.4 Kb) were PCR amplified from the genomic DNA of 3D7 and cloned in pGEM-T vector. Using T7 and SP6 primers, cysteine protease genes from the pGEM-T vector were amplified along with the flanking T7 and SP6 promoter sequences (Fig.1). With the help of T7 and SP6 Polymerase, both sense and antisense strands were prepared. dsRNAs were obtained by annealing both the complementary strands. These dsRNA were analyzed on 1% agarose gel to ensure that they were predominantly double-stranded (Fig.2).

The hallmark of RNAi is its specificity, simplicity, and the ease with which it can be applied to an organism. RNAi was first demonstrated in *C. elegans* by soaking the worm in dsRNA solution (Fire *et al.*, 1998). Later, RNAi was applied in various organisms by using dsRNA as well as siRNA to study the function of the genes. In *Drosophila* S2 cell lines; RNAi was used for dissecting the signal transduction pathway by just adding dsRNA to the

culture medium (Clemens *et al.,* 2000). Previous studies have shown that DNA/oligonucleotides molecules can be introduced into parasitized RBC by either electroporation or by their simple addition to the culture medium (Rapaport *et al.*, 1992, Bakers *et al.*, 1996). Since, electroporation procedure has been shown to cause death of large number of the parasite, we decided to use the soaking procedure i.e., by adding dsRNA to the culture medium. To determine the efficiency of this procedure, we first studied the uptake of labeled dsRNA by malaria parasite. In order to carryout this, synchronized *P. falciparum* cultures were incubated with 10 nM $^{32}$P labeled dsRNA made from either of the *falcipains*. After 24 h of incubation, parasitized erythrocytes were collected by centrifugation, lysed using saponin and washed three times to obtain a pure parasite pellet. Level of radioactivity was measured in different fractions of parasitized red blood cells. Approximately 0.1-0.15% of the input labeled dsRNAs were found to be associated with purified parasite pellet, suggesting that dsRNA was taken up by the parasites.

### 4.1b Effect of *falcipain 1&2* dsRNA on the growth of parasites

To determine the effect of *falcipain 1&2* dsRNA on the growth of parasite, synchronized *P. falciparum* parasites were incubated with dsRNA for each of the *falcipains* separately and in combinations at three different concentrations (10 µg/ml, 20 µg/ml, 50 µg/ml). Treatment of parasites with *falcipain 1&2* dsRNA led to the distinct morphological changes, which included swollen food vacuole with the accumulation of malarial pigment (Fig.3). Similar morphological effects were also seen when parasitized erythrocytes were treated with E-64. Rosenthal and co-workers have also reported such kind of food vacuole abnormalities on treatment with E-64 (Rosenthal, 1995). Maximum effects were seen, when the parasites were incubated with *falcipain1&2* together at a concentration of 25 µg/ml and 50 µg/ml than at 10 µg/ml concentration of either of *falcipain1*or *2* separately and in combination. Approximately, 60 % of the parasites showed swollen food vacuole abnormalities on incubation with both the *falcipains* dsRNA at 50 µg/ml concentration where as 30 % of parasitized erythrocytes showed food vacuole abnormalities on treatment of the parasites separately with each of the *falcipains* at both 25 and 50 µg/ml concentrations. Addition of the two

dsRNAs together to the parasite culture also drastically reduced the formation of new rings as well as total parasitemia. E-64 at a concentration of 100 nM was used as a positive control and dsRNA made from *Aminopeptidase-N* (insect origin) was used as a negative control (Table 1). We also studied the effect on the growth of the parasite by using $^{3}$[H]-hypoxanthine uptake assay, as the inhibition of growth is proportional to the uptake of hypoxanthine. We followed the protocol described by Rosenthal *et al.* (1995). The parasites at ring stage were treated with dsRNA and incubated for 24 h. Approximately 1μCi of $^{3}$[H]-hypoxanthine was added to the cultures and further incubated for 24 h. We observed 50-60 % reduction in the uptake of hypoxanthine in the case of *falcipain 1&2* dsRNA treated parasites at 50 μg/ml concentration, whereas only 30 % reduction was observed when the parasites were incubated separately with *falcipain 1&2* dsRNA (Fig.4). Parasites treated with E-64 gave complete inhibition in the uptake of $^{3}$[H]-hypoxanthine and *aminopeptidase* dsRNA treated parasites showed no inhibition in the $^{3}$[H]-hypoxanthine uptake, thus exemplifying the specificity of RNAi.

### 4.1c Inhibition of hemoglobin degradation by *falcipain* dsRNAs in *P. falciparum*

It has been shown that the morphological effects observed in parasites after the treatment with E-64, a broad-spectrum cysteine protease inhibitor are due to the accumulation of toxic hemoglobin (Rosenthal *et al.,* 1995). It has also been shown that recombinant *falcipain 1&2* have the ability to degrade hemoglobin. To investigate whether the observed morphological abnormalities in *falcipain 1&2* dsRNA treated parasites were predominantly due to the accumulation of hemoglobin, parasites were treated with both the *falcipain 1&2* dsRNAs separately and in combination at 50 μg/ml concentration and the total parasite proteins were subsequently analyzed by SDS-PAGE. Incubation of parasites with *falcipains* dsRNAs resulted in the accumulation of hemoglobin (Fig.5, lane 2-4). E-64 treated parasites were used as a positive control (Fig.5, lane 6). Parasites treated with DEPC water showed almost complete degradation of hemoglobin (Fig.5, lane 1). These results suggested that the addition of *falcipain 1&2* dsRNA to the *P. falciparum* culture leads to

the inhibition of hemoglobin degradation and subsequent accumulation of hemoglobin in the food vacuole of the parasite.

### 4.1.d *Falcipains* dsRNA mediated cleavage of its corresponding mRNA

By definition, RNAi is a process by which dsRNA brings about the silencing of a gene by cleaving its cognate mRNA. To determine whether the effects seen on *P. falciparum* were due to the *falcipain 1&2* mRNA degradation, we assessed the levels of *falcipains* mRNA in *falcipains* dsRNA treated parasites by Northern analysis. Parasites treated with *falcipain 1* dsRNA showed the *falcipain 1* specific mRNA degradation as compared to the untreated, *falcipain 2* dsRNA treated and *aminopeptidase* dsRNA treated parasites (Fig.6, panel A). Similarly, parasites treated with *falcipain 2* dsRNA showed loss of *falcipain 2* mRNA in comparison to control parasites or parasites treated with nonspecific dsRNA (Fig.6, panel B). These results clearly showed that the effects seen on parasites upon treatment with *falcipains* dsRNA treatment were because of degradation of its corresponding mRNA

### 4.1e RNAi machinery is conserved in *P. falciparum*

Several studies on the mechanism of RNAi have suggested a two-step mechanism for RNAi. The first step involves, degradation of dsRNA by RNAse III like enzyme, referred to as DICER, into small RNA species of lengths 21-25nt (siRNA). The second step involves, binding of siRNA to RNA induced silencing complex (RISC), subsequent activation of RISC, which is mediated by unwinding of siRNA, followed by the degradation of mRNA complementary to one of the strands of siRNA in the RISC (Agrawal *et al.*, 2003). To determine whether *falcipain 1&2* dsRNAs were also processed into small RNA, the cultured parasites were treated with $^{32}$P–labeled dsRNA corresponding to either of the two *falcipains* used in this study. After 24 h of incubation, parasite pellets were processed to get total RNAs and, subsequently, small RNA species were isolated from the total RNA. These were then analyzed on 15 % denaturing PAGE. Both *falcipain 1&2* dsRNAs were processed to 25 nt siRNA species (Fig.7, lane1 and 2) whereas treatment of normal RBCs with radiolabeled dsRNA did not generate 25nt siRNA

species (Fig.7, lane 3-4). We also observed an additional RNA species of approx 70nt, which could have been generated as intermediates during the processing of dsRNA.

In *Drosophila*, an *in vitro* system was developed to understand the different steps involved in the mechanism of RNAi (Elbashir *et al.,* 2001; Zamore *at al.,* 2000). In this system, it has been demonstrated that preincubation of the dsRNA with the *Drosophila* lysates results in potential nuclease activity for the target mRNA degradation (Tuschl *et al.,* 1999; Zamore *et al.,* 2000). To find out whether similar nuclease activity can be generated in *P. falciparum*, the *P. falciparum* culture was treated with *falcipain 2* dsRNA. The extracts prepared from treated vs. untreated cultures were analyzed for nuclease activity at different time points. Extracts from *falcipain 2* dsRNA treated parasites efficiently degraded *falcipain 2* mRNA whereas extracts from untreated parasites did not degrade mRNA at all (Fig.8). The degradation process was completed within 30 min of incubation of labeled *Falcipain 2* mRNA, whereas extracts from untreated parasites did not show any degradation of mRNA even after 3 h of incubation.

In summary, all these results showed that RNAi operates in *P. falciparum* and can be used to carryout functional genomic analysis in *P. falciparum.*

## 4.2 siRNA mediated gene silencing of *P. falciparum*

### 4.2a Effect of siRNA on the growth and morphology of *P. falciparum*

The hallmark of RNAi and the related phenomenon are generation of small RNA species of approximately 21-25 nt; named small interfering RNAs (siRNA), which in association with protein complex functions as a guide for the degradation of target transcripts. Elbhashir *et al.* (2001) provided the first evidence for the siRNA mediated sequence specific gene silencing and showed that the dicing step can be bypassed by the introduction of siRNA into the cells. Tuschl and coworkers for the first time applied siRNA to silence the genes in mammalian cells (Tuschl, 2002). Since then, siRNA is being used efficiently for wide scale functional genomic studies. Considering the above facts, we initiated studies using siRNA corresponding to *falcipain 1&2* and investigated their effects on the growth as well as morphology of *P.*

*falciparum* parasites *in vitro*. The sequences for *falcipain 1&2* siRNA were carefully selected by using the software on the Ambion site. Fig.9. shows the target sequence and its corresponding siRNA sequence.

### 4.2a.1 Growth Inhibition

We have previously shown that silencing of *falcipain1 and 2* genes using corresponding dsRNAs brings about substantial inhibitory effects on the parasite growth. To determine whether similar effects could also be produced by siRNAs corresponding to these genes synchronized *P. falciparum* parasites at the late ring stage were treated with siRNAs corresponding to *falcipain1&2* genes separately, as well as in combination at two different concentrations of 10 µg/ml and 100 µg/ml. Unrelated siRNA corresponding to insect aminopeptidase gene was used as a negative control. Reduction in [$^3$H]-hypoxanthine uptake was observed at both, 10 µg/ml and 100 µg/ml concentrations in comparison to the untreated parasites. However, at 100 µg/ml concentration of individual *falcipain* siRNA, pronounced reduction in the uptake of [$^3$H]-hypoxanthine was observed (50-60%). Maximum inhibition (~85%) in [$^3$H]-hypoxanthine uptake was seen when the parasites were treated with both the *falcipain* siRNAs at 100 µg/ml concentration (Fig. 10). No reduction in [$^3$H]-hypoxanthine uptake was observed when parasites were treated with *aminopeptidase-N* siRNA.

### 4.2a.2 *Falcipain 1&2* siRNAs treatment affects total parasitemia and the merozoite release

We also investigated the effects of *falcipains* siRNAs (*falcipain 1& 2*) on the parasite development as well as on the total parasitemia after 56 h of treatment. Treatment of parasites with either of the two *falcipains* siRNAs considerably reduced the total parasitemia (~60-65 %) (Fig. 11 A), thereby suggesting that these enzymes play an important role in the parasite survival. Different stages of the parasite i.e. ring as well as schizonts stages were also analyzed separately in the treated cultures. In the control culture, most of the parasites were at the new ring stage. *Falcipain 1* siRNA treated culture showed 60 % reduction in the formation of new rings in comparison to the control

culture (Figs.11 B and C). Few or no schizonts were seen in the *falcipain* 1 siRNA treated culture. However, cultures treated with the *falcipain 2* siRNA showed large number of schizonts along with some new ring stage parasites. These schizonts appeared as spherical clusters of merozoites, enclosed in a delicate membrane covering (Fig. 12). Parasites in the *Falcipain 2* siRNA treated culture seemed to be arrested at the schizont stage. Similar kind of morphological clusters have been reported on treatment of *P. falciparum* culture with different protease inhibitors (Wickham *et al.,* 2003; Salmon *et al.,* 2001; Lyon and Haynes, 1986).

### 4.2a.3 Silencing of *falcipain 2*

To determine whether the effects seen upon addition of *falcipain 2* siRNA were due to RNAi, we performed Western analysis of the total protein extract from treated and untreated parasites to assess the reduction in Falcipain 2 protein. To assess the level of Falcipain 2, total protein was extracted from the parasite and separated on 12% SDS- PAGE. Western analysis was done by using Falcipain 2 specific antibodies. As shown in the (Fig.13), *falcipain 2* siRNA treated parasites showed 3-4 fold reduction in the amount of Falcipain 2 in comparison to the *aminopeptidase-N* (insect origin) siRNA treated parasites and untreated parasites. Anti HRPII antibody was used to confirm equal loading in different wells.

These results indicated that *Falcipain 2* siRNA treatment specifically reduces the Falcipain 2 production in *P. falciparum, which* in turn inhibits the growth of the parasite and merozoite release from RBCs.

### 4.2b Determination of origin of membrane surrounding the incompletely ruptured schizonts

Previously it has been shown that the treatment of parasite with different cysteine protease inhibitors results in the accumulation of a large number of incompletely ruptured schizonts, which are either surrounded by PVM or erythrocyte membrane depending on the type of cysteine protease inhibitor used for the study. Since, we observed a large number of incompletely ruptured parasites in *falcipain 2* siRNA treated cultures we decided to determine the origin of the limiting membrane surrounding these

incompletely ruptured schizonts. To do so, we followed the experimental design of Wickham *et al.* (2003). These authors used GFP expressing lines (3D7+His) to ascertain the selective inhibition of PVM or erythrocyte membrane rupture by different protease inhibitors. In these parasite lines when PVM rupture was inhibited and the RBC membrane rupture proceeded, the GFP fluorescence was lost. However, when PVM was cleaved and the RBC membrane remained intact, GFP fluorescence diffused through the whole RBC around the merozoites. Upon treatment with *falcipain 2* siRNA, these transgenic parasites exhibited diffused fluorescence throughout the infected RBC, thereby suggesting that the RBC membrane in these parasites remained intact (Fig.14A). This result showed that RBC membrane rupture is inhibited in parasite lines treated with *falcipain 2* specific siRNA. To further confirm the erythrocytic origin of the membrane surrounding the merozoite clusters in *falcipain 2* siRNA treated parasites, immunofluorescence assays using antibodies to one of the parasite protein (PfEMP-1), and an erythrocyte membrane protein (Band 3), were carried out. PfEMP-1 is a parasite protein that has been shown to be localized on the host erythrocyte membrane during trophozoite and schizont stages. Incompletely ruptured schizonts in *falcipain 2* siRNA treated cultures showed the presence of PfEMP1, as well as Band 3 in the membrane surrounding them (Fig.14, B and C). This indicated that the limiting membrane surrounding the incompletely ruptured merozoite clusters in *falcipain 2* siRNA treated parasite is indeed the erythrocyte plasma membrane. Results of these experiments showed that Falcipain 2 plays an important role in the rupture of the erythrocyte membrane and the release of merozoites.

### 4.2c Localization of Falcipain 2 protein in different stages of parasite growth by immunofluorescence assay

Earlier studies conducted by Rosenthal's group on the localization of Falcipain 2 have shown that Falcipain 2 protein is predominantly localized in the food vacuole (Shenai *et al.,* 2001). Recently, Dhawan *et al.* (2003) showed the localization of the Falcipain 2 protein in the PVM as well as in the erythrocyte cytosol. In order to gain insight into the functional role of Falcipain 2, we

carried out immunolocalization studies using Falcipain 2 specific antibodies raised in mice. If Falcipain 2 is involved in the erythrocyte plasma membrane rupture, it probably should be transported to the erythrocyte cytoplasm from the parasite. Immuno-localization studies were carried out using confocal microscopy on the untreated parasites using Falcipain 2 specific antibodies. To obtain highly specific antibodies against Falcipain 2 protein, we first treated the Falcipain 2 specific mice antisera with recombinant Falcipain1 protein in order to eliminate the cross-reacting antibodies, common to Falcipain 1&2 proteins. Synchronized parasite cultures at the ring, trophozoite and schizont stages were immuno-stained to localize the Falcipain 2 protein. At the trophozoite stage, Falcipain 2 labeling was confined to the parasite cytosol and it co-localized with the endoplasmic reticulum resident protein (PfERC) (Fig. 15, panel A). At an early segmenter stage (schizont stage), intense Falcipain 2 labeling appeared in the food vacuole as well as around individual merozoites. Remarkably, Falcipain 2 labeling appeared comparable to the merozoite surface protein 1 (MSP-1) labeling in each segment (Fig. 15, panel B and C), although MSP-1 and Falcipain 2 did not merge totally; labeled Falcipain 2 appeared as a red rim circumventing the green MSP1 label at certain places. Absence of Falcipain 2 staining in the free merozoites suggested that it is not associated with the merozoite surface (Fig. 15, panel D). Considerable reduction in Falcipain 2 labeling in the parasites treated with *falcipain 2* siRNAs was observed (Fig. 16).

### 4.2d Fractionation of infected erythrocyte to localize Falcipain 2 protein

If Falcipain 2 is transported to the erythrocyte, it should either be associated with the membrane network or it should lie free in the cytoplasm. To further confirm the localization of Falcipain 2, infected erythrocytes were treated with Saponin and Streptolysin O, which was followed by western analysis using Falcipain 2 specific antibody. Saponin and Streptolysin O treatment allows the analysis of membrane–associated proteins as well as the exported proteins. Saponin dissolves both the erythrocyte plasma membrane and parasitophorous vacuolar membrane, while Streptolysin O permeabilises only the erythrocyte membrane, leaving the parasitophorous membrane intact. Upon lysis of parasitized erythrocyte with Saponin, Falcipain 2 was found both in the supernatant and in the pellet while in Streptolysin O treated

parasites, Falcipain 2 was present only in the pellet but not in the supernatant (Fig.16. panel A). These results clearly provided evidence for the association of Falcipain 2 protein with the tubolo-vesicular network (TVN) in the erythrocyte cytosol (Fg.16, panel B). These studies revealed that Falcipain 2 protein at schizont stage is exported from the parasite to the RBC cytosol through the vesicular network and remains associated with the TVM in the cytosol of the RBC.

### 4.2e Effect of Brefeldin A on the trafficking of Falcipain 2

In eukaryotes, the proteins, which are either secreted outside the cell or to the surface of the plasma membrane, are transported by a vesicle-mediated pathway or also known as classical pathway, which involves the Golgi apparatus. Treatment of eukaryotic cells with Brefeldin A leads to the collapse of Golgi network, which in turn blocks the transport of secretory proteins. In *Plasmodium*, it has been previously shown that most of the parasite proteins like KHARP, PfEMP-1 and PfEMP-3 that are exported out from the parasite through the vesicle mediated pathway are blocked by treatment with Brefeldin A. To determine whether the Falcipain 2 protein is transported to the cytosol by the vesicle-mediated pathway i.e., "classical pathway" or some other alternate pathway; we treated infected erythrocyte with Brefeldin A at the early trophozoite stage and studied the localization of Falcipain 2 after 24 h of treatment by using immunofluorescence microscopy. Trafficking of Falcipain 2 in the treated parasite was affected and it was found to be associated to a contracted compartment in the parasite cytosol, which co-localized with PfERC, suggesting that Falcipain 2 was retained in the ER in the treated parasite (Fig.18) From these results we can conclude that Falcipain 2 is transported to erythrocytic cytosol via the classical pathway and its transport is affected by the treatment with Brefeldin A.

### 4.2f Immuno-electron microscopy confirming the localization of Falcipain 2

To further confirm the sub cellular localization and transport of Falcipain 2, immuno-electron microscopy was performed using anti Falcipain 2 specific antibody. The Falcipain 2 protein showed localization in the food

vacuole of the parasite as well as in the cytosol of the infected erythrocyte (Fig.19). Many of the immunogold particles were associated with the membrane structure in the erythrocyte cytosol and in the Maurer's clefts, which are considered to be the transient depot during the export of the parasite protein (Fig.20, panel A and B; Fig.21). Thus, these studies provide substantial evidence regarding the transport of Falcipain 2 to the erythrocyte membrane through a tubulo-vesicular network and the Maurer's cleft.

Based on the results presented in this study, it can be suggested that in addition to its role in hemoglobin degradation, Falcipain 2 plays an important role in erythrocyte membrane rupture.

## 4.3. *In vivo* RNAi in mouse malaria model- *P. berghei*

Over the years RNAi has evolved into a powerful tool for manipulating gene expression in number of organisms with a potential utility for investigating gene function, for high–throughput, function based genetic screens and potentially for development as a therapeutic tool. Recently, a number of studies have shown the use of siRNA for genetic based therapies especially in viral infections, cancers and inherited genetic disorders. A strong evidence for the therapeutic potential of RNAi has come from a recent publication describing the prevention of Fas-mediated Hepatitis in mice following the intravenous delivery of naked siRNA by high-pressure injection. However, the effects of siRNAs in adult animal and their potential to treat or prevent disease are yet to be fully investigated. In this present study, we explored the possibility to carry out RNAi on circulating parasite *in vivo.*

### 4.3a Uptake of siRNA by circulating *P. berghei* parasite in mice

To investigate the role of falcipains' orthologues from rodent malaria parasite *P. berghei* and to study the *in vivo* efficacy of siRNA to trigger RNAi in circulating parasites, we used siRNA of *berghepain1&2* (Selected from Ambion website; Fig.22) to silence the *berghepain* genes in the circulating *P. berghei* parasites. The major obstacle in the use of siRNA in mice model is the route of delivery. The reported routes of delivery tried in different organisms are soaking, feeding, microinjection, viral vector, and intravenous injection.

We followed intravenous route for the delivery of siRNA to assess the effect of *bergheipains'* siRNA on the circulating parasites. Initially, we assessed the uptake of $^{33}$P-labeled siRNA by the circulating parasites in the blood of the mice. Eighteen hours after the injection of $^{33}$P-labeled siRNA, parasitized RBCs were collected by centrifugation; lysed using saponin and low molecular weight (LMW) RNAs were isolated. These RNAs were blotted to a nylon membrane and the uptake of labeled RNAs was assessed by autoradiography. We could detect a small amount of input labeled siRNA in these parasites (Fig.23). Presence of labeled siRNA in the parasitized erythrocyte indicated their uptake by the parasites but at a very low amount.

### 4.3b Effect of *berghepain* siRNA on *P.berghei* parasite

In the previous section, we have demonstrated the effect of *falcipains'* siRNA on the morphology and growth of the *P. falciparum* culture. To determine whether *berghepains'* siRNA trigger similar effects *in vivo,* we conducted a series of experiments using two different concentrations of *berghepains'* siRNAs, 5 µg and 25 µg. In all these experiments, siRNA was dissolved in 200 µl 1X PBS and injected intravenously into the *P. berghei* infected mice. SiRNAs were introduced into the mice at the onset of 1% parasitemia or just after 8 h of inoculation of parasite into the mice. Mice were treated with siRNA once a day for three days consecutively. *GFP* siRNA was used as a negative control. Forty-eight hours after the last injection, Geimsa stained smears were made and examined for morphological abnormalities in *P. berghei* parasite. Mice injected with 25 µg of *berghepains'* siRNA produced maximum abnormalities in circulating parasites, which included swollen food vacuole with the accumulation of malaria pigment in *P. berghei* parasites (Fig.24). These effects were similar to those observed in *P. falciparum cultures* after treatment with either E-64, a cysteine protease inhibitor or *falcipain 1&2* dsRNA.

### 4.3c Accumulation of hemoglobin in *berghepain* siRNA treated parasites

It has been previously reported that the food vacuole abnormalities seen in malaria parasites after treatment with cysteine protease inhibitors are mainly due to blockage of hemoglobin degradation. To assess the effects of

*berghepains'* siRNA on the accumulation of hemoglobin, *bergheipains'* siRNA treated parasitized erythrocytes were collected from mice blood, lysed using saponin and the parasite proteins were analyzed on 15 % SDS-PAGE. A 2-3 fold increase in the hemoglobin level was observed in *berghepains'* siRNA treated parasites in comparison to the control parasites (Fig.25). These results showed that Berghepains like Falcipains are involved in the degradation of hemoglobin and siRNAs can be effectively used to study the function of genes in *P. berghei*-a mouse malaria parasite.

### 4.3d. Northern analysis to show the reduction of *berghepain* mRNA level in *berghepains'* siRNA treated parasites

To determine whether the effects observed due to the treatment of parasites with *berghepains'* siRNA were due to the degradation of *berghepain* mRNA, we performed Northern analysis by using *berghepains'* DNA as a probe. For Northern analysis, total RNA was extracted from the treated and untreated parasites fractionated on 1.2 % denaturing agarose gel and transferred onto the nylon membrane. The membrane was hybridized with $^{32}P$ labeled *berghepain 1&2* DNA. The parasites treated with *berghepains'* siRNAs resulted in the reduction of >40 % of *berghepain 1* mRNA (Fig.26, panel A) and >50 % of *bergheipain 2* mRNA (Fig.26, panel B) as compared to the *GFP* siRNA treated and untreated parasites. Ethidium bromide stained gel showing rRNA was used as a loading control (Fig. 26, panel C).

### 4.3e Generation of Low molecular weight (LMW) RNA species

As mentioned earlier the hallmark of RNAi reaction is the generation of 25nt RNA species. Earlier, we have reported the generation of small RNA species resulting from the degradation of dsRNA. To investigate whether small RNA species were also formed in *berghepain* siRNA treated parasite, we isolated small RNA species from *berghepains'* siRNA treated parasite and analyzed them on 15 % denaturing PAGE, followed by their detection using labeled *berghepain* RNA probe. We observed generation of 25nt species in *berghepain* siRNA treated parasites, while untreated and *GFP* siRNA treated parasites did not show the generation of any small RNA species (Fig.27).

Along with 25nt species we also observed additional small RNA species >25nts. These longer species might represent the intermediates during mRNA degradation as reported in the previous studies in different organisms. From these results, it is clear that RNAi is functional in *P. berghei* parasite and siRNA can be used to silence genes of *P. berghei* in the mice by intravenous injection of siRNA.

In conclusion the results of the present study have shown that it is possible to carry out RNAi in malarial parasite both *in vitro* as well as *in vivo*. The RNAi can be an important tool for functional genomic studies for malarial parasites.

The malaria parasite is an intracellular organism, which has co-evolved in mosquitoes and vertebrates for millions of years. Designing drugs or vaccines that substantially and persistently interrupt the life cycle of this complex parasite will require a comprehensive understanding of its biology. Annotation of *P. falciparum* genome led to the identification of 5,267 genes, out of which 65 % are hypothetical. It is essential to understand the function of these genes in order to design a better drug /vaccine. With the development of resistance against most of the currently used antimalarials, there is an urgent need to identify new drug targets and design new drugs based on these targets. To design better/new drugs, better understanding of parasite biology is required. Hence, there is an urgent need to develop a molecular tool to study the function of the genes in less time and with less labour. The reverse genetic tools represent one of the best tools to decipher the function of the genes. The existing reverse genetic tools like gene knockouts using homologous recombination, ribozyme technology and antisense approaches have worked with some limitations. RNAi has emerged as an efficient tool to silence the genes and study their phenotypes. In the present study, we have successfully demonstrated RNAi in *P. falciparum* by using dsRNA of cysteine proteases, *falcipain1&2* of *P. falciparum*, which are involved in hemoglobin degradation pathway. Even though protease inhibitors have been shown to inhibit parasite growth since 1980, Rosenthal *et al.,* in 1989, for the first time reported a cysteine protease activity in the malaria parasite. Subsequently, cysteine protease gene (*falcipain1*) was cloned, expressed in *E. coli* and baculo vectors and was shown to have hemoglobinase activity (Salas *et al.*, 1995).

Rosenthal and co-workers, based on cysteine protease inhibitor studies, have proposed that Falcipains is involved in the initial step of hemoglobin degradation (Gomboa-de-Domingoez and Rosenthal, 1996). Involvement of Falcipains in the first step of globin digestion was inferred from the observation that the treatment of parasite with E-64 results in the accumulation of undigested globin in the food vacuole of parasites (Rosenthal, 1995). However, for the active cleavage of hemoglobin, cysteine proteases need reducing environment (Salas *et al.*, 1995) which was suggested to be provided by the uptake of glutathione from erythrocyte cytosol during the formation of food vacuole (Rosenthal et al., 1998). Goldberg and co-workers showed that the cysteine proteases isolated from the food vacuole cannot degrade native hemoglobin under non reducing conditions, but under same conditions

can degrade denatured hemoglobin (Francis *et al.*, 1996). These results clearly indicated that the denaturation of hemoglobin is an essential step for the cysteine proteases activity on hemoglobin and the reducing environment is brought about by the glutathione present in the food vacuole. These authors also suggested that the levels of catalase present in the food vacuole may be sufficient to protect hemoglobin from thiol mediated denaturation and the initial degradation was accredited to another class of proteases, Plasmepsin I & II belonging to the family of aspartic proteases (Goldberg *et al.*, 1991). By using aspartic protease inhibitors, it was shown that these proteases are essential for the degradation of hemoglobin (Goldberg *et al.*, 1990). With the completion of genome sequencing and its annotation, large number of new proteases (Cysteine, aspartic and metallo proteases) in the genome of *P. falciparum* has been identified. Ten different aspartic proteases have been described now and of these four have been implicated in hemoglobin degradation. Like wise, four different cysteine protease have been described and all have been implicated in hemoglobin degradation. Two different models have been proposed for the process of hemoglobin degradation. Goldberg and co-workers believe that the hemoglobin degradation is an ordered process in which initial cleavage event is performed by aspartic proteases and subsequent digestion within the food vacuole is the result of the synergistic action of Plasmepsin I, Plasmepsin II and Falcipains. Small peptides generated because of the digestion of cysteine and aspartic proteases are transported to the parasite cytosol, and are further digested to amino acids by the action of cytosolic exopeptidases. On the contrary, Rosenthal's group demonstrated that the hemoglobin can be cleaved by cysteine protease under non reducing conditions but at a slow rate and proposed that both the proteases (aspartic and cysteine proteases) act together on the native hemoglobin in a co-operative manner leading to the initial cleavage of hemoglobin. Due to the lack of gene disruption studies of individual genes, the precise role of these proteins is highly speculative.

In this present study, we made an attempt to pinpoint the exact role of two cysteine protease genes in the hemoglobin degradation by using RNAi. We initially tried different methods for the uptake of dsRNA by the parasite, which include 1) transfection by using different transfection reagents like oligofectamine, lipofectamine and Exygen-500, 2) Electroporation 3) Soaking. Since, transfection by using transfection reagents and electroporation at high voltage led to the excessive death of parasite, we decided to carry out RNAi experiments using soaking procedure

by incubating parasite with dsRNA in absence of serum. Another important aspect is the uptake of dsRNA by the parasite, which was initiated by addition of dsRNA to the parasite culture in serum free medium. We assessed the entry of radiolabeled dsRNA into the parasite which was surrounded by three membranes. Our results clearly showed that dsRNA can be taken up by the parasite at very low levels.

In present investigation, the addition of each of the two *falcipain* dsRNA to *P. falciparum* culture resulted in the inhibition of parasite growth, development and enlargement of food vacuoles. These effects were more pronounced when both the *falcipains* dsRNA were used together. Moreover, each of the *falcipain* dsRNA produced a significant block in the degradation of hemoglobin in the parasite. A non-specific dsRNA from *aminopeptidase-N* gene of insect origin did not produce any of these effects. The additive effect produced by two *falcipains* dsRNA suggested that hemoglobin hydrolysis in the parasites is probably a co-operative process involving a number of different enzymes together. The inhibitory effects produced by dsRNAs treatment of parasites coincided with a marked reduction in the levels of endogenous mRNA homologous to the dsRNA in these parasites, which is one of the hallmarks of RNAi. We also compared the effects produced by *falcipain* dsRNAs on malaria parasite with the effects of E-64, a well known cysteine protease inhibitor. Our study showed that various morphological as well as biochemical effects seen on malaria parasite after treatment with *falcipain1&2* dsRNAs were similar to those shown by E-64 (Rosenthal, 1995). These results indicate that RNAi can be an important tool in investigating metabolic events in the parasite life cycle and adds *P. falciparum* to the list of organisms in which RNAi has been shown to work successfully. The block in hemoglobin degradation observed by silencing the activity of the *falcipain* genes in the present study suggested that both the Falcipain proteins are required for initial cleavage event.

Based on various studies related to the RNAi mechanism in *C. elegans* and *Drosophila*, a two-step mechanism for RNAi has been proposed (Bernstein *et al.*, 2000; Hammond *et al.*, 2001). The first step involves cleavage of dsRNA to siRNA of 21-25 nt length by RNaseIII like enzyme, called as DICER. In a second step, which is also referred as an effector step siRNA produced in the first step serve as a guide for a ribonuclease complex, RISC (RNA induced silencing complex), which cleaves the homologous single stranded mRNA (Agrawal *et al.*, 2003). We wondered whether the mechanism of RNAi is similar in *P. falciparum*. Analysis of small RNA species in *P.*

*falciparum* culture treated with labeled *falcipain* dsRNAs showed the generation of 25nt labeled RNA species. We also showed that parasite extracts prepared from the *P. falciparum* culture treated with *falcipain2* dsRNA had the ability to cleave *falcipain2* mRNA whereas untreated parasites did not show this nuclease activity. These results supported the two-step mechanism and the components of dsRNA–induced gene silencing in a diverse group of organisms.

As siRNAs are the true intermediates of RNAi reaction, they are now being used successfully to study the function of the genes in large number of organisms including mammals where dsRNA cannot be used. We also assessed the efficiency of siRNA in an RNAi reaction in *P. falciparum*. In the present study, we used siRNA of *falcipains* 1& 2 genes to silence the corresponding *falcipain* genes. Treatment of parasites with *falcipains* siRNA individually and in combination resulted in drastic reduction in the growth of the parasite and the effects seen were similar to that of *falcipain's* dsRNA treated parasites. The effects of these siRNAs were better than those reported earlier with *falcipain* dsRNAs. This might be due to the efficient uptake of siRNA as compared to dsRNA. In addition to the effects seen on the parasite growth after the *falcipains* siRNAs treatment, we also observed accumulation of merozoite clusters in *falcipain2* siRNA treated cultures indicating abrogation of RBC's rupture. These merozoites appeared morphologically normal but were locked within a transparent membrane. These effects were not seen with *falcipain1 siRNA* treated parasites. Similar kind of morphologically abnormal structures have been earlier reported upon treatment of parasites with <10uM of E-64 or upon treatment with Leupeptin or Leupaptin+antipain. Based upon the E-64 inhibitor studies, Salmon *et al.,* (2001) proposed a two step mechanism for the merozoite release that involves erythrocyte membrane rupture followed by PVM rupture. Later, Wickham *et al.*, (2003) proposed a model, which is contradictory to the above proposed model. They showed by using different protease inhibitors that different proteases are involved in selective inhibition of PVM as well as erythrocyte membrane. Based on studies using transgenic parasite lines (-His GFP and +His GFP parasite lines), they proposed a two step egress model for the release of merozoites, which involved an initial rupture of PVM followed by erythrocyte membrane rupture. Since we have observed a block in erythrocyte rupture in *falcipain 2* siRNA treated parasites, we decided to probe it further by studying the origin of membrane surrounding the incompletely ruptured merozoites in *falcipain 2* siRNA treated parasites. To know whether the membrane

# Chapter 5: Discussion

surrounding these merozoites is PVM or erythrocyte membrane, we followed the approach of Wickham *et al.,* (2003) using transgenic parasite line, +His GFP. Treatment of parasites with falcipain2 siRNA resulted in the accumulation of incompletely ruptured schizonts showing diffused GFP fluorescence throughout RBC cytoplasm, thereby suggesting that erythrocyte membrane remains intact in these parasites. We confirmed these observations by carrying out immunofluorescence studies using antibodies against PfEMP1 and band 3. PfEMP1 is a parasite protein, which is exported out to the surface of the erythrocyte membrane whereas Band3 is a major erythrocyte membrane protein which is also referred as "Anion channel protein".

Our results using *falcipain 2* siRNA taken together with the studies using cysteine protease inhibitors suggest that falcipain2 is involved in the second step of merozoite release i.e., in the rupture of RBC membrane. In order to rupture RBC membrane, it is essential for Falcipain 2 to be transported to RBC. To confirm this, we carried out immunolocalization studies using Falcipain 2 specific antibodies as well as antibodies raised against some well known parasite proteins (MSP-$1_{19}$, ERC). Results based on co-localization studies using confocal microscopy showed that Falcipain 2 exists in the food vacuole as well as in the parasite cytosol at the trophozoite stage, whereas in the segmenter stages, Falcipain 2 is localized in the parasitophorous vacuole and also in the erythrocyte cytoplasm. Detergent solubility studies using saponin and streptolysin O showed association of Falcipain 2 with membrane network. These results indicate that Falcipain 2 is transported to erythrocyte cytoplasm and remains associated with the membrane network. In higher eukaryotes, Brefeldin A has been shown to block the export of proteins by disrupting the organization of Golgi complex (Lippincott-schwartz *et al.*, 1998). Even in malarial parasite, it has been shown that Brefeldin A treatment of parasitized erythrocyte results in the collapse of Golgi like vesicular network in the parasite and the Golgi markers were localized within the compartment made up of ER (Elmendorf and Haldar, 1993). The trafficking of Falcipain 2 protein was affected in Brefeldin A treated parasites and it showed the same distribution pattern as seen with other parasite exported proteins, PfEMP-1 and KHARP. These studies thus provide clear evidence regarding the trafficking of Falcipain 2 via membrane network.

Immunolocalization studies using electron microscopy further confirmed trafficking and localization of Falcipain 2 to erythrocyte cytoplasm. Falcipain 2 was

found to be associated with the membrane as well as with the Maurer's clefts. Recent evidences suggests that the Maurer's clefts are parasite-derived membranous system in the cytosol of the RBC and are crucial components for parasite protein sorting as well as trafficking of proteins outside the parasite into the host cell. It has also been proposed that the Maurer's cleft are associated with the host cytoskeleton (Wickham *et al.*, 2003, Przyboski *et al.*, 2003). Based on our results, we propose a model for the new role of Falcipain 2. According to this model, Falcipain 2 is transported to the erythrocyte via PVM through the classical secretory pathway and finally resides in the Maurer's cleft. During the time of RBC lysis, Falcipain 2 in the Maurer's cleft might be re-orienting in such a way that it comes in contact with the cytoskeletal proteins without being released from the Maurer's cleft and then degrades the cytoskeletal proteins leading to the lysis of RBC membrane, an essential step in the merozoite release.

Molecular analysis of erythrocyte membrane has revealed that erythrocyte cytoskeleton forms dense fibrillar shell under plasma membrane. This cytoskeleton network is attached to integral membrane proteins present in the erythrocyte plasma membrane at many points that provide strength and flexibility to the plasma membrane. Recently, Hanspal and co-workers showed that recombinant Falcipain 2 cleave ankyrin and band 4.1 proteins, which are associated with erythrocyte plasma membrane. They also showed that the peptides made from the ankyrin binding site of Falcipain2 can inhibit the cleavage activity. Addition of this peptide to the *P.falciparum* culture at the ring stage blocked the release of merozoites from the RBC (Dhawan *et al.*, 2003). Based on the work of Hanspal's group and the results presented in this study, it can be proposed that Falcipain 2 has dual function i.e., hemoglobin degradation and rupture of RBC membrane. Recently, it was shown that Plasmepsins, which are aspartic proteases were also involved in the cleavage of proteins present in the RBC membrane (Goldberg *et al.*, 2002). Thus it appears that not only cysteine proteases but aspartic proteases are also involved in the lysis of RBC membrane. It will be interesting to see whether these proteases act together or individually on the erythrocyte membrane proteins for the rupture of RBC membrane.

In conclusion, we have shown that RNAi is functional in *Plasmodium falciparum* and can be used as an efficient tool to study the function of genes at a faster rate in comparison with the existing techniques. By using dsRNA/siRNA of *falcipains*, we showed that the Falcipains are involved in hemoglobin degradation and

Falcipain 2 is also involved in the rupture of RBC membrane other than hemoglobin degradation.

## *Invivo* gene silencing of *berghepain* genes in *P. berghei* using *berghepain siRNA*

Earlier, RNAi was shown to have limited applicability. DsRNA were shown to produce non-specific inhibition in mammalian cells. One of the main reasons for the non-specific effects of dsRNA was the induction of γ-interferon response, which activates protein kinase-R (PKR). The activated PKR induces RNAse L, which in turn brings about the degradation of mRNA (Clarke and Mathews, 1995). However, discovery by Tuschl's group that the small interfering RNAs of 21-25 nt length do not produce interference response led to the large scale application of RNAi in mammals (Elbhashir *et al.*, 2002). At present siRNAs are not only being used as a tool to study gene functions, they have also been shown to have therapeutic potential. SiRNAs have been used successfully to block viral replication as well as prevent leukemia in culture cell lines. Recently, RNAi has also been successfully applied in the adult mice to silence Fas receptor genes, which in turn prevented liver cell inflammation and death in mice carrying fulminant hepatitis (McCaffery *et al.*, 2001). Based on these studies, we made an attempt to use siRNAs in adult mice against *P. berghei*-mouse malaria. We intravenously injected siRNAs of *berghepains,* an orthologues of *falcipains* into infected mice to evaluate the *in vivo* efficacy of siRNAs to trigger RNAi in circulating parasites. The effects of these siRNAs were found to be similar to the one seen with *falcipains* dsRNA/siRNA. However, fewer parasites showed morphological abnormalities in comparison to the *in vitro* cultures of *P. falciparum* treated with *falcipain* dsRNA. These results confirmed that Berghepains like Falcipains are also involved in the degradation of hemoglobin. As shown in dsRNA study on *in vitro P. falciparum* cultures, we also showed generation of 25 nt species in *berghepains'* siRNA treated parasites. These results thus demonstrated that RNAi can be applied *in vivo* in *P. berghei*–a mice malaria model.

Thus, through *in vivo* experiments on a mouse model, we have provided evidence that RNAi holds the potential for an attractive therapeutic strategy for the control of parasitic diseases. Nonetheless, many caveats still exist for the therapeutic use of siRNAs in the control parasitic diseases. One of our major concerns is the

efficacy of RNAi under *in vivo* conditions. We could observe only 40-50% reduction in *berghepains'* mRNA levels in the treated parasites. This could be due to the poor delivery of siRNAs to all the circulating parasites or due to the choice of siRNA sequences. It has been shown that all siRNAs along the length of the gene are not equally effective for RNAi (Holen *et al.*, 2002). Even though there was significant reduction in mRNA levels, we did not observe any significant reduction in parasitemia in siRNA treated parasites in comparison to the control. This could be due to the redundancy existing in the hemoglobin degradation pathway. Three different classes of enzymes: aspartic proteases (ten Plasmepsins), cysteine proteases (four Falcipains), and metallo proteases (one Falcilysin) have been shown to be involved in hemoglobin degradation (Padmanaban *et al.*, 2002). *In vivo* studies using *P. vinckei* murine malaria model have shown that inhibitors of any of the three classes of enzymes alone were not effective in curing malaria. However, the combined protease inhibitors cured the majority of the infected animals, suggesting that the combination of these inhibitors act synergistically in the inhibition of *Plasmodium* hemoglobin degradation *in vivo* and in the treatment of murine malaria (Semenov *et al.*, 1998). Based on these facts, it can be argued that mixture of siRNA corresponding to all three classes of enzymes involved in hemoglobin degradation may be required to show efficient antiparasitic effect in this model system.

The use of RNAi in *P. falciparum* to dissect gene function has several advantages over methods requiring the introduction of DNA into cells. Transfection experiments require knowledge of the full sequence of the gene and its flanking regions, whereas for RNAi, dsRNA corresponding to any gene fragments is sufficient to confer the interference effect. Particularly, it is difficult to carry out knockouts in intracellular parasites like *Trypanosomes* and *Plasmodium* (Ullu and Tschudi, 2000). Moreover use of RNAi in *P.falciparum* as well as in other organisms is technically simple and quick, and result of gene silencing can be obtained within 2-3 days. This contrasts sharply with the time necessary to produce selective gene knockouts by transfection technology. With the availability of large amount of sequence information for *P. falciparum,* RNAi-based functional genomics can thus be useful technique for investing the biological functions of novel genes. Finally, given the gene specific feature of RNAi and the ease of inducing RNAi, this methodology may also play an important role in the development of therapeutic applications against malaria.

In conclusion, the present study demonstrates that the RNAi can be a useful tool to study gene functions in *Plasmodium*. In case of pathways, which are essential for the parasite survival, gene targeting by homologous recombination is not possible. RNAi can be an important tool to study the function of genes involved in such pathways. RNAi can also be used in case of redundant pathways where gene-knock out may not permit any useful functional information. In addition to its use in functional studies in *Plasmodium*, our study also demonstrated potential applicability of siRNA as a therapeutic agent. But in this regard, one of the major issues to be taken care of is the delivery of siRNA to the specific tissue, while ensuring appropriate level of efficacy with minimum toxicity.

# Chapter 7: Summary and Conclusion

Malaria remains a public health problem of enormous magnitude, affecting over 500 millions people every year. In the fight against malaria, there is an urgent need to develop new antimalarials and effective malaria vaccine because of wide spread resistance to common antimalarials. The progress in the development of effective antimalarials/ vaccine has been slow due to the lack of understanding of the functions of different parasite proteins. Reverse genetics is one of the ways to assess the function of parasite genes. Gene function elucidation by antisense approach, ribozyme technology and gene disruption technology has met with limited success. Even though transfection technology to knock out different genes has been developed for different malaria parasites, they are not being applied routinely due to extensive selection procedures. With the availability of malaria genome sequence, there is an urgent need to develop an effective molecular tool to carry out functional genomic studies in malaria.

Recently, RNAi has emerged as a powerful and quick tool to study the gene function in a wide variety of organisms. RNA silencing is a sequence specific RNA degradation process mediated by double stranded RNA. It differs from other methods of reverse genetics in terms of specificity and simplicity. Extensive genetic and biochemical studies have revealed a three-step mechanism for RNAi induced gene silencing. The first step involves degradation of dsRNA in to small interfering RNA (siRNA) of 21-25nt long. An RNAse III like enzyme called DICER has been shown to be involved in this step. In the second step, RISC (**R**NA **i**nduced **s**ilencing **c**omplex) is activated by forming a complex with siRNA. The third step involves recognition of corresponding mRNA by siRNA-RISC complex and degradation of that mRNA by nucleases present in the complex. Currently RNAi is being used extensively in *C.elegans*, *Drosophila*, in cultured insect and mammalian cell lines to elucidate the function of the genes. In *C.elegans* RNAi has been successfully used to carry out large-scale functional analysis of several genes.

**Effect of dsRNA on *in vitro Plasmodium falciparum culture***

To carry out RNAi in malaria parasite, initially procedures to make dsRNA corresponding to falcipain genes were developed. Double-stranded RNAs for these genes were synthesized using T7 and SP6 polymerases. Agarose gel electrophoresis was carried out to analyze the synthesized dsRNA. Since malaria parasite is an

intracellular parasite, in the beginning, uptake of labeled dsRNA by the malaria parasite was examined. Careful analysis of different fractions of malaria parasite showed that approximately 0.1-0.15 % of dsRNA was taken up by the parasite. To determine the effects of *falcipains* dsRNA on *P. falciparum cultures*, synchronized parasites at late ring stage were incubated with dsRNA for each of the two *falcipains* separately, as well as in combination, at different concentrations. Inclusion of dsRNAs at conc. 50 μg/ml in the parasite culture showed distinct morphological changes, the most notable of which was the abnormally swollen food vacuole that contained an accumulation of malaria pigment. Addition of each dsRNA as well as in combination also brought reduction in the formation of new rings and as well as total parasitemia. These studies showed that cysteine proteases play an important role in parasite development and growth. The effect of *falcipain* dsRNA on the parasite growth was also confirmed by $^{3}$[H] hypoxanthine uptake assay. To investigate whether the observed morphological abnormalities in *falcipains* dsRNA treated parasites were indeed caused by a blockage in hemoglobin degradation, protein extracts were prepared from the treated as well as untreated parasite cultures and subsequently analyzed on SDS-PAGE. Parasites treated with *falcipains* dsRNAs showed significant accumulation of hemoglobin, when compared with the untreated parasites. The fate of *falcipain* mRNAs in dsRNA treated parasites was examined by Northern blot analysis. Parasites treated with *falcipains* dsRNA showed significant degradation of endogenous *falcipain* mRNAs in comparison to untreated parasites. One of the salient features of RNAi machinery is the formation of small RNA species called siRNAs. Analysis of small RNA species in the *P. falciparum* culture, treated with labeled *falcipain* dsRNAs showed the generation of 25 nt labeled RNA species. This shows that the mechanism of RNAi is conserved in *Plasmodium*. Parasite extracts prepared from the *P. falciparum* culture treated with *falcipain* dsRNA showed ability to cleave *falcipain* mRNA, while untreated parasite extract did not show any such nuclease activity. These results demonstrated the conservation of RNAi machinery in the malaria parasite.

**SiRNA mediated gene silencing in *P. falciparum***

SiRNA are the true intermediates of an RNAi reaction and have been used as an effective initiator for an RNAi reaction. SiRNA of *falcipain 1&2* were also used for an efficient RNAi in malaria parasite. Parasites treated with *falcipains* siRNAs

showed considerable growth reduction as evident from geimsa staining and $^{3}$[H] hypoxanthine assay. After 56hrs of treatment with *facipains'* siRNAs, individually and both together, considerable reduction in the number of new ring formation was observed. *Falcipain*2 siRNA treated parasite culture also showed large number of abnormal parasites arrested at schizont stage. Morphological examination of such parasites under light microscopy showed spherical clusters of merozoites, each enclosed in a delicate membranous covering. The delicate membrane surrounding these spherical clusters of merozoites was found to be of RBC origin based on immunofluorescence studies using PfEMP1 antisera. Previous studies have suggested that Falcipain 2 is only localized in the food vacuole. If Falcipain 2 plays a role in the merozoite release from RBCs, it should also be transported to RBC cytoplasm. Immunofluorescence studies using confocal microscopy and electron microscopy showed that Falcipain 2 is transported to erythrocyte cytoplasm in the parasitized RBC through Maurer's cleft, a pathway shown to be responsible for the export of parasite proteins. These results thus demonstrate the potential use of siRNA to study the function of the genes and also suggest the role of Falcipain 2 in the release of merozoites besides hemoglobin degradation.

**RNA interference in *P. berghei*, a mouse malaria model**

Recently, siRNA have been shown to hold great potential as gene therapeutic agents. In cultured cell lines, siRNA have been successfully used to inhibit expression of onco-proteins. Moreover, RNAi has also been used to silence the expression of genes in adult mice. Based on these facts, silencing effects of siRNAs corresponding to *berghepain* genes on *Plasmodium berghei* in adult mice were investigated. Three siRNA corresponding to *bergheipain 1&2* and GFP were used in the present study. GFP siRNA was used as a negative control for these studies. Mice were intravenously injected with siRNA once a day for three days and after 48hrs of last injection, Geimsa stained blood smears were examined for the morphological abnormalities. We observed similar kind of abnormalities as in the case of *falcipain* dsRNA treated *in vitro P. falciparum* culture. To assess the effects of *berghepains'* siRNAs on the accumulation of hemoglobin, treated parasitized erythrocytes were collected from mice blood, lysed with saponin and parasite extracts were analyzed on 15% SDS-PAGE. Accumulation of undegraded hemoglobin in comparison to the control parasites was observed in siRNA treated parasites. These siRNA treated parasites also

showed generation of 25mers. These results thus provide evidence that RNAi holds the potential for an attractive therapeutic strategy for the control of parasitic diseases. Based on the above observations, following conclusions can be drawn.

1. RNAi mechanism exists in *Plasmodium.*
2. dsRNA/ siRNA can be used in *P. falciparum* to study the functions of the genes.
3. Cysteine proteases are essential for survival of the parasite.
4. Falcipain 1 and 2 act at the initial stage of the hemoglobin degradation pathway.
5. Falcipain 2 is exported out from the parasite to the erythrocyte cytosol and resides in the maurer's cleft
6. siRNA mediated silencing of Falcipan 2 revealed that Falcipain 2 other than its role in hemoglobin degradation also helps in the release of the merozoites from the RBC by the rupture of erythrocyte membrane.
7. siRNA can be used as a potential therapeutic agent against malaria.
8. Bergheipains like Falcipains are involved in hemoglobin degradation.

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# Illustrations

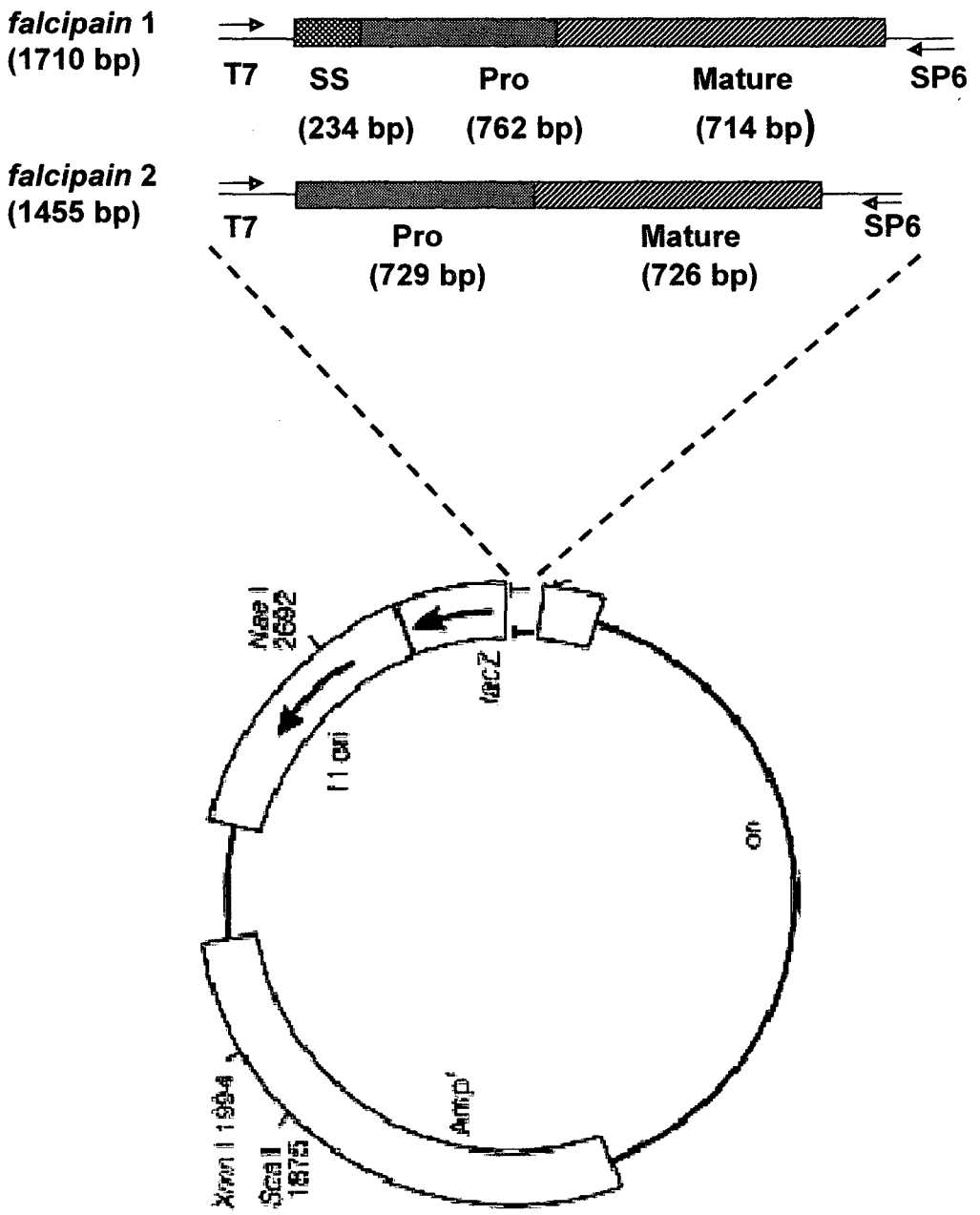



**Fig.1** The vector map of pGEMT with *falcipain* gene cloned in the multiple cloning site, which is flanked on either sides with T7 and SP6 promoter sequences. The insert shows the schematic representation of *falcipain* genes. SS, signal sequence: Pro, pro region.

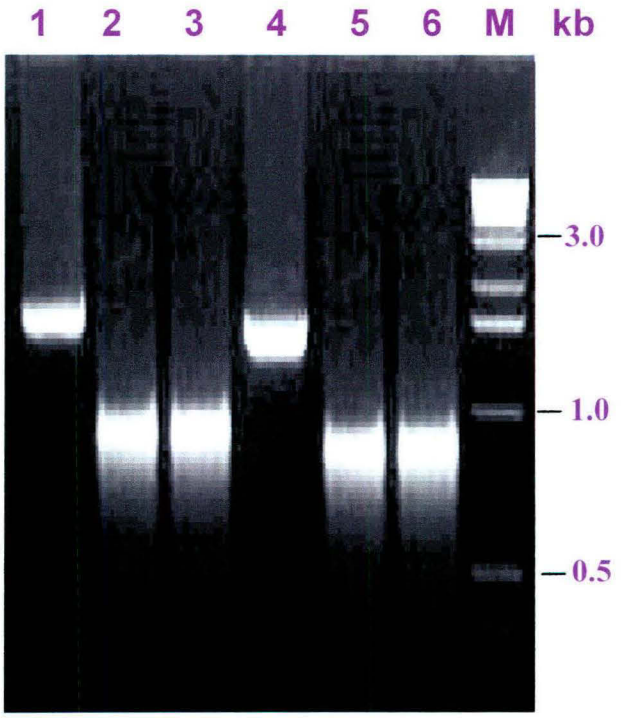
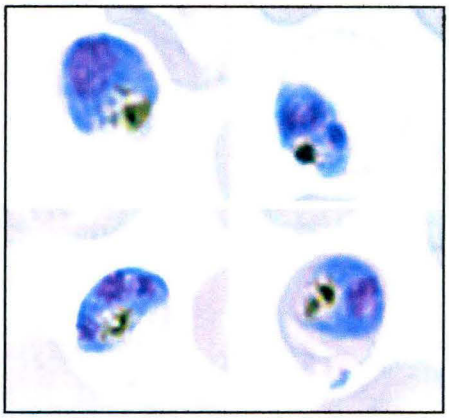



**Fig.2 Agarose gel electrophoresis of ssRNAs and dsRNAs of *falcipain 1&2* synthesized using T7 and SP6 polymerase.** Sense strand (ss) RNA and anti sense strand (as) RNA were synthesized using polymerases and were mixed, the mixture was incubated at 65°C and cooled slowly to obtain double stranded RNA (dsRNA). *falcipain1* dsRNA (Lane 1), *falcipain 1* ssRNA (Lane 2), *falcipain 1* asRNA (Lane 3), *falcipain 2* dsRNA (Lane 4), *falcipain 2* ssRNA (Lane 5), *falcipain 2* asRNA (Lane 6), 1kb marker (M).

| Treatment | Concentration | Food vacuole abnormality | % ring parasites |
|---|---|---|---|
| *falcipain*-1 dsRNA | 10μg/ml (9.0nM) | + | 82 |
| | 25μg/ml (23.0nM) | ++ | 60 |
| | 50μg/ml (45.5nM) | ++ | 65 |
| *falcipain*-2 dsRNA | 10μg/ml (11.0nM) | + | 90 |
| | 25μg/ml (27.0nM) | ++ | 70 |
| | 50μg/ml (50.0nM) | ++ | 77 |
| *falcipain*-1 & 2 dsRNAs | 25μg/ml of each | +++ | 35 |
| | 50μg/ml of each | ++++ | 30 |
| Control (DEPC water) | - | - | 100 |
| E-64 | 100μM | ++++++ | 0 |
| *aminopeptidase*-N (*Spodoptera litura*) dsRNA | 50μg/ml | - | 99 |

**Table 1:** Effects of Falcipains dsRNAs and E-64 on the morphology and viability of cultured parasite

A



B

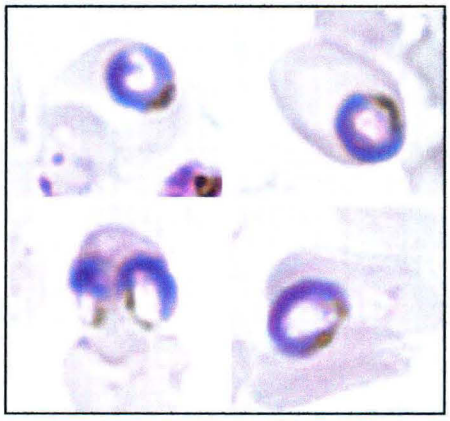


**Fig.3 Effects of falcipains dsRNA on *P. falciparum* parasite morphology**; *P. falciparum* cultures were treated with *falcipains* dsRNA and the smears were made from the treated cultures. Smears were Geimsa stained and visualized for morphological effects. Treated parasites (panel B) at trophozoite stage showed swollen food vacuole where as untreated parasites (panel A) were normal.

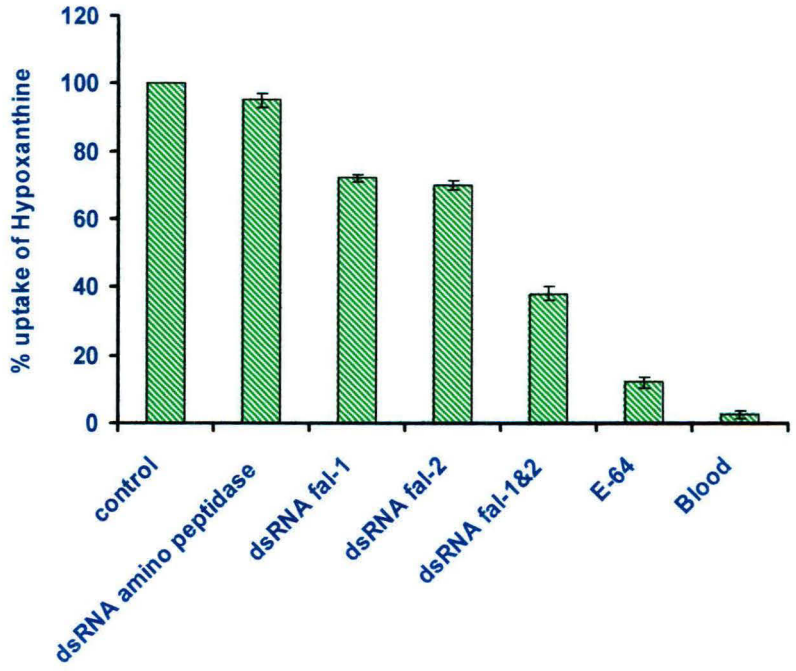



**Fig.4 Inhibition of $^{3}$[H]-hypoxanthine uptake in cultured parasites by *falcipains* dsRNA**. *P. falciparum* culture of volume (100µl) at ring stage were treated with *falcipains* dsRNA for 24 h. Approximately, 1µCi of $^{3}$[H]-hypoxanthine was added to each well and cultures were maintained for additional 24 h. The cells were harvested and $^{3}$[H]-hypoxanthine was quantified using scintillation counter. *Aminopeptidase-N* dsRNA (*Spodoptera litura*) was used as a control.

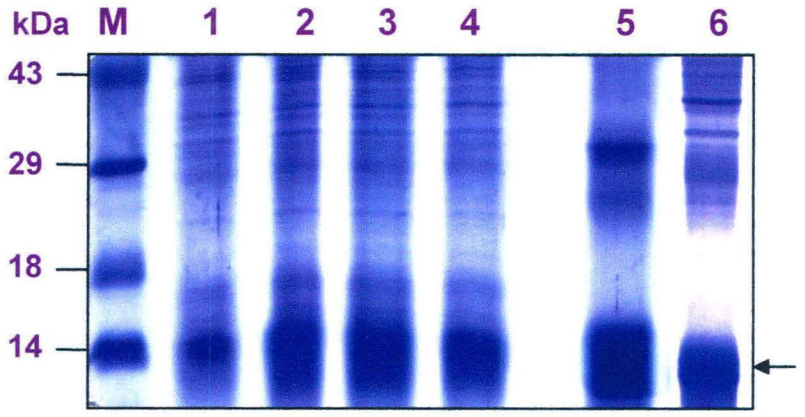



**Fig.5 Block in hemoglobin hydrolysis in *P. falciparum* parasite after treatment with *falcipains* dsRNA.** Cultured parasites were incubated for 48 h with control (DEPC water) and *falcipains* dsRNA. After treatment, parasitized erythrocytes were lysed with saponin and washed thoroughly to remove erythrocyte cytosolic components. Parasite pellet were solubilised in reducing SDS-PAGE sample buffer and parasite proteins were resolved on 15% SDS-PAGE. Negative control (Lane 1), dsRNA from *falcipain 1&2* (Lane 2), dsRNA *falcipain* 1 (Lane 3), dsRNA from *falcipain 2* (Lane 4), hemoglobin control (Lane 5), E-64 treated parasite (Lane 6). The arrow indicates the hemoglobin accumulation.

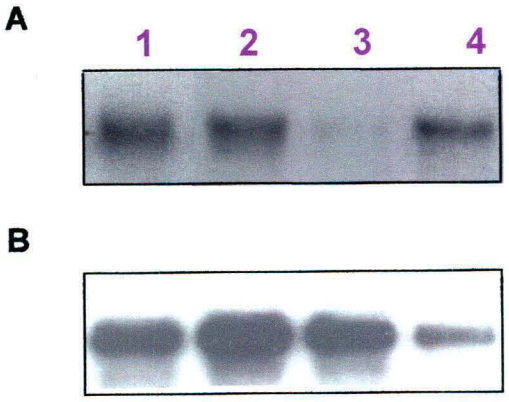



**Fig.6 Northern blot analysis demonstrates the effects of *falcipains* dsRNA treatment on levels of endogenous RNA transcripts**. Total RNA was extracted from control parasites (Lane 1), *aminopeptidase* dsRNA treated parasites (Lane 2), *falcipain 1* dsRNA treated parasites (Lane 3) and *falcipain 2* dsRNA treated parasites (Lane 4) and separated on 1.2% agarose gel for Northern blotting. Blots were hybridized with random primer labeled *falcipain 1* DNA probe (A) and *falcipain 2* DNA probe (B).

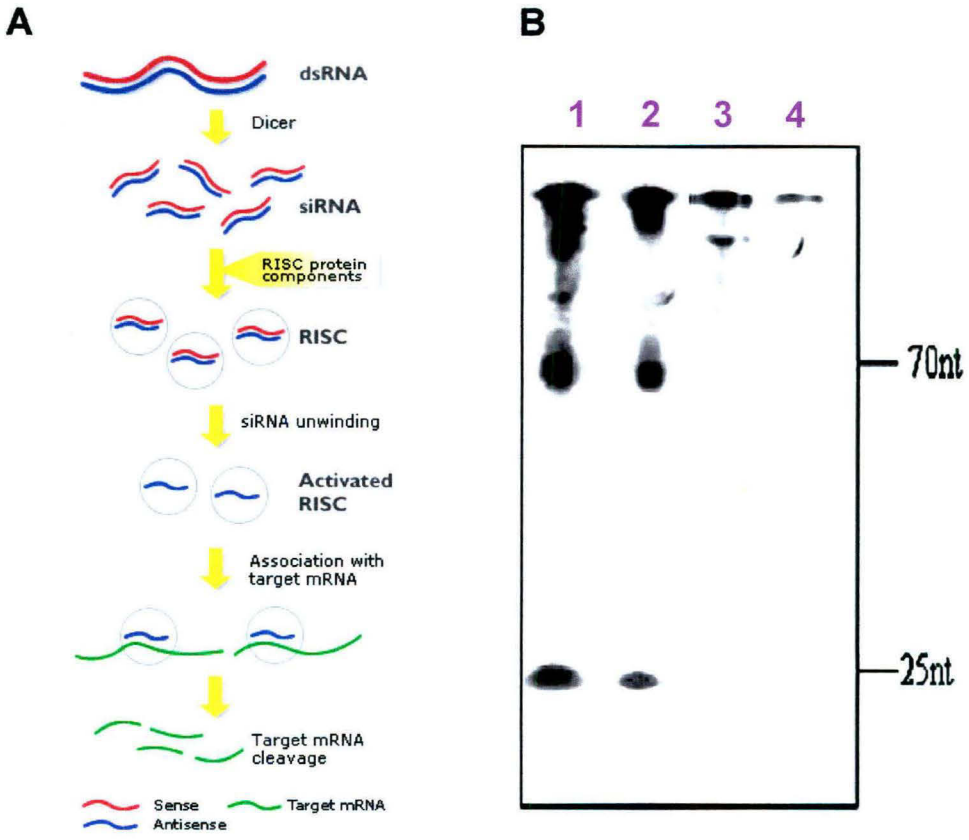



**Fig.7 Generation of 25nt small RNA species, a characteristic feature of RNAi, upon incubation with *falcipain* dsRNAs in parasite culture**. (A) Schematic representation of mechanism of RNAi. (B) $^{32}P$ labeled dsRNAs made from each *falcipain* were added to *P.falciparum* cultures and incubated for 40 h. As a control, labeled dsRNA was added to normal RBCs for the same period. Total RNA was isolated from the parasites and low molecular weight RNA was purified from total RNA. *falcipain 1* dsRNA treated parasite (Lane 1), *falcipain 2* dsRNA treated parasites (Lane 2), untreated parasites (Lane 3), *falcipain* dsRNA added to the RBC (Lane 4) .

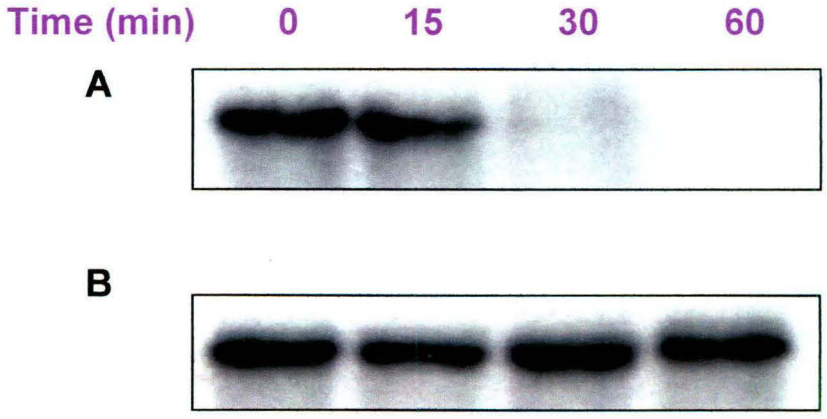



**Fig.8 Denaturing agarose gel showing time dependent degradation of radio labeled mRNA by parasite extract.** *P. falciparum* cultures were treated with dsRNA. To the extract made from the treated cultures, $^{32}P$ labeled mRNA was added and incubated for different time periods and analyzed on agarose gel. *falcipain 2 dsRNA* treated parasites (panel A) and untreated parasites (panel B).

***falcipain 1* (M81341)**

Target sequence : 5' CGA AGA AAA CAG GAAGAG GTT 3'

Sense strand: 5' CGA AGA AAA CAG GAA AGA GUU 3'
Anti sense strand: 3' UU GCU UCU UUU GUC CUU UCU C 5'

***falcipain 2* (AF239801)**

Target sequence : 5' GTA GAA TCA CAA TAT GCT AT 3'

Sense strand: 5'GUA GAA UCA CAA UAU GCU AU T T 3'
Anti sense strand: 3' T T CAU CUU AGU GUU AUA CGA UA 5'

***Aminopeptidase*-N (AF320769)**

Target sequence : 5' GAG CTC ATG AAG CGT TCA C 3'

Sense strand: 5' GAG CUC AUG AAG CGU UCA CUU 3'
Anti sense strand: 3'UU CUC GAG UAC UUC GCA AGU G 5'

**Fig.9 Target sequences identified from *falcipain* genes and their corresponding siRNA.** The gene accession numbers are indicated next to the gene names mentioned above.

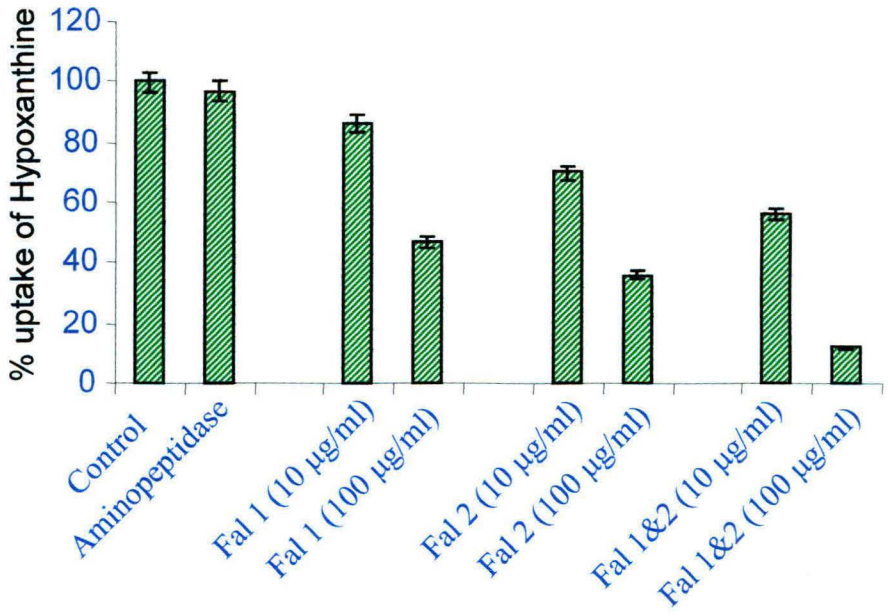



**Fig.10 Graph showing the effect of *falcipains* siRNA on the uptake of $^{3}$[H] hypoxanthine**. *P. falciparum* cultures were incubated with *falcipains* siRNA for 24 h. Approximately 1µCi of $^{3}$[H] hypoxanthine was added to the cultures and incubated further for 24 h. *Aminopeptidase* siRNA (*Spodoptera litura)* was used as a negative control.

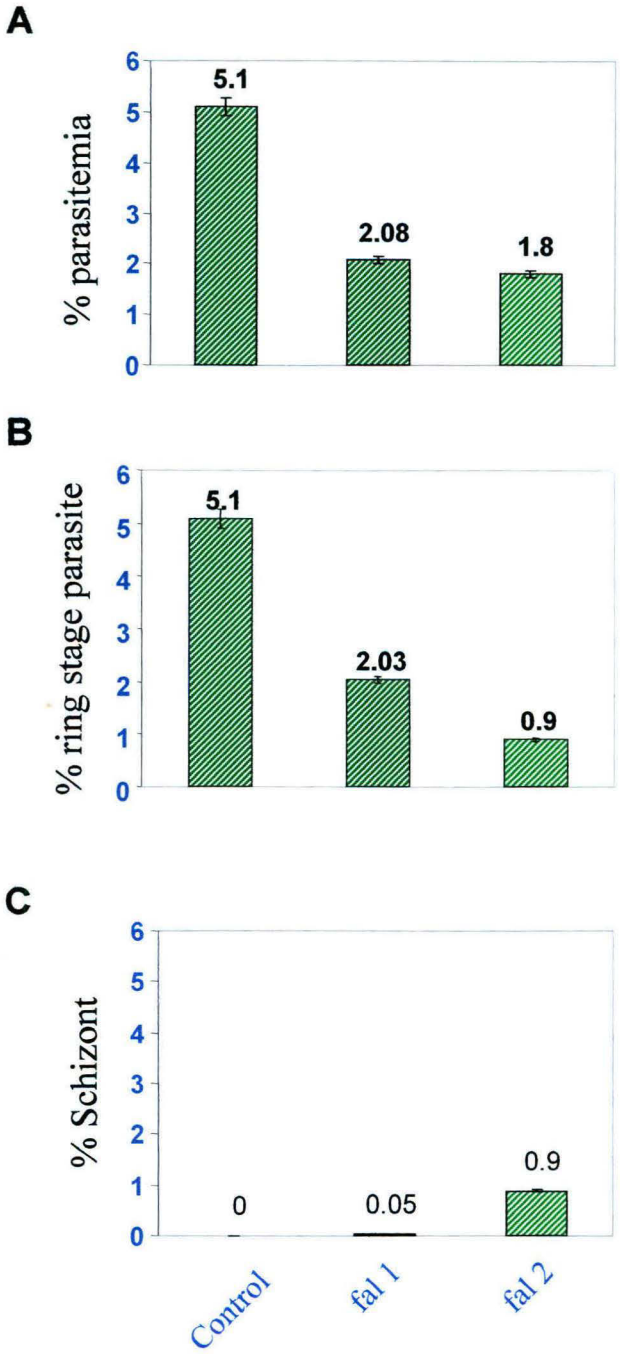
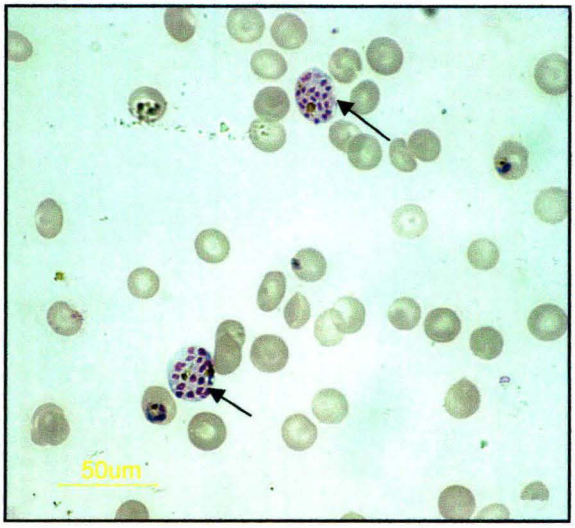
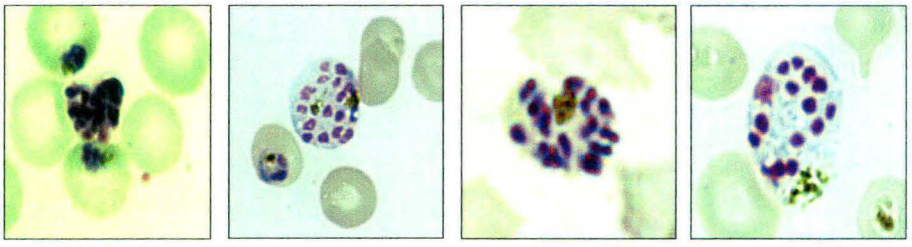



**Fig.11 Graphs showing the effect of siRNAs on total parasitemia as well as on different stages of parasite.** *P. alciparum* cultures were incubated with 50 µg of *falcipain 1&2* siRNA seperately and after 56 h of incubation total parasitaemia (panel A), parasites at new rings stage (panel B) and at schizont stage (panel C) were counted.

A




B



Control (untreated) | E-64 treated | Falcipain 2 siRNA treated

**Fig.12 Effect of *falcipain 2* siRNA and E-64 on the morphology of *P.falciparum* parasites at schizont stage.** Morphological examination of *P. falciparum* cultures treated with *facipain2* siRNA and E-64 under light microscopy. Overview of siRNA treated parasites (panel A). Spherical clusters of merozoites were surrounded by a delicate membrane in case of E-64 and siRNA treated samples while schizonts in the control were normal (panel B). Arrow indicates abnormal parasites.

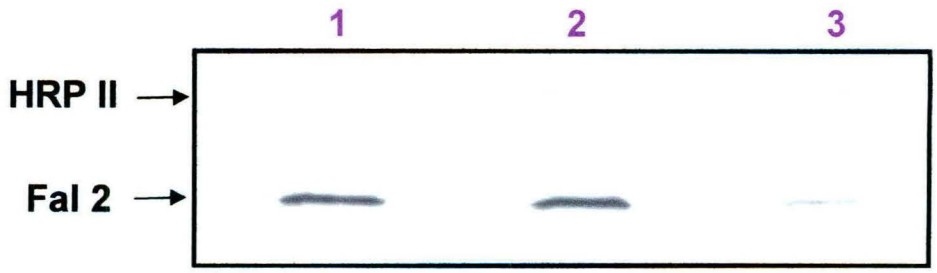



**Fig.13. Western blot analysis showing reduction in the amount of Falcipain 2 protein in treated parasites.** *P. falciparum* cultures were treated with *falcipain 2* siRNA and Western blot analysis of treated and untreated parasite extract was carried out using Falcipain 2 antibody. Control (untreated parasites) (Lane 1), *Aminopeptidase* siRNA treated parasites (Lane 2), *falcipain 2* siRNA treated parasites (Lane 3). HRPII antibody was used to show loading control.

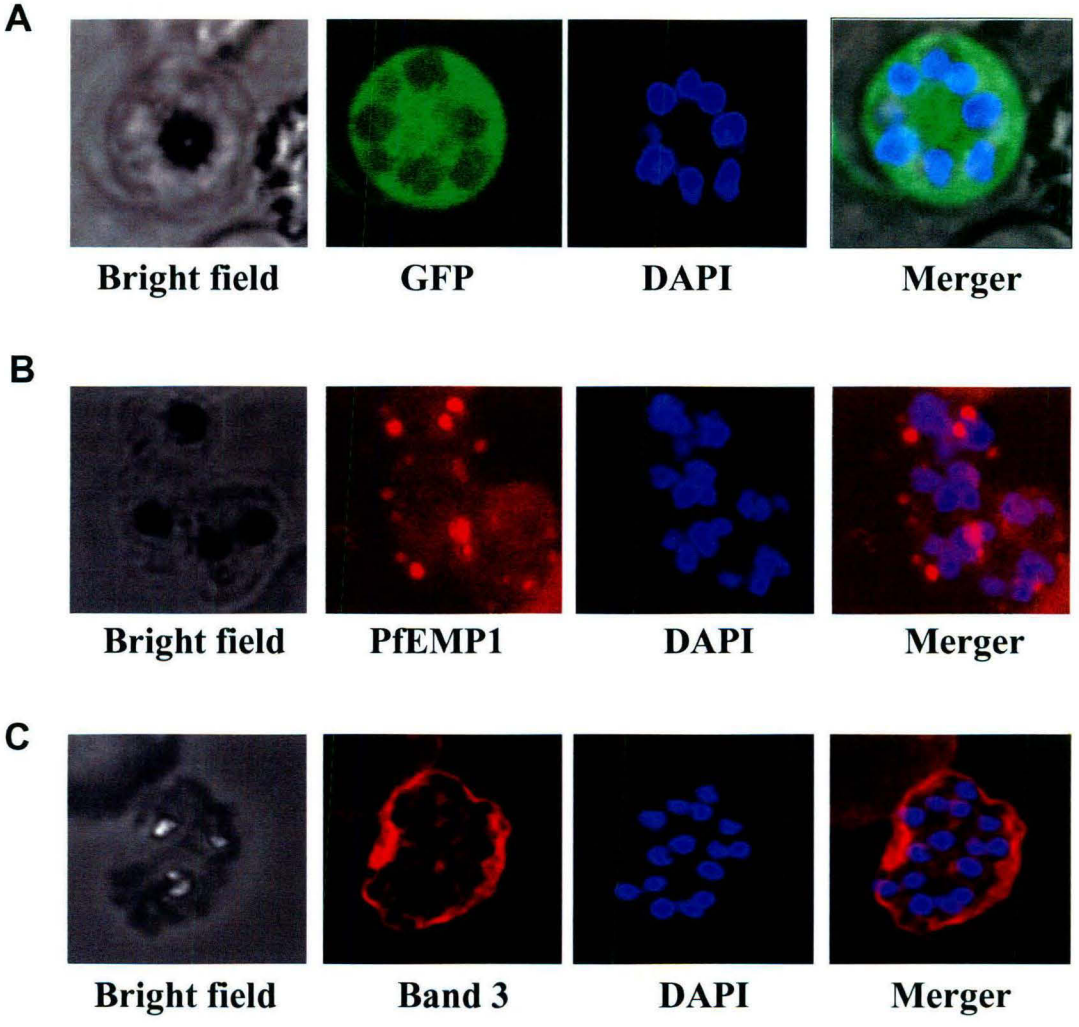



**Fig.14 Rupture of erythrocyte membrane is blocked in incompletely ruptured schizonts.** Fluorescent microscopic images of incompletely ruptured schizonts in 3D7+His parasites after 56 h siRNA treatment, showing the localization of GFP fluorescence immediately surrounding the daughter merozoites (panel A). Immuno-fluorescence images of incompletely ruptured schizonts using PfEMP1 antibodies (red) (panel B). Immuno-fluorescence images of incompletely ruptured schizonts using Band3 antibody (Erythrocyte surface protein) (panel C). Parasite nuclei were stained with DAPI.

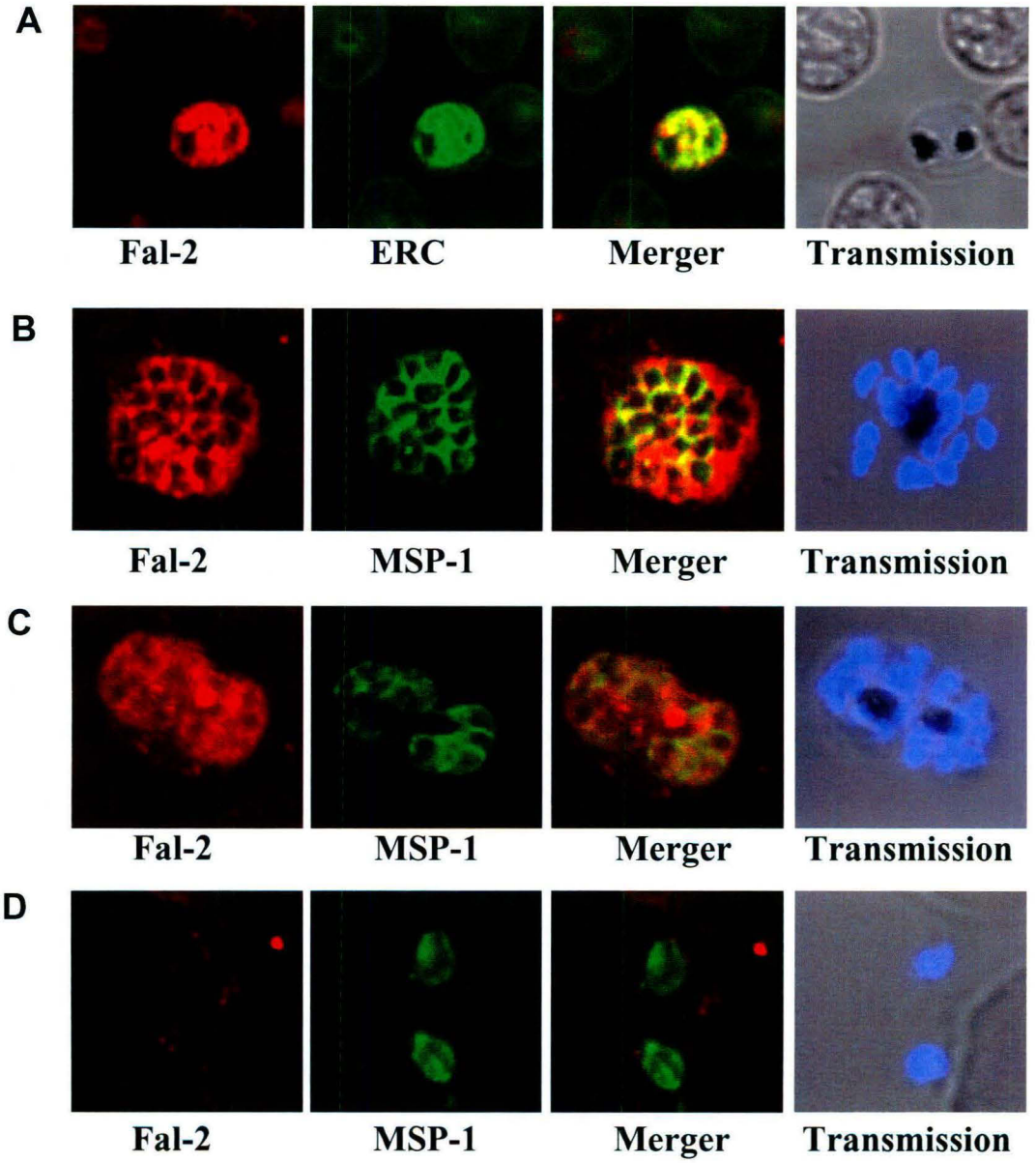



**Fig.15 Confocal microscopic images showing the localization of Falcipain 2 in different stages of the parasite growth in erythrocytes.** Trophozoite (Panel A), early and late schizont (panel B and C) and free merozoites (panel D). Falcipain 2 is indicated by red, DAPI by blue and MSP-1 by green staining respectively. Green staining represents PfERC in panel A.

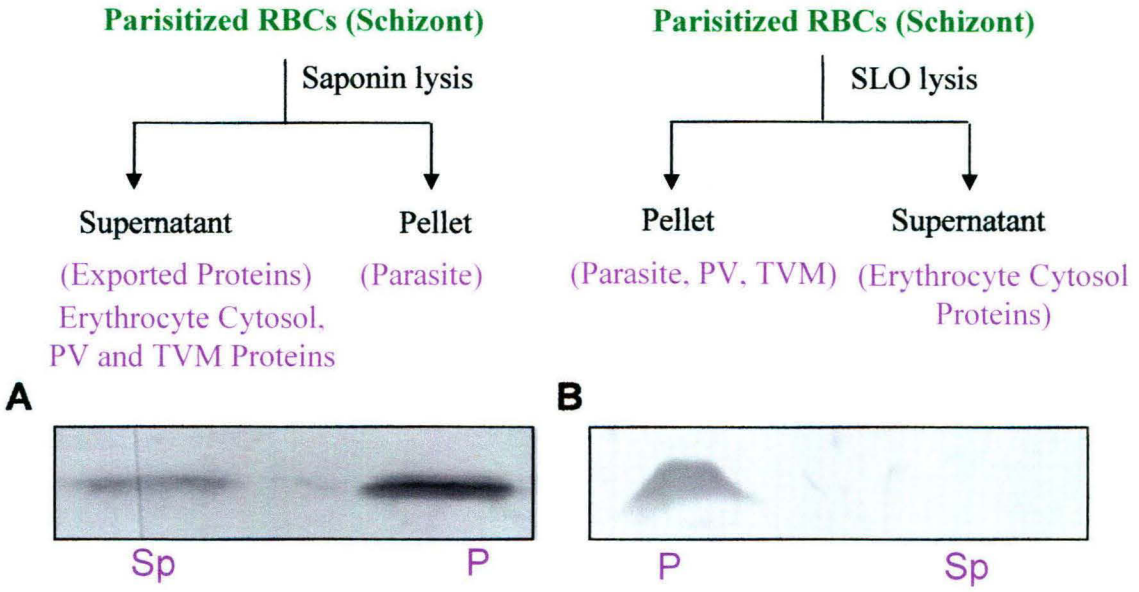



**Fig.16 Localization *falcipain* 2 in different parasite fractions**. Parasitized erythrocytes at schizont stage were treated with saponin and SLO separately. Both the supernatant and the pellet fractions were mixed with SDS-PAGE dye and run on 10% SDS-PAGE under reducing conditions. The gel was transferred on to the nitrocellulose membrane and incubated with mice anti falcipain2 antibody. The bands were developed by using ECL kit. Panel A shows saponin treated parasitized erythrocyte, where falcipain 2 protein was localized in both supernatant and pellet, panel B represents SLO treated parasitized erythrocytes, where falcipain 2 was localized only in pellet but not in the supernatant.

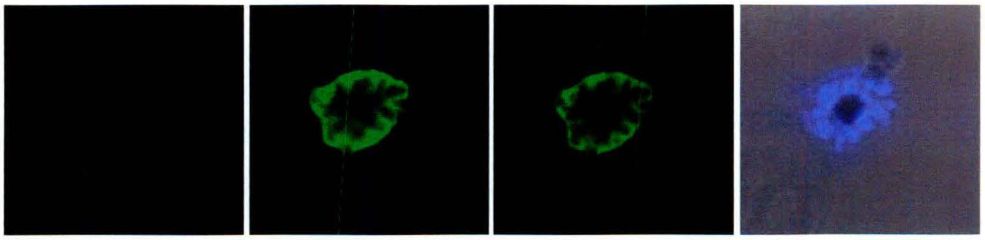



**Fig.17 Immuno fluorescence images showing the absence of fluorescence in *falcipain 2* siRNA treated parasites**. *Falcipain2* siRNA treated parasites were stained with mice anti *falcipain* 2 antibody and rabbit anti MSP1 antibody (green). DAPI was used to show nuclear staining (blue staining). MSP staining was used as a positive control.

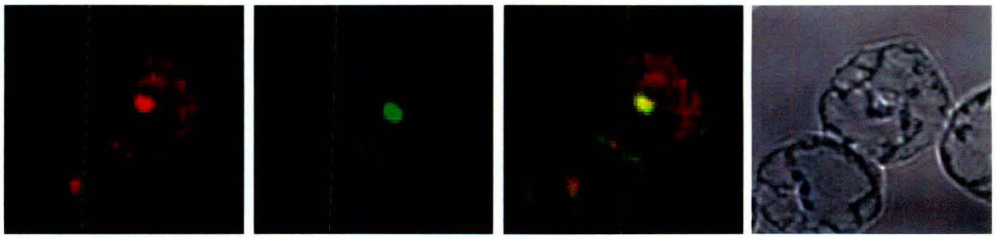



**Fig.18 Trafficking of falcipain 2 through brefaldin A sensitive vesicle mediated secretary system.** Confocal microscope image showing immuno localization of falcipain 2 protein in brefeldin A treated parasites. Synchronous parasites were treated with brefaldin A for 16 h, smears of these parasites were immuno-stained with falcipain 2 antibodies (red) and anti ERC antibodies (green) and visualised using confocal microscope.

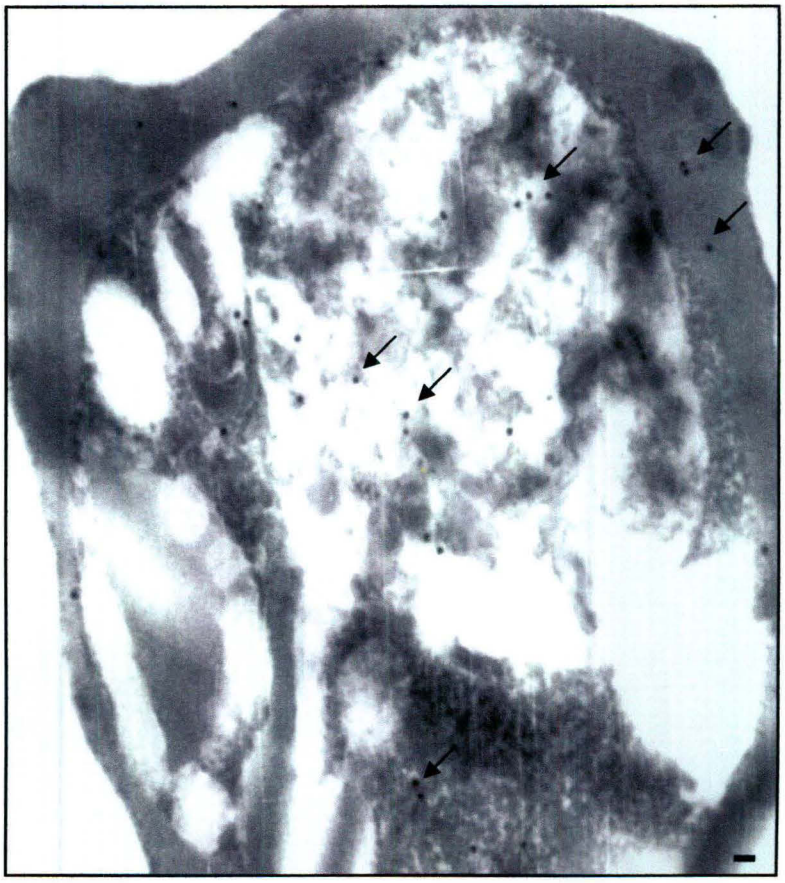


**Fig.19 Immuno electron microscopic image showing localization of falcipain 2 protein in trophozoite**. The trophozoite stages were isolated by percoll gradient centrifugation, fixed in 1% glutaraldehyde and stained with anti falcipain 2 antibody followed by secondary antibody conjugated to gold particle. Falcipain 2 protein at trophozoite stage was localized in food vacuole, ER and the parasitophorous vacuole. Less amount of protein was also localized to the surface of the RBC. Arrow marks point out the gold labeled particle. Bar 100μm

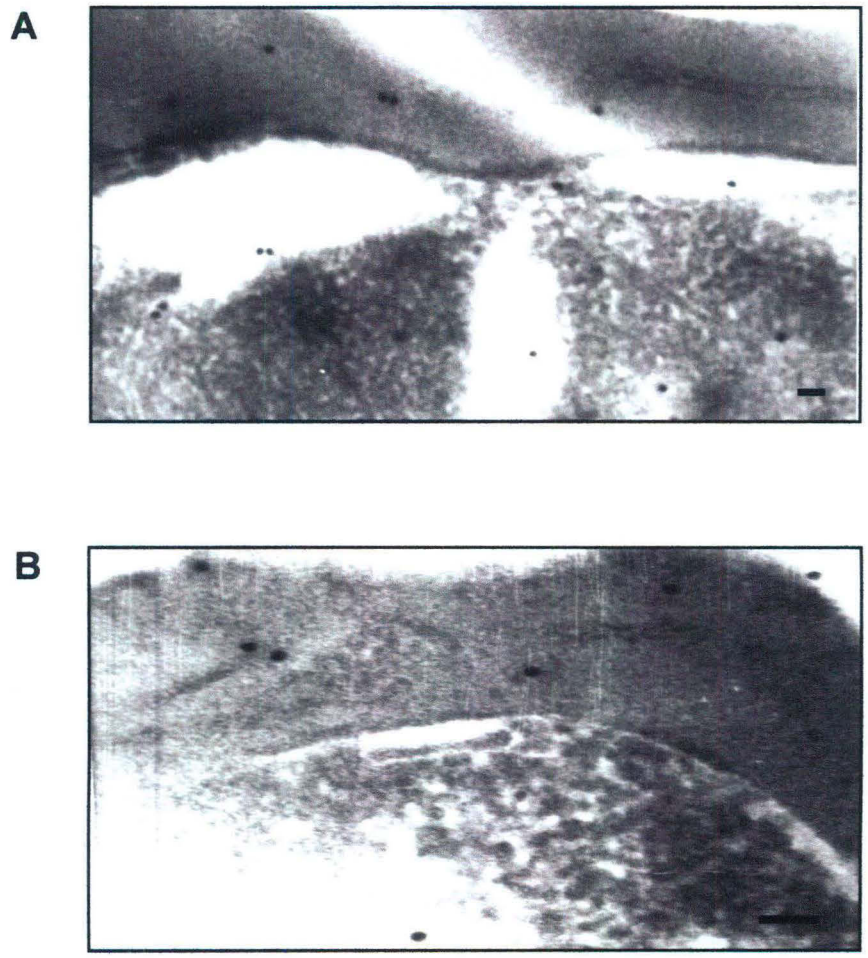



**Fig.20 Immuno electron microscopic image showing localization of falcipain 2 protein in schizont stage.** The schizont stages were isolated by percoll gradient centrifugation, fixed in 1% glutaraldehyde and stained with anti falcipain 2 antibody followed by secondary antibody conjugated to gold particle. Localization of Falcipain 2 protein in the parasitophorous vacuole and the vesicular net work outside the parasite in the erythrocyte cytosol (panel A). The localization of falcipain2 protein in the vesicular net work in the erythrocyte cytosol and close to the surface of the RBC membrane (panel B). Bar 100 μm.

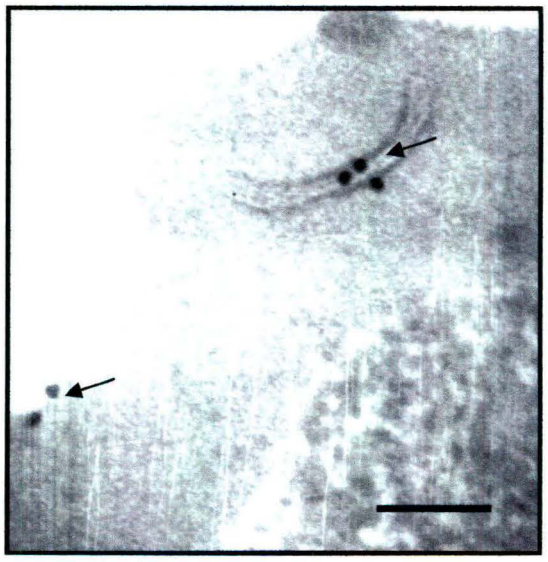


**Fig.21 Immuno electron microscopic image showing localization of falcipain 2 protein in schizont stage.** The schizont stages were isolated by percoll gradient centrifugation, fixed in 1% glutaraldehyde and stained with anti falcipain 2 antibody followed by secondary antibody conjugated to gold particle. falcipain2 protein was localized in the maurer's cleft and close to the surface of the RBC membrane. Bar 100μm.

***bergheipain 1* (987bp)**

Target sequence 5' AAT AAT GGG GTA TGC TTT GGC 3'

Sense siRNA UAA UGG GGT ATG CUU UGG CUU
Anti sense siRNA UU AUU ACC CCA UAC GAA ACC G

***bergheipain 2* (1448bp)**

Target sequence 5' AAG ATC TTA TAG ACA TGG ATG 3'

Sense siRNA GAU CUU AUA GAC AUG GAU GUU
Anti sense siRNA UU CUA GAA UAU CUG UAC CUA C

***GFP* (727bp )**

Target sequence 5' AAG GTT ATG TAC AGG AAA GAA 3'

Sense siRNA GGU UAU GUA CAG GAA AGA A AA
Anti sense siRNA UU CCA AUA CAU GUC CUU UCU U

**Fig.22 Target sequences identified from berghepains' and GFP genes and their corresponding siRNA.**

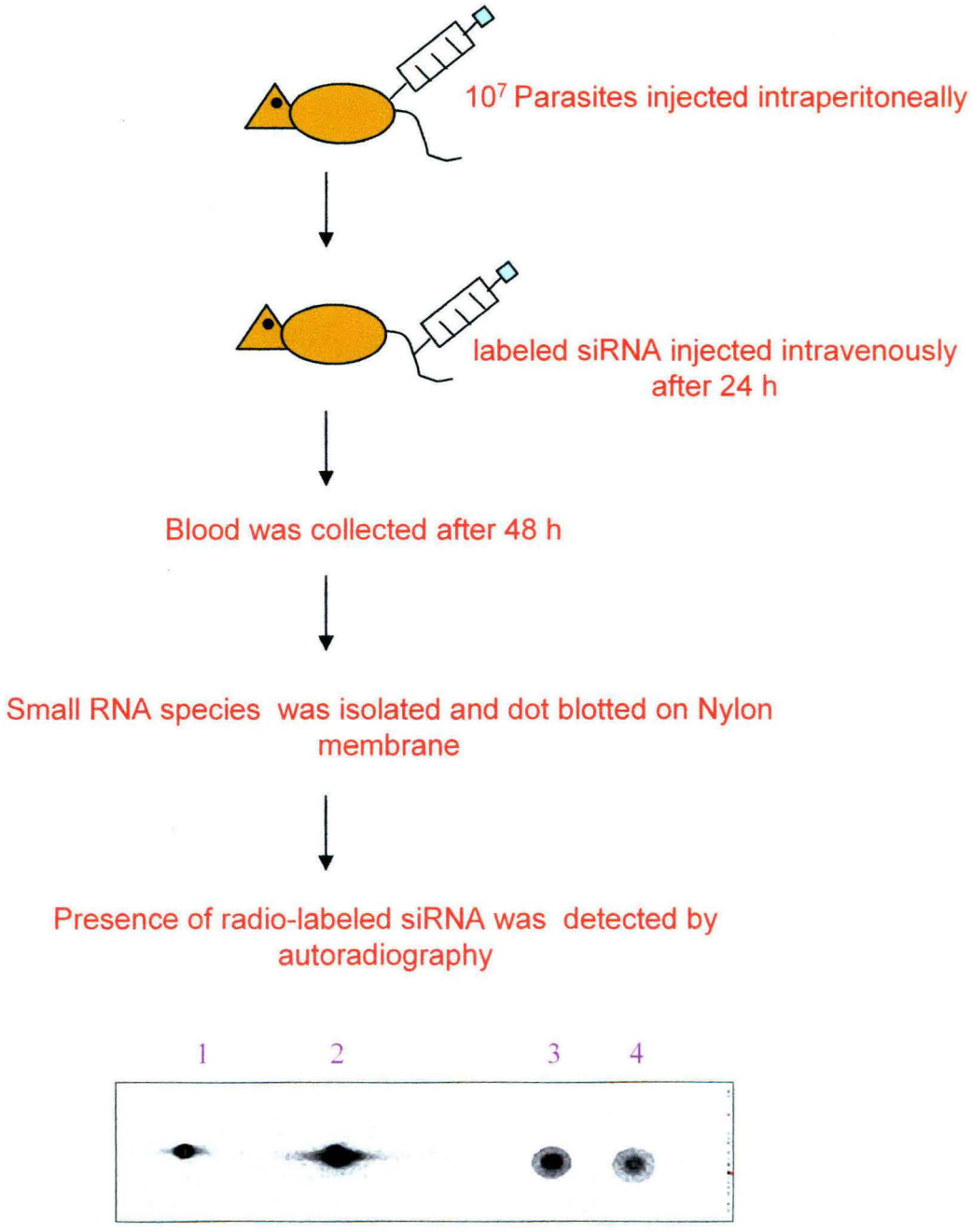



**Fig.23 Autoradiograph of low molecular weight (LMW) RNAs from parasites to show uptake of labeled siRNA by the parasites**: Labeled *BP1*-siRNA (spot1), labeled *BP2*-siRNA (spot 2), LMW-RNAs from parasite treated with labeled *BP1* siRNA (spot3), and with labeled *BP2* siRNA (spot 4).

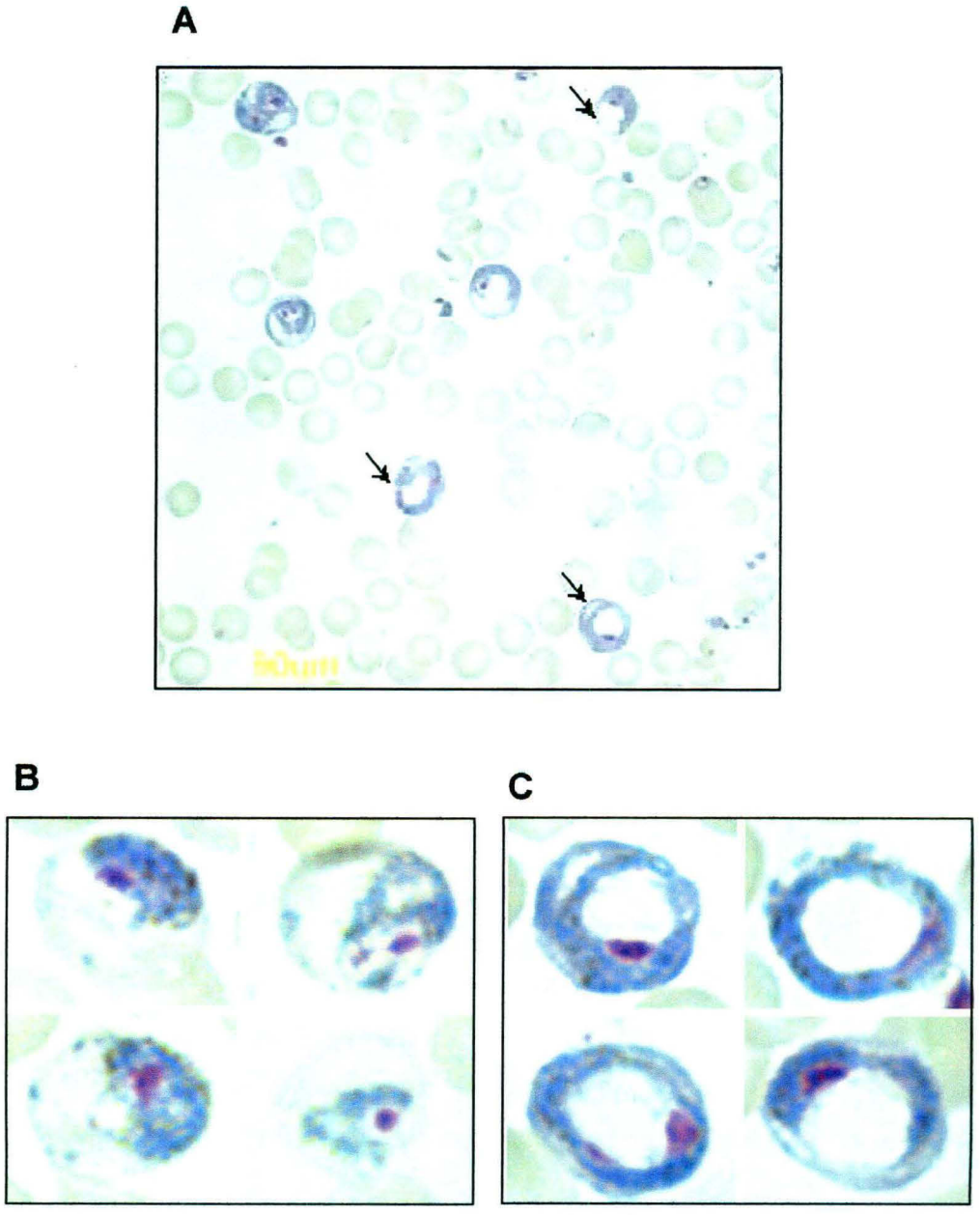



**Fig.24 Morphological abnormalities in *berghepain's* siRNA treated parasites:** Micrographs of Geimsa stained *berghepains'* siRNA treated parasites. Overview of siRNA treated parasites, abnormal parasites are indicated (panel A). Magnified view of control parasites (panel B) and magnified view of parasites showing an abnormally enlarged food vacuole after siRNA treatment (panel C).

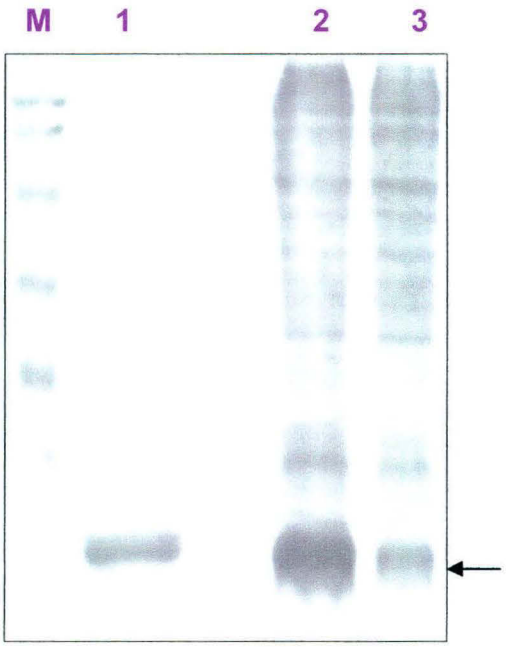



**Fig.25 Coomassie stained gel showing accumulation of hemoglobin in parasites treated with berghepain siRNAs**. Total proteins from treated parasites (Lane 2) and from untreated parasites (Lane 3) was resolved on SDS-PAGE, showing accumulation of hemoglobin in treated parasites. Lane 1 indicates control hemoglobin and M stands for marker lane. Arrow mark indicates hemoglobin accumulation.

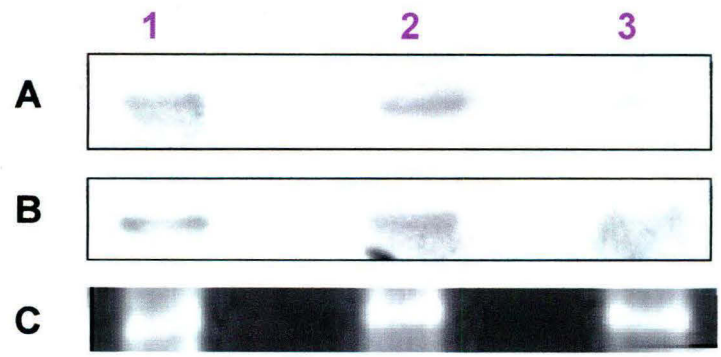



**Fig.26 Northern blot anlaysis showing the degradation of mRNA in *berghepain* siRNA treated *P. berghei* parsites**. Northern blot analysis of total RNA isolated from untreated (Lane 1*) GFP* siRNA treated (Lane 2), and *berghepains*' siRNA treated (Lane 3) parasites. The membranes were probed with *bergheipain1* (panel A) and *bergheipain2* (panel B) DNA probes. Equal loading in all the wells was confirmed by ethidium bromide staining of rRNA (panel C).

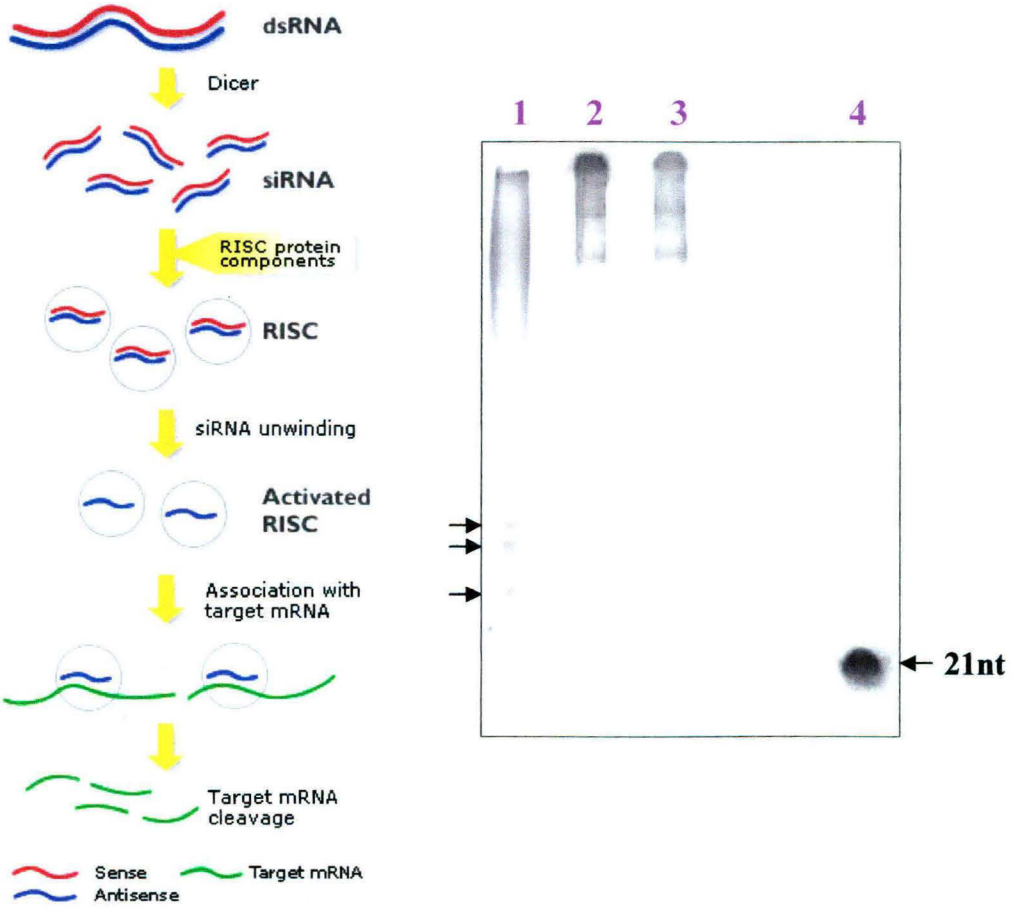



**Fig.27 Schematic representation of mechanism of RNAi (A). Generation of small interfering RNA (approx 25) corresponding to *berghepains* by RNAi (B):** Total LMW RNAs isolated from *berghepains'* siRNA treated (Lane 1), *GFP* siRNA treated (Lane 2), and untreated (Lane 3) parasites were separated on PAGE and probed with *berghepian 2* RNA probe. LMW RNA bands detected are marked. Lane 4 indicates the 21nt *BP2* siRNA as a marker.

Molecular Microbiology (2002) 45(5), 1245–1254

# Double-stranded RNA-mediated gene silencing of cysteine proteases (falcipain-1 and -2) of *Plasmodium falciparum*

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## Summary

**Malaria remains a public health problem of enormous magnitude, affecting over 500 million people every year. Lack of success in the past in the development of new drug/vaccines has mainly been attributed to poor understanding of the functions of different parasite proteins. Recently, RNA interference (RNAi) has emerged as a simple and incisive technique to study gene functions in a variety of organisms. In this study, we report the results of RNAi by double-stranded RNA of cysteine protease genes (*falcipain*-1 and -2) in the malaria parasite, *Plasmodium falciparum*. Using RNAi directed towards falcipain genes, we demonstrate that blocking the expression of these genes results in severe morphological abnormalities in parasites, inhibition of parasite growth *in vitro* and substantial accumulation of haemoglobin in the parasite. The inhibitory effects produced by falcipain double-stranded (ds)RNAs are reminiscent of the effects observed upon administering E-64, a cysteine protease inhibitor. The parasites treated with falcipain's dsRNAs also show marked reduction in the levels of corresponding endogenous *falcipain* mRNAs. We also demonstrate that dsRNAs of falcipains are broken into short interference RNAs ≈ 25 nucleotides in size, a characteristic of RNAi, which in turn activates sequence-specific nuclease activity in the malaria parasites. These results thus provide more evidence for the existence of RNAi in *P. falciparum* and also suggest possibilities for using RNAi as an effective tool to determine the functions of the genes identified from the *P. falciparum* genome sequencing project.**

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## Introduction

In the fight against malaria, there is an urgent need to develop new antimalarials and an effective vaccine because of widespread resistance to common antimalarials (Ridley, 2002). The progress in the development of effective antimalarials/vaccine has been slow because of a poor understanding of the functions of different parasite proteins (Cowman *et al.*, 2000). One of the direct ways to determine the biological function of a protein is to examine the phenotype of the organism that contains mutations in the coding gene or to knock out the gene by homologous recombination. In recent years, methodologies have been developed that permit transfection of *Plasmodium falciparum*, *Plasmodium berghei* and *Plasmodium knowlesi*, as well as the deletion of specific parasite genes in order to study their function (Waters *et al.*, 1997). However, malaria transfection studies, together with previously employed biochemical approaches, have provided limited insight into our understanding of the molecular basis of parasite infection (Cowman *et al.*, 2000). With the completion of the malaria genome project, there is an urgent need for additional methods for assessing gene functions in the malaria parasite.

Recently, RNA interference (RNAi) has emerged as a powerful method for studying gene functions in a wide range of organisms, both unicellular organisms such as *Trypanosoma brucei* and *Plasmodium falciparum* (Ngo *et al.*, 1998; McRobert and McConkey, 2002) and multicellular organisms such as *Caenorhabditis elegans*, *Drosophila*, *Planaria*, *Hydra*, zebrafish and mouse (Cogoni and Maino, 2000). The term RNAi refers to the sequence-specific degradation of mRNA by its homologous double-stranded (ds)RNA (Fire, 1999). Although RNAi was first demonstrated in nematodes (Fire *et al.*, 1998), similar phenomena, called post-transcriptional gene silencing (PTGS) in plants (Baulcombe, 1999) and 'quelling' in fungi (Cogoni *et al.*, 1996), have been known for many years. Recently, RNAi was applied successfully to study the functions of a large number of genes on a

genomic scale in *C. elegans* (Fraser *et al.*, 2000; Gonczy *et al.*, 2000; Maeda *et al.*, 2001).

Given the fact that gene knock-out experiments in *P. falciparum* are difficult to carry out and RNAi has been used to study gene functions in variety of organisms (Zamore *et al.*, 2000), we explored the possibility of using this technique in *P. falciparum*. We used dsRNAs of the two cysteine protease genes of *P. falciparum* to evaluate their metabolic roles in the erythrocytic stages of the parasite. Degradation of haemoglobin is a prerequisite for the establishment and multiplication of the parasite. A series of experiments on haemoglobin degradation pathways in *P. falciparum* led to the identification of three cysteine proteases (also known as falcipain 1, falcipain 2 and falcipain 3), 10 aspartic proteases (called plasmepsins) and metalloproteases involved in haemoglobin degradation (Coombs *et al.*, 2001; Eggleson *et al.*, 1999; Sijwali *et al.*, 2001). However, the exact roles of these *Plasmodium* proteases in the haemoglobin degradation pathway are not clearly understood. To understand the precise roles of falcipains in the haemoglobin degradation pathway, we carried out silencing of two *falcipain* genes by their respective dsRNAs. These experiments revealed that the treatment of parasites by *falcipain*-1 and -2 dsRNAs not only results in marked reduction in the levels of cognate cellular messenger RNA but also brings about several morphological and biochemical changes, which are reminiscent of changes induced by other specific inhibitors of cysteine proteases. Detailed analysis of the mode of RNAi action revealed that these effects are mediated by the formation of an ≈ 25 nucleotide (nt) RNA species, a feature characteristic of all PTGS phenomena. These results have important implications for carrying out large-scale functional genomic studies in *P. falciparum*.

## Results

### *RNAi functions in* P. falciparum

The hallmarks of RNAi are its specificity, simplicity and the ease by which RNAi can be applied to an organism. RNAi has been induced in most organisms by microinjections (Fjose *et al.*, 2001). However, in *C. elegans*, large-scale gene silencing by RNAi was carried out successfully simply by soaking worms in dsRNA containing solution or by feeding worms with *Escherichia coli* that express dsRNA (Maeda *et al.*, 2001). In *Drosophila* S2 cell lines, RNAi was used for dissecting signal transduction pathways just by adding dsRNA to the culture medium (Clemens *et al.*, 2000). Based on these observations, we decided to study RNAi in *P. falciparum* by adding dsRNAs to the culture medium using the protocol described by Clemens *et al.* (2000). We have successfully carried out gene silencing of aminopeptidase-N gene in *Spodoptera frugiperda* 21 (Sf21) cell lines using the same protocol (unpublished results). All the dsRNAs used in the present study were analysed on 1% agarose gel to make sure that these were predominantly double stranded (Fig. 1B). As *P. falciparum* is an intracellular parasite, at the beginning of our analysis of RNAi, we studied the uptake of $^{32}$P-labelled dsRNAs of *falcipains* by malaria parasites. To do this, synchronized *P. falciparum* cultures were incubated with 10 nM $^{32}$P-labelled dsRNA from either of the *falcipains*. After 24 h of incubation, parasitized erythrocytes were collected by centrifugation, lysed with saponin and washed three times to obtain a pure parasite pellet. Levels of radioactivity were measured in different fractions of parasitized red blood cells (RBCs). Approximately 0.1–0.15% of the input labelled dsRNAs were found to be associated with the purified parasite pellets, suggesting that dsRNAs were taken up by the parasite. This result was in line with the earlier observations by Rapaport *et al.* (1992), who showed uptake of labelled oligodeoxynucleotides by *P. falciparum*-infected erythrocytes. Uptake of antisense oligodeoxynucleotides (ODNs) from the culture medium and their inhibitory effects have also been reported in *P. falciparum* (Barker *et al.*, 1996).

To determine the effects of *falcipain* dsRNAs on *P. falciparum*, synchronized parasites at late ring stages were

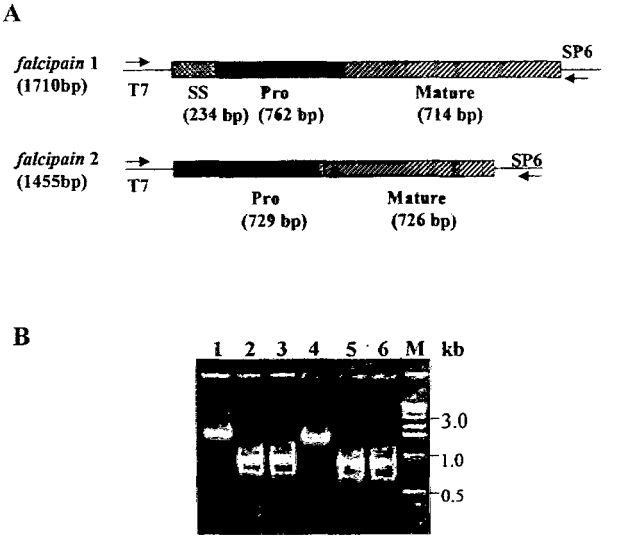


**Fig. 1.** A. Schematic representation of the two *falcipain* genes of *P. falciparum* used to produce single-strand RNA and dsRNA *in vitro* (SS, signal sequence; Pro, pro region; T7/SP6, promoter sequences). B. Agarose gel electrophoresis of ssRNAs and dsRNAs of falcipain-1 and -2. To prepare dsRNA, antisense RNAs (asRNAs) and sense RNAs (ssRNAs) were synthesized using T7 and SP6 polymerases. ssRNA and asRNA from each of the *falcipains* were mixed, incubated at 65°C and cooled slowly to obtain their corresponding dsRNAs. Lane 1, *falcipain*-1 dsRNA; lane 2, *falcipain*-1 ssRNA; lane 3, *falcipain*-1 asRNA; lane 4, *falcipain*-2 dsRNA; lane 5, *falcipain*-2 ssRNA; lane 6, *falcipain*-2 asRNA.

**Table 1.** Effects of Falcipain dsRNAs and E-64 on the morphology and viability of cultured parasites.

| Treatment | Concentration | Food vacuole abnormality[a] | Percentage ring parasites |
|---|---|---|---|
| Falcipain-1 dsRNA | 10 μg ml$^{-1}$ (9.0 nM) | + | 82 |
| | 25 μg ml$^{-1}$ (23.0 nM) | ++ | 60 |
| | 50 μg ml$^{-1}$ (45.5 nM) | ++ | 65 |
| Falcipain-2 dsRNA | 10 μg ml$^{-1}$ (11.0 nM) | + | 90 |
| | 25 μg ml$^{-1}$ (27.0 nM) | ++ | 70 |
| | 50 μg ml$^{-1}$ (50.0 nM) | ++ | 77 |
| Falcipain-1 and -2 dsRNAs | 25 μg ml$^{-1}$ each | +++ | 35 |
| | 50 μg ml$^{-1}$ each | ++++ | 30 |
| Control (DEPC water) | – | – | 100 |
| E-64 | 100 mM | ++++++ | 0 |
| Aminopeptidase-N (*Spodoptera litura*) dsRNA | 50 μg ml$^{-1}$ | – | 99 |

**a.** Percent abnormal parasites: –, 0%; +≤, 10%; ++≤, 30%; +++≤, 40%; ++++≤, 60%; ++++++≤, 75%

incubated with dsRNA for each of the two falcipains separately, as well as in combination, at three different concentrations of 10 μg ml$^{-1}$, 25 μg ml$^{-1}$ and 50 μg ml$^{-1}$, corresponding to approximate final concentrations of 10 nM, 25 nM and 50 nM respectively. Inclusion of dsRNAs in the parasite culture caused distinct morphological changes, the most notable of which was the abnormally swollen food vacuoles that contained an accumulation of malaria pigment (Fig. 2A). These effects were similar to that seen upon incubation of parasites with a well-established cysteine protease inhibitor, E-64 (Rosenthal, 1995). As shown in Table 1, at a 10 μg ml$^{-1}$ concentration of each of these dsRNAs, morphological effects seen on the cultured parasites were marginal. However, at 25 μg ml$^{-1}$ and 50 μg ml$^{-1}$ concentrations of dsRNA of *falcipains*-1 or -2, up to 30% of parasites developed food vacuole abnormalities. Significantly, when dsRNAs of both *falcipains* were included at the above concentrations, about 60% of the parasites showed food vacuole abnormalities. Addition of the two dsRNAs together to the parasite culture also reduced the formation of new rings as well as total parasitaemia. In contrast, addition of dsRNA from aminopeptidase-N (50 μg ml$^{-1}$) of insect origin, used as a control in the present study, had no affect on either parasite morphology or its development.

We confirmed further the inhibitory effects of *falcipain* dsRNAs on *P. falciparum* by an assay that uses [$^3$H]-hypoxanthine uptake as a measure of parasite growth (Fig. 2B). In this assay, the aminopeptidase-N dsRNA was used as a negative control, whereas E-64 was used as a positive control. At a 50 μg ml$^{-1}$ concentration of individual *falcipain* dsRNA, there was a 25–35% reduction in the uptake of [$^3$H]-hypoxanthine. However, when the two *falcipain* dsRNAs were combined, the reduction in the uptake of [$^3$H]-hypoxanthine was enhanced to the level of ≈ 65%. In comparison, E-64 at 50 μM concentration almost completely inhibited the uptake of [$^3$H]-hypoxanthine, whereas aminopeptidase-N dsRNA at this concentration had no effect at all (Fig. 2B). These results were thus consistent with earlier observations, which showed that various inhibitors of cysteine proteases affect parasite metabolism and development.

### *Inhibition of haemoglobin degradation by falcipain dsRNAs in* P. falciparum *parasites*

Cysteine protease activities have mainly been identified in the extracts of trophozoites, an erythrocytic parasite stage during which most haemoglobin degradation occurs and most antimalarial drugs have been shown to act (Rosenthal, 1995). Various studies using either native falcipains or recombinant falcipains have demonstrated a central role for the falcipains in the haemoglobin degradation pathway (Sijwali *et al.*, 2001). It has been suggested that morphological abnormalities displayed by malaria parasites after treatment with different cysteine protease inhibitors result from inhibition of haemoglobin degradation (Rosenthal, 1995). To investigate whether the observed morphological abnormalities in *falcipain* dsRNA-treated parasites were indeed caused by a blockage in haemoglobin degradation, parasite cultures were treated with dsRNA from the two *falcipains*, separately or together, at 50 μg ml$^{-1}$ concentration, and the parasite proteins were subsequently analysed by SDS-PAGE. Incubation of parasites with dsRNAs for each of the *falcipains* caused the accumulation of large quantities of undegraded haemoglobin in these parasites (Fig. 3A, lanes 2–4). E-64, a general cysteine protease inhibitor, was used as a positive control (Fig. 3A, lane 6). In the control parasite culture containing no inhibitor, a minimum level of undegraded haemoglobin was observed (Fig. 3A, lane 1). The block in haemoglobin degradation produced by each *falcipain* dsRNA indicated that these dsRNAs were able to effect the functioning of these genes, i.e. these dsRNAs were able to induce specific gene silencing.

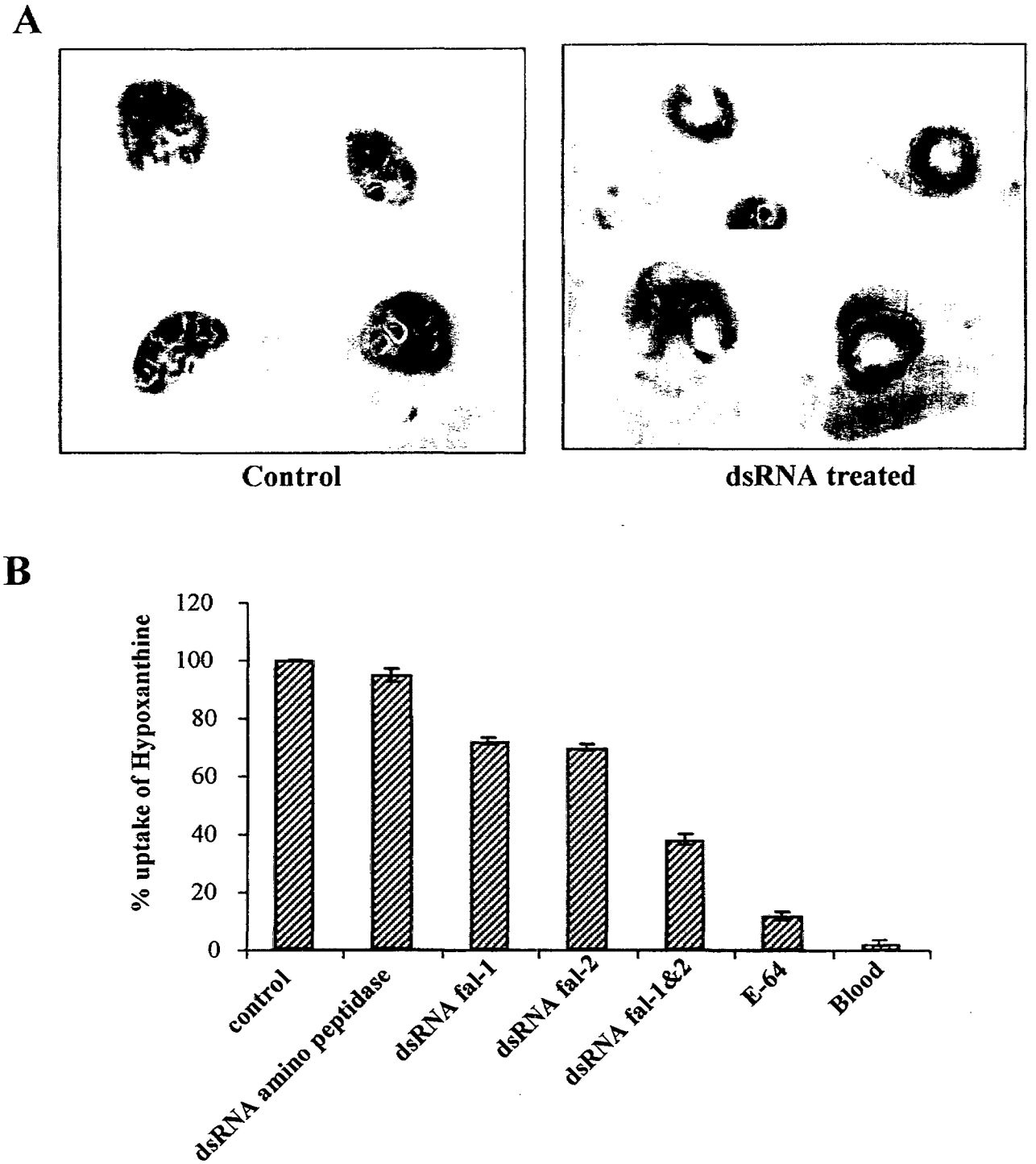


**Fig. 2.** A. Effects of *falcipain* dsRNA treatment on *P. falciparum* parasite morphology; note the abnormally swollen food vacuoles with malaria pigment in the treated parasites.
B. Inhibition of [$^3$H]-hypoxanthine uptake in cultured parasites by Falcipain-dsRNAs. Microwell cultures of ring-stage parasites were incubated with Falcipain dsRNAs for 24 h. Approximately 1 μCi of [$^3$H]-hypoxanthine was added to each well, and cultures were maintained for an additional 24 h. The cells were harvested, and [$^3$H]-hypoxanthine was quantified using a scintillation counter. Aminopeptidase-N (*Spodoptera litura*) dsRNA was used as a control.

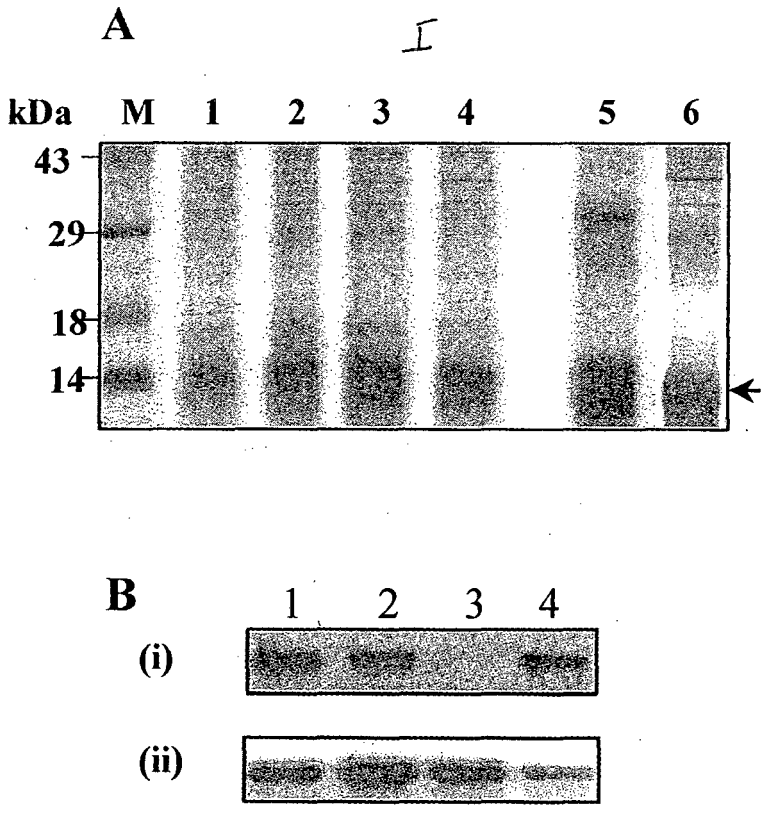


**Fig. 3.** A. Block in haemoglobin hydrolysis in *P. falciparum* parasites after treatment with *falcipain* dsRNAs. Cultured parasites were incubated for 48 h with: lane 1, water (control); lane 2, dsRNAs from *falcipains*-1 and -2; lane 3, dsRNA from *falcipain*-1; lane 4, dsRNA from *falcipain*-2; lane 6, E64. Then, parasitized erythrocytes were lysed with saponin, and parasites were washed extensively to remove erythrocyte cytosolic components. Parasite pellets were solubilized in reducing SDS-PAGE sample buffer, and parasite proteins were resolved on 10% SDS-PAGE. Haemoglobin protein was run as standard (lane 5), undegraded globin appeared as a dimer at ≈16 kDa. Lane M, molecular weight markers.
B. Northern blot analyses showing effects of *falcipain* dsRNA treatment on levels of endogenous RNA transcripts. Total RNA was extracted from control parasites (lane 1), aminopeptidase dsRNA-treated parasites (lane 2), *falcipain*-1 dsRNA-treated parasites (lane 3) and *falcipain*-2 dsRNA-treated parasites (lane 4) and separated on 1.2% agarose gel for Northern blotting. Blots were hybridized with random prime-labelled *falcipain*-1 DNA probe (i) and *falcipain*-2 DNA probe (ii).

*Falcipain dsRNAs induce degradation of homologous messenger RNA*

To determine whether morphological and biochemical effects induced by *falcipain* dsRNAs resulted from RNAi, we examined the fate of *falcipain* mRNAs in dsRNA-treated parasite cultures by Northern blot analysis. Parasites treated with the *falcipain*-1 dsRNA showed diminished endogenous *falcipain*-1 mRNA compared with the untreated, *falcipain*-2 and *aminopeptidase*-N dsRNA-treated parasites [Fig. 3B(i)]. Similarly, treatment of parasites with *falcipain*-2 dsRNA caused marked reduction in *falcipain*-2 mRNA compared with control parasites, whereas the level of *falcipain*-1 mRNA in *falcipain*-2 dsRNA-treated parasites was comparable with that in control parasites [Fig. 3B(ii)]. Overall, in *falcipain*-1 or *falcipain*-2 dsRNA-treated parasites, there was a significant reduction in the levels of cognate mRNAs compared with their levels in untreated parasites. These results indicated that inhibitory effects seen in parasite cultures after treatment with *falcipain* dsRNAs are caused specifically by RNAi.

*Dissection of the mechanism of RNAi in* P. falciparum

A striking paradigm to emerge from the study of RNAi/PTGS in plants and animals is that gene silencing in these organisms occurs as a result of the generation of small RNA called guide RNA or small interfering RNA (siRNA). A number of publications addressing the mechanism of RNAi have suggested that, during RNAi, dsRNA breaks into small guide RNAs, and these small RNAs activate and get associated with RNase complex that degrades the target mRNA (Zamore *et al.*, 2000; Elbashir *et al.*, 2001). To determine whether *falcipain* dsRNAs are also processed into small RNAs *in vivo*, the cultured parasites were treated with $^{32}$P-labelled dsRNA from either of the two *falcipains* used in this study. After 24 h of incubation, parasite pellets were processed to get total RNAs and, subsequently, small RNA species were isolated from the total RNAs. The small RNA species were then analysed by polyacrylamide gel electrophoresis. As shown in Fig. 4A, both *falcipain* dsRNAs were processed to ≈ 25 nt species. We also observed an additional RNA species of ≈ 70 nt in the dsRNA-treated parasites. Such intermediate/longer RNA species have also been observed in other studies, although their role in RNAi has not yet been elucidated (Grishok *et al.*, 2001).

With the development of the *Drosophila in vitro* system, different steps underlying the mechanism of RNAi have been recapitulated *in vitro* (Zamore *et al.*, 2000; Elbashir *et al.*, 2001). In the *in vitro* system, it has been demonstrated that preincubation of the dsRNA in the *Drosophila*

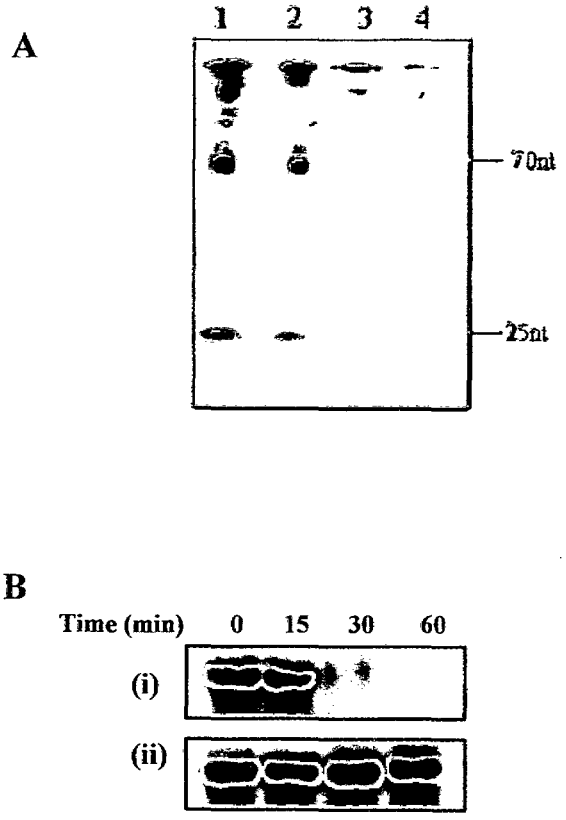


**Fig. 4.** A. Generation of PTGS characteristic ≈ 25 nt RNA upon incubation with *falcipain* dsRNAs in parasite culture. $^{32}$P-labelled dsRNA from each *falcipain* was added to *P. falciparum* cultures and incubated for 40 h. As a control, labelled dsRNAs were added to normal RBCs for the same period. Total RNAs were isolated from parasites, and small RNAs were purified from total RNA. The small RNA species were resolved on 15% denaturing acrylamide gel. Lane1, *falcipain*-1 dsRNA-treated parasites; lane 2, *falcipain*-2 dsRNA-treated parasites; lane 3, control labelled dsRNA from *falcipain*-1; and lane 4, control labelled dsRNA from *falcipain*-2.
B. RNAi *in vitro*. Denaturing agarose gel analysis of radiolabelled *falcipain*-2 mRNA incubated for the times indicated with falcipain-2 dsRNA-treated (i) and untreated parasite lysate (ii).

lysate results in potentiation of nuclease activity for target mRNA degradation (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). To investigate whether similar specific activity was generated in *P. falciparum*, cellular extracts from *falcipain-2* dsRNA-treated and untreated parasites were prepared and incubated with $^{32}$P-labelled *falcipain*-2 mRNA in the presence of ATP. Samples from these reaction mixtures were taken at different time points and analysed by agarose gel. As shown in Fig. 4B, extracts from dsRNA-treated parasites efficiently degraded *falcipain*-2 transcript, whereas extracts from untreated parasites did not degrade mRNA at all. Indeed, extracts from treated samples degraded mRNA very quickly, i.e. within 30 min of incubation, most of the mRNA was degraded, whereas extract from untreated parasites did not show any mRNA degradation activity up to 3 h.

## Discussion

The discovery of RNAi is one of the most exciting advances in molecular genetics in recent years (Fire, 1999; Sharp, 2001). In the present study, we attempted RNAi in *P. falciparum*, an intracellular parasite using dsRNAs from the two parasite-specific cysteine protease genes (*falcipain*-1 and *falcipain*-2). We chose these genes because the effects of different cysteine protease inhibitors on the morphology and biochemistry of malaria parasites, i.e. enlargement of the food vacuole with large amounts of haemozoin in it and block in haemoglobin degradation, have been well documented (Rosenthal, 1995; Rosenthal *et al.*, 1996; Sijwali *et al.*, 2001). Recombinant Falcipain proteins have also been shown to degrade haemoglobin *in vitro* (Sijwali *et al.*, 2001). More recently, the role of cysteine proteases in the release of malaria parasites from host erythrocytes has also been postulated (Salmon *et al.*, 2001). In the present investigation, the addition of each of the two *falcipain* dsRNAs to *P. falciparum* culture resulted in inhibition of parasite growth, development and enlargement of food vacuoles. These effects were more pronounced when two *falcipain* dsRNAs were put together. Moreover, each of the *falcipain* dsRNAs produced a significant block in the degradation of haemoglobin in the parasite. A non-specific dsRNA from an aminopeptidase-N gene of insect origin did not produce any of these effects. The additive effects produced by two *falcipain* dsRNAs suggested that haemoglobin hydrolysis in parasites is probably a co-operative process involving a number of different enzymes together. The inhibitory effects produced by dsRNA treatment of parasites coincided with a marked reduction in the levels of endogenous mRNA homologous to the dsRNA in these parasites, which is one of the hallmarks of RNAi. To confirm further that these effects resulted from falcipain dsRNAs, we simultaneously studied the uptake of labelled *falcipain* dsRNAs by the malaria parasites. Indeed, we observed the accumulation of radioactivity in the saponin-lysed parasite pellets, suggesting uptake of dsRNA by the parasites. Although we observed low levels of uptake of radioactive *falcipain* dsRNAs compared with total input counts, we believe that it is sufficient to produce RNAi. DsRNA molecules do not seem to act stoichiometrically to silence gene expression. In *C. elegans*, it has been shown that two molecules of dsRNA per cell were able to silence an abundantly expressed gene effectively (Fire *et al.*, 1998; Cogoni and Maino, 2000). We also compared the effects produced by *falcipain* dsRNAs on malaria parasites with the effects of E-64, a well-known cysteine

protease inhibitor. Our study showed that various morphological as well as biochemical effects seen on malaria parasites after treatment with *falcipain* dsRNAs were similar to those shown by E-64. These results indicate that RNAi can be an important tool in investigating metabolic events in the parasite life cycle and add *P. falciparum* to the list of organisms in which RNAi has been shown to work successfully. Although substantial inhibitory effects were observed with the highest concentrations of *falcipains* in the present study, we could never achieve 100% efficacy. This could either be attributed to technical limits of the RNAi application or suggest the existence of hidden cysteine proteases in addition to the two taken up in the present study.

The block in haemoglobin degradation observed by silencing the activity of the *falcipain* genes in the present study suggested that both the falcipain proteins are required for initial cleavage of haemoglobin. Several different enzymes present in the food vacuole of *P. falciparum* appear to be involved in haemoglobin degradation, but their precise role and hierarchy in the proteolysis of haemoglobin are not clearly understood. It has been suggested that haemoglobin degradation is an ordered process with aspartic proteases leading the proteolytic process, followed by cysteine and other proteases (Goldberg *et al.*, 1990). However, this model has been a matter of debate, and results contrary to the above hypothesis have been reported (Rosenthal, 1995; Shenai *et al.*, 2000). Taken together, the results of the present study also suggest that cysteine proteases might be involved in the initial cleavage of haemoglobin and that their inhibition causes its accumulation in the parasite. In this regard, our results support the work of Rosenthal (1995), who showed that cysteine proteases are involved right from the beginning in the proteolysis of haemoglobin. It is quite possible that both classes of proteases are playing a dominant role initially. However, regardless of whether cysteine, aspartic or both classes of proteases cleave haemoglobin initially inside the food vacuole, the haemoglobin hydrolysis pathways remain an attractive target for the quest for new antimalarials.

Recently, on the basis of studies related to the RNAi mechanism, a two-step RNA degradation process has been proposed. The first step involves a dsRNA endonuclease [ribonuclease III (RNase III)]-like activity that processes dsRNA into RNAs 21–25 nt long called siRNA. In a second step, siRNAs produced in the first step serve as a guide for a different ribonuclease complex, RISC (RNA-induced silencing complex), which cleaves the homologous single-stranded mRNA (Metzke *et al.*, 2001). We wondered whether the mechanism of RNAi is similar in *P. falciparum*. Analysis of small RNA species in the *P. falciparum* culture treated with labelled *falcipain* dsRNAs showed the generation of ≈ 25 nt labelled RNA species. We also showed that parasite extracts prepared from the *P. falciparum* culture treated with *falcipain-2* dsRNA have the ability to cleave *falcipain-2* mRNA, whereas the untreated parasite did not show this nuclease activity. These results thus supported the two-step mechanism of RNAi and also suggested a conservation of both mechanisms and the components of dsRNA-induced gene silencing in a diverse group of organisms. Recent genetic, molecular and mutational studies have provided further support for this hypothesis. Two sets of genes coding for proteins implicated in RNAi have been identified in different organisms. The first set of proteins is the one with homology to tomato RNA-directed RNA polymerase (RdRP): *Neurospora* QDE-1, *C. elegans* EGO-1 and *Araabidopsis* SGS-2/SDE-1. The second set of proteins involved in RNAi includes *Arabidopsis* AGO-1, *Neurospora* QDE-2 and *C. elegans* RDE-1 (Fagard *et al.*, 2000; Elbashir *et al.*, 2001; Grishok *et al.*, 2001). A BLAST search at the PlasmoDB.org site was carried out using tomato RdRP and *Arabidopsis* AGO-1 sequences. A gene related to AGO-1 was identified on chromosome 10 (19186) of *P. falciparum*, which showed 31% identity to putative translation factor EIF-2B. We also found putative RNase III and RdRP sequences in the *P. falciparum* genome, using the *Plasmodium* genome BLASTX text query tool at the PlasmoDB.org site, which showed some homology (29% and 24% respectively) to that of *Dictyostelium discoideum*. The significance of these findings remains to be investigated.

The use of RNAi in *P. falciparum* to dissect gene functions has several advantages over methods requiring the introduction of DNA into cells. Transfection experiments require knowledge of the full sequence of the gene and its flanking regions, whereas for RNAi, dsRNA corresponding to any gene fragments is sufficient to confer the interference effect (Ullu and Tschudi, 2000). Moreover, use of RNAi in *P. falciparum* as well as in other organisms is technically simple and quick, and the result of protein silencing can be obtained within 2–3 days. This contrasts sharply with the time necessary to produce selective gene 'knock-outs' by transfection technology. RNAi in *P. falciparum* also provides a tool for studying the functions of a number of genes together, as shown in the present study. With the availability of a large amount of sequence information for *P. falciparum*, RNAi-based functional genomics can be a useful technique for investigating the biological functions of novel genes. Finally, given the gene-specific feature of RNAi and the ease of inducing RNAi, this methodology may also play an important role in the development of therapeutic applications against malaria. In conclusion, the present study demonstrates that the RNAi technique can be used conveniently for studying

jene function in *P. falciparum*, and RNAi is mechanisti- :ally conserved in *P. falciparum* and other organisms.

## Experimental procedures

### RNA preparation

ndividual DNA fragments coding for both *falcipain*-1 (1.7kb) ınd *falcipain*-2 (1.4kb) genes (GenBank accession num- )ers AF239801 and M81341 respectively) (Fig. 1A) were ımplified by polymerase chain reaction (PCR) using *P. alciparum* genomic DNA as a template and oligonucleotide )rimer pairs Pf1(1) 5′-ATGGTTGCCATAAAAGAAATG-3′ ınd Pf1(2) 5′-TTACAAGATAGGATAGAAGAC-3′; and Pf2(1) 5′-ATGGATTACAACATGGATTAT-3′ and Pf2(2) 5′- TATTCAATTAATGGAATGAA-3′. The PCR products were jel purified and cloned into pGEM-T vector (Promega) to jenerate pGFal-1 and pGFal-2 clones. The clones were sequenced by dideoxy sequencing reactions and used as a emplate to amplify individual *falcipain* genes using universal ınd reverse primers (Promega). The PCR products were )urified using a PCR purification kit (Qiagen). The purified PCR products were used as a template to generate sense RNA (sRNA) and antisense RNA (asRNA) using T7 and SP6 RNA polymerases (Ambion). To make dsRNAs, equal ımounts of ssRNAs and asRNAs were mixed, heated to 65°C and annealed by slow cooling over several hours. ndividual RNAs and their dsRNAs were analysed on 1% ıgarose gel. A 0.9 kb dsRNA from the unrelated aminopep- idase-N gene of insect *Spodoptera litura* was prepared in ı similar way to use as a control. To make $^{32}$P-labelled dsRNAs, either sense or antisense strands of *falcipain* genes were internally labelled using 0.3 μM [α-$^{32}$P]-UTP (3000 Ci mmol$^{-1}$) and mixed with unlabelled complementary strands. [$^{32}$P]-dsRNAs were treated with RNase $T_1$ to elimi- ınate ssRNA contaminants.

### Parasite culture and RNAi

*Plasmodium falciparum* (strain 3D7) were cultured with ıuman erythrocytes with 5% haematocrit in RPMI media Gibco) supplemented with 10% human serum using a pro- ocol described previously (Trager and Jensen., 1976). Par- ısite cultures were synchronized using sorbitol (Lambros and /anderber, 1979). For analysis of the effects of dsRNAs or -transepoxy-succinyl-leucylamido-(4-guanidino)-butane (E- 64), the synchronized cultures were adjusted to 5% haema- ocrit with 1% infected red blood cells (RBCs), and 1 ml of hese cultures was treated in 24-well culture plates in tripli- ate in serum-free medium for 30 min with intermittent nixing. Subsequently, human serum was added to these ultures to a final concentration of 10%, and parasites were naintained further for 24 h or 48 h. For the microscopic anal- sis, smears were made from each well, stained with Giemsa ınd examined for abnormalities as well as for the number f ring-stage parasites. The number of ring-stage parasites er 1500 RBCs was determined for each well. Parasites vith enlarged food vacuoles and haemozoin accumulation vere scored as abnormal. To assess the effects of various dsRNAs/inhibitors on parasite metabolic activity, a [$^{3}$H]-hypoxanthine uptake assay was performed as described previously (Rosenthal *et al.*, 1996). Briefly, 100 μl of parasite cultures (0.8–1.0% parasitaemia) at late ring stage were incubated with dsRNAs in triplicate for 24 h, [$^{3}$H]-hypoxanthine (Dupont-NEN) was added (1.2 μCi per well), and cultures were maintained for an additional 24 h. Cultures of mature parasites were frozen and thawed to lyse the infected RBCs. Lysed cultures were harvested on glass-fibre filters, which were subsequently washed with water, dried with ethanol and counted on a scintillation counter. Uptake of [$^{3}$H]-hypoxanthine by dsRNA-treated parasites was compared with that of control cultures treated with diethyl pyrocarbonate (DEPC)-treated water.

### Analysis of endogenous falcipain RNA transcripts

Northern blot analysis was carried out to determine the levels of endogenous *falcipain* transcripts after *falcipain* dsRNA treatment for 48 h. Total RNAs were prepared from treated and untreated malaria parasites using the Qiagen RNA isolation kit. Total RNA (1 μg) was fractionated on 1.2% agarose gel, transferred to a nylon membrane (Hybond N$^{+}$) and hybridized with random prime-labelled *falcipain*-1 DNA probe according to a previously described protocol (Kyes *et al.*, 2000). The blot was exposed to X-ray film (Kodak). After exposure, the membrane was deprobed, hybridized with random prime-labelled *falcipain*-2 DNA probe and re-exposed to X-ray film.

### Analysis of globin hydrolysis in P. falciparum parasites

To determine the effects of *falcipain* dsRNAs or E-64 on haemoglobin degradation in the malaria parasite, *P. falciparum* cultures were treated with these inhibitors for 24 h. After the treatment, parasite-infected erythrocytes were collected by centrifugation and lysed with 0.1% saponin in PBS at 37°C for 15 min. The lysed samples were centrifuged, and the parasite pellets were washed three times with ice-cold PBS to remove erythrocyte cytoplasmic contents. The washed parasite pellets were solubilized in SDS sample buffer containing β-mercaptoethanol, boiled and separated on 15% SDS-PAGE. The gels were stained with Coomassie blue.

### Analysis of falcipain dsRNA processing in P. falciparum

To determine whether *falcipain* dsRNAs inside *P. falciparum* parasites are processed to small RNAs, as shown for RNAi in the case of other organisms (Zamore *et al.*, 2000), parasite cultures were treated with $^{32}$P-labelled dsRNAs of *falcipain*-1 and -2 separately as described earlier. Treated parasitized erythrocytes were pelleted by centrifugation. Total RNAs were extracted as described earlier. Low-molecular-weight RNAs were extracted from total RNA by precipitation with 5% polyethylene glycol–0.5 M NaCl (Hamilton and Baulcombe, 1999). Low-molecular-weight RNAs were subsequently analysed by electrophoresis through 15% polyacrylamide–7 M urea–0.5% Tris-borate gel. The gel was dried under vacuum and exposed to X-ray film (Kodak).

### Plasmodium falciparum *extract preparations and degradation of mRNA* in vitro

DsRNA-mediated gene silencing occurs as a result of activation of RNA-dependent nucleases inside the cells by dsRNA. To show that similar activation is also responsible for RNAi in *P. falciparum*, extracts were prepared from *falcipain-2* dsRNA-treated as well as DEPC water-treated parasites. For the preparation of extracts, intact parasites were collected by saponin lysis and washed three times in ice-cold PBS to remove erythrocyte cytoplasmic components. The washed parasite pellets were lysed by sonication in 200 µl of lysis buffer [100 mM phosphate acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate and 5 mM dithiothreitol (DTT), pH 7.4]. The lysate were centrifuged for 30 min at 15 000 *g* at 4°C, and the supernatants were flash frozen in liquid nitrogen and stored at –80°C. The supernatants were used for an *in vitro* assay. Typically, the reaction mixtures (50 µl) contained 25 µl of parasite extract and 50 000 c.p.m. of synthetic mRNA as a substrate in a buffer containing 20 mM HEPES, pH 7.3. 110 mM KOAc, 1 mM Mg $(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1 mM DTT and 1 mM ATP. The reaction mixtures were incubated for different time periods at 37°C. Reactions were quenched by the addition of 10 volumes of 2% PK buffer [200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 300 mM NaCl and 2% (w/v) SDS]. Proteinase K was added to a final concentration of 465 µg $ml^{-1}$. The reactions were extracted with phenol–chloroform–isoamyl alcohol and precipitated with an equal volume of isopropanol. Reactions were analysed by electrophoresis in a formaldehyde–agarose 0.8% gel. mRNA degradation was detected by exposing the agarose gel dried under vacuum to X-ray film (Kodak).

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# In vivo gene silencing in *Plasmodium berghei*—a mouse malaria model

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**Abstract**

RNA interference (RNAi) has emerged as a specific and efficient tool to silence gene expression in a variety of organisms and cell lines. An important prospect for RNAi technology is its possible application in the treatment of diseases using short interfering RNAs (siRNAs). However, the effect of siRNAs in adult animals and their potential to treat or prevent diseases are yet to be fully investigated. The main goal of the present study is to find out whether it was possible to carry out RNAi on circulating malaria parasite in vivo. To trigger RNAi in mouse malaria parasite, we used siRNAs corresponding to cysteine protease genes of *Plasmodium berghei* (*berghepain*-1 & 2). Intravenous injections of *berghepains*' siRNAs in infected animal resulted in characteristic enlargement of food vacuole in circulating parasites. Protein analysis of these treated parasites showed substantial accumulation of hemoglobin, which is reminiscent of the effect observed upon treating *Plasmodium falciparum* with different cysteine protease inhibitors. Parasites treated with *berghepain* 1 & 2 siRNAs showed marked reduction in the levels of their cognate mRNAs, thereby suggesting specific inhibition of *berghepains*' gene expression in vivo. We also observed the generation of ~25 nt RNA species from *berghepains*' mRNAs in the treated parasites, which is a characteristic of an RNAi phenomenon. These results thus provide evidence that beyond its value for validation of gene functions, RNAi may provide a new approach for disease therapy.


*Keywords:* *Plasmodium berghei*; RNAi; siRNA; Cysteine protease; *Berghepain*

Despite the great advances in combating the infectious diseases over the last three decades, malaria remains a major and growing threat to the public health and economic development of the countries in the tropical and subtropical regions of the world. Millions of deaths, predominantly among children, occur in Africa every year due to malaria [1]. With the spread of resistance to current anti-malarials, there is a need to identify new drug and vaccine targets and develop new pharmacophore/vaccines [2]. Completion of *Plasmodium* genome sequences has provided new avenues/opportunities to understand parasite biology in detail and explore new control measures [3]. In order to utilise this wealth of information, new tools are being explored to carry out large-scale genome analysis. Recently, RNA interference (RNAi) has emerged as a powerful reverse genetic method to carry out functional genomic studies [4]. RNA interference is the process of sequence-specific post-transcriptional gene silencing triggered by double-stranded RNA (dsRNA) homologous to the silenced gene [5]. The existence of RNAi has been demonstrated in various organisms including flies, worms, protozoa, vertebrates, and higher plants [6]. In *Caenorhabiditis elegans* and *Drosophila*, RNAi has been successfully applied to understand the functions of gene/genes on a genome-wide scale [7,8].

Studies on the mechanism of RNAi have demonstrated that short interfering RNAs (siRNAs) of 21–25 nt length are an intermediary in RNAi reaction, and synthetic siRNAs can be effectively used to carry out sequence-specific mRNA degradation [9,10]. Moreover, in mammalian cell lines it has been shown that only siRNAs and not the long dsRNAs can be used for a RNAi reaction [11]. Since then siRNAs have become powerful reagents for genome-wide analysis of gene functions in cultured cell. Because of their exquisite

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specificity, siRNAs are now being considered emerging agents for gene therapy. In fact, siRNAs have been successfully used in cultured mammalian cell lines, for inhibiting the replication of viruses and for the treatment of virus-induced cancer [12]. A strong evidence for the therapeutic potential of RNAi has come from a recent paper describing the prevention of Fas-mediated hepatitis in mice following the intravenous delivery of naked siRNAs by high-pressure injection [13]. However, further development is needed to carry out the clinically acceptable efficient delivery of siRNAs in vivo.

In the present study, we investigated the silencing effects of siRNAs corresponding to *berghepain* genes on *Plasmodium berghei* in adult mice. *Berghepains* are papain-family cysteine-proteases of *P. berghei*, which are the homologues of *falcipains*, the key cysteine proteases of *Plasmodium falciparum*. Studies in *P. falciparum* and *Plasmodium vinckei* using cysteine protease inhibitors have suggested that cysteine proteases are appropriate new chemotherapeutic targets [14,15]. This was further confirmed by RNAi studies using long dsRNAs corresponding to *falcipains* 1 & 2 with in vitro *P. falciparum* culture [16]. Intravenous injections of *berghepains'* siRNA in mice resulted in considerable reduction in corresponding mRNA transcripts in the parasite, which resulted in accumulation of hemoglobin. Analysis of small molecular weight RNA species in siRNA treated parasites showed degradation of mRNAs and generation of ~25 nt RNA species. Results of the present study thus suggest that siRNAs can be used to trigger RNAi in circulating malaria parasites and thus can be used as gene-specific anti-parasitic agents.

## Materials and methods

*siRNA design and synthesis.* Two siRNAs—BP1 and BP2, each of 21 nucleotides, were designed for *berghepain* 1 and *berghepain* 2 genes, respectively, following the programme developed by Elbashir and Tuschl [17]. In addition, a siRNA corresponding to green fluorescent protein gene (GFP) was also designed to use as a control. All siRNAs were obtained from Dharmacon Research (Colorado) in annealed and lyophilised form. These siRNAs were suspended in RNase–DNase free water at a concentration of 5 μg/μl. For the uptake assay, siRNAs were end-labelled using T4-kinase (NEB) following manufacturer's recommendations.

*Parasite culture and injection of siRNAs.* Female BALB/c mice, of 4–6 weeks age and weighing 20–25 g, were used. Mice were infected by intravenous injection of $10^5$ parasitised erythrocytes. After 8–10 h, desired amounts of siRNAs were suspended in 200 μl PBS and injected intravenously into the infected mice in three replicates. The injections were repeated after 24 and 48 h. Control mice were injected with GFP-siRNA or PBS. Blood was collected from mice 24 h after the last injection. Blood smears stained with Giemsa stain were analysed for parasite morphology and total parasitemia was counted.

*Isolation of parasites and analysis of globin hydrolysis.* Blood collected from siRNAs treated and control mice was passed through CF11 (Whatmann) column to remove white blood cells. Erythrocytes were lysed by 0.1% saponin at 37 °C for 15 min. The parasite pellets were collected after centrifugation and washed three times with PBS to remove erythrocyte cytoplasmic content. The parasite pellets were suspended in SDS sample buffer with β-mercaptoethanol, boiled, and separated on 15% SDS–PAGE. The gel was stained with Coomassie blue R250.

*Isolation of total RNA and Northern blot analysis of endogenous mRNA.* Total RNAs were isolated from parasites collected from the treated and untreated mice using Qiagen RNA isolation kit. Total samples (2 μg of each) were separated on 1.2% denaturing agarose gel, transferred to nylon membrane (Hybond $N^+$ Amersham), and hybridised with random labelled *berghepain* 1 DNA probe following the procedure of Kyes et al. [18]. The blot was washed and exposed to X-ray film. The membrane was deprobed, hybridised with *berghepain* 2 DNA probe, and re-exposed to X-ray film (Kodak).

*Low molecular weight (LMW) RNA extraction and detection.* For the siRNA uptake assay and to detect the formation of low molecular weight (LMW) RNAs, mice were pre-infected with $10^5$ parasitised erythrocytes. After the parasitemia reached 2%, $\gamma^{33}$P-labelled ($10^5$ cpm) or unlabelled (25 μg) siRNAs were injected intravenously into these mice. After 12–14 h, blood was collected and processed for the extraction of total RNAs. Low molecular weight RNAs were extracted from total RNA by precipitation with 5% polyethylene glycol–0.5 M NaCl following the protocol described by Hamilton and Baulcombe [19]. For the uptake assay, the labelled LMW RNAs were detected directly by blotting on nylon membrane (Hybond $N^+$). For the analysis of generation of 25 nt species, LMW RNA species were separated on a 15% PAGE with 7 M Urea in 0.5× Tris–borate buffer and transferred onto nylon membrane (Hybond $N^+$) by electroblotting. The blot was hybridised with *berghepain* 2 DNA probe and exposed to X-ray film.

## Results and discussion

Over the years, small interfering RNAs have emerged as an important tool to ascertain efficiently the function of novel genes and to validate targets for drug discovery [12,20]. In malaria, murine models are routinely used for in vivo screening of potential anti-malarials [21]. In order to evaluate the role of *falcipains'* orthologues from rodent malaria parasite *P. berghei* and to study the in vivo efficacy of siRNAs to trigger RNAi in circulating malaria parasites, we sought to specifically inhibit the expression of *berghepain* 1 & 2 genes by using their respective siRNAs. Three siRNAs corresponding to *berghepain* 1 & 2 and GFP genes were used in the present study and their sequences are shown in Fig. 1A. The reported routes for delivery of siRNAs in different organisms are soaking, feeding, microinjection, viral vectors, and intravenous injections [12]. We followed the intravenous route for the delivery of siRNAs in mice. We first explored the uptake of $^{33}$P-labelled *berghepains'* siRNA by the circulating parasites. Eighteen hours after the injection of siRNAs, parasitised RBCs were collected by centrifugation, lysed with saponin, and LMW RNAs were isolated. These RNAs were blotted on nylon membrane and the uptake of labelled RNA by the parasite was assessed by autoradiography. As shown in Fig. 1B, we could detect ~0.01% of the input labelled siRNAs in these parasites. Even though this uptake of $^{33}$P-labelled siRNAs was very low, it did, however, confirm the entry of siRNAs into the parasite in vivo

A

***bergheipain 1* (987bp)**

Target sequence 5' AAT AAT GGG GTA TGC TTT GGC 3'

Sense siRNA UAA UGG GGT ATG CUU UGG CUU
Anti sense siRNA UU AUU ACC CCA UAC GAA ACC G

***bergheipain 2* (1448bp)**

Target sequence 5' AAG ATC TTA TAG ACA TGG ATG 3'

Sense siRNA GAU CUU AUA GAC AUG GAU GUU
Anti sense siRNA UU CUA GAA UAU CUG UAC CUA C

***GFP* (727bp )**

Target sequence 5' AAG GTT ATG TAC AGG AAA 3'

Sense siRNA GGU UAU GUA CAG GAA AGA A AA
Anti sense siRNA UU CCA AUA CAU GUC CUU UCU U

B

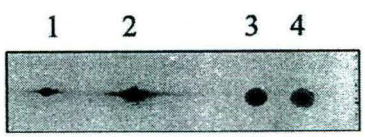


Fig. 1. Uptake of siRNAs by the parasites. (A) Target sequences identified from *berghepains*' and GFP genes and their corresponding siRNAs. (B) Autoradiograph of low molecular weight (LMW) RNAs from parasites to show the uptake of labelled siRNAs by the parasites: labelled BP1-siRNAs (spot 1), labelled BP2-siRNA (spot 2), LMW-RNAs from parasite treated with labelled BP1 siRNA (spot 3), and with labelled BP2 siRNAs (spot 4).

condition. A recent report has also shown an efficient delivery of siRNAs into mouse liver, spleen, kidney, lung, and pancreas by high-pressure tail vein injection [22].

We have earlier demonstrated in *P. falciparum* that double-stranded RNA-mediated gene silencing of *falcipain* 1 & 2 genes brings about distinct morphological as well as biochemical effects such as abnormally swollen food vacuoles with the accumulation of malarial pigment [16]. To determine whether *berghepains*' siRNAs trigger similar effects in vivo, we carried out a series of experiments using two different concentrations of siRNAs, 5 and 25 μg. In all these experiments, siRNAs were dissolved in 200 μl PBS and injected intravenously. SiRNAs were introduced into mice either at the onset of 1% parasitemia or just 8 h after the delivery of parasites into the mice. Mice were treated with siRNAs once a day for three days. Control mice were treated with GFP-siRNAs in an identical manner. Forty-eight hours after the last injection, Giemsa-stained blood smears were examined for morphological abnormalities in siRNA treated parasites. Of these different groups of mice, mice injected with 25 μg of berghepain's siRNAs 8 h after the parasite infection showed abnormal parasites. As shown in Figs. 2A and C, these treated parasites showed enlargement of food vacuole. These effects were similar to those observed in *P. falciparum* after treatment with E-64, a cysteine protease inhibitor or *falcipain* 1 & 2 long dsRNAs [16,23]. Specificity of these effects for *berghepains*' siRNA was confirmed by the absence of these morphological effects in parasites treated with GFP-siRNA (Fig. 2B). To determine whether the morphological effects seen in parasites treated with *berghepains*' siRNAs were a consequence of RNAi, we examined the fate of *berghepains*' mRNAs in these treated parasites by Northern blot analysis. As shown in Fig. 3A, we observed a reduction in both *berghepain* 1 & 2 mRNAs. *Berghepain* 1 mRNA was <40% of that in control mice, while *berghepain* 2 mRNA was <50% of that present in control mice. These results suggested that inhibitory effects seen in parasite isolated from mice after treatment with *berghepains*' siRNAs were due to RNAi. It has been previously reported that the food vacuole abnormalities seen in malaria parasites after treatment with cysteine protease inhibitors are mainly due to block in hemoglobin degradation [15,16,23]. To assess the effects of berghepain's siRNAs on the accumulation of hemoglobin, treated parasitised erythrocytes were collected

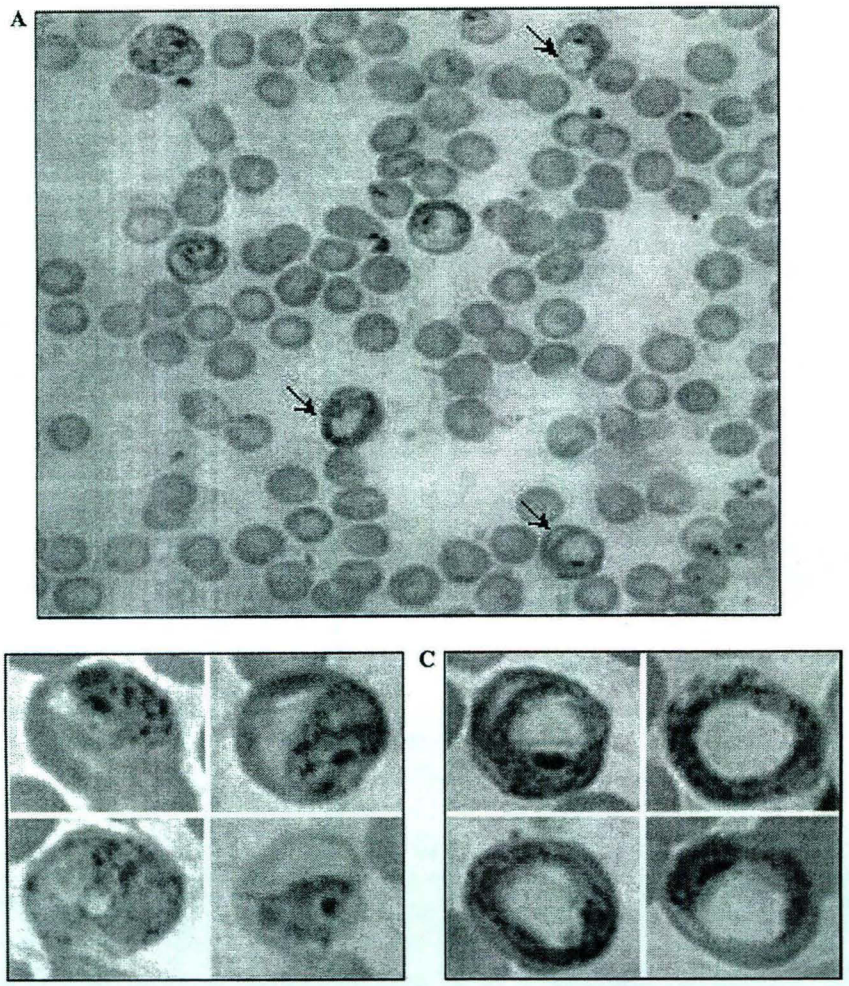


Fig. 2. Morphological abnormalities in siRNA treated parasites: micrographs of Giemsa-stained *berghepains*' siRNA treated parasites. (A) Overview of siRNA treated parasites, abnormal parasites are indicated. (B) Magnified view of control parasites and (C) magnified view of parasites showing an abnormally enlarged food vacuole after siRNA treatment.

from mice blood and lysed with saponin and the parasite proteins were analysed on 15% SDS–PAGE. As shown in Fig. 3B, a ~2- to 3-fold increase in the hemoglobin level was observed in *berghepains*' siRNA treated parasites in comparison to the control parasites. Our results using RNAi technology thus add to the prior evidences using different inhibitors that cysteine proteases are an important component of the hemoglobin degradation pathway in malaria parasites.

An important feature of RNAi-mediated gene silencing is the generation of 21–25 nt RNA species followed by mRNA degradation [9–11]. We also investigated the formation of the LMW RNA species in *berghepains*' siRNA treated parasites on PAGE followed by Northern blot analysis. As shown in Fig. 4, we observed the generation of ~25 nt species in *berghepains*' siRNA treated parasites, while untreated and GFP-siRNA treated parasites did not show these RNA species. We have previously reported the generation of ~25 nt long siRNAs in *P. falciparum* after the treatment of parasites with long dsRNAs corresponding to *falcipain* 1 & 2 [16]. These siRNAs are a few nucleotides longer than siRNAs produced in *Drosophila* and mammalian extracts, which are 20–22 nt long [9–11], but interestingly very similar in size to that of small RNA produced in *Trypanosoma* [24] and in plants [19]. Generation of ~25 nt RNA species thus clearly established the occurrence of RNAi in malaria parasites in vivo condition. Along with ~25 nt RNA species, we also observed additional RNA species of >25 nt. These longer species may represent the intermediate RNAs generated during mRNA degradation. Such intermediates have also been reported in previous studies [16,25].

We have previously shown that RNAi phenomenon exists in *P. falciparum* in vitro [16] and can be an important tool to investigate molecular and biochemical events in parasite life cycle. By in vivo experiments on a mouse model, we now provide evidence that RNAi

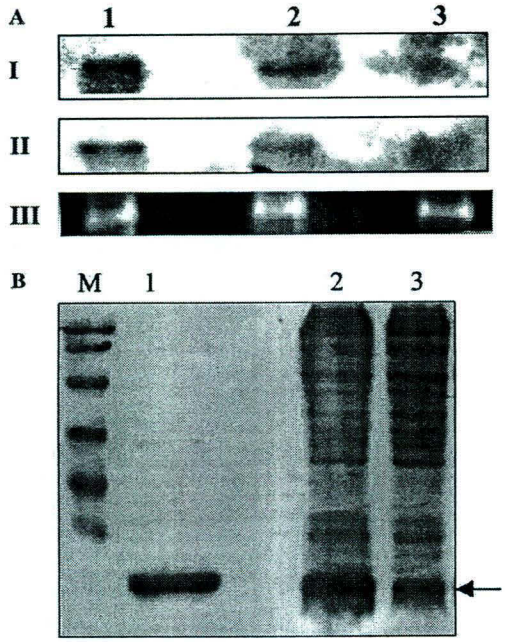


Fig. 3. Silencing of *berghepains* in siRNA treated parasites. (A) Northern blot analyses of total RNAs isolated from untreated (lane 1), GFP-siRNA treated (lane 2), and *berghepains*' siRNA treated (lane 3) parasites. The membranes were probed with *berghepain* 1 (panel I) and *berghepain* 2 (panel II) DNA probes. Equal loading in all the wells was confirmed by ethidium bromide staining of rRNAs (panel III). (B) Total proteins from treated (lane 2) and untreated parasites (lane 3) resolved on SDS–PAGE, showing accumulation of hemoglobin in treated parasites. Lane 1 indicates control hemoglobin.

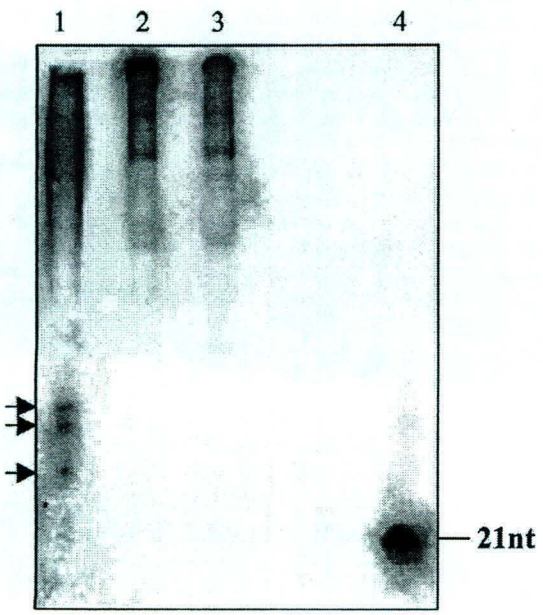


Fig. 4. Generation of small interfering RNAs (~25 nt) corresponding to *berghepains* by RNAi: total LMW-RNAs isolated from *berghepains*' siRNA treated (lane 1), GFP-siRNA treated (lane 2), and untreated (lane 3) parasites were separated on PAGE and probed with *berghepain* 2 DNA probe. Lane 4 indicated the 21 nt BP2 siRNA as marker.

holds the potential for an attractive therapeutic strategy for the control of parasitic diseases. Nonetheless, many caveats still exist for the therapeutic use of siRNAs in the control of parasitic diseases. One of our major concerns is the efficacy of RNAi under in vivo conditions. We could observe about 40–50% reduction in *berghepains*' mRNA levels in the treated parasites. This could be due to the poor delivery of siRNAs to all the circulating parasites or due to the choice of siRNA sequences. It has been shown that all the siRNAs along the length of a gene are not equally effective for RNAi [26]. Even though there was significant reduction in the mRNA levels, we did not observe any significant reduction in parasitemia in siRNA treated parasites in comparison to the control. This could be due to redundancy existing in the hemoglobin degradation pathway in malaria parasites. Three different classes of enzymes: aspartic proteases (10 plasmepsins), cysteine proteases (four falcipains), and metallo-proteases (one falcilysin) have been shown to be involved in hemoglobin degradation [2,3,27]. In vivo studies using *P. vinckei* murine malaria model have shown that inhibitors of any of these three classes of enzymes alone were not effective in curing malaria. However, the combined protease inhibitors cured the majority of the infected animals, suggesting that a combination of different inhibitors works synergistically in the inhibition of *Plasmodium* hemoglobin degradation in vivo and in the treatment of murine malaria [28]. Based on this fact, it can be argued that a mixture of siRNAs corresponding to all three classes of enzymes involved in hemoglobin degradation may be required to show an efficient anti-parasitic effect in this model system. Nonetheless, the present study provided evidence for the use of siRNAs to trigger RNAi in circulating malaria parasites.

Since siRNAs are stable, highly sequence specific, and are of low molecular weight, they represent new avenues for parasitic/gene therapies. There are a large number of studies that show gene-specific therapeutic potential of siRNAs in different cell lines [12]. However, in vivo studies to demonstrate siRNA potential in adult animal for the treatment/prevention of diseases are limiting. Results of the present study along with two other elegant studies [12,22] clearly demonstrate that siRNAs may be an attractive alternative to other commonly used therapeutic strategies. However, there is a long road ahead to enable us to use siRNA for therapeutic purposes, clinically acceptable delivery procedures, and strategies to select siRNAs need to be developed for this purpose.

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# RNA Interference: Biology, Mechanism, and Applications

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## INTRODUCTION

RNA silencing is a novel gene regulatory mechanism that limits the transcript level by either suppressing transcription (transcriptional gene silencing [TGS]) or by activating a sequence-specific RNA degradation process (posttranscriptional gene silencing [PTGS]/RNA interference [RNAi]). Although there is a mechanistic connection between TGS and PTGS, TGS is an emerging field while PTGS is undergoing an explosion in its information content. Here, we have limited our discussion to PTGS/RNAi-related phenomena.

Pioneering observations on PTGS/RNAi were reported in plants, but later on RNAi-related events were described in almost all eukaryotic organisms, including protozoa, flies, nematodes, insects, parasites, and mouse and human cell lines, as shown in Table 1. Three phenotypically different but mechanistically similar forms of RNAi, cosuppression or PTGS in

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TABLE 1. Eukaryotic organisms exhibiting RNAi-related phenomena

| Kingdom | Species | Stage tested | Delivery method | Reference(s) |
|---|---|---|---|---|
| Protozoans | *Trypanosoma brucei* | Procyclic forms | Transfection | 52 |
| | *Plasmodium falciparum* | Blood stage | Electroporation and soaking | 143, 150 |
| | *Toxoplasma gondii* | Mature forms in fibroblast | Transfection | 4 |
| | *Paramecium* | Mature form | Transfection and feeding | 14 |
| | *Leishmania donovanii* | | Tried but not working | 183 |
| Invertebrates | *Caenorhabditis elegans* | Larval stage and adult stage | Transfection, feeding bacteria carrying dsRNA, soaking | 26, 31 |
| | *Caenorhabditis briggsae* | Adult | Injection | 79 |
| | *Brugia malayi* (filarial worm) | Adult worm | Soaking | 1 |
| | *Schistosoma mansoni* | Sporocysts | Soaking | 23 |
| | *Hydra* | Adult | Delivered by micropipette | 49 |
| | *Planaria* | Adult | Soaking | 49 |
| | *Lymnea stagnalis* (snail) | Adult | Injection | 122 |
| | *Drosophila melanogaster* | Cell lines, adult, embryo | Injection for adult and embryonic stages, soaking and transfection for cell lines | 96, 114, 155 |
| | *Cyclorrphan* (fly) | Early embryonic stages | Injection | 200 |
| | Milkweed bug | Early embryonic stages | Injection | 102 |
| | Beetle | Early embryonic stages | Injection | 27 |
| | Cockroach | Larval stage | Injection | 146 |
| | *Spodoptera frugiperda* | Adult and cell line | Injection and soaking | 176, 215 |
| Vertebrates | Zebra fish | Embryo | Microinjection | 224 |
| | *Xenopus laevis* | Embryo | Injection | 162 |
| | Mice | Prenatal, embryonic stages, and adult | Injection | 31, 229 |
| | Humans | Human cell lines | Transfection | 42 |
| Plants | Monocots/dicots | Plant | Particle bombardment with siRNA/transgenics | 88 |
| Fungi | *Neurospora crassa* | Filamentous fungi | Transfection | 51 |
| | *Schizosaccharomyces pombe* | Filamentous fungi | Transgene | 178 |
| | *Dictyostelium discoideum* | | Transgene | 147 |
| Algae | *Chlamydomonas reinhardtii* | | Transfection | 231 |

plants, quelling in fungi, and RNAi in the animal kingdom, have been described. More recently, micro-RNA formation, heterochromatinization, etc., have been revealed as other facets of naturally occurring RNAi processes of eukaryotic cells.

During the occurrence of RNAi/PTGS, double-stranded RNA (dsRNA) molecules, which cleave the inducer molecules into smaller pieces first (16) and eventually destroy the cellular or viral cognate mRNA molecules (called the target) (17) act as inducers or activators of this process. As a result, the target mRNAs cannot accumulate in the cytosol, although they remain detectable by nuclear run-on assays (73). In certain instances, the DNA expressing the target mRNA also undergoes methylation as a by-product of the degradation process (226).

The natural functions of RNAi and its related processes seem to be protection of the genome against invasion by mobile genetic elements such as viruses and transposons as well as orchestrated functioning of the developmental programs of eukaryotic organisms. There are several excellent recent reviews which deal with different aspects of RNAi separately (95, 191). Here, we have put together the various aspects of the RNAi process known to date, identified the mechanistic similarities and differences operating in various forms of eukaryotic life, and focused on the experimental results that have led to conceptual advancements in this field.

## UNRAVELING RNA SILENCING

In order to understand the process of homology-dependent RNA silencing, it would be prudent to overview the process itself and describe its important features. In the later part of this review, the genetics, biochemistry, and potential therapeutic applications of the process will be dealt with.

### PTGS in Plants

In plants, the RNA silencing story unfolded serendipitously during a search for transgenic petunia flowers that were expected to be more purple. In 1990, R. Jorgensen's laboratory wanted to upregulate the activity of a gene for chalcone synthase (*chsA*), an enzyme involved in the production of anthocyanin pigments. Surprisingly, some of the transgenic petunia plants harboring the *chsA* coding region under the control of a 35S promoter lost both endogene and transgene chalcone synthase activity, and thus many of the flowers were variegated or developed white sectors (163). The loss of cytosolic *chsA* mRNA was not associated with reduced transcription, as demonstrated by run-on transcription tests in isolated nuclei (216). Jorgensen coined the term cosuppression to describe the loss of mRNAs of both the endo- and the transgene.

Around the same time, two other laboratories (105, 217) also reported that introduction of the transcribing-sense transgenes could downregulate the expression of homologous endogenous genes. Subsequently, many similar events of cosuppression were reported in the literature. All cases of cosuppression resulted in the degradation of endogene and transgene RNAs after nuclear transcription had occurred (120). Since posttranscriptional RNA degradation was observed in a wide range of transgenes expressing the plant, bacterial, or viral sequences, it was rechristened posttranscriptional gene silencing (PTGS). PTGS could be initiated not only by sense transgenes but also by antisense transgenes, and biochemical evidence suggests that similar mechanisms might operate in both cases (81). It is worthwhile to point out that although the cosuppression phenomenon was originally observed in plants, it is not restricted to plants and has also been demonstrated in metazoans and mammals (98).

In keeping with the times, the observed alterations in the PTGS-related phenotypes were attributed to multiple-site integrations, aberrant RNA formations, repeat structures of the transgenes, etc. Later on, it became clear that the expression of the transgene led to the formation of dsRNA, which, in turn, initiated PTGS. For example, in the case of cosuppressed petunia plants, *chsA* mRNA formed a partial duplex, since there are regions of self-complementarity located between *chsA* 3′ coding region and its 3′ untranslated region (154). This was revealed by DNA sequence analysis and experimental detection of in vitro-transcribed, RNase-resistant duplex *chsA* RNA. In an independent study, a p35S-ACC (1-aminocyclopropane-1-carboxylate [ACC] oxidase) sense transgene carrying a small inverted repeat in the 5′ untranslated region was introduced into tomato to test the role of dsRNA structure as an inducer of PTGS. Cosuppression of the endogenous *acc* gene occurred at a higher frequency in these plants than in those harboring only the p35S-ACC sense transgene without the inverted repeat (93).

Reports from several laboratories in the past few years have established that the loss in steady-state accumulation of the target mRNA is almost total if the designed transgene construct of the transgenic plant produces the nuclear transcript in the duplex conformation. Very recently it was reported that the expression of self-cRNA of plum pox virus under the control of *rolC* promoter caused degradation of transgenic viral RNA and as a result, the systemic disease resistance to challenge inoculum of plum pox virus occurred with a high frequency in transgenic *Nicotiana benthamiana* (170). This evidence points out that the production of dsRNA is required to initiate PTGS in plants. Based on this, plants carrying strongly transcribing transgenes in both the sense and antisense orientations are currently being produced that show strong PTGS features. These transgenic plants can silence endogene, invading viral RNA, or unwanted foreign genes in a sequence-specific and heritable manner.

Generally, the sense and antisense components of the above-mentioned transgenes are separated only by an intron to increase the efficacy of PTGS (43, 198). For example, *Arabidopsis thaliana* and *Lycopersicon esculentum* (tomato) plants were transformed with a transgene construct designed to generate self-complementary *iaaM* and *ipt* transcripts. *iaaM* and *ipt* are oncogenes of agrobacteria that are responsible for crown gall formation in infected plants. The transgenic lines retained susceptibility to *Agrobacterium* transformation but were highly refractory to tumorigenesis, providing functional resistance to crown gall disease by posttranscriptional degradation of the *iaaM* and *ipt* transcripts (72).

## Quelling and RNAi

While reports of PTGS in plants were piling up, homology-dependent gene silencing phenomena were also observed independently in fungal systems. These events were called quelling. Quelling came to light during attempts to boost the production of an orange pigment made by the gene *al1* of the fungus *Neurospora crassa* (50). An *N. crassa* strain containing a wild-type $al1^+$ gene (orange phenotype) was transformed with a plasmid containing a 1,500-bp fragment of the coding sequence of the *al1* gene. A few transformants were stably quelled and showed albino phenotypes. In the *al1*-quelled strain, the level of unspliced *al1* mRNA was similar to that of the wild-type strain, whereas the native *al1* mRNA was highly reduced, indicating that quelling and not the rate of transcription affected the level of mature mRNA in a homology-dependent manner.

The phenomenon of RNAi first came into the limelight following the discovery by Fire et al. (78), who unequivocally demonstrated the biochemical nature of inducers in gene silencing by introducing purified dsRNA directly into the body of *Caenorhabditis elegans*. The investigators injected dsRNA corresponding to a 742-nucleotide segment of *unc22* into either the gonad or body cavity region of an adult nematode. *unc22* encodes an abundant but nonessential myofilament protein, and the decrease in *unc22* activity is supposed to produce an increasingly severe twitching phenotype. The injected animal showed weak twitching, whereas the progeny individuals were strong twitchers. The investigators showed that similar loss-of-function individuals could also be generated with dsRNAs corresponding to four other nematode genes. The phenotypes produced by interference by various dsRNAs were extremely specific.

This experiment paved the way for easy production of null mutants, and the process of silencing a functional gene by exogenous application of dsRNA was termed RNA interference (RNAi). RNAi in *C. elegans* was also initiated simply by soaking the worms in a solution containing dsRNAs or by feeding the worms *Escherichia coli* organisms that expressed the dsRNAs (209). This is a very potent method, requiring only catalytic amounts of dsRNA per cell to silence gene expression. The silencing spread not only from the gut of the worm to the remainder of the body, but also through the germ line to several generations. These phenomena of RNAi have also been demonstrated to occur in *Drosophila melanogaster* and many other invertebrates and vertebrates.

## Insights from Virus-Infected Plants (Virus-Induced Gene Silencing)

Besides the processes mentioned above, homology-driven RNA degradation also occurs during the growth of viral genomes in infected plants (73). Viruses can be either the source, the target, or both the source and the target of silencing. PTGS

mediated by viruses can occur with RNA viruses, which replicate in the cytoplasm, and also with DNA viruses, which replicate in the nucleus (71). As early as in the 1920s, it was known that plants could be protected from a severe virus by prior infection with a mild strain of a closely related virus. Although the mechanism of such cross protection in plants remained unknown for a long time, such phenomena could be explained partly in terms of PTGS that could be induced by the mild strain and targeted later against the virulent viral genome. It was also found that transforming plants with virus-derived transgenes gave protection against the challenge viruses even when no transgene protein was produced (132).

Analyses of these virus-resistant plants revealed that the transgenes were highly transcribed in the nucleus, whereas the steady-state level of cytoplasmic mRNA was very low. Further analysis suggested that some of the transgenic mRNA molecules assumed the conformation of dsRNA, which triggered sequence-specific degradation of self and other homologous or cRNA sequences in the cytoplasm. Thus, in the virus-resistant lines, not only the transgene mRNAs but also the mRNA from the homologous endogenous gene and the invading viral RNA (with homology to the transgene) were degraded.

Another form of virus-induced gene silencing is the phenomenon of viral recovery itself. When *Brassica napus* was inoculated with cauliflower mosaic virus (a DNA virus), lesions at the site of virus entry were visible 5 to 7 days postinoculation. Symptoms of systemic infections were apparent by 10 to 14 days postinoculation. Symptoms were most prominent at 30 to 40 days postinoculation and declined thereafter (i.e., the plants recovered), with the newly emergent leaves remaining asymptomatic at 50 days postinoculation (5).

Figure 1 diagrammatically illustrates the systemic spread of RNAi in plants. Such recovery occurred by a PTGS-like mechanism because 19S and 35S RNAs encoded by the cauliflower mosaic virus were degraded while cauliflower mosaic virus DNA was still replicating in the nucleus. Induction of PTGS was visualized if the cauliflower mosaic virus infection and subsequent recovery were followed up in a transgenic *B. napus* expressing a p35S-GUS (β-glucuronidase) transgene. At the site of inoculation, GUS silencing associated with local lesions was first observed 7 days postinoculation. GUS silencing eventually spread systemically, and the GUS activity of the entire plant was suppressed by 50 days postinoculation. In this particular example, cauliflower mosaic virus acted as the inducer of PTGS for the transgenes sharing homology with the virus within the transcribed region. However, the virus itself was also the target of the induced PTGS, since 19S and 35S RNAs were found degraded.

A similar example of virus-induced gene silencing was found when *Nicotiana clevelandii* was infected with an RNA nepovirus, tomato black ring virus (179). RNA viruses make abundant dsRNA during intracellular replication of their genomes and thus elicit cellular PTGS degradative activity. Virus-induced gene silencing also occurs with viruses that do not undergo recovery. When a DNA geminivirus, tomato golden mosaic virus (TGMV), infected *N. benthamiana*, a high level of viral DNA replication in the nucleus and accumulation of viral RNA in the cytoplasm occurred. An infection by a recombinant TGMV carrying the coding sequence of the sulfur (*su*) gene of the host plant in either the sense or antisense orientation led to the bleaching of leaves due to PTGS of the endogenous *su* gene, but the DNA of the recombinant did not fail to replicate (117). Here, TGMV acted as an inducer of PTGS but was not itself a target of PTGS. Thus, plant viruses elicit PTGS but sometimes can escape the degradative PTGS activity.

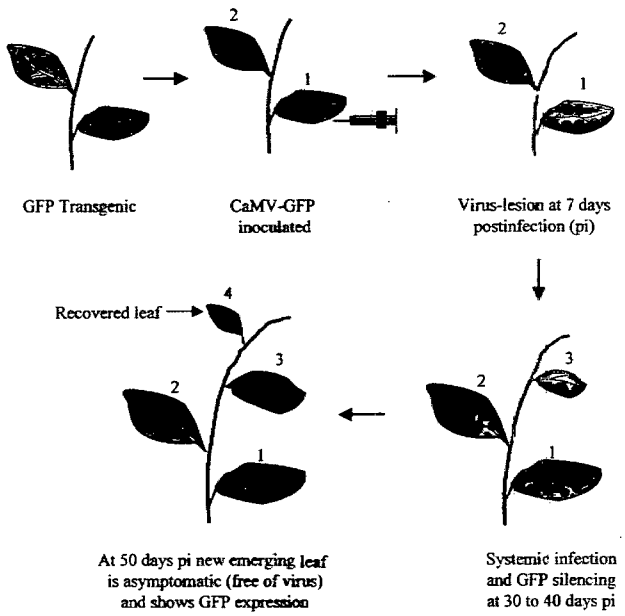



FIG. 1. Schematic illustration of systemic viral spread as well as RNAi and subsequent viral recovery in plants. Green and red indicate the presence and loss of GFP fluorescence, respectively, and orange denotes the presence of both colors. The red dots on leaves show viral lesions. The bold arrows indicate the stages of plant growth, and the leaves are numbered accordingly. An arrow with a thin line shows a newly emerged leaf recovered from viral attack.

Based on the principles of virus-induced gene silencing, vectors designed with the genome sequence of RNA viruses tobacco mosaic virus, potato virus X, and tobacco rattle virus are being widely used to knock down the expression of host genes. The characteristics of many plant genes were revealed by observing the loss-of-function-related phenotypic changes when the recombinant vectors incorporating the concerned host genes were introduced into plants (136). Of these vectors, the TRV-based are more promising because these are capable of inducing-meristematic gene silencing, which has not been possible to achieve with other RNA virus-based vectors. Meristematic gene silencing employing TGMV vectors has also been reported (173). Thus, virus-induced gene silencing-based techniques are extremely useful for studies related to functional genomics in plants.

## IMPORTANT FEATURES OF RNA SILENCING

Independently of one another, investigations on diverse organisms, labeled variously as PTGS in plants, RNAi in animals, quelling in fungi, and virus-induced gene silencing, have converged on a universal paradigm of gene regulation. The critical common components of the paradigm are that (i) the inducer is the dsRNA, (ii) the target RNA is degraded in a homology-dependent fashion, and, as we will see later, (iii) the degrada-

tive machinery requires a set of proteins which are similar in structure and function across most organisms. In most of these processes, certain invariant features are observed, including the formation of small interfering RNA (siRNA) and the organism-specific systemic transmission of silencing from its site of initiation.

### siRNA

The key insight in the process of PTGS was provided from the experiments of Baulcombe and Hamilton (92), who identified the product of RNA degradation as a small RNA species (siRNA) of ≈25 nucleotides of both sense and antisense polarity. siRNAs are formed and accumulate as double-stranded RNA molecules of defined chemical structures, as mentioned later. siRNAs were detected first in plants undergoing either cosuppression or virus-induced gene silencing and were not detectable in control plants that were not silenced. siRNAs were subsequently discovered in *Drosophila* tissue culture cells in which RNAi was induced by introducing >500-nucleotide-long exogenous dsRNA (96), in *Drosophila* embryo extracts that were carrying out RNAi in vitro (240), and also in *Drosophila* embryos that were injected with dsRNA (236). Thus, the generation of siRNA (21 to 25 nucleotides) turned out to be the signature of any homology-dependent RNA-silencing event.

The siRNAs resemble breakdown products of an *E. coli* RNase III-like digestion (13). In particular, each strand of siRNA has 5′-phosphate and 3′-hydroxyl termini and 2- to 3-nucleotide 3′ overhangs. Interestingly, in vitro-synthesized siRNAs can, in turn, induce specific RNA degradation when added exogenously to *Drosophila* cell extracts (69). Specific inhibition of gene expression by these siRNAs has also been observed in many invertebrate and some vertebrate systems (67). Recently, Schwarz et al. (189) provided direct biochemical evidence that the siRNAs could act as guide RNAs for cognate mRNA degradation.

### Amplification and Systemic Transmission

Besides the formation of siRNAs, another intriguing characteristic of homology-dependent gene silencing is that the inducer dsRNA molecules do not act stoichiometrically. It was estimated that only two molecules of dsRNA per cell were able to induce RNAi of an abundantly expressed *C. elegans* gene such as *unc22*. In another report, injection of dsRNA into the intestine of a *C. elegans* hermaphrodite generated RNAi, which could be stably inherited to the $F_2$ generation. These two findings led to the proposal that RNAi signals could be systemic and amplifiable in nature (78). The similar systemic effects of RNAi have also been demonstrated in the planarian *Schmidtea mediterranea* and the cnidarian *Hydra magnipapillata* (140).

Similar evidence is also available for plant PTGS. The new tissues growing from a GUS-expressing scion grafted onto a GUS-silenced rootstock show progressive silencing of GUS expression (168). The silencing signal seems to spread by a nonmetabolic, gene-specific diffusible signal, which travels both between cells, through plasmadesmata, and long distances via the phloem (75). In the case of virus-induced gene silencing, the systemic character has also been revealed (185). To account for the gene specificity of a systemic signal, it has been proposed that the signal could be an RNA molecule (228). However, such processes are not universal, as these are not found in flies and mammals.

## COMPONENTS OF GENE SILENCING

Both genetic and biochemical approaches have been undertaken to understand the basis of silencing. Genetic screens were carried out in the fungus *Neurospora crassa*, the alga *Chlamydomonas reinhardtii*, the nematode *Caenorhabditis elegans*, and the plant *A. thaliana* to search for mutants defective in quelling, RNA interference, or PTGS. Analyses of these mutants led to the identification of host-encoded proteins involved in gene silencing and also revealed that a number of essential enzymes or factors are common to these processes. Some of the components identified serve as initiators, while others serve as effectors, amplifiers, and transmitters of the gene silencing process. In the years to come, many other components as well as their interrelations will be revealed. Here we outline what is known so far.

### Dicer

RNase III family members are among the few nucleases that show specificity for dsRNAs (164) and cleave them with 3′ overhangs of 2 to 3 nucleotides and 5′-phosphate and 3′-hydroxyl termini (69). Bernstein et al. (17) identified an RNase III-like enzyme in *Drosophila* extract which was shown to have the ability to produce fragments of 22 nucleotides, similar to the size produced during RNAi. These authors showed that this enzyme is involved in the initiation of RNAi. Owing to its ability to digest dsRNA into uniformly sized small RNAs (siRNA), this enzyme was named Dicer (DCR). These nucleases are evolutionarily conserved in worms, flies, fungi, plants and mammals. Dicer has four distinct domains: an amino-terminal helicase domain, dual RNase III motifs, a dsRNA-binding domain, and a PAZ domain (a 110-amino-acid domain present in proteins like Piwi, Argo, and Zwille/Pinhead), which it shares with the RDE1/QDE2/Argonaute family of proteins that has been genetically linked to RNAi by independent studies (34, 203). Cleavage by Dicer is thought to be catalyzed by its tandem RNase III domains. Some DCR proteins, including the one from *D. melanogaster*, contain an ATP-binding motif along with the DEAD box RNA helicase domain.

The predicted *C. elegans* Dicer homologue, K12H4.8, was referred as DCR1 because it was demonstrated to be the functional ortholog of the *Drosophila* Dicer protein (173). The 8,165-bp DCR1 protein has a domain structure similar to that of the *Drosophila* Dicer protein. *dcr1* mutants of *C. elegans* showed defects in RNAi of germ line-expressed genes but no effect on the RNAi response of somatic genes. These mutants were found to be sterile, suggesting the important role of this gene in germ line development apart from RNAi (119). CAF has been identified as a Dicer homologue in *A. thaliana*, but it is not involved in PTGS activity. The structure of CAF1 shows the presence of the four distinct domains that were identified in the *Drosophila* Dicer protein (17, 36, 108). Dicer homologues from many different sources have been identified; some

recombinant Dicers have also been examined in vitro, and phylogenetic analysis of the known Dicer-like proteins indicates a common ancestry of these proteins (83).

Complete digestion by RNase III enzyme results in dsRNA fragments of 12 to 15 bp, half the size of siRNAs (235). The RNase III enzyme acts as a dimer and thus digests dsRNA with the help of two compound catalytic centers, whereas each monomer of the Dicer enzyme possesses two catalytic domains, with one of them deviating from the consensus catalytic sequences.

Recently, the crystal structure of the RNase III catalytic domain was solved, and this led to the model for generation of 23- to 28-mer diced siRNA products (20). In this model, the dimeric Dicer folds on the dsRNA substrate to produce four compound catalytic sites so that the two terminal sites having the maximum homology with the consensus RNase III catalytic sequence remain active, while the other two internal sites bearing partial homology lose functional significance. Thus, the diced products appear as the limit digests of the RNase III enzymes and are double the size of the normal 12- to 15-mer fragments. Such a model also predicts that certain changes in Dicer structure might modify the spacing between the two active terminal sites and thus generate siRNAs of variable sizes bearing species-specific imprints (98). Clearly, the crystal structure of Dicer is necessary to authenticate this model.

### Guide RNAs and RNA-Induced Silencing Complex

Hammond et al. (96) determined that the endogenous genes of *Drosophila* S2 cells could be targeted in a sequence-specific manner by transfection with dsRNA, and loss-of-function phenotypes were created in cultured *Drosophila* cells. The inability of cellular extracts treated with a $Ca^{2+}$-dependent nuclease (micrococcal nuclease, which can degrade both DNA and RNA) to degrade the cognate mRNAs and the absence of this effect with DNase I treatment showed that RNA was an essential component of the nuclease activity. The sequence-specific nuclease activity observed in the cellular extracts responsible for ablating target mRNAs was termed the RNA-induced silencing complex (RISC) (96).

After partial purification of crude extracts through differential centrifugation and anion exchange chromatography, the nuclease cofractionated with a discrete ≈25-nucleotide RNA species. These results suggested that small RNAs were associated with sequence-specific nuclease and served as guides to target specific messages based upon sequence recognition. In another report, the multicomponent RNAi nuclease was purified to homogeneity as a ribonucleoprotein complex of ≈500 kDa (97). One of the protein components of this complex was identified as a member of the Argonaute family of proteins and was termed Argonaute2 (AGO2). AGO2 is homologous to RDE1, a protein required for dsRNA-mediated gene silencing in *C. elegans*. AGO2 is a ≈130-kDa protein containing polyglutamine residues, PAZ, and PIWI domains characteristic of members of the Argonaute gene family. The Argonaute family members have been linked both to the gene-silencing phenomenon and to the control of development in diverse species. The first link between Argonaute protein and RNAi was shown by isolation of *rde1* mutants of *C. elegans* in a screen for RNAi-deficient mutants. Argonaute family members have been shown to be involved in RNAi in *Neurospora crassa* (QDE3) as well as in *A. thaliana* (AGO1) (75).

Recently, two independent groups identified additional components of the RISC complex. Hammond and group showed the presence of two RNA binding proteins, the Vasa intronic gene and dFMR proteins, in the RISC complex isolated from *Drosophila* flies (35). Of these, dFMR is a homologue of the human fragile X mental retardation protein. In a parallel study, Siomi and group also isolated a novel ribonucleoprotein complex from the *Drosophila* lysate that contained dFMRI, AGO2, a *Drosophila* homologue of p68 RNA helicase (Dmp68), and two ribosomal proteins, L5 and L11, along with 5S rRNA (106). Both of these groups showed not only the presence of these components in the RISC complex, but also interactions among these proteins in vitro. Other components of RISC have not been clearly established yet. Nevertheless, some of the proteins mentioned below could very well constitute the RISC complex.

### RNA and DNA Helicases

Aberrant RNA elimination surveillance seems to be common to most eukaryotic organisms. However, a diverse array of proteins specific for each organism seem to carry out such surveillance. Broadly, they fall in the biochemically similar group of RNA-DNA helicases. A mutant strain (*mut6*) of *C. reinhardtii* was isolated in which a gene required for silencing a transgene was disrupted (232). This RNAi-resistant mutant also showed an elevated transposition activity. The *mut6* gene was cloned and sequenced. The deduced MUT6 protein contains 1,431 amino acids and is a member of the DEAH box RNA helicase family. It also has a glycine-rich region that includes several RGG repeats, resembling an RGG box, a motif implicated in RNA binding and protein-protein interactions. MUT6 also has three putative nuclear localization signals and is predicted to be nuclear by PSORT analysis (161). MUT6 RNA helicase may be involved in degradation of misprocessed aberrant RNAs and thus could be a part of an RNAi-related surveillance system.

In *Neurospora crassa*, three classes of quelling-defective mutants (*qde1*, *qde2*, and *qde3*) have been isolated (46). The *qde3* gene has been cloned, and the sequence encodes a 1,955-amino-acid protein (48). The protein shows homology with several polypeptides belonging to the family of RecQ DNA helicases, which includes the human proteins for Bloom's syndrome and Werner's syndrome (238). In addition, QDE3 is believed to be involved in the activation step of gene silencing. The DNA helicase activity of QDE3 may function in the DNA-DNA interaction between introduced transgenes or with a putative endogenous gene required for gene-silencing activation by unwinding the double-stranded DNA. These interactions may induce changes in methylation or chromatin structure, producing an altered state that could result in aberrant RNA production. Thus, QDE3 protein may be more important for the transcriptional part of gene silencing, i.e., TGS.

When the RNAi sensitivity of several existing *C. elegans* mutants was examined, two mutant strains, *mut2* and *mut7*, that had previously shown elevated levels of transposon mobilization also showed resistance to RNAi. Ketting et al. (116) identified a mutator gene, *mut7*, in *C. elegans* and character-

ized it at the molecular level. MUT7 was found to be homologous to proteins with 3′-5′ exonuclease domains, such as Werner's syndrome protein and *E. coli* RNase D. It contained all the key catalytic residues for nuclease activity. A model was proposed in which MUT7 was speculated to play a role in repressing transposition by degrading the target mRNA with its exonuclease activity.

*smg* (suppressor of morphological effects on genitalia) mutants of *C. elegans*, defective in a process called nonsense-mediated decay, have been isolated (63). Seven *smg* genes which are involved in nonsense-mediated decay have been identified (29, 100). Since this process also involves RNA degradation, the function of these genes, if any, in the RNAi process was examined. Animals mutant for a subset of these genes, *smg2*, *smg5*, and *smg6*, were initially silenced by dsRNA but later showed rapid recovery from the effects of RNAi, unlike the wild-type worms, which remained silenced. Thus, these genes might affect the persistence of RNA interference. On the other hand, *smg1*, *smg3*, and *smg4* mutant animals behaved like wild-type worms and did not recover from RNAi at all, indicating that these genes are not required for RNAi persistence. The *smg5* and *smg6* genes have not been cloned, but the *smg2* gene shows homology to *Saccharomyces cerevisiae upf1*, which encodes an ATPase with RNA-binding and helicase activities.

The SMG proteins could unwind dsRNA to provide a template for amplification activity. In this way, the three SMG proteins might facilitate amplification of the silencing signal and cause persistence of the silenced state. Alternatively, SMG proteins could increase the number of dsRNA molecules by promoting endonucleolytic cleavage of existing dsRNA molecules, which has been observed in *Drosophila* flies. No SMG2 homologues have been identified in plants or fungi. However, a search of the *A. thaliana* genome sequence database revealed a number of candidates with either helicase and/or RNase domains.

In a recent report, Tijsterman et al. (208) showed that unlike sense oligomers, single-stranded oligomers of antisense polarity could induce gene silencing in *C. elegans*. The antisense RNA-induced gene silencing was explained by proposing that RNA synthesis was primed on the mRNA by antisense RNA, resulting in dsRNAs, which acted as substrates for Dicer-dependent degradation. Antisense RNAs showed a requirement for the mutator/RNAi genes *mut7* and *mut14* but acted independently of the RNAi genes *rde1* and *rde4* of *C. elegans*. The *mut14* gene was cloned by genetic mapping and subsequent candidate gene approach. The MUT14 protein is a member of the family of putative RNA helicases that contain the signature DEAD box motif. These proteins are involved in diverse cellular functions. The helicase activity of MUT14 might thus act to permit de novo RNA synthesis on the target.

Dalmay et al. (54) identified an *sde3* locus in *A. thaliana* plants which is required for the PTGS phenotype. They proposed that SDE3 protein might be involved in the production of dsRNA. SDE3 differs markedly from QDE3/MUT7 and has slight similarity to MUT6 in the helicase motif. Although it is highly similar to Upf1p and SMG2, it is unlikely that SDE3 is the functional homologue of Upf1p and SMG2 because it lacks important motifs (167). Notably, no SDE3 homologue was found in *C. elegans*, suggesting that SDE3-like proteins are regulators rather than essential cofactors of PTGS and are not used in *C. elegans*. This is further supported by the observation that *sde3* mutant plants exhibit only partial loss of PTGS (55). The closest homologue of SDE3 as identified by BlastP was a mouse protein encoded by *gb110* (91, 159). These SDE3 homologues have RNA helicase motifs that are quite distinct from those of the DEAD, DEAH, and Ski2p types of RNA helicase (134). It has been speculated that SDE3 and SMG2 are multifunctional RNA helicases involved in PTGS.

### Translation Initiation Factor

Mutants of *C. elegans* showing resistance to dsRNA-mediated RNAi were selected by Tabara et al. (203). They genetically mapped seven mutant strains that were placed in four complementation groups. One of the groups, *rde1*, consisted of three alleles. Gene *rde1* is a member of a large family which includes *Drosophila* homologues (*piwi* and *sting*) and *Arabidopsis* homologues (*argonaute* and *zwille*) and rabbit *eIF2C*. The full-length cDNA sequence for *rde1* was determined, and the deduced protein, consisting of 1,020 amino acids, was referred to as RDE1. The RDE1 protein is homologous to the product of the quelling deficiency (*qde2*) gene in *Neurospora crassa* (75). The initiation step of RNAi might be affected in the *rde1* mutant, as it completely lacks an interference response to several dsRNAs. It does not show any increase in transposon mobilization and or any effect on growth and development.

### RNA-Dependent RNA Polymerase

The effects of both RNAi and PTGS are potent and systemic in nature. This has led to a proposed mechanism in which RNA-dependent RNA polymerases (RdRPs) play a role in both triggering and amplifying the silencing effect. Transgenic and virus-infected plants show an accumulation of aberrant transgenic and viral RNAs. The RdRP enzymes might recognize these aberrant RNAs as templates and synthesize antisense RNAs to form dsRNAs that are finally the targets for sequence-specific RNA degradation (45, 47, 56, 133).

Genetic screens of *Neurospora crassa* (QDE1) (48) and *A. thaliana* (SDE1/SGS2) (54, 160) led to the identification of proteins which are similar to tomato RdRP (77, 187) and are required for quelling and PTGS, respectively. This testifies to the importance of RdRP in gene silencing. Cogoni et al. (45) cloned the *qde1* gene from *N. crassa*. It encodes a 158-kDa protein which lacks the typical signal peptide or a transmembrane domain, indicating its intracellular location. Dalmay et al. (54) found that the 113-kDa *Arabidopsis* RdRP is encoded by *sde1*. It is a plant homologue of QDE1 in *N. crassa* and EGO1 in *C. elegans*, which are required for quelling and RNAi, respectively. The SDE1 protein is required for transgene silencing but not for virus-induced PTGS, suggesting that SDE1 might be required to produce dsRNA, the initiator of PTGS (54).

The dsRNA produced as an intermediate in virus replication by virus-encoded RdRP might induce PTGS itself, and thus SDE1 may not be required for virus-induced PTGS. Plants with the *sde* mutation grow and develop normally, excluding a role for *sde* in development or basic cellular function. Two PTGS-controlling genes, *sgs2* and *sgs3*, were identified in *A.*

TABLE 2. Components of posttranscriptional gene silencing

| Phenomenon | Organism | Mutation causing defective silencing | Gene function | Developmental defect |
|---|---|---|---|---|
| Posttranscriptional gene silencing | Plant (*Arabidopsis thaliana*) | *sgs2/sde1* | RdRP | None |
| | | *sgs3* | Unknown function | None |
| | | *sde3* | RecQ helicase | |
| | | *ago1* | Translation initiation factor | Pleiotropic effects on development & fertility |
| | | *caf1* | RNA helicase & RNase III | |
| Quelling | Fungus (*Neurospora crassa*) | *qde-1* | RdRP | None |
| | | *qde-2* | Translation initiation factor | None |
| | | *qde-3* | RecQ DNA helicase | |
| RNA interference | Worm (*Caenorhabditis elegans*) | *ego-1* | RdRP | Gametogenetic defect & sterility |
| | | *rde-1* | Translation initiation factor | None |
| | | *rde-2, rde-3, rde-4, mut-2* | Unknown function | None |
| | | K12H4.8 (*dcr-1*) | Dicer homologue RNA helicase & RNase III | Sterility |
| | | *mut-7* | Helicase & RNase D | None |
| | | *mut-14* | DEAD box RNA helicase | |
| | | *smg-2* | Upflp helicase | |
| | | *smg-5* | Unknown function | |
| | | *smg-6* | Unknown function | |
| | | *sid-1* | Transmembrane protein | |
| | Alga (*Chlamydomonas reinhardtii*) | *mut-6* | DEAH box RNA helicase | |

*thaliana* by another group of workers (160). Later, it was found that *sgs2* and *sde1* are different descriptions of the same gene. On comparing the protein sequence of all the RdRPs, a conserved block was identified which seems to be crucial for RdRP function in PTGS and RNAi. *sgs3* mutants have the same molecular and phenotypic characteristics as *sgs2* mutants, but the SGS3 protein shows no significant similarity with any known putative proteins.

In *C. elegans*, EGO1, a protein required for RNAi, was found to be similar to tomato RdRP and the QDE1 protein of *Neurospora crassa* (197), as mentioned earlier. For a number of germ line-expressed genes, *ego1* mutants were resistant to RNA interference. The *ego1* transcript is found predominantly in the germ line. *ego1* is thus yet another example of a gene encoding an RdRP-related protein with an essential developmental function. RdRP is speculated to play a role in the amplification of the dsRNA signal, allowing its spread throughout the organism (50, 77, 168, 221). The RdRP is also perhaps responsible for sustaining PTGS at the maintenance level even in the absence of the dsRNA that initiates the RNAi effect.

In spite of its omnipresence in different kinds of eukaryotic cells, RdRP homologues are not coded by either the *Drosophila* or human genome. Though the systemic characteristics of RNAi have not been revealed yet in either flies or humans, the amplification of siRNAs may be an essential step of RNAi even in these systems. Hence, it is important to know how these steps of RNAi are biochemically carried out in the absence of RdRP activity.

### Transmembrane Protein (Channel or Receptor)

The systemic spread of gene silencing from one tissue to another has been well established in *C. elegans* and plants. To investigate the mechanism of systemic RNAi, Winston et al. (231) constructed and used a special transgenic strain of *C. elegans* (HC57). They identified a systemic RNA interference-deficient (*sid*) locus required to transmit the effects of gene silencing between cells with green fluorescent protein (GFP) as a marker protein. Of the 106 *sid* mutants belonging to three complementation groups (*sid1*, *sid2*, and *sid3*), they isolated and characterized *sid1* mutants. The *sid1* mutants had no readily detectable mutant phenotype other than failure to show systemic RNAi. Interestingly, these mutants also failed to transmit the effect of RNAi to the progeny.

The SID1 polypeptide is predicted to be a 776-amino-acid membrane protein consisting of a signal peptide and 11 putative transmembrane domains. Based on the structure of SID1, it was suggested that it might act as a channel for the import or export of a systemic RNAi signal or might be necessary for endocytosis of the systemic RNAi signal, perhaps functioning as a receptor. No homologue of *sid1* was detected in *D. melanogaster*, which may be consistent with the apparent lack of systemic RNAi in the organism (80, 174). However, the presence of SID homologues in humans and mice might hint at the systemic characteristics of RNAi in mammals.

### Genetic Mutations with Unknown Function

The three other complementation groups identified by Tabara et al. (203) in *C. elegans* are *rde2* and *rde3*, with one allele each, and *rde4*, with two alleles. *rde4* mutants behaved like the *rde1* strain in not showing any increase in transposon mobilization and no effect on growth and development. The product of *rde2* remains to be identified. *mut2*, *rde2*, and *rde3* exhibited high-level transposition similar to *mut7*. This suggests a possible biological role of RNAi in transposon silencing (203).

Mello and colleagues (87) have proposed that *rde1* and *rde4* respond to dsRNA by producing a secondary extragenic agent that is used by the downstream genes *rde2* and *mut7* to target

specific mRNAs for PTGS. According to this view, *rde1* and *rde4* act as initiators of RNAi whereas *rde2* and *mut7* are effectors. Various components of gene silencing have been listed in Table 2.

## MECHANISM OF RNA INTERFERENCE

As the various pieces of the RNAi machinery are being discovered, the mechanism of RNAi is emerging more clearly. In the last few years, important insights have been gained in elucidating the mechanism of RNAi. A combination of results obtained from several in vivo and in vitro experiments have gelled into a two-step mechanistic model for RNAi/PTGS. The first step, referred to as the RNAi initiating step, involves binding of the RNA nucleases to a large dsRNA and its cleavage into discrete ≈21- to ≈25-nucleotide RNA fragments (siRNA). In the second step, these siRNAs join a multinuclease complex, RISC, which degrades the homologous single-stranded mRNAs. At present, little is known about the RNAi intermediates, RNA-protein complexes, and mechanisms of formation of different complexes during RNAi. In addition to several missing links in the process of RNAi, the molecular basis of its systemic spread is also largely unknown.

### Processing of dsRNA into siRNAs

Studies of PTGS in plants provided the first evidence that small RNA molecules are important intermediates of the RNAi process. Hamilton and Baulcombe (92), while studying transgene-induced PTGS in five tomato lines transformed with a tomato 1-aminocyclopropane-1-carboxyl oxidase (ACO), found accumulation of *aco* small RNAs of 25 nucleotides. More direct evidence about the generation of siRNAs in RNAi came from an in vitro cell-free system obtained from a *Drosophila* syncytial blastoderm embryo by Tuschl et al. (212). These authors were able to reproduce many of the features of RNAi in this system. When dsRNAs radiolabeled within either the sense or the antisense strand were incubated with *Drosophila* lysate in a standard RNAi reaction, 21- to 23-nucleotide RNAs were generated with high efficiency. Single-stranded $^{32}$P-labeled RNA of either the sense or antisense strand was not efficiently converted to 21- to 23-nucleotide products. The formation of the 21- to 23-nucleotide RNAs did not require the presence of corresponding mRNAs.

The role of the small RNAs in RNAi was confirmed independently by Elbashir et al. (69), who showed that synthetic 21- to 23-nucleotide RNAs, when added to cell-free systems, were able to guide efficient degradation of homologous mRNAs. To assess directly if the siRNAs were the true intermediates in an RNAi reaction, Zamore et al. (240) fractionated both the unprocessed dsRNAs and processed dsRNAs from the *Renilla luc* dsRNA-treated cell-free *Drosophila* system and showed that only the fractions containing native siRNAs were able to bring about the cognate RNA degradation and their ability to degrade RNA was lost when these fractions were treated at 95°C for 5 min. These in vivo and in vitro studies thus provided the evidence that siRNAs are the true intermediates of the RNAi reaction.

Together with the experiments to identify siRNAs as the key molecules for the RNAi effect, several investigators carried out the logical search for polypeptides that could generate such molecules. Based on the binding and cleavage properties of *E. coli* RNase III enzymes, Bass (13) for the first time predicted the involvement RNase III-type endonucleases in the degradation of dsRNA to siRNAs. The RNase III enzyme makes staggered cuts in both strands of dsRNA, leaving a 3′ overhang of 2 nucleotides. The first evidence for the involvement of RNase III enzyme in RNAi was provided by T. Tuschl's group, who chemically analyzed the sequences of the 21- to 23-nucleotide RNAs generated by the processing of dsRNA in the *Drosophila* cell-free system. They showed the presence of 5′-phosphate, 3′-hydroxyl, and a 3′ 2-nucleotide overhang and no modification of the sugar-phosphate backbone in the processed 21- to 23-nucleotide RNAs (69).

Two groups recently identified candidate enzymes involved in degradation by scanning the genomes of *D. melanogaster* and *C. elegans* for genes encoding proteins with RNase III signatures (17, 115). Bernstein et al. (17) showed that one of these identified genes, *dicer* in *Drosophila*, codes for the RNA processing enzyme that fragments dsRNA into 22-nucleotide fragments in vitro. An antiserum raised against Dicer could also immunoprecipitate a protein from the *Drosophila* extract or from S2 cell lysate, and these Dicer protein immunoprecipitates were able to produce RNAs of about 22 nucleotides from the dsRNA substrate. The direct correspondence in size of these RNAs with those generated from dsRNA by cell extract suggested a role of this protein in dsRNA degradation. The role of Dicer in RNAi was further confirmed by the fact that the introduction of Dicer dsRNA into *Drosophila* cells diminished the ability of the transfected cells to carry out RNAi in vitro. Similar experimental studies were carried out with *C. elegans* extract, and an ortholog of Dicer named DCR1 was identified.

A number of in vivo and in vitro experimental studies have shown that the production of 21- to 23-nucleotide RNAs from dsRNA requires ATP. The rate of 21- to 23-nucleotide RNA formation from corresponding dsRNAs has been shown to be six times slower in the *Drosophila* extract depleted for ATP by treatment with hexokinase and glucose (165). Bernstein et al. (17) and Ketting et al. (115) showed that the Dicer immunoprecipitates from *D. melanogaster* as well as S2 cell extracts and DCR1 immunoprecipitates from *C. elegans* extract required ATP for the production of 22-nucleotide RNAs (17, 115). Recently, Nykanen et al. (165) reduced ATP levels in *Drosophila* extract by 5,000-fold with a sensitive ATP depletion strategy and showed considerable reduction in the rate of siRNA production in the *Drosophila* cell extract. These experiments suggest that ATP controls the rate of siRNA formation. However it is still unclear whether ATP is absolutely rate limiting for the production of siRNAs from dsRNA.

The RNase activity and dsRNA binding of 218-kDa recombinant human Dicer have also been examined in vitro (175). The enzyme generated siRNA products from dsRNA quite efficiently in the presence of $Mg^{2+}$ and the absence of ATP. The RNase activity was sensitive to ionic interactions, whereas the dsRNA binding was quite effective in presence of high salt and did not require $Mg^{2+}$ at all. The dsRNA binding domain is located at the C terminus of Dicer, which is separable from the helicase and PAZ motifs. Human Dicer expressed in mammalian cells colocalized with calreticulin, a resident protein o

the endoplasmic reticulum. In other systems. Dicer has also been found to complex with various other proteins (35, 106). Hence, it is possible that the Dicer RNase activity functions as a complex of proteins in vivo.

### Amplification of siRNAs

One of the many intriguing features of RNA interference is the apparently catalytic nature of the phenomenon. A few molecules of dsRNA are sufficient to degrade a continuously transcribed target mRNA for a long period of time. Although the conversion of long dsRNA into many small siRNAs results in some degree of amplification, it is not sufficient to bring about such continuous mRNA degradation. Since mutations in genes encoding RNA-dependent RNA polymerase (RdRP) affect RNAi, it was proposed that this type of polymerase might replicate siRNAs as epigenetic agents, permitting their spread throughout plants and between generations in *C. elegans*. Recent studies by Lipardi et al. (135) and Sijen et al. (193) provided convincing biochemical and genetic evidence that RdRP indeed plays a critical role in amplifying RNAi effects.

Lipardi et al. (135), while investigating the dsRNA-dependent degradation of target mRNA in a *Drosophila* embryo cell extract system, showed the generation of full-length cognate dsRNAs from labeled siRNAs at early time points. Both single-stranded RNAs (equivalent to target mRNA) and dsRNAs served as templates for copying by RdRP. New full-length dsRNAs were formed rapidly and cleaved. They also showed a strict requirement for the 3′-hydroxyl group and 5′-phosphate group on siRNAs for primer extension in the RdRP-mediated reaction (135).

Sijen et al. (193) further revealed the role of RdRP activity in RNAi. In an RNAi reaction, they observed the formation of new siRNA species corresponding to target mRNAs but different from trigger dsRNAs. They named these new siRNAs secondary siRNAs. With a primary trigger dsRNA specific for the *lacZ* region of the target mRNA that encoded a GFP-LacZ fusion protein, these authors demonstrated the degradation of a separate GFP mRNA target. This kind of RNAi induced by secondary siRNAs was named transitive RNAi. These authors demonstrated the requirement for the *rrf1* gene, a *C. elegans* gene with sequence homology to RdRP, in the generation of secondary siRNAs and transitive RNAi (193).

Amplification of siRNAs might occur at various stages of the RNAi reaction and has been documented in plants, *C. elegans*, *N. crassa*, and *Dictyostelium discoideum* but not in flies and mammals (66). Though the RdRP activity is present in *Drosophila* embryo extract, as mentioned earlier, it is surprising that the fly genome does not code for RdRP. Additionally, numerous experiments also suggest that RdRP is not required for RNAi in *D. melanogaster* (98).

### Degradation of mRNA

In the effector step of RNAi, the double-stranded siRNAs produced in the first step are believed to bind an RNAi-specific protein complex to form a RISC. This complex might undergo activation in the presence of ATP so that the antisense component of the unwound siRNA becomes exposed and allows the RISC to perform the downstream RNAi reaction. Zamore and colleagues (240) demonstrated that a ≈250-kDa precursor RISC, found in *Drosophila* embryo extract, was converted into a ≈100-kDa complex upon being activated by ATP. This activated complex cleaved the substrate. The size and constitution of the precursor as well as the activated RISC might vary depending on the choice of system (98). The antisense siRNAs in the activated RISC pair with cognate mRNAs, and the complex cuts this mRNA approximately in the middle of the duplex region.

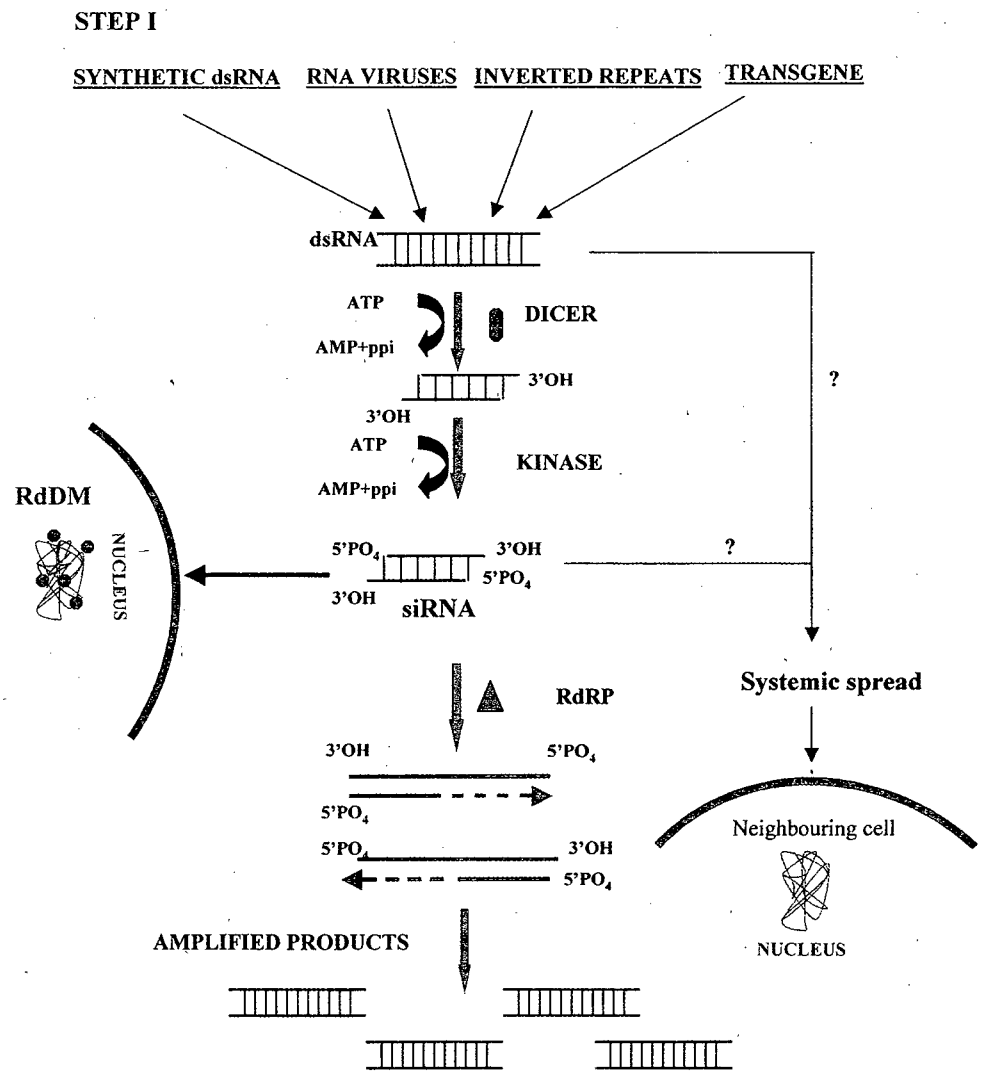
A few independent studies demonstrated the importance of the RISC complex in this part of RNAi reactions. The mRNA-cleaving RNA-protein complexes have also been referred to as siRNP (small interfering ribonucleoprotein particles). It is widely believed that this nuclease is probably different from Dicer, judging from the substrate requirements and the nature of the end products. Since the target cleavage site has been mapped to 11 or 12 nucleotides downstream of 5′ end of the guide siRNA, a conformational rearrangement or a change in the composition of an siRNP ahead of the cleavage of target mRNA is postulated. Finally, the cleaved mRNAs are perhaps degraded by exoribonucleases (96).

A part of cleaved fragments of mRNA at the end of step 2 might also be converted to the duplex forms by the RdRP-like activity. These forms might have siRNA-like functions and eventually enter the pool of the amplification reaction. Thus, it is likely that amplification of the RNAi reaction takes place at both step 1 and step 2 of RNAi. In another model, it has been proposed that siRNAs do not act as primers for the RdRP-like enzymes, but instead assemble along the length of the target RNA and are then ligated together by an RNA ligase to generate cRNA. The cRNA and target RNA hybrid would then be diced by the DCR protein. All these models were summarized by Schwarz et al. (189). Most of the steps involved in the mechanism of RNAi have been illustrated schematically in Fig. 2.

## RNA SILENCING FOR GENOME INTEGRITY AND DEFENSE

Considerable evidence indicates that PTGS has evolved as a protective mechanism against parasitic DNA sequences such as transposons and the RNA sequences of plant viruses. DNA methylation and transcriptional gene silencing (TGS) are mainly responsible for keeping the transposition frequency at a minimum. However, PTGS also provides additional protection against the genomic instability caused by transposons. Mutations in the *C. elegans mut-7* gene increase the transposition frequency in the germ line and downregulate RNAi as well (58), implicating RNAi in the control of transposons. Recently, Djikeng et al. (61) cloned and sequenced the siRNA products of an RNA interference event occurring in *Trypanosoma brucei*. By sequencing over 1,300 siRNA-like fragments, they observed abundant 24- to 26-nucleotide fragments homologous to the ubiquitous retrotransposon INGI and the site-specific retroposon SLACS. Thus, they convincingly demonstrated that RNAi is involved in silencing the retroposon transcript.

In plants, PTGS has been widely linked with RNA virus resistance mechanisms (219, 227). Plant RNA viruses are, in fact, both inducers and targets for PTGS and gene-silencing-




2. Two-step model for the mechanism of gene silencing induced by double-stranded RNA. In step I, dsRNA is cleaved by the Dicer to produce siRNAs. A putative kinase seems to maintain 5′ phosphorylation at this step. The siRNAs have also been proposed to be ble for nuclear DNA methylation (●) and systemic spread of silencing. Amplification might occur due to the presence of RdRP (▲). Ir he siRNAs generated in step I bind to the nuclease complex (RISC). A helicase present in the complex might activate RISC by unwinding NAs. The antisense component of siRNA in the RISC guides the complex towards the cognate mRNA (—), resulting in endonucleolytic ( ↓ ) of the mRNA. RdDM, RNA-dependent DNA methylation.

ve mutants of plants show increased sensitivity to viral ons (160). The direct role of dsRNA in inhibiting viral on has recently been demonstrated by Tenllado and uiz (207). They showed that dsRNAs derived from viral se sequences could interfere with virus infection in a ce-specific manner by directly delivering the dsRNAs to ls either by mechanical coinoculation with the virus or *Agrobacterium*-mediated transient-expression approach. ful interference with the infection of plants by repre- ve viruses belonging to the tobamovirus, potyvirus, and virus genera has been demonstrated. These results sup- e view that a dsRNA intermediate in virus replication an efficient initiator of PTGS in natural virus infections. clinching support for the notion that PTGS has evolved ntiviral mechanism has come from reports that plant viruses encode proteins that are suppressors of PTGS (8, 25 222). These suppressors have evolved to save the viral RNA genomes from the PTGS degradative machinery of host plants Different types of viral suppressors have been identifiec through the use of a variety of silencing suppression assays Suppressors HC-PRO, P1, and AC2 are one type (encoded by potyviruses, rice yellow mottle sobemovirus, and geminiviruses of subgroup III, respectively) that is able to activate GFP expression in all tissues of previously silenced GFP-expressing plants (222). HC-PRO reduces target mRNA degradation and is thus responsible for reduced accumulation of siRNAs (137 145). The second type of suppressors include movement pro teins, i.e., p25 of potato virus X, which are involved in curbing the systemic aspect of transgene-induced RNA silencing (220) The third type includes cytomegalovirus 2b protein, which is

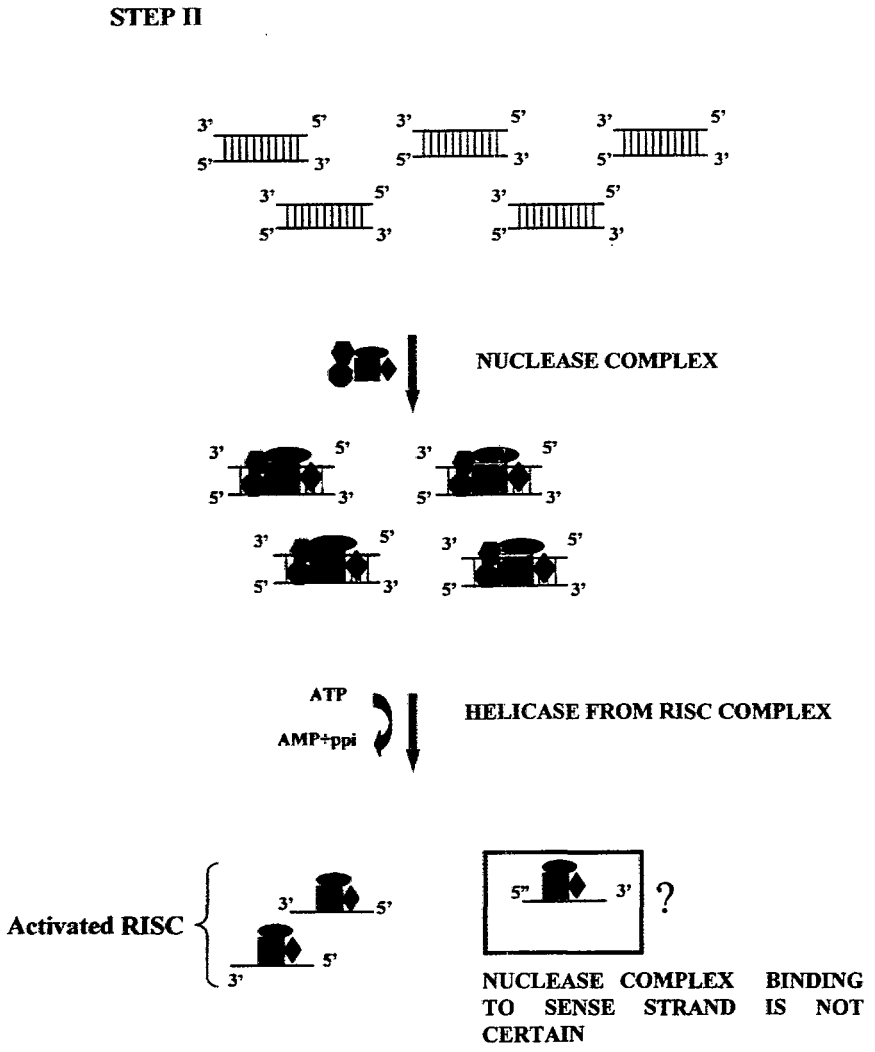



FIG. 2—*Continued.*

involved in systemic signal-mediated RNA silencing (60). The cytomegalovirus 2b protein is nucleus localized and also inhibits salicylic acid-mediated virus resistance (141). Other types of viral suppressors with undefined biochemical activities are also known (128). These findings not only provide the strongest support that PTGS functions as a natural, antiviral defense mechanism, but also offer valuable tools for dissecting the biochemical pathways of PTGS (128).

The PTGS degradative machinery can both detect and inactivate repetitive DNA sequences, suggesting it controls the expansion of repetitive elements, including endogenous genes (18). Although RNAi occurs in mammals and mammalian cell cultures, its role in animal virus protection is not clear. In mammals, dsRNA induces RNAi as well as interferon-mediated nonspecific RNA degradation and other nonspecific responses leading to blockage in protein synthesis and cell death (2). Thus, mammals seem to have evolved multiple mechanisms to detect and target dsRNA and to fight viruses. These various mechanisms may have different specificities or can function in distinct tissues or during development (210). A few other roles of RNAi in development and genome maintenance will be discussed in later sections.

## MECHANISTIC DIFFERENCES AMONG THE BIOSYNTHETIC PATHWAYS OF siRNA

Although the functional parallelism of gene silencing is quite apparent in plants and animals, a few unique attributes separate the pathways in these groups. For example, systemic spreading of the RNAi reaction from the site of initiation is known to occur in plants and worms (74, 79), but not in flies or mammals. The noteworthy distinct molecules that have been identified to cause differences at the pre-Dicer, Dicer, and post-Dicer stages of gene silencing pathways are mentioned below.

**Pre-Dicer stage.** Plant proteins such as SGS2 (RdRP), SGS3 (coiled protein), AGO1 (responsible for plant development), and HEN1 (enhancer of floral *hua1* mutation) are required for

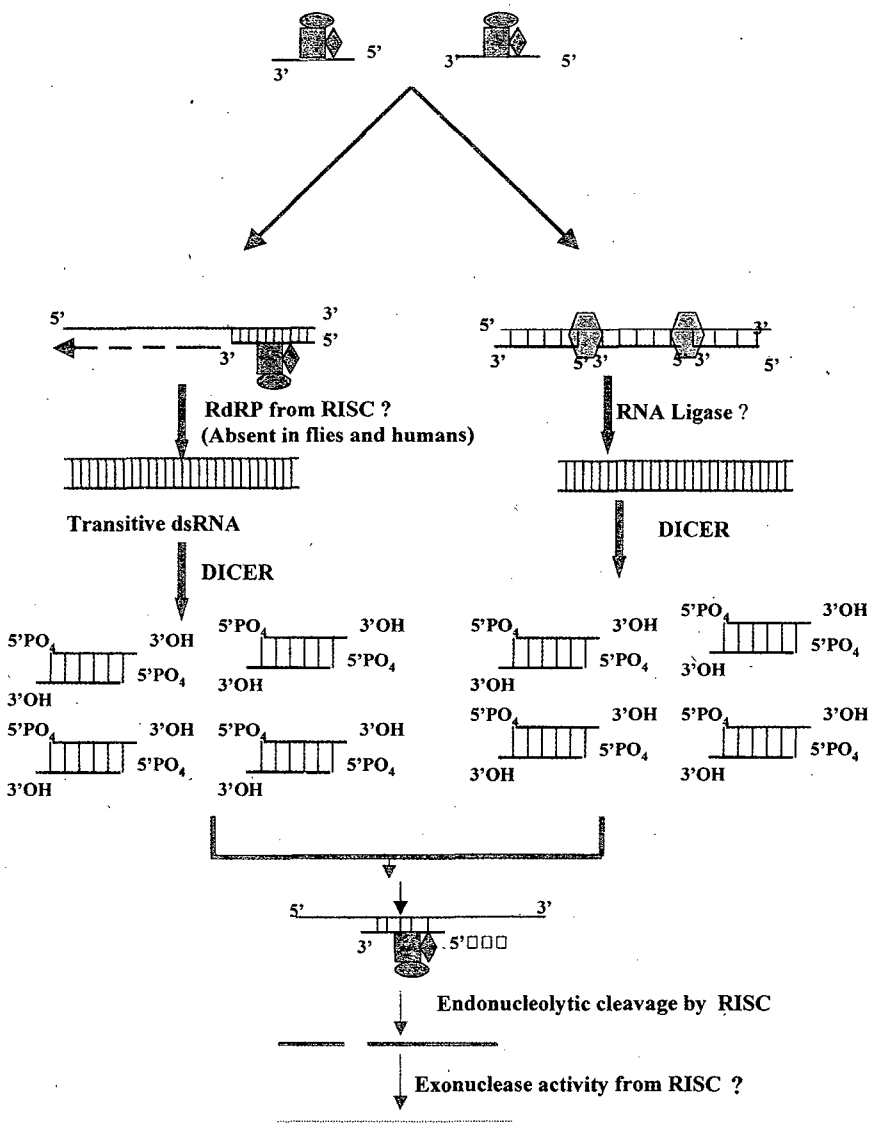



FIG. 2—*Continued.*

PTGS activities induced by the sense transgenes. But if the transgenes are in the form of hairpins expressing the panhandle dsRNA, the absence of or defects in the above-mentioned proteins do not play any role in altering the PTGS/cosuppression function. Hence, those proteins supposedly play a role upstream of dicing of dsRNA and may be involved in the formation and stabilization of dsRNA (22).

Homologues of SGS3 are unknown beyond the plant world. Even though HEN1 analogs are known in bacteria, yeasts, and animals, their roles in sense PTGS have not yet been identified. Likewise, SGS2 homologues are known in *C. elegans*, *N. crassa*, and *Dictyostelium discoideum*, but their roles at the pre-dicing stage have not been established yet in those systems. The equivalents of SGS2 in other animal systems are nonexistent both structurally and functionally (205). The role of worm AGO1 protein, i.e., RDE1, is also unique, as described earlier. AGO1 homologues are present in all eukaryotes, but they mostly function as a component (AGO2) of the animal RISC complex (32). The plant HEN1 protein is believed to be nuclear because of the presence of the nuclear localization signal at its N-terminal region (40). Since HEN1 is essential for plant PTGS (cosuppression), which is supposedly a cytoplasmic activity, the exploration of its subcellular distribution is of utmost importance. Boutet et al. (22) speculated that HEN1 could be a dsRNA stabilizing protein, and since many such proteins are known in the animal kingdom, it would be of interest to find animal analogs of the plant HEN1 protein. In fungus as well as the animal system, sense transgene-induced PTGS phenomena are known, but the machinery operative at the pre-dicing stage is still elusive.

The roles of plant SGS2, SGS3, AGO1, and HEN1 proteins may be limited at the stage of production of dsRNA from the transcript of sense transgenes, but no mechanism has been established regarding the presentation of the dsRNA to Dicer for the generation of siRNA. However, such a mechanism has been reported in *C. elegans*. The RDE4 and RDE1 (AGO1)

proteins of *C. elegans* were reported as initiators of RNAi and speculated to have no mechanistic role in the downstream processes of RNAi (87, 203). Unlike the *Arabidopsis* AGO1 and HEN1 proteins, RDE4 and RDE1 proteins are required for RNAi even when the dsRNAs are produced intracellularly in transgenic worms (203), but the defects in RDE4 and RDE1 are of no consequence if exogenous siRNAs or short antisense RNAs drive the RNAi reaction (208). RDE4 binds tightly to dsRNA (during the RNAi reaction) by virtue of its two RNA-binding domains and is always found in a tight complex with RDE1 protein even in absence of the RNAi reaction. During RNAi, RDE4 is found in a complex with RDE1, Dicer (DCR1), and a conserved DEXH-box RNA helicase (DRH1/DRH2). Based on these observations and other genetic evidence, Tabara and coworkers postulated that RDE4 and RDE1 functioned together to detect and retain foreign dsRNA and present the dsRNA to DCR1 for processing into siRNAs (202). Analogs of the RDE4 and DRH proteins are found in many eukaryotes, including plants and humans, but their roles have not been defined yet.

**Dicer stage.** The plant Dicer responsible for biosynthesis of plant siRNA is not known yet, whereas the Dicers of *C. elegans*, *D. melanogaster*, and humans as effectors for siRNA have been well characterized. The *A. thaliana* and rice genomes both encode at least seven RNase III-like proteins, of which at least four are putative homologues of Dicer, conveniently called DCLs (i.e., DCL1, DCL2, DCL3, and DCL4). The genetic evidence rules out that the *Arabidopsis* DCL1 (or CAF1) could be competent for siRNA formation (76). The roles of other DCL proteins are still to be revealed.

Interestingly, both in vivo and in vitro data suggest that the end products of plant dicing activities are different from those of the animal Dicers. When uniformly $^{32}$P-labeled dsRNA was incubated with wheat germ extract, Zamore et al. (205) found that the dsRNA was chopped into siRNAs of two discrete size classes, one ≈21 nucleotides and the other 24 to 25 nucleotides long, whereas *D. melanogaster* and human Dicers generated only the 21-nucleotide siRNAs. Two similar size classes were also produced with cauliflower extract and were found independently in the set of 423 endogenous small RNAs cloned from *A. thaliana*. Thus, in plants, dicing activity leads to the generation of two distinct classes of siRNAs.

With specific synthetic siRNAs that supposedly bind tightly to and inhibit Dicer as competitors, Zamore et al. concluded that a different Dicer-like enzyme was responsible for the generation of each class of siRNA. These two distinct classes of siRNAs were reported first in vivo from transgenic plants bearing the silenced GFP sense transgenes (94). With an array of plant virus-encoded suppressors of gene silencing, Baulcombe et al. proposed that the 21-mer siRNAs controlled localized PTGS via mRNA degradation and the 24-mer siRNAs triggered systemic silencing and methylation of the homologous DNA. It remains to be seen whether this kind of dual dicing activity reflects any novel pathway intrinsic to plant RNAi. Interestingly, the longer (≈25-mer) siRNAs have also been detected in the natural RNAi biology of *Trypanosoma brucei* (61).

**Post-Dicer stage.** RISC has been isolated from *D. melanogaster*, *C. elegans*, and humans, and only some of its components have been characterized biochemically and genetically. Both mammalian and *Drosophila* RISC contain AGO2 proteins, whereas the GEMIN3 (a DEAD box helicase) and GEMIN4 proteins are found only in mammalian RISC (103). Similarly, dFXR, a homologue of the human fragile X mental retardation protein, is found only in *Drosophila* RISC (35). However, there is no report on the isolation of RISC in plants. Hence, mechanistically little is known about postdicing activity, especially in plants. A worthwhile question to address is whether there is any anchoring site for the occurrence of RNAi in the cytoplasm. Recently, it was reported that *E. coli* RNase III binds to the 70S ribosome and is functionally modified after binding (6). It is widely believed that the RISC associates with eukaryotic ribosomes (96). Hence, the exploration of ribosome association of the RNAi activities, especially of dicing and postdicing leading to mRNA degradation, might shed light on RNAi mechanisms in the future. The various affinities of ribosome-binding complexes might also reveal interesting system-specific features.

RdRP-dependent siRNA amplification and systemic spreading from the site of origin is another area where many system-specific variations have been noticed. RdRP homologues are not present in many organisms, so the mechanisms by which sense transgene-mediated PTGS are effected in those organisms remain a mystery (98). In other systems where RdRP is present, the biochemical steps and details of siRNA amplification may not necessarily be the same.

In *C. elegans*, RRF1 (a putative homologue of RdRP), along with other proteins, is required for RNAi even when the trigger dsRNA is expressed directly from the hairpin transgene in the nuclei of somatic tissues, whereas SGS2 (*Arabidopsis* RdRP) is dispensable for PTGS activity if induced directly by hairpin sense transgenes in *A. thaliana*. This suggests that the RdRP-mediated putative amplification steps of worms are different from those of plants (37). In plants, the SGS2-dependent spreading of silencing occurs from the region homologous to the trigger dsRNA into both the adjacent nonhomologous 5′ and 3′ regions of a target transgene (214). In contrast, spreading occurs only in the 5′ region in worms and fungi, which is consistent with the primer-dependent 5′-3′ copying activity of RdRP. Hence, in plants, the spread of silencing requires other activities (such as chromatin modification) in addition to that of RdRP (37).

In worms, tissue-specific variations of RdRP-dependent RNAi have also been reported, but not in plants or other systems. EGO1 is essential for RNAi in the germ line of *C. elegans*, whereas another RdRP homologue, RRF1, is required for silencing in soma (193, 197). Another intriguing observation is that the loss of function of RRF3 (third putative RdRP of worms) is responsible for the enhancement of sensitivity to RNAi in several tissues of *C. elegans*. Here, RRF3 acts as a negative regulator of RNAi, a fact difficult to reconcile with the postulated activity of RdRP (195). For systemic transmission of gene silencing, the membrane-bound SID1 protein of *C. elegans* and the plasmodesmatal connections of plants are implicated, but in both cases, the molecular nature of the moving signal has not been ascertained yet. An association between Dicer and the RdRP has been suspected in the case of *Dictyostelium discoideum* and *C. elegans*, but conclusive evidence is still lacking (37).

## siRNA: SYNTHESIS, DELIVERY, AND GENE KNOCKDOWN

The natural RNAi biology of eukaryotic cells offers a protection mechanism against foreign nucleic acids; however, only in the recent past has the exploitation of its mechanistic details sparked a revolution in the investigation of cellular gene functions. Transcriptional regulation with the dsRNA technology provides an easy means to identify cellular characteristics in response to both internal and external cues. However, the application of RNAi in higher eukaryotes, particularly mammalian cells, has been hampered by the presence of a number of dsRNA-triggered pathways that mediate nonspecific suppression of gene expression (152). These nonspecific responses to dsRNA are not triggered by dsRNAs shorter than 30 bp, including the siRNA duplexes. Moreover, studies in *C. elegans* and *D. melanogaster* have clearly demonstrated that synthetic siRNAs can produce effects similar to those of the long dsRNAs (69, 236). Based on these experimental analyses, siRNAs are now being optimized for systematic exploration of the function of genes in a variety of organisms.

Prior to the siRNA era, approaches such as gene targeting by homologous recombination, ribozymes, and antisense technologies were commonly used to determine gene functions. All such approaches have their limitations, and none can be applied universally (201). The dawn of siRNA-directed knockdown approaches facilitated studies of gene function in a rapid and inexpensive way. This siRNA technology has the potential to decipher the function of virtually any gene that is expressed in a cell type- or pathway-specific manner. In the span of only a few years, large-scale functional analysis of almost all the ≈19,000 genes of *C. elegans* has been carried out with the siRNA-directed knockdown approach. A fairly detailed account of this technology has recently been reviewed by Dykxhoorn et al. (66).

Here, some of the salient aspects of the technology are summarized. In brief, the application of siRNA for gene silencing involves a careful consideration of the following variables: (i) selecting the siRNA sequence in the target gene; (ii) synthesis of siRNAs or construction of plasmids bearing DNA sequence encoding for siRNAs; (iii) optimizing transfection of the siRNAs or the plasmids expressing siRNAs in the target cells; and (iv) monitoring the efficacy of gene silencing.

### Selection and Generation of siRNA

Several siRNAs synthesized against different regions of the same target mRNA show different silencing efficiencies (101). A number of groups have analyzed several parameters for optimizing siRNA-induced gene silencing, and these include the length, secondary structure, sugar backbone, and sequence specificity of the siRNA duplex. The efficacy of these parameters has been tested on several occasions for induction of RNAi in *D. melanogaster* and human cells (69, 189). No consensus on choosing the siRNA sequence has evolved. A line of thinking seems to suggest the following. The sequence should be selected in the region 50 to 100 bp downstream of the start codon. The 5′ or 3′ untranslated regions and regions near the start codon should be avoided, assuming that untranslated region-binding proteins and translation initiation complexes may interfere with the binding of siRNP or RISC endonuclease complex. The GC content of the siRNAs should be kept between 30 and 70%. The computer programs developed by Lin (Jack Lin's siRNA sequence finder; www.lc.sunysb.edu/stu/shiklin/rnai.html) and by Ambion (www.ambion.com) offer helpful guidelines to select potential siRNA sequences and determine whether these selected sequences match mRNA sequences other than those of intended target.

Based on different experimental approaches, a few guidelines have been laid for the synthesis of siRNAs. A general rule is that the sequence of one strand should be $AA(N_{19})TT$, where N is any nucleotide, i.e., these siRNAs should have a 2-nucleotide 3′ overhang of uridine residues. The siRNAs should be 21 nucleotides long. The siRNAs should have 5′-phosphate and 3′-hydroxyl group for efficiency. Compared to antisense or ribozyme technology, the secondary structure of the target mRNA does not appear to have a strong effect on silencing. The 21-nucleotide siRNAs can be chemically synthesized with appropriately protected ribonucleoside phosphoramidites and a conventional synthesizer and thus are widely available commercially. However, the use of chemically synthesized siRNA in RNAi has been restricted because of the high synthesis cost. Due to the paucity of information on the selection of siRNAs and their structures, these general guidelines are suggestive and do not guarantee the silencing effect. To overcome the siRNA selection ambiguity, Yang et al. (235) incubated dsRNA with the *E. coli* RNase III enzyme to generate a random array of siRNAs. The introduction of such a reaction soup resulted in the silencing of the target gene.

The exorbitant cost of synthesizing siRNAs and their lack of amplification in mammalian cells have compelled investigators to explore alternative strategies to generate a continuous supply of a battery of siRNAs. Several groups have devised strategies to synthesize short RNAs in vitro (64) or by introducing plasmids with the ability to make de novo siRNAs inside the cell (235, 239). DNA-based plasmid vectors have been designed by cloning siRNA templates downstream of an RNA polymerase III transcription unit, which normally encodes the small nuclear RNA U6 or human RNase H1.

Two approaches have been developed for expressing siRNAs. In the first, sense and antisense strands constituting the siRNA duplex are transcribed by individual promoters (64), and in the second, siRNAs are expressed as fold-back stem-loop structures that give rise to siRNAs with a small loop. A stretch of four to five thymidines is added at the end to the siRNA template that acts as a transcription termination signal. Many of these plasmid-based vectors, such as pSilencer 1.0 (Ambion) and pSuper (DNA Engine), are now commercially available. These vectors provide advantages over chemically synthesized siRNAs, but use of these plasmid vectors also remains limited due to numerous disadvantages, including the transient nature of siRNA expression and low as well as variable transfection efficiency.

To circumvent these problems. virus-based high-efficiency siRNA delivery systems are also being developed. A retrovirus-based system developed by Devree and Silver (59) is cited here as an example. The U6 promoter along with the siRNA-generating hairpin construct was cloned upstream of the 3′ long terminal repeat of the commercially available pMSCV-puro vector. The in vitro-packaged recombinant virus was allowed to

transfect HeLa cells with high efficiency in the presence of puromycin selection, and a dramatic downregulation of the target gene product was observed. A downregulation of this extent was not possible with the plasmid-based delivery system. Such virus-based vectors or their improved variants hold the promise to efficiently detect the function of any gene in virtually any cell type, provided that the production of recombinant virus is not a limitation.

Most of the siRNA expression vectors produced to date use RNA polymerase III regulatory units, which do not allow tissue-specific siRNA expression. However, Shiagawa and Ishiid reported a polymerase II promoter-based plasmid encoding a dsRNA expression system that could eventually express siRNA in a tissue-specific manner (192). In their novel scheme, a pDECAP vector was used, which expressed long dsRNAs corresponding to the *ski* gene (encoding a transcriptional repressor) in the form of a hairpin. The engineered hairpin RNA expressed from a cytomegalovirus promoter lacked the 7-methylguanosine cap structure at its 5′ end and a poly(A) tail at its 3′ end. The transcript of such a design did not exit the nucleus to reach the cytoplasm and thus prevent the interferon pathway-mediated nonspecific antiviral response. The double-stranded transcript was diced in the nucleus, and the siRNAs were subsequently released into the cytoplasm to mediate the gene-specific silencing. The silencing was specific, since the level of a related protein, SNO, remained unaffected.

The same vector was also used to create *ski* knockdown mice, the phenotype of which was similar to that of *ski* knockout embryos, which exhibited defects in neural tube and eye formation. Later generations of such vectors may use more tissue-specific *cis*-acting elements in the employed promoter to stringently knock down gene functions in the animal system. It is pertinent to highlight here that because plants do not elicit an interferon-mediated antiviral response, the dsRNA/siRNA delivery system need not be as complex as the pDECAP system.

### Transfection of siRNA and Detection of Gene Silencing

An attempt to understand a gene's function in diverse organisms necessitates optimization of protocols for efficient delivery of siRNAs into cells. A number of transfection reagents are being employed for transfecting siRNA into different cell lines. Lipofectamine 2000 and Oligofectamine (Invitrogen) are being routinely used for siRNA delivery. A few newer transfection reagents such as TransIT-TKO (Mirus) and Ambion's Siport Amine and Siport, have also been used successfully in cultured cell lines. Electroporation has been used to transfect siRNAs in cell lines as well as in parasites such as *Trypanosoma brucei* and *Plasmodium falciparum* (150, 213). In adult mice, naked siRNAs have been delivered by hydrodynamic transfection methods to combat hepatitis C virus infection in the intact liver (151). The transfecting siRNAs have been used successfully for studying the role of proteins in DNA damage response and cell cycle control, general cell cycle metabolism, signaling, the cytoskeleton and its rearrangement during mitosis, membrane trafficking, transcription, and DNA methylation (211). These molecules have also been used to differentiate between housekeeping and other genes (112).

The preferred way to detect specific gene knockdown by RNAi is to study the depletion of the target protein by immunofluorescence and Western blotting with the specific antibody. In addition, the knockdown phenotype and Northern blot analysis can also be used to detect the effects of siRNA. If the gene is essential, cellular growth is delayed or arrested, and [$^3$H]thymidine uptake can also be used to assign the function of a particular gene (70).

### siRNA Introduction into Plants

siRNAs have been delivered into tobacco plants by biolistic pressure to cause silencing of GFP expression. Silencing occasionally was detected as early as a day after bombardment, and it continued to potentiate up to 3 to 4 days postbombardment. Systemic spread of silencing occurred 2 weeks later to manifest in the vascular tissues of the nonbombarded leaves that were closest to the bombarded ones. After a month or so, the loss of GFP expression was seen in nonvascular tissues as well. RNA blot hybridization with systemic leaves indicated that the biolistically delivered siRNAs induced the de novo formation of siRNAs, which accumulated to cause systemic silencing (118).

## MICRO-RNA

Since the RNAi machinery is present constitutively within eukaryotic cells, it is important to explore and understand the metabolic advantages that are accorded by RNAi-related proteins during the intrinsic normal growth of cells and development of organisms. The natural RNAi machinery not only keeps the mobile transposable elements from disrupting the integrity of genomes, as was suggested by analyses in lower plants, *A. thaliana*, *C. elegans*, *D. melanogaster*, and animals (9, 94, 138, 203, 232), but also participates in organism development. Genetic defects in *C. elegans* RNAi genes *ego1* and *dicer* cause known, specific developmental errors (87, 119, 197). Similarly, the Argonaute family of genes of *A. thaliana* (especially the ZWILLE proteins) is also responsible for plant architecture and meristem development (32), and the Dicer homologue of *A. thaliana*, CAF1, is required for embryo development (83). Thus, genetic evidence illustrates the role of the RNAi machinery as a controller of development-related genes. The mechanistic details of these developmental processes are beginning to emerge.

In 1991, Ambros and coworkers first isolated a *lin4* mutant of *C. elegans* which was arrested at the first larval stage (127). Later on, the *let7* mutation was isolated in the same system, which was responsible for development through the fourth larval stage. Both *lin4* and *let7* encode short 22-nucleotide mature RNAs and were called short temporal RNA because they control the temporal development program of *C. elegans*. The mature *lin4* RNA defines (negatively regulates) the mRNA expression of the *lin14* and *lin28* heterochronic genes with the antisense-mediated repression mechanism of translation initiation and thus specifies the fate of cells during the first three larval stages. Recent studies have revealed that the short temporal RNAs are actually members of a group of tiny RNAs (21 to 28 nucleotides) called the micro-RNAs, isolated members of which could easily run to a few hundreds. Some of the components of the RNAi machinery have also been clearly

established as the effector proteins for the maturation of micro-RNAs.

### Identification and Biogenesis

A range of biochemical techniques have been applied to clone the 21- to 28-nucleotide RNAs that are present during the normal cellular development of many organisms, for exploring the abundance and complexity of micro-RNA. Micro-RNAs have been found to be abundant and phylogenetically extensive in plants, flies, worms, and humans. In *D. melanogaster*, *C. elegans*, plants, and humans, more than 600 micro-RNAs have been identified (123, 125, 126, 137). Bioinformatic analyses of the complete genome sequences have been extremely useful for identification studies. The genome sequences of a variety of organisms revealed the authenticity of these micro-RNAs, the nature of the precursor RNAs, the genomic locations of micro-RNA genes, and the evolutionarily conserved character of some of these micro-RNAs. With the RNA folding program mfold (148) and Northern analyses of micro-RNA, it has been universally inferred that most micro-RNAs arise from the imperfectly annealed 70-nucleotide hairpin precursor RNA whose expression is often developmentally regulated. These micro-RNAs are thus predicted to be processed from multiple bulged and partially duplex precursors, like the short temporal RNA precursors (186).

The identification of micro-RNAs is the first major hurdle in micro-RNA-related research. The first step in computational identification of micro-RNAs from genome sequences is identification of sequences forming hairpin loops (stem-loop sequences). For this purpose, software such as srnloop (85) and RNA fold (130) is used. These are Blast-like software packages which identify short complementary sequences within a specified distance on the genome. The hairpin sequences obtained by this analysis are then evaluated as candidate micro-RNAs based on different criteria, such as GC content and minimum free energy, and by passing through different filters, such as short-repeat filters and structure quality filters (85).

Another important criterion that has been used for the identification of candidate micro-RNAs is the correspondence of a hairpin of one species with that of another species. Two hairpins are said to be in correspondence if a short sequence (>19 nucleotides) in the stem of one hairpin is also present in the stem of another hairpin, although the two hairpins may have otherwise variable sequences. If a hairpin from one species has correspondence with a hairpin from one or more other species, this strengthens its status as a candidate to be a micro-RNA. The homology of hairpins with known micro-RNAs is also considered a useful criterion to select candidate micro-RNAs.

D. P. Bartel's laboratory has developed a computational procedure, called MiRscan, to identify micro-RNAs based on their homology to known micro-RNAs with respect to the characteristic features in the stem region. MiRscan evaluates the stem-loops by passing a 21-nucleotide window along the stem region and assigning a likelihood score to each window that measures how well its attributes resemble those of the previously experimentally identified and validated micro-RNAs (129, 130). The candidate micro-RNAs identified by these procedures are experimentally validated by Northern blot assay of total small RNAs with the stem region of the candidate as a probe or by a more sensitive PCR assay of the amplified small RNA library (85, 130). Detection of a 21- to 24-nucleotide band in these assays validates a candidate micro-RNA, whereas a ≈70-nucleotide band is detected in Dicer-deficient mutants, further confirming that the micro-RNAs arise from a ≈70-nucleotide precursor.

An analysis of micro-RNA expression in cell lines and tissues suggests cell- or tissue-specific expression. For example, micro-RNA 1 (miR1) is specifically expressed in human heart tissues and stage-specifically in mouse embryogenesis (126). *A. thaliana* small RNA 39 is detected exclusively in inflorescence tissues and downregulates the expression of a Scarecrow-like transcription factors (137, 139). Considering the diverse functions in which micro-RNAs have been implicated, micro-RNAs have also been named variously, i.e., micro-RNAs which mediate spatial development are referred to as sdRNAs, while cell cycle micro-RNAs are referred to as ccRNAs, etc. The regulated expression patterns of these micro-RNAs are suggestive of their functions in developmental control. However, many micro-RNAs are uniformly expressed, suggesting their role in general gene regulation (186). Downregulation of micro-RNAs leads to serious developmental defects, as evidenced by isolation of various micro-RNA mutants. Recent reports reveal that *miR15* and *miR16* are located in human chromosome 13q14, a region which gets deleted in more than half of B-cell chronic lymphocytic leukemias. Detailed deletion and expression analyses point out that these two micro-RNAs are located within a 30-kb region of loss in chronic lymphocytic leukemias, and both genes are deleted or downregulated in a majority (≈68%) of chronic lymphocytic leukemia cases (30).

A majority of micro-RNAs occur in relatively short (≈70-nucleotide) and single stem-loop precursor structures. However, in both animals and plants, some micro-RNAs are arranged in clusters. The genes in the tandem clusters are coexpressed, for example, in the germ line and early embryos of *C. elegans* and *D. melanogaster* (123, 125) and in the inflorescence tissues of *A. thaliana* (137). A set of seven highly related *C. elegans* micro-RNA genes that are coexpressed are so tightly clustered within 1-kb region that they are predicted to form a precursor from which all the seven mature micro-RNAs are processed (186). Similarly, several micro-RNAs originate from each of the five chromosomes of *A. thaliana* containing clusters of two to four micro-RNAs spaced irregularly within the intergenic region. Interestingly, three of the clusters contain micro-RNA sequences of both sense and antisense polarities, a scenario not found in the animal system yet. Such variations in the precursor structures of micro-RNAs may point towards distinct mechanisms of biosynthesis of micro-RNAs, although all micro-RNAs originate by transcription events that are independent of adjacent conventional genes.

### Apoptosis-Related Micro-RNA

The proliferation of tissues and organs of any organism requires careful coordination between cell proliferation and cellular death. The proliferation processes of a cell include active inhibition of the apoptotic process. Recently, two micro-RNA genes, *bantam* and *mir14*, that suppress cell death by inhibiting the translation of apoptotic messages have been isolated from *D. melanogaster*. Expression of the *bantam* 21-nu-

cleotide micro-RNA is temporally and spatially regulated in response to patterning cues. The proapoptosis gene *hid* has been identified as a target for regulation by *bantam* micro-RNA (24). *bantam* deletion mutants grow poorly and die as early pupae, whereas *mir14* mutants are viable but stress sensitive and cursed with a reduced life span. The *mir14* suppresses death induced by expression of Rpr, Hid, Grim, or the apical caspase Dronc (234). *mir14* also regulates fat metabolism by decreasing the levels of triacylglycerol and diacylglycerol. *bantam* is related to *mir80* and *mir82* of *C. elegans*, indicating that the *mir80* family of RNAs might be involved in apoptosis in worms (33). Identification of these micro-RNAs promises discovery of similar micro-RNAs in other systems and reveals the hidden treasure of knowledge relating to micro-RNA-controlled biological functions (11).

### Kinship of siRNA- and Micro-RNA-Related Pathways

Since micro-RNAs are derived from their precursor dsRNAs and are similar in size to siRNAs, the biogenesis of siRNAs and micro-RNAs is similar. In fact, both siRNAs and micro-RNAs are processed by Dicer activities in animals as well as in plants (86, 96, 104, 115, 240). Human recombinant Dicer can process pre-*let7* RNA to mature *let7* quite efficiently in vitro (175). Recent work by D. P. Bartel's group (181) has also shown that *caf1* (*dicer* homologue) mutants of *A. thaliana* fail to process micro-RNAs. The genetic and biochemical data point toward interaction between Dicer and the Argonaute group of proteins in *C. elegans* and *D. melanogaster* for processing the micro-RNAs (86, 95). The similar interaction is possibly also present in plants between Dicer on one hand and PNH (zwille/pinhead) on the other to generate plant micro-RNAs (83). Additionally, both forms of small RNA, micro-RNAs and siRNAs, were found integrally associated with riboprotein complexes containing a member of the PIWI/PAZ domain family, siRNAs in the RISC and micro-RNAs in the microribonucleoprotein complexes (96).

In fact, recent evidence suggests that at least for some micro-RNAs, the microribonucleoprotein and the RISC complex could be the same entity (103, 137, 139, 182). Though the same or similar DCR and subsequent ribonucleocomplexes are required to process mature forms of the micro-RNAs, in some cases, such as *C. elegans lin4* and *let7*, the ≈22-nucleotide form is processed from the 5′ part of the stem, and in other cases, such as *miR1* and *miR58*, maturation results from the 3′ part of the precursors. Thus, there is a gene specificity of micro-RNA processing and/or stabilization (126). Since the biosynthetic pathways of micro-RNAs and siRNAs are somewhat similar, the viral suppressors that inhibit siRNA formation are also expected to interfere in the biogenesis of micro-RNAs. A detailed understanding of this suppression process may unravel the hitherto unknown molecular basis of virus-induced development-related diseases in eukaryotes, especially in plants.

An RNA-silencing suppressor, PI/HC-PRO of turnip mosaic virus, induces a number of developmental defects in the vegetative and reproductive organs of *A. thaliana*. Many of these defects are reminiscent of observed defects in Dicer-like mutants of *A. thaliana*. The PI/HC-PRO suppressor interferes with the formation of *miR171*, and as a result the downstream target mRNAs accumulate instead of being cleaved, causing developmental errors (113). Thus, it is interesting that the counterdefensive strategy of the viruses has evolved not only to protect the viral RNA genome from the host degradative machinery but also to subvert the cellular development program in favor of the virus.

However, it is important to mark the distinctions among the pathways leading to the formation as well as the activities of siRNAs and micro-RNAs. Although over 600 micro-RNAs from various organisms have been identified (33) only about 3% of them are fully complementary to the target mRNA sequences. All known micro-RNAs are derived in vivo from dsRNA precursors which are imperfectly annealed. Since the biosynthesis and activities of the micro-RNAs do not require perfect complementarity, noncanonical pathways of RNAi may be involved for the micro-RNAs because the usual RNAi calls for extensive complementarity of the dsRNA. It is only because of this characteristic mismatch between the sequences of micro-RNA and cognate mRNA that the in silico identification of the target mRNA is so difficult (182). The imperfect nature of annealing between the two partners is viewed as the prime cause for translational repression of the target mRNA (172).

Second, the mature micro-RNAs are always found in the single-stranded conformation in nature for some unknown reason, whereas siRNAs are double-stranded when detected. Third, unlike siRNAs, micro-RNAs enter riboprotein complexes with differing PPD proteins (PAZ and Piwi domains), depending on the specificity of the micro-RNA or its precursor with the cognate PPD proteins (86). The sequence or structure of a micro-RNA or its precursor might ensure that it functions as a translational repressor and not as a trigger of RNAi. It is widely speculated that the siRNAs and micro-RNAs are distinguished following their biosyntheses, and these two are then allowed to form related but distinct ribonucleoprotein complexes that target downstream substrates for degradation or translation repression, respectively. This hypothesis is based on the observation that siRNAs or exogenously supplied hairpin RNAs containing even a single mismatch with their substrate fail to repress the target mRNAs and do not simply shift their regulatory mode to translation inhibition (98).

Fourth, a viral suppressor of RNA silencing, the HC-PRO protein of potato virus Y, has been found to differentially regulate the accumulation of siRNAs and micro-RNAs in tobacco (144). The HC-PRO protein prevents accumulation of siRNAs of the silenced genes and thus releases silencing in a universal manner, but the same protein helps accumulation of all micro-RNAs tested, namely, *miR167*, *miR164*, and *miR156* of tobacco, in vivo. This result indicates that the dicing complexes for siRNA and micro-RNA may not be exactly similar in biochemical features, and as a result the biochemical functions of the complexes are different in response to this particular HC-PRO protein. Lastly, not all RNAi pathway mutants are developmentally aberrant, whereas micro-RNA pathway mutants are expected to be defective in organism architecture and development. For example, an RNA-dependent RNA polymerase-defective mutant of *A. thaliana*, the *sgs2/sde1* mutant, shows defects only in cosuppression phenomena (a form of RNAi) but is perfectly normal in phenotypic development (54, 160). This observation raises the question of whether the

RdRP-dependent amplification step is required at all for micro-RNAs.

### Functional Classifications

A number of micro-RNAs, including *let7*, are conserved across all organisms throughout evolution. About 12% of the micro-RNAs identified so far in animal systems are conserved at least among nematodes, *D. melanogaster*, and humans. Interestingly, a majority of the micro-RNAs are speculated to control development-related genes. However, the mechanisms of such control are not quite established yet. Micro-RNAs probably employ a variety of mechanisms to downregulate target genes. Micro-RNAs such as *C. elegans lin4* and *let7* have been shown to imperfectly anneal to the 3′ untranslated region of the target mRNA.

A vast majority of micro-RNAs probably belongs to this category. Due to imperfect complementarity, some micro-RNAs may also anneal to a host of different target mRNAs either simultaneously or in a temporally controlled manner. On the other hand, there are micro-RNAs, located mostly in the *A. thaliana* intergenic region, which have perfect or nearly perfect complementarity to the target mRNAs. Such micro-RNAs might trigger site-specific cleavage of the mRNA after being incorporated into a functional RISC-like complex. In such a situation, micro-RNAs act like siRNAs. The *A. thaliana* inflorescence-specific small RNA 39 cleaves the middle part of mRNA of the three scarecrow gene family members in a similar fashion (137).

On the basis of nearly perfect complementarity with the micro-RNAs, numerous *A. thaliana* mRNA targets have been predicted, and these targets have also been phylogenetically conserved in rice. Fifteen cleavage-type targets were validated recently by in vitro or in vivo micro-RNA-guided cleavage assays. The majority of these predicted mRNA targets encode members of large family of transcription factors, including Phavoluta (PHV), Phabulosa (PHB), cup-shaped cotyledon 1 (CUC1), CUC2, etc. These transcription factors are required for meristem identity, cell division, organ separation, and organ polarity (33). On the other hand, *mir172* likely acts in cell fate specifications as a translational repressor of APETALA2 in *Arabidopsis* flower development (39).

There are other varieties of micro-RNA which also interact with target mRNAs affecting the posttranscriptional steps, such as RNA splicing, mRNA localization, and RNA turnover. In *D. melanogaster*, many micro-RNAs are known to be complementary to the 3′ untranslated region sequence motifs, which are responsible for mediating negative posttranscriptional regulation. These sequence motifs include the K box (CUGUGAUA), the B-rd box (AGCUUUA), and the recently found GY box (UGUCUUCC). All micro-RNAs showing complementarity to these motifs are expressed either broadly throughout development or in the narrow window of embryogenesis of *D. melanogaster* (124). It is possible to have even a fourth class of micro-RNAs, which may serve as guides for modification of chromosomal DNA and control the epigenetic processes of nuclear genomes (225).

### Genetic Diversity in Species-Specific Biosynthesis of Micro-RNA

The siRNA and micro-RNA pathways closely parallel each other. It has been mentioned earlier that the biosynthesis of siRNAs have interesting system-specific features. Hence, system-specific features of micro-RNAs would also be of no surprise. Here, we illustrate some of those features. In *C. elegans*, *D. melanogaster*, and other animals, the Dicer proteins responsible for siRNA formation are also involved in the biosynthesis of micro-RNAs; but in *A. thaliana*, DCL1 (or CAF1) is responsible for micro-RNA but not for siRNA formation (76). Interestingly, the DCL1 mRNA is predicted to be a micro-RNA target, indicating that the micro-RNA-related apparatus in plants is regulated by a negative feedback loop (233). In plants, HEN1 is required for both siRNA and micro-RNA formation (22). Such a role for HEN1 orthologues in other systems is not known yet. Both CAF1 and HEN1 have nuclear localization sequence signals, raising the question of whether plant micro-RNAs are made intranuclearly.

The functions of plant micro-RNAs may be different from those of their animal counterparts in some events. The animal micro-RNAs act as translational repressors, whereas some plant micro-RNAs act on the target mRNA posttranscriptionally, like siRNAs (139). In animals, the majority of the AGO family members tightly regulate the biosynthesis of micro-RNAs (32), whereas in plants, especially *A. thaliana*, only one member of the 10 constituents of the AGO family, Ziwelle, alone contributes to the synthesis of micro-RNA. Surprisingly, though *A. thaliana ago1* mutants show a strong hypermorphic phenotype, AGO1 protein is not responsible for plant micro-RNA formation (22). However, AGO1 is required for initiation of PTGS, whereas Zwielle is not. Recently, Vauchert et al. also isolated a few *ago1* alleles of *A. thaliana* which were hypomorphic in nature (157).

The biogenesis of some plant micro-RNAs seems to be different from that of their animal counterparts. Most of the *Arabidopsis* micro-RNAs belong to group1, as their precursor forms are detected poorly or not at all. Despite the absence or greatly reduced abundance of the mature micro-RNAs, accumulations of pre-micro-RNA are never detected in Dicer-defective *caf1* mutants (171). However, the pre-micro-RNAs accumulate to a higher level in *C. elegans* and metazoans in which Dicer activity is abolished or reduced (86). Very few *Arabidopsis* micro-RNAs belong to group II, including the micro-RNAs *miR176*, *-177*, *-178*, and *179*, the pre-micro-RNA transcripts of which are, however, detectable. The levels of these precursor transcripts do not change in either the *caf1* or *hen1* mutant background. Such facts indicate that even within the same plant, the biosynthesis pathways of micro-RNAs might vary depending on the particular micro-RNA. The tissue specificity of micro-RNAs is well known. Hence, micro-RNAs specific to tissues that are unique either to animals (e.g., brain) or plants (roots, for example) might exemplify variant pathways of biosynthesis of micro-RNAs.

The discovery of micro-RNAs has been branded one of the top discoveries in developmental molecular biology. The survey of micro-RNAs is still at a subsaturated stage. The future will witness the discovery of hundreds of new micro-RNAs and

their corresponding mRNA targets, and the mysteries of developmental pathways from embryogenesis to adulthood will be unfolded.

## SMALL-RNA-MEDIATED EFFECTS ON CHROMOSOMAL DNA

The siRNAs work not only at the posttranscriptional stage but also leave their indelible marks on the genomes to repress the gene transcription activity or selectively remove portions of the genomes, especially of protozoans. These stunning discoveries have been reported only in the span of the last 2 years, the detailed mechanisms of which are still to be revealed and have been reviewed in two recent articles (57, 109). In the present review, we describe these effects briefly with special emphasis on plant systems, since the genetics and biochemistry of some of these processes are better illustrated in plants.

Broadly speaking, the siRNAs bring about three different biochemical end products with the chromatin DNA: DNA methylation, as revealed mostly in plant systems; heterochromatin formation; and programmed elimination of DNA. DNA methylation had been reckoned a major source of transcriptional gene silencing (TGS), and mechanistically TGS had been viewed very distinctively from PTGS in the past. But recent developments have caused a blurring in the identity between these two pathways (218), and some of these developments will be highlighted below. The discoveries of such epigenetic changes have ignited a revolution not only in the field of gene regulation but also in gene maintenance and gene evolution.

### RNA-Dependent DNA Methylation

A role for RNA in guiding de novo cytosine methylation of homologous DNA sequences was first discovered in viriod-infected plants and subsequently also in nonpathogenic plant systems (194). When the dsRNA degradation mediated PTGS occurs in plants, the genomic DNA regions homologous to dsRNA are often found methylated at almost all the sensitive cytosine residues. This process is generally referred to as RNA-dependent-DNA methylation and the corresponding part of the genome, especially the promoter region might remain transcriptionally silent. The initiator of RNA-dependent DNA methylation/TGS could be either the transgene-derived dsRNA or the consequent siRNA (110, 111, 214). Depending on the sequence information of the dsRNA, RNA-dependent DNA methylation was found to occur at the open reading frame and/or the promoter region of the genome (10, 149). If methylation occurred only at the open reading frame, TGS did not result. However, RNA-dependent DNA methylation at the promoter sequences induced TGS, which, unlike PTGS, was stable and heritable (98). RNA-dependent DNA methylation within the host genes has also been found to occur preponderantly during virus-induced gene silencing, a type of RNAi that is generally initiated by plant virus vectors carrying portions of host genes, as has been described earlier (214).

It was demonstrated that the movement of transposons was controlled by transcriptional suppression (TGS) and that methylation also played a role in this suppression, depending on the nature of the transposon (226). In animals and lower plants, siRNAs corresponding to the transposable elements were discovered and cloned earlier (9, 232), and in *A. thaliana* and *Nicotiana* species, the siRNAs corresponding to retroelements have recently been discovered (94). These siRNAs are perhaps responsible for the methylation of the homologous DNA.

There are also conflicting data in the literature concerning the cause-and-effect relationship between PTGS and DNA methylation. In some examples, there is no correlation between PTGS and DNA methylation (153). In other events, as mentioned earlier, the correlation is strong (137). Llave et al. (137) showed that a viral protein, HC-PRO, that suppresses PTGS/RNAi, when introduced into GUS-silenced tobacco, inhibited the maintenance of small RNAs and caused a concomitant decrease in methylation of the GUS sequence in the plant genome. This study suggested that DNA methylation of the silenced gene could be directly correlated with PTGS. However, in a contrasting study carried out by Mette et al. (153), HC-PRO was found to increase the methylation of a target promoter DNA when gene silencing was induced by the promoter dsRNA. The later study also revealed that the amount of promoter siRNA was elevated fivefold in the presence of HC-PRO. Taken together, both of these studies indicate that the level of target DNA methylation is directly related to the amount of siRNA present in the cell, and thus the apparent differences between these observations can be resolved. In other words, the availability of siRNA may determine the level of RNA-directed DNA methylation. In the events of RNA-dependent DNA methylation, the chromodomain containing DNA methylases acts either alone or in combination with other proteins, such as piwi-containing proteins, to form complexes with the siRNAs and cause sequence-specific RNA-dependent DNA methylation, finally resulting in TGS (10).

Evidence of cross talk between PTGS and TGS has been obtained from the mutational analysis of *A. thaliana* and *D. melanogaster*. Two types of *A. thaliana* mutants, *ddm1* (deficient in DNA methylation) and *met1* (methyl transferase), were isolated from a screen of mutations causing a reduction in global methylation of the genome. The locus *ddm1* encodes an SNF2/SW12-like chromatin-modeling protein, whereas MET1 is a major DNA methyltransferase. Both of these mutants exhibit marked reduction in PTGS activity, as measured by the accumulation of transgene transcripts (10, 218). Although the patterns of reduction are different with these mutants, these studies highlight the strong correlation between PTGS and TGS.

In *D. melanogaster*, polycomb protein-dependent TGS is also affected by mutations in PIWI, a family of proteins required for RNAi (169). Other evidence includes the *argonaute4* gene of *A. thaliana*, which controls both locus-specific siRNA accumulation and DNA methylation (241); the *Arabidopsis sde4* locus, which is of unknown biochemical function but is responsible for (retroelement TS SINE-specific) siRNA formation (94); and the *Arabidopsis rts1* (RNA-mediated transcription silencing) mutation, which causes a ≈50% reduction in target promoter DNA methylation (10). However, not all TGS mutations affect the PTGS pathways and vice versa, suggesting that the two pathways diverge at some point (218).

RNA-dependent DNA methylation has been reported only in plants until now. Aufsatz et al. (10) have also shown that

asymmetric non-CpG methylation is mostly affected by RNA-dependent DNA methylation, but the existence of non-CpG methylation in mammals has always been a contentious issue. Mammalian DNA is methylated mostly at symmetric CpG or CNG sites by various forms of DNA methyltransferases. However, using a dual-labeling nearest-neighbor technique and the bisulfite genomic sequencing methods, Ramsahoye et al. (177) found that the genomes of embryonic stem cells but not that of somatic tissues harbored non-CpG methylation, which accounted for 15 to 20% of total cytosine methylation. This methylation is perhaps caused by the methylase Dnmt 3a, which is highly expressed in embryonic stem cells but poorly expressed in somatic tissues (179). Other studies have also revealed that in *D. melanogaster* and mammals, non-CpG methylation is an early embryonic event (10), and this methylation can be catalyzed by Dnmt 2, which is primarily active at the initial stages of development (142).

In the above-mentioned studies, however, no connection between non-CpG methylation and any homologous RNA has been shown. Hence, if RNA-dependent DNA methylation occurs at all in animals, it might be limited to the early developmental stages when the effector proteins may be found in abundance. In contrast, RNA-dependent DNA methylation is observed throughout plant development, implying the continuous availability of the appropriate plant DNA methyltransferases. This feature also explains the ease of RNA-dependent DNA methylation detection in plants (10).

### Heterochromatin Formation

Even for organisms in which RNA-dependent DNA methylation is supposedly absent, there is growing evidence that RNAi processes cause chromatin modifications leading to TGS. This evidence reveals that the connections between TGS and PTGS are strong across all layers of eukaryotic life. For example, in *C. elegans*, in which DNA methylation has not been detected, some PTGS mutations, namely *mut7* and *rde2*, derepress transgenes which are affected by polycomb-dependent TGS (203). The polycomb group of proteins are known to keep the chromatin in the closed or compact conformation. Conversely, it has also been found recently that the polycomb proteins MES3, MES4, and MES6 are required for RNAi, at least under some experimental conditions (65, 121).

Generally, in eukaryotic systems, histone modifications make the chromatin structure inert to transcription by heterochromatin formation, which is modulated greatly by the RNAi processes, as recent discoveries have revealed. In almost all organisms heterochromatin formation requires that histone H3 of the chromatin be deacetylated and then methylated at lysine 9. The SET domain of a special group of histone methyltransferases carries out this function. This methylated lysine is subsequently bound by a heterochromatin binding protein, HP1. The binding of the chromodomain containing HP1 to Met H3-K9 is highly specific and of very high affinity (12). This binding may be followed by multimerization of HP1 and complex formation with other chromatin-remodeling proteins. As a result of this multicomplex formation, the chromatin becomes condensed and locked in a transcriptionally repressed heterochromatic state.

Once formed, the heterochromatin spreads a large distance due to cooperative protein-protein interactions of chromatin-remodeling factors, the components of which have not been fully identified yet. However, these structures are generally initiated at places containing repeated DNA sequences, for example, centromere, telomere, mating locus, and elsewhere in the genome containing repetitive DNA in the fission yeast *Schizosaccharomyces pombe* (7). These repeats are responsible for producing dsRNAs, which are processed by the RNAi machinery. D. P. Bartel's group has discovered abundant species of centromeric repeat-specific siRNAs from *S. pombe* (180). Volpe et al. (223) demonstrated that these siRNAs are blocked and instead, large noncoding RNAs (≈1.4 to 2.4 kb) homologous to the centromere repeats accumulate in *dcr1*, *ago1*, and *rdrp1* mutants of *S. pombe*. These mutant cells also do not show the heterochromatin-mediated silencing of a *ura4*$^+$ gene inserted into the outer and inner repeats that flank the central core of the centromeres. A corresponding reduction in Met-H3 K9 is also observed in the outer repeats of these mutant cells (223). This loss in gene silencing is phenotypically similar in cells lacking the histone methyltransferase (*clr4*) or the HP1-like (*swi6*) activity.

That the DNA repeats are central to the RNAi-like processing of dsRNA and concomitant heterochromatin formation was clearly established by the findings of Hall and colleagues (89), who inserted a 3.6-kb centromere H repeat, normally present at the silent mating type domain, in a euchromatic position (*ura4* locus). The introduction of this repeat was sufficient to turn on the silencing of a linked reporter gene and induce H3-K9 methylation and recruitment of HP1-like factors (Swi6) (89). The link between the RNAi machinery and heterochromatin formation has also been established by a recent finding in *A. thaliana*. From a large screen of mutants, Zilberman et al. (241) found that the *ago4* gene is responsible for the RNAi-related silencing of the *A. thaliana superman* gene, which is implicated in flower formation. The *ago4-1* mutation reactivates the silent *superman* allele and decreases non-CpG as well as H3-K9 methylation. Significantly, the same mutation also blocks DNA methylation and the accumulation of siRNA corresponding to the retroelement at SN1 (241).

The above-mentioned facts are put together in a model (Fig. 3) showing a link between siRNA and heterochromatin formation. In the wild-type scenario, one strand of the centromeric region is constitutively expressed, whereas the complementary strand, which is subjected to heterochromatic repression, is occasionally transcribed (57). Such transcription will lead to the formation of dsRNA, which will be processed by the RNAi machinery. This processing might even be a nuclear step, since a component of this machinery, the RdRP, was found to be physically bound to the outer repeats of the centromeric region in a chromatin immunoprecipitation assay (223). The siRNA thus formed might enter a complex containing the histone methyltransferase enzyme. This complex could be a nuclear equivalent of the RISC complex (Nu.RISC of Fig. 3) lacking nuclease activity (98). Such a complex would be guided to the appropriate DNA region following the DNA-RNA base pairing rules, and the histone H3-K9 of the region might be methylated to eventually generate the heterochromatin structure. Since RdRP is found locally, the spread of the heterochromatic structure may be associated with the extension of the 3′ end of the siRNA primer. It has also been shown in *N. crassa* and *A.*

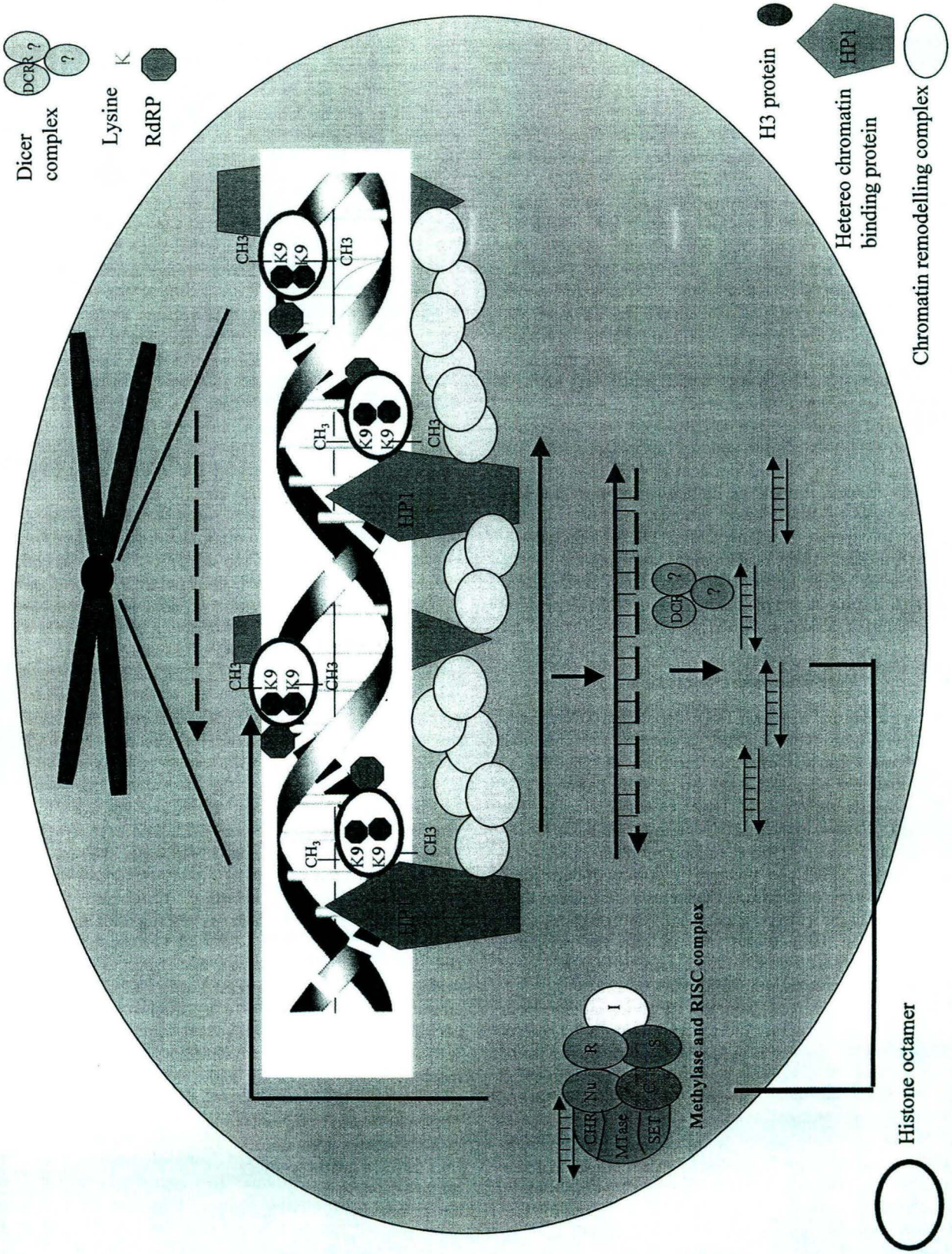



FIG. 3. Role of the RNAi process in heterochromatinization of nuclear DNA. The methylated (H3-K9) histone and many chromatin components are involved in the cross talk in several epigenetic regulatory pathways within the nucleus. The centromeric region (black oval) of the chromatin (thick purple line) might be responsible for the production of dsRNA transcripts (continuous red line and broken blue line). The transcript coming out of the DNA strand subjected to heterochromatinization is represented by broken blue lines. The siRNAs of the nucleus join the complex, of which the histone methyltransferase is a constituent. The siRNA binding the chromodomain (CHR), methyltransferase (MTase), and the SET domain of the methyltransferase are indicated.

*thaliana* that H3-K9 methylation directs DNA methylation (107, 204). The methylated DNA could be complexed further with the methyl-binding proteins. Following these binding events, the chromatin structure will be extremely compact and condensed and would remain transcriptionally inert.

### DNA Elimination

The most dramatic effect of siRNA-mediated heterochromatin formation followed by chromosomal DNA elimination and rearrangement has been recorded in the ciliated protozoan *Tetrahymena pyriformis* (156, 206). Among unicellular organisms, *T. pyriformis* is unique because of its nuclear dimorphism. The two nuclei, the micronucleus and macronucleus, serve different functions. The polyploid macronucleus is the transcription center of the cell during vegetative growth, whereas the diploid and transcriptionally inert micronucleus acts as the germ line nucleus. During conjugation, the micronucleus gives rise to the macronucleus, and this transition is accompanied by two interesting and peculiar recombinant events. First, approximately 6,000 internal eliminated sequences of five pairs of micronucleus chromosomes, accounting for about 15% of genomic micro-DNA, are removed. Second, the remaining parts of these chromosomes are broken into 200 to 300 minichromosomes concomitant with the deletion of <50 nucleotide breakage eliminated sequences. The mechanisms of removal of internal eliminated sequences and breakage eliminated sequences remained elusive for a long time but were recently unveiled, courtesy of the awareness of the siRNA world.

Mochizuki et al. (156) showed that in wild-type cells of *Tetrahymena pyriformis*, siRNAs of about ≈26 to ≈31 nucleotides were produced which hybridized to micronuclear genomic DNA and not the macronuclear DNA, indicating that these siRNAs could be internal eliminated sequence/breakage eliminated sequence-specific and are referred to as scan RNAs. These scan RNAs were not made in *twi1* mutants, and the production of internal eliminated sequence/breakage eliminated sequence elements was also impaired by the *twi1* mutation. *twi1* produces a Piwi-related protein during the sexual cycle and can transmit the RNA-encoded information from the micronucleus to the old macronucleus and finally to the new macronucleus to mark the sequences to be eliminated (57). The defect in accumulation of the scan RNAs in the *twi1* mutant was similar to the case of another mutant, *pdd1* (156). In a related report, Taverna et al. (206) showed that the protein PDD1 was the effector protein for DNA excision and that PDD1 along with Met H3-K9 was associated preferentially with the internal eliminated sequence/breakage eliminated sequence elements in the new macronucleus that developed from the micronucleus during the sexual cycle. PDD1 contains two chromodomains and an additional RNA-binding domain (3).

The above data and the model presented in the earlier section lead to a straightforward and interesting scheme for programmed DNA degradation in *Tetrahymena pyriformis*. The bidirectional transcription that occurs across the internal eliminated sequence repeats (38) may form the dsRNA, which would give rise to the scan RNAs following the action of RNAi-related Dicing complexes that perhaps also include the Twi1 and PDD proteins. These scan RNAs eventually may be associated with the nuclear equivalents of RISC factor in the new macronucleus to provide heterochromatic sites at the internal eliminated sequence/breakage eliminated sequence regions. The chromodomain containing PDD proteins may remain bound to the scan RNA and thus guide to destroying the cognate DNA. As an extension of this work, Yao et al. found that a similar RNAi process recognized and deleted a foreign neomycin resistance gene of bacterial origin which was integrated in a *Tetrahymena* chromosome (237). These two studies together strongly suggest an siRNA- (or scan-RNA)-based mechanism that controls genome-wide DNA arrangements and provides genomic surveillance against invading foreign DNAs.

Thus, the *Tetrahymena pyriformis* as well as *S. pombe* data show how dramatic the epigenetic consequences of the genome could be following the formation of siRNA molecules in cells. Discovery of the link between the RNAi processes and the epigenetic chromatin modification as well as chromosome behavior is probably the most fascinating and novel face of regulation of gene silencing mechanism. The RNAi machinery is reported to control many explosive features of cellular biology, namely stem cell maintenance (53), cell fate determination (21), nonrandom chromosome segregation (188), etc. A recent report established that the fission yeast RNAi-related genes *ago1*, *dcr1*, and *rdp1* also control the fidelity of chromosome segregation during mitosis and meiosis. As discussed earlier, these gene products are required to maintain centromeric silencing. The report also demonstrated that the chromosome missegregation of the RNAi mutants occurred due to the loss of centromeric cohesion, suggesting a clear link between centromeric silencing and cohesion. This report broadly hinted that the regulation of chromosomal dynamics could be largely traced to the natural RNAi biology of the eukaryotic cells (90).

It is not difficult to imagine that we might witness RNAi-related unifying signals in diverse chromosome behaviors, namely X-chromosome inactivation, satellite-repeat contraction and expansion, hybrid dysgenesis in *D. melanogaster*, chromatin diminution in ascarid nematodes, nuclear dominance in plants, and so on in the not so distant future (57).

## APPLICATIONS OF RNAi

Besides being an area of intense, upfront basic research, the RNAi process holds the key to future technological applications. Genome sequencing projects generate a wealth of information. However, the ultimate goal of such projects is to accelerate the identification of the biological function of genes. The functions of genes can be analyzed with an appropriate assay, by examining the phenotype of organisms that contain mutations in the gene, or on the basis of knowledge gained from the study of related genes in other organisms. However, a significant fraction of genes identified by the sequencing projects are new and cannot be rapidly assigned functions by these conventional methods.

RNAi technology is proving to be useful to analyze quickly the functions of a number of genes in a wide variety of organisms. RNAi has been adapted with high-throughput screening formats in *C. elegans*, for which the recombination-based gene knockout technique has not been established. Chromosomes I

and III of *C. elegans* have been screened by RNAi to identify the genes involved in cell division and embryonic development (82, 84). Recently, a large-scale functional analysis of ≈19,427 predicted genes of *C. elegans* was carried out with RNA interference. This study identified mutant phenotypes for 1,722 genes (112). Similarly, in *D. melanogaster*, RNAi technology has been successfully applied to identify genes with essential roles in biochemical signaling cascades, embryonic development, and other basic cellular process (44). In plants, gene knockdown-related functional studies are being carried out efficiently when transgenes are present in the form of hairpin (or RNAi) constructs. Plant endotoxins could also be removed if the toxin biosynthesis genes are targeted with the RNAi constructs. Recently, the theobromine synthase of the coffee plant was knocked down with the hairpin construct of the transgene, leading to the production of decaffeinated coffee plants (166). Virus-induced gene silencing has also been proven to be a successful approach for plant genetics (15).

Given the fact that RNAi is easy to apply, whole-genome screens by RNAi may become a common method of choice in the near future. RNAi may facilitate drug screening and development by identifying genes that can confer drug resistance or genes whose mutant phenotypes are ameliorated by drug treatment, providing information about the modes of action of novel compounds. Although RNAi is unlikely to replace the existing knockout technology, it may have a tremendous impact for those organisms that are not amenable to the knockout strategy. It may also be a method of choice to study the simultaneous functions of a number of analogous genes in organisms in which redundancy exists with respect to a particular function, because many of these genes can be silenced simultaneously.

Given the gene-specific features of RNAi, it is conceivable that this method will play an important role in therapeutic applications. Since siRNAs direct cellular RNAi biology, these are potential therapeutic reagents because of their power to downregulate the expression pattern of mutant genes in diseased cells. However, central to this hypothesis is the assumption that the effect of exogenous siRNA applications will remain gene specific and show no nonspecific side effects relating to mismatched off-target hybridization, protein binding to nucleic acids, etc. Though it was demonstrated that mismatches of more than even one nucleotide within the 19- to 20-mer siRNAs effectively disrupted proper degradation of the target mRNA (68), the gene specificity of siRNAs needs to be confirmed on a genome-wide scale.

Recently, Chi et al. (41) reported that the GFP siRNA-induced gene silencing of transient or stably expressed GFP mRNA was highly specific in the human embryonic kidney (HEK) 293 cell background. The specific silencing did not produce secondary changes in global gene expression, as detected by the DNA microarray experiment. They also failed to detect the presence of transitive RNAi in experimentally engineered human cell lines (41). In their own experiments, Semizarov et al. (190) reached a similar conclusion while using siRNAs corresponding to *akt1*, *rb1*, and *plk1* in the human non-small cell lung carcinoma cell line H1299. These experiments prove that siRNAs could be used as highly specific tools for targeted gene knockdown and can be used in high-throughput approaches and drug target validation. This exquisite sequence-specific effect of siRNAs has also been exploited in silencing the mutant allele of the diseased gene while not affecting the wild-type allele of the healthy version of the same gene (158).

siRNAs have been shown to inhibit infection by human immunodeficiency virus, poliovirus, and hepatitis C virus in cultured cell lines (152). Bitko and Barik (19) successfully used siRNAs to silence genes expressed from respiratory syncytial virus, an RNA virus that causes severe respiratory disease in neonates and infants. siRNA treatment has also been shown to reduce the expression of the BCR-ABL oncoprotein in leukemia and lymphoma cell lines, leading to apoptosis in these cells (230). With respect to future medical applications, siRNA-based therapy seems to have a great potential to combat carcinomas, myeloma, and cancer caused by overexpression of an oncoprotein or generation of an oncoprotein by chromosomal translocation and point mutations (211).

Recently, the therapeutic potential of the siRNA technique has been demonstrated in vivo in mouse models. McCaffrey et al. (151) and Song et al. (199) demonstrated effective targeting of a sequence from hepatitis C virus and the *fas* gene by RNA interference in mouse liver (199). An epiallelic series of p53 hypomorphs created by RNAi have been shown to produce distinct tumor phenotypes in mice in vivo, suggesting that RNAi can stably suppress gene expression (99). Song et al. (199) have shown that treatment with *fas* siRNA abrogated hepatocyte necrosis and inflammatory infiltration and protected mice from liver fibrosis and fulminant hepatitis. Rubinson et al. (184) showed highly specific, stable, and functional silencing of gene expression in transgenic mice with the lentivirus system for the delivery of siRNAs.

Although the delivery of siRNAs to a proper site remains problematic for gene therapy, chemical modifications of siRNAs such as changing the lipophilicity of the molecules or the methods previously developed for the application of antisense oligonucleotides or nuclease-resistant ribozymes might help the entry and stability of siRNAs within the transfected cells or tissues. The absence of specific micro-RNAs has been demonstrated in carcinoma cells, implying that cancer development could be arrested by introduction of the missing micro-RNAs. The micro-RNAs could be supplied in the form of siRNAs, since the function of micro-RNAs can be mimicked by the exogenous siRNA (62). However, independent of its biomedical applications, RNAi appears to be a forthcoming method for functional genomics.

## CONCLUDING REMARKS

In the footsteps of the discovery of the double-helical structure of DNA, some outstanding discoveries have been recorded, but few of them really match the explosive content and implication of dsRNA-mediated gene silencing. This homology-dependent silencing has established a novel paradigm with far-reaching consequences in the field of transcription regulation. The regulatory mechanism offers cellular protection against parasitic nucleic acid sequences, carries out epigenetic as well as genetic alterations on the one hand, and governs organisms architecture and development on the other. Capitalizing on the basic principles of silencing, large-scale functional genomics have come into play in diverse organisms.

Studies conducted at the laboratory level have revealed the tremendous power of siRNAs as therapeutics and have demonstrated the potential of micro-RNAs to reverse cellular developmental aberrations.

The new paradigm has a lot more to offer than it has delivered already. The stepwise detailed mechanism of RNAi and its related processes is waiting to be explored. The rationale for many unexplained genetic findings of RNAi in worms, plants, and other organisms will be revealed in the wake of further mechanistic discoveries. The cytoplasmic location of RNAi is evident, but the evidence of nuclear connections of RNAi and related events are also too many. Surprisingly, there are some components of RNAi, GEMIN3 and GEMIN4 of humans, which partition in both the nuclear and cytoplasmic compartments. Hence, clarification of the subcellular locations of the RNAi processes is required. Hopefully, the detailed biochemical framework of RNAi would provide such clarifications.

As we gain more insight into the mechanisms, more effective methods for analysis of gene functions may evolve. We may learn more about geriatrics, nervous diseases, genetic imprints, nuclear dominance in plants, and so on and thus might wield control over such processes in the future. Meanwhile, the knockdown technology might improve vastly with better-designed plasmid- or virus-based vectors for delivery of siRNAs to the appropriate tissues at the appropriate time. Such technology is bound to give a new shape to therapeutic gene silencing as well. The science and technology of RNAi has given us a cultural ocean of virtually bottomless depth.

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