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# Development and Characterization of an Inducible RNAi System in *Toxoplasma gondii*

by Biju Vasavan

A Thesis  
Submitted to the Faculty of Graduate Studies  
through the Department of Chemistry and Biochemistry  
in Partial Fulfillment of the Requirements for the  
Degree of Master of Science at the  
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Windsor, Ontario, Canada

2009

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## ABSTRACT

*Toxoplasma gondii* is a ubiquitous protozoan parasite that can infect and replicate within any nucleated mammalian or avian cell. The ability of double stranded RNA to downregulate gene expression has been demonstrated in *T. gondii*. We have constructed a tetracycline based inducible RNAi vector which can be used for regulating gene expression in *T. gondii*. Upon the addition of anhydrotetracycline, the production of double stranded RNA homologous to the target gene is induced. The inducible vector was characterized using the marker gene, uracil phosphoribosyl transferase. The inducible vector was stably transfected into RHtetR strain of the parasite and specifically lowered the expression of uracil phosphoribosyl transferase detected by RT-PCR, uracil incorporation assay and 5-fluoro-2'-deoxyuridine resistance. Subsequently, the inducible RNAi vector was used to elucidate the role of microneme protein 3 (MIC 3), which is an invasion related gene. We generated a stable transgenic parasite line in which the expression of MIC 3 was knocked down detected by RT-PCR. However, we did not detect any changes in the growth of the parasite or its invasion ability, as monitored by nucleotide uptake assay and growth assay.

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## LIST OF ABBREVIATIONS

ATC	anhydrotetracycline
ATP	adenosine triphosphate
bp	base pair
BAPTA-AM	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CAT	chloramphenicol acetyl transferase
DEPC	diethylpyrocarbonate
DFBS	dialyzed fetal bovine serum
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DPBS	Dulbecco's phosphate-buffered saline
dsRNA	Double-stranded RNA
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetra-acetic acid disodium salt
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
FBS	fetal bovine serum
FDUR	5 fluoro-2'-deoxyuridine
GFP	green fluorescent protein
HFF	human foreskin fibroblasts
hr	hours
HXGPRT	Hypoxanthine-guanine phosphoribosyltransferase
LDH	lactate dehydrogenase
MIC 3	microneme protein 3
M-MLV RT	Moloney murine leukemia virus-reverse transcriptase
mM	Millimolar
MPA	mycophenolic acid
mRNA	Messenger RNA
ORF	open reading rame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PTGS	post-transcriptional gene silencing
PVM	parasitophorous vacuole
RNA	ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RT	reverse transcription
RT-PCR	reverse transcription PCR
RISC	RNA induced silencing complex

siRNA	small interfering RNA
SAG	surface antigen
SOC	super optimal broth with catabolite repression
SRS	surface antigen-related sequence
TAE	Tris-Acetate-EDTA
<i>Taq</i>	<i>Thermus Aquaticus</i>
TE	Tris-EDTA
<i>T.gondii</i>	<i>Toxoplasma gondii</i>
<i>T.brucei</i>	<i>Trypanosoma brucei</i>
TET	Tetracycline
TetO	Tetracycline operator
TetR	Tetracycline repressor protein
tTA	Transactivator
TCA	trichloroacetic acid
TMP	Thymidine monophosphate
TUB	Tubulin
6TX	6-thioxanthine
UMP	Uridine monophosphate
UPRT	uracil phosphoribosyl transferase

## CHAPTER 1

# Introduction

### 1.1 *Toxoplasma gondii*

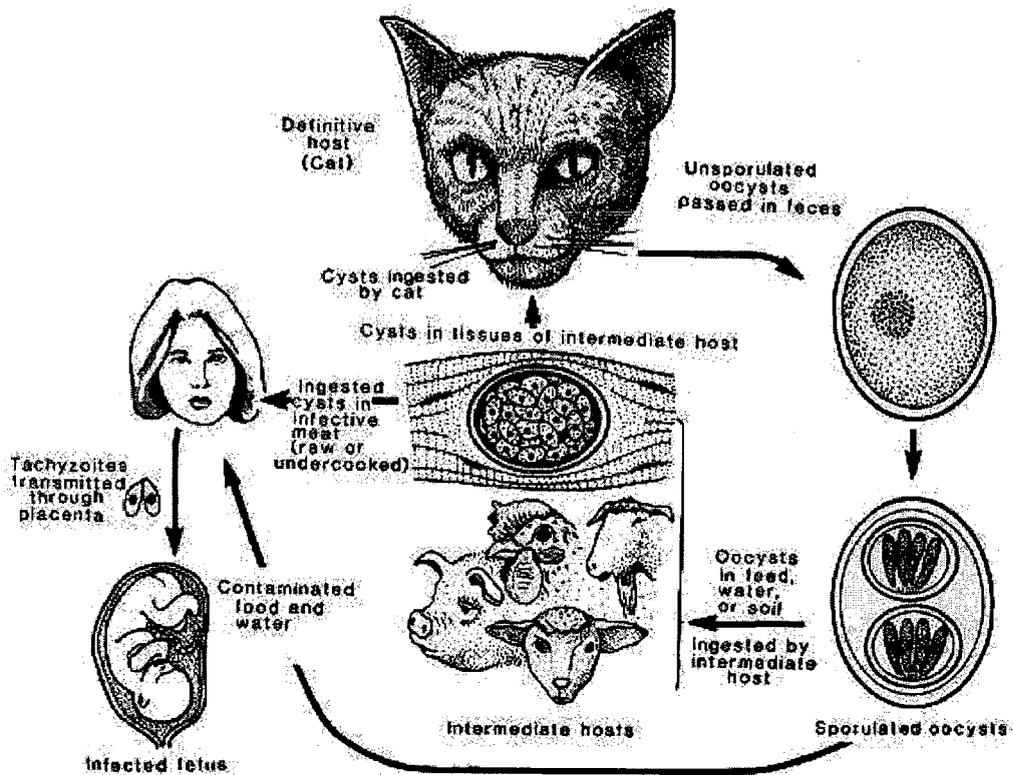
*T. gondii* is a unicellular apicomplexan protozoan. The apicomplexan parasites are characterized by the presence of a plastid acquired by secondary endosymbiosis of algae, which is essential for parasite replication (McFadden and Roos, 1999; Coppens and Joiner, 2001). *T. gondii* is globally distributed and infects a wide variety of mammals and birds (Frenkel and Dubey, 1972). It was discovered in 1908 by Nicholle and Manceaux from the liver of African rodent *Ctenodactylus gondii* (Sukthana, 2006).

In immuno-competent hosts, the parasite produces only mild clinical symptoms and progresses to a chronic state without major complications (Krahenbuhl and Remington, 1982). In immuno-compromised humans, recipients of organ transplants and newborn babies, the infection can be life threatening (Kafsack *et al.*, 2004). Primary infection during pregnancy can cause abortion, neurological damage, pre-term birth and visual impairment (Gilbert *et al.*, 2001; Remington *et al.*, 2001; Wallon *et al.*, 2004; Beghetto *et al.*, 2006). *T. gondii* infection occurs as a result of ingestion of oocysts from definitive hosts, cats or through tissue cysts present in undercooked meat. The transition from acute to chronic toxoplasmosis occurs through a stage conversion of fast replicating tachyzoite form to the cyst-forming dormant bradyzoite form. This stage conversion is marked by a significant change in mRNA and protein expression profiles and helps the parasite to persist in the host tissue (Dzierszinski and Knoll, 2007; Tomavo and Weiss, 2007; Friesen *et al.*, 2008).

### 1.1.1 Life cycle of *T. gondii*

*T. gondii* can infect and replicate within any nucleated mammalian or avian cell (Wong and Remington, 1993; Dubey, 1998; Black and Boothroyd, 2000). *T. gondii* has a complex life cycle. The sexual life cycle takes place only in members of the felidae (cats), which is the parasite's primary host. The gametes are formed in the small intestinal epithelium of the cat. Unsporulated oocysts are released into the intestinal lumen and passed into the environment with the faeces. Sporogony occurs outside the host and leads to the development of infectious oocysts which contains two sporocysts, each containing four sporozoites (Dubey and Beattie., 1988; Jackson and Hutchison., 1989; Tenter *et al.*, 2000). The asexual life cycle takes place in warm-blooded animals and birds. Depending on whether the infection is in the acute or chronic phase, the asexual components consist of two distinct stages, the fast growing tachyzoite and the slow growing bradyzoite (Black and Boothroyd, 2000).

*T. gondii* can enter the host either in the form of an oocyst or as a tissue cyst. The ingested cysts multiply in the small intestine and liberate sporozoites from the oocysts or brazyzoites from the tissue cyst. Sporozoites and bradyzyzoites infect the intestinal epithelium and differentiate into fast growing tachyzoites which then spread throughout the body via monocytes and macrophages (Sumyuen *et al.*, 1995; Carruthers 2002). In order to escape the host immune response, *T. gondii* differentiates from tachyzoites to bradyzoites, which build a cyst wall to encapsulate and fortify themselves within host cell (Bohne *et al.*, 1993, 1994).



**Figure 1.1** Life cycle of *T. gondii*. The definitive hosts (feline) of *T. gondii*, ingests sporulated oocysts and becomes infected (sexual cycle) and further infects other warm blooded animals and birds (asexual cycle). *Toxoplasma* can also be transmitted to intermediate hosts through oocysts, by carnivorous, or transplacentally. The most common route of infection in sheep and humans is through trans-placental infection (Dubey, 1986).

### 1.1.2 Host cells and parasite strains

Excellent culture systems are prerequisites for *T. gondii* research (Grimwood *et al.*, 1979; Huges *et al.*, 1986; Roos *et al.*, 1994). HeLa cells (cervical epithelial cells) have been used for the culture of tachyzoites. The two major disadvantages of using these cells are inconsistent yield of tachyzoites and difficulty in predicting the time of lysis of these cells (Evans *et al.*, 1999; Chatterton *et al.*, 2002). *T. gondii* are also cultured using Vero cells (kidney epithelial cells obtained from African green monkey) (Cleary *et al.*, 2002). However, most commonly used host cells are the human foreskin fibroblasts (HFF). The main advantages include the fact that these cells grow easily, even in the presence of growth inhibiting drugs that are used to select stably transfected parasites. Furthermore, the large surface area of HFFs allows the parasite to complete multiple cycles within the cells before they are lysed (Roos *et al.*, 1994; Freyre, 1995).

Several strains of *T. gondii* are available. The virulence, replication rates and cyst forming capabilities differ from one strain to the others (Soete *et al.*, 1993; Fryere., 1995). For the generation of tachyzoites, the RH strain is commonly used in laboratory. The advantage of using the RH strain is that it replicates faster as compared to other strains and efficiently lyses host cells releasing a large number of parasites. The main disadvantage of the RH strain is its poor cyst-forming abilities. For generation of bradyzoites, PLK stain is used which is known for good cyst forming capabilities (Soete *et al.*, 1993; Boothroyd *et al.*, 1997).

### 1.1.3 *Toxoplasma* as a model for apicomplexan phylum

The genera of phylum Apicomplexa include pathogens such as *Cryptosporidium*, *Eimeria*, *Neospora*, *Plasmodium* and *Theileri* (Saeij *et al.*, 2005). *Cryptosporidium* and *Eimeria* are enteric pathogens; *Neospora*, and *Theileri* causes veterinary diseases while *Plasmodium* causes malaria. *T. gondii* is considered to be an intracellular apicomplexan model because it can be easily cultured, transfection can be achieved easily and genetic studies can be done *in vitro* and *in vivo* (Kim and Weiss, 2004). Among apicomplexa, it was *T. gondii* in which transfection was first reported (Kim *et al.*, 1993; Soldati and Boothroyd, 1993; Donald and Roos 1993). The efficiency of transient transfection is high (above 50%) as compared to other apicomplexans. Stable transformation can also be achieved in *T. gondii*. In malarial parasites the transfection efficiency is low and it can take months to generate a stable transformant (Donald and Roos 1993). *Cryptosporidium* and *Eimeri* cannot be maintained in tissue culture for a long time and thus it is very difficult to perform important genetic studies. Furthermore, transient transfections can be achieved in *Eimeri* but phenotypic studies cannot be done successfully since it cannot be cultivated *in vitro* successfully (Kelleher and Tomly, 1998).

Large numbers of tools have been developed for genetic manipulation of *T. gondii*. Gene expression has been downregulated using RNA-based techniques (Al-Anouti and Ananvoranich, 2002; Al-Anaouti *et al.*, 2003; Sheng *et al.*, 2004). Since it is very difficult to genetically manipulate most apicomplexans, *T. gondii* has been used as a model expression system to study the biochemical role of certain proteins that cannot be expressed in other organisms. The *Plasmodium* protein, circumsporozoite protein (CSP) has been successfully expressed in *T. gondii* after codon optimization. Codon optimization

is done to overcome the A/T codon bias of *Plasmodium* compared G/C preference of *T. gondii* (Charest *et al.*, 2000). Sporozoite surface antigen gp40/15 of *C. parvum*, which is also rich in A/T has also been successfully expressed in *T. gondii* after codon optimization (O'Connor *et al.*, 2003).

#### **1.1.4 Genetic Manipulation of *T. gondii***

The *Toxoplasma* genome has been successfully manipulated by DNA transfection (Striepen and Soldati, 2007). DNA is introduced into tachyzoites by electroporation. The use of cytomix, which has a composition that resembles the ionic composition of the cells, ensures maximum survival of the cells after electroporation (Van den Hoff *et al.*, 1992). Nearly 80% tachyzoites were able to successfully invade host cells after electroporation with cytomix (Soldati and Boothroyd, 1993).

##### **1.1.4.1 Transgenic expression in *T. gondii***

A transient gene expression occurs if the plasmids are maintained for a few days in the parasite as episomal DNA after electroporation whereas stable gene expression takes place if the plasmid integrates into the genome of the parasite. Stable transformation can take place by homologous or non-homologous recombination of the plasmid into the parasite genome (Donald and Roos, 1994).

Episomal vectors, which are able to replicate autonomously in *T. gondii* without integrating into the genome have also been developed (Black and Boothroyd, 1998). These vectors avoid the possibility to introduce mutations into the genome by non-homologous recombination.

#### 1.1.4.2 Selectable markers for stable transformation

The markers used to select stable transformants in *T. gondii* include hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) (Donald *et al.*, 1996), uracil phosphoribosyltransferase (UPRT) (Donald and Roos, 1995), chloramphenicol acetyltransferase (Kim *et al.*, 1993), phleomycin resistance (Messina *et al.*, 1995), dihydrofolate reductase-thymidilate synthase (Donald and Roos, 1993), and tryptophan synthase (Sibley *et al.*, 1994).

*T. gondii* is dependent on host cells for purines as it is incapable of synthesizing them on its own (Schwartzman and Pfefferkorn, 1982) but has the ability to interconvert purine nucleotide that it salvages. HXGPRT can convert hypoxanthine to inosine monophosphate (IMP), xanthine to xanthosine monophosphate (XMP) and guanine to guanine monophosphate (GMP) (Pfefferkorn *et al.*, 2001). IMP can be converted to XMP and XMP can be converted to GMP with the help of enzymes IMP-dehydrogenase and GMP-synthase respectively. If IMP-dehydrogenase is blocked by mycophenolic acid (MPA), then HXGPRT is required to convert xanthine to GMP. Thus MPA can be used for positive selection for HXGPRT. HXGPRT converts 6-thioxanthine (6-TX) to 6-thioxanthosine 5'-phosphate which is toxic to parasite. Therefore, 6-TX can be used for negative selection for HXGPRT (Donald *et al.*, 1996).

UPRT is a parasite specific enzyme. This enzyme is used by the parasite in pyrimidine salvage to convert uracil to UMP. In the presence of fluorouracil, phosphoribosylation of fluoruridine leads to production of F-dUMP and results in the inhibition of TMP synthesis. 5-fluorodeoxyuridine is metabolized to 5-fluorouracil by

UPRT. Thus it can be used to select parasite strains that lack a functional UPRT gene (Donald and Roos, 1995).

## **1.2 Genes responsible for virulence of *T. gondii***

The preliminary attachment of *T. gondii* by its apical pole involves interactions between surface antigens of the parasite and cell surface receptors on the host cell (Bonhomme *et al.*, 1999). Five major surface antigens, SAG 1-5 have been identified on the surface of *T. gondii* (Couvreur *et al.*, 1998). SAG 1 and SAG 2 are tachyzoite specific antigens that play an important role in attachment of parasite to host cell during the early stage of invasion. Three surface antigen related sequences, SRS 1, SRS 2 and SRS 3 have been identified but their functions are still unknown (Boothroyd *et al.*, 1997). After the parasite is attached to the host cell membrane, a tight junction forms between the attached parasite and the host cell membrane, probably through surface membrane components attached to the cytoskeletal microtubules of *T. gondii*. The actin-myosin motor provides the driving force to the parasite to glide through the tight junction.

After the parasite is apically oriented, micronemal proteins (MICs) are released (Achbarou *et al.*, 1991). MIC 1 and MIC 2 proteins help the parasite to intimately attach itself to the host cell surface (Fourmaux *et al.*, 1996). MIC 3 has an epidermal growth factor (EGF)-like domain which suggests that micronemes contain possible ligands for host cell receptors. After the microneme proteins are released, the rhoptries which are club-shaped organelles excrete their contents that help to decrease the viscosity of the host cell membrane and this helps the parasite in invagination and further enhances the invasion process (Joiner *et al.*, 1990).

The rhoptry proteins also help the parasite to build the parasitophorous vacuole. These vacuoles protect the parasites from phagocytes by preventing acidification of the internal environment thereby avoiding the normal endocytosis process (Sibley *et al.*, 1993). The parasitophorous vacuoles also provide an environment for parasite growth and development. The release of rhoptry protein is believed to be regulated by an endogenous  $\text{Ca}^{2+}$ -dependent phospholipase (Saffer and Schwartzman, 1991). Ten different rhoptry proteins have been discovered so far but ROP 1 and ROP 2 are believed to play the major role (Saffer *et al.*, 1992). ROP 1, also known as penetration enhancing factor, is a 61 kDa protein. ROP 2, a 54 kDa protein is initially associated with parasitophorous vacuole but disappears as the vacuole matures (Beckers *et al.*, 1994).

Dense granules are molecules that are secreted into the parasitophorous vacuole from the time these vacuoles are formed till the parasites mature. These molecules help to support the intravacuolar network of the membranous tubules within the lumen of the parasitophorous vacuole. Dense granule proteins GRA 3 and GRA 5 are distributed to the vacuolar membrane while GRA 1, GRA 2, GRA 4 and GRA 6 are distributed to the intravacuolar network (Sibley *et al.*, 1995). Thus, dense granule proteins that are released into parasitophorous vacuole may help the parasite to replicate and evade the host immune response.

### **1.2.1 Microneme protein 3 (MIC 3)**

*T. gondii* invade the host cell in a sequential manner with the aid of proteins secreted from three secretory organelles (Carruthers and Sibley, 1997). MICs are discharged first and they help the parasite to attach to the host cells as they can recognize

and effectively bind to the host cell receptors (Soldati *et al.*, 2001). After the secretion of MICs, rhoptry proteins are released. These proteins help in the formation of parasitophorous vacuole membrane (PVM) in the host cells in which the parasite resides (Saffer *et al.*, 1992). Finally, the proteins from the dense granules are released. Dense granule proteins are released during the process of invasion as well as after the invasion process is complete. These proteins help the parasite to survive in the intracellular environment as well as help them during replication (Zhou *et al.*, 2005).

Microneme protein MIC 3 is an important adhesion protein secreted by *T. gondii* (Cerede *et al.*, 2002). This protein can be observed in tachyzoite, bradyzoite and sporozoite stage of the parasite (Ismael *et al.*, 2003). It is synthesized as a 40 KDa protein and then undergoes N-terminal cleavage resulting in the formation of a 38 KDa protein, which further undergoes dimerization to form a 90 kDa active protein. MIC 3 has five epidermal growth factor-like (EGF) domains and a lectin-like domain. EGF domain helps in exposing the binding domain as well as binding to its escort, MIC 8 (Meissner *et al.*, 2002). The lectin-like domain helps either in protein-protein interaction or protein-carbohydrate interaction (Garcia-Reguet *et al.*, 2000). The protein is translocated to the apical region of the parasite during the process of invasion and then it migrates to the posterior part of the parasite during invasion. The release of the protein is regulated by the calcium ions present inside the cytoplasm of the parasite (Carruthers and Sibley, 1999). The parasite can be stimulated to release microneme proteins even in the absence of host cells by using thapsigargin, ammonium chloride or ethanol (Carruthers *et al.*, 1999). BAPTA-AM can inhibit the secretion of microneme proteins by chelating the calcium ions. The secretion of this protein can also be inhibited by

staurosporine, which is a protein kinase inhibitor. This implies that the secretion of microneme is dependent on phosphorylation which is regulated by calcium ion dependent pathway (Tomley and Soldati, 2001).

### **1.3 RNA interference**

Fire and co-workers coined the term “RNAi” or RNA interference (Fire *et al.*, 1998; Zamore *et al.*, 2000). It is a mechanism that regulates the level of transcripts by degrading the RNA in a sequence specific manner. Three phenotypes of the mechanism are observed and they are referred to as co-suppression or post-transcriptional gene silencing (PTGS) in plants, quelling in fungi and RNAi in animals (Agarwal *et al.*, 2003). It is believed that it is a mechanism through which the cells keep a check on the movement of transposable elements and protect themselves from viral attack. Apart from protective functions, some of the genes involved in RNAi, such as argonaute and dicer play a pivotal role in the development of a eukaryotic organism.

#### **1.3.1 Discovery of RNA interference**

Richard Jorgensen observed that when an exogenous transgene was employed to deepen the purple colour in *Petunia* plants by over expressing the chalcone synthase enzyme, instead of obtaining deep purple flowers, the flowers were either variegated or colourless. Since the introduction of an exogenous transgene resulted in the suppression of endogenous homologous gene and transgene in the colourless flower, they coined the term “co-suppression” to explain this observation (Napoli *et al.*, 1990).

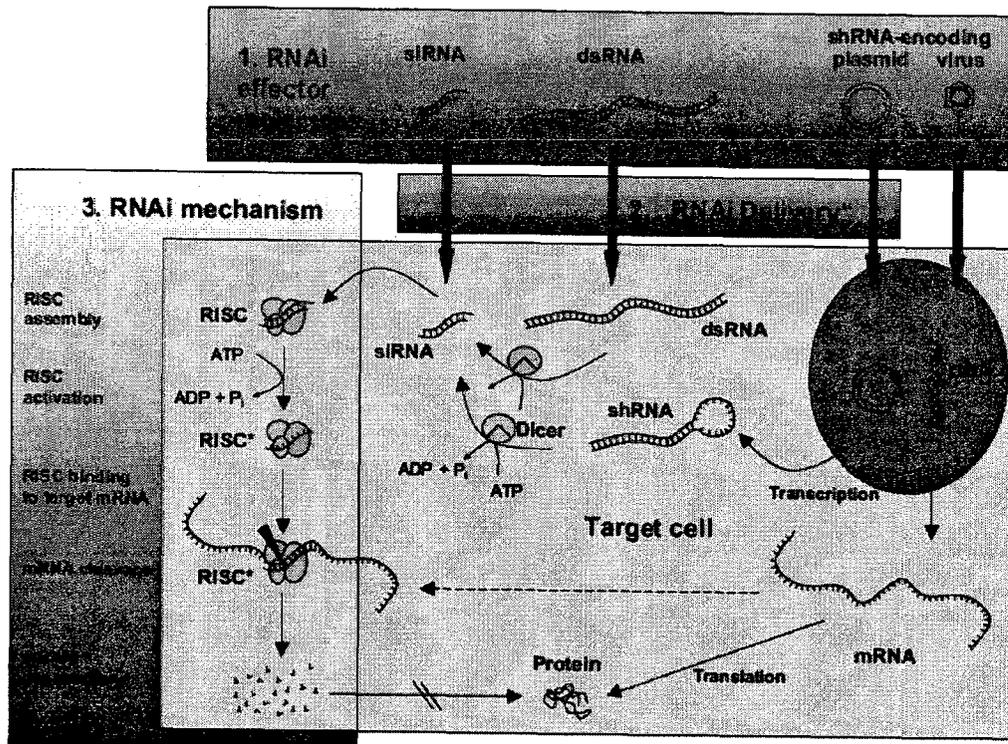
The mechanism of downregulation of gene by RNA interference was established by Fire and co-workers when they discovered that introduction of double-stranded RNA (dsRNA) could block gene expression in the nematode worm *Caenorhabditis elegans* (Fire *et al.*, 1998).

### **1.3.2 Mechanism of RNA interference**

Recent studies have shown that the mechanism of RNA interference is a two step process (Agarwal *et al.*, 2003). The initiating step involves the attack on a long double stranded RNA (dsRNA) by ribonuclease III enzymes (Zamore *et al.*, 2000). This enzyme cleaves the dsRNA to 21 to 25 nucleotide small dsRNA fragments called small interfering RNA (siRNA). Long dsRNA are more effective in down-regulation compared to short dsRNA. In order to produce the same effect as a 81-bp dsRNA, 250 times higher concentration of 21-bp dsRNA has to be used. An effective downregulation is observed when there is >90% homology between the dsRNA and the gene to be knocked down (Sharp, 2001).

In the next step called the effector step (Fig. 1.2), small interfering RNA binds to a protein complex known as RNA-induced silencing complex (RISC). RISC complex is also known as small interfering ribonucleoprotein particles (siRNP) (Agarwal *et al.*, 2003). RISC is a complex of siRNA and proteins with nuclease activity that can specifically degrade target mRNAs (Hannon, 2002). Partial purification of *Drosophila* RISC complex has identified argonaute 2 as one of the components. Argonaute belongs to the PPD family of proteins, which have characteristic PAZ and PIWI domains (Jaronczyk *et al.*, 2005). Dicer binds to Argonaute by interacting with the PIWI domain

and then transfers the siRNA to the RISC complex. An ATP dependent process then unwinds the siRNA duplex and activates the RISC complex (Nykanen *et al.*, 2001). Once the RISC is activated, ATP is not required to cleave the target (Hammond *et al.*, 2000). Argonaute then preferentially cleaves the passenger or sense strand. The RISC complex guided by an anti-sense strand also called guide strand, then cleaves the target mRNA between the tenth and eleventh nucleotide from the antisense (guide) strand's 5'end (Elbashir *et al.*, 2001; Rand *et al.*, 2005). RNA-dependent RNA polymerase can use double stranded siRNA as primers and can synthesize long strand of dsRNA. This step further amplifies the effect of RNA interference, resulting in the lowering of target mRNA and subsequently the level of target protein (Shuey *et al.*, 2002).



**Figure 1.2** Mechanism of RNA interference. Different effector molecules can induce RNA interference. The double stranded RNA (dsRNA) is loaded into the RISC complex. Argonaute then cleaves the sense strand (passenger RNA), utilizing ATP in the process and then RISC complex guided by the anti-sense (guide) strand cleaves the target mRNA. The RISC complex, apart from degrading RNA, can inhibit translation and also take part in chromatin remodelling (Aigner, 2007).

### 1.3.3 RNA interference in *T. gondii*

RNA tools have been extensively used for downregulation of gene expression in many organisms including *T. gondii* (Nakaar *et al.*, 1999, 2000; Al-Anouti and Ananvoranich, 2002; Sheng *et al.*, 2004). *T. gondii* can be subjected to genetic manipulation using various RNA tools including antisense RNA, ribozyme and dsRNA (Al-Anouti and Ananvoranich, 2002; Al Riyahi *et al.*, 2006). These RNA tools allow for the analysis of the function of essential genes because they do not disrupt gene expression at the DNA level, unlike DNA transformation (Lamond and Sproat, 1993). Moreover, these RNA tools can be used in suppressing gene expression and phenotypic studies.

Antisense RNA functions by base-pairing with mRNA to interrupt the downstream process (translation) or the destruction of the mRNA by RNase H (Hostomsky *et al.*, 1994). It was employed to modify the expression of triphosphate hydrolase (NTPase) as well as HXGPRT in *T. gondii* (Nakaar *et al.*, 1999). Ribozyme, first discovered by Cech and Altman in 1980s, is a RNA molecule that can catalyze RNA cleavage in site-specific fashion (Tanner, 1999). In *T. gondii*, engineered delta ribozyme was successfully utilized to reduce expression of UPRT and HXGPRT (Sheng *et al.*, 2004). Adenosine kinase which is an important enzyme in *T. gondii* purine salvage pathway has been successfully downregulated using Adenosine kinase specific siRNAs (Yu *et al.*, 2009).

## **1.4 Inducible System**

In order to evaluate the phenotype in a loss of function study, it is essential to have an inducible system where one can switch 'on' and 'off' the gene as required. In an optimal inducible system, the gene regulation should be tight or the system should not be 'leaky' so that the background expression is minimal. In a good inducible system, high level of expression of the desired gene should take place only when the inducer is present and should be specific to the gene of interest. The inducer should not be toxic to the organism and the most important feature of an inducible system is that the induced gene should revert back to wild type once the inducer is removed. The inducible system should work equally well in both *in vitro* and *in vivo* conditions (Mills, 2001).

Different eukaryotic-based inducible systems have been developed based on heat shock, interferon  $\gamma$ , metallothionein and hormone-dependent promoters, but the major problems of these systems include pleiotropic effects caused by the inducer in this systems, low level of induction and high basal transcription. Regulatable systems from prokaryotes are being used now days to reduce the pleiotropic effect and toxicity resulting from a eukaryotic-based inducible system. One of the widely used prokaryotic-based inducible systems is the tetracycline (Tet) regulatable system found in *Escherichia coli* (*E.coli*) (Yamamoto *et al.*, 2001).

### **1.4.1 Tetracycline-regulatable system**

The three main components of a tetracycline-inducible system are (1) transcriptional modulator (2) tetracycline-responsive promoter and (3) an antibiotic that belongs to tetracycline family. In this system tetracycline repressor protein (TetR)

from *E. coli* which binds to DNA in a Tet-dependent manner is used as a transcription modulator. Tetracycline-responsive promoter is the tetR binding sequence or tetracycline operator (TetO). Tetracycline family of drugs like doxycycline and anhydrotetracycline (ATC) are widely used as inducers (Orth *et al.*, 2000). Tetracycline repressor (TetR) is used by the gram-negative bacteria to obtain resistance from antibiotic tetracycline.

TetR regulates the transcription of TetA protein that helps to export tetracycline – magnesium complex  $[MgTc]^+$  out of the cell. High expression levels of TetA lead to nonspecific cation export from the cytoplasm leading to a change in the osmotic potential and collapse of cells. Therefore, the expression of TetA has to be tightly regulated. As soon as an optimal level of the level of TetA is reached, TetR binds to TetO and stops the transcription of tetA. This strong regulatable system is modified and made use in a Tet inducible system. TetR proteins and its mutants have been used to generate different types of Tet-inducible systems. One of the tet-inducible systems consists of tetracycline-controlled trans-activator (tTA), which consists of an activating domain of *Herpes simplex* virion protein 16 (VP16) bound to TetR protein of *E. coli* (Gossen and Bujard, 1992; Meissner *et al.*, 2001). In the absence of tetracycline tTA binds to TetO and in presence of tetracycline, tTA binds to tetracycline and this result in a conformational change, because of which TetR can no longer bind to TetO. The disadvantage of this system is that tetracycline has to be present in the cell all the time to repress tTA protein and constitutive expression of tTA is toxic to the eukaryotic cell. To remove the toxic effect due to constitutive expression of tTA, an auto-regulatory system was established wherein TetO sequence was inserted within tTA promoter (Shockett *et al.*, 1995). A different version of Tet-inducible system uses reverse trans-activator (rtTA) that binds to

TetO in the presence of doxycycline and as a result the gene of interest is transcribed in the presence of doxycycline (Mills, 2001).

The most commonly used tetracycline inducible system in protozoan parasite takes advantage of the wild TetR protein. The TetR system has been used to control gene expression in protozoan parasites like *Trypanosoma brucei* (Wirtz and Clayton, 1995), *Entamoeba histolytica* (Hamann *et al.*, 1997), *Giardia lamblia* (Sun and Tai, 2000) and *Toxoplasma gondii* (Meissner *et al.*, 2001).

### **Objectives**

The main goal of this project is to develop and characterize a tetracycline repressor based inducible RNAi system in *T. gondii*. An inducible system would allow us to study the function of various genes in *T. gondii*. This system can be further used to identify various genes responsible for pathogenesis of *T. gondii*.

## CHAPTER 2

# Materials and Methods

### 2.1 Chemicals

The chemicals used in this research work are listed below.

#### **Bio-Rad Laboratories**

Bromophenol blue, Coomassie Brilliant Blue R-250, xylene cyanol

#### **BioBasic**

Ethidium bromide, tris base

#### **BioExpress**

Agarose

#### **Fermentas**

Restriction enzymes *Hinf* I, *Not* I, *Pvu* II, *Sac* I, *Xho* I

#### **GibcoBRL Invitrogen**

Agar, Dulbecco's modified essential medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), minimum essential medium (MEM).

#### **Hyclone**

Cosmic calf serum

#### **Invitrogen corporation**

Antibiotic-antimycotic, proteinase K, RNase Out ribonuclease inhibitor

#### **NewEngland Biolabs Inc.**

Restriction enzymes *Alu* I, *Bgl* I, *EcoR* I, *Kpn* I, *Mbo* II, *Xmn* I.

**Promega**

Molony murine leukemia virus reverse transcriptase (M-MLV), RQ1 RNase free DNase ,  
1kb DNA ladder 1Kb, 100 bp DNA ladder, Restriction enzymes *BamH* I, *Bgl* II, *EcoR* V,  
*Hind* III, *Not* I

**Qiagen**

Midi prep kit for plasmid DNA isolation .

**Sigma-Aldrich**

Acetic acid, diethyl pyrocarbonate (DEPC), 95% and anhydrous ethanol, calcium chloride,  
chloramphenicol, EGTA, formaldehyde, hydrochloric acid, lauryl sulphate, mycophenolic  
acid (MPA), sodium citrate, sodium chloride, sodium acetate, streptomycin,  
trichloroacetic acid, xanthine.

**TaKara Bio Inc.**

Restriction enzymes *Pvu* I, *Sac* II.

**USB**

Glycerol, magnesium chloride, phenol, RNase A, sodium dodecyl sulphate (SDS)

**Nucleopore**

Polycarbonate filters (3  $\mu$ m)

**Wisent**

Dialysed fetal bovine serum (DFBS)

## 2.2 Generation of transgenic parasites

### 2.2.1 Strain of parasites

RH and RHtetR parasites were used for the present study. RH parasites were obtained from NIH through the AIDS Research and Reagent program. The RH strain was initially isolated from a 6 year-old boy in the year 1938 from Cincinnati, Ohio (Sabin, 1941). The child who had the initials RH was hit with a baseball bat and died 30 days after the incident. Homogenate of his cerebral cortex when inoculated into mice revealed the presence of *T. gondii*. The isolate has since then been called the RH strain. Since 1938, the strain has been passaged in mice in different labs and due to this prolonged passage its pathogenicity in mice has been stabilized (Dubey, 1977) and the strain no longer produce any oocysts in cats (Frenkel *et al.*, 1976). The RH parasites were cultured in confluent HFF monolayer containing ED1 medium (DMEM supplemented with L-glutamine, 10% dialyzed fetal bovine serum (DFBS) and 5 ml antibiotic-antimycotic (10000 unit/ml penicillin, 10000 µg/ml streptomycin, and 25 µg/ml fungizone).

RHtetR strain was donated by Markus Meissner (Heidelberg, Germany). To construct the RHtetR strain, a plasmid p5RT70tetR was created by cloning the TetR sequence between *EcoR*I and *Pac*I sites of plasmid p5RT70/HX. Hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) was used as a selection marker to express the transgene stably in *T. gondii*. RHtetR strain was grown on confluent HFF monolayer supplemented with ED1 medium supplemented with 25 µg/ml mycophenolic acid and 50 µg/ml xanthine (ED1/MPA/X). RHtetR parasites that were stably transfected with

various expression vectors were grown in ED1/MPA/X medium and selected using 20 mM Chloramphenicol.

### **2.2.2 Host cell**

Cell culture was carried out in MSC12 class II type A/B3 biosafety cabinet (Jouan). The parasites were grown in HFF monolayer obtained from NIH through AIDS Research and Reagent program. The host cells were grown in D10 media (Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Invitrogen) adjusted to pH 6.8, supplemented with L-glutamine, 10% iron fortified Cosmic calf serum (Hyclone), 5 ml antibiotic-antimycotic containing 10000 unit/ml penicillin, 10000 µg/ml streptomycin, and 25 µg/ml fungizone (Gibco, invitrogen). The cells were incubated at 37 °C in 5% CO<sub>2</sub> incubator (ThermoForma Model 310). The host cells were sub-cultured by adding 1 ml Trypsin-EDTA solution (0.25% trypsin, 0.35% EDTA) into a 60 mm tissue culture plate containing confluent HFF monolayer, incubating at 37 °C for 1 minute and then gently tapping to release the and disperse the trypsin treated cells. Fresh D10 media was added to trypsin treated plates and the contents were split into 4 plates.

### **2.2.3 Transfection**

Basic technique of transfection of *Toxoplasma gondii* was used for generating stable transfectants (Soldati and Boothroyd, 1993; Donald and Roos, 1993). Briefly, freshly released tachyzoites from lysed host cells were pelleted down by centrifugation at 1500x g for 15 minutes. The pellet was washed with sterile Dulbecco's phosphate-buffered saline supplemented with 0.1 g/l CaCl<sub>2</sub> (DPBS) before electroporation. The

pellet was then re-suspended in 300  $\mu$ l filter sterilized Cytomix buffer (120 mM KCL, 0.15 mM CaCl<sub>2</sub>, 10 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 7.6, 25 mM HEPES, pH 7.6, 2 mM EGTA, pH 7.6, 5 mM MgC<sub>2</sub>) to which 2 mM ATP and 5 mM glutathione was added immediately before use. 50  $\mu$ g of required plasmid which was linearized with appropriate restriction enzyme and precipitated with ethanol-sodium acetate, was re-dissolved in 100  $\mu$ l cold Cytomix. The 100  $\mu$ l of cold plasmid was then mixed with 300  $\mu$ l Cytomix containing parasites in a 1.5 ml centrifuge tube. The entire solution was transferred to a 2 mm BTX cuvette. The cuvette was tapped gently to release any air bubbles and then quickly subjected to electroporation. The electroporation was done by a single 1.5 kEV pulse at a resistance setting of 25  $\Omega$  and 25  $\mu$ F, using a BTX model 600 Electro Cell manipulator.

After electroporation, the cuvette was left undisturbed for 15 minutes at room temperature. The electroporated parasites were then inoculated into a confluent monolayer containing the appropriate selection medium.

#### **2.2.4 Genomic DNA isolation**

Genomic DNA was isolated using DNAzol<sup>®</sup> reagent. RH or RHtetR parasites were pelleted down by centrifugation at 5000x g for 5 minutes. The media was aspirated out; pellets were washed with 1 ml PBS and were again pelleted down at 5000x g for 5 minutes. 1 ml DNAzol<sup>®</sup> reagent was added to the pellet and pipetted up and down gently to lyse the cells. The sediments from the homogenate were removed by centrifugation at 10,000x g at 4 °C for 10 minutes. This step removes the cell debris and the RNA. The supernatant was transferred to a fresh 1.5  $\mu$ l centrifuge tube and the DNA

was precipitated with 500  $\mu$ l of 100% ethanol. The sample was mixed by inversion and stored at room temperature for 3 minutes. The precipitated DNA was collected by centrifugation at 12000x g for 5 minutes at room temperature and washed twice with 1 ml 75% ethanol. The DNA was pelleted by centrifugation at 7500x g for 5 minutes at room temperature. The DNA pellet was then air dried for 1 minute, re-suspended in 200  $\mu$ l of 8 mM NaOH and pH was adjusted to 7 by adding 32  $\mu$ l of 0.1 M HEPES. The quality of the isolated DNA was determined by agarose gel electrophoresis and the quantity was determined by spectrophotometric quantification.

### **2.2.5 Polymerase Chain Reaction**

Different PCR conditions were set up to amplify the required fragments. In general 25  $\mu$ l PCR reactions were set up containing 2.5  $\mu$ l of 10X PCR buffer (100 mM Tris-HCL, pH 8 at 25 °C, 500 mM KCL and 1% Triton<sup>®</sup> X-100), 2.5  $\mu$ l of 50 mM magnesium chloride, 0.25  $\mu$ l of 2.5 mM dNTPs, 0.5  $\mu$ l of each primer (10 pmol), 1  $\mu$ l of DNA and 1 unit of Taq polymerase. The PCR was set for 30 cycles (95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute).

### **2.2.6 Cloning (Gateway Cloning)**

Gateway cloning technique was used to generate the required clones. With the aid of gateway technology it is possible to do parallel transfer of any number genes into or out of multiple expression systems by simply mixing DNA, adding the required enzymes and incubating the reaction mixture. One of the major advantages of this technique is that DNA segments transferred using this technology maintain reading frame and orientation.

In a LR reaction, the sequence of interest is cloned in an 'entry vector' that is transcriptionally silent, spectinomycin resistant ( $spn^r$ ) and flanked by two recombination sites (*att* L1 and *att* L2). The sequence of interest is then transferred to a 'destination vector'. The destination vector contains all of the sequence information required for expression of the required gene of interest and is ampicillin resistant ( $Amp^r$ ). This plasmid contains two recombination sites (*att* R1 and *att* R2) that flanks a gene of negative selection, *ccdB*. Entry and destination vector in the presence of LR Clonase™ reacts directionally to yield 'expression vector' and a donor vector. Selection on ampicillin eliminates the entry vector and negative selection through the *ccdB* gene eliminates the donor vector.

pCR®8/GW/TOPO® vector was used to construct the entry clones. pCR®8/GW/TOPO® is a linear vector with single 3'-thymidine (T) overhangs so that it can be used for TA cloning. It has topoisomerase covalently bound to it and thus it is referred to as an activated vector. This vector is generated by treating plasmid with Vaccinia viral topoisomerase I which binds to DNA duplex containing the sequence CCTT and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy generated from the broken phosphodiester backbone is conserved in a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and the enzyme can be subsequently attacked by the 5' hydroxyl of the original cleaved strand or by any DNA strand with free 5' hydroxyl group, reversing the reaction and thereby releasing topoisomerase. TOPO® Cloning takes advantage of this reaction to efficiently clone PCR products.

### **2.2.6.1 Generation of Entry clones**

The gene of interest to be cloned into pCR®8/GW/TOPO® vector was PCR amplified using Taq polymerase. 1 µl salt solution (1.2 M NaCl, 0.06 M MgCl<sub>2</sub>) was mixed with 4 µl PCR product and then 0.5 µl TOPO® vector was added. The reaction was mixed gently and incubated at room temperature for 5 minutes. After 5 minutes the reaction was placed on ice until the transformation was performed using One Shot®Mach1™-T1<sup>R</sup> Chemically Competent *E. coli* cells.

### **2.2.6.2 Generation of Expression Clones**

To set up the LR reaction, 0.5 µl of the required entry vector was mixed with 0.5 µl of destination vector (pT5784 att<sub>2</sub> 2.2 mCAT) and the volume was made up to 4 µl with TE buffer. The LR Clonase™ enzyme mix was thawed on ice for 2 minutes and vortexed twice for 2 sec to mix the contents. 1 µl LR Clonase™ enzyme was added to the mixture of entry and destination vectors and the reaction was incubated at 25 °C for 1 hour. After 1 hour 1 µl of Proteinase K solution was added to the reaction and incubated at 37 °C for 10 minutes to break down the LR Clonase™ enzyme. 1 µl of this reaction was used for transformation in One Shot®Mach1™-T1<sup>R</sup> Chemically competent *E. coli* cells.

### **2.2.6.3 Transformation in *E. coli***

#### **2.2.6.3.1 Competent cell preparation**

Transformation efficient DB 3.1 cells were streaked on LB plate to obtain isolated single colonies. One single colony was picked using a sterile toothpick, inoculated into a

Falcon 2059 culture tube containing 5 ml LB broth and was grown for 12-16 hr in a shaker incubator (200 rpm) at 37 °C. This 5 ml culture was then transferred into a culture flask containing 200 ml pre-warmed LB medium and incubated at 37 °C in a shaker incubator (200 rpm) until an O.D of 0.6 was reached. The flask was then chilled on ice for 30 minutes and then the bacterial culture was aliquoted into four, 50 ml Falcon tubes. The tubes were centrifuged at 3000x g for 15 minutes at 4 °C in a swing bucket rotor centrifuge. The tubes were carefully transferred to an ice bucket after discarding the media. Nearly 5 ml media was retained in the Falcon tubes to re-suspend the pellet. 30 ml of ice cold 100 mM calcium chloride solutions was added to the Falcon tubes, the contents were mixed by inverting and the tubes were then centrifuged at 4 °C and 4000 rpm for 15 minutes. The pellet washed with 100 mM calcium chloride, three times the same fashion as described above. After the last wash all of the supernatant was removed from the Falcon tubes. The pellet in each Falcon tube was re-suspended in 1.5 ml of 200 mM calcium chloride and 30% glycerol. The competent cells were aliquoted and placed in -80 °C immediately.

#### **2.2.6.3.2 Transformation**

A slightly modified protocol from Sambrook *et al.*, 1989 was used for bacterial transformation. For each transformation 20 µl of competent cells were aliquoted into a 1.5 µl microcentrifuge tube. 1 µl of the plasmid to be transformed was added to the competent cells and mixed by gentle tapping of the microcentrifuge tube. The tube was incubated on ice for 30 minutes and subjected to 30 sec of heat-shock at 42 °C without shaking. 280 µl of room temperature SOC medium was added and incubated in a shaker

incubator (200 rpm) at 37 °C for 1 hour. 50 µl of this bacterial culture was spread on LB agar plates containing the appropriate antibiotics and incubated at 37 °C for 12-16 hr.

#### **2.2.6.4 Identification of transformed colonies by colony PCR**

Bacterial colonies were picked from agar plates using sterile 200 µl pipette tips or toothpicks and inoculated into a 0.5 ml centrifuge tube containing 50 µl sterile water. The tube was vortexed to evenly distribute the bacterial cells. The centrifuge tube was then placed in boiling water or heating block that was heated to 95 °C for 5 minutes to lyse the bacterial cells and denature DNase. The tube was centrifuged at 12000x g for 1 minute to remove the cell debris. 10 µl of the supernatant was transferred to a fresh 0.5 µl centrifuge tube and left on ice until PCR reaction was set up. A 40 µl PCR reaction was set up containing 1 µl of 2.5 mM dNTP mix, 1 µl each of upstream and downstream primer (5 pmol/µl each), 5 µl of 10X PCR buffer (100 mM Tris-HCL, pH 8 at 25 °C, 500 mM KCL and 1% Triton<sup>®</sup> X-100), 3 µl of 25 mM MgCl<sub>2</sub>, 0.25 µl of Taq DNA polymerase (home made) and 28.8 µl of sterile autoclaved double distilled water.

#### **2.2.6.5 Isolation of Plasmid**

Bacterial colony harbouring the right plasmids, confirmed by colony PCR were inoculated into a Falcon 2059 culture tube containing 3 ml LB medium with appropriate antibiotics using a sterile toothpick. The culture was incubated for 12 hr at 37 °C in a shaker incubator at (200 rpm). 1ml bacterial culture was transferred to a micro centrifuge tube and centrifuged at 12000x g for 10 minutes. Alkaline lysis method of plasmid isolation was followed to further isolate plasmids. After centrifugation, the medium was

aspirated to leave the pellet as dry as possible. The pellet was re-suspended in 100  $\mu$ l of 'Re-suspension-solution 1' (50 mM Glucose, 25 mM Tris-HCL pH 8, 10 mM EDTA) by pipetting up and down until all the pellet was completely dispersed. 200  $\mu$ l of 'Lysis Buffer-solution II' (0.2 N NaOH, 1% SDS) was added to completely lyse the bacterial cells followed by 100  $\mu$ l of 'Precipitation Buffer-solution III' (3 M NaOAc, pH 5.2). The precipitate formed was mixed by inversion and then centrifuged at 12000x g for 15 minutes at room temperature. The supernatant was carefully poured into a fresh 1.5  $\mu$ l centrifuge tube and 400  $\mu$ l phenol:chloroform solution was added. The tube was vortexed and centrifuged at 12000x g for 1 minute at room temperature for phase separation. The aqueous phase was transferred to a fresh 1.5  $\mu$ l centrifuge tube, mixed with 800  $\mu$ l ice cold 95% ethanol and centrifuged at 12000x g for 10 minutes at 4 °C. The supernatant was decanted and the pellet was washed with 400  $\mu$ l ice cold 70% ethanol. The tube was centrifuged at 12000x g for 10 minutes and the ethanol supernatant was discarded. The pellet was further re-suspended in 30  $\mu$ l TE buffer containing 20  $\mu$ g/ml RNase A and incubated at 37 °C for 15 minutes to degrade RNA. The quality of the DNA obtained was analysed by gel electrophoresis and later subjected to restriction analysis or PCR.

#### **2.2.6.6 Analysis of restriction profile to confirm the plasmid**

Restriction digestions of the plasmids were performed to confirm the plasmid. The predicted restriction profiles with respect to specific enzymes were first generated using, Vector NTI bioinformatics tool. The restriction digestion was performed in a total volume of 10  $\mu$ l containing 2  $\mu$ l plasmid DNA, 1  $\mu$ l 10x buffer, 0.2  $\mu$ l enzyme and 6.8  $\mu$ l autoclaved Millipore water. The reaction was mixed gently by pipetting and incubated

overnight at 37 °C. The reaction was run on 1% agarose gel and visualized using AlphaImager 2200 documentation and analysis system.

## **2.3 Gel Electrophoresis**

Genomic DNA, RNA, PCR and RT-PCR products were resolved on 1% (w/v) agarose gels.

### **2.3.1 Agarose gel electrophoresis**

Agarose was dissolved in Tris-acetate-EDTA (TAE) buffer (40 mM Tris acetate and 1 mM EDTA) containing 10ng/ml ethidium bromide. Melted agarose was poured into gel-casting tray with appropriate combs and allowed to cool. The combs were removed and the gel was placed in an agarose gel electrophoresis chamber. The gel was completely submerged with TAE buffer. 1 µl of sample to be loaded into the well was mixed 5 µl of DNA/RNA loading dye 0.25% (w/v) Bromophenol Blue, 0.25% (w/v) xylene cyanol FF and 30% glycerol in water. The agarose gel was run for 15-30 minutes at 120V. The gel was then visualized using AlphaImager 2200 documentation and analysis system and the picture was analyzed using Chemilmager V5.5.

## **2.4 Reverse transcription-polymerase chain reaction (RT-PCR)**

### **2.4.1 Isolation of total RNA**

Total RNA from all strains of *T. gondii* that was used in the study, was extracted using TRI Reagent®. The parasites that had lysed the HFF monolayer were passed through 26G<sup>3/8</sup> (PrecisionGlide®) syringe needle and filtered through 3 µm filter to break

and remove host cell debris. Centrifugation for 10 minutes at 5000x g was used to pellet down the parasites. The pellets were further washed with 1ml phosphate buffered saline (PBS) to wash out all the media from the parasite pellet. The pellet was lysed and homogenized by adding 1 ml TRI Reagent® and incubating at room temperature for 5 minutes. 200 µl chloroform was added, mixed vigorously and the sample was incubated at room temperature for 3 minutes. The sample was then centrifuged at 12000x g for 15 minutes at room temperature for phase separation. The aqueous phase was transferred to a new microcentrifuge tube. 500 µl isopropanol was added to the aqueous phase, mixed and stored at room temperature for 10 minutes to precipitate total RNA. Precipitated RNA was collected by centrifugation at 12000x g for 10 minutes. The RNA pellet was further washed with 1 ml 75% ethanol, centrifuged for 5 minutes at 7,500x g at room temperature and air dried for 5 minutes. The RNA pellet was then dissolved in 50µl of DEPC treated water and incubated at 55 °C for 10 minutes. 1 µl of isolated RNA was run on agarose gel to determine the integrity of the isolated RNA and to check for DNA contamination. The total RNA after isolation was treated with DNase to remove DNA contamination. For this a 25 µl reaction was set up containing 16.5 µl of total RNA, 2.5 µl of 10X DNase buffer, 5 µl of 5X transcription buffer and 1 µl of DNase enzyme. The reagents were mixed properly by pipetting and incubated at 37 °C for 15 minutes. In order to reduce loss of RNA during phenol:chloroform extraction, the volume was made up to 200 µl by adding DEPC treated and autoclaved water. 200 µl of TE-buffered phenol:chloroform:isoamyl alcohol (25:24:1) was added, the sample was vortexed to denature DNase and centrifuged at 12000x g for 5 minutes for phase separation. The aqueous phase was transferred to a fresh 1.5 µl centrifuge tube and 0.1 volume 3 M sodium acetate and 2

volumes of ice cold 95 % ethanol were added. The solution was mixed properly, placed at -20 °C for 30 minutes and centrifuged at 12000x g for 5 minutes. The pellet obtained was washed with 70% ethanol; air dried for 2 minutes and dissolved in 50 µl DEPC treated autoclaved water.

#### **2.4.2 Quantification of RNA**

RNA has an absorbance peak at 260 nm. 2 µl total RNA was diluted into 500 µl DEPC water placed inside the quartz cuvette. The spectrophotometer was first blanked using DEPC water and then the absorbance of the diluted RNA was recorded. The RNA was quantified using the following equation:

$$\text{RNA } [\mu\text{g}/\mu\text{l}] = \text{A}_{260} \times 500 / 2 \mu\text{l of RNA assayed} \times 40$$

#### **2.4.3 RT-PCR**

One microgram DNase treated total RNA from stable transformants and wild type parasites were used as templates for the RT reaction. Briefly, 1 µg total RNA was mixed with 0.5 µg of the required primer and the volume was made up to 15 µl. The reaction mixture was incubated at 70 °C for 5 minutes to melt the secondary structure within the template. The tube was immediately cooled on ice to prevent the secondary structure from reforming. The tube was then centrifuged briefly to collect the solution at the bottom of the tube. To this tube 50 mM Tris-HCL (pH 8.3), 75 mM KCL, 3 mM MgCl<sub>2</sub> and 10 mM DTT were added followed by 5 µl of 10 mM dNTPs, 25 units of RNase inhibitor and 200 units of M-MLV RT enzyme. The volume was made up to 25 µl using DEPC treated autoclaved water. The reagents were mixed by gentle flicking and incubated for 60

minutes at 42 °C. The reverse transcriptase enzyme was inactivated by heating the reaction mixture at 95 °C for 2 minutes.

## **2.5 Nucleotide incorporation assay**

Freshly harvested parasites were counted using a haemocytometer and 10000 parasites were inoculated per well in a 24-well plate containing a confluent HFF monolayer. The RH parasites were grown in ED1 media, RHTetR parasites were grown in ED1 media containing mycophenolic acid and xanthine while stable transfectants in RHTetR parasites were grown in media containing ED1 with mycophenolic acid xanthine and chloramphenicol. After 24 hr, 1  $\mu$ Ci [5, 6 -<sup>3</sup>H]-uracil or hypoxanthine was added per well and incubated for 4 hr at 37 °C in 5% CO<sub>2</sub> incubator. The 24-well plate was chilled at 20 °C for 2 minutes and then 1 ml ice cold 0.6 N trichloroacetic acid (TCA) was added per well into the existing medium. The plate was incubated for 1 hr to fix the HFF monolayer. The TCA solution was pipetted out into the radioactive waste container. To each well 500  $\mu$ l Millipore water was added and the plate was put on a shaker to rinse out the TCA solution and unincorporated radioactive material. This step was repeated at least five times to ensure proper washing of the host cells. In order to ensure that the cells were properly washed, 250  $\mu$ l of the final wash solution was added to a scintillation tube and mixed with 3 ml scintillation fluid and read using a scintillation counter. The washes were done until a reading of less than 300 cpm was observed for the washes. After proper washing of the cells the plate was dried and 500  $\mu$ l of 0.1 N NaOH was added per well. The plate was then incubated at 37 °C for 60 minutes to dissolve the TCA precipitates. 250  $\mu$ l of the sample per well was mixed with 3 ml scintillation fluid and the radioactive

count was determined using the scintillation counter. Each experiment was carried out in triplicate.

## **2.6 Tolerance of transgenic UPRT knockdown parasite for 5-fluoro-2'-deoxyuridine**

5-fluoro-2'-deoxyuridine (FDUR) is converted to 5-fluorouracil by the parasite inhibiting the synthesis of TMP. Therefore assimilation of FDUR is toxic to the parasite. A microtiter assay was carried out to study the ability of UPRT knockdown parasite to grow in the presence of 5-fluoro-2'-deoxyuridine. 6-thioxanthine was used as a control to rule out drug resistance due to mutation. Briefly, FDUR and 6-thioxanthine were serially diluted in EDI media supplemented with mycophenolic acid and xanthine using a 96-well plate. The effect of the drug was studied under both induced and uninduced condition. 10000 parasites were added per well and the plate was incubated at 37 °C in a CO<sub>2</sub> incubator. The HFF monolayer with parasite and devoid of drug, which acted as the control, was observed daily for the progression of parasite growth. When the cell monolayer was completely destroyed in control wells, the media was aspirated and the host cells were rinsed with PBS, fixed with methanol and stained with crystal violet (5x stock: 25 g crystal violet dissolved in 250 ml methanol was added to 1000 ml 1% ammonium oxalate. The intensity of the stained monolayer was recorded using Alpha Imager. Control wells containing parasite, devoid of drug were assigned an arbitrary value of 100%. Values of other wells were calculated in comparison to control well. LD<sub>50</sub> value was calculated from the graph that was plotted based on the obtained value.

## **2.7 Analysis of the role of MIC 3 in invasion and metabolism of the parasite**

RHtetR parasites that were stably transfected with MIC 3 were grown in HFF cells containing ED1 media supplemented with mycophenolic acid and xanthine, with and without anhydrotetracycline. Freshly lysed parasites from individual plates were counted using haemocytometer and 10000 parasites were inoculated into separate plates with and without anhydrotetracycline. The parasites were allowed to grow for 24 hr. The infected monolayers were fixed with 3% paraformaldehyde, and their nuclei were stained using 100  $\mu$ M Hoechst stain to enable the visualization of intracellular parasites and host cells, respectively. The number of vacuoles and number of parasites per vacuole was counted.

## CHAPTER 3

### Results

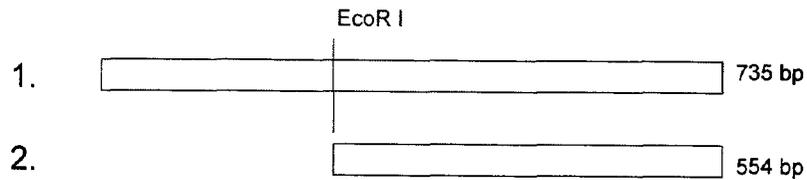
The aim of this project was to develop and characterize a tetracycline repressor based inducible RNAi system in *T. gondii*. RNA tools including antisense RNA, ribozyme and dsRNA have been previously used to downregulate the expression of *T. gondii* genes (Nakaar *et al.*, 1999, 2000; Al-Anouti and Ananvoranich, 2002; Sheng *et al.*, 2004). These tools have proved to be very efficient for the study of the gene function through loss-of function studies. An inducible system would further help us to study the function of various genes in *T. gondii*. The tetracycline based inducible system will be a valuable tool to control the expression of gene in this protozoan parasite for the study of its pathogenesis.

The results obtained from this study are described in two sections. The first section describes characterization of a tetracycline based inducible RNAi system that was targeted to downregulate the marker gene uracil phosphoribosyl transferase (UPRT). UPRT is an enzyme which is used by the parasite in the pyrimidine salvation pathway by converting free uracil to uridine monophosphate (Schwartzman and Pfefferkorn, 1977). The second section describes the generation of a tetracycline inducible RNAi expression system that targets an invasion-related gene called microneme protein 3 (MIC 3). MIC 3 is required for the parasite's attachment to the host cells through recognition and effective binding of host cell receptors (Soldati *et al.*, 2001).

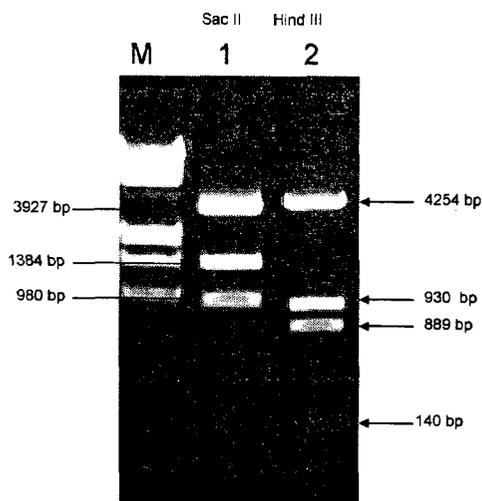
### **3.1 Inducible RNAi system against UPRT**

#### **3.1.1 Plasmid construct**

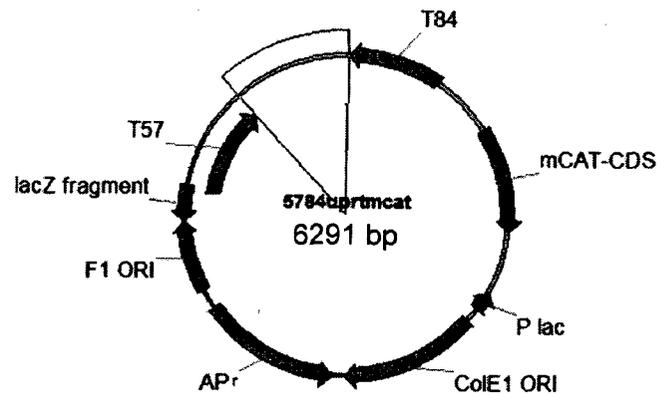
To create the plasmid used for the study, a 554 bp fragment of the UPRT gene was cloned between two head-to-head tetracycline inducible promoters. The resultant plasmid called p5784uprtmcat was digested with restriction enzymes for confirmation. The open reading frame (ORF) of UPRT is 735 bp long (Fig.3.1.A1). The ORF was digested with *EcoR* I and the resulting fragment from 182 bp to 735 bp (554 bp) were cloned between the head-to head promoters (Fig.3.1.A2). The plasmid was individually digested with *Sac* II and *Hind* III to verify the construct (Fig.3.1.B). *Sac* II sites are located at 624 bp, 2008 bp and 5935 bp on the plasmid. The presence of bands of 3,927 bp, 13,884 bp and 980 bp confirm the plasmid. *Hind* III sites are located on 423 bp, 563 bp, 641 bp, 4895 bp, and 5,784 bp. The bands of 4,254 bp, 930 bp, 889 bp, 140 bp, 78 bp further confirmed the validity of the construct.



A



B

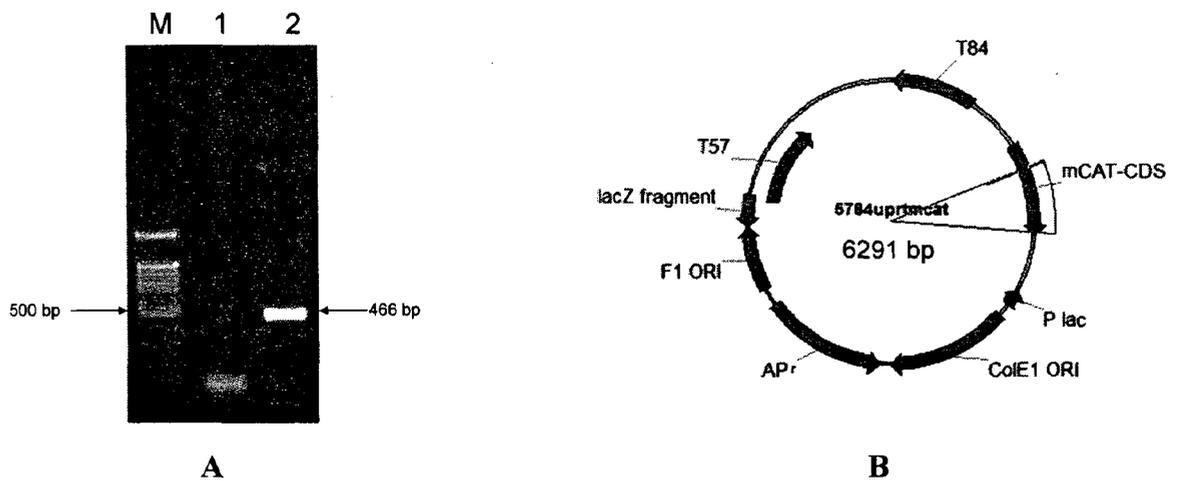


C

**Figure 3.1** Confirmation of plasmid p5784uprtmcat by restriction digestion. (A) 1: 735 bp ORF of UPRT gene; 2: 554 bp fragment of the ORF that was cloned to create the p5758uprt plasmid (B) Lane 1: plasmid p5784uprtmcat Sac II digest; Lane 2: plasmid p5784uprtmcat Hind III digest (C) Vector map of plasmid p5784uprtmcat. The region where the 554 bp UPRT gene fragment is cloned is shown by a pie section.

### **3.1.2 Generation and confirmation of transgenic parasites containing inducible RNAi system against UPRT gene**

To generate transgenic parasites with the inducible system, the plasmid p5784uprtmcat was introduced into the RHtetR strain of *T. gondii* through electroporation. Genomic DNA was extracted from the transgenic parasites and subjected to PCR amplification to confirm stable integration of the plasmid. The 5784uprtmcat plasmid contains a selectable gene that codes for chloramphenicol acetyl transferase (CAT). CAT gene is absent in *T. gondii* therefore, only parasite strains stably transfected with the plasmid will harbour this selectable gene. PCR was done using the CAT\_EcoRI<sub>mut</sub> and SPHI3'CAT primers and the presence of a 466 bp product confirmed the presence of CAT fragment in the transgenic parasite line (Fig. 3.2). This stable line of transgenic parasite was named RHtetRuprt.



**Figure 3.2** Confirmation of transgenic integration of plasmid p5784uprtmcat. A 466 bp fragment was PCR amplified from the CAT coding sequence using primers CAT\_EcoRI<sub>mut</sub> and SPHI3'<sub>CAT</sub>. (A) Lane 1: PCR amplified product from DNA isolated from RHtetRuprt; Lane 2: PCR amplified product from plasmid p5784uprtmcat which was used as a positive control. (B) Vector map of plasmid p5784uprtmcat. The CAT coding sequence that was amplified from the integrated plasmid is marked by the pie section.

### **3.1.3 Confirmation of the expression of RNA homologous to UPRT in RHtetRuprt**

#### **Parasites**

RT-PCR procedure was performed using primer set 1 (Fig. 3.3 A, B), to verify if the antisense strand of UPRT is generated by the RHtetR parasite. The absence of any product corresponding to antisense RNA against UPRT confirmed that antisense RNA against UPRT is absent in these parasites. The transgenic RHtetRuprt strain which harbours the dsUPRT generating plasmid should be able to generate dsUPRT and thus antisense RNA for UPRT when the head-to-head promoters are induced. RT-PCR was used to amplify antisense RNA from induced and uninduced RHtetRuprt parasites using primer set 2. No product was obtained when antisense strand was amplified from uninduced RHtetRuprt parasites. When antisense RNA was amplified from induced RHtetRuprt parasites, the expected fragment (280 bp) was observed. These results indicated that antisense RNA against UPRT mRNA was not expressed naturally. Further, antisense RNA and thus dsUPRT in RHtetRuprt parasites were expressed only when they were subjected to induction (Fig. 3.3.B).

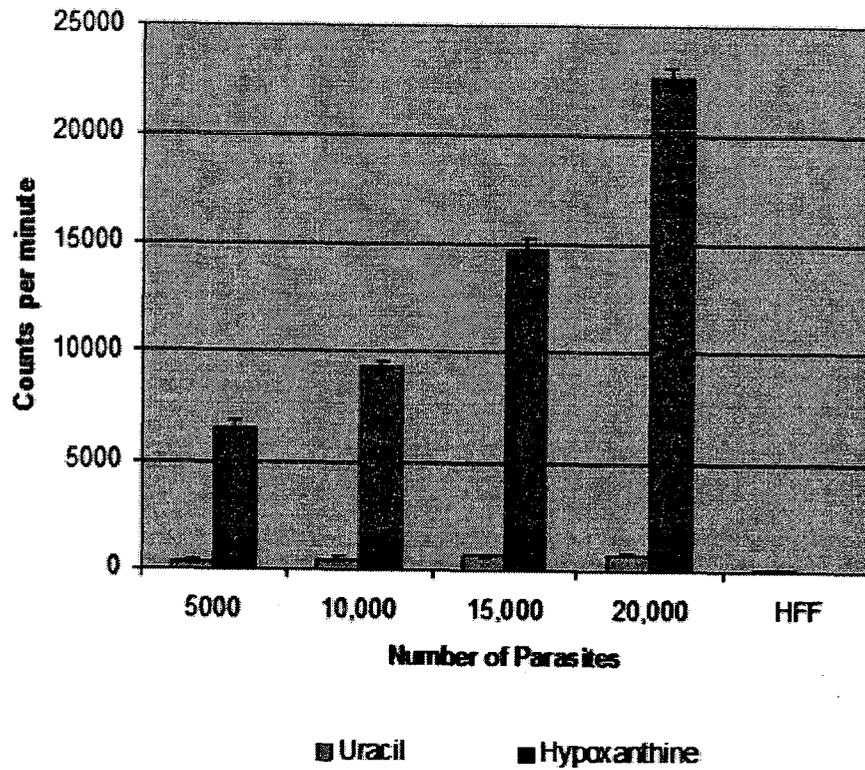


### **3.1.4 Confirmation that the expression of UPRT was altered in transgenic RHtetRuprt parasites**

#### **3.1.4.1 Validation of UPRT downregulation in RH strain**

To check the efficiency of the plasmid to downregulate UPRT, the plasmid was stably transfected in the RH strain of *T. gondii*. The RH strain does not have the ability to produce tetracycline repressor protein. Thus, constitutive expression of dsUPRT should take place in the RH parasites. The UPRT dsRNA will trigger the RNAi pathway and will result in downregulation of UPRT expression. Consequently, the parasites' ability to salvage free uracil will be diminished.

When  $^3\text{H}$  uracil was introduced to the medium, it was observed that the ability of the transgenic RH parasites to salvage uracil was considerably low (Fig. 3.4). This result confirmed that endogenous UPRT was downregulated in the transgenic RH parasites containing plasmid. To demonstrate the specificity of gene knockdown, the activity of the HXGPRT gene, which is responsible for hypoxanthine uptake, was monitored by measuring the salvage of  $^3\text{H}$  hypoxanthine by the parasite. The results showed that the uptake of hypoxanthine increased with increasing number of parasites and the presence of plasmid p5784uprtmcat did not impair HXGPRT activity. This confirmed the specificity of the knockdown.



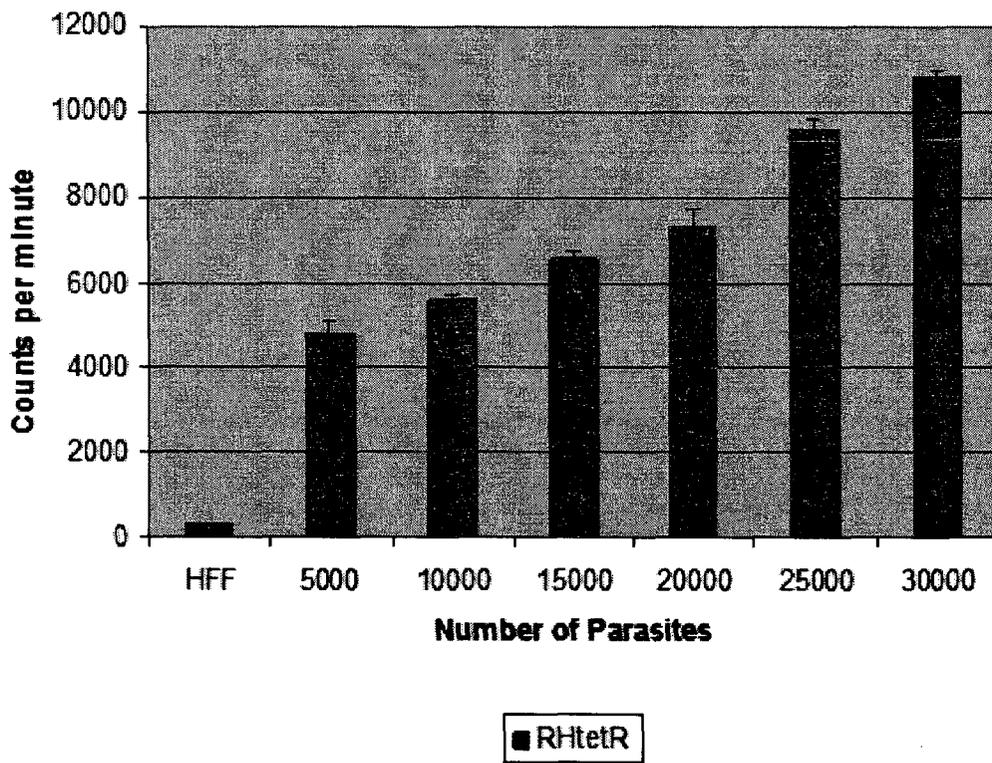
**Figure 3.4** Nucleotide uptake assay. The UPRT gene was downregulated in RH strain and the ability of these parasites to assimilate  $^3\text{H}$ -uracil and  $^3\text{H}$ -hypoxanthine was analysed.

### **3.1.4.2 Characterization of the inducible system in RHtetraprt strain**

#### **3.1.4.2.1 Optimization of number of transgenic RHtetR parasites**

To characterize the inducible system, it was essential to work with an optimum number of parasites. Particularly for nucleotide uptake assays, infected monolayers are required to be intact so that the fixing and washing steps can be performed. Large number of parasites will lyse the host cells but too few parasites results in low radioactive emission readings that are difficult to interpret. One way to increase the radioactive emission readings is to increase the concentration of radioactive material but this is not advised as radioactive materials are costly.

To optimize the number of parasites to be used, different amounts of (5,000 to 30,000) parasites were inoculated into host cells. As seen in Fig. 3.5, the uptake of <sup>3</sup>H-uracil increased with increasing number of parasites. The host cells lysed when more than 10,000 parasites were administered at 24 hr post-infection. Therefore, it was concluded that 10,000 parasites were optimal for the nucleotide uptake assays.

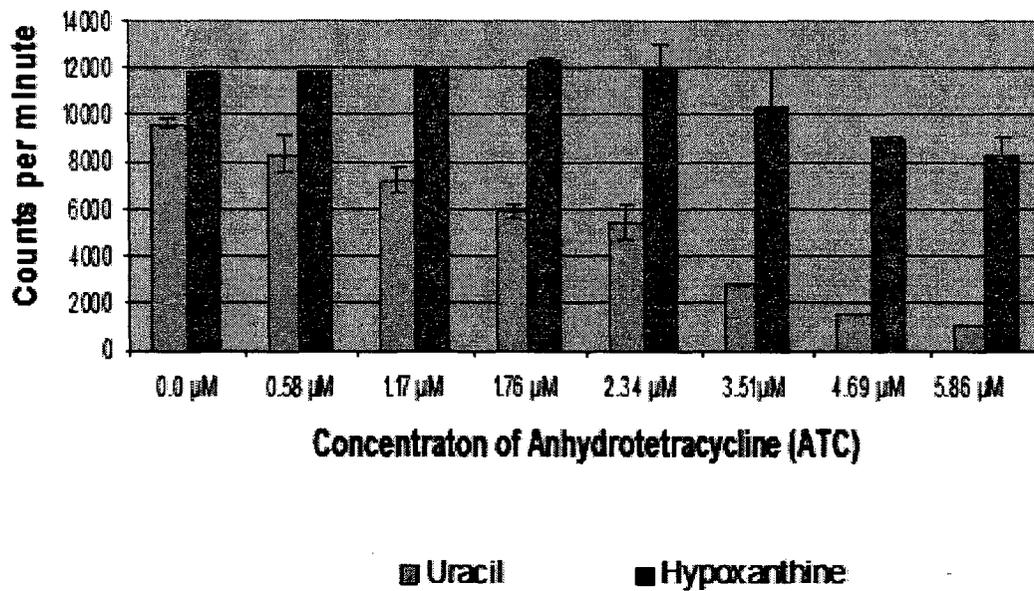


**Figure 3.5** Optimization of number of the RHtetR parasites. In order to optimize the maximum number of parasites that would grow without lysing the host cell in 24 hr, <sup>3</sup>H-uracil uptake assay was done with different number of RHtetR parasites.

### 3.1.4.2.2 Optimization of the concentration of inducer

Anhydrotetracycline (ATC) is toxic to the parasites and host monolayer at high concentration so it was essential to optimize the amount required for the experiment. Freshly lysed, 10,000 parasites were inoculated into each well of a 24-well plate containing confluent HFF monolayer. Various concentrations (0  $\mu\text{M}$  to 5.86  $\mu\text{M}$ ) of ATC were added to the selection media, and the nucleotide uptake assay was done using the transgenic RHtetRuprt parasites. The nucleotide uptake assay was performed with  $^3\text{H}$  uracil and  $^3\text{H}$  hypoxanthine.  $^3\text{H}$  hypoxanthine was included in the study to measure the toxic effect of ATC. Uptake of  $^3\text{H}$  hypoxanthine remained constant until 1.76  $\mu\text{M}$  ATC but it gradually decreased beyond that concentration due to the toxic effect of ATC. The uptake of  $^3\text{H}$  uracil decreased as the concentration of ATC was increased. The decrease in uptake of  $^3\text{H}$  uracil could be either due to knockdown of endogenous UPRT or due to the toxic effect of ATC.

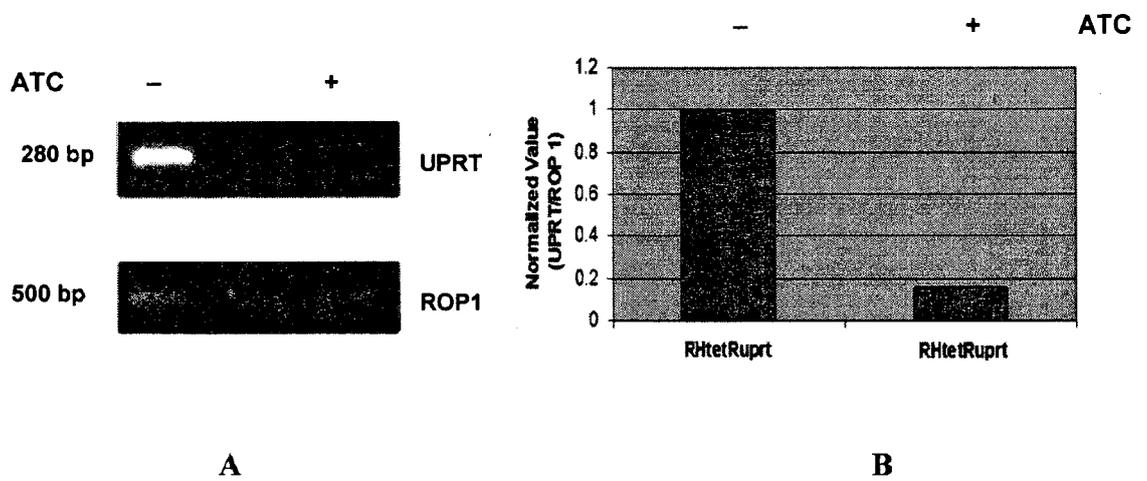
The result shows that 1.76  $\mu\text{M}$  ATC results in a maximal induction with minimum toxicity to the parasite (Fig. 3.6). This concentration was used for remainder of the study.



**Figure 3.6** Optimization of inducer. Different concentration of ATC was added to 10,000 parasites to check for its lethal effect on the parasite. Induction ability is depicted by the amount of <sup>3</sup>H-uracil uptake as UPRT is down-regulated upon induction. As a control <sup>3</sup>H-hypoxanthine was used as parasite viability is directly proportional to <sup>3</sup>H-hypoxanthine uptake.

### **3.1.4.3 The UPRT expression profile of RHtetRuprt strain**

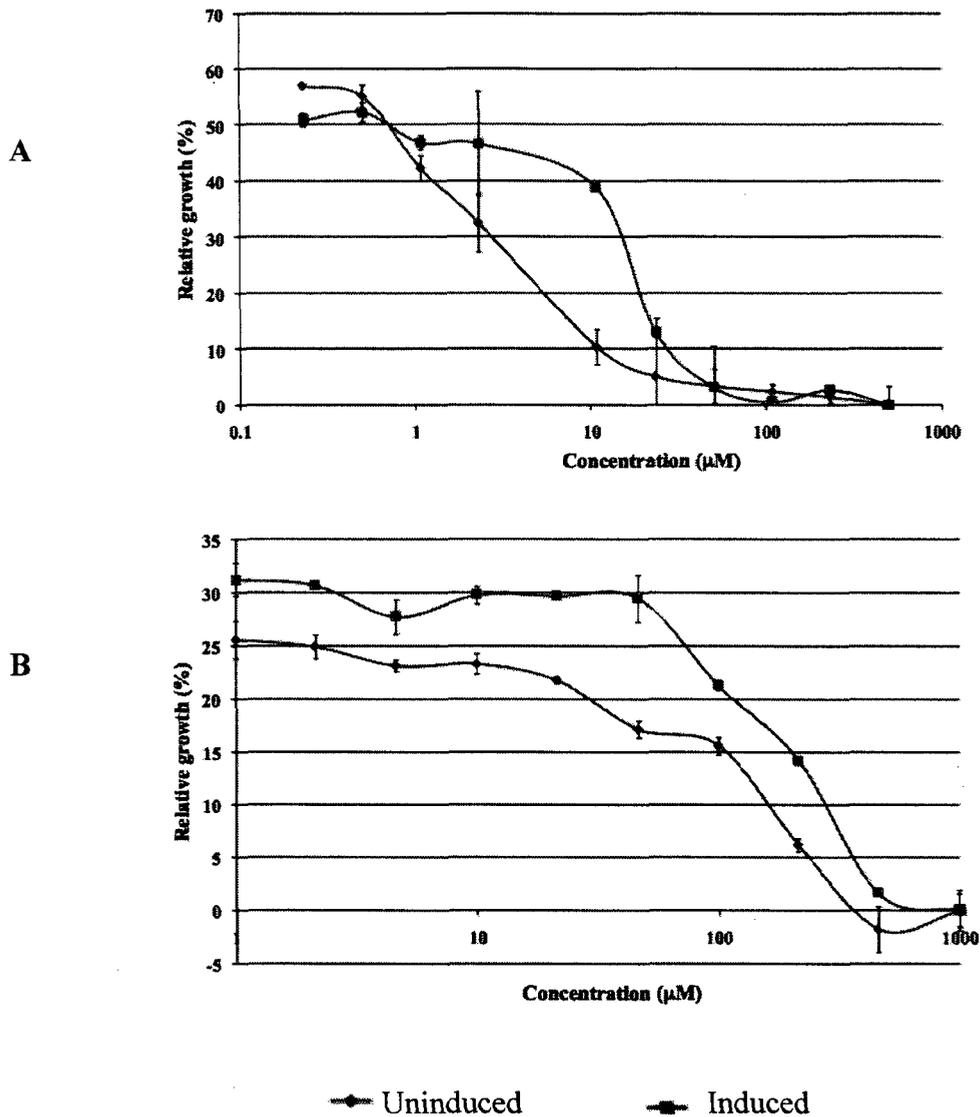
The stable line of transgenic parasite RHtetRuprt was designed to express UPRT dsRNA upon induction with ATC. After confirming the optimum concentration of ATC that resulted in maximum induction with minimum toxicity, we verified the extent to which the target gene was knocked down. Freshly lysed RHtetRuprt parasites were grown in the presence or absence of 1.76  $\mu$ M ATC. Induced and uninduced parasites were collected after host cells had been completely lysed. Total RNA was extracted from these parasites and subjected to RT-PCR using primers upstream\_HincII and EcoRI\_3'UPRT. The gene expression level of UPRT and Rhoptry 1 (ROP 1) in induced and uninduced RHtetRuprt parasites is shown in the Fig. 3.7.A. ROP 1 was used as an internal control. The ratio of signals of UPRT/ROP 1 was calculated and the normalized UPRT expression level in induced and uninduced parasites is shown in Fig. 3.7.B. The result shows that the expression of endogenous UPRT mRNA level was decreased in induced RHtetRuprt parasites as compared to uninduced parasites. Thus, these results showed that we have been successful in generating a transgenic line of parasites called RHtetRuprt that contains an inducible RNAi system against the UPRT gene.



**Figure 3.7** Gene expression level in transgenic RHtetRuprt parasites. These parasites were grown without or with ATC. (A) RT-PCR of UPRT and ROP 1. Lane 1: RT-PCR of UPRT and ROP1 gene in uninduced parasites; Lane 2: RT-PCR of UPRT and ROP1 gene in induced parasites. (B) Expression profile of UPRT normalized against ROP 1.

### 3.1.5 Tolerance of RHtetR parasite for 5-fluro-2'-deoxyuridine

5-fluro-2'-deoxyuridine (FDUR) can be taken up into the parasite by the UPRT enzyme from the media and metabolized to 5-fluorouracil, which is toxic to the parasite (Donald and Roos, 1995). Thus, FDUR can be used to select parasite strains that lack a functional UPRT gene. Since endogenous UPRT in RHtetRuprt parasites can be knocked down by inducing them with ATC, their ability to tolerate FDUR was measured. Dose-dependent studies were conducted to determine the concentration of FDUR that results in 50% parasite lethality ( $LD_{50}$ ). The ability of the UPRT knock down parasite to grow in the presence of 5-fluro-2'-deoxyuridine (Fig. 3.8.A) or 6-thioxanthine (Fig. 3.8.B) under induced and uninduced conditions was observed. 6-thioxanthine was included in the study as a control for FDUR resistance incurred as a result of mutation due to electroporation. The  $LD_{50}$  values for FDUR under uninduced and induced conditions were found to be  $3\pm 0.5 \mu\text{M}$  and  $18\pm 0.5 \mu\text{M}$  respectively. The  $LD_{50}$  values for 6-thioxanthine was  $110\pm 0.5 \mu\text{M}$  and  $115\pm 0.5 \mu\text{M}$  in the uninduced condition and induced condition respectively.



**Figure 3.8** Tolerance of RHtetRuprt for FDUR. RHtetRuprt parasites were grown in the presence of FDUR (Figure A) or 6-thioxanthine (Figure B) under induced and uninduced conditions. The LD<sub>50</sub> values for FDUR under uninduced and induced condition were found to be 3±0.5 µM and 18±0.5 µM. The LD<sub>50</sub> values for 6-thioxanthine under uninduced and induced conditions were found to be 110±0.5 µM and 115±0.5 µM respectively.

## Section 1 Summary

Thus, from the above experiments it can be concluded that we have been successful in generating a transgenic parasite line, RHtetRuprt that contains a tetracycline inducible RNAi system against the UPRT gene. The head-to-head promoters are efficient in producing dsRNA which can downregulate UPRT gene expression. The tetracycline inducible system can effectively modulate the level of gene expression depending on the amount of inducer added. An optimal level of induction was achieved at a concentration of 1.76  $\mu$ M anhydrotetracycline. Furthermore, an LD<sub>50</sub> assay has shown that when RHtetRuprt parasites are induced they become more tolerant to FDUR. This finding has confirmed that the UPRT gene is downregulated in these induced parasites and thus the inducible RNAi system is functional. Therefore it can be concluded that this tetracycline inducible system can be used to downregulate genes in *T. gondii* and can prove to be a valuable tool to study gene function through loss-of-function studies and phenotype analysis.

### **3.2 Inducible RNAi system directed against an invasion related gene: MIC3**

When the parasite comes in contact with any nucleated cell, microneme proteins (MICs) are the first proteins to be released (Soldati *et al.*, 2001). Microneme proteins possess domains that recognize the host cell receptors and help the parasite to effectively bind to them thus contributing to the virulence of the protein. MIC 3 is an adhesion protein in the microneme family of protein that is secreted by *T. gondii* (Cerede *et al.*, 2002). MIC 3 was downregulated to study the resulting phenotype.

#### **3.2.1 Generation of MIC 3 expression vector**

We generated a tetracycline inducible RNAi vector called pCR8GWMIC3, that can produce dsRNA homologous to the MIC 3 gene and could be used to downregulate endogenous MIC 3 mRNA. To create this vector a 404 bp MIC 3 fragment was PCR amplified from genomic DNA (Fig. 3.9.A, B) and cloned to pCR8GWTOPO to generate an entry vector. The entry vector was digested with *Pvu* II to confirm the validity of the plasmid (Fig. 3.9.C). *Pvu* II sites are located on 122 bp, 2,310 bp and 3,018 bp on the entry vector. The presence of bands of 2,188 bp, 708 bp and 325 bp confirmed the correct plasmid. The Gateway cloning strategy was used to create the expression vector and is described in detail in “Material and Methods” section 2.2.6. The destination vector used generate the expression clone was also digested for its confirmation (Fig. 3.9.D). The destination vector was digested individually with *Hind* III, *Pvu* II and *Bgl* I. *Hind* III sites are located on 423 bp, 563 bp, 641 bp and 4,895 bp on the destination vector. The digestion resulted in four bands of 4,254 bp, 3,346 bp, 140 bp and 78 bp. Bands of 4,254 bp and 3,356 bp can be clearly seen on the gel. Bands of 140 bp and 78 bp were too small

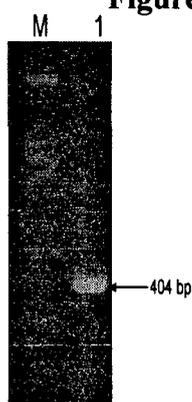
to be observed clearly. *Pvu* II sites are located on 1,114 bp, 2,235 bp, 4,748 bp and 7,306 bp. *Pvu* II digest yielded four bands of 2,558 bp, 2,513 bp, 1636 bp and 1,121 bp. *Bgl* I sites are located on 3,424 bp, and 4,691 bp. This digestion resulted in two bands of 6,561 bp and 1,267 bp. In order to obtain the expression vector an LR recombination reaction of destination vector and entry vector was done using LR Clonase™. The expression vector pCR8GWMIC3 obtained was analysed by restriction digestion (Fig. 3.9.G). Plasmid pCR8GWMIC3 was individually digested with *Age* I and *Pvu* I. *Age* I sites are located on 401 bp, 1,963 bp, 4,890 bp and 5,120 bp. Bands of 2,930 bp, 1,562 bp, 1,325 bp and 227 bp confirmed the validity of the expression vector. *Pvu* I sites are located on 4,737 bp, and 3,692 bp and the bands of 4,999 bp and 1,045 bp further confirmed the validity of the pCR8GWMIC3 plasmid.

**Figure 3.9** Construction and validation of MIC 3 entry vector (A) Genomic DNA was isolated from the RH strain. Lane M: 1.5 Kb ladder; Lane 1: Genomic DNA. (B) MIC 3 was amplified from genomic DNA isolated from the RH strain. Lane M: 100 bp ladder; Lane 1: PCR amplified 404 bp fragment of MIC 3. (C) To validate the entry vector, it was digested with restriction enzyme *Pvu* II. Lane 1-4: MIC 3 entry vector digested with *Pvu* I; Lane 5: undigested vector. (D) The Destination vector was digested with restriction enzymes, individually with *Hind* III, *Pvu* II and *Bgl* I. Lane 1: undigested destination vector; Lane 2: destination vector *Hind* III digest; Lane 3: destination vector *Pvu* II digest; Lane 4: destination vector *Bgl* I digests. (E) Vector map of destination vector. (F) The vector was digested with restriction enzymes *Age* I and *Pvu* I. Lane 1: undigested plasmid pCR8GWMIC3; Lane 2: plasmid pCR8GWMIC3 *Age* I digest; Lane 3: plasmid pCR8GWMIC3 digested with *Pvu* I. (G) Vector Map of expression vector pCR8GWMIC3.

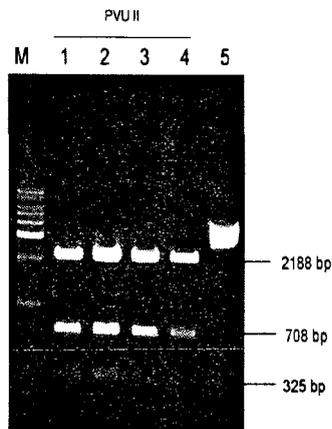
**Figure 3.9**



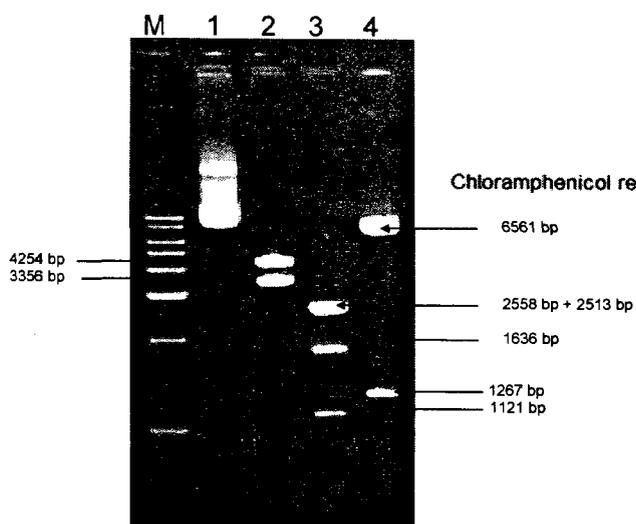
**A**



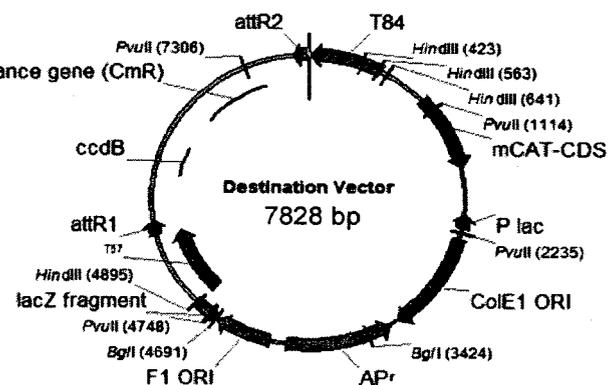
**B**



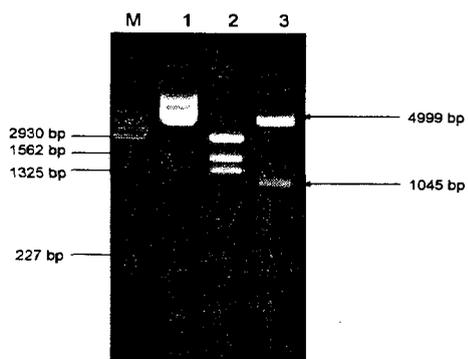
**C**



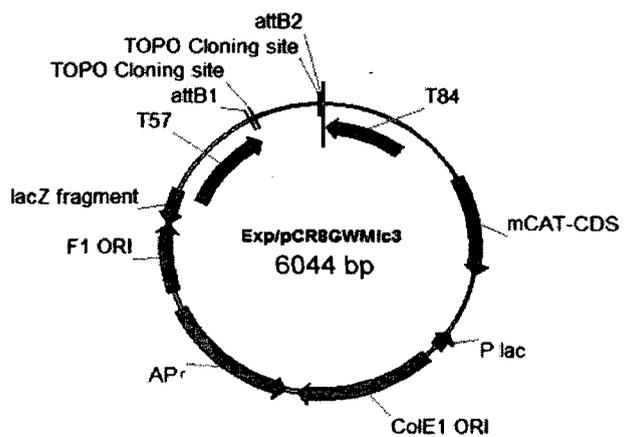
**D**



**E**



**F**

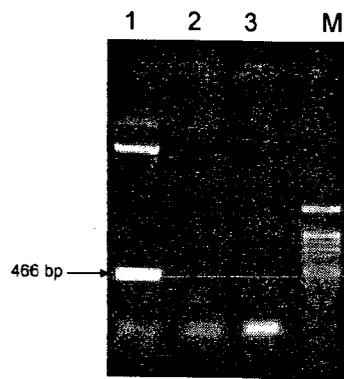


**G**

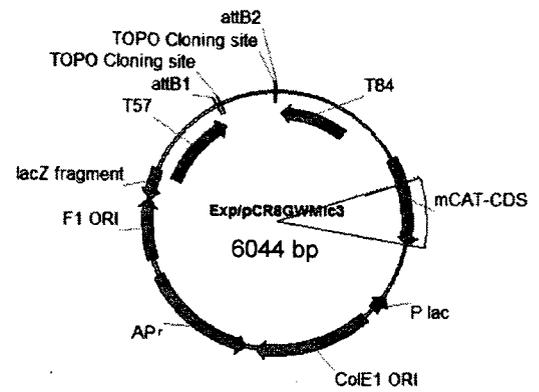
### **3.2.2 Generation and confirmation of transgenic parasites containing inducible**

#### **RNAi system against MIC 3 gene**

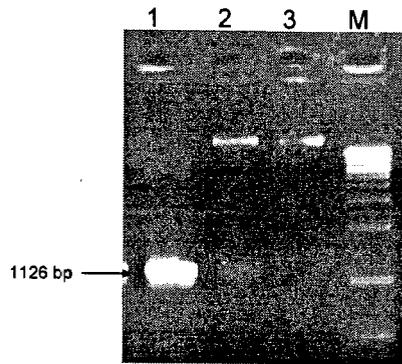
The pCR8GWMIC3 plasmid was electroporated into the RHtetR parasite to obtain a stable line of transgenic parasites called RHtetRmic3. To confirm the stable integration of plasmid pCR8GWMIC3, genomic DNA was extracted from the transgenic RHterRmic3 and RHtetR parasites and subjected to PCR amplification of the CAT fragment. PCR was done using primers CAT\_EcoRI<sub>mut</sub> and SPHI3'CAT. No PCR product was observed in the lane corresponding to RHtetR while the presence of a 466 bp amplified product confirmed the presence of CAT in the transgenic parasite RHterRmic3 (Fig. 3.10.A). To further validate the stable transformation, DNA was extracted from the transgenic RHterRmic3 and RHtetR parasites and subjected to PCR amplification of the fragment located between the tub 5 promoter and MIC 3 fragment. PCR was done using primers BamH1 5'pTUB5 and MIC3\_sense. No PCR product was observed in the lane corresponding to RHtetR while the presence of 1126 bp amplified product confirmed the presence of the plasmid in transgenic RHterRmic3.



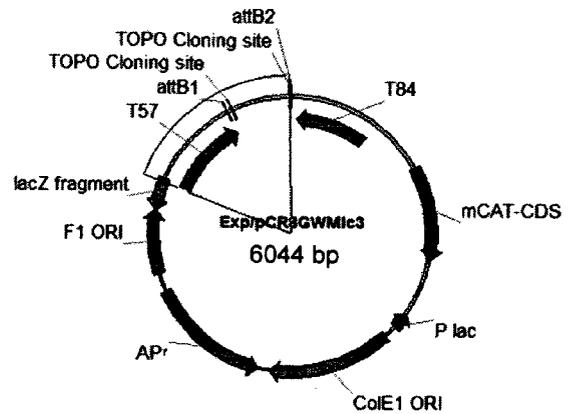
A



B



C



D

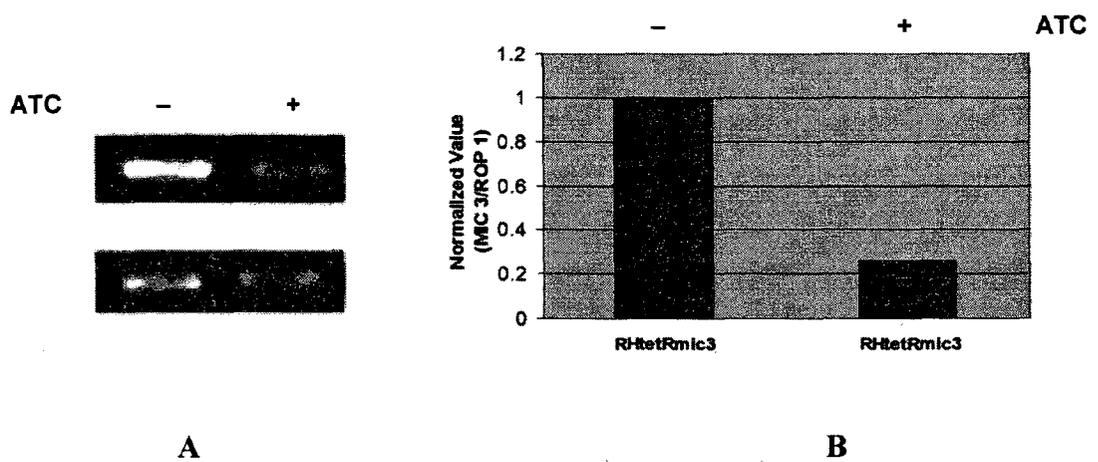
**Figure 3.10 Confirmation of transgenic integration of plasmid pCR8GWMIC3.**

A 466 bp fragment was PCR amplified from the CAT coding sequence using primers CAT\_EcoRI<sup>mut</sup> and SPH13'CAT (A) Lane 1: PCR amplified product from plasmid pCR8GWMIC3, used as a positive control; Lane 2: PCR amplified product from DNA isolated from RHtetRmic3; Lane 3: PCR amplified product from DNA isolated from RHtetR parasites, used as negative control. (B) Vector map of plasmid pCR8GWMIC3. The part of the CAT coding sequence that was amplified from the integrated plasmid is marked by the pie section (C) A 1126 bp fragment was amplified spanning tubulin 5 promoter regions and MIC 3 fragment cloned into pCR8GWMIC3 plasmid using primers BamH1 5'pTUB5 and MIC3\_sense, Lane 1: PCR amplified product from plasmid pCR8GWMIC3; Lane 2: PCR amplified product from DNA isolated from RHtetRmic3 parasites; Lane 3: PCR amplified product from DNA isolated from RHtetR parasites, used as negative control (D) Vector map of plasmid pCR8GWMIC3. The 1126 bp fragment that was amplified from the integrated plasmid is marked by the pie section.

### **3.2.3 Modulation of MIC 3 expression level in transgenic RHtetRmic3 parasites**

After confirming the stable integration of the plasmid, experiments were conducted to check if these transgenic parasites are capable of downregulating endogenous MIC 3 mRNA levels upon ATC induction. Freshly lysed RHtetRmic3 parasites were grown in the presence or absence of 1.76  $\mu$ M ATC. Induced and uninduced parasites were collected after the host cells had been completely lysed. Total RNA was extracted from these parasites and subjected to RT-PCR to check the expression of endogenous MIC 3 mRNA transcripts. The gene expression levels of MIC 3 and ROP 1 in induced and uninduced RHtetRmic3 parasites is shown in the Fig. 3.11.A. ROP 1 was used as an internal control. The ratio of signals of UPRT/ROP 1 was calculated and the normalized MIC 3 expression levels in induced and uninduced parasites are shown in Fig. 3.11.B.

The normalized expression level for MIC 3 in uninduced RHtetRmic3 parasites was given an arbitrary value of 1. Compared to uninduced parasites, the induced parasite had a value of 0.26. These results confirmed successful generation of a transgenic line of parasites called RHtetRmic3 that contains an inducible RNAi system against the MIC 3 gene.



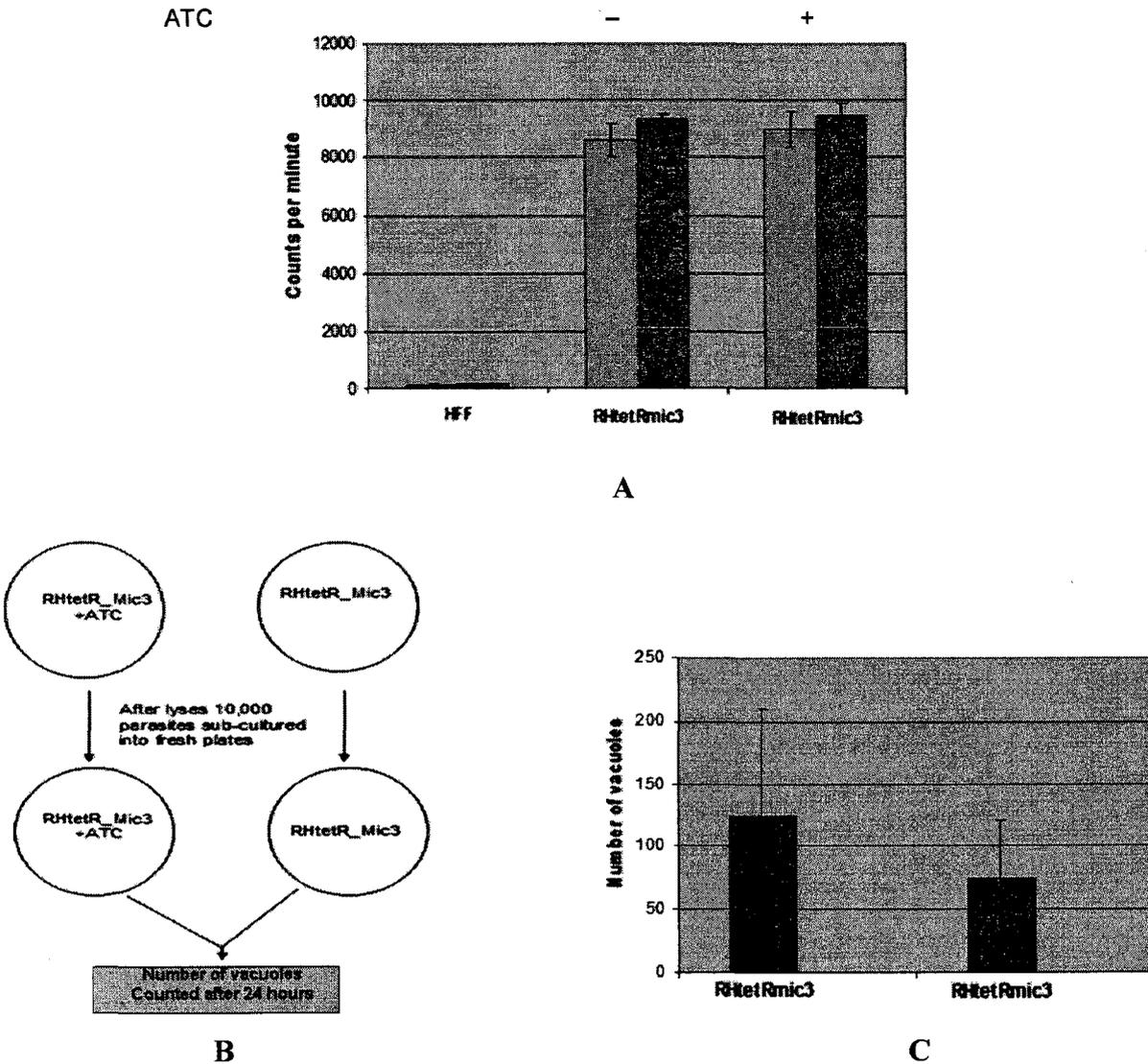
**Figure 3.11** Gene expression level in transgenic RHtetRmic3. The parasites were grown without or with ATC (A) RT-PCR of MIC 3 and ROP 1. Lane 1: RT-PCR of MIC 3 and ROP 1 gene in uninduced parasites; Lane 2: RT-PCR of UPRT and ROP1 gene in induced parasites. (B) Expression profile of UPRT normalized against ROP 1.

### **3.2.4 The effect of MIC 3 downregulation on invasion ability of the parasite**

The MIC 3 gene was downregulated to study its possible role in parasite invasion. RHtetRmic3 parasites were grown in presence and absence of ATC. Freshly lysed parasites were counted using a haemocytometer and 10,000 induced and uninduced parasites were inoculated into each well of a 24-well plate containing confluent HFF monolayer. The parasites that were initially induced were further allowed to grow in media containing 1.76  $\mu$ M ATC while the uninduced were grown without ATC. A nucleotide uptake assay was done using  $^3\text{H}$  uracil and  $^3\text{H}$  hypoxanthine. These radioactive nucleotides served as metabolic labels to measure the viability of the parasites. If MIC 3 plays a role in invasion, down regulation of the gene would have resulted in decreased invasion ability compared to uninduced parasites thus the uptake of the nucleotide should have decreased accordingly. The results show that both the induced and uninduced parasites were able to salvage almost equal amounts of nucleotides from the media; this is indirect evidence that almost equal number of induced and uninduced parasites invaded the host cell (Fig. 3.12.A). This shows that MIC 3 does not play a role in invasion of the parasite and thus does not contribute to the virulence of the parasite. Since the nucleotide uptake assay is an indirect method to check the invasion ability of the parasite, a more direct method we employed to analyse the invasion ability of the parasite was to count the number of vacuoles formed by the parasites over a given period of time.

In order to analyze the number of vacuoles formed by the parasites, they were grown in 60mm plates in the presence and absence of ATC. After they lysed the host cells, 10000 parasites were introduced into fresh host cells, allowed to grow with or without the inducer and the number of vacuoles formed in 24 hr were counted (Fig. 3.12.B). Ideally,

if MIC 3 plays a role in parasite invasion, the number of vacuoles produced by induced parasites should have been less than the number of vacuoles produced by uninduced parasites. The results showed that there is a negligible difference in the number of vacuoles formed by the induced and the uninduced parasites. The P value of 0.478 obtained from the graph suggests that there is no statistical significance in the difference between the number of vacuoles obtained from the induced and uninduced parasites (Fig. 3.12.C).

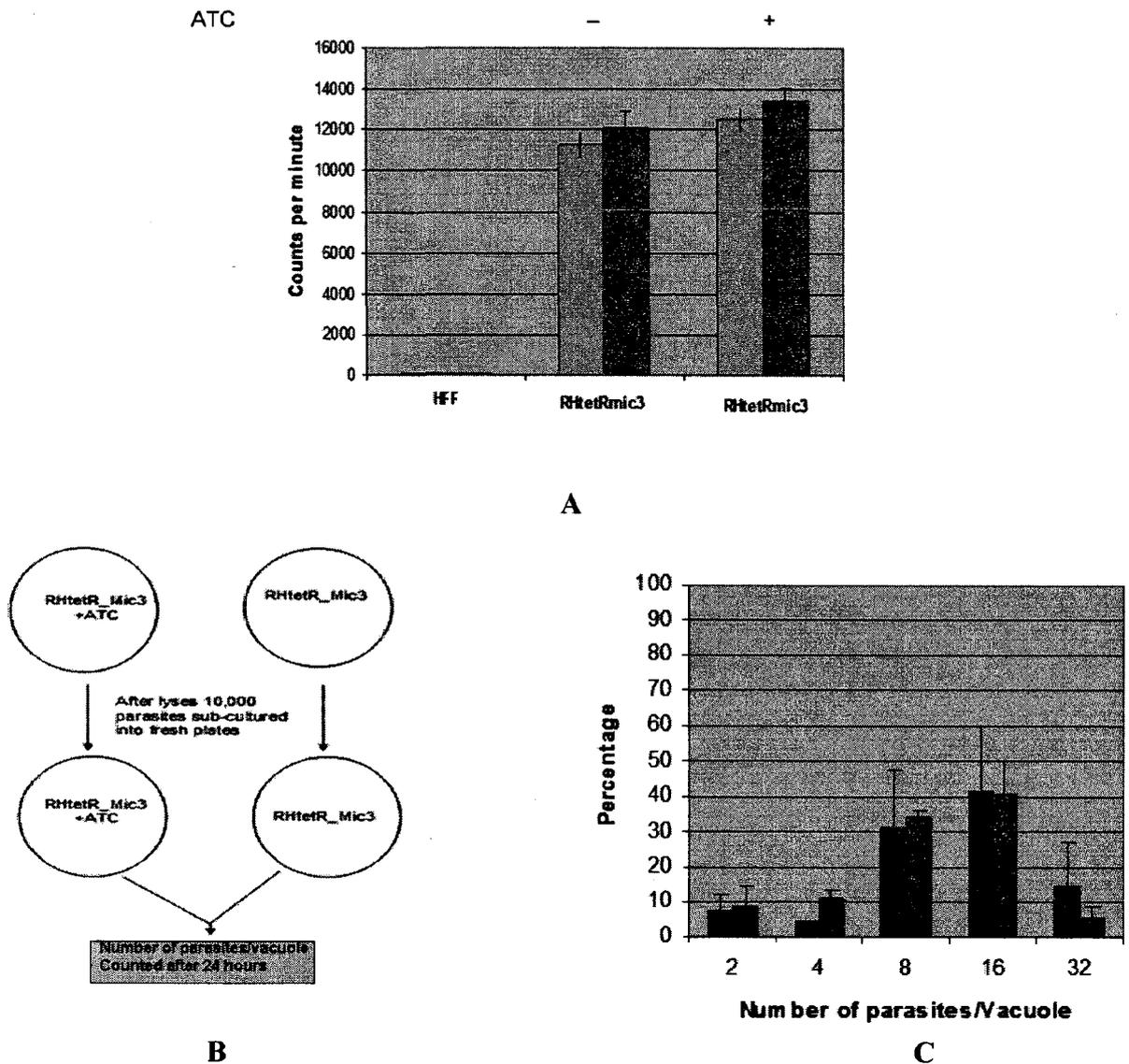


**Figure 3.12** Effect of MIC 3 downregulation on parasite's invasion ability. (A) RHtetRmic3 were grown in the presence and absence of ATC and allowed to invade host cells. The amount of  $^3\text{H}$ -uracil (■) and  $^3\text{H}$ -hypoxanthine (■) assimilated is directly proportional to the number of parasites and indirectly indicates the number of parasites that could invade the host cells. (B) Schematic representation of invasion assay. (C) RHtetRmic3 parasites were grown in the presence (■) or absence (■) of ATC and allowed to invade host cells and then total numbers of vacuoles were counted (P value = 0.478).

### **3.2.5 The effect of MIC 3 downregulation on parasite metabolism**

To check if MIC 3 has a possible role in the metabolism of the parasite, it was downregulated by induction. Freshly lysed parasites were counted using a haemocytometer and 10,000 parasites were inoculated per well of a 24-well plate containing confluent HFF monolayer. The parasites were allowed to successfully invade the host cells for 45 minutes. Induction was initiated by adding 1.76  $\mu$ M ATC to the media containing the parasites. A nucleotide uptake assay was done using  $^3\text{H}$  uracil and  $^3\text{H}$  hypoxanthine as a measure of metabolic activity: if MIC 3 played a role in metabolism of the parasite, induced parasites would be less metabolically active compared to the uninduced and would assimilate less nucleotide. The results showed that both induced and uninduced parasites have the same metabolic rate as they assimilated almost equal amount of nucleotide (Fig. 3.13.A).

Freshly lysed parasites were counted using a haemocytometer and 10,000 parasites were grown in 60 mm plates in the presence and absence of ATC. Upon lysis, the parasites were re-introduced into fresh host cells, allowed to grow with or without the inducer and the number of parasites per vacuole was counted after 24 hrs (Fig. 3.13.B). If MIC 3 had a role in metabolism, downregulation of the gene would have affected the growth and division of the parasites, and thus the number of parasites per vacuoles would have been less when compared to uninduced parasites. The results show that there is a negligible difference in number of parasites per vacuole in induced and uninduced parasites, displaying that the knockdown of MIC 3 does not affect metabolism of the parasite (Fig. 3.13.C).



**Figure 3.13** Effect of MIC3 downregulation on parasite metabolism. (A) RHtetRmic3 parasites were grown in the presence and absence of ATC and allowed to invade host cells. The amount of <sup>3</sup>H-uracil (■) and <sup>3</sup>H-hypoxanthine (■) assimilated is directly proportional to the number of parasite and indirectly indicates the metabolic rate of the parasite. (B) Schematic representation of the assay. (C) RHtetRmic3 parasites were grown in the presence (■) or absence (■) of ATC and allowed to invade host cells and then total numbers parasites per vacuole was counted.

## **Section 2 Summary**

Microneme protein MIC 3 is an important adhesion protein secreted by *T. gondii*. A tetracycline based inducible system was used to downregulate this gene in RHtetR strain of *T. gondii*. A stable line of the transgenic parasite, RHtetRmic3 was successfully created that produces dsRNA homologous to MIC 3 upon induction with ATC. The MIC 3 gene was downregulated to study its affect in transgenic RHtetRmic3 parasites. The results suggest that though MIC 3 protein has an adhesive domain, it does not directly play a role in invasion. A possible role of this protein in metabolism was also analyzed. The results suggest that MIC 3 does not directly play a role in parasite metabolism.

## CHAPTER 4

### Discussion

*T. gondii* is a protozoan parasite which has a worldwide distribution (Luft and Remington, 1992). Toxoplasmosis, the disease caused by *T. gondii*, leads to congenital diseases as well as results in abortion in humans and animals. In immuno-compromised people such as AIDS patients, *T. gondii* causes encephalitis. The treatment of the disease is very difficult as the drugs that are currently available are very toxic.

A considerable amount of research has been done in recent years to develop a vaccine against *Toxoplasma* for both human and animals (Buxton and Innes, 1995). Currently, there is no vaccine available that could be used by humans against this protozoan parasite. A commercial vaccine has been developed against ovine toxoplasmosis using a live attenuated S48 strain of *T. gondii* (Araujo, 1994). This type of vaccine is not suitable for human use as these attenuated parasites may revert back to wild type and can lead to infection. Various surface antigens present during the tachyzoite stage of the parasite life cycle such as SAG 1, SAG 2 and SAG 3 have been identified as potential candidates for vaccines and research is going on to develop a human vaccine (Couvreur *et al.*, 1988).

In order to develop vaccines that can be used by humans or drugs that could treat toxoplasmosis, it is essential to have a thorough knowledge about the parasite itself. Determination of genes responsible for the pathogenesis associated with toxoplasmosis and further elucidation of roles of these genes, would allow production of drugs and vaccines against this protozoan parasite. Genes can be downregulated to decipher their

potential role and RNA tools have been extensively used for this purpose in many organisms including *T. gondii* (Nakaar *et al.*, 1999, 2000; Al-Anouti and Ananvoranich, 2002; Sheng *et al.*, 2004). In *T. gondii*, genetic manipulation can be done using various RNA tools including antisense RNA, ribozymes and dsRNA (Al-Anouti and Ananvoranich, 2002). To study the role of a gene or the phenotype as a result of loss-of-function, it is desirable to have an inducible system where one can switch the gene ‘on’ and ‘off’ as required (Mills, 2001). The tetracycline inducible system has been successfully used to control expression of genes in various protozoan parasites, including *T. gondii* (Meissner *et al.*, 2001).

#### **4.1 Characterization of inducible promoter using marker gene, UPRT**

The inducible RNAi vector has two main components as follows: 1. Head-to-head promoters that would generate the double stranded RNA which would take part in the presumed RNAi pathway to knock down the gene of interest that is cloned between them and 2. The transcription of the desired genes could be induced. The head-to-head promoter system consists of two promoters that are arranged in inverted repeats with the gene of interest to be downregulated cloned between them. The inverted promoter would allow the transcription of both strands of DNA sequence placed between them. The head-to-head promoter system has been previously used in *Trypanosoma brucei* to downregulate genes like TUB, FLA1 and GFP (LaCount *et al.*, 2000). The head-to-head promoter system was also previously used in our laboratory to knock down UPRT gene using SAG 1 promoters (Al-Anouti *et al.*, 2003). Tetracycline based inducible system has

been previously used in *T. gondii* to downregulate myosin A protein (Meisner *et al.*, 2001).

The tetracycline repressor protein (TetR) from *E. coli* was cloned into *T. gondii*, which binds to tetracycline operator elements (tetO) placed between the tub promoters, preventing the transcription. This system is a modified form of the tightly regulated, tetracycline-controlled gene expression system described by Gossen and Bujard (1992). When the system is induced with ATC, dsRNA is expressed, which results in suppression of the gene targeted by the dsRNA through RNAi. Thus, the system tightly regulates the expression of dsRNA and the gene that the dsRNA targets-in response to the concentration of ATC.

We have constructed a tetracycline inducible RNAi vector with head-to-head tub promoters with TetO placed between them. A stable line of RHtetRuprt parasites was generated containing the inducible RNAi vector. PCR using DNA extracted from transgenic RHtetRuprt parasites showed stable integration of the inducible vector. Uracil incorporation data indicated that uracil incorporation was lowered when RHtetRuprt parasites were induced with ATC to produce dsRNA against UPRT.

A major disadvantage of TetR dependent inducible system is the leakiness, which is determined by the affinity strength of TetR/TetO, within the inducible promoter (Zhang *et al.*, 2007). Interestingly, in our study it was observed that the background or leaky expression of dsRNA was extremely low in the absence of the inducer (Fig. 3.6).

To further characterize the phenotype of RHtetRuprt parasites, a drug tolerance assay was conducted for 5-fluoro-2'-deoxyuridine (FDUR). The UPRT knockdown parasites were six times more resistant to the drug compared to the uninduced parasites.

6-thioxanthine was included in the study as a control to confirm that the resistance to FDUR was not a result of mutation due to electroporation. Both induced and uninduced parasites were less sensitive to 6-thioxanthine compared to FDUR. Detection of antisense RNA and downregulation of mRNA in these parasites show that double stranded RNA is produced and at the same time hints to the presence of an RNAi like mechanism of downregulation in *T. gondii*.

Taken together, our results indicate that the prokaryotic tetracycline-inducible expression system is highly suitable for controlled gene expression in *T. gondii*. The inducible RNAi system was able to effectively downregulate the UPRT gene upon induction and tight regulation of the tetracycline repressor promoters showed that this system could be used to study the function of any gene that is cloned between them through loss-of-function studies.

#### **4.2 Phenotype analysis of MIC 3 using an inducible RNAi vector**

Microneme proteins are secreted by *T. gondii* in the early stage of invasion (Soldati *et al.*, 2001). These proteins contain adhesive domains that help them to bind host cell surface receptors and thus help in invasion. MIC 3 has a lectin-like domain as well as several epidermal growth factor-like domains that can help the parasite to bind to the host cell surface receptor.

The nucleotide uptake assay is done to determine the ability of the parasite to salvage nucleotides from the surrounding medium. The results obtained can be interpreted to find out the relative number of parasites that have successfully invaded the host cells as well as the metabolic rate of the parasites. The nucleotide uptake assay shows that upon

downregulation of MIC 3, there was no significant difference in the uptake of nucleotides in downregulated RHtetRMIC3 parasites as compared to wild type RHtetR parasites. The result obtained here agrees with the results obtained from complete knockout of the MIC 3 gene (Ismael *et al.*, 2006). There was about same number vacuoles as well as parasites per vacuole in induced and uninduced parasite.

The endogenous MIC 3 mRNA level was effectively knocked down using the tetracycline inducible RNAi vector but there was no change in either invasion or metabolism of MIC 3 knocked down parasites compared to the wild type RHtetR parasite. Due to the presence of lectin-like domain, MIC 3 has strong affinity towards the host cell surface (Cerede *et al.*, 2002). Apart from MIC 3, other MICs like MIC 1 and MIC 8 also have lectin-like domain. Therefore, when we observed that MIC 3 downregulation did not alter the invasion ability of the parasites, we hypothesized that other MICs may have compensated for the loss of MIC 3. Previous studies have shown that single deletion of either MIC 1 or MIC 3 slightly reduced virulence *in vivo* but when both MIC 3 and MIC 1 were knocked out, the parasites were severely impaired in virulence (Cerede *et al.*, 2005).

#### **4.3 Generation of mini inducible RNAi library**

In order to study the possible genes that might be responsible for pathogenesis in *T. gondii* using the inducible RNAi vector a mini RNAi library was generated. The mini library consisted of various surface antigens, microneme proteins and lactate hydrogenase gene 1 (LDH 1). Various genes used to construct the mini library and verification of these constructs has been explained in detail in Appendix II and Appendix III.

## **Future work**

In this study we have shown that the tetracycline inducible system is able to effectively knockdown the gene of interest upon induction. Thus, the inducible vector can be further used to screen genes responsible for virulence of *T. gondii*. We have not been able to show here that the induction is reversible. The main aim of creating an inducible system is to study reversible regulation of gene expression. Thus future work should be aimed to study the reversibility of induction.

## References

- Agrawal N, Dasaradhi PV, Mohammed A, Malhotra P, Bhatnagar RK, Mukherjee SK. RNA interference: biology, mechanism, and applications. *Microbiol Mol Biol Rev* 2003; 67 (4):657-85.
- Achbarou A, Mercereau-Pujalon O, Autheman JM, Fortier B, Camus D, Dubremetz JF. Characterization of microneme proteins of *Toxoplasma gondii*. *Mol Biochem Parasitol* 1991; 47 (2):223-33.
- Aigner A. Applications of RNA interference: current state and prospects for siRNA-based strategies *in vivo*. *Appl Microbiol Biotechnol* 2007; 76 (1):9-21.
- Al-Anouti F and Ananvoranich S. Comparative analysis of antisense RNA, double-stranded RNA, and delta ribozyme-mediated gene regulation in *Toxoplasma gondii*. *Antisense Nucleic Acid Drug Dev* 2002; 12 (4):275-81.
- Al-Anouti F, Quach T, Ananvoranich S. Double-stranded RNA can mediate the suppression of uracil phosphoribosyltransferase expression in *Toxoplasma gondii*. *Biochem Biophys Res Commun* 2003; 302 (2):316-23.
- Al Riyahi A, Al-Anouti F, Al-Rayes M, Ananvoranich S. Single argonaute protein from *Toxoplasma gondii* is involved in the double-stranded RNA induced gene silencing. *Int J Parasitol* 2006; 36 (9):1003-14.
- Araujo FG. Immunization against *Toxoplasma gondii*. *Parasitol Today* 1994; 10 (9):358-60.

Beghetto E, Spadoni A, Bruno L, Buffolano W, Gargano N. Chimeric antigens of *Toxoplasma gondii*: toward standardization of toxoplasmosis serodiagnosis using recombinant products. J Clin Microbiol 2006; 44 (6):2133-40.

Black MW and Boothroyd JC. Development of a stable episomal shuttle vector for *Toxoplasma gondii*. J Biol Chem 1998; 273 (7):3972-9.

Black MW and Boothroyd JC. Lytic cycle of *Toxoplasma gondii*. Microbiol Mol Biol Rev 2000; 64 (3):607-23.

Beckers CJ, Dubremetz JF, Mercereau-Puijalon O, Joiner KA. The *Toxoplasma gondii* rhoptry protein ROP 2 is inserted into the parasitophorous vacuole membrane, surrounding the intracellular parasite, and is exposed to the host cell cytoplasm. J Cell Biol 1994; 127 (4):947-61.

Bohne W, Heesemann J, Gross U. Induction of bradyzoite-specific *Toxoplasma gondii* antigens in gamma interferon-treated mouse macrophages. Infect Immun 1993; 61 (3):1141-5.

Bohne W, Heesemann J, Gross U. Reduced replication of *Toxoplasma gondii* is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion. Infect Immun 1994; 62 (5):1761-7.

Bonhomme A, Bouchot A, Pezzella N, Gomez J, Le Moal H, Pinon JM. Signaling during the invasion of host cells by *Toxoplasma gondii*. FEMS Microbiol Rev 1999; 23 (5):551-61.

Boothroyd JC, Black M, Bonnefoy S, Hehl A, Knoll LJ, Manger ID, Ortega-Barria E, Tomavo S. Genetic and biochemical analysis of development in *Toxoplasma gondii*. Philos Trans R Soc Lond B Biol Sci 1997; 352 (1359):1347-54.

Buxton D and Innes EA. A commercial vaccine for ovine toxoplasmosis. *Parasitology* 1995; 110.

Carruthers VB. Host cell invasion by the opportunistic pathogen *Toxoplasma gondii*. *Acta Trop* 2002; 81 (2):111-22.

Carruthers VB, Moreno SN, Sibley LD. Ethanol and acetaldehyde elevate intracellular [Ca<sup>2+</sup>] and stimulate microneme discharge in *Toxoplasma gondii*. *Biochem J* 1999; 342 (2):379-86.

Carruthers VB and Sibley LD. Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. *Mol Microbiol* 1999; 31 (2):421-8.

Carruthers VB and Sibley LD. Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *Eur J Cell Biol* 1997; 73 (2):114-23.

Cerede O, Dubremetz JF, Bout D, Lebrun M. The *Toxoplasma gondii* protein MIC3 requires pro-peptide cleavage and dimerization to function as adhesin. *Embo J* 2002; 21 (11):2526-36.

Cerede O, Dubremetz JF, Soète M, Deslée D, Vial H, Bout D, Lebrun M. Synergistic role of micronemal proteins in *Toxoplasma gondii* virulence. *J Exp Med* 2005; 201(3):453-463.

Charest H, Sedegah M, Yap GS, Gazzinelli RT, Caspar P, Hoffman SL, Sher A. Recombinant attenuated *Toxoplasma gondii* expressing the *Plasmodium yoelii* circumsporozoite protein provides highly effective priming for CD8<sup>+</sup> T cell-dependent protective immunity against malaria. *J Immunol* 2000; 165 (4):2084-92.

Chatterton JM, Evans R, Ashburn D, Ashburn D, Joss AW, Ho-Yen DO. *Toxoplasma gondii* *in vitro* culture for experimentation. J Microbiol Methods 2002; 51 (3):331-5.

Cleary MD, Singh U, Blader IJ, Brewer JL, Boothroyd JC. *Toxoplasma gondii* asexual development: identification of developmentally regulated genes and distinct patterns of gene expression. Eukaryot Cell 2002; 1 (3):329-40.

Coppens I, Joiner KA. Parasitehost cell interactions in toxoplasmosis: new avenues for intervention? Expert Rev Mol Med 2001; 2001:1-20.

Couvreur G, Sadak A, Fortier B, Dubremetz JF. Surface antigens of *Toxoplasma gondii*. Parasitology 1988; 97:1-10.

Donald RG, Carter D, Ullman B, Roos DS. Insertional tagging, cloning, and expression of the *Toxoplasma gondii* hypoxanthine-xanthine-guanine phosphoribosyltransferase gene. Use as a selectable marker for stable transformation. J Biol Chem 1996; 271 (24):14010-9.

Donald RG and Roos DS. Stable molecular transformation of *Toxoplasma gondii*: a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. Proc Natl Acad Sci 1993; 90 (24):11703-7.

Donald RG and Roos DS. Insertional mutagenesis and marker rescue in a protozoan parasite: cloning of the uracil phosphoribosyltransferase locus from *Toxoplasma gondii*. Proc Natl Acad Sci 1995; 92 (12):5749-53.

Donald RG and Roos DS. Homologous recombination and gene replacement at the dihydrofolate reductase-thymidylate synthase locus in *Toxoplasma gondii*. Mol Biochem Parasitol 1994; 63 (2):243-53.

Dubey JP. Advances in the life cycle of *Toxoplasma gondii*. Int J Parasitol 1998; 28 (7):1019-24.

Dubey JP. *Toxoplasma, Hammondia, Besnoitia, Sarcocystis*, and other tissue cyst-forming coccidia of man and animals. In: Kreier JP (ed) Parasitic Protozoa Vol. III. Academic Press, New York San Francisco London, 1977; 101–238.

Dubey JP. Toxoplasmosis. J Am Vet Med Assoc 1986; 189 (2):166-70.

Dubey JP and Beattie CP. Toxoplasmosis of animals and man. In: CRC Press, Boca Raton, FL 1988; 1–200.

Dzierszynski F, Knoll LJ. Biology of bradyzoites. In: Weiss LM, Kim K, editors. *Toxoplasma gondii*—The Model Apicomplexan: Perspectives and Methods. London, UK: Academic Press 2007: 303–20.

Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21 and 22 nucleotide RNAs. Genes Dev 2001; 15 (2):188-200.

Evans R, Chatterton JM, Ashburn D, Joss AW, Ho-Yen DO. Cell-culture system for continuous production of *Toxoplasma gondii* tachyzoites. Eur J Clin Microbiol Infect Dis 1999; 18 (12):879-84.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 1998; 391 (6669):806-11.

Fourmaux MN, Garcia-Réguet N, Mercereau-Puijalon O, Dubremetz JF. *Toxoplasma gondii* microneme proteins: gene cloning and possible function. Curr Top Microbiol Immunol 1996; 219:55-8.

Frenkel JK, Dubey JP. Toxoplasmosis and its prevention in cats and man. J Infect Dis 1972; 126 (6):664-73.

Frenkel JK, Dubey JP, Hoff RL. Loss of stages after continues passage of *Toxoplasma gondii* and *Besnoitia jellisoni*. J Protozool 1976; 23:421-424.

Freyre A. Separation of *Toxoplasma* cysts from brain tissue and liberation of viable bradyzoites. J Parasitol 1995; 81 (6):1008-10.

Friesen J, Fleige T, Gross U, Bohne W. Identification of novel bradyzoite-specific *Toxoplasma gondii* genes with domains for protein-protein interactions by suppression subtractive hybridization. Mol Biochem Parasitol 2008; 157 (2):228-32.

Garcia-Reguet N, Lebrun M, Fourmaux MN, Mercereau-Puijalon O, Mann T, Beckers CJ, Samyn B, Van Beeumen J, Bout D, Dubremetz JF. The microneme protein MIC3 of *Toxoplasma gondii* is a secretory adhesin that binds to both the surface of the host cells and the surface of the parasite. Cell Microbiol 2000; 2 (4):353-64.

Gilbert R, Dunn D, Wallon M, Hayde M, Prusa A, Lebech M, Kortbeek T, Peyron F, Pollak A, Petersen E. Ecological comparison of the risks of mother-to-child transmission and clinical manifestations of congenital toxoplasmosis according to prenatal treatment protocol. Epidemiol Infect 2001; 127 (1):113-20.

Gossen M and Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci 1992; 89 (12):5547-51.

Grimwood BG, Hechemy K, Stevens RW. *Toxoplasma gondii*: purification of trophozoites propagated in cell culture. Exp Parasitol 1979; 48 (2):282-6.

Hamann L, Buss H, Tannich E. Tetracycline-controlled gene expression in *Entamoeba histolytica*. Mol Biochem Parasitol 1997; 84 (1):83-91.

Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 2000; 404 (6775):293-6.  
Hannon GJ. RNA interference. *Nature* 2002; 418 (6894):244-51.

Hostomsky Z, Hughes SH, Goff SP, Le Grice SF. Redesignation of the RNase D activity associated with retroviral reverse transcriptase as RNase H. *J Virol* 1994; 68 (3):1970-1.  
Hughes HP, Hudson L, Fleck DG. *In vitro* culture of *Toxoplasma gondii* in primary and established cell lines. *Int J Parasitol* 1986; 16 (4):317-22.

Ismael AB, Sekkai D, Collin C, Bout D, Mévélec MN. The MIC3 gene of *Toxoplasma gondii* is a novel potent vaccine candidate against toxoplasmosis. *Infect Immun* 2003; 71 (11):6222-8.

Ismael AB, Dimier-Poisson I, Lebrun M, Dubremetz JF, Bout D, Mevelec MN. Mic1-3 knockout of *Toxoplasma gondii* is a successful vaccine against chronic and congenital toxoplasmosis in mice. *J Infect Dis* 2006; 194 (8):1176-83.

Jackson MH and Hutchison WM. The prevalence and source of *Toxoplasma* infection in the environment. *Adv Parasitol* 1989; 28:55-105.

Jaronczyk K, Carmichael JB, Hobman TC. Exploring the functions of RNA interference pathway proteins: some functions are more RISCy than others? *Biochem J* 2005; 387 (3):561-71.

Joiner KA, Fuhrman SA, Miettinen HM, Kasper LH, Mellman I. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science* 1990; 249 (4969):641-6.

Kafsack BF, Beckers C, Carruthers VB. Synchronous invasion of host cells by *Toxoplasma gondii*. *Mol Biochem Parasitol* 2004; 136 (2):309-11.

Kelleher M and Tomley FM. Transient expression of beta-galactosidase in differentiating sporozoites of *Eimeria tenella*. Mol Biochem Parasitol 1998; 97 (1-2):21-31.

Kim K, Soldati D, Boothroyd JC. Gene replacement in *Toxoplasma gondii* with chloramphenicol acetyltransferase as selectable marker. Science 1993; 262 (5135):911-4.

Kim K and Weiss LM. *Toxoplasma gondii*: the model apicomplexan. Int J Parasitol 2004; 34 (3):423-32.

Krahenbuhl JL and Remington JS. The immunology of *Toxoplasma* and toxoplasmosis. In: Cohen S, Warren KS (eds) Immunology of parasitic infections. Blackwell, London, 1982; pp 356–421.

LaCount DJ, Bruse S, Hill KL, Donelson JE. Double-stranded RNA interference in *Trypanosoma brucei* using head-to-head promoters. Mol Biochem Parasitol 2000; 111 (1):67-76.

Lamond AI and Sproat BS. Antisense oligonucleotides made of 2'-O-alkylRNA: their properties and applications in RNA biochemistry. FEBS Lett 1993; 325 (1-2):123-7.

Luft BJ and Remington JS. Toxoplasmic encephalitis in AIDS. Clin Infect Dis. 1992; 15 (2):211-22.

Mateus-Pinilla NE, Hannon B, Weigel RM. A computer simulation of the prevention of the transmission of *Toxoplasma gondii* on swine farms using a feline *T. gondii* vaccine. Prev Vet Med 2002; 55 (1):17-36.

McFadden GI, Roos DS. Apicomplexan plastids as drug targets. Trends Microbiol 1999; 7 (8):328-33.

Meissner M, Brecht S, Bujard H, Soldati D. Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in *Toxoplasma gondii*. *Nucleic Acids Res* 2001; 29 (22):E115.

Meissner M, Reiss M, Viebig N, Carruthers VB, Toursel C, Tomavo S, Ajioka JW, Soldati D. A family of transmembrane microneme proteins of *Toxoplasma gondii* contain EGF-like domains and function as escorts. *J Cell Sci* 2002; 115 (3):563-74.

Messina M, Niesman I, Mercier C, Sibley LD. Stable DNA transformation of *Toxoplasma gondii* using phleomycin selection. *Gene* 1995; 165 (2):213-7.

Mills AA. Changing colors in mice: an inducible system that delivers. *Genes Dev* 2001; 15 (12):1461-7.

Nakaar V, Beckers CJ, Polotsky V, Joiner KA. Basis for substrate specificity of the *Toxoplasma gondii* nucleoside triphosphate hydrolase. *Mol Biochem Parasitol* 1998; 97 (1-2):209-20.

Nakaar V, Ngo EO, Joiner KA. Selection based on the expression of antisense hypoxanthine-xanthine-guanine-phosphoribosyltransferase RNA in *Toxoplasma gondii*. *Mol Biochem Parasitol* 2000; 110 (1):43-51.

Nakaar V, Samuel BU, Ngo EO, Joiner KA. Targeted reduction of nucleoside triphosphate hydrolase by antisense RNA inhibits *Toxoplasma gondii* proliferation. *J Biol Chem* 1999; 274 (8):5083-7.

Napoli C, Lemieux C, Jorgensen R. Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. *Plant Cell* 1990; 2 (4):279-289.

- Nykanen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* 2001; 107 (3):309-21.
- O'Connor RM, Kim K, Khan F, Ward HD. Expression of Cpgp40/15 in *Toxoplasma gondii*: a surrogate system for the study of *Cryptosporidium* glycoprotein antigens. *Infect Immun* 2003; 71 (10):6027-34.
- Orth P, Schnappinger D, Hillen W, Saenger W, Hinrichs W. Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. *Nat Struct Biol* 2000; 7 (3):215-9.
- Pfefferkorn ER, Bzik DJ, Honsinger CP. *Toxoplasma gondii*: mechanism of the parasitostatic action of 6-thioxanthine. *Exp Parasitol* 2001; 99 (4):235-43.
- Rand TA, Petersen S, Du F, Wang X. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* 2005; 123 (4):621-9.
- Remington JS, McLeod R, Thulliez P, Desmonts G. Toxoplasmosis. In Remington JS, Klein JO (eds): *Infectious diseases of the fetus and newborn infants*, 5<sup>th</sup> ed. Philadelphia, WB Saunders, 2001: 205.
- Remington JS, Thulliez P, Montoya JG. Recent developments for diagnosis of toxoplasmosis. *J Clin Microbiol* 2004; 42 (3):941-5.
- Roos DS, Donald RG, Morrissette NS, Moulton AL. Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Methods Cell Biol* 1994; 45:27-63.
- Sabin AB. Toxoplasmic encephalitis in children. *JAMA* 1941; 116:801-807.

Saeij JP, Boyle JP, Boothroyd JC. Differences among the three major strains of *Toxoplasma gondii* and their specific interactions with the infected host. Trends Parasitol 2005; 21 (10):476-81.

Saffer LD, Mercereau-Pujalon O, Dubremetz JF, Schwartzman JD. Localization of a *Toxoplasma gondii* rhoptry protein by immunoelectron microscopy during and after host cell penetration. J Protozool 1992; 39 (4):526-30.

Saffer LD and Schwartzman JD. A soluble phospholipase of *Toxoplasma gondii* associated with host cell penetration. J Protozool 1991; 38 (5):454-60.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd ed. Cold spring harbour laboratory press, Cold spring harbour, NY 1989.

Schwartzman JD and Pfefferkorn ER. Pyrimidine synthesis by *Toxoplasma gondii*. J Parasitol 1977; 67:150-156.

Schwartzman JD and Pfefferkorn ER. *Toxoplasma gondii*: purine synthesis and salvage in mutant host cells and parasites. Exp Parasitol 1982; 53 (1):77-86.

Seeber F, Boothroyd JC. *Escherichia coli* beta-galactosidase as an *in vitro* and *in vivo* reporter enzyme and stable transfection marker in the intracellular protozoan parasite *Toxoplasma gondii*. Gene 1996; 169 (1):39-45.

Sharp PA. RNA interference 2001. Genes Dev 2001; 15 (5):485-90.

Sheng J, Al-Anouti F, Ananvoranich S. Engineered delta ribozymes can simultaneously knock down the expression of the genes encoding uracil phosphoribosyltransferase and hypoxanthine-xanthine-guanine phosphoribosyltransferase in *Toxoplasma gondii*. Int J Parasitol 2004; 34 (3):253-63.

Shockett P, Difilippantonio M, Hellman N, Schatz DG. A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc Natl Acad Sci* 1995; 92 (14):6522-6.

Shuey DJ, McCallus DE, Giordano T. RNAi: gene-silencing in therapeutic intervention. *Drug Discov Today* 2002; 7 (20):1040-6.

Shuman S. Site-specific interaction of *vaccinia* virus topoisomerase I with duplex DNA. *J Biol Chem* 1991; 266 (30):20576-7.

Sibley LD, Pfefferkorn ER, Boothroyd JC. Development of genetic systems for *Toxoplasma gondii*. *Parasitol Today* 1993; 9 (10):392-5.

Sibley LD, Messina M, Niesman IR. Stable DNA transformation in the obligate intracellular parasite *Toxoplasma gondii* by complementation of tryptophan auxotrophy. *Proc Natl Acad Sci* 1994; 91 (12):5508-12.

Sibley LD, Niesman IR, Parmley SF, Cesbron-Delauw MF. Regulated secretion of multi-lamellar vesicles leads to formation of a tubulo-vesicular network in host-cell vacuoles occupied by *Toxoplasma gondii*. *J Cell Sci* 1995; 108 (4):1669-77.

Soete M, Fortier B, Camus D, Dubremetz JF. *Toxoplasma gondii*: kinetics of bradyzoite-tachyzoite interconversion *in vitro*. *Exp Parasitol* 1993; 76 (3):259-64.

Soldati D and Boothroyd JC. Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. *Science* 1993; 260 (5106):349-52.

Soldati D, Dubremetz JF, Lebrun M. Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. *Int J Parasitol* 2001; 31 (12):1293-302.

Striepen B and Soldati D. Genetic manipulation of *Toxoplasma gondii*. In: Weiss LM, Kim K, editors. *Toxoplasma gondii*—The Model Apicomplexan: Perspectives and Methods. London, UK: Academic Press 2007: 303–20.

Sukthana Y. Toxoplasmosis: beyond animals to humans. *Trends Parasitol* 2006; 22 (3):137-42.

Sumyuen MH, Garin YJ, Derouin F. Early kinetics of *Toxoplasma gondii* infection in mice infected orally with cysts of an avirulent strain. *J Parasitol* 1995; 81 (2):327-9.

Sun CH and Tai JH. Development of a tetracycline controlled gene expression system in the parasitic protozoan *Giardia lamblia*. *Mol Biochem Parasitol* 2000; 105 (1):51-60.

Tanner NK. Ribozymes: the characteristics and properties of catalytic RNAs. *FEMS Microbiol Rev* 1999; 23 (3):257-75.

Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma gondii*: from animals to humans. *Int J Parasitol* 2000; 30 (12-13):1217-58.

Tomavo S and Weiss LM. *Toxoplasma* gene regulation and bradyzoite development. In: Ajioka JW, Soldati D, editors. *Toxoplasma*—Molecular and Cellular Biology. Norfolk, UK: Horizon Bioscience 2007: 285–302.

Tomley FM and Soldati DS. Mix and match modules: structure and function of microneme proteins in apicomplexan parasites. *Trends Parasitol* 2001; 17 (2):81-8.

Van den Hoff MJ, Moorman AF, Lamers WH. Electroporation in 'intracellular' buffer increases cell survival. *Nucleic Acids Res* 1992; 20 (11):2902.

Wallon M, Kodjikian L, Binquet C, Garweg J, Fleury J, Quantin C, Peyron F. Long-term ocular prognosis in 327 children with congenital toxoplasmosis. *Pediatrics* 2004; 113 (6):1567-72.

Wirtz E and Clayton C. Inducible gene expression in *trypanosomes* mediated by a prokaryotic repressor. *Science* 1995; 268 (5214):1179-83.

Wong SY and Remington JS. Biology of *Toxoplasma gondii*. *Aids* 1993; 7 (3):299-316.

Yamamoto A, Hen R, Dauer WT. The ons and offs of inducible transgenic technology: a review. *Neurobiol Dis* 2001; 8 (6):923-32.

Yu L, Gao YF, Li X, Qiao ZP, Shen JL. Double-stranded RNA specific to adenosine kinase and hypoxanthine-xanthine-guanine-phosphoribosyltransferase retards growth of *Toxoplasma gondii*. *Parasitol Res* 2009; 104 (2):377-83.

Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 2000; 101 (1):25-33.

Zhang J, Wang C, Ke N, Bliesath J, Chionis J, He QS, Li QX, Chatterton JE, Wong-Staal F, Zhou D. A more efficient RNAi inducible system for tight regulation of gene expression in mammalian cells and xenograft animals. *RNA* 2007; 13 (8):1375-83.

Zhou XW, Kafsack BF, Cole RN, Beckett P, Shen RF, Carruthers VB. The opportunistic pathogen *Toxoplasma gondii* deploys a diverse legion of invasion and survival proteins. *J Biol Chem* 2005; 280 (40):34233-44.

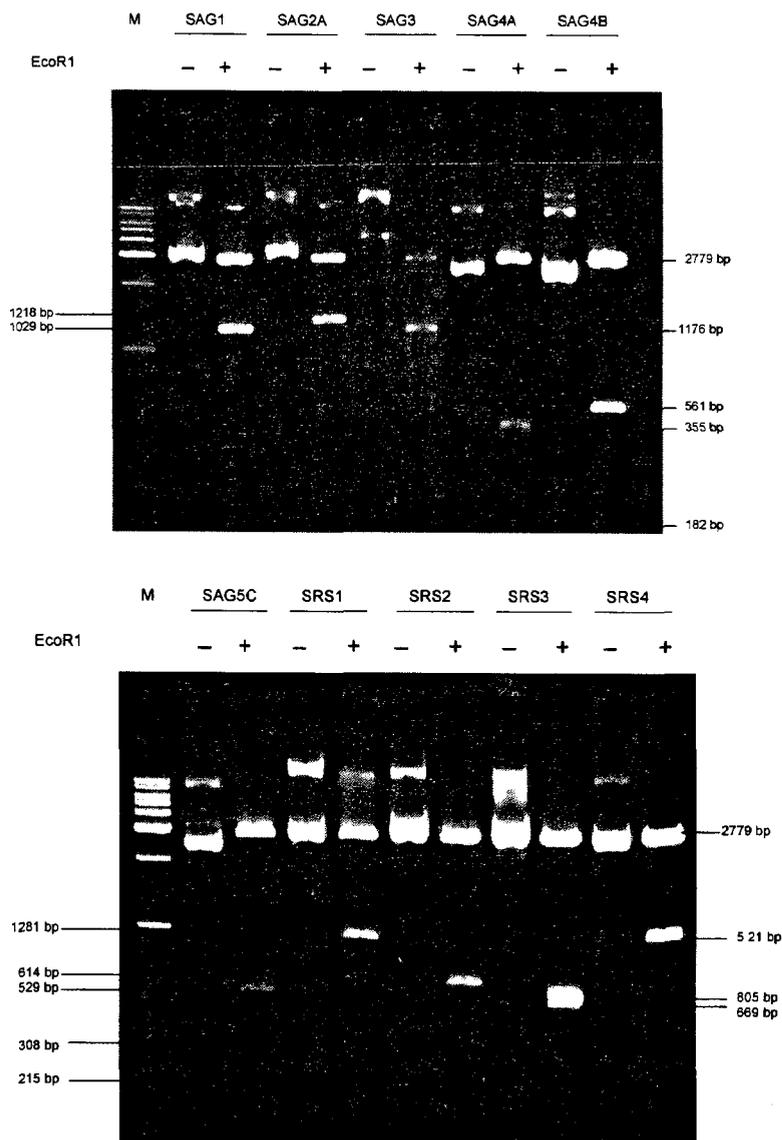
# Appendix I

## List of oligonucleotide primers used in the study

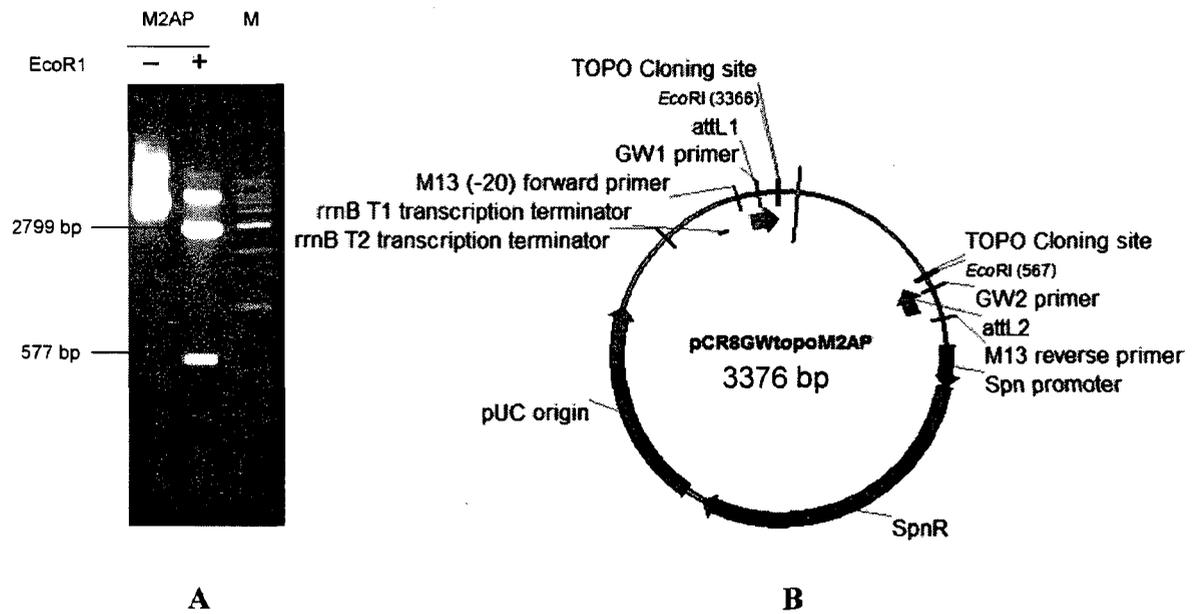
CAT EcoRI mut 5'	GATGAATGCTCATCCGGAGTCCGTATGGCAATGAAAGACG
SPH13'CAT	AAAGCATGCCCCGCCCTGCCACTC
ROP1-forward	GGAACATGGGCCACAG
ROP1-reverse	CGCCGAAAGCGTCTCTG
Nsi I-UPRT	TGCATGCATATAGGCGCAGGTCCCAGCGAGC
upstream HincII	CGACATTAGAGATCGCTGGGT
EcoRI on 3'UPRT	AAAGAATTCCAAAGTACCGGTCACCG
MIC3 antisense	AGTGAAGGCAAGCCATGTTCGAAAC
MIC3 sense	CATGTGTAGCTGACTGAATCGACG
LDHORF (sense)	ATGGCACCCGCACTGGT
LDHORF (antisense)	CGTTTCGGCTCCACTCG
BamHI 5' Ptub5	AATTGGGATCCGGTCGACGG

## Appendix II

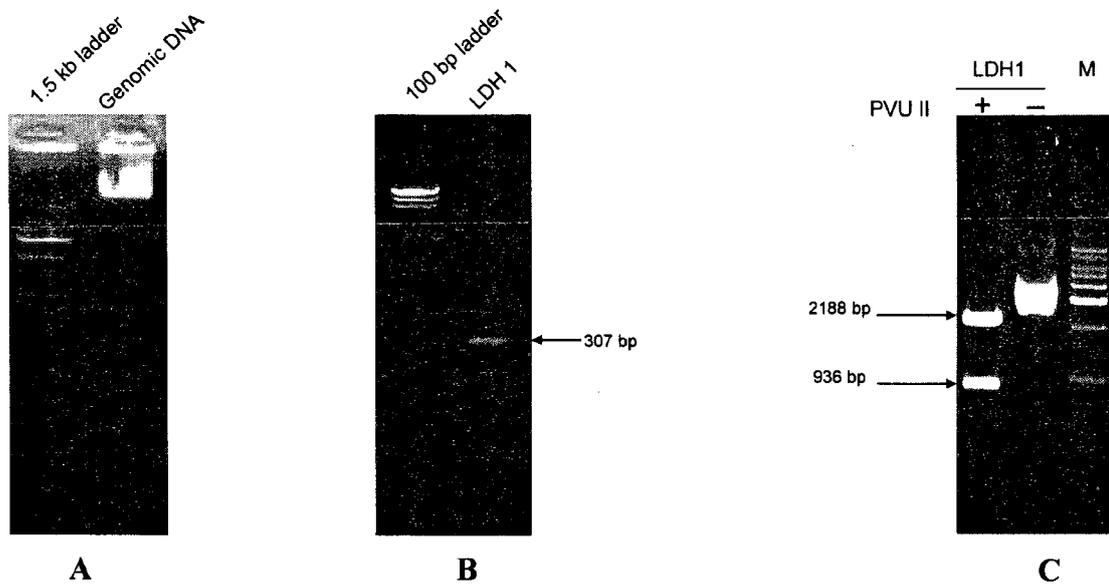
### Restriction profile of entry vectors



**Fig A.1 Restriction profile of entry vectors of various surface antigens (SAG).** Genes coding for various surface antigens were cloned into plasmid pCR8GWTOPO to create their respective entry vectors. Restriction digestion with *EcoR1* was done to confirm these clones. 2779 bp product represent the pCR8GWTOPO vector backbone



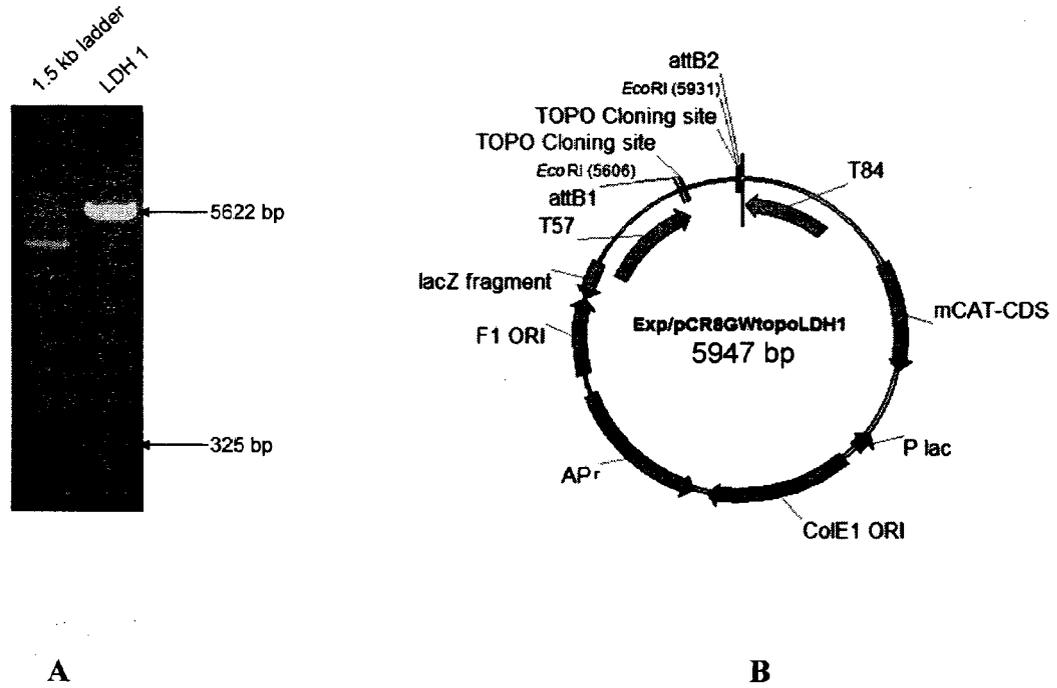
**Fig A.2 Restriction profile of M2AP entry vector.** (A) Genes coding for M2AP was cloned into plasmid pCR8GWTOPO to create the M2AP entry vector. Restriction digestion with *EcoRI* was done to confirm the clones. 2779 bp product represent the pCR8GWTOPO vector backbone (B) Vector map of M2AP entry vector



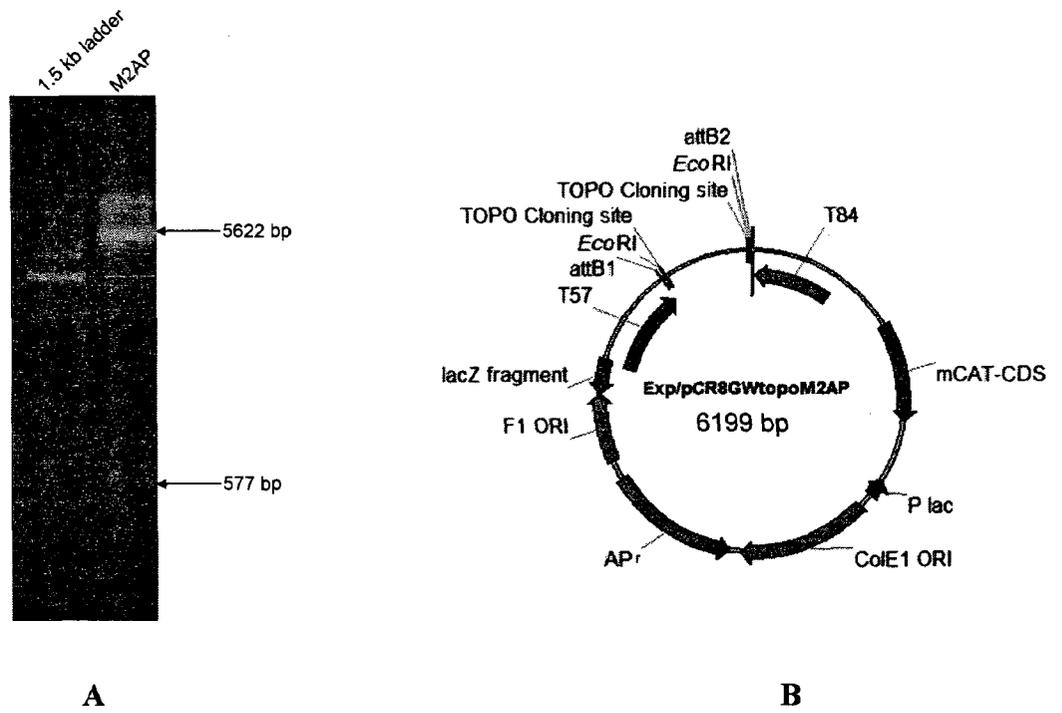
**Fig A.3 Restriction profile of LDH 1 entry vector.** (A) Genomic DNA was isolated from RH strain of *T.gondii* and resolved on 1% agarose gel. (B) 317 bp fragment of LDH 1 gene was PCR amplified from genomic DNA using the primer pair LDHORF (sense) and LDHORF (antisense) (C) 317 bp fragment of LDH 1 gene was cloned into plasmid pCR8GWTOPO to create LDH 1 entry vector. Restriction digestion with *Pvu* II was done to confirm this clone.

# Appendix III

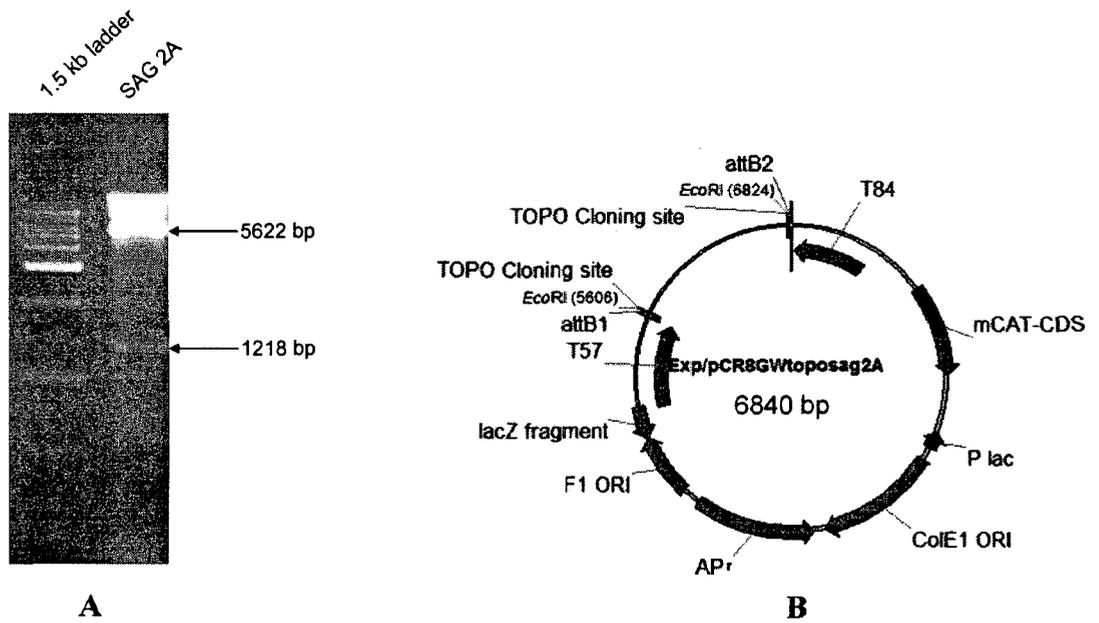
## Restriction profile of expression vectors



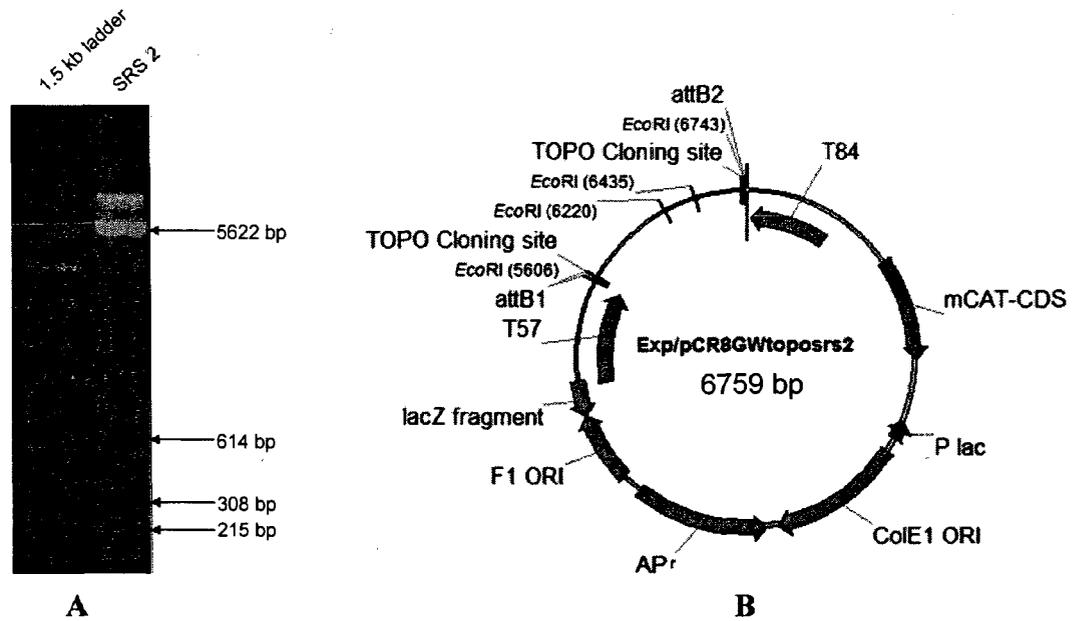
**Fig A.4 Restriction profile of LDH 1 expression vector. (A)** LDH 1 expression vector was digested with EcoR 1 to confirm the validity of the vector. The digested product was resolved on 1 % agarose gel **(B)** Vector map of LDH 1 expression vector



**Fig A.5 Restriction profile of M2AP expression vector.** (A) M2AP expression vector was digested with EcoR 1 to confirm the validity of the vector. The digested product was resolved on 1 % agarose gel (B) Vector map of M2AP expression vector



**Fig A.6 Restriction profile of SAG 2A expression vector. (A)** SAG 2A expression vector was digested with EcoR 1 to confirm the validity of the vector. **(B)** Vector map of SAG 2A expression vector



**Fig A.7 Restriction profile of SRS 2 expression vector.** (A) SRS 2 expression vector was digested with EcoR 1 to confirm the validity of the vector. The digested product was resolved on 1 % agarose gel (B) Vector map of SRS 2 expression vector.

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