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Investigation of the Molecular Mechanism Involved in *Lactate dehydrogenase* Gene Expression during Stage Conversion in *Toxoplasma gondii*

by

Ghazala Shamim

A Thesis
Submitted to the Faculty of Graduate Studies and Research
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ABSTRACT

Toxoplasma gondii, an obligate intracellular parasite, has the ability to differentiate between actively replicating tachyzoites and encysted bradyzoites. Bradyzoites within the tissue cysts are resistant to both the immune system and the chemotherapy. They are not only responsible for the transmission of the parasites via carnivorism, but also the source for recrudescence of infection in immunocompromised patients. Molecular mechanisms triggering the differentiation between the two forms are not clear. However, studies have shown that *T.gondii* expresses two distinct lactate dehydrogenases (LDH) during its stage conversion; LDH1 during the tachyzoite and LDH2 during the bradyzoite stage. *LDH2* shows transcriptional regulation by being exclusively expressed during bradyzoite stage that resembles anaerobic condition. Whereas *LDH1* transcription is found in both stages and indicates a translational suppression of LDH1 in bradyzoites. Regulatory activities of 5′ and 3′untranslated region have been reported in other eukaryotic systems indicating their role in gene expression. Aim of this study is to investigate the role of upstream and downstream sequences of *LDH1* in translational suppression and to observe the effect of removal of suppression of *LDH2* gene on the parasite's phenotype. For the first part of this study, transgenic parasite strains were generated to express GFP under the influence of upstream and/or downstream sequence of *LDH1*. GFP expression and localization was measured during both stages of the parasites and was found decreased in bradyzoite stage. These results indicates that the upstream and down stream sequences of LDH1 could be responsible for the lowered GFP expression. Possible effects on phenotype and future work are discussed.

DEDICATIONS

To Allah

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
Ble	Bleomycin
bp	base pair
BSA	bovine serum albumin
CAT	Chloramphenicol acetyl transferase
CIAP	Calf Intestinal Alkaline Phosphatase
cDNA	Complementary DNA
DAPI	4', 6diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DHFR-TS	Dihydrofolate reductase thymidylate synthase
DMEM	Dulbeco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DPBS	Dulbecco's phosphate-buffered saline
E.Coli	*Escherichia Coil*
EDTA	ethylenediaminetetra-acetic acid sodium salt
ENO	Enolase
FBS	fetal bovine serum
FITC	Fluorescein iso-thiocyanate
GFP	Green fluorescent protein
G_6PI	Glucose-6-phosphate isomerase
HFF	Human foreskin fibroblast
HXGPRT	Hypoxanthine-guanine phosphoribosyltransferase
LDH	Lactate dehydrogenase
Min	Minutes
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
mM	Millimolar
MPA	mycophenolic acid
mRNA	messenger RNA
nt	nucleotide
NTP	nucleotide triphosphate
Oligo	oligonucleotide
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	Ribonucleic acid
RNase	ribonuclease
ROP1	Rhoptry protein
Rpm	rotations per minute
RT	Reverse transcription
RT-PCR	Reverse transcription PCR
RFU	relative fluorescence unit

SAG1	Surface antigen 1
SDS	Sodium dodecyl sulphate
Sec	second
TAE	Tris-acetate-EDTA
Taq	*Thermus Aquaticus*
T.gondii	*Toxoplasma gondii*
TCA	trichloroacetic acid
TEMED	N,N,N′,N′-Tetramethyethylenediamine
Tub	Tubulin
Trp	Tryptophan
UPRT	Uracil phosphoribosyltransferase
UV	ultraviolet
UTR	untranslated region

CHAPTER 1

INTRODUCTION

1.1 *Toxoplasma gondii*

Toxoplasma gondii is an unusually promiscuous intracellular protozoan parasite that has the ability to infect virtually any vertebrate. It belongs to phylum Apicomplexa and is closely related to other protozoan parasites e.g. *Plasmodium* and *Cryptosporidium* (Dubey, 1994). Based on its worldwide distribution, *T. gondii* is the most ubiquitous parasite of the vertebrates that infects up to 50% of the human population (Dubey, 1977).

Infection occurs through ingestion of viable tissue-cyst in undercooked meat or by contact with the oocysts shed in cat's feces. Infection in healthy individuals remains asymptomatic except for some mild clinical symptoms. However, this infection progress to a chronic state without complications, persists for the life time of the host, and remains clinically unapparent. On the contrary, primary infection during pregnancy cause trans-placental transmission of the parasite and can lead to spontaneous abortion or severe congenital birth defects such as blindness, mental retardation, and hydro encephalopathy. Severity of infection depends on stage of pregnancy (Luft and Remington, 1992). Being an opportunistic pathogen, *T. gondii,* can reactivate the chronic infection in conditions where host immune system is compromised e.g. patients receiving cytotoxic chemotherapy or with HIV/AIDS. Unrestricted parasite growth leads to severe toxoplasmosis in such patients (Luft and Remington, 1988; Mills, 1986). In recent years, there has been a dramatic increase in the population of immunocompromised individuals and *T. gondii* can cause intracerebral focal lesions, leading to toxoplasmic encephalitis in this group (Wong and Remington, 1993).

Infection in the intermediate host (humans, cattle, birds) involves stage conversion between the two asexual forms of *T. gondii* i.e. rapidly multiplying tachyzoites which is responsible for acute toxoplasmosis and slowly dividing bradyzoites which is dormant encysted form found predominantly in brain and muscles. Tachyzoites are effectively cleared away by host's immune system (Dubey, 1994) while bradyzoites remain protected from immune response for the remaining life span of the host (Soete *et al.*, 1993) and act as a source of infection for transmission via carnivorism. This stage conversion is reversible and is responsible for the reactivation of a chronic infection into an acute one during period of lowered immunity (Freyre, 1995). The differentiation of these two forms of *T. gondii* involves morphological and molecular biological changes like stage specific antigen expression and alterations in metabolism (Yang and Parmley, 1997).

Current medicinal regimen (Sulfonamide and pyrimethamine) can effectively eliminate the tachyzoites but do not remove chronic bradyzoite stage (Cooper *et al.*, 1991). No effective treatment is available for chronic toxoplasmosis to date (Boothroyd *et al.*, 1997; Black and Boothroyd, 2000). Inability to completely eliminate the infection and toxic side effects of the existing drugs represents a critical need for a safer and more effective chemotherapeutic agent. Hence we need to decipher more about *T.gondii* cell biology in order to develop new strategies that could help in elimination of parasite infection.

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1.1.1 Parasite strains and host cells

Toxoplasma gondii can be grown and maintained in virtually any mammalian cell type e.g. HeLa, Vero, macrophages, T cells, and human foreskin fibroblast (HFF) (Boothroyd *et al.*, 1997; Mavin *et al.*, 2004). Parasites infect monolayer cultures more efficiently as compared to suspension cultures, probably because contact and invasion is easy in monolayers. HFF are the most commonly used host cells as they give high parasite titer with minimal cell debris, and are resistant to metabolic inhibitors e.g. drugs used for parasite selection. Being human cells they also provide comparability to clinical infections (Roos *et al.*, 1994; Freyre, 1995).

Many *T.gondii* laboratory strains are used and show differences in their rate of replication, virulence, and ability to form cysts. RH strain is easiest to grow both in cell-cultures and in animals and most commonly used because of its high virulence, rapid replication, and efficient host cell lysis (Sibley and Boothroyd, 1992). However, RH strain has weak ability to differentiate into bradyzoite stage or to from tissue cysts. RH is also incapable of making gametes in feline hosts (Freyre, 1995). Two other common strains are CEP derived from cat (Pfefferkorn *et al.*, 1977) and PLK from sheep (Kasper and Ware, 1985). PLK strain is capable of producing tissue cysts and is ideal for bradyzoite differentiation and development studies. PLK which is a subclone of ME-49 strain can complete sexual cycle of parasite in feline host. Replication rate of PLK strain, however, is much slower than RH strain (Soete *et al.*, 1993; Boothroyd *et al.* 1997).

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1.1.2 *Toxoplasma gondii* as experimental model for phylum Apicomplexa

Unlike other members of the phylum apicomplexa that are difficult to culture or propagate in the laboratory, *T. gondii* is amenable to genetic manipulations, easy to propagate using standard culture procedures and has emerged as a model for studying intracellular parasitism. Haploid genome of the parasite during asexual cycle has facilitated the generation of loss-of-function mutants (Black and Boothroyd, 1998). Data base of *T.gondii* genome is also well established (http://toxodb.org; Kissinger *et al.*, 2003).

1.1.3 Genetic manipulations in *T.gondii*

For the introduction of different forms of nucleic acids, electroporation is one of the most efficient mean for *T.gondii* transformation. Electric pulse generated during electroporation is believed to form small pores within the parasite cell membrane and facilitates the uptake of plasmid or nucleic acid. Once taken up by the parasites these plasmids serve as vehicle for stable or transient gene expression depending on whether it integrate into parasite genome or not (Boothroyd *et al.*, 1995 and Roos *et al.*, 1994).

Stable DNA transformation results in permanent expression of transgenes by random or directed integration into the parasite's genome (Donald and Roos, 1994). Episomal vectors are used for stable transformation without integration into the parasite's genome, eliminating the possibility of mutation due to random integration. Several strategies have been established for stable expression of transgene. One such stable DNA transformation is based on complementation of naturally occurring tryptophan auxotrophy in *T.gondii*. Prototroph was achieved by expressing *E.Coli trpB* gene

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encoding tryptophan synthase that catalyzes formation of tryptophan from supplemented indole or serine and eliminates the tryptophan dependency of *T.gondii* on host cells. Efficient *T.gondii* selection was made by tryptophan-depleted media and cytokines (IFNγ) treated host cells (Pfefferkorn, 1984). Tryptophan starvation was enhanced by IFNγ treatment. Transforming vector encoding *trpB* gene integrated into parasite genome by non homologous recombination (Sibley *et al.*, 1994) and the transformants were stable in the absence of continued selection.

Transient gene expression, on the other hand, is achieved when a plasmid fails to integrate into the parasite genome (Roos *et al.*, 1994). Expression is most efficient when it is under the control of *T.gondii* gene promoters such as *β*-tubulin (TUB1), rhoptry protein (ROP1), and surface antigen (SAG1) (Roos *et al.*, 1994; Soldati *et al.*, 1995), TUB1 being the strongest and SAG1 being the weakest promoter. Limitations to transient expression are short duration of expression and significant variation in the amount of genetic material taken up by the cell resulting into heterogenous population. Several reporter genes have been successfully used for transient gene expression such as *β*-galactosidase (*lacZ*), *β*-glucuronidase (*gus*), chloramphenicol acetyltransferase (*cat*), and luciferase. However, *cat* appears to function more effectively in *T.gondii* as compare to other reporter genes.

Vectors having small sequences of genomic DNA usually integrate by nonhomologous or random recombination (Tomavo, 2001; Sibley *et al.*, 1994). Transformation vectors based on sequences from *dihydrofolate reductase-thymidyle synthase* gene (DHFR-TS) have been developed for *T. gondii* genetic transformation (Donald and Roos, 1993). DHFR-TS gene was engineered to harbor mutations analogous

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to those found in DHFR-TS gene of pyrimethamine-resistant *Plasmodium falciparum* to introduce resistance for pyrimethamine in *T.gondii*. In more than 5% of the transformed parasites, DHFR-TS gene was reported to integrate into parasite genome by non homologous recombination. DHFR-TS mini gene has been used by several investigators to disrupt non essential genes by insertional mutagenesis (Donald and Roos 1995; Donald *et al*, 1996). Insertion at the hypoxanthineguanins phosphoribosyl transferase (HXGPRT) and Uracil phosphoribosyl transferase (UPRT) loci, which code for enzymes of purine and pyrimidine salvage pathway respectively, has been successfully generated.

As with transient expression, insertional mutagenesis has its own limitations, Such as the function of an essential gene could be compensated due to complementation. 500-bp sequence has been reported to permit the maintenance of bacterial plasmid as episomal DNA (Black and Boothroyd, 1998). This sequence could incorporate into vectors bearing fragments of the parasite genome and vectors can replicate autonomously with in *T.gondii*. On the other hand, Pyrimethamine resistant parasites pose a serious hazard because antifolates are used for the treatment of acute toxoplasmosis. Moreover, resistant strains should not be passed through a feline host to prevent the formation of oocysts having pyrimethamine resistance. Alternative selectable markers such as expression of *cat* that mediates CAT resistance (Kim *et al.*, 1993) expression of *ble* that mediates bleomycin resistance (Perez *et al.*, 1989) and complementation of tryptophan auxotrophy using *trp*B (Sibley *et al.*, 1994) are available, however they exhibit low transformation frequency as compare to expression of *dhfr* that mediates pyrimethamine resistance.

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Bleomycin resistance has been used as a selectable marker in *T.gondii*. *ble* resistance gene encodes a small acidic protein BLE which binds to the bleomycin family of glycopeptide antibiotic with strong affinity (1:1 complex) and prevents toxicity. Tn5 *ble* from Tn5 transposon (Messina *et al.*, 1995) and *Sh ble* from *Streptoallateichus hindustanus* (Soldati *et al.*, 1995) have been used to express BLE in *T.gondii*. Bleomycin provides efficient selection for extracellular tachyzoites, but has minimal effects on intracellular parasites thus selection is repeated twice to prevent leakiness. Also higher concentration of the drug can not be used because of its mutagenic effects.

Two non-essential enzymes of nucleotide salvage pathway are also used as selectable markers for *T. gondii* transformation (Donald and Roos, 1994). These enzymes are Hypoxanthine-xanthine-guanine phosphoribosyl trasnferase (HXGPRT) that is essential for purine salvage and Uracil phosphoribosyl trasnferase (UPRT) that is important to pyrimidine salvage. HXGPRT has been used to select the transgenic parasite strains during this study. This enzyme catalyzes the conversion of xanthine to xanthine monophosphate (XMP), which is then converted to guanine monophosphate (GMP). XMP can also be synthesized from inosine monophosphate (IMP) by the enzyme IMP-dehydrogenase, in the absence of the substrate i.e. xanthine or the enzyme HXGPRT. The drug mycophenolic acid (MPA) can inhibit IMP-dehydrogenase enzyme and prevents the conversion of xanthine to XMP and subsequently to GMP.

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Figure 1.1

Purine salvage pathway
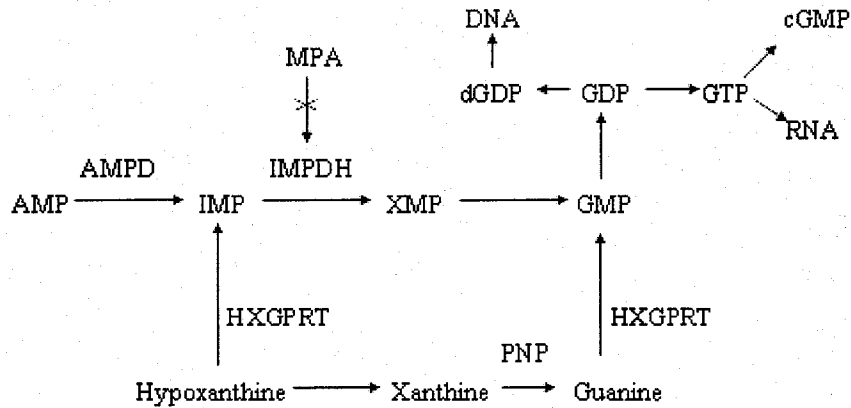
(Adapted from www.amg.gda.pl/~essppmm/metabolism.html)

AMPD   Adenosine Monophosphate Deaminase

HXGPRT Hypoxanthine Guanine Phosphoribosyl Transferase

IMPDH   Inosine Monophosphate Dehydrogenase

MPA   Mycophenolic Acid (Inhibitor of IMPDH)

PNP   Purine Nucleoside Phosphorylase

Figure 1.1 Purine salvage pathway. This pathway is important for DNA and RNA synthesis. HXGPRT deleted parasite strain is unable to synthesize GMP from guanine or hypoxanthine. Selection is made by the drug MPA which inhibits IMPDH enzyme. Parasites having HXGPRT as selectable marker would be able to synthesize GMP using xanthine as a substrate.

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HXGPRT deletion mutant (PLKΔHX) was used in this study for the generation of transgenic parasites. When PLKΔHX parasites were transformed with the plasmid that expresses the HXGPRT gene, the selection of transgenic parasites was carried out by supplementing the medium with MPA and xanthine. Parental strain is unable to use xanthine as a substrate due to deletion of HXGPRT gene. Other route of xanthine formation i.e. from IMP to XMP is also blocked due to inhibition of IMP-dehydrogenase by the drug MPA. On the contrary, transgenic parasites can use xanthine due to the plasmid insertion that has HXGPRT gene.

1.1.4 Sexual and asexual life cycle of *T.gondii*

Toxoplasma gondii has the ability to infect and replicate in virtually any nucleated mammalian or avian cell (Dubey, 1998). *T.gondii* life cycle is divided into two phases i.e. sexual and asexual phase. Sexual cycle exclusively occurs in feline intestinal epithelia, with the cat being a definitive host (Frenkel, 1973). This phase begins when a cat ingests infected intermediate hosts e.g. rats and birds that have encysted parasites present in their tissues (Dubey, 1994; Soete *et al.*, 1993). Bradyzoites are released from the cyst in the small intestine and undergo sexual reproduction. The differentiation of bradyzoites into micro and macro gametes and later fusion of these gametes results in the formation of oocyst. The oocyst later develops a thick impermeable cyst wall and excreted in the feces (Soete *et al.*, 1993). Upon exposure to atmosphere, each oocyst undergoes sporulation producing eight sporozoites. The mature haploid oocyst is very stable and remains infectious for months in cold and dry climates (Dubey, 1994).

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Figure 1.2

Toxoplasma gondii Life cycle

warm-blooded intermediate   *feline*

bradyzoite   gamete

3   1   2   6   5   4

tachyzoite   7   oocyst
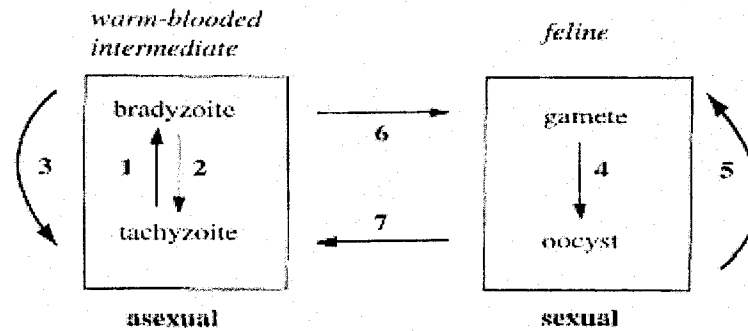
asexual   **sexual**

(Figure adapted from Boothroyd *et al.*, 1997)

Figure 1.2 Asexual cycle occurs in large number of warm blooded animals. Asexual phase involves two forms of parasite i.e. tachyzoites and bradyzoites (1, 2). Environmental factors especially the host immune response determines the balance between the two forms: strong immune response results in encystation whereas reactivation results upon lowered immune response. Transmission within asexual phase occurs through ingestion of meat infected with encysted bradyzoites (3). Asexual cycle involves gametogenesis and fertilization in gut epithelium of feline host (4). Transmission within the sexual phase is by ingestion of oocysts in feaces (5). Crossover between the two cycles (6, 7) results from accidental ingestion of the mature oocyst or bradyzoite infected meat.

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The asexual life cycle begins with the ingestion of either sporulated oocysts from cat's feces or viable tissue cysts in raw or undercooked meat. Ingested cysts are ruptured as they pass through the digestive tract and results in bradyzoite release. Released bradyzoites then infect the epithelium of the intestinal lumen and differentiate back to actively dividing tachyzoite form, enters the macrophages for the dissemination through out the body, and complete the asexual phase of the life cycle. The immune response forces the tachyzoites to shift to the encysted bardyzoites that persist for the remaining life span of the host (Dubey, 1994; Soete *et al.*, 1993).

1.1.4.1 Stages of asexual life cycle

The asexual phase of *T.gondii* life cycle consists of two distinct stages of growth i.e. tachyzoite stage or bradyzoite stage. Tachyzoite have an approximate length of 5μm and width of 2μm (Smith, 1995) and represents rapidly growing form of parasite that is found during acute phase of toxoplasmosis. The first step of infection is parasite attachment to the host cell which leads to invasion into parasitophorous vacuole (PV). Precise origin of the vacuole is not known yet. However, this invasion process involves clearing of rhoptries (specialized structure located at the apical end of parasite), and secretion from dense granules (Carruthers and Sibley, 1997). Unlike many other Apicomplexan parasites that replicate multiple times before assembly of daughter cells, toxoplasma replicates by endodyogeny i.e. produce two daughter cells in each division. In a given vacuole parasites divide synchronously and number of tachyzoites in any vacuole is always a power of two. With a generation time of 6-8 hours (in vitro) tachyzoites keep replicating with in the cell until they rupture the host cell to infect

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neighboring cells (Radke and White, 1998). Parasite exit is facilitated by a twisting motility. Ionic changes in host cell membrane due to mechanical strain from growing parasitophorous vacuole could induce parasite exit (Endo *et al.*, 1987; Endo and Yatiga, 1990).

Tachyzoites differentiate into bradyzoites that represent slowly dividing form capable of making tissue cyst and appears 7-10 days after infection. These cysts are found predominantly in the central nervous system and muscles where they reside for the life of the host. Formation of tissue cysts throughout the body defines the chronic stage of asexual life cycle. Bradyzoites can be distinguished from tachyzoites by the difference in their ultra structure. Bradyzoites accumulates numerous amylopectin granules, have increased number of micronemes, have electron dense rhoptries and have their nucleus located posteriorly. Tachyzoites, on the other hand normally lack amylopectin granules, have fewer micronemes and the nucleus is centrally located (Ferguson and Hutchison 1987; Matsukayashi and Akao 1963; Dubey, 1993). The host-parasite relationship is also different for both. Tachyzoites proliferates with in a parasitophorous vacuole that has a single unit membrane and a tubular network is formed from the invaginations of the membrane into the vacuole. Bradyzoites, on the contrary, develop with in the cyst that is surrounded by a cyst-wall. The differentiation of tachyzoites to bradyzoites occurs when the immune system attacks in order to eliminate the proliferating tachyzoites.

Toxoplasma gondii not only modifies its morphology, but presumably its metabolism also during stage conversion in order to adapt to the environmental changes. Mechanism of stage conversion between two stages of *T. gondii* as well as factors regulating the conversion is not fully understood. Stage specific gene product has been

used as a marker to understand the kinetics of stage conversion both *in vitro* and *in vivo* models (Burge *et al.*, 1988; Prince *et al.*, 1990; Gazzinelli *et al.*, 1993; Bohne *et al* 1994; and Soete *et al.*, 1994). Multiple factors of both host and parasite origin are most likely responsible for stage conversion. Recent studies have shown that small population of *T.gondii* undergoes spontaneous stage conversion *in vitro* (Tomavo *et al.*, 1991; Soete *et al.*, 1993). The percentage of bradyzoites in the population can be increased by altering the pH, adding cytokines such as IFN-γ, and treating with mitochondrial inhibitors like antimycinA (Bohne *et al.*, 1993; Soete *et al.*, 1994). Production of nitric oxide from activated macrophages has been suggested to induce conversion to bradyzoite in vivo possibly by interfering with parasite electron transport system (Bohne *et al.*, 1993; 1994). By altering the parasite mitochondrial function such as energy production, the host tries to control parasite proliferation. In response to host action, parasite transforms into slow replicating and less energy-consuming stage to maintain its viability. It is also well established that high amount of DNA damage can inhibit replication and delay the entry of cell into mitosis (Nasmyth, 1996). Immunological and environmental stresses involved in *T.gondii* differentiation could cause increased DNA damage/repair and result in slow replication of encysted bradyzoites. DNA damage may not be the only reason moreover it could be the result of nutrient deprivation as a form of stress. Parasites have been reported to express stage specific proteins e.g. surface antigens or enzymes, which are probably required for the adaptation (Tomavo *et al.*, 1991; Yang and Parmley, 1995; Yang and Parmley, 1997; Dzierszinski, 1999). Molecular cloning of the genes encoding stage specific enzymes is important and could be helpful in investigating the molecular mechanics involved in *T. gondii* stage conversion from tachyzoite to bradyzoite.

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1.1.4.2 Stage specific difference in metabolism

Mitochondrial inhibitors such as antimycin A, myxothiazol, and oligomycin have been reported to increase the expression of bradyzoite-specific antigens indicating a correlation between mitochondrial function and stage conversion (Bohne *et al.* 1994; Tomavo and Boothroyd, 1995). Impaired oxidative phosphorylation due to mitochondrial dysfunction induces bradyzoite formation *in vitro*. Key enzymes of TCA cycle and oxidative phosphorylation have been reported to be missing in bradyzoites (Denton *et al.*, 1996), while three isoenzymes (lactate dehydrogenase, glucose 6-phosphate isomerase, and enolase) involved in glycolysis have been reported to express stage specifically (Yang and Parmley, 1997: Dzierszinski *et al.*, 1999). These observations suggests that tachyzoites use both mitochondria and glycolytic pathway as ATP generating source, whereas encysted bradyzoites having impaired mitochondrial oxidative phosphorylation mainly rely on anaerobic glycolysis (Fulton and Spooner, 1960; Melo *et al.*, 1992; Vercesi *et al.*, 1998; Pfefferkorn and Borotz, 1994).

1.1.4.3 Stage specific isoenzymes

Lactate dehydrogenase (LDH), Enolase (ENO), Glucose 6-phosphate isomerase (G6-PI), and phospatidylinositol synthase (PIS) have been reported to express stage specifically in *T.gondii*. Except for phospatidylinositol synthase, all are important enzymes for glycolysis. Stage specific expression of LDH, main focus of the present study, will be discussed in detail in this section.

Lactate dehydrogenase (L-lactate: oxidoreductase, EC 1.1.1.27) uses NAD^+ as a coenzyme and catalyzes the interconversion of pyruate to lactate during glycolysis. Gene

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encoding tachyzoite LDH has been cloned and named as LDH1 and bradyzoite LDH as LDH2. These two LDH genes show 64% nucleotide identity in their coding region and both have an intron at the same relative position. Size of the intron is 538nt and 588nt in LDH1 and LDH2 respectively. Amino acid sequences of the two LDH proteins share 71.1% identity. Using RT-PCR, Yang and Parmley (1997) have demonstrated that mRNA of LDH1 is found equally in both stages of the *T. gondii* while mRNA of LDH2 is only detected in bradyzoite stage. The absence of LDH2 mRNA in tachyzoites suggests that LDH2 transcription is suppressed during transition from bradyzoite to tachyzoites. These data also suggest that LDH1 is exclusively expressed in tachyzoites. LDH2 mRNA levels have been reported to increases predominantly when tachyzoite to bradyzoite differentiation is induced. LDH2 mRNA stability was not determined during the study, thus it is not clear whether this increament in LDH2 mRNA is due to transcriptional activation of the LDH2 gene or an increase in mRNA stability.

Yang and Parmley (1997) also demonstrated that LDH1 enzyme is only detectable in tachyzoites using two dimensional PAGE. Difference in isoelectric points i.e. 5.96 for LDH1 and 7.08 for LDH2 has been exploited to show that only one LDH protein is expressed in each stage. The possibility of higher isoelectric point of bradyzoite LDH due to tyrosoine phosphorylation was ruled out as it would only make tachyzoite LDH more acidic (Cooper *et al.*, 1983). Post-translational modifications that make LDH more basic have not been reported yet. These results suggest that two distinct forms of LDH proteins are produced in each stage i.e. LDH1 in tachyzoite stage and LDH2 in bradyzoite stage. Putative promoter sequences of respective LDHs have been used to express CAT reporter construct. Approximately 1500 nt upstream and downstream

sequence of LDH1 were used for CAT expression. High CAT activity observed in tachyzoites was reduced to four fold when parasites were switched to bradyzoite stage. Similarly 993 nt upstream and 1750 nt downstream sequence of LDH2 were used for CAT expression. No activity was detected during tachyzoite stage while CAT expression was increased to forty five fold with the differentiation to bradyzoites. This indicates that the production of LDH2 seems to be under transcriptional control due to the appearance of LDH2 mRNA only in bradyzoites, while production of LDH1 appears to be under translational control as mRNA of LDH1 is present in both stages but it is translated only in tachyzoites. Roos *et al.*, 1997 has also showed that putative LDH2 promoter sequences are involved in stage specific LDH2 expression during bradyzoite differentiation. Using cNTPase (Nucleotide triphosphatase lacking N-terminal signal) as a reporter they demonstrated that the reporter expression is transcriptionally controlled with the stage conversion.In conclusion, the process of developmental regulation of the two LDH genes appears to be different for each stage. Studies have shown that *LDH1* and *LDH2* genes are found on different chromosomes i.e. LDH1 on chromosome VIII and LDH2 on chromosome IX. When the expression of both the LDHs was knocked down using dsRNA, growth rate in both the stages varied as compared to parental strain and parasite differentiation (in-vitro) was impaired. Tachyzoites were unable to form significant number of cysts *in vivo,* to establish a chronic infection (Fatme Al-Anouti *et al.*, 2004). These results indicate that LDH expression is important for *T.gondii* cell cycle.

1.2 Eukaryotic gene regulation

Eukaryotic gene expression is primarily controlled at the level of transcription initiation (Lemon and Tijan, 2000). Housekeeping genes are expressed constitutively and are shut down in response to extreme environmental condition such as heat shock or starvation (Prikkala *et al.*, 2001). Facultative genes, on the contrary are expressed in response to specific physiological, hormonal or environmental stimuli (Benecke *et al.*, 2001). Eukaryotic genes that code for protein are transcribed by RNA polymerase II hcloenzyme which is composed of 10-12 proteins (Lee and Young, 2000). All the genes that are transcribed by the RNA polymerase have two core promoter elements, the TATA box and the initiator sequence. Promoters can not generate significant level of mRNA by itself, they determine transcription start site and initiate low or basal level of transcription (Kuras and Struhl, 1999). Transcriptional machinery assembles on the promoter which serves as a docking site for the transcriptional complex. Most of the proteins that bind to basal promoters are ubiquitously expressed and have little regulatory specificity (Lemon and Tijan, 2000). These proteins are called general transcription factors. In addition to the promoter, some regulatory sequences are also needed for accurate regulation of transcription and are usually located upstream of the promoter sequence. For example, the consensus sequences of two regulatory elements CCAAT and GGGCGG (called GC box) are located 100 base pair upstream of the TATA box. These regulatory sequences are found in many eukaryotic genes and were first identified during the promoter studies of the gene encoding thymidine kinase in herpes simplex virus. Some regulatory sequences known as enhancers are located far from the promoter (could be 10 kbp away) and were first described by Walter Schaffner in 1981, during the studies of promoter of

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another virus SV40. Activity of enhancer is independent of distance or orientation (Blackwood and Kadonaga, 1998). On the contrary, upstream repressor sequences have been reported to inhibit transcription when bound to specific factors (Hanna-Rose and Hansen, 1996). Inhibition occurs through various mechanisms such as interference in activator binding or modification in chromatin structure. The silencer, on the other hand, is the DNA sequence that represses promoter activity regardless of its orientation and position (Guarente, 1999). In higher eukaryotes a CpG dinucleotide motif is reported to be involved in gene silencing through DNA methylation (Antequera and Bird, 1999).

1.2.1 Regulation of transcription by chromatin structure

Eukaryotes maintain and control their huge genetic content by packing DNA into nucleosomes (Luger et al., 1974). Packaging of promoter into chromatin is a key determinant in transcription initiation (Grunstein, 1997). Studies have shown that transcriptional activators are associated with histone acetyl transferase (Wolffe and Pruss, 1996) while transcriptional repressors are linked with histone deacetylase (Pazin and Kadonaga, 1997). Protein complexes named as nucleosome remodeling factors facilitate the binding of transcription factors to the DNA. By catalyzing the histone octamer along the DNA, they reposition the nucleosome and help binding of transcription factors to the DNA. DNA methylation at cytosine residues that precedes Guanine (CpG dinucleotide) is associated with reduced transcription. A protein MeCP2 binds to methylated DNA and represses transcription (Geoffrey MC, 2000).

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1.2.2 Post-transcriptional gene regulation

RNA processing involves addition of 5'cap, addition of 3'polyA tail and removal of introns and represents another level of gene regulation. The sequence AUUUA in 3'UTR is the signal for early degradation and short lifetime of mRNA. The higher the frequency of occurrence of this sequence, the shorter the life span of mRNA. Since the lifespan is encoded in nucleotide sequence, it is different for each mRNA and can not be varied. Information about the life span and stability of RNA is found in 3'UTR (untranslated region) of gene. 3'UTR have been reported to affect gene expression by modulating RNA stability and translation (Casey *et al.*, 1988 and Hentze *et al.*, 1988). Role of 3'UTR in suppression of basal and growth-hormone induced transcription has also been reported (Le Cam and Legraverend, 1995). UTRs of other genes have been reported to function as regulatory elements of the genes and affect transcription efficiency. Expression of human aggrecan gene has been reported to depend on the sequence present in 5'UTR (Wilmot *et al.*, 1998) which is thought to serve as the binding site for general transcription factors that are needed for the assembly of transcription initiation complex. On the contrary, erythropoietin gene expression was reported to be enhanced upon removal of 5' and 3' UTRs (Park *et al.*, 2001).

1.2.3 Translational gene regulation

Factors affecting mRNA translation includes m^7G cap, length of 5'UTR, position and context of initiator codon (AUG), and high order structure within mRNA (Nicola and Matthias, 1994). Structural elements in mRNA control translation initiation. Stability and position of secondary structure introduced into the 5'UTR of mRNA has been reported to

inhibit its translation (Lawson *et al.*, 1986). When a secondary structure 12 nucleotide away from the m^7G cap was introduced into mRNA, it strongly diminishes translation *in vitro*. Same structure, when placed 52 nucleotide downstream of the m^7G cap, had no effect on translation. It is suggested that when the distance between the m^7G cap and secondary structure was sufficient enough to allow 43S entry, progression of 43S pre-initiation complex and associated factors occurred which disrupted the secondary structure and resulted in translation (Lawson *et al.*, 1986). Various cellular mRNAs that code for transcription factors or growth factors have unusually long 5UTRs with upstream AUG, small ORF and strong secondary structures that result in poor translation (Kozak, 1991). For example mRNA of human platelet-derived growth factor-2 (PDGF2) has 5′UTR of 1022 nucleotides with 140 nucleotide GC-rich regions. GC-rich region immediately preceding AUG was found to be responsible for the poor translation of PDGF2. Role of secondary structure in 3UTR in regulation mRNA translation has not been established. However, long range interaction between 5′UTR and 3′UTR has been reported to regulate mRNA translation in Zea mays (Spena and Dobberstein, 1985).

Secondary structure in various mRNAs serve as binding sites for repressor protein and provides translational suppression and rapid regulation of gene/mRNA in response to cellular or extracellular stimuli. For example expression of ferritin (an iron storage protein) is regulated at translational level. Regions in the 5′UTR termed as iron responsive element (IRE) were capable of forming stem-loop structure and sufficient to control translation of ferritin mRNA in response to cellular iron concentration. (Hentze *et al.*, 1987; Hentze *et al.*, 1988). IRE is a highly conserved stem-loop structure which is important in mediating translational control *in vivo* and *in vitro* (Gray *et al.*, 1993). A

similar translational regulation has been identified in haem synthesis enzyme, erythroid 5-aminolevulinic acid synthase (eALAS). An IRE was found in 5′UTR of eALAS mRNA to regulate the translation (Melefors *et al.*, 1993). Multiple IRE have been reported in 3UTR of mRNA encoding transferrin receptor (involved in iron uptake in cell). However, translation is controlled here via change in mRNA stability (Klausner *et al.*, 1993). Human thymidylate synthase (TS) mRNA has been shown to undergo translational autoregulation *in vitro* (Chu *et al.*, 1993). A 29 nucleotide stem-loop motif in 5UTR of the mRNA, specifically interact with TS protein. Similarly human dihydrofolate reductase mRNA translation is also autoregulated. Role of RNA elements in such translational regulation is to provide access sites for repressor protein. Ribosomal protein synthesis is translationally regulated in *Saccaromyces cerevisiae*, *Xenopus laevis*, and mammalian cells. These mRNAs contain short 5′UTR (35-50 nucleotides), have a polypyrimidine tract (8-14 nucleotide) in cap-proximal position. 5′UTR of *Xenopus laevis* S19 and murine S16/L30 mRNA are sufficient for regulation of translation. The polypyrimidine tract is necessary but has not been reported to be sufficient alone (Levy *et al.*, 1991).

Elements in 3UTR which serve as protein binding site can also regulate mRNA translation by mediating changes in poly-A tail length. Erythroide lipoxygenase LOX mRNA is expressed at early stage and must be translationally silenced in immature erythroide cells. 3′UTR (576 nucleotide) of rabbit (LOX) mRNA has ten repeats of 19 nucleotide pyrimidine rich element and starts 50 nucleotide down stream from the translation termination codon (Fleming *et al.*, 1989). Human reticulocyte LOX mRNA has four such repeats and sufficient for regulation. These pyrimidine repeats specifically

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interact with LOX-BP protein (48kDa) which represses LOX mRNA translation. UA-rich sequences in 3′UTR (associated with mRNA instability) also serve as binding site for repressor proteins and regulate translation of tumor necrosis factor mRNA translation (Goodwin *et al.*, 1993).

Objectives

From the previous studies it has been established that mRNA of LDH1 is present in both stages of asexual life cycle of *T.gondii* i.e. rapidly replicating tachyzoites and encysted bradyzoites. However, LDH1 protein is expressed only in tachyzoites but not in bradyzoites, suggesting a post-transcriptional and/or a translational suppression of LDH1 in bradyzoites. On the other hand LDH2 mRNA is present only in bradyzoites and is translated to LDH2 protein, showing a transcriptional regulation of the *LDH2* gene (Yang and Parmley, 1997). Governing mechanism involved in distinct expression of two LDHs i.e. *LDH1* in tachyzoites and *LDH2* in bradyzoites, is still unclear. Putative promoter and downstream sequence of LDHs have been reported to play a role in differential expression of LDHs during parasite stage conversion (Yang and Parmley 1997; Boothroyd et al., 1997). The study was divided into two parts, using plasmid pTub8mycHisGFP-HX as control in both parts of the study.

1. To refine the search for the putative promoter and downstream sequence for LDH1 and create a tool for the long term goal, using GFP reporter system, followings were the objectives.

a) To construct three different plasmids from the control and to generate four transgenic parasite strains from these plasmids. In transgenic control strain GFP reporter

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expression is under TUB8 promoter with a 3UTR of SAG1 downstream GFP. Among the three test transgenic strains, one strain has upstream sequence of *LDH1* alone, other strain has both upstream and downstream sequence of *LDH1*, and the last strain has downstream region of *LDH1* alone, such that the first two strains are under the control of putative *LDH* promoter and the last strain is under *TUB8* promoter control.

b) To determine GFP levels and its localization in both stages of *T.gondii* to establish the role of upstream and downstream sequence of *LDH1* in translational suppression.

2. To create tools for the long term goal for the understanding of the mechanism behind transcriptional regulation of LDH2,

a) To generate two transgenic parasite lines using myc*LDH1* and myc*LDH2* as reporter, from the same control plasmid i.e. reporter expression is under the influence of Tub8 promoter and has 3′UTR of SAG1 downstream of the reporter.

b) To determine the levels of constitutive mycLDH1/LDH2 expression by western blot.

c) To monitor the effect of *LDH* misregulation on the phenotype of the both stages of the parasite.

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CHAPTER 2

MATERIALS AND METHODS

2.1 Parasite strain and chemical supplies.

PLKΔHX strain, used in this study was obtained from the AIDS Research and Reference Reagent Program, NIH. The *T.gondii* strain PLKΔHX was derived from the slowly multiplying strain PLK. The gene encoding hypoxanthine-xanthine-guanine phosphoribosyltransferase has been silenced in the PLKΔHX strain. This silencing allows the selection of the transgenic parasites (Donald and Roos, 1995; Donald *et al.*, 1996).

Following is the list of chemicals and reagents used in this study along with the name of the supplier:

Amersham-Pharmacia Biotech (Baie d'Urfe, Quebec)

2' -deoxyribonucleoside 5'-triphosphate (dNTPs), nitrocellulose membranes.

Baxter Diagnostics Corp. (Toronto, ON)

Nalgene™ disposable 25mm syringe filters (0.2 μm pore size) and glass Pasteur pipettes.

Bio-Rad Laboratories (Mississauga, ON)

Bromophenol Blue, Coomassie brilliant blue R-250, Xyelene Cyanol, protein molecular weight standards (medium range), and protein assay dye reagents.

Carolina Biological Inc

Restriction enzyme BamHI

Gelman Sciences (Ann Arbor, MI)

The Vacucap™ disposable bottle-top filter for sterilization of media.

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GibcoBRL Invitrogen (Burlington, ON)

Cell culture media Dulbeco's modified essential medium (DMEM), minimal essential medium (MEM), Dulbeco's phosphate buffered saline (DPBS) supplemented with calcium, trypsin, penicillin-streptomycin (10 μg/ml), L-glutamine.

Hyclone (Logan, UT)

10% cosmic calf serum.

Invitrogen Corporation (Burlington, ON)

Proteinase K, Trizol reagent, and RNase out ribonuclease inhibitor.

NewEngland Biolabs Inc

Restriction enzymes KpnI, NsiI, PstI.

Promega (Madison, WI)

Agarose, Mung Bean Nuclease, T4 DNA ligase, Moloney murine leukemia virus reverse transcriptase (M-MLV), RQ1 RNase free DNase, DNA ladder 1kb and 100 bp, Multicore buffer, EcoRI, HindIII.

Qiagen (Mississauga, ON)

QIAEX® I and plasmid midi prep kits for DNA purification.

Roche Diagnostics (Laval, Québec)

Taq DNA polymerase.

Sigma-Aldrich (Oakville, ON)

Acetic acid, ampicillin, tetracycline, chloroform, 4′,6 diamidino-2-phenylindole (DAPI), *Dolichos biflorus* conjugated to fluorescein isothiocyanate, dimethylsulfoxide (DMSO), dithiothreitol (DTT), diethylpolycarbonate (DEPC), ethanol, agar, ethidium bromide, formaldehyde, glycine, glycerol, hydrochloric acid, mycophenolic acid (MPA),

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polyoxyethylene sorbitan monolaurate (Tween-20), 2-propanol, phenyl methyl sulfonyl fluoride (PMSF), sodium citrate, sodium chloride, sodium acetate, tris-hydromethylaminomethane (Tris), Triton X-100, Trichloroacetic acid, Xanthine, midi-prep plasmid purification kit.

Stratagene (La Jolla, CA)

XL1BL bacterial strain.

TaKara Bio Inc

Restriction enzyme HincII

USB (Cleveland, OH)

Acrylamide, bis-acrylamide, ammonium persulfate (APS), equilibrated phenol, RNaseA, Sodium dodecyl sulphate (SDS)

Nucleopore (ON)

Polycarbonate filters (3μm pore size)

Wisent (Montreal)

Dialyzed fetal bovine serum (FBS)

pTUB8mycHisGFP-HX plasmid (kindly donated by Dr. Dominique Soldati)

2.2 Apparatus and Instrumentation

Miniature Horizontal Gel System MLB-06 from Tyler Research Instruments was used for DNA agarose gel electrophoresis. Gels were viewed and images were captured using AlphaImager™ 2200 Light Imaging system with AlphaEase software. Vertical gel electrophoresis system, including all glasses, Teflon combs and spacers from BRL

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(Bethesda Research Laboratories) were used to run Sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE).

J2-HS centrifuge (Beckman), desktop Eppendorf Model 5415C microcentrifuge from Desaga (Sarstedt Gruppe, Germany) or BR4i Centrifuge (Jouan, SA) were used to carry out centrifugations.

Protein quantification was performed using Shimadzu UV-Visible Recording spectrophotometer, Hadley Tekscience.

Polymerase chain reaction (PCR) was carried out using 20-well Techgene Thermal Cycler (Techgene. Cambridge, UK). Ligation reactions were performed in 48-well Perkin Elmer Cetus DNA thermal Cycler (Perkin Elmer. Norwalk, CT).

Cell and parasite cultures were conducted in the class II type A/B3 Biosafety cabinet (Jouan, SA). All cultures were maintained in a CO_2 incubator (Thermoforma). Electroporations were performed using BTX model; 600 Electro Cell Manipulator (Genetronics). All Bacterial cultures were grown in NEW BRUNSWICK SCIENTIFIS G-25R shaking incubator.

All glassware, broth solutions were autoclaved prior to use in order to maintain sterile conditions throughout bacterial culturing.

2.3 General methods

2.3.1 Agarose gel electrophoresis

Miniature Horizontal Gel System MLB-06 (Tyler Research Instruments, Edmonton) was used for agarose gel electrophoresis. 1% (w/v) Agarose gel was made by dissolving solid agarose in Tris-acetate EDTA (TAE) buffer, 40 mM Tris-acetate, 1mM

EDTA. Ethidium bromide was later added to a final concentration of 10ng/ml. The gel was first allowed to solidify in the electrophoresis tray and than immersed into the electrophoresis tank filled with 1x TAE buffer. DNA samples were mixed with the gel loading buffer (0.025% Bromophenol blue, 0.025% Xyelene Cyanol FF, 10mM EDTA pH 8.0 and 98% deionized formamide) and were loaded into the wells. DNA marker was loaded and the gel was electrophoresed for 30-45 min at 100V (Sambrook 1989). The gels were visualized using Benchtop Ultraviolet Transillumoinator (VWR Scientific. Mississauga, ON). AlphaImager 2200 Light Imaging System with AlphaEase Software was used to capture gel images and for the photography of ethidium bromide stained agarose gels (Alpha Innotech Corporation, San Leandro).

2.3.2 Reverse Transcription (RT)

Reverse transcription reaction was carried out by mixing 2μg of extracted RNA with 50 pico moles of oligo dT. This was incubated at 70°C for 5 minutes to remove RNA secondary structure and then chilled on ice for 1 minute. The mixture was completed to 10 μl by adding in order, 2μl of M-MLV reverse transcriptase 5 x buffer, 1.5μl of dNTPs (2.5 mM), 0.5μl of RNaseOut (40 U/μl), 1μl of M-MLV reverse transcriptase (50U/μl) and incubated for 1 hr at 37°C. Reverse transcriptase was then heat inactivated at 95°C for 5 minutes. 4μl of the RT-mixture was used as a template for subsequent PCR amplification. Any genomic DNA contamination was checked by running a PCR using ROP1 primer. No PCR amplification indicated that RNA sample is un-contamination.

2.3.3 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was performed using 20-well Techgene thermal cycler (Techne, Cambridge, UK). The PCR was performed in a total volume of 50μl containing 75mM Tris-HCl pH 8.8, 50 mM KCl, 2mM $MgCl_2$, 50 mM dNTPs, 2 U of *Taq* DNA polymerase and 0.5 μl of oliginucleotide primers (100 pmol/μl). The amplification reaction included 35 cycles each consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45sec. 1μl of genomic DNA, as a positive control and autoclaved water, as a negative control were used as templates in the amplification reaction. Amplified product was resolved in a 1% (w/v) agarose gel.

2.3.4 Host cell culture

Human foreskin fibroblasts (HFF; obtained from Dr.Roos, University of Pennsylvania) were used as a host for the intracellular parasite *T.gondii*. These HFF cells were grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5ug/ml streptomycin, 5units/ml penicillin and 10% cosmic calf serum, in a 5% CO_2 atmosphere.

To propagate the host cells, confluent HFF cultures were detached by adding 1 ml of Trypsin EDTA solution (0.25% trypsin, 0.3%EDTA) followed by incubation at 37°C for 45 seconds. Detached cells were suspended in fresh media and split into 3-4x the area of old culture.

To freeze the host cells for later use, cells were pelleted after trypsin treatment. These pellets were resuspended in cell freezing medium containing 25% dimethyl

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sulfoxide (DMSO) and 20% dFBS (dialysed fetal bovine serum) in DMEM and were immediately placed in cryovials and were stored at -80°C.

2.3.5 *Toxoplasma gondii* Culture

Toxoplasma gondii was propagated in HFF grown in MEM medium with L-glutamine, supplemented with 1% dialyzed fetal bovine serum. Parasites scraped from infected HFF monolayers were used to infect a new monolayer of HFF grown in MEM medium (Donald and Roos, 1994).

2.3.6 Parasite Pelleting

Freshly released tachyzoites from HFF monolayer were scraped, passed through a 27 G-1/2 syringe needle to release parasites from vacuole, filtered through a $3\mu m$ polycarbonate filter to remove all the host cell debris, and than pelleted by centrifugation at 2000-3000 rpm for ten minutes. Parasite pellet were stored at -80°C for later use for genomic DNA, RNA or protein isolation.

2.3.7 Genomic DNA Isolation

Genomic DNA was extracted by resuspending the parasite pellet in 0.2 ml lysis buffer (1mM EDTA, 10mM Tris pH 8.0, 0.5% SDS, $0.2\mu g/\mu l$ Proteinase K, $0.125\mu g/\mu l$ RNase) followed by incubation at 56° C for 4-6 hrs. The suspension was extracted twice with 0.2 ml of phenol: chloroform: isoamyl alcohol and once with chloroform. Genomic DNA was then recovered from the aqueous phase and precipitated with 0.1 volume 3M sodium acetate and 2.2 volume 95% ethanol followed by centrifugation at 7500 rpm for

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10 min. Genomic DNA pellet was washed using 70% ethanol and dried followed by resuspension in 20μl autoclave water.

2.3.8 RNA extraction from *T.gondii*

Parasite pellet was lysed with 500μl Triziol reagent and incubated for 5 min at room temperature to permit a complete dissociation of nucleoprotein complexes. 100μl of chloroform was added to the mixture and was shaken vigorously for 15 seconds and incubated at room temperature for 2 min. Phase separation was carried out by centrifugation at 12000 rmp at 4°C for 15 minutes. The upper aqueous layer containing RNA was precipitated by 250μl of isopropyl alcohol and incubated at room temperature for 10 minutes and then centrifuged at 10000 rpm for 10 minutes at 4°C. Precipitated RNA was washed with 500μl of 70% ethanol and resuspended in DEPC-treated water. Any genomic DNA contamination was removed by treating the RNA with 4 units of RQ1 RNase-free DNase for 20 minutes at 37°C. DNase was subsequently inactivated by heating at 75°C for 5 minutes.

2.3.9 Protein quantification by Bradford Assay

Bradford assay was used to determine protein concentration using the Agilent UV-visible spectrophotometer (Shimadzu). Parasite pellets were completely resuspended in 200 μl of lysis buffer (HEPES 50μM, PMSF 5μM, Triton X100 0.025%, Glycerol 20%) and centrifuged at 13000 rpm for 10 min and the supernatant having protein in it, was collected in a separate tube. The protein sample was mixed with 0.7 ml of working Bradford reagent and incubated for 10 min at room temperature. Using the Bradford

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reagent as blank, spectrophotometric measurements were carried out at 595 n.m. A standard curve made from standard protein solution of BSA was used to determine the concentration of the unknown protein in the test samples (Sambrook 1989).

2.3.10 Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis

A discontinuous buffer system was used to conduct sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The stacking and resolving gels were caste in a vertical gel electrophoresis system from BRL (Bethesda Research Laboratories). The resolving solution was poured first and left to polymerize, then stacking gel was overlaid on to it. Samples were mixed with the gel loading buffer (Appendix) and boiled for 5 min, centrifuged at 13000 rpm for 30 sec and then loaded into the wells. The gel was run with Tris-Glycine running buffer (25mM Tris, 192 mM Glycine and 1%SDS) at 130 volts until the bromophenol dye came out of the gel.

2.3.11 Electro-blotting and immunodetection

The proteins were transferred to nitrocellulose membrane at 130 mA for 2 hrs, in the transfer buffer (20mM Tris pH8.0, 150mM Glycine and 20% methanol) using electro-blotting (BIO-RAD) apparatus. The blot was incubated with the blocking buffer (5% non-fat dry milk in TBS) for 1 hr at room temperature on a shaker, followed by one wash with TBS buffer. The blot was then incubated with the primary antibody solution (GFP/LDH1/His antiserum, diluted to 1:500 in 2.5% non-fat dry milk in TBS) for over night on a shaker at 4°C. Blot was washed 3x5 minutes with TBST (TBS containing 0.1% Tween20) and incubated with anti-rabbit/goat IgG-HRP conjugate (diluted 1:10,000 in

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2.5% non-fat dry milk in TBS) for 1 hr at room temperature on a shaker. Finally, the blot was washed twice with TBST and the product was revealed using ChemiGlow West detection kit (Alpha Innotech).

2.3.12 Immunofluorescence assay

Host cells, HFF, were grown as monolayer in a culture plate with glass cover slips. Using the selection medium, these monolayers were infected with the following parasite strains i.e. negative control (PLKΔHX), transgenic control (mycHisGFP-HX) and the three test strains (5′UTRGFP-HX, 3′5′UTRGFP-HX, 3′UTRGFP-HX), and allowed to grow for 4-5 days. After aspirating off the culture media, the cells were washed thrice with Dulbeco's phosphate buffered saline (DPBS) and were fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes. Cells were permeabilized with 0.25% Triton X-100 in PBS for 10 minutes. After washing twice with PBS, the cells were blocked with 5% Bovine serum albumin (BSA) in PBS for an hour at room temperature (Yahiaoui et al., 1999). Cover slips were then incubated with the primary antibody (Goat GFP anti-serum diluted 1:200 Rockland Inc Gilbertsville, PA) in a humidity chamber for 2 hrs at room temperature. After three washes with PBS, cells were incubated with rabbit anti-goat IgG conjugated to rhodamine (diluted 1:300, Rockland Inc Gilbertsville, PA) in a dark humidity chamber for 1 hour and washed with the PBS again. For cyst staining, cells were incubated with *Dolichos biflorus* agglutinin conjugated to FITC (diluted 1:300, Sigma) for 1 hour. Cellular nuclei were stained by incubating the cells for 10 minutes with 4′, 6 diamidino-2-phenylindole (DAPI, Sigma), followed by three washes with PBS. Cover slips were placed carefully on the glass slides

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and were allowed to dry and finally overlaid with fluoromount and examined with a Leica DMIRB microscope. All images were taken with a cooled Q-Imaging CCD camera using Improvision Openlab software.

2.4 Plasmid construction and purification

For genetic studies in *T. gondii*, electroporation is the most efficient means of transformation. Parent plasmid pTUB8mycHis GFPHX (6418-bp) has GFP as a reporter gene. The sequence and map of this plasmid can be found in Appendix A. It has 3´UTR (untranslated region) of SAG1 (surface antigen) downstream GFP. Under the control of tubulin promoter it can produce GFP fused to Myc and 6xHis tags at the N-terminus. This plasmid carries an ampicillin resistant gene for bacterial selection and an expression cassette of hypoxanthine-xanthine-guanine phosphoribosyltransferase (HX) that allows the selection of stably transformed parasites when media is supplemented with the drug mycophenolic acid and xanthine as a substrate. Parent plasmid pTUB8mycHisGFP-HX was used to generate two sets of plasmids.

2.4.1 Construction of plasmid with GFP reporter gene

To investigate the role of 5´UTR and 3´UTR of LDH1 (lactate dehydrogenase) or either one of them, in translational suppression of LDH1 enzyme during bradyzoite stage, the parent plasmid was modified to produce three different plasmids as follows,

1. For the investigation of the role of 5´UTR of LDH1 alone, Tub 8 promoter was replaced by upstream sequence of LDH1 (626-bp) and the plasmid was named p5´UTRL1GFP-HX. This plasmid still has 3´UTR of SAG1 downstream GFP. The

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sequence and map of this plasmid can be found in Appendix A. The parent plasmid was digested by KpnI and EcoRI to remove the Tub8 promoter (495-bp) from the parent plasmid and then gel purified. EcoRI site was made blunt by treating with mung bean nuclease. In order to remove 5′PO$_4$ group from the linearized plasmid, it was treated with alkaline phosphatase to help in later ligation reaction. Again using genomic DNA as a template, the upstream region of LDH1 (626-bp) was amplified, and extracted from the gel as mentioned above. KpnI and HindIII restriction sites were introduced using specific nucleotide primers named as HindIII dws 600 LDH1 and KpnI ups 600 LDH1 respectively. The sequences of the primers are listed in Appendix A. Phosphorylation of the insert was done to help in ligation. HindIII site was made blunt by mung bean treatment. Phenol chloroform extraction and ethanol precipitation was done. This insert was ligated into the resulting plasmid p5UTRL1GFP-HX. Parasite strain generated from this plasmid was 5UTRGFP-HX

2. For the investigation of the role of 3′UTR of LDH1 alone, 3′UTR of SAG1 was replaced by downstream sequence of LDH1 and the plasmid was named p3′UTRL1GFP-HX. This plasmid is still under the influence of Tub8 promoter. The sequence and map of this plasmid can be found in Appendix A. The insert, downstream sequence of LDH1 (794-bp), was made by PCR from genomic DNA. PacI and BamHI restriction sites were introduced by specific nucleotide primers named BamHI 3UTR dws 78LDH1 and PacI 3UTR ups 780 LDH1. The sequences of the primers are listed in Appendix A. Restriction sites were introduced for later sticky end ligation reaction. PCR product was run on gel and 794-bp band was excised from the gel and purified using QIAEX®II Gel Extraction kit from QIAGEN. Parent plasmid was digested with BamHI

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and PacI enzymes. 3′UTR of SAG1 (320-bp) was removed from the parent plasmid. The digested plasmid was run on gel, and 6098-bp band was excised and gel purified as described above. Using purified plasmid, 794-bp insert was ligated in. This plasmid was named p3UTRL1GFP-HX. Parasite strain generated from this plasmid was 3UTRGFP-HX.

3. For the investigation of the role of both 3′and 5′UTR of LDH1, 3′UTR of SAG1 from plasmid 5′UTRGFP-HX was replaced by the downstream sequence of LDH1 and named p3′5′UTRL1GFP-HX. Using p3′UTRL1GFP-HX plasmid, Tub8 promoter (495-bp) was digested and removed using KpnI and EcoRI enzymes. EcoRI site was made blunt and the insert was ligated as described above. Parasite strain generated from this plasmid was 35UTRGFP-HX. The sequence and the map of this plasmid can be found in Appendix A.

Parent plasmid pTUB8mycHisGFP-HX served as a positive control. Gene bank accession numbers for LDH1 and LDH2 mRNA are U35118 and U23207 respectively.

2.4.2 Construction of plasmid with mycLDH1/LDH2 reporter gene

Using the same parent plasmid i.e. pTUB8mycHisGFP-HX, another set of plasmid was generated to investigate that if the above hypothesis is true (3′and 5′UTR of LDH1 do play a role in translational suppression) then a) can the removal of suppression (3′and 5′UTR of LDH1) in bradyzoites result in continuous LDH1 expression and b) can the overexpression of LDH2 in tachyzoites force encystation. Two following plasmids were generated from the same parent plasmid.

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1. Genomic DNA from *T.gondii* was used as a template for reverse transcription reaction to get complementary DNA (cDNA) and then PCR was done to amplify open reading frame (ORF) of LDH1 (990-bp). Specific primers were used to introduce NsiI and PacI sites and named as 3LDH PacI and 5LDHx NsiI respectively. Specific sequences of the primers are listed in Appendix A. The parent plasmid, pTUB8mycHisGFP-HX was digested with NsiI and PacI enzymes and GFP was removed. ORF of LDH1 was ligated in the plasmid by sticky end ligation. In this plasmid GFP reporter was replaced with open reading frame of LDH1 enzyme and the plasmid was named pmycLDH1-HX. The parasite strain generated from this plasmid was mycLDH1-HX.

2. The second plasmid was made in a similar fashion as the one described above. In this plasmid, however, ORF of LDH2 (982-bp) was ligated in the plasmid at NsiI and PacI sites. GFP reporter was replaced with open reading frame of LDH2 enzyme and the plasmid was named pmycLDH2-HX. The sequence and the map of this plasmid are listed in Appendix A. The parasite strain generated from this plasmid was mycLDH2-HX. Both of these plasmids are under the control of Tub8 promoter, have ten amino acid myc-tag, 8 x His tag, ampicillin resistance, and HX as selectable marker. The parent plasmid pTUB8mycHisGFP-HX served as a positive control again in this set of experiment.

2.5 Plasmid transformation into *E.coli*

2.5.1 Preparation of competent bacteria

XL$_1$BL *E.Coli* cells were grown on agar plate containing tetracycline (0.1g/l). A single colony was picked from the plate & inoculated into 5mls of LB with tetracycline

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and incubated overnight in the shaker incubator at 37 °C. Next day it was transferred to 500 ml LB and incubated for 3 hrs of vigorous shaking. The bacteria were then chilled in ice for 15 min and then pelleted at 5000 rpm for 15 min at 4°C. Pellet was resusupended in 100 ml of Cold sterile $CaCl_2$ (100mM) and then centrifuged again at 5000 rpm for 15 min at 4°C. The pellet was washed three times with 50ml of $CaCl_2$ solution and centrifuged as described. Finally the pellet was resuspended in 200mM $CaCl_2$ and 25% glycerol and then aliquoted into microfuge tubes and stored at -80°C for later use (Sambrook, 1989).

2.5.2 Transformation of competent bacteria

5µl of the ligation reaction product was mixed with 25 µl of freshly thawed competent *E.Coli* cells and incubated on ice for 20 min. The cell suspension was heat-shocked for 45 sec at 42°C and incubated on ice for another 2min. 400µl of LB broth was added to the cells and incubated for 30 min at 37°C with shaking. Cells were centrifuged at 1000 rpm for 30-45 sec. 300µl of supernatant was discarded and the remaining 100µl was used to spread on LB agar plate containing 0.1 g/l ampicillin. Plates were then incubated for overnight at 37°C (Sambrook, 1989).

2.5.3 Plasmid isolation from transformed bacteria

After the positive clones were identified, recombinant plasmids from transformed bacteria were isolated by plasmid mini-preparations (Sambrook, 1989). Confirmation of ligation was done by restriction mapping. Large scale plasmid preparation was performed

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using Gen Elute™ HP Plasmid Midiprep kit from sigma for later electroporation of plasmid into the parasites.

2.6 Generation of stable transgenic parasite lines

Two sets of transgenic parasite lines were generated using the above mentioned plasmids. In the first set, parent and three modified plasmids were electroporated into PLKΔHX strain. As a result, four transgenic parasite lines were generated to investigate the transcriptional regulation of LDH1 in tachyzoites and bradyzoites. In the second set, the parent plasmid and the two modified plasmids were electroporated into PLKΔHX strain. As a result, three transgenic parasite lines were generated to investigate the translational suppression of LDH2 in tachyzoites.

Freshly lysed out *Toxoplasma gondii* tachyzyotes from confluent HFF monolayer were scraped from culture plates, syringed through a 21-G needle to release the parasite from host parasitophorous vacuole, filtered through a 3 μm membrane to remove host cell debris and then sedimented by centrifugation at 2000-3000 rpm for 10 min at room temperature. The collected parasite pellet was washed with Dulbecco's phosphate buffered saline supplemented with 0.1 g/l Ca^{+2}(DPBS) prior to electroporation. The washed pellet was resuspended in 400 μl cytomix buffer (120mM KCl, 0.1mM $CaCl_2$, 10mM K_2HPO_4, $KH2PO_4$, pH 7.6, 2 mM EDTA, 5Mm $MgCl_2$) freshly supplemented with 5mM glutathione and 2 mM ATP. Electroporation makes the cells sensitive to the osmolarity and ionic composition of the medium. Use of potassium phosphate-based cytomix buffer resembles the ionic composition of the cytosol and help increasing the cell survival to a greater extent. 10μg of the purified Plasmid/transforming DNA was

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added to the parasite suspension in a 4mm sized electroporation cuvette. Using BTX model 600 Electro Cell Manipulator (Genetronics) (25Ω current, 1.8 kEV, 25uF), this mixture was subjected to electroporation and then incubated for 30 min at room temperature. The electroporated parasites were then inoculated to a fresh confluent HFF cells (Donald and Roos 1994). Stable populations were selected using 25μg/ml mycophenolic acid as an inhibitor to inosine monophosphate dehydrogenase (IMPDH) and 50μg/ml xanthine as a substrate. Both of these were added to the culture media (Donald and Roos 1998).

2.6.1 Differentiation of the parasites

HFF monolayers were infected with 4×10^4 of the transgenic parasites and allowed to grow in ED1 medium. After four hours, all the remaining extra cellular parasites were removed by aspirating off the ED1. Culture medium was replaced with RPMI 1640 medium, having 5% fetal bovine serum (FBS), 50mM HEPES, 20mg of antimycotic/L and with pH 8.2. Parasites were allowed to grow in low CO_2 environment (0.4%) for five days. RPMI medium lacks growth factors and has a high pH. This, along with low CO_2 induces switching of tachyzoites into bradyzoites.

2.7 Confirmation of transgene integration by PCR

Confirmation of clones with GFP reporter plasmids, and mycLDH1/LDH2 plasmids was done using PCR as described in general methods. PCR products were visualized in 1.5% agarose gel.

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2.7.1 Confirmation of the UTR clones

Different set of primers were designed for the confirmation of the clone. Primer set A has primers named 5PL1 and 3GFP1491 and was designed to amplify a 500-bp long DNA sequence, encompassing an area from upstream region of *LDH1* to GFP for the confirmation of 5'UTRGFP-HX and 3'5'UTRGFP-HX clones. The parent plasmid p5'UTRGFP-HX was used as a positive control. Primer set B consists of primers named 5GFP1667 and 3UTRL1 and was designed to amplify a 600-bp long DNA sequence that covers the area from GFP to downstream region of *LDH1* and was used for the confirmation of 3'UTRGFP-HX and 3'5'UTRGFP-HX clones. Primer set C, consisting of primers 5GFPmyc and 3GFP1940 was designed to amplify 700-bp band from GFP reporter. Parent plasmid p3'UTRGFP-HX was used as positive control. Wild type parasite strain i.e. PLKΔHX served as a negative control for all of these primer sets.

2.7.2 Confirmation of the mycLDH1/LDH2 clones

In order to confirm the transgenic LDH1 and to differentiate it from the native LDH1 different pairs of primers were designed. Primer set D, that has primers LDH-ORF sense and LDH-ORF antisense was designed to amplify a 300-bp band from the open reading frame (ORF) of the LDH1 that was ligated in place of GFP. The genomic *LDH1* of toxoplasma however, has an intron of approximately 537-bp size within its ORF. Primers could amplify a 300-bp band from transgenic *LDH1* or 837-bp band using genomic *LDH1* as their template. Similarly mycLDH2 clone confirmation was carried out using primer set E, having primers LDH2 forward and LDH2 reverse that amplifies a 480-bp band from the open reading frame of *LDH2* that was ligated in the parent plasmid

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in place of GFP. Again the genomic *LDH2* of toxoplasma has an intron of 587-bp size with in its ORF. The primers could amplify 480-bp band when transgenic *LDH2* was used as a template and a 1067-bp band when genomic *LDH2* was used as template. Sequences of these primers are listed in Appendix A.

2.7.3 mycLDH1/LDH2 clone confirmation by restriction digestion

In order to further confirm the transgenic LDH expression, restriction enzymes specific to *LDH1* and *LDH2* were used. PCR product (100μl) of transgenic *LDH1* and *LDH2* were first phenol: chloroform extracted and then ethanol precipitated. Respective DNA pellets were resuspended in 10 μl autoclave water and used for restriction digestion. Restriction digestion was performed in a 15μl total volume having 2μl DNA, 1.5μl of 10x enzyme buffer, 0.5μl enzyme and 11μl of sterile water. This mixture was incubated at 37°C for overnight and then visualized in 1.5% agarose gel. For the confirmation of mycLDH1 clone, HincII enzyme that cuts the ORF of *LDH1* was used, while PstI enzyme that cuts the ORF of *LDH2* was used for the confirmation of mycLDH2 clone.

2.8 Expression of reporter gene in *T.gondii*

Expression of GFP and mycLDH1/LDH2 were measured using western blot as described in general methods.

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2.8.1 Expression of GFP

In order to examine the expression of GFP gene products, a western blot analysis was performed using polyclonal antibodies, in wild type and transgenic parasite lines. Equal amounts of protein extracts from PLHΔHX, mycHisGFP-HX, 35UTRGFP-HX, 3UTRGFP-HX, and 5UTRGFP-HX were immunoblotted and incubated with affinity purified antibody specific to GFP raised in goat (1:500) Rockland Inc. Antigoat IgG-HRP conjugate was used in 1:1000 dilution. Proteins from host HFF cells were also loaded in equal amounts to eliminate the possibility of cross contamination. In order to compare GFP expression with native LDH1 expression, the same blot was stripped with 0.1 M Glycine. HCl buffer (pH 2.5-3), washed twice with TBST and a western blot was performed using rabbit antiLDH1serum (1:500) from Sigma. Anti-rabbit IgG-HRP conjugate (Sigma) was used as the secondary antibody.

2.8.2 Expression of mycLDH1/LDH2

Equal amounts of protein extracts from PLKΔHX, mycLDH1, mycLDH2 were immuno-blotted and incubated with affinity purified antibody specific to 6xHis tag (1:500) raised in rabbit (Rockland Inc). Anti rabbit-IgG-HRP conjugate (1:10,000) was used as the secondary antibody. Protein extracts from human foreskin fibroblasts (HFF) were also subjected to western blotting to make sure there is no cross contamination. Equivalent amounts of the same protein preparations were subjected to western blot analysis using antibody against LDH1 to ensure that comparable amounts of cell free lysates were loaded and probed.

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2.9 Localization of GFP

Subcellular localization and abundance of GFP was monitored by measuring immunofluorescence. Transgenic parasite lines as well as the wild type PLKΔHX were subjected to immunofluorescence assay using GFP-specific polyclonal antisera raised in goats (1:200) from Rockland Inc. Anti goat IgG-rhodamine conjugated was used to reveal the location of GFP. Nuclear staining was done using DAPI (1:15) which was used as an internal control.

2.9.1 GFP localization in tachyzoites

In order to estimate the abundance of GFP among three test transgenic parasite lines as compared to the transgenic positive control and the wild type, DAPI was used as an internal control. Intensities of rhodamine fluorescence signals and that of DAPI were quantified. The relative fluorescence was determined by the ratio of GFP signals with reference to DAPI signals.

2.9.2 GFP localization in bradyzoites

HFF monolayers were grown in plates with cover slips; parasites were inoculated and were allowed to invade for four hours in ED1 medium. After that ED1 was replaced with RPMI medium that lacks growth factors and have high pH and parasites were allowed to grow in low CO_2 environment for five days to induce switching to bradyzoites as described in general methods. Parasites reside within a cyst in bradyzoite stage. Fluorescein isothiocyanate (FITC)-conjugated *Dolichos biflorus* lectin (1:300) that specifically binds to the N-acetylglucosamine moiety within the cyst wall was used as a

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marker for cyst wall development (Tomavo, 2001). Localization and abundance of GFP was monitored, along with the presence of cyst wall, using GFP specific polyclonal antisera raised in goat. A rhodamine conjugated anti goat was used to reveal the signals of GFP as was done in tachyzoites. DAPI was used again as an internal control to stain the nuclei.

2.10 Growth assay

In order to assess the growth rate and infectivity of parasites, plaque assays were done. Freshly released parasites from wild type and all transgenic strains were stained with 0.4% Trypan blue and counted under microscope. Live parasites were able to pump out the blue dye and appeared transparent while dead parasite showed blue color. Confluent HFF monolayers in 35mm culture plates were inoculated with 4-5 x 10^4 parasites from control and test strains. Parasites were allowed to grow undisturbed in ED1 medium for 8-10 days and then media was aspirated off. The infected monolayer was rinsed with PBS, fixed with methanol (95%) for five minutes and then stained with 1x crystal violet (5x stock: 25g crystal violet in 250 ml methanol, volume made up to 1000ml with 1% ammonium oxalate). Monolayers were finally washed with PBS and air-dried. Parasite plaques appear as irregular clear areas against violet background of HFF monolayer.

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CHAPTER 3

RESULTS

In order to investigate the mechanism behind differential expression of *LDH1* and *LDH2* during stage conversion the study was divided into two parts. The first part was designed to elucidate the influence of 5′ upstream and 3′downstream sequence of *LDH1* on the expression of LDH1 using GFP as a reporter system. The experiments in the first part were aimed at understanding translational suppression of LDH1. The second part was aimed at the investigation on how the expression of LDH2 in the tachyzoites and that of LDH1 in bradyzoites, will influence the life cycle of *T.gondii*.

3.1 Translational suppression of *LDH1*

Four transgenic parasite lines were generated, as described in Materials and Methods section 2.4.1, for the investigation of the translational suppression of *LDH1* during stage conversion. These parasite strains were referred to as mycHisGFP-HX, 5′UTRGFP-HX, 3′5′UTRGFP-HX, and 3′UTRGFP-HX. The strain mycHisGFP-HX was used as a positive control while the remaining three were tested strains. Their names reflect the genetic material carried by them. For example, the strain 5′UTRGFP-HX has a 626-bp upstream region of *LDH1* to control the expression of reporter GFP and HXGPRT as a selectable marker. All parasite lines were generated using PLKΔHX. Thus PLKΔHX served as a negative control for the study.

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3.1.1 Confirmation of transgene integration

To determine that the GFP reporter system was integrated into the genome of the transgenic parasites, PCR analysis was performed using genomic DNA as starting material. In order to amplify a specific segment of the reporter system, several pairs of PCR primers were designed as shown in Figure 3.1 A. Using primer set A (Material and Methods 2.7.1) a 500-bp band was amplified from 5′UTRGFP-HX and 3′5′UTRGFP-HX as shown in left part of Figure 3.1 B. This band was not amplified when genomic DNA from PLKΔHX, mycGFP-HX or 3′UTRGFP-HX was used as template. This further confirmed the specificity of the primers for the clones. Parent plasmid p3′5′UTRGFP-HX was used as a positive control for PCR and gave a very bright prominent band as compared to the clonal parasite strains depending upon level of expression.

Similarly when the genomic DNA from all transgenic and wild type parasite strains were subjected to PCR amplification using primer set B (Materials and Methods 2.7.1) that uses GFP-3′UTR of LDH1 as a template, a 600-bp band was amplified from 3′5′UTRGFP-HXand 3′UTRGFP-HX as shown in right part of Figure 3.1 B. Again this band did not appear when the genomic DNA from wild type, mycHisGFP-HX, and 5′UTRGFP-HX were used as template again indicating the specificity of the primers. Parent plasmid p3′5′UTRGFP-HX was used as a positive control and produced a prominent band as compared to the parasite clones. Primer set C somehow did not produce any band when used with genomic DNA from transgenic strains, even though it amplified a 700-bp band from parent plasmid pTub8mycHisGFP-HX data not shown here.

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Figure 3.1

Confirmation of transgene integration

Legend

A. Schematic presentation of the primer design. Primer set A has 5′ and 3′ primers named 5PL1 and 3GFP1491 that were designed to amplify a 500-bp band from DNA segment encompassing an area from 5′UTRLDH1 to GFP. This set of primers was used for the confirmation of upstream region of the clones. Primer set B has 5′ and 3′ primers named 5GFP1667 and 3UTRL1 and were designed to amplify a 600-bp band using DNA segment from GFP to 3′UTRLDH1 as their template. This set of primer was used for the confirmation of down stream region of the clones. Primer set C, with the primers 5GFPMYC and 3GFP1940, was designed to amplify 700-bp is also shown.

B. Image of an agarose gel that resolved the PCR product. The set of primers are labeled on top of the name of the parasite strain tested. The expected band migrated at ~500-bp when primer set A was used and ~600-bp when primer set B was used. Genomic DNA was used as a template from wild type (PLKΔHX) and transgenic strains i.e. mycHisGFP-HX (GFP), 5′UTRGFP-HX (5H), 3′5′UTRGFP-HX (35C) and 3′UTRGFP-HX (3B) respectively. Parent plasmid p3′5′UTRGFP-HX (p35GFP) was used as a positive control for the PCR for both primer sets.

48

Figure 3.1

A.

B.

49
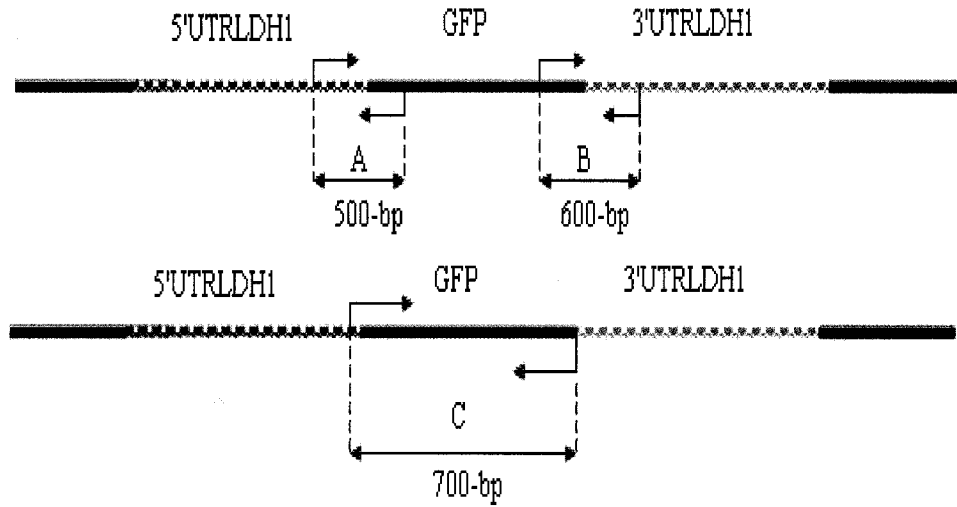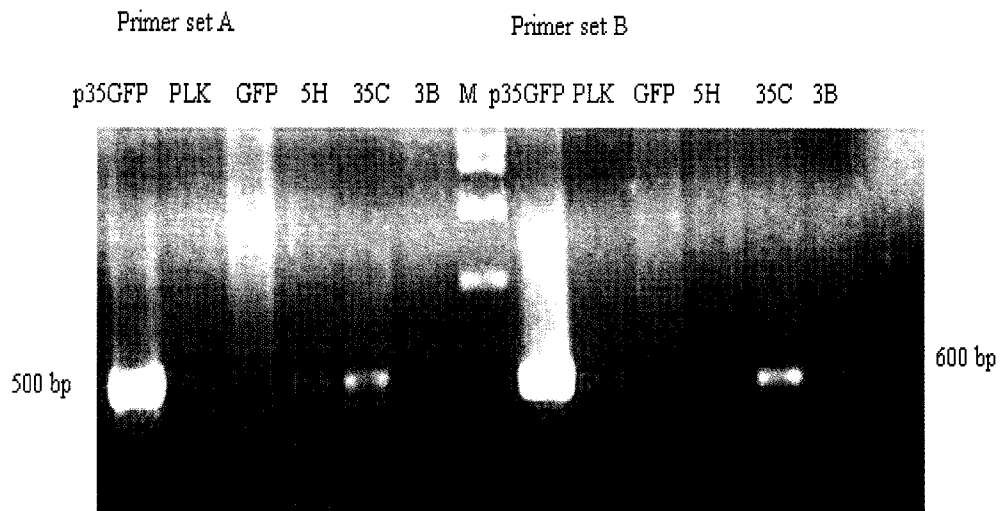
3.1.2 Expression profile of GFP under the control of upstream and downstream regions of *LDH1*

In order to evaluate whether the GFP reporter system is being expressed under the control of LDH1 upstream and downstream sequences, a western blot analysis was performed using polyclonal antibody raised against GFP. Protein lysates were prepared from wild type PLHΔHX (negative control), and transgenic control (mycHisGFP-HX) and transgenic tests (5′UTRGFP-HX, 3′5′UTRGFP-HX, and 3′UTRGFP-HX). Equal amounts of protein extracts from each strain were analyzed by immunobloting and incubated with affinity purified antibody specific to GFP. Another parasite strain (HEDL), which constitutively expresses GFP fusion protein was used as positive control in early experiments (Figure 3.2 A). Low GFP expression was observed in all transgenic tests as compared to that of transgenic control (Tub8mycHisGFP-HX). Negative control PLHΔHX did not show any GFP band, as expected. In addition, no band was detected in protein samples from HFF host cells as shown in Figure 3.2 B. Size of the GFP band showed approximately at 57 kDa while the expected GFP band has 35 kDa molecular weight.

In order to compare GFP expression with native LDH1, the same blot was stripped and western blot was performed using antibody against LDH1. LDH1 with a molecular weight of 35 kDa gave very prominent band in each parasite strain tested indicating that the amount of protein loaded was equal in each lane and the difference in GFP expression observed in transgenic tests could be due to the difference in promoter and downstream sequences.

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Figure 3.2

Expression profile of GFP under upstream and downstream region of *LDH1*

Legends

Images of three different western blot analyses are shown.

A. Upper panel shows GFP expression performed on wild type (PLHΔHX), transgenic positive control i.e. mycHisGFP-HX (GFP), test strain 35UTRGFP-HX (35C) and another parasite strain HEDL that expresses a GFP fusion protein and was used as positive control for the experiment. HEDL and transgenic control mycHisGFP-HX showed prominent bands. Transgenic test, 35C however did not show any band. Lower panel is showing the expression of LDH1 in the same strains when equal amount (20μg) of protein was probed with anti LDH1 antibody. All the strains gave strong LDH1 bands at 37kDa.

B. Upper panel shows GFP band appeared at 57 kDa when negative control (PLKΔHX), transgenic positive control mycHisGFP-HX (GFP), and three transgenic test strains 5UTRGFP-HX (5H), 35UTRGFP-HX (35C), and 3UTRGFP-HX (3B) were probed with anti-GFP antibody. Protein lysates from host HFF were also subjected to western blot analysis to rule out the possibility of antibody cross reaction with host proteins. Transgenic positive control gave very prominent bands as compare to the three transgenic test strains. Negative control and HFF did not show any bands. Lower panel shows LDH1 expression in all the strains tested.

Figure 3.2

A.

HEDL 35C GFP PLKΔHX

GFP ————————————————— 57kDa

LDH1 ————————————————— 37kDa

B.

3B 35C 5H GFP HFF PLKdHX

GFP ————————————————— 57kDa

LDH1 ————————————————— 35kDa

52
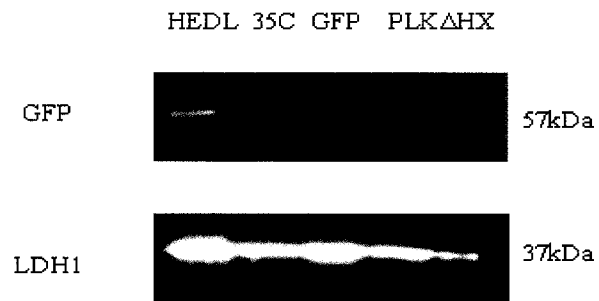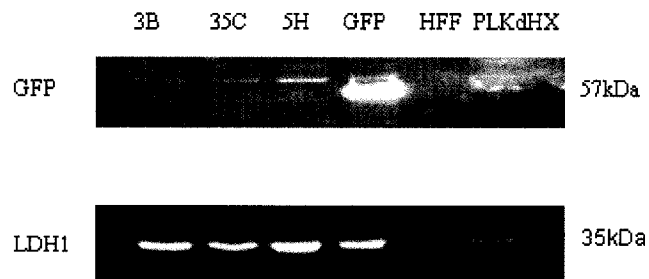
3.1.3 mRNA levels

In order to determine the level of mRNA among transgenic test strains and to compare it with the wild type, RNA was isolated from wild type and transgenic strains and reverse transcription was performed. In order to check the quality of cDNA, the resulting RT product from transgenic control mycHisGFP-HX (GFP) was subjected to PCR using primer set C and another primer set LDH (sense and antisense). Primer set LDH was able to amplify DNA from the complimentary DNA of mycHisGFP-HX (GFP) while primer set C did not produce any band from the same cDNA template. Plasmid pmycLDH1(pmycL1) was used as a positive control for the PCR reaction using the same primer LDH. Several attempts were made but did not work. Another primer set 5′T7GFPmut2 and 3′T7GFPmut was also tested with the genomic DNA from wild type and transgenic tests, but a non specific band appeared (data not shown here). This band appeared with genomic DNA from RHΔHX also. Thus primer set C could not be used for PCR from cDNA of transgenic Control.

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Figure 3.3

Measurement of mRNA level

Legend

Image of an agarose gel showing RT-PCR from wild type (PLKΔHX), mycHisGFP-HX (GFP), and 3UTRGFP-HX (3B) strains. Primer set C and primer LDH were used. Primer set LDH successfully amplified 300-bp band from mycHisGFP-HX cDNA. No product was made using primer set C.

3.1.4 Localization of GFP gene product

In order to determine whether the GFP reporter was expressed in a similar fashion as that of LDH1, levels of GFP expression were monitored by immunofluorescence and its subcellular localization was determined using immunolocalization in both stages of parasite life cycle (Figure 3.4). Transgenic parasite strains, as well as the wild type (PLKΔHX) were subjected to immunofluorescence assay using GFP-specific polyclonal antisera raised in goats. A rhodamine conjugated secondary antibody was used to reveal the abundance and location of GFP since a direct green fluorescence from the transgenic strains could not be detected. Nuclear staining was performed using DAPI. When merged to DAPI images, Rhodamine signals were limited to cytoplasm indicating that GFP has a cytoplasmic localization.

3.1.4.1 GFP localization in tachyzoites

In order to estimate the abundance of GFP among the three test transgenic parasite lines as compared to the transgenic positive control and that of wild type, DAPI was used as an internal control. Intensities of rhodamine fluorescence signals and that of DAPI were quantified. The relative fluorescence was determined by the ratio of GFP signals with reference to DAPI signals. The positive control and the three test strains showed GFP in more or less same amount. The negative control showed some background noise signals.

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Figure 3.4

Localization of GFP in Tachyzoites

Legend

A. Immunofluorescence assay performed with transgenic and wild type parasites in tachyzoite stage. Phase contrast micrographs are shown in upper panel and those of immunofluorescence labeling in the lower panels. DAPI was used to label nucleus of human foreskin fibroblasts and of parasites. Rhodamine-labeled sheep anti-goat IgG was used to reveal the immunoreactive protein recognized by anti-GFP. All transgenic strains showed cytoplasm localization of GFP when compared with nuclear staining by DAPI.

B. GFP fluorescence measured and recorded by the software provided with the microscope. GFP levels were compared with that of DAPI as an internal control is shown in graphical form. Level of GFP expression is approximately the same in all transgenic strains. The negative control also showed some noise signals. The error bars are representing standard deviation.

56

Figure 3.4

A.

B.

57
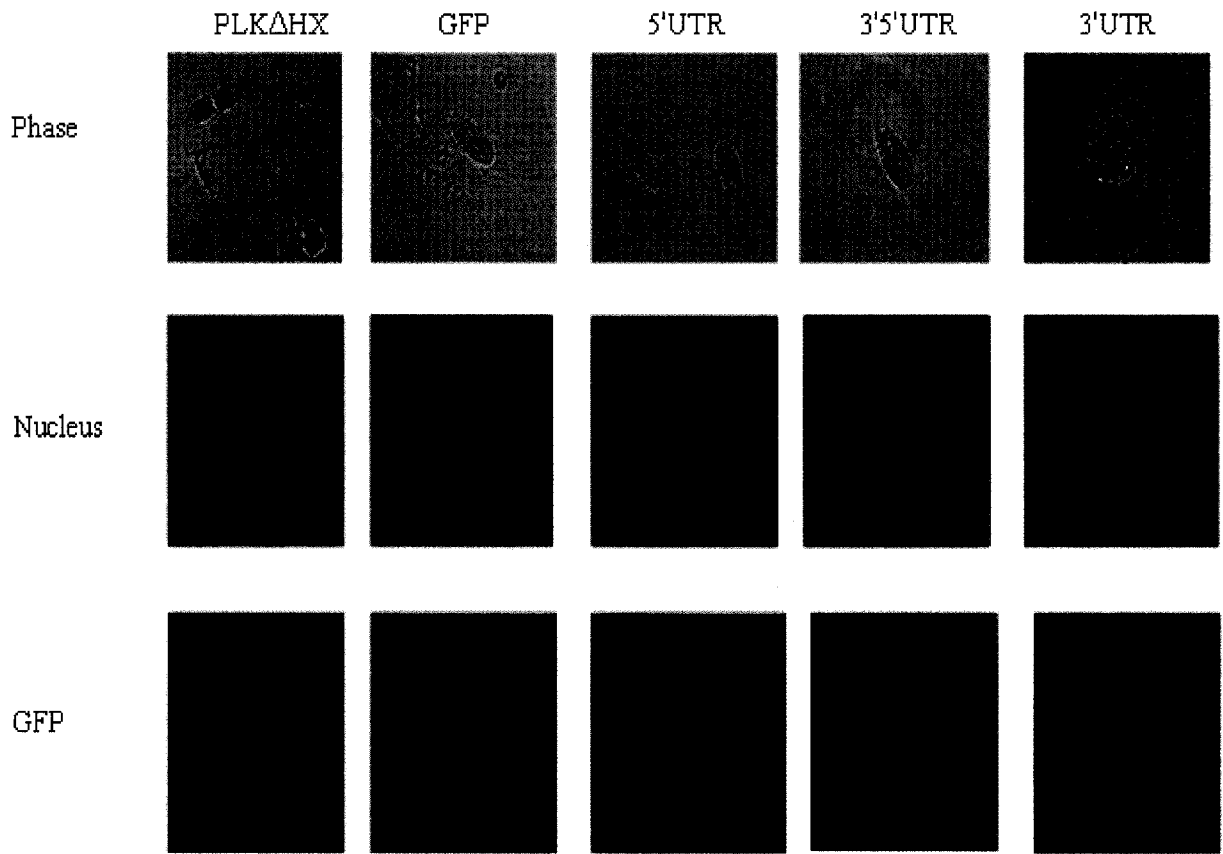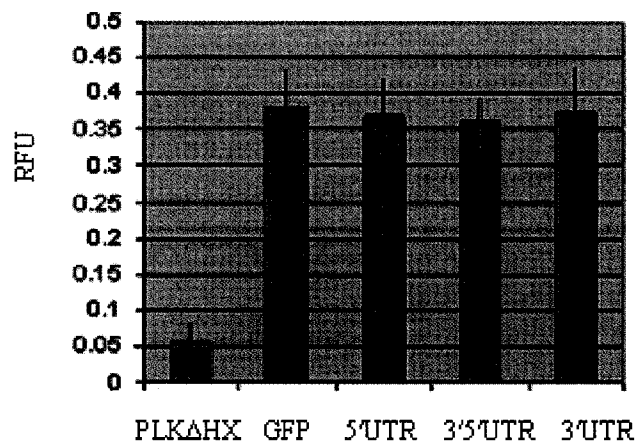
3.1.4.2 GFP localization in bradyzoites

In order to determine the effect of upstream and downstream regions of *LDH1* on GFP expression during bradyzoite stage, the tachyzoites were switched to bradyzoite. The differentiation was done by changing ED1 medium to RPMI and growing the parasites in low CO_2 environment. Once the parasite switches to bradyzoites they form a cyst around them and the rate of parasite replication slows down. Parasite metabolism changes with the differentiation and instead of LDH1, the parasite now expresses LDH2, even though the mRNA of LDH1 is reported to be found in both stages. Fluorescein isothiocyanate (FITC)-conjugated *Dolichos biflorus* lectin that specifically binds to the N-acetylgalactosamine moiety within the cyst wall was used as a marker for cyst wall development (Tomavo, 2001). FITC conjugated *Dolichos biflorus* lectin was used to stain the cyst wall and as an indicator of bradyzoite differentiation. Rhodamine-labeled sheep anti-goat IgG was used to reveal the immunoreactive protein recognized by anti-GFP. Localization and abundance of GFP was monitored, along with the presence of cyst wall. DAPI was used again as an internal control to stain the nuclei (Figure 3.5). GFP levels were found decreased with the bradyzoite differentiation.

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Figure 3.5

Localization of GFP in bradyzoites

Legend

A. Immunofluorescence assay performed with PLKΔHX and all the transgenic strains. Phase contrast micrographs are shown in upper panel and those of immunofluorescence labeling in the lower panels. Nuclear staining was done by DAPI, shown in panel 2, and was used as internal control for GFP expression and cyst wall staining. Cyst wall development was measured and shown in panel 3. GFP localization and abundance was measured by the rhodamine conjugated anti-GFP and shown in the last panel. Transgenic strains showed a lowered level of GFP expression but comparatively high levels of N-acetylgalactoseamine that served as an indicator of cyst wall development. Rhodamine fluorescence as a measure of GFP expression and FITC fluorescence as a measure of N-acetylgalactoseamine expression were compared with that of DAPI as an internal control to calculate the relative fluorescence units (RFU). Fluorescence was measured and recorded by the software provided with the microscope. Level of GFP expression was approximately the same in all transgenic strains and was much lower when compared to the N-acetylgalactoseamine expression.

B.. Comparison of GFP levels (RFU values) in both stages is shown in graphical form. GFP levels were decreased significantly in all transgenic strains when tachyzoites were switched to bradyzoites. The negative control (PLKΔHX) also showed noise signals for GFP expression. Error bars are showing standard deviation.

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Figure 3.5

A.

	PLKΔHX	GFP	5'UTR	3'5'UTR	3'UTR
Phase					
Nucleus					
Cyst-Wall					
GFP					

B.

GFP levels in Tachyzoites and Bradyzoites

■ GFP in Tachy
■ GFP in Brady

60
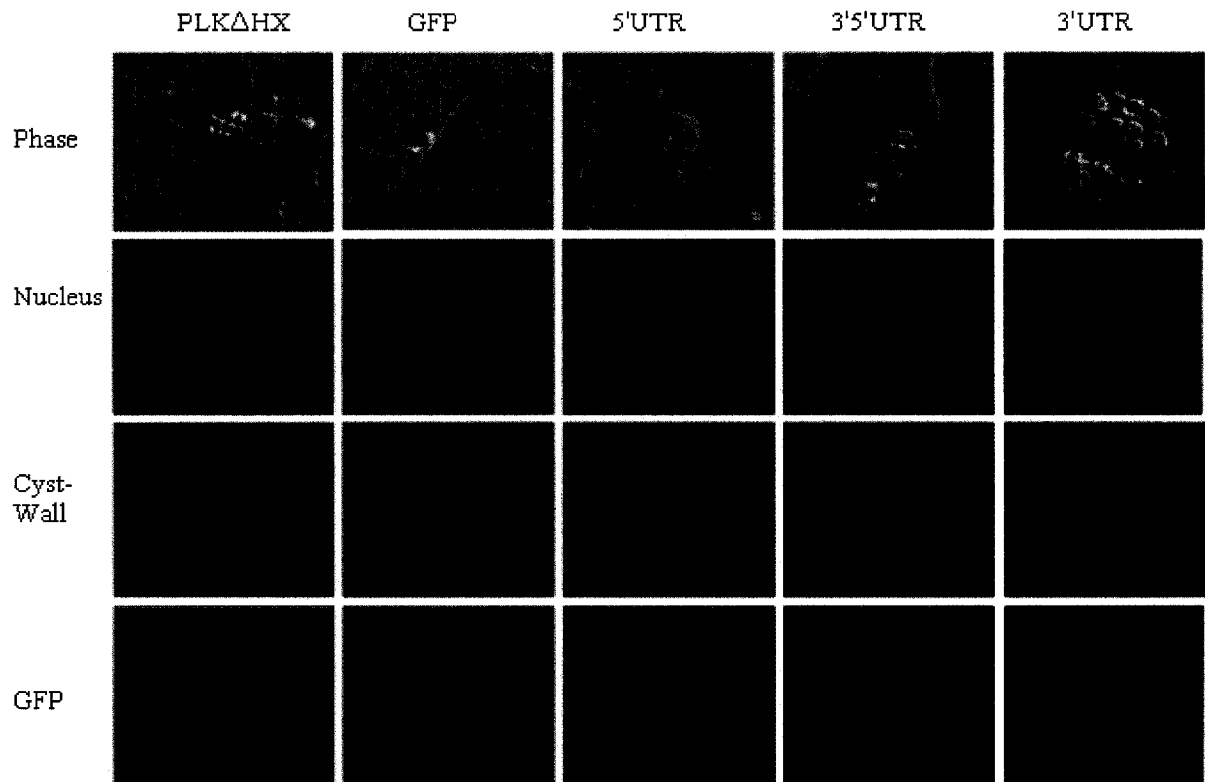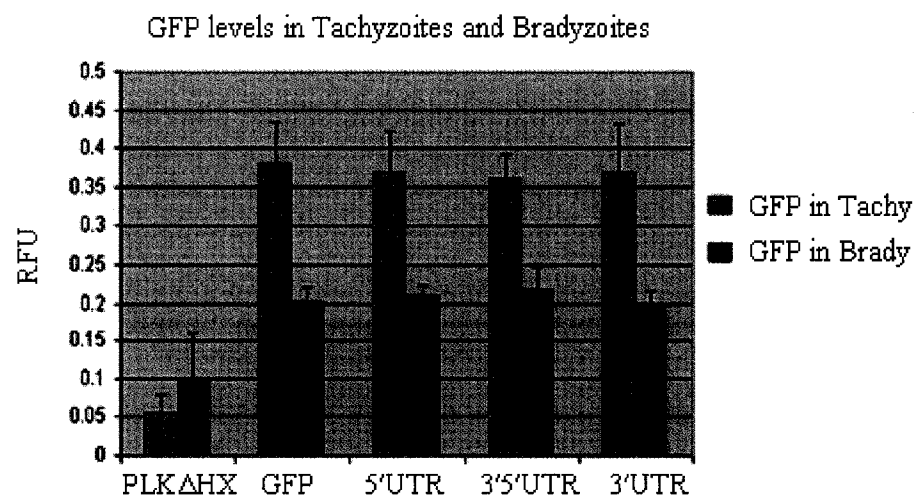
3.1.5 Growth Assay:

In order to determine the effect of plasmid insertion on parasite growth, infectivity and growth of transgenic parasite strains was determined by performing plaque assay. Equal number of parasites ($4\text{-}5 \times 10^4$) was inoculated and allowed to grow undisturbed for 8-10 days. Then number of plaques formed were counted and are shown in Figure 3.6. Since parasite invasion is coupled with its infectivity, the number of plaques formed is taken as a measure of parasite growth.

Figure 3.6

Measurement of growth

Legend

Number of plaques formed by each parasite strain is shown in graphical form. Wild type and transgenic strains were allowed to grow for 8-10 days. Growth rate is almost similar in all strains showing that plasmid insertion did not affect the growth rate of the parasites. The error bars represent standard deviation.

Growth Assay

Figure 3.6

62
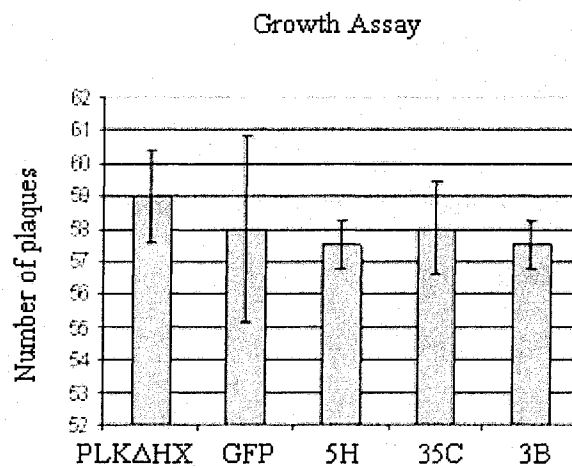
3.2 Transcriptional regulation of *LDH2*

Two transgenic parasite lines were generated to study transcriptional regulation of *LDH2* in tachyzoites. These transgenic strains were named mycLDH1-HX and mycLDH2-HX respectively. Parasite strain mycHisGFP-HX served as negative control for the study.

3.2.1 Confirmation of transgene integration

To determine that the LDH1/LDH2 was integrated into the genome of the transgenic parasites, PCR analysis was performed using genomic DNA as the starting material. In order to amplify a specific segment of the reporter system, different pair of PCR primers were designed (Figure 3.7 A). The parasite has genomic LDH1 and LDH2 also, but both of them have an intron at same relative position and approximately of the same size i.e. 538·nucleotides in LDH1 and 588 nucleotides in LDH2. This fact was exploited to differentiate between transgenic and native LDH1 and LDH2 expression. In order to confirm the transgenic LDH1 expression and differentiate it from the native LDH1, primer set D was used. Transgenic parasite line produced both 300 and 837-bp band when subjected to PCR amplification. PLKΔHX and mycGFP-HX could only give 837-bp band but not the 300-bp band, which further confirms the specificity of the primers (Figure 3.7 B).

For the confirmation of mycLDH2 clones, primer set E was used that amplifies a 480-bp band from the ORF of transgenic LDH2. The two transgenic test strains have both genomic and transgenic LDH2 and produced both the bands when subjected to PCR amplification. On the other hand, PLKΔHX, being a negative control could amplify only

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1067-bp band but not the 480-bp band further confirming the specificity of these primers. Plasmids pmycLDH1 and pmycLDH2 were used as a positive control and gave very intense bands when used with primer set for LDH1 and LDH2 respectively (Figure 3.7 B).

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Figure 3.7

Confirmation of transgene integration

Legend

A. Schematic presentation of primer design. Two different set of primers were designed for clone confirmation. Primer set D was designed to synthesize a 300-bp band using LDH1 ORF as template as shown below. Primer set E was designed to amplify a 480-bp band using LDH2 ORF as template.

B. Image of an agarose gel resolving PCR product. The set of primers are labeled under the name of the parasite strain tested. The expected band migrated at 837-bp in all the strains and 300-bp migrated only in transgenic mycLDH1 strain, when primer set D was used. Similarly the expected band migrated at 1067-bp in all strains and 480-bp band migrated only in transgenic mycLDH2 strain when primer set E was used. Plasmid pmycLDH1-HX (pL1) and pmycLDH2-HX (pL2) were used as positive control for the PCR for primer set D and E respectively. Genomic DNA amplification from wild type PLKΔHX, transgenic control mycGFP-HX (GFP) and transgenic test strains mycLDH1-HX (L1C) and mycLDH2-HX (L2B) are shown in order.

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Figure 3.7

A.

Primer set D

300-bp

TUB8promoter   LDH1 ORF (987 nt)   3UTRSAG1

Primer set E

480-bp

TUB8promoter   LDH2 ORF (978 nt)   3UTRSAG1

B.

Plasmid   Clonal parasite

pL1 pL2 PLKΔHX GFP   L1C   L2B   M

Primer set → D E   D E   D E   D E   D E

480 bp
300bp

DNA amplified from,

◊ genomic LDH2

* genomic LDH1

Δ transgenic LDH1

° transgenic LDH2

66
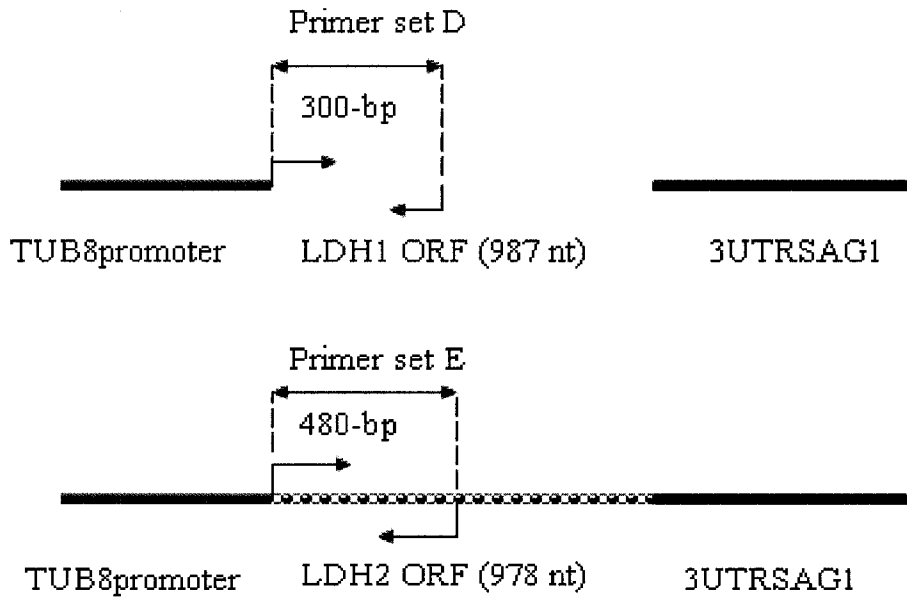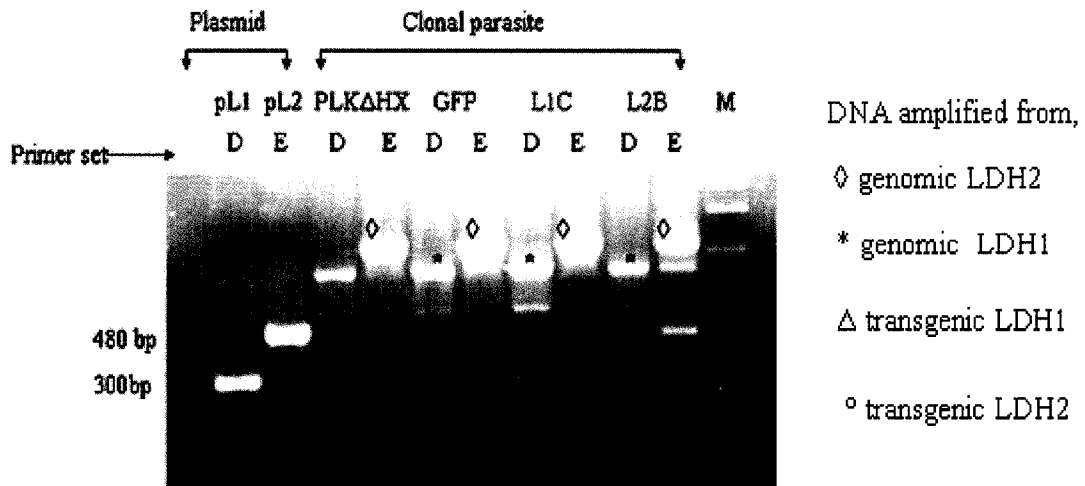
3.2.2 Confirmation of transgene by restriction digestion

In order to further confirm the transgenic LDH expression, restriction enzymes were used. PCR product from transgenic strains and that of wild type were first phenol: chloroform extracted and then ethanol precipitated. The respective DNA pellets were resuspended in water and used for later digestion reaction.

3.2.2.1 Confirmation of mycLDH1

For the confirmation of mycLDH1, HincII enzyme was used that cuts the 300-bp band amplified from transgenic *LDH1* at the 180th nucleotide, resulting into two bands of 180 and 127-bp. HincII cuts at nucleotide 180 cleaving the 837-bp band amplified from genomic *LDH1* into 180 and 657 nucleotide bands. Transgenic strain, mycLDH1 shows both 180-bp and 657-bp bands confirming the presence of transgenic LDH1 in parasites. PLKΔHX showed only the 657-bp band but not the 180 bp band.

Figure 3.8

mycLDH1 Clone confirmation by restriction digestion

Legend

HincII-digested (D) and undigested (U) samples are shown from the parent plasmid pmycLDH1 (pL1), transgenic parasite strain mycLDH1 (mycL1C) and the negative control (PLKΔHX). Digestion of 837 nucleotide DNA strand from genomic *LDH1* resulted into 180 and 657 nucleotide bands in both the negative control and the transgenic strain. However, 300-bp from transgenic *LDH1* was digested into two bands of 180 and 127 nucleotides transgenic strain.

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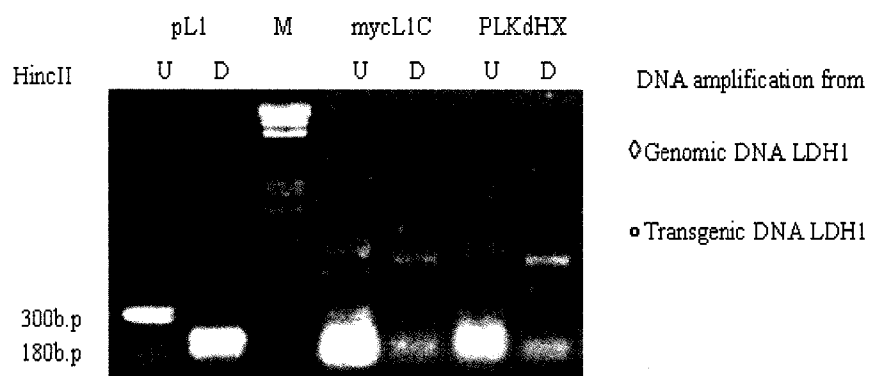
3.2.2.2 Confirmation of mycLDH2

PstI enzyme was used for the further confirmation of mycLDH2 expression. PstI cuts the 500-bp transgenic *LDH2* at nucleotide 252 cleaving it into two bands of approximately 250-bp size, while the 1087 nucleotide long native *LDH2* is cleaved into 252 nt and 835 nt long DNA strands. PCR product from PLKΔHX and mycLDH2-HX, using primer set E, was subjected to PstI digestion. 1087-bp native LDH2 from both wild type and transgenic strain was cut into 835 and 252bp band. PstI cut 500-bp transgenic LDH2 into two bands of approximately 250-bp size. PCR product from parent plasmid pmycLDH2 (pL2) was used as positive control for the digestion reaction and 500-bp band was cut into 250-bp band as shown in Figure 3.8.

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Figure 3.9

mycLDH2 clone confirmation by restriction digestion

Legend

PstI-digested (D) and undigested (U) samples are shown under the name of the parasite strains i.e. wild type (PLKΔHX) and transgenic strain mycLDH2-HX (mycL2B). 1087-bp native LDH2 band from both wild type and transgenic strain was digested by PstI and the resulting bands migrated at 835 and 250-bp. While 500-bp transgenic LDH2 band was digested into 250-bp band. 500-bp PCR product from parent plasmid (pL1) was also digested into 250-bp band. PCR product from the parent plasmid pmycLDH2-HX (pL2) served as positive control for the digestion reaction.

	pL2		PLKdHX		M	mycL2B		DNA amplification from
PstI	U	D	U	D		U	D	◊ Genomic LDH2
								○ Transgenic LDH2
500 b.p								
250 b.p								

70
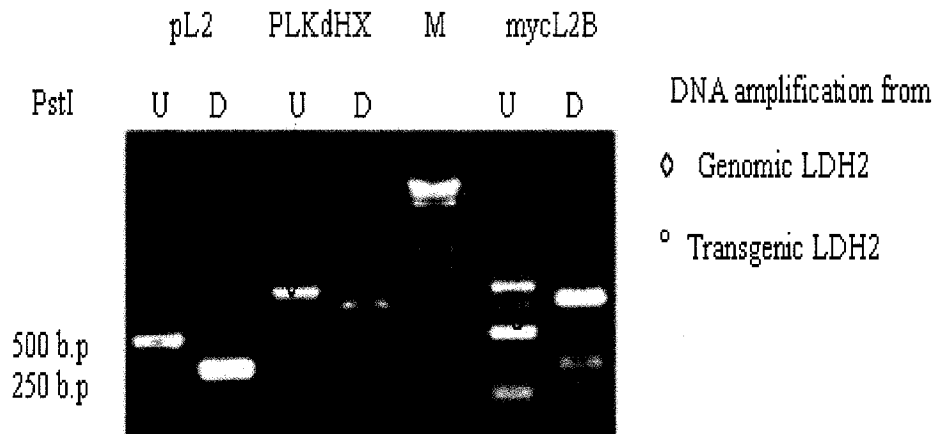
3.2.3 Expression profile of mycLDH1

In order to examine the expression of the mycLDH1 gene product, western blot analysis was performed. Equal amounts of protein extracts from PLKΔHX, mycLDH1, and mycLDH2 were immuno-blotted and incubated with affinity purified antibody specific to 6xHis tag. Protein extracts from human foreskin fibroblasts (HFF) were also subjected to western blotting to rule out the possibility of cross contamination. A strong band was detected in mycLDH1 as compared to mycLDH2 at approximately 37kDa. However, when the same experiment was repeated after several passages, did not show this band using myc-antibody.

Equivalent amounts (10-15µg) of the same protein preparations were subjected to western blot analysis using antibody against LDH1 to ensure that comparable amounts of cell free lysates were loaded and probed. All of them gave the same intensity band for LDH1 at 37kDa.

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Figure 3.10

mycLDH1 expression

Legend

Images of three different western blots analyses are shown.

A. upper panel shows western blot of the transgenic and wild type parasites using anti-His antibody. Two different amounts of mycLDH1 were loaded. A strong band appeared at 37kDa for mycLDH1 strain while a faint band appeared in mycL2 strain. The band appeared in PLKΔHX was due to overflow of mycL1 sample in the adjacent well. The protein lysates from host HFF did not show any band. The lower panel shows the expression of LDH1 in all these strains when equal amount of protein was loaded and probed with anti-LDH1. Equal intensity bands appeared at 37 kDa. No band was detected in HFF using either antibody.

B. Western blot that was repeated after several parasite passages (15-20) using anti-His antibody. No signals were revealed, however when the same blot was stripped and reprobed with anti-LDH1 antibody gave prominent bands at 37 kDa showing LDH expression.

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Figure 3.10

A.

mycL2   M   mycL1 mycL1 PLKdHX HFF

His   37 kDa

LDH1   37 kDa

B.

mycL2 mycL1   PLKΔHX   HFF

His   37 kDa

LDH1   37 kDa

73
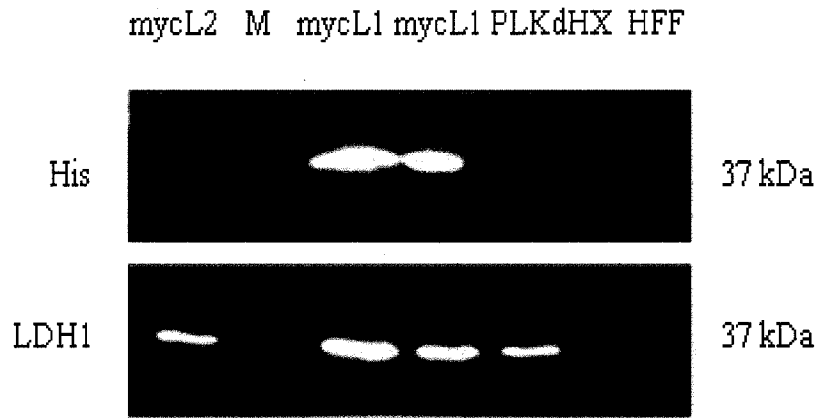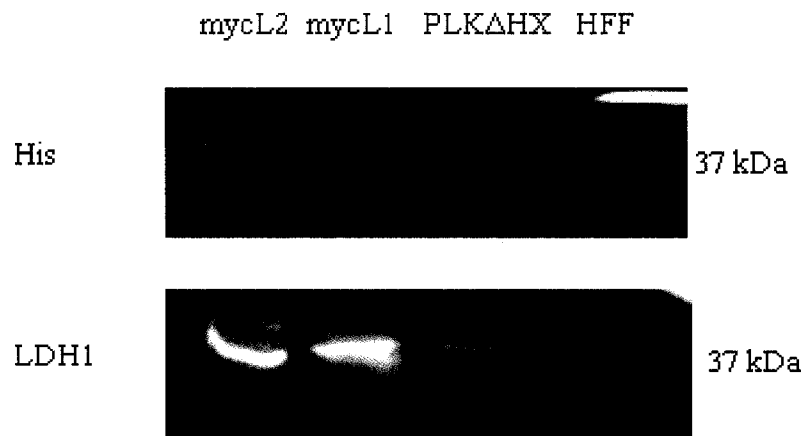
CHAPTER 4

DISCUSSION

The differentiation between actively replicating tachyzoites and slowly dividing/ encysted bradyzoites plays an important role in the pathogenesis of *T. gondii,* especially in patients with lowered immunity. From the previous studies it has been established that *T. gondii* expresses two distinct lactate dehydrogenases (LDH) at different stages: LDH1 during tachyzoite and LDH2 during bradyzoite stage (Yang and Parmley, 1997). Mechanism of distinct expression is not well understood, however. LDH is an important glycolytic enzyme which catalyzes the interconversion of lactate and pyruate using NADH as a coenzyme. Under anaerobic conditions when glycolysis becomes the only pathway to generate energy, LDH plays an essential role. Yang and Parmely (1997) have clearly shown that mRNA for *LDH1* is present in both stages but translated only in tachyzoites not in the bradyzoites, indicating a translational suppression of LDH1 in bradyzoites. On the contrary, mRNA for *LDH2* is present only in bradyzoites but not in tachyzoites suggesting a transcriptional regulation of *LDH2* during this stage. LDH2 mRNA stability was not measured in this study, hence it is not clear whether high mRNA levels are due to an increase in mRNA stability or because of transcriptional activation of *LDH2* gene. Using a CAT reporter with the putative promoter sequences of *LDH1* and *LDH2* they also demonstrated that in the tachyzoites, CAT expression under *LDH1* promoter sequence was high and reduced four fold with the differentiation to bradyzoites. On the other hand no CAT activity was found in tachyzoites when CAT expression was under *LDH2* promoter control. However, it increased to forty-five fold upon switching to

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bradyzoites. Studies done in other eukaryotic systems also suggest a possible role of untranslated regions in gene expression (Park *et al.*, 2001). Regulatory activities of 5′ and 3′ untranslated regions have been reported in human aggrecan gene where untranslated regions exerted differential effects on two different types of promoters (Wilmot *et al.*, 1998).

4.1 Translational suppression of LDH1

The expression of GFP under the control of upstream and downstream sequence of *LDH1* was determined by western blot analysis. GFP was tagged with ten amino acids of myc-epitope and 8x His tag at its amino terminus. Anti-myc antibody and anti-His antibody were used in determining GFP expression but signals were not detected (data are not shown). A possible reason could be that these short amino acid tags were masked by the folding of GFP during translation. However, GFP was detected using the anti-GFP antibody, even though the signals were not very strong, especially in transgenic strains, as compared to the transgenic control line, mycHisGFP-HX (Figure 3.2). This could be due to the low abundance of GFP. The observed low level of GFP could be due to the difference in the controlling promoter. *TUB8* promoter is a stronger promoter than the upstream region of LDH1 used in 5UTRGFP-HX and 35UTRGFP-HX. Moreover, the expression of GFP in the control line could also be controlled by the 3′-UTR isolated from the major surface antigen (SAG1). 35UTRGFP-HX and 3UTRGFP-HX that has the downstream sequence of LDH1 also showed low levels of GFP. Attempts were made to determine the levels of GFP mRNA by RT-PCR, however these were unsuccessful in obtaining any PCR product using primer set C, even though other primer sets worked

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with the cDNA from RT reaction, for example primer set D (Figure 3.3). The primer set

C (Material and Method, section 2.7.1) was aimed to amplify whole GFP including myc-

tag. This primer set did not give any PCR product when it was used with genomic DNA

of transgenic parasites also. A new set of primer should be made aiming to amplify first

the 350 nt from GFP starting from 1243 nt to 1600 nt in the parent plasmid and can be

used for both amplifying GFP from genomic DNA and measuring mRNA level from

cDNA. Northern blot analysis can also be used to determine the mRNA levels of GFP,

but it requires more starting materials, could be less sensitive than RT-PCR and slight

degradation of RNA sample would greatly affect the ability to quantitate the mRNA

expression. Without measuring mRNA level, it is not clear if the lowered GFP expression

is due to alteration in mRNA stability and subsequent translation. The expected size of

the band was 37 kDa, while the GFP band was detected at approximately at 57 kDa. This

band can not be regarded as false positive because it was visible when run along with the

protein lysates from HEDL strain which expresses a GFP fusion protein. The difference

in the molecular weight could be due to the readthrough translation of mRNA. Stop

codon in Colorado tick fever virus (CTFV) has been reported to be a "leaky terminator"

allowing readthrough translation. As a result truncated termination protein and full length

readthrough protein are made and serve as structural and non-structural component of the

virus respectively (Fauziah et al., 2004). There are three stop codons in 3'UTR of SAG1

at nucleotide position 72, 81 and 135 that could give three different proteins with

molecular weights 48, 49 and 57 kDa respectively in mycGFP and 5'UTRGFP-HX

strains. There are four stop codons in 3'UTRLDH1 at nucleotide positions 216, 411, 651

and 663 which could give three different proteins with the molecular weight 48, 58, and

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70 kDa respectively in 3´UTRGFP-HX and 3´5´UTGFP-HX transgenic strains. There are different possibilities in case the readthrough occur but why 57 kDa protein appeared in particular is debatable.

GFP localization was found to be cytosolic in all transgenic strains, which is in accordance with the endogenous LDH that is a cytosolic enzyme. Levels of GFP expression among transgenic and control strains during tachyzoite stage were not significantly different, even though the control strain showed the highest expression. The detection of GFP expression was observed to be better using IFA analysis as compared to that of western blot analysis. This could be due to the high sensitivity of detection of IFA technique. Negligible amount of noise signals were detected in wild type which could be due to the variation in signal recording. The transgenic parasite strains, their respective upstream and down stream sequences and the resulting GFP expression and localization are shown in tabular on next page. Up arrow indicates an increase and down arrow indicates the decrease in intensity of expression of GFP by the detection method used.

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Parasite strain	Controlling elements		Detection methods		
	Upstream	Downstream	WB	IFA (Tachy)	IFA (Brady)
mycGFP-HX	TUB8 promoter	3UTR SAG1	↑↑↑	↑↑↑↑	↓↓
3UTRGFP-HX	TUB8 promoter	Downstream (794-bp) sequence of *LDH1*	↑	↑↑↑	↓↓
5UTRGFP-HX	Upstream sequence (626-bp) of *LDH1*	3UTR SAG1	↑↑	↑↑↑	↓↓
35UTRGFP-HX	Upstream sequence (626-bp) of *LDH1*	Downstream (794-bp) sequence of *LDH1*	↑↑	↑↑↑	↓↓

GFP expression and localization during tachyzoite stage is the highest in the control where the controlling elements are TUB8 promoter and 3UTR SAG1. The intensity of expression was affected when the controlling elements were replaced with upstream and/or downstream sequence of LDH1.

Levels of GFP were found greatly reduced when measured in bradyzoite stage as shown in Figure 3.5. Bradyzoite stage resembles to anaerobic condition where parasite metabolism is changed significantly to adapt to the surrounding environment. The reduction in GFP expression during bradyzoite stage could be due to the upstream and downstream region of LDH1. However, from these results it is hard to say that the decrease in expression is due to low transcription or low translation. If the level of

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mRNA in all transgenic strains is measured and is found equal, it would clearly establish that lowering of expression is occurring at translation level.

Growth rate was found approximately the same in all transgenic strains indicating that the plasmid insertion by electroporation does not affect the growth and infectivity of the parasites. Growth rate of the wild type was found to be a bit higher as compared to the transgenic control and test strains.

4.2 Transcriptional regulation of LDH2

The other two transgenic strains were generated to investigate the transcriptional regulation of *LDH2* in tachyzoites. PCR analysis from the genomic DNA of transgenic parasites showed the presence of transgenes, however it is not clear that if the transgene is integrated into the parasite genome and replicated with the parasite chromosome. The plasmid integrates into the genome by homologous recombination which could results in random integration, insertional mutagenesis, difference in copy number and silencing of the introduced gene or nearby genes by *de novo* methylation. Western blot analysis was performed to determine the expression of ORF of *LDHs* using anti-His antibody. Strong signals were revealed from mycLDH1 strain while very faint signal appeared in mycLDH2 strain even though both the reporters were under the control of TUB8 promoter and should have given constitutive expression. This could be due to difference in copy number of the transgene. However, when the same experiment was repeated after several parasite passages (15-20), these signals were not detected. Because of the selection pressure, only the transformed parasite could survive and the untransformed would gradually die with the continued drug selection, even if they have survived during

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first few passages. However, the parasites kept growing but the reporter expression vanished i.e. selectable marker is being expressed in the parasites but not the reporter. How this partial transgene expression could have happened is difficult to explain in *T.gondii.*

4.3 Future Directions

Translational suppression

In order to further confirm and clearly establish the role of upstream and down stream sequences of *LDH1* in translational suppression followings could be done,

1. To quantitate the level of GFP mRNA by RT-PCR to establish that the decrease in expression is at translational level.

2. Determining mRNA Stability to rule out the possibility of mRNA being degraded before transport to cytosol.

LDH2 misregulation

In order to better understand the transcriptional regulation of LDH2, unregulated LDH expression and its effect on parasite phenotype would be monitored. The following experiments could be done,

1. To investigate the effect of misregulation, parasites can be inoculated into the mice to test their virulence. Characteristic symptoms of the disease will be monitored along with their ability to make brain cyst as compared to the control.

2. To monitor if the overexpression of transgenic LDH2 in tachyzoites could force parasite encystations, IFA could be done for the detection of cyst wall development.

3. To differentiate the native LDH1 from myc-tagged transgenic LDH2, antibodies against both of them conjugated to different fluorescent probe can be used. Co-localization of both, if any, can be monitored by doing IFA

4. To determine the effects of transgenic LDH1 during bradyzoite stage, again phenotype change can de monitored. To differentiate native LDH2 from myc-tagged transgenic LDH1, specific antibodies conjugated to different fluorescent probe can be used. IFA can be done to determine if any co-localization exists.

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APPENDIX A

List of Oligonucleotides and plasmids

	Primers	Sequence
Set A	5PL1	5′ CACACTGGGTAGTGTCC 3′
	3GFP1491	5′GTCGTGCCGCTTCATATG 3′
Set B	5GFP1667	5′ GAATACAACTACAACTCC 3′
	3UTRL1	5′ CTCATGCTTGCACGCGTT 3′
Set C	5GFPMYC	5′ATGCAGGAGCAGAAGCTC 3′
	3GFP1940	5′ GCCATGTGTAATCCCAGC 3′
Set D	LDH-ORF sense	5′ ATGGCACCCGCACTGGT 3′
	LDH-ORF antisense	5′ GCGGAAGCGACCAGTCGA 3′
Set E	LDH2 forward	5′ATGACGGGTACCGTTAG 3′
	LDH2 reverse	5′ AAATCGTGCCGAATCTA 3′
	3′LDH PacI	5′CCCTTAATTAAGTGCCAAGCTTAAGA 3′
	5′LDHx NsiI	5′ GATTTCAGAATTCGGATCC 3′
	BamHI 3′UTR dws 780 LDH1	5′ AAAGGATCCTCTATGTATCATGGGC 3′
	PacI 3UTR ups 780LDH1	5′ CCCTTAATTAAGCGTTGGCAAAACAGG 3′
	HindIII dws 600 LDH1	5′ AAGCTTGCTGACTAAAAATGAGC 3′
	KpnI ups 600 LDH1	5′ CCCGGTACCTGCTGTGCGAATGTGTC 3′
	5′ T7 GFPmut2	5′ AAGAATTCTAATACGACTCACTA 3′
	3′ T7 GFPmut	5′ GTGTCCAAGAATGTTTCC 3′

95

Restriction map for control plasmid (pTUB8mycHisGFP-HX)
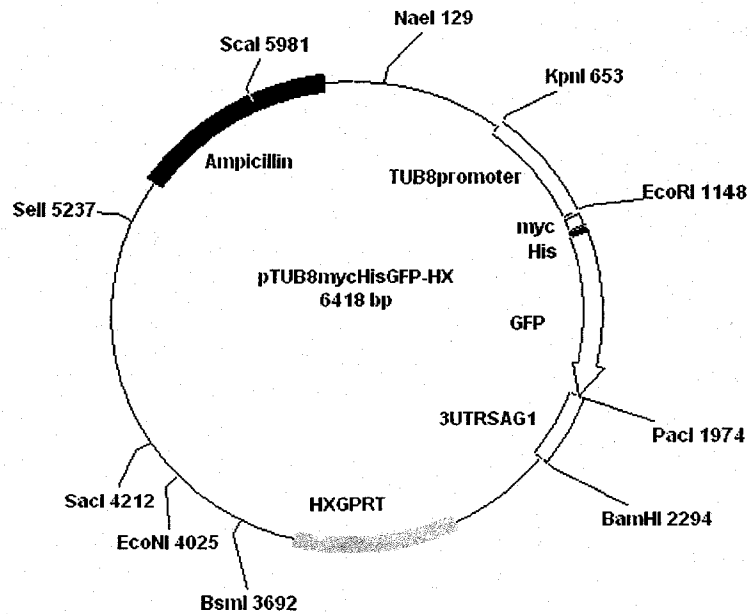
Sequence of TUB8 promoter (495-bp)

KpnI (653)

GGTACCGGGC	CCCCCCTCGA	CGGTATCGAT	AAGCTTAACC	ACAAACCTTG
AGACGCGTGT	TCCAACCACG	CACCCTGACA	CGCGTGTTCC	AACCACGCAC
CCTGAGACGC	GTGTTCTAAC	CACGCACCCT	GAGACGCGTG	TTCTAACCAC
GCACCCTGAG	ACGCGTGTTC	AA GCTTGCCT	GCATTGGGTG	CGGTTGGTGA
TCCTGGTTGG	ACCGGTGGAG	ATGCGCGCGC	ACGAAGGGGA	TGTGTCAGAA
ACATTTTGTT	TGTTCTCTGT	GAACTTTTAG	ATGTGTTAAA	GGCGGCGAAT
ATTAGCAGAG	AGTCCTCCTT	GTTCCATTCT	CTCTTGAATT	TCGCCCTTTC
CTTCTCTTTG	CGAGTGTGGT	AGAGAACAAG	CACTCGTTCG	CCGTCCCTGA
CGACGCAACC	CGCGCAGAAG	ACATCCACCA	AACGGTGTTA	CACAATCACC
TTGTGTGAAG	TTCTTGCGGA	AAACTACTCG	TTGGCATTTT	TTCTTGAATT
C				

EcoRI (1148)

Sequence of myc and His tags (75-bp)

30 nt Myc and 24 nt His tags (in bold) are shown in order.
CCTTTTTCGA CAAAAAATGC AGGAGCAGAA GCTCATCTCC GAGGAGGACC
TGGCCATGGC CATGCAG**CAC CACCATCACC ACCATCACCA T**

96

Sequence of GFP (734-bp)

NsiI (1243)

ATGCATAGTA	AAGGAGAAGA	ACTTTTCACT	GGAGTTGTCC	CAATTCTTGT
TGAATTAGAT	GGTGATGTTA	ATGGGCACAA	ATTTTCTGTC	AGTGGAGAGG
GTGAAGGTGA	TGCAACATAC	GGAAAACTTA	CCCTTAAATT	TATTTGCACT
ACTGGAAAAC	TACCTGTTCC	ATGGCCAACA	CTTGTCACTA	CTTTCTCTTA
TGGTGTTCAA	TGCTTTTCAA	GATACCCAGA	TCATATGAAG	CGGCACGACT
TCTTCAAGAG	CGCCATGCCT	GAGGGATACG	TGCAGGAGAG	GACCATCTTC
TTCAAGGACG	ACGGGAACTA	CAAGACACGT	GCTGAAGTCA	AGTTTGAGGG
AGACACCCTC	GTCAACAGGA	TCGAGCTTAA	GGGAATCGAT	TTCAAGGAGG
ACGGAAACAT	CCTCGGCCAC	AAGTTGGAAT	ACAACTACAA	CTCCCACAAC
GTATACATCA	TGGCCGACAA	GCAAAAGAAC	GGCATCAAAG	CCAACTTCAA
GACCCGCCAC	AACATCGAAG	ACGGCGGCGT	GCAACTCGCT	GATCATTATC
AACAAAATAC	TCCAATTGGC	GATGGCCCTG	TCCTTTTACC	AGACAACCAT
TACCTGTCCA	CACAATCTGC	CCTTTCGAAA	GATCCCAACG	AAAAGAGAGA
CCACATGGTC	CTTCTTGAGT	TTGTAACAGC	TGCTGGGATT	ACACATGGCA
TGGATGAACT	ATACAAAGCT	GCAGATGGTG	CTTAA	

PacI (1974) continued...

Sequence of 3UTR SAG1 (320-bp)

PacI (1974)

TTAATCACCG	TTGTGCTCAC	TTCTCAAATC	GACAAAGGAA	ACACACTTCG
TGCAGCATGT	GCCCCATTAT	AAAGAAACTG	AGTTGTTCCG	CTGTGGCTTG
CAGGTGTCAC	ATCCACAAAA	ACCGGCCGAC	TCTAAATAGG	AGTGTTTCGC
AGCAAGCAGC	GAAAGTTTAT	GACTGGGTCC	GAATCTCTGA	ACGGATGTGT
GGCGGACCTG	GCTGATGTTG	ATCGCCGTCG	ACACACGCGC	CACATGGGTC
AATACACAAG	ACAGCTATCA	GTTGTTTTAG	TCGAACCGGT	TAACACAATT
CTTGCCCCCC	CGAGGGGGGA	TCC		

BamH I (2294)

Sequence of HXGPRT

ATGGCGTCCA	AACCCATTGA	AGACTACGGC	AAGGGCAAGG	GCCGTATTGA
GCCCATGTAT	ATCCCCGACA	ACACCTTCTA	CAACGCTGAT	GACTTTCTTG
TGCCCCCCCA	CTGCAAGCCC	TACATTGACA	AAATCCTCCT	CCCTGGTGGA
TTGGTCAAGG	ACAGAGTTGA	GAAGTTGGCG	TATGACATCC	ACAGAACTTA
CTTCGGCGAG	GAGTTGCACA	TCATTT GCAT	CCTGAAAGGC	TCTCGCGGCT
TCTTCAACCT	TCTGATCGAC	TACCTTGCCA	CCATACAGAA	GTACAGTGGT
CGTGAGTCCA	GCGTGCCCCC	CTTCTTCGAG	CACTATGTCC	GCCTGAAGTC
CTACCAGAAC	GACAACAGCA	CAGGCCAGCT	CACCGTCTTG	AGCGACGACT
TGTCAATCTT	TCGCGACAAG	CACGTTCTGA	TTGTTGAGGA	CATCGTCGAC
ACCGGTTTCA	CCCTCACCGA	GTTCGGTGAG	CGCCTGAAAG	CCGTCGGTCC

97

```
CAAGTCGATG   AGAATCGCCA   CCCTCGTCGA   GAAGCGCACA   GATCGCTCCA
ACAGCTTGAA   GGGCGACTTC   GTCGGCTTCA   GCATTGAAGA   CGTCTGGATC
GTTGGTTGCT   GCTACGACTT   CAACGAGATG   TTCCGCGACT   TCGACCACGT
CGCCGTCCTG   AGCGACGCCG   CTCGCAAAAA   GTTCGAGAAG   TAA
```

98

Restriction map for plasmid p5UTRGFP-HX
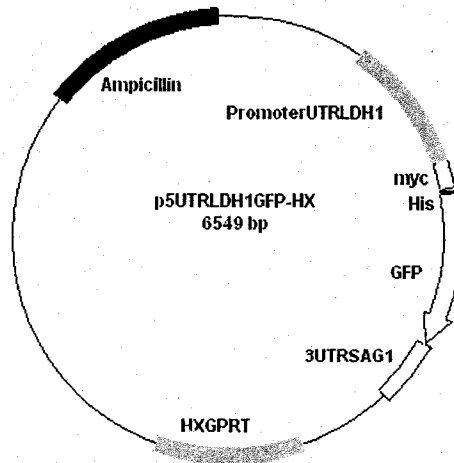
Upstream sequence of *LDH1* (629-bp)
(KpnI was introduced at 5′ primer and HindIII was introduced on 3′primer. HindII was made blunt for cloning)

```
CCTGCTGTGC  GAATGTGTCG  CGTCGTTTAA  AAACTGAACG  TGGAAACCGT
TTTCGTGCGA  TCCCAGTTAC  TCGGCGGCGT  CTGAAGTGTG  TCCCTCTTCT
CCACTTCAAG  ACTGGGACGG  GAACGAGAGT  TGGACGACCA  CTGGAAGCGG
GCGCTCGAAT  TTTCTAAAGT  TGGCCTCGGG  TGTTCGTCCG  GTATCTCGCA
GCAGATGACG  CAGCACTCCC  CGCTGTTTAA  GGAACTGGAA  TCTTGAACTG
TGCCCCTCTA  ACATCGCACC  GTCTCTGCGC  ACCGTCGCTG  TTCCGCGACT
CCCGTCAGAG  CGATATCGCG  TTGCGTCACG  TCATCGTTAT  TCGGTTTTTG
TTCGGGAAAA  TGGCAGCAGC  GGGACGAACA  CACTGGGTAG  TGTCCACGCC
GCTTCCGTCC  CACCTGCGAA  GGTGCGTCCA  CCACAACTCA  CGCGACTAGG
TGCTGCGGCT  GCGAATTCGC  GTTCCCACAC  TCGACCAGAT  TTTTTTGATT
TTCGATTTCC  AAACGCGATT  TATTCCAATC  CGCCTTGTAG  CAGACTAACC
GGTGCACTTT  TGCACGTGTA  CGCAAGACAG  CTTCGCAGAC  AACATCTGGC
AGCCTCCCGC  TCATTTTTAG  TCAGCAAAA
```

99

Restriction map for plasmid p3UTRGFP-HX
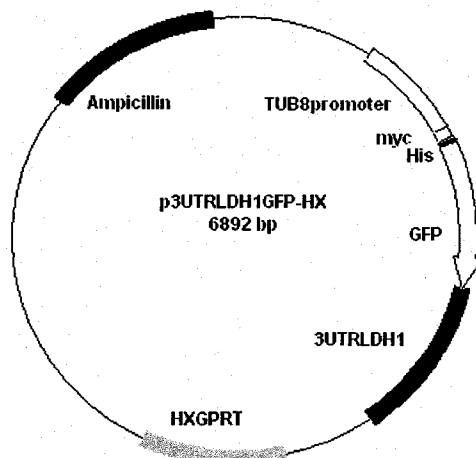
Downstream sequence of *LDH1* (796-BP)
(PacI was introduced on 5′primer and BamHI was introduced on 3′primer for cloning)

```
CGTTGGCAAA   ACAGGAGCGG   AATGCCACTT   TACTGCGCGG   GGCCCATGAT
TTATACACGC   GTTTGCAACG   GAAGCGAAAA   GACGGTTCCG   GTTCGCACCA
CGCGCTCGTC   CCGAAAAAGG   GAAGTCGCGG   CGCTGTCGGT   CAACGCTGTG
CGGGTTGCAG   GTGCGTGCTT   AAGCATCACA   AAGTGGCAGA   GCCATTTTGT
CCAGGGAAGT   AGCGTCTCAA   ACAGGTGAAC   GCGTGCAAGC   ATGAGAGGCA
TCCGTCGCTG   CGTTCGCTCA   CATATCCGAA   GGCAACAGAT   TTTGGTCGGC
AAAAGCCTCT   GCACAACCGT   GGCAAGATGA   TGGAAACAGT   TCGTGTTTGG
ACAGCAACCG   CGTTCGCTCT   CACTCAAAAC   CCTGTAGTCG   AGAGGGGTGT
CGCATGACTT   GGCTTTTGTG   GGAGTGCCCA   AATCGTCTGT   GTTCGAGGTG
AAGATCACAT   GTCCGCTGCA   GTACTGAAAA   ACACTTGGTG   CGCAGAGGCG
AGCGATAGGT   GCGCTGACTT   TTTTTTTGTT   TGTTCAAGGA   AGGCATCTTT
TTTTTTTTAC   CGGTTGTCCA   CTGTCATGTC   GAAAACGTAG   TCCGTGTGAA
GTGGTTGGTC   CCCTGTTGTC   CTT TGTCTAG   CCGCGCGTGT   CTCATGGAGC
ATTTTTCAAC   GTTGCTTCAA   ACGAATGCTG   GTTTCTCGAG   TGCACCTGCA
CCGAAAGCTG   CGGGAAATTC   AAAATCTTTC   CCGACAATGC   GGAGCCGTAC
GAAAGCCGAC   GTGGGTTATT   TGGCCCATGA   TACATAGAGG   AACTGG
```

Restriction map for plasmid p35UTRGFP-HX
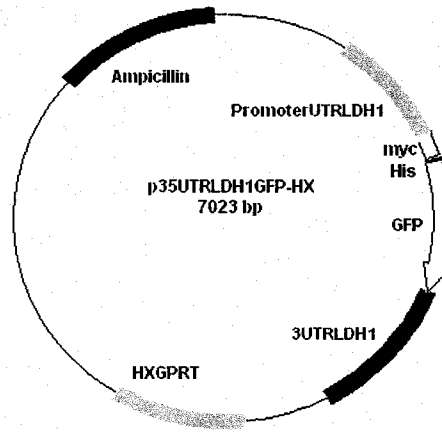
Upstream sequence of *LDH1* (629-bp)
(Primer 5PL1 is highlighted and was used for clone confirmation)

```
CCTGCTGTGC  GAATGTGTCG  CGTCGTTTAA  AAACTGAACG  TGGAAACCGT
TTTCGTGCGA  TCCCAGTTAC  TCGGCGGCGT  CTGAAGTGTG  TCCCTCTTCT
CCACTTCAAG  ACTGGGACGG  GAACGAGAGT  TGGACGACCA  CTGGAAGCGG
GCGCTCGAAT  TTTCTAAAGT  TGGCCTCGGG  TGTTCGTCCG  GTATCTCGCA
GCAGATGACG  CAGCACTCCC  CGCTGTTTAA  GGAACTGGAA  TCTTGAACTG
TGCCCCTCTA  ACATCGCACC  GTCTCTGCGC  ACCGTCGCTG  TTCCGCGACT
CCCGTCAGAG  CGATATCGCG  TTGCGTCACG  TCATCGTTAT  TCGGTTTTTG
TTCGGGAAAA  TGGCAGCAGC  GGGACGAACA  CACTGGGTAG  TGTCCACGCC
GCTTCCGTCC  CACCTGCGAA  GGTGCGTCCA  CCACAACTCA  CGCGACTAGG
TGCTGCGGCT  GCGAATTCGC  GTTCCCACAC  TCGACCAGAT  TTTTTTGATT
TTCGATTTCC  AAACGCGATT  TATTCCAATC  CGCCTTGTAG  CAGACTAACC
GGTGCACTTT  TGCACGTGTA  CGCAAGACAG  CTTCGCAGAC  AACATCTGGC
AGCCTCCCGC  TCATTTTTAG  TCAGCAAAA
```

Sequence of myc and His tags
(Primer 5GFPmyc is highlighted)

```
AATGCAGGAG  CAGAAGCTCA  TCTCCGAGGA  GGACCTGGCC  ATGGCCATGC
AGCACCACCA  TCACCACCAT  CACCAT
```

Sequence of GFP (734-bp)
(Primer 3GFP1491, 5GFP1667, and 3GFP1940 are highlighted in order. All were used for clone confirmation)
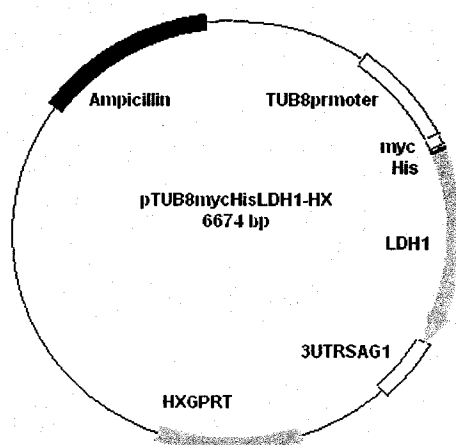
101

```
ATGCATAGTA   AAGGAGAAGA   ACTTTTCACT   GGAGTTGTCC   CAATTCTTGT
TGAATTAGAT   GGTGATGTTA   ATGGGCACAA   ATTTTCTGTC   AGTGGAGAGG
GTGAAGGTGA   TGCAACATAC   GGAAAACTTA   CCCTTAAATT   TATTTGCACT
ACTGGAAAAC   TACCTGTTCC   ATGGCCAACA   CTTGTCACTA   CTTTCTCTTA
TGGTGTTCAA   TGCTTTTCAA   GATACCCAGA   TCATATGAAG   CGGCACGACT
TCTTCAAGAG   CGCCATGCCT   GAGGGATACG   TGCAGGAGAG   GACCATCTTC
TTCAAGGACG   ACGGGAACTA   CAAGACACGT   GCTGAAGTCA   AGTTTGAGGG
AGACACCCTC   GTCAACAGGA   TCGAGCTTAA   GGGAATCGAT   TTCAAGGAGG
ACGGAAACAT   CCTCGGCCAC   AAGTTGGAAT   ACAACTACAA   CTCCCACAAC
GTATACATCA   TGGCCGACAA   GCAAAAGAAC   GGCATCAAAG   CCAACTTCAA
GACCCGCCAC   AACATCGAAG   ACGGCGGCGT   GCAACTCGCT   GATCATTATC
AACAAAATAC   TCCAATTGGC   GATGGCCCTG   TCCTTTTACC   AGACAACCAT
TACCTGTCCA   CACAATCTGC   CCTTTCGAAA   GATCCCAACG   AAAAGAGAGA
CCACATGGTC   CTTCTTGAGT   TTGTAACAGC   TGCTGGGATT   ACACATGGCA
TGGATGAACT   ATACAAAGCT   GCAGATGGTG   CTTAA
```

Downstream sequence of *LDH1* (796-BP)
(Primer 3UTRL1is highlighted and was used for clone confirmation)

```
CGTTGGCAAA   ACAGGAGCGG   AATGCCACTT   TACTGCGCGG   GGCCCATGAT
TTATACACGC   GTTTGCAACG   GAAGCGAAAA   GACGGTTCCG   GTTCGCACCA
CGCGCTCGTC   CCGAAAAAGG   GAAGTCGCGG   CGCTGTCGGT   CAACGCTGTG
CGGGTTGCAG   GTGCGTGCTT   AAGCATCACA   AAGTGGCAGA   GCCATTTTGT
CCAGGGAAGT   AGCGTCTCAA   ACAGGTGAAC   GCGTGCAAGC   ATGAGAGGCA
TCCGTCGCTG   CGTTCGCTCA   CATATCCGAA   GGCAACAGAT   TTTGGTCGGC
AAAAGCCTCT   GCACAACCGT   GGCAAGATGA   TGGAAACAGT   TCGTGTTTGG
ACAGCAACCG   CGTTCGCTCT   CACTCAAAAC   CCTGTAGTCG   AGAGGGGTGT
CGCATGACTT   GGCTTTTGTG   GGAGTGCCCA   AATCGTCTGT   GTTCGAGGTG
AAGATCACAT   GTCCGCTGCA   GTACTGAAAA   ACACTTGGTG   CGCAGAGGCG
AGCGATAGGT   GCGCTGACTT   TTTTTTTGTT   TGTTCAAGGA   AGGCATCTTT
TTTTTTTTAC   CGGTTGTCCA   CTGTCATGTC   GAAAACGTAG   TCCGTGTGAA
GTGGTTGGTC   CCCTGTTGTC   CTT TGTCTAG   CCGCGCGTGT   CTCATGGAGC
ATTTTTCAAC   GTTGCTTCAA   ACGAATGCTG   GTTTCTCGAG   TGCACCTGCA
CCGAAAGCTG   CGGGAAATTC   AAAATCTTTC   CCGACAATGC   GGAGCCGTAC
GAAAGCCGAC   GTGGGTTATT   TGGCCCATGA   TACATAGAGG   AACTGG
```

102

Restriction map for plasmid pmycLDH1-HX

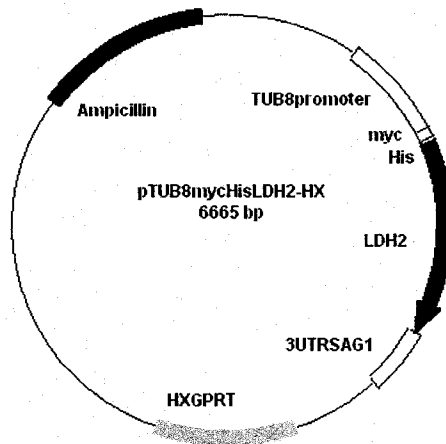**ORF of LDH1** (GenBank accession number U35118)
The LDH1 cDNA (990 nt) fragment was cloned at NsiI and PacI sites.
Primers LDHORF sense and LDHORF antisense are highlighted and were used for clone confirmation

```
 121 CCGCTCATTT TTAGTCAGCA AAAATGGCAC CCGCACTTGT GCAGAGGAGA AAGAAGGTGG
 181 CCATGATTGG CTCTGGCATG ATTGGTGGCA CTATGGGCTA CCTGTGCGCT CTCCGTGAGC
 241 TCGCTGACGT CGTTCTCTAC GATGTTGTCA AAGGTATGCC CGAGGGTAAG GCTCTTGACC
 301 TGAGCCATGT GACCTCCGTG GTCGACACCA ACGTTTCCGT CCGTGCTGAG ACTCTTACG
 361 AGGCCGCGCT CACCGGTGCG GACTGCGTTA TCGTTACCGC CGGTCTGACC AGGTGCCGG
 421 GCAAGCCCGA CTCCGAGTGG AGCCGAAACG ATCTGCTCCC GTTCAACTCG AGATCATTC
 481 GCGAGATCGG TCAGAACATC AAGAAGTACT GCCCCAAGAC CTTCATCATC TGGTCACCA
 541 ACCCGCTGGA CTGCATGGTC AAGGTTATGT GCGAGGCCTC TGGCGTCCCG CCAACATGA
 601 TCTGCGGTAT GGCCTGCATG CTCGACTCTG GTCGCTTCCG CCGATACGTC CCGACGCGC
 661 TGTCTGTCTC TCCCCGCGAC GTCCAGGCCA CCGTCATCGG CACACACGGC ACTGCATGG
 721 TCCCGCTTGT CCGGTACATT ACCGTGAACG GCTACCCGAT CCAGAAGTTC TCAAGGACG
 781 GCGTAGTCAC GGAGAAGCAG CTCGAGGAGA TCGCTGAGCA CACCAAAGTG CTGGCGGCG
 841 AGATCGTCCG CTTCCTCGGC CAGGGTTCCG CTTACTACGC CCCCGCCGCA CCGCTGTCG
 901 CCATGGCAAC ATCCTTCTTG AACGACGAAA AGCGCGTCAT CCCGTGCAGT TGTACTGCA
 961 ACGGAGAGTA CGGCTTGAAG GACATGTTCA TTGGTCTCCC GGCCGTCATT GAGGCGCCG
1021 GCATCGAGCG CGTCATCGAG CTCGAGCTGA ACGAGGAGGA GAAGAAGCAG TCCAGAAGT
1081 CCGTCGACGA CGTCATGGCG CTCAACAAGG CGGTTGCTGC TCTTCAGGCG AAGCGTTGG
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Restriction map for plasmid pmycLDH2-HX

ORF of LDH2 (GenBank accession number U23207)
The LDH2 cDNA (981 nt) fragment was cloned in place of GFP.
Primers LDH2 forward and LDH2rewerse are highlighted and were used for clone confirmation.

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 241 TGCTCGGCAT TCGTACTTCA CTTCCACCAT GACGGGTACC GTTAGCAGAA AAAAAAGAT
 301 TGCAATGATT GGTTCTGGCA TGATAGGAGG AACCATGGGC TACCTTTGTG GCTTCGGGA
 361 GCTAGCCGAC GTCGTACTAT TCGACGTTGT AACAGGCATG CCAGAAGGGA GGCGTTGGA
 421 TGATTCACAG GCGACAAGCA TTGCTGACAC GAACGTGAGC GTGACGAGCG AAACCAGTA
 481 TGAGAAGATC GCCGGATCGG ATGTCGTAAT AATAACTGCA GGGCTGACCA GGTACCGGG
 541 GAAGAGTGAC AAGGAGTGGA GCAGAAACGA CCTTTTACCG TTCAATGCAA AATCATTCG
 601 AGAGGTAGCG CAGGGAGTGA AGAAGTACTG CCCGCTTGCT TTTGTAATTG AGTGACAAA
 661 CCCGCTTGAC TGCATGGTAA AGTGCTTTCA TGAAGCGAGC GGGTTACCAA AAACATGGT
 721 TTGCGGAATG GCGAATGTGT TAGATTCGGC ACGATTTAGA CGCTTCATTG GGATCAGCT
 781 GGAGATATCA CCTCGAGATA TTCAAGCTAC TGTTATCGGC ACACACGGTG CCACATGCT
 841 GCCTCTCGCG CGTTATGTGA CGGTGAGTGG TTTCCCACTG CGAGAATTCA TAAAAAAGG
 901 AAAAATGACT GAGGCAAAAC TGGCAGAGAT CGTAGAGCGT ACAAAGAAAG TGGGGGGGA
 961 GATTGTGAGA CTTTTGGGGC AAGGGTCAGC GTACTATGCT CCAGCCCTTT TGCTATAAC
1021 AATGGCCCAG GCATTCTTAA AAGACGAGAA GAGAGTGCTC CCGTGCAGTG GTATTGCCA
1081 GGGTGAGTAT GGGCTTCACG ATATGTTTAT TGGTTTGCCG GCAGTTATAG CGGTGGGGG
1141 AATCGAGCAG GTCATCGAAC TGGAGCTGAC ACATGAGGAA CAAGAGTGTT CAGGAAGTC
1201 AGTTGATGAC GTGGTGGAAT TGAATAAGAG TTTAGCGGCG CTGGGTTGAA AAGTACGCA
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List of Buffers and Solutions

Cytomix Buffer	120mM KCl, 0.15 mM $CaCl_2$, 10mM K_2HPO_4, KH_2PO_4 pH7.6, 25mM HEPES pH7.6, EDTA 2mM, $MgCl_2$ 5mM
D-10 medium	10% cosmic calf serum,0.1% antimycotic in DMEM
Dulbecco's phosphate buffered saline (DPBS)	PBS buffer supplemented with 0.1g/L Ca^{++}
DNA gel loading buffer	0.025% Xyelene Cyanol FF, 0.025% Bromophenol Blue, 10mM EDTA pH 8.0 and 98% deinonized formamide
Ed1 medium	Eagle's MEM, 1%dFBS, 0.1%l antimycotic
HFF freezing medium	25%DMSO, 20% dFBS in DMEM
Lauri Bertani broth (LB broth)	10g/L tryptone, 5g/L yeast extract and 0.17 M NaCl. The broth was sterilized by autoclaving for 15 min.
Phosphate-buffered saline (PBS)	0.14M NaCl, 2.7 mM KCl, 10mM Na_2HPO_4 and 1.76mM KH_2PO_4 K_2HPO_4 pH7.4
Protein lysis buffer	50μM HEPES, 5μM PMSF, 0.025% Triton x100, 20% glycerol
Ponceau S	0.1 % Ponceau in 5% acetic acid solution
Resolving gel	9% acrylamide/bisacrylamide (37.5:1), 0.39 M Tris pH 8.8, 0.1% SDS, 0.1% APS and 0.04% TEMED

RPMI1640	50mM HEPES pH8.2, 5% FBS, 20mg antimycotic/L
Running Buffer for protein gel	25mM Tris base, 192mM Glycine, 0.037% SDS pH 8.4
SDS-PAGE destaining solution	45% methanol and 10% glacial acetic acid (v/v)
SDS-PAGE loading buffer	0.5M Tris-HCl pH 6.8, Glycerol, 14.3M 2-merceptoethanol, 10% SDS, 0.05% bromophenol blue.
SDS-PAGE staining solution	2.5g/L Coomassie blue in 40% methanol and 10% glacial acetic acid (v/v)
Stripping buffer	0.25 M Glycine pH2.0 and 1% SDS
TBS	200mM NaCl, 20mM Tris pH 7.4
TBST	200mM NaCl, 20mM Tris pH 7.4 and 0.2 % Tween (Polyoxyethylene Sorbitan Monolaurate)
T.gondii freezing medium	25% DMSO and 20% dFBS in ED1
Transfer buffer	25mM Tris base, 192mM Glycine, 20% Methanol (v/v) pH 8.4.
Tris EDTA buffer (TE)	10 mM Tris pH 8.0, and 1mM EDTA
Tris-Acetate-EDTA (TAE)	40mM Tris-acetate and 1mM EDTA

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VITA AUCTORIS

Name:	Ghazala Shamim
Place of Birth:	Karachi, Sindh. Pakistan.
Year of Birth:	1969
Education:	A.P.W.A College, Karachi. Pakistan
	1983-1985 Intermediate
	Federal Govt. Urdu Science College, Karachi. Pakistan.
	1986-1988 B.Sc. Chemistry, Biochemistry & Microbiology
	University of Karachi, Pakistan
	1989-1991 M.Sc. Biochemistry
	University of Karachi, Pakistan
	1994-1998 M.Phil. Biochemistry
	Institute of Business Administration, Karachi. Pakistan
	1996-1997 MBA (3 semesters)
	University of New Orleans, LA. USA
	2000-2001 BS (Computer Science, 3 semesters)
	University of Windsor, Ontario. Canada
	2004-2006 M.Sc.

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