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Unraveling the RNAi-like Activity in Toxoplasma Gondii

Ionut Pricop
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UNRAVELING THE RNAi-LIKE ACTIVITY IN *TOXOPLASMA GONDII*

by

Ionut Viorel Pricop

A Thesis
Submitted to the Faculty of Graduate Studies
to the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
2009
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RNA-mediated silencing is a conserved mechanism by which the cleaving of double stranded RNA (dsRNA) causes the degradation of homologous mRNA within the cell. Ribonuclease activity within the RNAi mechanism has been thoroughly described in model organisms such as *Caenorhabditis elegans*. However, RNAi is still largely a mystery in Apicomplexan parasites. Our group previously has reported that specific gene silencing via the introduction of dsRNA in *Toxoplasma gondii* can be achieved. Furthermore, a key protein has been characterized, namely the Argonaute protein. In this study, through the use of bioinformatic tools, several putative proteins that may act upon the RNAi mechanism have been identified. Furthermore *in vitro* dsRNA-directed ribonuclease activity and dsRNA-specific ribonuclease activity in *Toxoplasma gondii* lysates were assessed. Lastly, using a His construct, the Argonaute protein in *T. gondii* was successfully isolated from inclusion bodies and characterized in terms of its interaction with RNA species. This study demonstrates the presence of an RNAi-like mechanism in *T. gondii* and can aid in determining future drug targets in this parasite.
DEDICATIONS

To the most amazing parents, grandparents and sister
ACKNOWLEDGEMENTS

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A special thank you to Marlene Bezaire for all her support and help throughout my years in the department. You are the foundation to our department and I am indebted to you for your friendship and the numerous reminders to sign up for classes.

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<td>Ago</td>
<td>Argonaute protein</td>
</tr>
<tr>
<td>asRNA</td>
<td>Antisense RNA</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>DDE</td>
<td>Aspartate, aspartate, glutamate</td>
</tr>
<tr>
<td>DDH</td>
<td>Aspartate, aspartate, histidine</td>
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<td>dH$_2$O</td>
<td>Distilled water</td>
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<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleoside triphosphate</td>
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<tr>
<td>dsRBD</td>
<td>Double stranded RNA binding domain</td>
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<td>dsRNA</td>
<td>Double stranded RNA</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>E.coli</td>
<td>Escherichia coli</td>
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<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid disodium salt</td>
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<td>EtOH</td>
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<tr>
<td>FDUR</td>
<td>5-fluoro-2’-deoxyuridine</td>
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<tr>
<td>GST tag</td>
<td>Glutathione tag</td>
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<tr>
<td>HFF</td>
<td>Human foreskin fibroblasts</td>
</tr>
<tr>
<td>His tag</td>
<td>Histidine tag</td>
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<tr>
<td>HX</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
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<tr>
<td>IB</td>
<td>Inclusion body</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MCAC</td>
<td>Metal chelate affinity chromatography</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>-----------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>Oligo</td>
<td>Oligonucleotide</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
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<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RITS</td>
<td>RNA-induced transcriptional silencing</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>RNase</td>
<td>Ribonuclease</td>
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<tr>
<td>rpm</td>
<td>Rotations per minute</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>ssRNA</td>
<td>Sense strand RNA</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
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<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’,N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TB</td>
<td>Tris Borate</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris Borate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline Tween</td>
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<td>T. gondii</td>
<td><em>Toxoplasma gondii</em></td>
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<tr>
<td>TgAgo</td>
<td><em>Toxoplasma gondii</em> Argonaute</td>
</tr>
<tr>
<td>U</td>
<td>Enzyme unit</td>
</tr>
<tr>
<td>UPRT</td>
<td>Uracil phosphoribosyltransferase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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CHAPTER I
INTRODUCTION

1.1 Toxoplasma gondii

*Toxoplasma gondii* is an obligate intracellular parasite belonging to the phylum Apicomplexa. *T. gondii* as a parasite was first described in 1908 by Nicolle and Manceaux while working in North Africa on rodents and by Splendore working on rabbits in Brazil (Nicolle, 1908). The name of the parasite was adopted due to its bow-like crescent shape (toxon = bow, plasma = form) and the rodent it was isolated from *Ctenodactylus gundii* (Black and Boothroyd, 2000).

It has been suggested that nearly 30% of North Americans are carriers of *T. gondii*. However, in most of the cases, infection is asymptomatic and individuals lead a normal lifestyle. In immuno-compromised individuals, severe toxoplasmosis can arise due to the lack of a functional immune system, which often results in brain and eye damage. *T. gondii* infections in pregnant women can also affect the fetus tremendously, resulting in spontaneous abortions, fetal death and severe birth defects (Luft and Remington, 1992).

Like many other intracellular parasites, upon cell invasion, it was shown that *T. gondii* prevents the host cell from undergoing apoptosis by inactivating the caspase cascade (Kim *et al.*, 2006).

The life cycle of *T. gondii* is made up of two distinct replication cycles, namely the sexual and the asexual cycles. The sexual cycle of *T. gondii* takes place in felines, the definitive host of these parasites. Cats usually become infected upon ingestion of prey such as rats or birds that are carriers of the parasite. Once the encysted parasites reach the small intestine of cats, they are released and infection usually starts with the epithelial
cells. Due to the slow immune response in cats, the parasites are able to start dividing and are able to produce millions of oocytes, which are then released in the environment in the feces of the animal. Sporulation then occurs within 1 to 5 days after excretion and these oocytes remain infectious for months regardless of the severity of the climate (Dubey, 1994).

The asexual cycle may take place in a plethora of species, including land and sea mammals, as well as humans (Mikaelian et al., 2000). Infections in these species usually occurs by direct ingestion of cat feces or poorly cooked meat containing the viable cysts, after which the tachyzoites distribute themselves in the body of the host until an immune response is reached. This immune response causes the fast replicating tachyzoites to shift to the bradyzoite stage, in which the parasite continues the slow replication process through the remainder of the host’s life (Black and Boothroyd, 2000).

During the infectious stages T. gondii has the ability to convert between a rapidly replicating form named tachyzoite and a slower growing form termed bradyzoite. The advantages of this stage inter-conversion process is that in the bradyzoite stage the parasite requires less energy to survive and it is also able to escape the host’s immune response while the tachyzoites are readily recognized and eliminated (Freyre, 1995). Recurring acute infections are likely the result of the inability of the host’s immune response to detect the bradyzoite cysts (Gross et al., 1996). The current available treatment for this condition is in the form of a antimicrobial cocktail aimed at inhibiting the function of dihydropteroate synthetase and dyhydrofolate reductase. This chemotherapy is fairly effective in the acute stages of the infection however the treatment described may cause severe side effects and toxicity in the patients and are inefficient against bradyzoites (Black and Boothroyd, 2000).

2
1.1.2 Parasite strains and culture conditions

*Toxoplasma gondii* can be safely cultured *in vitro* in a plethora of cell types, although Human Foreskin Fibroblasts (HFF) is generally the preferred cell line. This is due to the fact that HFF cells allow for a greater number of replication cycles due to their morphology and surface area producing a higher parasitic yield with reduced debris derived from cell lysis. Other advantages for the utilization of HFF cells is that they are very robust by comparison with other cell lines, allowing for culturing and maintenance of these monolayers for an extended period of time before infection occurs. In addition, HFF cells are resistant to many known metabolic inhibitors and facilitate drug selection of parasites. Some disadvantages are their slow growth rate at high passages and a limited lifespan with respect to replication (Roos *et al.*, 1994).

There are several *T. gondii* strains that are commonly used in the laboratory setting that fall under the category of Type I, II or III based on their virulence. Type I parasites are the most virulent due to what is thought to be their higher rate of reinvasion rather than faster replication (Saeij *et al.*, 2005). Some common parasitic strains include RH, VEG, ME49 and a sub-clone of the latter termed PLK. The RH strain is characterized by its ability to replicate quickly and it has been described as highly virulent. It is one of the most commonly cultured strains and is generally used to characterize drug resistance and virulence (Roos *et al.*, 1994). This particular strain does not have the ability to actively convert to the bradyzoite stage, and it also lacks the ability to undergo sexual reproduction in felines (Freyre, 1995). The trend of virulence seems to be inversely proportional to the ability to produce cysts. The ME49 strain is less virulent than the RH strain and it has the capability of converting into bradyzoites under stress with high efficiency, making it an ideal candidate when studying differentiation of the parasite from
tachyzoites to bradyzoite. The PLK sub-clone of ME49 has slower replication rates and is commonly used to study the life cycle due to the ability to produce bradyzoite cysts and being able to undergo full sexual reproduction (Boothroyd et al., 1997).

### 1.1.3 T. gondii as an Apicomplexan model

A large number of pathogenic species belong to the phylum Apicomplexa, and the current drug therapies available are at risk due to emerging drug resistance in these parasites. Some Apicomplexan parasites are also very difficult to maintain in the laboratory setting such as the malaria causing parasite *Plasmodium falciparum* (Sibley, 2003). This problem has not been encountered in *T. gondii* as it can be safely maintained using standard cell culture techniques in host cells that are rather robust and easily obtained. Another attractive feature of *T. gondii* in this regard is that it is susceptible to genetic manipulation, essentially making any gene within it a feasible study target. Molecular transformation through transient and stable transfection has been achieved and has become a very important tool in determining gene function in *T. gondii* (Kim and Weiss, 2004). Various genetic tools are now used to study gene expression in *T. gondii*, such as the tetracycline repressor system described by Meissner, as well as insertional mutagenesis (Donald and Roos, 1995; Nakaar et al., 2000; Meissner et al., 2001).

With recent advancements in bioinformatics, the genomic information of *T. gondii* is readily available via the genomic database ToxoDB (http://www.ToxoDB.org, Kissinger, et al., 2003). This may lead to not only a better understanding of this particular Apicomplexan parasite, but may also uncover new applications for future drug targets. Apicomplexans generally maintain the same orthologs within their enzyme arsenal, and the sequenced genome combined with ease of culturing and genetic manipulation,
Toxoplasma gondii emerges as the ideal Apicomplexan model. It should be noted however that greater dissimilarities have been witnessed between *T. gondii* and evolutionary distant *Cryptosporidium*. Therefore, *T. gondii* may not be a suitable model for such parasites however it still remains the ideal model in studying genetic functions for Apicomplexans such as *P. falciparum* (Nene *et al.*, 2000; Striepen and Kissinger, 2004).

1.2 RNA Interference

1.2.1 RNA interference phenomenon

RNA-mediated silencing is a conserved mechanism by which the cleaving of double stranded RNA (dsRNA) causes the degradation of homologous messenger RNA (mRNA) within the cell. Initially, the phenomenon of RNAi was observed in plants, and it was characterized as a defence mechanism against viral infections, by which double stranded RNA of viral origin, is disposed of in the cells. As more experimental attention was devoted to this mechanism, it was noticed that the expression of genes may be reduced in organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* upon the introduction of dsRNA with complementary sequences to endogenous mRNA molecules within those species. Remarkably, RNAi has become one of the leading techniques in studying gene function through knockdown and shows great promise as a tool in the therapeutic development against a plethora of infectious diseases (Novina and Sharp, 2004).
1.2.2 RNAi Mechanism

Extensive work has been done to determine the mechanism by which RNA interference transcends and it was determined that it involves an initiator step and an effector step. In the initiation step, the dsRNA introduced is cleaved into nucleotides segments varying from 21 to 23 base pairs in length by a member of the RNase III family of dsRNA specific RNAse III endonuclease, Dicer. Dicer recognizes the long dsRNA substrate through its double stranded RNA binding domain, producing siRNAs with a 5’ phosphate and 2 base pair overhang in the 3’ direction, which are believed to facilitate the target RNA cleavage (Elbashir et al., 2001). Dicer has also been shown to recognize hairpin RNA structures, preparing them into micro RNAs (miRNAs) causing translational suppression, with the miRNAs produced varying in complementarity to the target mRNA on a species-dependent manner. Some reports indicate that this process is an ATP dependant step (Zamore et al., 2000; Nykanen et al., 2001) however in humans this process is performed without a requirement for ATP (Zhang et al., 2003).

The effector step begins with the recruitment of a multi protein complex termed the RNA-induced silencing complex (RISC) which acts mainly through the Argonaute protein. Binding of Argonaute to the siRNAs takes place mainly through its PIWI-Argonaute-Zwille (PAZ) domain, while the PIWI portion of the protein acts as the slicer. Upon binding, Argonaute specifically cleaves the passenger strand or the sense strand on the siRNA structure which is determined thermodynamically according which strand that has the lowest thermodynamic stability in its 5’ end (Schwarz et al., 2003). The strand that is still associated with the RISC complex is termed the guide strand and it aids in the target mRNA recognition and binding. The binding between the guide strand and the RISC complex is rather robust by comparison to the interaction between the target mRNA
and the siRNA. This interaction facilitates the cleavage of the target mRNA, which is achieved in an ATP dependent manner followed by the ejection of the complementary mRNA molecule. The sliced mRNA fragments are discarded and completely degraded within the cytoplasm (Nykanen et al., 2001). In organisms such as C. elegans, siRNA propagation takes place in which RNA dependent RNA polymerase proteins recognize siRNA-mRNA duplexes and trigger the synthesis of a new dsRNA strand, which in turn is prepared into siRNAs by Dicer and signal amplification is achieved (Carmell and Hannon, 2004). The RNAi mechanism is illustrated in Figure 1.1.
Figure 1.1 The mechanism of RNA interference. The mechanism is initiated by the presence of long dsRNA, which is in turn processed by Dicer. This produces siRNAs 21-23 base pairs in length. Next, the Argonaute protein associates with the siRNAs produced and together they form a scaffold facilitating RISC formation. Upon RISC formation, the passenger strand is expelled, allowing for mRNA binding, triggering the slicing activity of Argonaute within RISC. The figure was adapted from Matzke and Matzke 2004.
1.2.3 Key features of RNAi and its components

Whether it is labelled post-transcriptional gene silencing (plants), RNA interference (mammals) or quelling (fungi), some common key features are recognized in all these mechanisms. One of the key features within the mentioned mechanisms is the ability of dsRNA to induce the particular response in fungi, plants and animals alike. Due to this, dsRNA is generally regarded as an inducer of RNAi. The second feature that is present in these mechanisms is the degradation of a target RNA species based on the complementarity to the inducer, and lastly, a third specific feature is the degradation of target RNA by a machinery comprised of proteins that maintain high similarity in structure and function within these organisms.

Genetic screens for RNAi-deficient mutants in model organisms such as *A. thaliana*, *Chlamydomonas reinhardtii*, *Neurospora crassa* and *C. elegans* has expanded the understanding of the RNAi pathway as a whole, revealing the most important players in this mechanism. The initiators were found to be RNase III family proteins such as Dicer, while the effectors were determined to be proteins from the Argonaute-PIWI family. Lastly, the amplifiers of RNAi have been identified to be RNA-Dependent RNA polymerases (Agrawal *et al.*, 2003).

1.2.3.1 RNase III protein Family and Dicer

A key feature of RNase III family proteins is their ability to specifically bind and cleave dsRNA. The members of this particular protein family all carry a signature ribonuclease domain, often referred to as the RNase III domain. Another characteristic feature of these proteins is the production of dsRNA with a specific two base pair overhang in the 3’ direction and a 5’ phosphate group (Gan *et al.*, 2006).
There are three main classes of RNase III proteins (Figure 1.2). Class 1 RNase III enzymes are of bacterial origin and are the smallest members of the RNase III protein family. These enzymes generally contain two domains, namely the ribonuclease domain (RNase III) and a double stranded RNA binding domain (dsRBD) connected through flexible linkers. Dimerization of Class 1 RNase III proteins is common and this interaction is thought to occur through the ribonuclease domains (Nagel et al., 2000). Through mutagenesis, Zhang determined that the active unit of RNase III enzymes is actually a ribonuclease domain dimer arranged opposite to one another that work independently on the particular 3’ or 5’ end of the dsRNA substrate (Zhang et al., 2004). Extensive work in *Saccharomyces cerevisiae* allowed for better understanding of its RNase III protein Rnt1, making it one of the best characterized eukaryotic Class 1 RNase III enzymes. The function of Rnt1 has been linked to processing of yeast pre-rRNAs and many small nuclear and nucleolar RNAs (MacRae et al., 2007). Although it has the ability to process many substrates in yeast, the common feature of these substrates is the specific hairpin structure of the dsRNA it binds containing the consensus sequence AGNN, and although it was thought that the adenine and guanine nucleotides participate in hydrogen bonding to the enzyme, it was shown that they simply have structural implication within the RNA hairpin molecule rather than offering an improved binding efficacy to the dsRBD of the protein (Wu et al., 2004; Leulliot et al., 2004). Through the recognition of the consensus sequence, the dsRBD is then able to position the hairpin molecule within the active site of the ribonuclease domain at a distance of 13-16 base pairs from the loop.

The second main class of RNase III proteins, Class 2 contains Drosha protein found in *Drosophila*. Although the structure of this protein has not been yet obtained, it has been
postulated that it acts as a monomer rather than the dimeric formation witnessed in Class 1 RNases. Drosha contains four well defined domains, namely a proline rich region, a dsRBD and two RNase III domains, denoted as RNase IIIa and RNase IIIb. The working hypothesis for the mode of action of Drosha is that the two RNase III domains interact to produce the catalytic site. Drosha is involved mainly in the processing of pre-rRNAs in humans and it plays a major role in miRNA maturation (Wu et al., 2000; Lee et al., 2003). Drosha normally cleaves pre-miRNA hairpins, which are then further processed by Dicer into mature miRNA. Studies using purified Drosha showed that the accuracy of excising pre-miRNA hairpins is lost and more specifically, it acts as a general RNase III protein. Substrate and cleavage specificity however were shown to be coupled to the interaction between Drosha and its partner DGCR8 (DiGeorge syndrome critical region gene 8). Such critical evidence moulded current thinking in that DGCR8 is the Drosha accessory protein which directs substrate selection and specificity (Han et al., 2006). Evidence of the interaction between DGCR8 and Drosha are seen at a structural level due to the proline binding WW domain that DGCR8 displays, facilitating the interaction with the proline rich N-terminal region of Drosha (Gregory et al., 2004). A DGCR8 orthologue is found in C. elegans named Pasha, which in conjunction with Drosha, play a significant role in the processing of pre-miRNAs.

The last class of RNase III enzymes is called Class 3 RNase III or simply Dicer. The Dicer enzymes act on a dsRNA substrate creating siRNAs, or they may act within the miRNA pathway in maturing these. Recently, the Dicer protein structure of Giardia intestinalis was resolved through X-ray crystallography and it revealed some important structural features that aid in understanding its function. Dicer enzymes possess a very
distinct helicase domain, namely the DExD/H-box helicase which is generally conserved in higher eukaryotes.

In *Giardia*, the crystallographic structure revealed that this lower eukaryotic organism does not harbour the DExD/H-box helicase, a DUF283 showing a distinct dsRNA binding fold, or a dsRBD domain. Its structure did reveal that the domains it possesses fulfil the minimum requirement for binding and processing dsRNA. The domains present are the PAZ and the two RNase III domains common in Class 2 and Class 3 RNase III proteins, with the PAZ domain acting in stabilizing and binding end of the incoming dsRNAs to be processed. As seen before, the endonuclease domains of *Giardia* Dicer behave similarly to Class 1 RNase III proteins in the sense that they dimerize internally producing the catalytic site (Macrae *et al.*, 2006; Zhang *et al.*, 2004). Metal ion dependency is often the case with proteins interacting with DNA or RNA structures. Dicer heavily relies on metal ions to properly arrange and stabilize the incoming dsRNAs, and it has been shown that the nature of the metal ions involved is usually of divalent nature, some examples of which are Mg$^{2+}$, Ni$^{2+}$ and Co$^{2+}$ (Macrae *et al.*, 2006). Products of the *Giardia* Dicer are 25-27 base pairs in length, which corresponds to the distance measured from the catalytic center produced by the RNase III dimer and the region that binds the 3’ end of the RNA on the PAZ domain. Human dicer however has been shown to produce 21 base pair products, indicating that the distance between the PAZ domain and the catalytic domain may be slightly shorter than witnessed in *Giardia*. 
Figure 1.2  **Visual representation of the RNase III family of enzymes.** Bacterial RNase III or Class 1 display a small protein containing an RNase III domain coupled with a double stranded RNA binding domain (dsRBD) and is thought to be active through dimerization. Class 2 Drosha proteins display their signature polyproline region which enables the interaction of Pasha (or DGCR8 protein in humans) along with 2 RNase III domains and the dsRBD domain. Lastly, the most complex RNase III enzymes, the Dicer of higher eukaryotes displays the signature DExD helicase domain as well as its distinct PAZ domain. The figure was adapted from MacRae, 2007.
1.2.3.2 Argonaute-PIWI protein Family

In recent years, Argonaute proteins have been subjected to extensive research due to their presumed importance in the RNAi mechanism and have since been recognized as the central component of the RNA-mediated silencing complexes. In 2007, the crystallographic structure of the Argonaute protein isolated from *Pyrococcus furiosus* revealed two distinct motifs which elucidated the mode of action of this particular protein. The PAZ domain acts as a single stranded nucleic acid binding motif, suggested to be actively participating in the binding of the 3’ end of the single stranded siRNA, while the PIWI domain has been coined as the catalytic domain of this protein, due to its RNase H fold. The PAZ domain depends heavily on its interaction with magnesium ions in order to coordinate its distinct Asp-Asp-Glu (DDE) motif, while it has been suggested that the PIWI domain relies on magnesium ions to properly arrange its active site of Asp-Asp-His (DDH) motif. This shows close similarities between Argonaute and other RNase H bearing domains (Tolia and Joshua-Tor, 2007).

It should be noted however that while Argonaute proteins are generally regarded as the catalytic engine of the RNAi mechanism in regard to mRNA degradation, for many Argonaute protein isoforms in several organisms, this does not hold true. In humans, there are 4 Argonaute isoforms, with Argonaute 2 being recognized as the only member of the subfamily that is able to exhibit exonuclease activity through its DDH catalytic triad. Although the DDH catalytic motif is present in human Argonaute 3, this isoform has been described as catalytically inactive (Liu *et al.*, 2004). In *C. Elegans*, there are 27 Argonaute isoforms that are divided into two groups of primary and secondary Argonautes. The primary Argonaute group is
responsible for the initial response through the small dsRNAs derived from the cleavage of larger dsRNAs by Dicer and are further processed into siRNA within the RISC complex resulting with the degradation of the target mRNA complementary to the siRNAs loaded within the complex. Upon the degradation of the target mRNA, an RNA-dependant RNA polymerase generates new dsRNAs which are then utilized by the secondary class of Argonaute proteins via an unknown mechanism, resulting in an amplified RNAi signal (Steiner et al., 2006).

Moreover, the family of Argonaute proteins has been further characterized into two distinct subfamilies, namely the Argonaute subfamily and the PIWI subfamily (Rodriguez et al., 2005) however no functional difference has been yet reported in these two subfamilies. The Argonaute subfamily members have been determined to resemble the *Arabidopsis* Ago1 protein, while the PIWI subfamily members have been shown to closely resemble the PIWI protein found in *Drosophila* (Carmell et al., 2002). A graphical representation of the Argonaute protein domains in several organisms is provided in Figure 1.3.
Figure 1.3 Alignment of various Argonaute proteins. The key aspect is the presence of the PIWI catalytic domain in all of them. TgAgo (T. gondii Argonaute) is the smallest compared to AfPiwi (A. fulgidus PIWI protein), HsAgo2 (human Argonaute 2), AtAgo1 (A. thaliana Argonaute 1) and TbAgo1 (T. brucei Argonaute 1). The figure was adapted from Al Riyahi et al., 2006.
1.2.3.3 RNA-Dependent RNA Polymerases

RNA-dependent RNA polymerases play a crucial role in the cytoplasmic and chromatin RNA silencing pathways in *C. elegans*, fungi and plants, however their presence in insects and mammals has yet to be shown (Baulcombe, 2004). RdRp was initially cloned from the tomato plant, where it is responsible for the production of dsRNAs from already present single strand RNA templates (Schiebel *et al.*, 1998). The amplification mechanism for RdRps proposes the recognition and extension of a siRNA-mRNA duplex forming new dsRNA, which in turn acts as the substrate for Dicer, perpetuating the RNA interference response. Several orthologues are found in *A. thaliana* suggesting the implication of these in several silencing pathways and specificity towards different targets might be the case. Tobacco plants with reduced RdRp1 levels showed an increased susceptibility towards infection with the tobacco mosaic virus, while RdRp6 mutants in *A. thaliana* are extremely vulnerable towards the cucumber mosaic virus, but not to the tobacco mosaic virus (Dalmay *et al.*, 2001; Xie *et al.*, 2001). Studies with *Arabidopsis* RdRp2 mutants resulted in the lack of endogenous retroelement-derived siRNAs, suggesting that perhaps this protein plays a role in the chromatin silencing pathway (Xie *et al.*, 2004). Along the same lines, studies in *S. pombe* revealed that RdRp is crucial for the RNAi mediated heterochromatin assembly (Sugiyama *et al.*, 2005). In *C. elegans* however, mutant RdRp studies showed that the RNAi response obtained in the mutant and the wild type worms are comparable (Sijen *et al.*, 2001).
1.2.4 RNA interference in prokaryotes

Although prokaryotes do not possess RNAi systems homologous to those found in eukaryotic cells, they do possess a system analogous in function to that of eukaryotes (Shabalina and Koonin, 2008). However, key proteins active in viral infections in the modern eukaryotic RNAi mechanism do have staggering prokaryotic connections. For example, one of the central proteins in the RNAi mechanism of eukaryotes, Dicer has two of its main catalytic domains linked to archaeal and bacterial ancestry. The catalytic domains in question are the RNase III and the Superfamily II RNA helicase domains. The helicase domain of eukaryotic Dicer is closely related to archaeal Hef proteins (Superfamily II helicases) which aid in DNA replication (Komori et al., 2004). The RNase III domain of Dicer, responsible for the nuclease activity, draws its origins from bacteria, where it processes ribosomal RNA (rRNA) and plays an active role in mRNA degradation (MacRae et al., 2007). Another key protein, Argonaute, the slicer protein of the RNAi mechanism also seems to have archaeal roots, and although the function of the archaeal orthologue is still elusive, it has been associated with chromatin remodelling in archaea (Ma et al., 2005; Aravind et al., 2000).

Nonhomologous protein orthologues linked to the RNAi pathway have been shown in prokaryotes however it was unclear how these protein orthologues aid in the regulation of gene expression of these early organisms until the discovery of small, noncoding regulatory RNAs in E. coli. These noncoding RNAs are similar to eukaryotic miRNAs, displaying partial complementarity to the mRNA target, resulting in mRNA degradation or inhibition of translation. Prokaryotes also display a siRNA-like system however its particular mechanism has not been yet elucidated. The basis of the prokaryotic RNAi system is the CRISPR loci (clustered regularly interspaced short
palindromic repeats) which are complementary to sequences of known prokaryotic viruses, transposons and plasmids (Bolotin et al., 2005; Makarova et al., 2006). Through bioinformatics, a number of CRISPR-associated genes (cas genes) have been predicted coding for helicases, RNA-binding proteins, nucleases and also a polymerase (Jansen et al., 2002; Makarova et al., 2002, 2006). Through extensive in silico research, it has been hypothesized that these predicted cas genes may actually comprise an RNAi-like system which aids in the defence of the prokaryotic genome. The hypothetical mode of action has been described by RNA sequences from the CRISPR loci associating with Cas proteins which in turn trigger the degradation of the invasive RNA structures Bolotin et al., 2005; Makarova et al., 2006). Experimentally, new evidence suggests the importance of these CRISPR loci in prokaryotic viral resistance. In 2007, Barrangou showed that alterations in a prokaryote’s CRISPR sequences lead to the destruction of a corresponding viral sequence (Barrangou et al., 2007), however the pathway by which this targeted degradation takes place has not been yet determined.

1.2.5 RNA interference in eukaryotes

The eukaryotic RNAi machinery is complex and plays a role in many different cellular processes. Through the elucidation of this mechanism, a better understanding of post-translational gene regulation can be achieved. It all commences with the production of small RNAs, 20-30 nucleotides in size that trigger the cascade. Currently, three main classes of small regulatory RNAs are recognized, namely short interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs). These classes differ from one another not only in structure, but also through their mode of action. Most of the proteins that participate in the RNAi pathway in eukaryotes show a distinct
conservation in their domains, most importantly for the Argonaute and Dicer proteins (Tolia and Joshua-Tor, 2007). Through the interaction of these important proteins with their specific small non-coding RNAs, genomic surveillance and protection against transposable elements and viruses is achieved. The first two classes of small RNAs rely on a similar pathway in which the active proteins are Dicer and Argonaute, however in the case of siRNAs the result is target mRNA degradation, while the production of miRNAs results in translational repression. The third class of small non-coding RNAs, piRNAs are found in animal germ cells and they generally silence retrotransposons and are highly active during spermatogenesis.

1.2.5.1 Post-Transcriptional Gene Silencing in Plants

The first time an RNAi-like mechanism was reported was in 1990 by Napoli and Jorgensen while studying chalcone synthase, a rate limiting enzyme in the anthocyanin biosynthesis pathway in petunias responsible for the violet colouration (Napoli et al., 1990). Through overexpression, they anticipated a deepening of the violet colour however the results showed white flowers with violet patches, a phenomenon coined “cosuppression” by the investigators.

Three main RNA silencing pathways occur in plants through siRNAs, miRNAs and chromatin modification. The first pathway, post-transcriptional gene silencing (PTGS) relies on the production of siRNAs that in turn trigger the degradation of a complementary mRNA substrate and it is induced through the production of dsRNAs from RNA viruses, transgenic inverted repeats and lastly RNA-dependent RNA polymerases (RdRps). The second pathway relies on the production of endogenous miRNAs produced by a Dicer like protein (DCL1) which act in the downregulation of
gene expression through translational repression or by triggering degradation of target mRNA. Lastly, the third pathway by which gene regulation is achieved in plants is through transcriptional gene silencing (TGS). This last pathway is initiated by siRNAs which couples with heterochromatin, leading to DNA methylation and chromatin remodelling ([Chan et al., 2005). Recent observations have linked the siRNA-directed DNA methylation in plants to the process of histone modification in fission yeast ([Zilberman et al., 2003]).

Studies in the model organism *Arabidopsis thaliana* unveiled the presence of four Dicer like proteins (DCL1-4), 10 Argonaute protein family members, 6 members of the RNA-dependent RNA polymerase family and five members of the double-stranded RNA-binding domain protein family (dsRBP). The Argonaute family members in *A. thaliana* have some overlap in their function, however, general consensus is that they are the slicer components of RISC or RITS complex (RNA-induced transcriptional silencing), the latter of which generally is present during specific developmental stages. The Argonaute 1 protein in *Arabidopsis* has been speculated to participate in the RISC complex of the post-translational gene regulation mechanism. Evidence supporting this hypothesis came through the research of Vaucheret, in which hypomorphic mutants that were created continued to accumulate miRNA, however slicing of the target mRNA was not observed (Vaucheret et al., 2004). The question of function overlap between Argonaute in *A. thaliana* was addressed through the creation of Ago1 mutants, which led to defective miRNA and siRNA defective phenotypes, suggesting that perhaps Ago1 participates in several pathways (Fagard et al., 2000; Vaucheret et al., 2004).

The Dicer family in *A. thaliana* contains four members, all of which may actively participate in the silencing of genes via different mechanisms. Even though four Dicer-
like proteins have been identified, only two of them, namely DCL1 and DCL3 have been characterized and linked to a specific function. DCL1 protein has been shown to act in the production of miRNAs, 21 nucleotides in size, from a source of dsRNA producing nearly perfectly matched miRNAs to the targeted sequence (Papp et al., 2003; Xie et al., 2004). DCL3 has been implicated in chromatin silencing through the production of transposon and retroelement-derived siRNAs. Moreover, the siRNA products prepared by DCL3 are 24 nucleotides in size (Baulcombe, 2004). The functions of the remaining two members of this family, namely DCL2 and DCL4 have been difficult to demonstrate. DCL2 has been associated with viral siRNA production however the function of DCL4 is still not known (Xie et al., 2004).

1.2.5.2 RNA interference in Invertebrates and Mammals

Upon the discovery of RNAi in plants, many genetic studies questioned whether or not the same machinery is conserved in higher eukaryotes such as invertebrates and mammals. The RNAi mechanism was first observed and characterized in C. elegans (Fire et al., 1998). A distinct phenomenon was observed in C. elegans, in which the RNAi response seems to be heritable and can be maintained for several generations. This phenomenon has not been encountered in other organisms (Grishok et al., 2000). PAZ-PIWI family proteins such as Rde-1 and Rde-4 were also identified and these dsRNA binding proteins were found to associate with the only Dicer, Dcr-1 (Kamath et al., 2003; Hammond et al., 2001). The Dicer protein identified in this organism contains a conserved DExH box and it is responsible for processing dsRNAs into siRNAs (Tabara et al., 2002). Other RNAi related proteins were also elucidated in through the help of mutants, with as many as 27 Argonaute proteins being identified and classified as primary
and secondary. The secondary Argonautes have been described to act in the RNAi amplification phenomenon found in worms (Steiner et al., 2006; Kamath et al., 2003; Vastenhouw et al., 2003).

The signal amplification was a result of template amplification, and the proteins responsible for creating these secondary substrates for the amplified RNAi signal were two RNA-dependent RNA polymerases. These RdRp were ego-1, found exclusively in the germline, and rrf-1 in somatic tissues (Smardon et al., 2000; Sijen et al., 2001). Mechanistically, it is thought that the products of the Dicer enzyme in concert with the mRNA template facilitate binding of these proteins leading to the generation of new dsRNA substrates for subsequent Dicer cleavages (Grishok, 2005).

A key component of the RNAi mechanism was eventually identified in Drosophila and its involvement in RISC has been linked to the slicing of target mRNA with the aid of siRNAs (Hammond et al., 2001). Further evidence on the importance of Argonaute involvement in the RNAi mechanism in flies came from embryos lacking Ago2, one of the two Argonaute isoforms in flies. Ago2 mutants were described to have the ability to degrade RNA targets using miRNAs specific to those, however they lacked the ability to undergo siRNA-directed degradation of such substrates (Okamura et al., 2004). Furthermore, Ago1 mutants have been reported to retain their ability to undergo siRNA induced degradation, however the miRNA directed target degradation is lost (Kataoka et al., 2001). Both these studies imply that slicer activity is related to both Argonaute isoforms, however the exclusive protein associated with target RNA cleavage through RNAi is Ago1 (Rand et al., 2004).

Similarities between the slicer components in flies and humans are remarkable. At the peptide level, Ago1 in flies is related to hAgo2 (human Argonaute 2). hAgo1,
although present, does not display slicer activity, even though it is able to bind single stranded and double stranded RNA. Also, the similarities between itself and its active counterpart, hAgo2, at the protein level are staggering (Meister et al., 2004; Liu et al., 2004). In 2002, siRNAs 21 base pairs long were shown to interact with human RISC as well as causing a distinct knockdown in the gene expression of HeLa cells. The same study also demonstrated that single stranded antisense siRNAs are also capable of gene silencing in these cells when phosphorylated at their 5’ end suggesting that the guide strand within RISC is a single stranded antisense RNA molecule (Martinez et al., 2002). MicroRNA processing was also witnessed using recombinant dicer protein and cytoplasmic extracts from HeLa cells in vitro. This study effectively demonstrated the processing of pre-miRNAs of various lengths into the mature miRNAs 21 base pairs in length (Leuschner et al., 2007). Recently, it was determined that the downregulation of Dicer expression in human endothelial cells leads to apoptosis, showing the importance of miRNA and siRNA biogenesis in mammalian cells (Asada et al., 2008).

Studies in other mammalian systems were aimed at elucidating the developmental roles of important genes such as Dicer at embryological stages, mirroring the importance of tightly regulating pre-miRNAs. It was shown that a loss of Dicer1 protein is lethal in the early developmental stages of mouse development. The study also demonstrated the importance of the Dicer1 protein since the group was not able to create embryonic stem cell null mutants (Bernstein et al., 2003). A recent mouse model study revealed that Dicer1 expression is crucial in the normal development of the reproductive tract, especially in females (Hong et al., 2008). Studies in mammalian systems suggest that although the RNAi machinery plays an important role in defending the genome, it plays
an even bigger part in the normal development of mammals, controlling key genes linked to apoptosis.

**1.2.6 Applications of RNA interference**

In the past decade, RNA interference has become a powerful tool in the inquiry of gene function in different model organisms as it allows for targeted downregulation of gene expression. Due to the simplicity of the method, it quickly became an alternative to gene-knockout methods or transgenic animals. RNAi also permits large-scale functional analysis such as a study in *C. elegans* which isolated mutant phenotypes for almost 1800 genes (Kamath *et al.*, 2003). Another successful application of RNAi was shown in *D. melanogaster* aimed at identifying essential signalling cascades and embryonic development (Clemens *et al.*, 2000).

Gene specific therapy may soon become a reality based on the high specificity of the target mRNA recognition in diseases in which aberrant protein production disrupts cellular processes. Highly specific siRNA-induced gene silencing was reported in trials using Human Embryonic kidney (HEK) 293 cells expressing GFP mRNA, showing that while siRNAs specific to the GFP mRNA expressed promotes the active degradation of this substrate, no other secondary changes were observed in overall HEK293 gene expression (Chi *et al.*, 2003). Another study showed that the complementarity of siRNAs can also be used to target mutant alleles, while allowing for proper expression of the wild type allele, offering more evidence of the importance of this technique (Moss, 2003). Evolutionarily, the RNAi mechanism was developed as a method to keep guard against viral invasion and several experiments have shown the ability of specific siRNAs to
inhibit infection by a myriad of viruses, including the Human Immunodeficiency Virus (HIV), hepatitis C virus and the poliovirus.

The main pitfall to this technique is the delivery of siRNAs to the necessary site or cells in vivo. With other organisms such as C. elegans, siRNAs can be introduced in the worm through feeding however in mammals the delivery of siRNAs becomes more problematic. Additionally, plasmid systems expressing dsRNA through the head-to-head promoter arrangement has been shown to cause downregulation of a target gene in organisms such as T. gondii and T. brucei (Al-Anouti et al., 2004; Tschudi et al., 2003). A 2007 study showed that the introduction of siRNAs in mammalian systems is recognized by the IFN-pathway, which in turn leads to inflammation through innate immunity activation. This phenomenon was observed regardless of the cell line or sequence of the siRNA used, determining that siRNAs activated both the IFN-alpha and IFN-beta cytokines (Sledz et al., 2003, Kariko et al., 2004). Due to this sensitivity, better delivery methods are necessary in mammalian systems. Reports of chemical modifications to uridines within the siRNA such as the addition of a good leaving group to the 2’ position such as fluorine (2’fluoro uridine) may escape detection by the immune system (Sioud, 2007). Other methods of evading the immune system include lipophillic vehicles and a new antibody delivery strategy (Song et al., 2005).

1.3 RNAi in protozoan parasites

1.3.1 Evidence of RNAi in protozoa

The first account of RNAi in a protozoan parasite came from T. brucei in which downregulation of a target gene was achieved, however at the time this phenomenon was poorly understood (Ngo et al., 1998). Upon this discovery, extensive work has been
done in protozoans in the generation of vectors capable of inducing an RNAi response. The most commonly used method involves the cloning of a gene of interest between opposing promoters arranged in a head to head fashion, yielding dsRNA upon expression. Other methods for RNA-induced downregulation include the expression of hairpin RNAs or the generation of an inducible system such as the tet-R system which allows for dsRNA expression only upon induction (Tschudi et al., 2003; Meissner et al., 2001). Through the use of bioinformatics, it was predicted that members of the Trypanosomatidae family such as *T. brucei* and *T. congoles*e appear to be RNAi positive, while other members of this family such as *T. cruzi* and *Leishmania major* do not posses the classical RNAi components (Shi et al., 2004; Ullu et al., 2004). Recent results show that Argonaute1 is an important component in the RNAi pathway of *T. brucei* since depletion of this protein caused an impairment of the RNAi response (Shi, et al., 2007). In apicomplexan parasites, *P. falciparum* was shown to exhibit dsRNA specific downregulation of dihydro-orotate dehydrogenase (DHODH), an important enzyme in the pyrimidine biosynthesis pathway (McRobert and McConkey, 2002). Database mining however failed to show the existence of classical RNAi related genes in *P. falciparum* such as Argonaute, Dicer and RdRp and the observed RNAi effects showed in 2002 may possibly be artifacts due to antisense RNA and not an effect of dsRNA. The antisense RNA molecule hybridizes with the mRNA molecule preventing the initiation of translation (Ullu et al., 2004).

In the case of *G. intestinalis*, an RNAi mechanism is evident from data base mining, with clear regions for Argonaute and Dicer like proteins being present. Recently, the Dicer protein of *Giardia* was isolated, and its crystallographic structure reveals the protein’s ability to generate siRNAs (Macrae et al., 2006).
1.3.2 RNAi in \textit{T. gondii}

As witnessed in other protozoans, the ability to initiate an RNAi response in the presence of dsRNA has been successfully identified, and database mining suggests that \textit{T. gondii} may possess the same ability. Scanning of ToxoDB resulted in the finding of a PIWI protein as well as several dsRBD proteins that may emerge as the Dicer protein in this organism. Furthermore, it was previously reported that the introduction of dsRNA in \textit{T. gondii} elicits the specific degradation of homologous mRNA (Al-Anouti and Ananvoranich, 2002; Al-Anouti \textit{et al.}, 2003). Moreover, a 2006 study showed that by lowering the expression of Argonaute protein in transgenic \textit{T. gondii}, the ability to carry out dsRNA induced gene silencing became impaired. Also, the study suggested that only one Argonaute protein may actively participate in the process of gene silencing via a dsRNA induced mechanism (Al Riyahi \textit{et al.}, 2006). Although the hallmark of RNAi was not demonstrated, namely the production of siRNAs, the study conclusively determined the involvement of a minimal Argonaute protein in dsRNA induced gene silencing.

1.3.3 RNAi Machinery in \textit{T. gondii}

1.3.3.1 \textit{T. gondii} Argonaute protein

Argonaute proteins are the catalytic components of the RNA induced silencing complex. These proteins are generally composed of four domains, namely the N-terminus, middle, PAZ and PIWI domains. The presence of a Argonaute protein in \textit{T. gondii} has been largely refuted, although it was demonstrated that an Argonaute ortholog may be present in \textit{T. gondii} which is able to participate in the double stranded RNA induced silencing (Al Riyahi \textit{et al.}, 2006). It was also reported that the \textit{T. gondii}
Argonaute protein has high similarities in its structural and functional features with eukaryotes as well as bacteria. The TgAgo cDNA is 1575 nucleotides which encode for a 524 amino acid peptide, with a molecular weight of 58.5 kDa and a theoretical isoelectric point of 9.4. By Argonaute standards, this protein appears to be very small however it is able to interact with dsRNA in the RNAi mechanism (Al Riyahi et al., 2006).

The lack of a PAZ domain in *T. gondii* has sparked much debate regarding its activity, however it has been shown that although the Argonaute protein of *A. fulgidus* (*AfPIWI*) does not contain a PAZ domain, its ability in binding single stranded or double stranded RNA and DNA is maintained. It has also been shown that structurally, *AfPIWI* is able to adopt an RNase H-like fold, explaining the binding capacity to single and double stranded RNA (Parker et al., 2004); (Ma et al., 2005). Furthermore, in *Drosophila*, studies have shown that recombinant PIWI domains maintain their slicer activity *in vitro* (Miyoshi et al., 2005).

### 1.3.3.2 Dicer (RNase III)

Our group has previously shown that database mining produces several candidates for the role of Dicer in *T. gondii*. Furthermore, a past member of our lab was also able to clone and express the RNase III domain that arose from *in silico* research. The production of siRNAs has yet to be shown in *T. gondii*, however experimental evidence suggests that through the introduction of dsRNA, gene specific knockdown can be achieved (Al-Anouti and Ananvoranich, 2002; Al-Anouti et al., 2003). This suggests that although this particular protein has not been yet characterized, the propensity of it carrying a classical role is high.
1.4 Objectives

From its discovery nearly a decade ago, RNAi has quickly become the leading tool in studying the function of unknown genes. Previous studies in our lab suggest that RNA interference is active in *T. gondii* with some of the pivotal proteins being recognized through a bioinformatics approach (Al-Anouti *et al.*, 2003; 2004). The first aim of this study was to use a more in depth bioinformatics based approach to identify other possible candidates in the RNAi pathway of *T. gondii* using well annotated genes from other organisms as well as to confirm previous bioinformatic data. The second aim was to show the presence of a double-stranded RNA mediated ribonuclease activity and to identify its requirements. The third objective was to present evidence of siRNA formation, therefore proving the existence of an RNAi-like pathway in *T. gondii*. The fourth aim of this study was to isolate and purify the Argonaute protein present in *T. gondii* and to characterize it.
CHAPTER II
MATERIALS AND METHODS

2.1 Chemicals and Reagents

Chemicals and reagents that were used in this study are listed below.

**Amersham-Pharmacia Biotech (Baie d’Urfe, Quebec).**
T7 RNA polymerase, 2’-deoxyribonucleoside 5’-triphosphates (dNTPs), ribonucleoside triphosphates (rNTPs), nitrocellulose membranes, and ECL western blot detection reagent.

**Bio-Rad Laboratories (Mississauga, ON)**
Xylene Cyanol, Bromophenol Blue, Coomassie brilliant blue R-250 and Bio-Rad protein assay dye reagents.

**Invitrogen Corporation (Burlington, ON)**
RNase Out Ribonuclease inhibitor, BL21 (DE3) competent cells

**MP Biomedicals (Ohio, US)**
Cyclohexylaminopropane sulfonic acid (CAPS), N-lauroyl sarcosine

**Promega (Madison, WI)**
Agarose, RQ1 RNase-free DNase, Multicore buffer.

**Roche Diagnostics (Laval, Quebec)**
*Taq* DNA polymerase

**Sigma-Aldrich (Oakville, ON)**
Acetic acid, ampicillin, chloroform, DNA ladders, dimethylsulfoxide (DMSO), dithiothreitol (DTT), diethylpolycarbonate (DEPC), ethanol, ethylenediaminetetra-acetic
acid disodium salt (EDTA), ethidium bromide, formamide, formaldehyde, glycine, glycerol, hydrochloric acid, methanol, polyoxyethylene sorbitan monolaurate (Tween-20), 2-propanol, phenylmethyl sulfonyl fluoride (PMSF), sodium chloride, sodium acetate, N,N,N',N’ tetramethyl ethylenediamine (TEMED), tris-hydroxymethylaminomethane (Tris), Triton X-100.

**USB (Cleveland, OH)**

Boric Acid, Acrylamide, bis-acrylamide, ammonium persulfate (APS), RNase A and sodium dodecyl sulphate (SDS).

### 2.2 Apparatus and instrumentation

Agarose gel electrophoresis of DNA and RNA was carried out using the Miniature Horizontal Gel System MLB-06 from Tyler Research Instruments. AlphaImager™ 2200 Light Imaging System with AlphaEase software was used to view gels and take images. Sodium dodecyl sulfute Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the vertical gel electrophoresis system, including all the glasses, Teflon combs and spacers, from Bethesda Research Laboratories (BRL).

Centrifugations were carried out in J2-HS Centrifuge (Beckman), the desktop Eppendorf Model 5415C microcentrifuge from Desaga (Sarstedt Gruppe, Germany), or the Br4i centrifuge (Jouan, SA). DNA, RNA and protein concentrations were quantified using the Genesys 10 UV-Visible spectrophotometer. The quartz cuvettes used were from Sigma.

PCR reactions were performed using the 20-well Techgene Thermal Cycler (Techne, Cambridge, UK).
2.3 Parasite strains and culturing conditions

Two different *T. gondii* strains were used for this study, namely the RH and Ui. RH is a laboratory strain and was obtained from the AIDS Research and Reference Reagent Program, NIH. The Ui parasite strain is a stable transgenic strain that was created in our laboratory from parental RH, designed to constitutively express double stranded RNA homologous to the Uracil phosphoribosyltransferase (*UPRT*) gene under selection with 5-fluoro-2’-deoxyuridine (FDUR), while parasites lacking the plasmid expressing dsRNA *UPRT* will perish under this selection.

The *T. gondii* parasites were grown in HFF host cells. These HFF cells were grown in Dulbecco’s Modified Eagle Media (DMEM) containing 10% cosmic calf serum at 37°C in a 5% CO₂ environment. The HFF media was also supplemented with 50 µg/µL of streptomycin and 50 units/mL of antibiotic/antimycotic mix. The HFF medium was replaced with minimum essential media (MEM) containing 1% dialyzed fetal bovine serum (FBS) with appropriate antibiotics prior to parasite infection. Upon lysis of infected HFF cells, the parasites were harvested by centrifugation at 4,000×g, washed with phosphate buffered saline (PBS), and stored at -20°C.

2.4 Plasmid DNA preparation

Single bacterial colonies were inoculated and grown in 3 mL of LB broth containing the appropriate antibiotic of concentrations of 100 µM/mL, after which it was incubated overnight at 37°C while shaking at 250 rpm. 1.5 mL of this culture was then transferred to a 1.5 mL centrifuge tube and centrifuged at 13000 rpm for approximately 2 minutes. The supernatant was aspirated and 100 µL of ice cold 25mM Tris-HCl with a pH of 8.0 containing 10 mM EDTA was added, and the pellet was resuspended. 200 µL
0.2 N NaOH with 1% SDS was added and then the tube contents were mixed by inversion, after which they were left on ice for 3 minutes. 150 µL of ice cold NaOAc (pH 5.2) was added to the tubes, which were then mixed by inversion and left on ice for another 5 minutes. The tubes were then centrifuged at 12000 rpm for 15 minutes at 4 °C after which the supernatant was transferred to a fresh tube. 400 µL of TE-buffered phenol:chloroform:isoamyl alcohol (25:24:1) was added to the supernatant and the tubes were vortexed for 30 seconds, after which they were centrifuged at room temperature at 12000 rpm for a duration of 5 minutes. The top aqueous layer was then transferred to a fresh tube and 800 µL of 95% ethanol were added to it, following centrifugation at 12000 rpm for 15 minutes at 4 °C. The supernatant was discarded and 400 µL 70% ethanol were added followed by centrifugation at room temperature for 10 minutes. The supernatant was discarded once again and the pellet was allowed to dry in the fumehood for 10 minutes. Once the pellet had been given enough time to dry, 30 µL buffer containing 20 µg/µL RNase A was added and the tubes were then incubated at 37°C for a period of 15 minutes.

2.5 Agarose Gel Electrophoresis

1% agarose gels were prepared (0.4 grams to 40 mL TAE buffer) in order to visualize the size of various DNA fragments. Ethidium bromide was used in order to visualize the DNA fragments on the gel and it was added directly to the TAE buffer. The DNA samples were first mixed with 6X gel loading buffer containing 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF and 40% w/v sucrose, after which the gel was run at a voltage of 120 mV and upon completion the gel was visualized on the AlphaImager Imaging System using the provided software.
2.6 Restriction Enzyme analysis

For a 10 µL reaction pool, 2 µL of DNA was incubated with 1 µL of 10X enzyme buffer and 0.2 µL enzyme along with 6.8 µL of autoclaved Millipore water. The reaction mixture was incubated overnight at 37°C. The reaction pool was adjusted accordingly depending on the purpose, and post incubation, the fragments may be viewed using the agarose gel electrophoresis, they may be gel purified or they may be precipitated using ethanol as described in section 2.1.

2.7 Polymerase chain reaction

PCR used to generate DNA templates for in vitro transcription was performed in a total volume of 50 µL containing 34.1 µL of dH2O, 5 µL of Taq DNA polymerase buffer (20 mM ammonium sulphate, 75 mM Tris-HCl, pH 8.8), 5 µL of 25 mM MgCl₂, 0.5 µL of 2.5 mM dNTP mix, 2 µL of 10 pmol/µL of each oligonucleotide primer (5’ and 3’), 2µL of cDNA template, and 1U of Taq DNA polymerase. The PCR reaction was carried out in the 20-well Techgene thermal cycler, in 25 cycles (94°C for 15 sec, 62°C for 15 sec, 72°C for 1 min) followed by a final extension step at 72°C for 5 min. The PCR products were purified by phenol:chloroform precipitation and stored at -20°C until use.

2.8 Phenol:Chloroform Extraction

DNA/RNA samples were purified by phenol:chloroform extraction. All samples were diluted in dH₂O to a total volume of 200 µL and mixed with an additional 200 µL volume of phenol:chloroform:isoamyl alcohol (25:24:1 (v/v)) by vortexing for 30 seconds. The samples were centrifuged at 12,000×g at for 2 minutes at room temperature, and upon completion, the top aqueous layer of the suspension was collected in a separate tube,
followed by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.2 volume of 95% (v/v) EtOH. The tubes were gently mixed and allowed to incubate at -20°C for 20 minutes causing the precipitation of nucleic acids. The samples were then centrifuged at 12,000×g for 15 minutes at 4°C to collect the nucleic acid pellets. The supernatant was discarded and the pellets were washed with 2.2 volume of 70% EtOH, followed by centrifugation at 12,000×g for 10 minutes at 4°C. The tubes were inverted and the pellets were air dried to remove EtOH traces followed by the addition of 20-30 µL of DEPC dH2O. PCR products that were used as templates for *in vitro* transcription reactions were also resuspended in DEPC treated dH2O.

### 2.9 *In vitro* transcription

In order to test the RNA interference capability in *T. gondii*, several RNA species were synthesised by PCR from the UPRT open reading frame (ORF) in T7GGpUC18 plasmid and the HXGPRT ORF in ptub8MycHisGFP-HX, respectively. The PCR primers and their expected size is shown in the table below. Upon visualization of the complete and correct PCR product, phenol:chloroform extraction was performed in order to ensure an RNAse free template for the subsequent steps of *in vitro* transcription. The *in vitro* transcription reaction was performed for 4 hours after which the product was visualized followed by phenol:chloroform purification. The concentrations of RNAs obtained from *in vitro* transcriptions were calculated by taking absorbencies at A260 according to the formula: 

\[
[RNA] = (A_{260\text{nm}})(\text{dilution factor})(40 \, \mu\text{g/mL}) \quad \text{for sense and anti sense RNA}
\]

and

\[
[RNA] = (A_{260\text{nm}})(\text{dilution factor})(50 \, \mu\text{g/mL}) \quad \text{for double stranded RNA}
\]
<table>
<thead>
<tr>
<th>RNA</th>
<th>Primer Name</th>
<th>Sequence 5'-3'</th>
<th>Size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPRT dsRNA</td>
<td>T7 antisense UP</td>
<td>TAATACGACTCACTATAGGGTTC CAAAGTACCGGT</td>
<td>735</td>
</tr>
<tr>
<td></td>
<td>T7 promoter GG</td>
<td>TAATACGACTCATATAGG</td>
<td></td>
</tr>
<tr>
<td>UPRT ssRNA</td>
<td>UPRT-Pac I</td>
<td>CCCTTAATTAACACATG GTTCCAAGTACC</td>
<td>735</td>
</tr>
<tr>
<td></td>
<td>T7 promoter GG</td>
<td>TAATACGACTCATATAGG</td>
<td></td>
</tr>
<tr>
<td>UPRT asRNA</td>
<td>T7 antisense UP</td>
<td>TAATACGACTCACTATAGGGTTC CAAAGTACCGGT</td>
<td>735</td>
</tr>
<tr>
<td></td>
<td>Nsi-UPRT</td>
<td>TGCATGCATATAGGCAGGTC CAGGAGG</td>
<td></td>
</tr>
<tr>
<td>HX dsRNA</td>
<td>T7 on 5' HX</td>
<td>TAATACGACTCACTATAGGATGGCGTTC CAAACCCATTG</td>
<td>690</td>
</tr>
<tr>
<td></td>
<td>T7 on 3' HX</td>
<td>TAATACGACTCACTATAGGACCGGTGTCGACGTTC</td>
<td></td>
</tr>
<tr>
<td>HX ssRNA</td>
<td>T7 on 5' HX</td>
<td>TAATACGACTCACTATAGGATGGCGTTC CAAACCCATTG</td>
<td>690</td>
</tr>
<tr>
<td></td>
<td>HXGPRT reverse</td>
<td>ACCGCTGTCGACGTTC</td>
<td></td>
</tr>
<tr>
<td>HX asRNA</td>
<td>T7 on 3' HX</td>
<td>TAATACGACTCACTATAGGACCGGTGTCGACGTTC</td>
<td>690</td>
</tr>
<tr>
<td></td>
<td>HXGPRT forward</td>
<td>ATGGCGTCCAAACCCATTG</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.1 Primers used in the study.** PCR primer pairs required for constructing the templates used in *in vitro* transcriptions.

as = antisense strand; ss = sense strand; ds = double strand
**Figure 2.2 Visualization of PCR and *in vitro transcription* products.** Production of RNA species from PCR products containing T7 promoter on their 3’, 5’ or both ends: In panel A, the PCR templates were run to validate the size of DNA template encoding for UPRT dsRNA (1), UPRT ssRNA (2) and UPRT asRNA (3). The products run at the expected size of approximately 735 base pairs. Panel B depicts *in vitro* synthesized UPRT asRNA, UPRT ssRNA and UPRT dsRNA respectively.
2.10 dsRNA directed ribonuclease assay

This assay was designed to test for the overall RNAi activity of *T. gondii*. Two different parasite strains, RH and Ui, were used for the assay. Freshly released parasites were collected, washed in PBS and resuspended in 60 µL of an assay buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM DTT in DEPC treated dH₂O and supplemented with 5 µL of PMSF protease inhibitor. Lysis was performed by sonication at level 5 for 2 pulses of 5 seconds each (sonication was performed on ice), followed by centrifugation at 13,000×g for 15 minutes at 4°C to remove cell debris. Protein concentrations were assessed by Bradford assay. Each assay contained 0.375 - 1 µg of lysate and 65 pmols of ssRNA, asRNA or dsRNA (HX or UPRT) respectively, in a total volume of 65 µL at 37°C. Samples were taken at different times and the reactions were stopped by the addition of loading dye containing EDTA. To check that the substrate was cleaved by *T. gondii* lysates, a negative control containing only the tested RNA species in assay buffer was incubated alongside the other reaction pools until the last time point was collected.

2.11 dsRNA specific ribonuclease assay

This assay was designed to test for the Dicer activity of *T. gondii*. dsRNA UPRT was prepared as described above, followed by incubation with RH lysates. The concentration of protein and RNA used was determined as described above, and the assay used a total of 1 µg of protein lysate with 90 pmols UPRT dsRNA in assay buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT in DEPC treated dH₂O). Time points were collected every hour up to 5 hours, followed by 1 aliquot of over night incubation (18 hours). The
samples were then ran on 15% Acrylamide gels at a voltage of 140 V for 1.5 hours, followed by Sybr Gold staining and visualization.

2.12 Recombinant protein purification

2.12.1 Transformation of competent bacteria with plasmids

BL21 competent cells were transformed with various expression plasmids, including those used for the induction and purification of the recombinant protein TgAgo. 25 µL competent cells were mixed with 2 µL plasmid and allowed to incubate on ice for 20 minutes. The transformation reaction was then heat shocked at a temperature of 42°C for 45 seconds, after which they were placed on ice for 2 minutes. 400 µL of SOB media was heated to 42°C and added to the transformation reaction, after which it was incubated at 37°C for 30 minutes, shaking at 250 rpm. After the incubation step was complete, the tubes were centrifuged at 5000 rpm in order to concentrate the transformation products to 100 µL, after which it was plated on plates containing the appropriate antibiotic at concentrations of 100 µg/µL. The plates were then incubated overnight at 37°C.

2.12.2 GST-TgAgo and HisTgAgo plasmids

A graphical representation of the plasmids used may be found in the Appendix section. These plasmids were constructed by Karen Cozens and by Ashley Dresser and the full information may be found in their respective thesis.
2.12.3 Optimizing protein induction

Initially, 15 mL LB media were incubated with a single colony of BL21 *E. coli* containing the plasmid of interest was grown overnight at 37°C with 250 rpm shaking, after which it was evenly distributed into three 250 mL flasks containing 100 mL LB media and allowed to grow until the cultures reached an optical density of 0.4. Once this point was reached, varying IPTG concentrations, namely 1 μM, 5 μM and 10 μM were added to each of the three flasks respectively and the cultures were allowed to grow in the same conditions for 3 hours.

2.12.4 Bacterial cell lysis

To confirm the success of induction, a 1 mL aliquot of induced cell culture was tested. The 1 mL aliquot was pelleted by centrifugation at 18,000×g for 5 minutes and the pellet was resuspended in 50 μL of MCAC buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 1 mM PMSF). The lysate was sonicated at level 5 for 3 pulses of 5 seconds each (sonication was performed on ice) and centrifuged at 18,030×g for 5 minutes at 4°C. The supernatant (soluble fraction) was collected, and the insoluble fraction was resuspended and sonicated once more following the same procedure as above.

2.12.5 Glutathione-agarose purification of GST tagged TgAgo

After the sonication step, the tubes containing the lysed pellets were centrifuged at 10000 rpm for 15 minutes at 4°C, after which the supernatant was collected. 1 mL of the supernatant was saved for SDS-PAGE analysis, while the rest was incubated with the Glutathione-agarose beads.
Initially, the Glutathione-agarose beads were washed in Binding Buffer made up of 10 mM Tris and 150 mM NaCl, after which the supernatant was incubated in the column for 3 hours to overnight at 4°C. When the incubation was complete, 1 mL of the incubated supernatant was saved for future SDS-PAGE analysis. The column was then washed three times with 10 mL of Binding buffer containing 1% Triton 100%, and 1 mL of each of these fractions was saved. Elution of the GST tagged proteins was achieved by washing the column with 10 mM reduced glutathione in 50 mM Tris-base (pH=9.0). 4 elution volumes of 1 mL each were saved for SDS-PAGE analysis. The column was then washed according to the manufacturer's protocol (Sigma).

2.13 Bradford assay

Protein concentration was determined by the Bradford method using the Genesys 10 UV-visible spectrophotometer. Protein samples were diluted in dH2O up to a volume of 0.5 mL and mixed with 1.5 mL of diluted Bradford reagent. Similarly, a set of standard protein solutions ranging from 0 - 10 µg/mL was prepared with diluted 100X BSA according to Bio-Rad’s Bradford reagent instructions. All absorbencies were measured at 595 nm. A standard curve was constructed to allow subsequent determination of the protein concentration in the tested samples (Appendix D).

2.14 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted with a discontinuous buffer system with the stacking and resolving gels cast vertically. The resolving gel solution (2.58 ml ddH2O, 1.8 ml 30% Acrylamide solution, 1.5 ml 1.0 M Tris pH 8.8, 60 µl 10%SDS, 60 µl 10% ammonium persulfate, 5 µl
TEMED) was poured first and left to polymerize before being overlaid by the stacking gel solution (1.46 ml ddH₂O, 0.25 ml 30% acrylamide solution, 0.25 ml 1.0M Tris-HCl pH 6.8, 20 µl 10% SDS, 20 µl 10% ammonium persulfate, 3 µl TEMED).

The protein samples were mixed with gel loading buffer containing 6.25 mM Tris-HCl (pH 6.8), 2% SDS, 10% glucose, 0.05% Bromophenol blue and 720 mM 2-mercaptoethanol, boiled for 5-10 minutes and loaded onto the SDS-PAGE gel. The gel was run in Tris-glycine running buffer containing 196 mM glycine, 25 mM Tris-HCl (pH 8.4), 0.1% SDS, at 140 volts until the bromophenol blue dye reached the end of the gel.

2.15 Coomassie brilliant blue staining

After SDS-PAGE, the gel was submerged in Coomassie staining solution (2.5 g/L Coomassie R-250 in 40% methanol and 10% (v/v) glacial acetic acid) and allowed to stain for 1 hour upon which it was transferred into destaining solution (40% (v/v) methanol and 10% (v/v) acetic acid) until protein bands were clearly seen. The gels were visualized and their images stored using AlphaImager 2200 Light Imaging system.

2.16 Solubilization of recombinant protein from inclusion bodies

A 50 mL aliquot of induced culture was pelleted by centrifugation at 6,500×g for 15 minutes at 4°C and the supernatant was discarded. The pellet was resuspended in 0.1 culture volume of IB wash buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% Triton X-100) and the cells were incubated on ice for 30 minutes with 100 µg/mL lysozyme. The mixture was then transferred to the French Press and the lysis of the suspended cells followed at 1200 psi. The supernatant was stored for future analysis. The pellet was then subjected to two successive steps of sonication, followed by centrifugation at 10,000×g
for 10 minutes and resuspension in 0.1 culture volume of IB wash buffer. The washed inclusion bodies were resuspended in IB solubilization buffer (50 mM CAPS, pH 11.0, 0.3% N-lauroyl sarcosine, 1mM DTT) to a final concentration of 10 mg/mL and incubated at room temperature for 15 minutes. Non-solubilized debris was precipitated by centrifugation at 10,000×g for 10 minutes at room temperature and the supernatant containing the solubilized protein was collected.

2.17 Protein refolding

In order to remove the N-lauroyl sarcosine and CAPS detergents, the solubilized protein was subjected to dialysis overnight in 100 volumes of dialysis buffer (20 mM Tris-HCl, pH 8.5, 0.1 mM DTT) at 4°C. A second dialysis step was performed with 100 volumes of dialysis buffer lacking DTT for 6 hours at 4°C followed by a third dialysis step overnight in the presence of 1 mM reduced and 0.2 mM oxidized glutathione to promote refolding into a native-like structure through disulfide bridge formation. PMSF was added to a final concentration of 10 µg/mL and the final dialysate was stored at 4°C, followed by a Bradford assay to determine the protein concentration.

2.18 Western blot analysis

Proteins resolved by SDS-PAGE were transferred onto a nitrocellulose membrane using the Mini-Electrophoretic Blotting System. Electro-blotting was carried out in transfer buffer (192 mM glycine, 25 mM Tris-HCl, pH 7.4, 20% (v/v) methanol) at 80 Volts for 1.5 hours under constant temperature of 1.5°C. The efficiency of the transfer was checked by staining the membrane in Ponceau S stain (30% (v/v) sulfosalicylic acid, 30% (v/v) trichloroacetic acid, 0.02% (w/v) Ponceau S), followed by blocking over night
at 4°C using blocking solution (5% (w/v) skim milk in Tris-Buffered Saline Tween-20 (TBST)). Two 5 minute wash steps in TBST were performed in order to remove excess milk proteins, followed by incubation for 1 hour at room temperature with primary antibody in 2% skim milk in TBST, then by a set of four washes in TBST for 15, 5, 5, and 5 minutes, respectively. The blot was then incubated for 30 minutes at room temperature in 2% milk in TBST containing secondary antibody specific to the primary antibody used. Another set of four washes in TBST for 15, 5, 5, and 5 minutes, respectively was carried out, followed by probing with chemiluminescence reagent.

2.19 Gel shift assay

In order to determine the binding capacity of the isolated recombinant protein to various RNA species, a gel shift assay was performed. 5 μg of isolated recombinant protein were incubated with RNA in a buffer consisting of 160 mm NaCl, 30 mM Tris (pH 8), 5 mM CaCl₂, 0.1 mM EDTA, 0.1 mM DTT and 5% (v/v) glycerol. The samples were first incubated at 37°C for 10 minutes, followed by a incubation period of 20 minutes on ice. The samples were resolved on a 6% polyacrylamide non-denaturing gel (acrylamide: bisacrylamide 80:1, 0.5X Tris Borate and 5 mM CaCl₂). The gel was run for 20 minutes at constant voltage (160 V), after which the samples were loaded and the gel was ran for an additional 2 hours. The gel was then stained using Sybr Gold and visualized.
2.20 RNase A protection assay

Purified TgAgo was incubated in the same binding buffer as described above with ssRNA, followed by the addition 1 μl of diluted RNase A (0.003μg/mL). The reaction mixture was then incubated for 30 minutes at 37°C followed by visualization on a 6% denaturing gel (acrylamide: bisacrylamide 80:1, 8 M urea, 1X Tris Borate EDTA and 5 mM CaCl₂). The gel was ran as described above and visualized using Sybr Gold.
CHAPTER III
RESULTS

3.1 Bioinformatic studies of RNAi components in *T. gondii*

RNAi related genes such as a PIWI-PAZ containing protein have been previously reported in *T. gondii* through bioinformatics studies. Our lab however obtained and characterized the Argonaute protein and it was established that it lacks a PAZ domain (Al-Riyahi *et al.*, 2006). Furthermore, preliminary data suggested that this homolog may be a ribonuclease slicer component of RNAi in this parasite. Using a bioinformatics approach other putative RNAi components of *T. gondii* were identified using the well described RNAi protein members of *C. elegans*, and from other organisms.

Sequences of well annotated genes were obtained by performing a search in the NCBI database and the resulting protein sequences were subsequently used to scan the ToxoDB database for putative homologs using the blastp feature. A descriptive list of the genes used and their homologs is displayed in Table 3.1. It should be noted that the searches yielded more than one protein at times however the lowest score was selected in choosing possible protein homologs since a low score indicates higher confidence. As it can be seen in Table 3.1, some proteins have high scores, and they can be disregarded as possible homologs.

The rsd-3 (RNAi spreading defective 3) protein which has been postulated to be a nucleoside transporter protein in *C. elegans* was initially used to scan ToxoDB for putative homologs. EPN3 (epsin 3) protein was identified with high confidence, displaying 31% identity to the input sequence. The database accession number for EPN3 protein was TGME49_014180. Even though the confidence score is relatively high and
there is some amino acid overlap, it is unlikely that the function of rsd-3 is similar to that of EPN3, with the latter being involved in lipid binding. Next, the dcr-1 (Dicer 1) protein sequence was used to obtain possible *T. gondii* homologs. 22 possible candidates were obtained, however most of the scores were largely insignificant. A putative helicase (TGME49_067030) was obtained with the top score and the predicted roles of this protein as described by the ToxoDB website include nucleic acid binding, ATP binding, helicase activity and ATP-dependent helicase activity. The same putative helicase was obtained when the drh-1 (Dicer related helicase 1) protein sequence was used however the score was slightly lower than the previous search. Moreover, the dicer (qde-3) sequence from *N. crassa* was used and it produced 30 possible candidates. The most significant candidates include 3 putative DEAD/DEAH box helicase (TGME49_064840, TGME49_106080 and TGME49_116300), one of the key feature of RNase III domain proteins, as well as a putative ATP-dependent DNA helicase (TGME49_059250) closely related to RecQ-like protein 5 in *Canis familiaris*. Although a total of 5 putative dicer candidates have been obtained, it is unlikely that they are all functional proteins, and it is expected that if one of these proteins participates the RNAi mechanism of *T. gondii*, it will most likely be one of the DEAD/DEAH box helicase candidates.

Next, the mut-14 (MUTator) protein sequence, responsible for transgene silencing, was analyzed resulting in 37 candidates, however the top protein score was obtained for a putative ATP-dependent RNA helicase (TGME49_026250). It is predicted that this putative protein is able to bind nucleic acids and ATP, carry out helicase activity and ATP-dependent helicase activity. Another RNAi component of *C. elegans*, namely the rha-1 (RNA helicase 1) protein was blasted in ToxoDB, yielding 8 putative homologs. The rha-1 protein is generally involved in regulation of transcription, translation and
RNA processing, possessing a well defined ATP-dependent DEAD/H box RNA helicase. The top candidate was a putative ATP-dependent RNA helicase (TGME49_112280) with similar predicted functions to the one described previously.

The egl-13 (EGg laying defective) protein sequence was inputted and produced 3 candidates in *T. gondii*. These 3 candidates were remarkably similar in that they were all characterized by an HMG-box. The accession number for the top hit was TGME49_063720 and the predicted function for this protein was associated with regulation of transcription. The gfl-1 (human gas41-like) protein has been associated with potent RNAi suppression upon its removal, suggesting that it is a very important RNAi component in *C. elegans*. A single *T. gondii* homolog to gfl-1 was identified to be a hypothetical protein (TGME49_015730). The predicted function of this hypothetical protein was linked to transcriptional regulation.

Several key proteins were also tested, namely the alg-1 (Argonaute like 1), alg-2, ppw-1 (PAZ-PIWI domain containing), ppw-2 and rde-1 (RNAi deficient 1). These proteins converged on the same *T. gondii* PIWI-PAZ domain containing protein (TGME49_110160). The Argonaute proteins alg-1 and alg-2 regulate the expression of RNA species involved in the proper development of the nematode and are also actively implicated in the RNAi mechanism. The ppw-1 and ppw-2 proteins are involved in transposon regulation, while the rde-1 protein has been postulated to participate downstream from the siRNA producing step. Moreover, the rde-1 protein in *C. elegans* is an absolute requirement for RNAi. It should be noted that the highest probability score for the PIWI-PAZ domain containing protein was obtained using the alg-1 sequence, suggesting that the Argonaute protein in *T. gondii*, much like the one in *C. elegans* emerged from a plant lineage.
Searches using the smg-2 (Suppressor with morphological effect on genitalia) protein sequence resulted in 8 putative homologs. Smg-2 in *C. elegans* is responsible for mRNA surveillance, and its top homolog in *T. gondii* was determined to be a putative regulator of nonsense transcripts UPF1 (TGME49_091820). Another key component of the RNAi mechanism surfaced upon searches using ego-1 (Enhancer of Glp-One), rrf-1 (RNA-dependent RNA polymerase family) and rrf-3 converging on a RNA-dependent RNA polymerase domain-containing protein (TGME49_017190). RNA-dependent RNA polymerases play an active role in *C. elegans* in terms of transitive interference and maintenance of somatic and germline RNAi, suggesting that although an amplification in the RNAi signal has not been documented in *T. gondii*, it may have the capability to generate dsRNA from ssRNA templates. Another interesting find was the emergence of a SET domain-containing protein (TGME49_057770) from the query of mes-2 (Maternal Effect Sterile) and mes-4. In *C. elegans*, these proteins actively participate in the repression of the X chromosome and chromatin regulation respectively. Moreover, this result is indicative of possible chromatin repression and transgene monitoring in *T. gondii*.

The tsn-1 (tudor staphylococcal nuclease) protein was also tested, and surprisingly yielded 2 homologs, the most significant of which was the tudor/staphylococcal nuclease domain-containing protein (TGME49_038050). Tsn-1 is generally associated with the RISC complex in *C. elegans* and is required from proper let-7 miRNA function, indicating that miRNAs may be present in *T. gondii* and more importantly, that the RNAi-like activity observed in this parasite may be due to a similar complex containing both the Argonaute and the tudor proteins.
Figure 3.1 RNAi related genes in *T. gondii*. Possible components of the RNAi mechanism in *T. gondii*. Protein sequences from various organisms were inputted in the blastp feature of ToxoDB and putative homologs in *T. gondii* were obtained. The putative functions of these proteins were also obtained.
3.2 dsRNA-directed ribonuclease activity in *T. gondii*

3.2.1 dsRNA-directed ribonuclease activity in Ui, RH and HFF lysates

To test for the ribonuclease activity, the parasite lines RH and Ui were used. The RH parasite line is the parental line of Ui and it is sensitive to FDUR. The Ui parasite strain is a stable transgenic strain that was created in our laboratory from parental RH, designed to constitutively express double stranded RNA homologous to the *UPRT* gene under selection with FDUR, while parasites lacking the plasmid expressing dsRNA UPRT will perish under this selection. Fresh parasite pellets were lysed in an assay buffer consisting of 20 mM Tris pH 7.5, 1 mM DTT and a Bradford assay was performed in order to quantify the protein concentrations. Equal protein concentrations of Ui and RH (1μg total lysate) were incubated with equal concentrations of ssRNA UPRT (100 pmols). Representative gels are displayed in Figure 3.2. Relative Fluorescence Units (RFU) readings were obtained by measuring subtracting the background reading from the sample using the ImageJ program. The dsRNA UPRT producing parasite line Ui exhibits a ribonuclease activity specific to ssRNA UPRT. Rapid cleavage of the substrate was observed within the first 10 minutes of the experiment, with an observed rate of 71.8 μM.min⁻¹.mg⁻¹ (upper panel). The observed rate in the Ui fraction over the course of the experiment was 20.9 μM.min⁻¹.mg⁻¹. On the other hand, the RH lysates did not show the same ribonuclease activity throughout the experiment (lower panel). This data indicated that the parasite unable to express dsRNA UPRT (RH) is unable to exhibit ribonuclease activity toward ssRNA UPRT substrate under the tested conditions. Therefore, these assay conditions were used for further studies to evaluate the dsRNA-directed ribonuclease activity of *T. gondii*. 
Figure 3.2 Cleavage profile of Ui and RH lysates. Assay conditions were initially tested to determine if Ui parasite line exhibits dsRNA-directed Ribonuclease activity towards the UPRT ssRNA substrate. UPRT ssRNA was incubated with lysates from Ui and RH parasites. The effect of the RNAi machinery on the UPRT ssRNA substrate is displayed. The above data represent an individual experiment only, and the initial rate of degradation of UPRT ssRNA substrate is displayed for the Ui parasite line.
*T. gondii*, an intracellular parasite, was cultured on HFF monolayers. Due to this, the observed rate in dsRNA-directed ribonuclease activity may be skewed due to the presence of host cell protein contamination. Although some preliminary data indicate that HFF cells displayed a weak dsRNA-directed ribonuclease activity, the capability of the HFF lysates to cleave UPRT ssRNA was tested. To achieve this, three reaction pools were set up, one for the Ui parasite line and two for the HFF cell line. The lysate for one of the HFF reactions was supplemented with the initiator (UPRT dsRNA) and incubated to allow the formation of potential siRNAs, while the other HFF reaction pool consisted of HFF lysate, buffer and UPRT ssRNA.

The results are displayed in Figure 3.3. HFF lysates that were not supplemented with UPRT dsRNA lacked the capability of cleaving the UPRT ssRNA substrate (middle panel). When HFF lysates were pre-incubated (primed) with UPRT dsRNA, degradation of target UPRT ssRNA was observed. The observed cleavage rate obtained was 16.8 μM.min⁻¹.mg⁻¹ (bottom panel). Furthermore, the Ui lysates displayed the highest observed dsRNA-directed ribonuclease rates calculated to be 48.8 μM.min⁻¹.mg⁻¹ (upper panel). The data suggests that the ribonuclease activity monitored here was due to that of the Ui lysates, with the HFF lysates displaying minimal activity when primed.
Figure 3.3 dsRNA-induced ribonuclease activity in HFF cells. Analysis of the dsRNA-directed ribonuclease activity in the Ui parasite line alongside the HFF host cells. 65 pmol of UPRT ssRNA substrate were added to 0.125 μg lysates from Ui parasites, as well as to primed HFF lysates (UPRT dsRNA pre-incubated) and HFF lysates. The data was obtained from one experiment and the observed rates were calculated for each lysate.
3.2.2 The requirements of dsRNA-directed ribonuclease activity

Earlier studies in model organisms such as *C. elegans* and *Drosophila* showed the importance of divalent metal ion cofactors, namely magnesium ions, in the classical RNAi pathway of these. To determine whether magnesium ions play a role in the dsRNA-directed ribonuclease activity observed in *T. gondii*, an experiment was designed in which three Ui cell free lysates were respectively (i) unsupplemented, (ii) supplemented with EDTA in order to chelate divalent ions present, and (iii) supplemented with magnesium chloride. The results are displayed in Figure 3.4. The chelation of divalent ions through the addition of EDTA suppressed the cleavage of UPRT ssRNA target (middle panel), while the addition of MgCl$_2$ enhanced the cleavage of UPRT ssRNA target (bottom panel) with respect to the untreated Ui lysates (top panel). Furthermore, the calculated cleavage rate for the MgCl$_2$ supplemented Ui lysates was 33.6 $\mu$M.min$^{-1}$.mg$^{-1}$ with respect to the 20 $\mu$M.min$^{-1}$.mg$^{-1}$ observed in the untreated reaction pool indicating that the addition of magnesium ions enhances the cleavage of UPRT ssRNA substrate.

The experiment was performed in triplicate however the results show some variation in their observed rates. The observed rates over the three trials were 33.6 $\mu$M.min$^{-1}$.mg$^{-1}$, 77.5 $\mu$M.min$^{-1}$.mg$^{-1}$ and 37.5 $\mu$M.min$^{-1}$.mg$^{-1}$ respectively. This significant deviation can be attributed to the quantification method used and the inaccuracy of the relative fluorescence unit readings. This can also be seen in the middle panel in which Ui lysates were pre-treated with EDTA, and although no cleavage is taking place, the fluorescence unit readings suggest an increasing trend. This increasing trend suggests that target RNA was added over the time course of the experiment however this is not the case.
Figure 3.4 Effects of divalent ions on dsRNA-directed ribonuclease activity. Lysates were pretreated for 30 minutes with EDTA (middle panel) or MgCl₂ (lower panel) and UPRT ssRNA cleavage was tested with respect to the control Ui (upper panel). 65 pmol of UPRT ssRNA substrate were added to 0.125 μg lysates from Ui parasites. Although the experiment was performed in triplicate, only representative gel images are shown above. Large variations within the readings were observed hence the rates were calculated for each individual experiment.
3.2.3 Specificity of dsRNA-directed ribonuclease activity in *T. gondii*

It has been previously reported that the dsRNA-directed ribonuclease in many organisms is highly specific and can be directed for homologous mRNA (Martinez *et al.*, 2002). In the second step of the classical RNAi mechanism, an Argonaute protein promotes RISC complex formation through binding to siRNAs (Ma *et al.*, 2005); (Preall *et al.*, 2006). Upon formation, the passenger strand (sense strand of siRNA) is expelled, leaving behind the guide strand (antisense strand). Moreover, it has been shown that this preferential loading takes place on the RISC complex and the targeted RNA specie is generally the sense strand RNA (Ma *et al.*, 2005).

In order to test the specificity in the dsRNA-directed ribonuclease activity of *T. gondii*, Ui lysates were incubated with full length UPRT ssRNA and UPRT asRNA respectively for 2 hours. Figure 3.5 shows representative gel images depicting the preferential cleavage by the dsRNA-directed ribonuclease activity, completely degrading the UPRT ssRNA, while leaving the UPRT asRNA intact for the course of the experiment. This specificity in cleavage activity can be attributed due to the fact that the UPRT ssRNA mimics the normal mRNA of this gene allowing its specific degradation (upper panel), while the UPRT asRNA is unable to bind to the siRNAs available (lower panel). Another observation that arises from this experiment is that due to ssRNA degradation, the siRNA moiety that aids in the degradation of the substrate has to be the antisense siRNAs produced in the Ui line. The observed cleavage rate in this experiment was 36 μM.min⁻¹.mg⁻¹. The triplicate average rate was 35.2 ± 2.4 μM.min⁻¹.mg⁻¹.
Figure 3.5 Substrate specificity for dsRNA-directed ribonuclease activity. Full length UPRT ssRNA (panel A) and UPRT asRNA (panel B) were tested with the Ui parasite line. This was done to determine whether or not preferential targeting of substrate RNA takes place within the dsRNA-directed ribonuclease activity of *T. gondii*. 65 pmol of UPRT ssRNA and UPRT asRNA substrate, respectively were added to 0.125 μg lysates from Ui parasites The gels above are representative of only one experiment performed. The observed cleavage rate in this experiment was 36 μM.min\(^{-1}\).mg\(^{-1}\). The triplicate average rate was 35.2 ± 2.4 μM.min\(^{-1}\).mg\(^{-1}\).
3.2.4 Inducing dsRNA-directed ribonuclease activity in RH lysates

The inability of the RH lysates to undergo dsRNA-directed ribonuclease activity towards a target RNA was documented in section 3.1. However the parental strain RH should have the capability to exhibit dsRNA-directed ribonuclease activity if the dsRNA precursors are provided. To verify this, RH lysates were pre-incubated with UPRT dsRNA 30 minutes prior to the addition of ssRNA. Additionally a reaction pool consisting of Ui lysate was used as a control. Representative gels are displayed in Figure 3.6. These gels distinctly show a cleavage of target UPRT ssRNA substrate in both the untreated Ui and the UPRT dsRNA primed RH reactions with calculated rates of 44 μM.min⁻¹.mg⁻¹ and 59.2 μM.min⁻¹.mg⁻¹ respectively. This suggests that once the dsRNA precursor is present, the RH lysates have the capability to recognize and cleave the ssRNA substrate, provided that it is homologous to the initiator.

A close correlation between the Ui and the primed RH lysates also appeared upon the completion of several trials. The observed average rate for the Ui lysates was calculated to be 44.09 ± 8.06 μM.min⁻¹.mg⁻¹, while the observed average rate for the primed RH lysates was 41.33 ± 8.67 μM.min⁻¹.mg⁻¹. The variation between the two observed rates is largely insignificant, suggesting that the cleavage of the UPRT ssRNA substrate is due to the production of siRNAs from a UPRT dsRNA precursor. Furthermore, this suggests that mechanistically, the same set of proteins act on the target RNA in both lysates given the proximity in the two rates observed.
Figure 3.6 Effect of priming RH lysates. Previously it was observed that pre-incubation of HFF lysates with UPRT dsRNA promotes dsRNA-directed Ribonuclease activity in these lysates, hence RH lysates were also tested for this by pre-incubating them with 0.5 pmols UPRT dsRNA. 65 pmol of UPRT ssRNA substrate were added to 0.125 μg lysates from Ui parasites and RH parasites respectively. Priming of RH lysates with UPRT dsRNA promotes cleavage of target UPRT ssRNA substrate (lower panel). The above gels were obtained from a single experiment, however this was performed in triplicate. The observed average rate for the Ui lysates was calculated to be 44.09 ± 8.06 μM.min⁻¹.mg⁻¹, while the observed average rate for the primed RH lysates was 41.33 ± 8.67 μM.min⁻¹.mg⁻¹.
To test whether this observed cleavage of the target UPRT ssRNA is a direct result of the added UPRT dsRNA, an experiment was designed in which the initiating dsRNA specie was tested. Two reaction pools containing 0.25μg total RH lysates were pre-incubated with UPRT dsRNA and HX dsRNA respectively. To these reactions, 50 pmols of HX ssRNA substrate were added. Gel representatives of this experiment are shown in Figure 3.7. In the upper panel of Figure 3.7, no cleavage was observed based on the relative fluorescence unit readings, which show an increasing trend as time elapses. The lower panel of the same figure however displays target HX ssRNA degradation caused by the homologous inducer, HX dsRNA. The observed rate for this last experiment was 36.6 μM.min⁻¹.mg⁻¹, suggesting that the value falls within the rates obtained previously for the Ui lysates and the RH lysates that were primed with UPRT dsRNA. This result indicates that the homology between the inducing specie and the target RNA specie is a key aspect to the ribonuclease activity observed.

Similarly, RH lysates were primed with HX dsRNA and tested against UPRT ssRNA and UPRT asRNA (Figure 3.8) in order to show the role of homology between the initiator molecule and the target molecule. Representative gels are shown Figure 3.8 and no ribonuclease activity is detected in either reaction. This is due to the incompatibility of siRNAs produced from HX dsRNA and the substrates tested, which serves as evidence for a highly specific RNAi like mechanism in T. gondii that relies heavily on the complementary of siRNAs and the specific target ssRNAs.
Figure 3.7. Initiator specificity in dsRNA-directed ribonuclease activity. Panel A depicts 0.125 μg RH lysates pre-treated with 0.5 pmols of UPRT dsRNA for 30 minutes, after which 50 pmol HX ssRNA substrate was introduced. 50 pmols HX ssUPRT substrate were then added to each reaction. Panel B shows RH lysates primed with 0.5 pmol HX dsRNA for 30 minutes, upon which HX ssRNA substrate was added to the reaction. Degradation is observed only in panel B. The above gel images are obtained from a single experiment.
Figure 3.8 Initiator and substrate specificity in dsRNA-directed ribonuclease activity. RH lysates were primed with 0.5 μg HX dsRNA and tested against UPRT ssRNA and UPRT asRNA in order to show the role of homology between the initiator molecule and the target molecule. RH lysates were pretreated with HX dsRNA for 30 minutes, after which 50 pmol UPRT ssRNA (top) and 50 pmol UPRT asRNA (bottom) substrates was introduced. No degradation is observed in either reaction.
3.3  dsRNA-specific Ribonuclease Activity in *T. gondii*

One of the hallmarks of the classical RNAi mechanism is the conversion of long double stranded RNA into siRNAs. A set of putative dicer proteins have been reported in *T. gondii* through bioinformatics, however they remain uncharacterized. Dicer like enzyme activity which produces the siRNA substrate was evaluated.

3.3.1  Production of siRNA from dsRNA

In this study, RH lysates were incubated with UPRT dsRNA over the course of 18 hours with aliquots removed at different time intervals. A total 1 µg of lysate was incubated with 35 pmols UPRT dsRNA in assay buffer containing 20 mM Tris, 1mM DTT, pH 7.5. The reaction was stopped by the addition of equal volume of loading dye.

Figure 3.9 displays the time course degradation of UPRT dsRNA. From this figure, a distinct band cannot be observed anywhere in the region of 21-26 nucleotides, however the smear is a direct result of the degradation. Furthermore, by comparison to earlier time points, the 18 hour mark displays a distinct accumulation of product between the primer derived marker region consisting of 20 and 39 base pairs respectively. The bottom part of the graph was further altered to display the observed accumulation effect (Panel A).

The same experiment was performed in panel B, however this yielded different results. Under the same assay conditions, a distinct accumulation of product accumulates (denoted by the asterix sign) in a region much higher than the expected region of 21-39 nucleotides, however no siRNAs are visible.
Figure 3.9 Production of siRNAs from long dsRNA precursors. Time course degradation of full length UPRT dsRNA in the presence of RH lysate was observed. A total 1 μg of lysate was incubated with 35 pmols UPRT dsRNA, upon which aliquots were removed at pre-determined times. The last lane consists of 2 primers of known size, 39 base pairs and 20 base pairs respectively which serves as a ladder. A distinct accumulation of siRNA-like molecules is observed above the 20 base pair primer in the last lane. Furthermore, the second gel depicts the formation of a intermediate RNA specie, possibly due to the dsRNA-specific Ribonuclease activity in *T. gondii*. The last lane was assigned to UPRT dsRNA that was not incubated with lysates.
3.4 TgAgo recombinant protein expression and purification

The Argonaute protein in *T. gondii* has not yet been characterized in detail. With the use of a GST-TgAgo and a His-TgAgo construct, we aimed to obtain the protein in high quantity and in its active state to test its binding capacity to RNA species and to obtain kinetic insight into the overall mechanism.

3.4.1 Optimization of GST-TgAgo induction in *E. coli*

In order to over-express the protein of interest, the IPTG inducer concentration was tested to determine the lowest effective concentration at which over-expression was taking place. To achieve this, different concentrations of IPTG, ranging from 5 µM to 100 µM, were used to induce equal volumes of *E.coli* cultures at an optical density of 0.6. Upon induction, the bacterial cultures were incubated for a further 3 hours at 37°C, after which they were collected by centrifugation. Each individual culture was then lysed by sonication and the supernatant and the pellet were isolated through centrifugation. A small aliquot of each supernatant and pellet were then treated with SDS loading dye, ran on a 10% polyacrylamide gel and stained with Coomasie Brilliant Blue in order to visualize the protein of interest. Initially, the experiments were carried out for a GST-tagged version of TgAgo, which can be seen at 89 kDa on the Coomasie stained gels. From this experiment, it was found that optimal induction was achieved using 5 µM of IPTG however a more worrisome outcome was the fact that the majority of the protein of interest was found in the pellet or inclusion bodies. As a control, an uninduced sample was visualized along side to the induced cultures, and as expected, no prominent bands around 89 kDa can be observed (Figure 3.10).
Figure 3.10 Optimization of IPTG concentrations. *E. coli* cells were grown to an OD of 0.6, after which they were subjected to different IPTG concentration treatments as indicated in the table above. It was observed that the induced protein localizes into inclusion bodies.
Due to the abundant formation of inclusion bodies containing the protein of interest, several other factors must be altered in order to achieve protein solubility since solubility would imply a native like structure for TgAgo. Another parameter that can be altered is the optical density at which the *E. coli* cells are grown. Bacterial cultures were grown to optical densities of 0.4, 0.6 and 0.8, measured at $A_{600}$, after which they were induced with the optimal 5 μM IPTG observed in the previous experiment. Upon induction, these cultures were allowed to incubate for the appropriate time upon which the bacteria were collected as described above. An aliquot of uninduced bacterial culture was saved and ran alongside the induced samples which would serve as an induction control. The induced samples were further separated in supernatant and pellets in order to check for the emergence of inclusion bodies containing the protein of interest.

From Figure 3.11, it is observed that optimal induction takes place at the first two optical densities of 0.4 and 0.6 however the TgAgo protein is still being packaged into inclusion bodies. Insignificant levels of TgAgo can be visualized in the supernatant fraction. To avert inclusion bodies, a third experiment was designed in which the induction temperature was tested. Hypothetically, if *E. coli* cells are induced at a minimal optical density with a minimal concentration of inducer at low temperatures, proteins that usually associate into inclusion body aggregates tend to become more soluble.

To test the effect of temperature on induction and to assess whether the protein of interest is becoming soluble, two *E. coli* cultures were grown to an OD of 0.4, followed by induction with 5 μM IPTG. Upon induction, the two cultures were incubated at room temperature (25°C) and at 37°C.
Figure 3.11 Optimization of bacterial growth and temperature. The optical density and temperature parameters were altered to determine if an increase in solubility is observed on TgAgo. It was observed that the induced protein localizes into inclusion bodies.
The Coomassie stained gel in Figure 3.11 reveals that there is no clear distinction between the supernatant samples of the induced cultures and the uninduced culture, with the protein of interest, TgAgo was still found in the inclusion body aggregates. Theoretically, the factors that affect the induction of a protein of interest in E. coli are optical density of the bacterial culture, the temperature at which the induction is taking place, the induction time and inducer concentration. The only parameter that remained to be tested was induction time. For this, bacterial cultures were grown to an optical density of 0.4, after which they were induced with 5 \( \mu \text{M} \) IPTG and further incubated for time periods of 3 hours, 5 hours, 7 hours and overnight, both at 37 °C as well as room temperature. The results revealed that the protein in question still localized into inclusion bodies.

Several experiments were then designed in order to obtain the protein of interest in the soluble fraction. The optical densities of cultures taken were 0.4, 0.6 and 0.8 respectively, all induced with varying IPTG concentrations, after which they were incubated at different times and different temperatures. This mix and match approach did not yield any significant results as the pattern of insoluble GST-TgAgo continued to emerge. It should be noted that the same experimental approach was taken when dealing with the His tagged version of the protein of interest and the results demonstrated that the His-TgAgo protein is packaged into inclusion bodies as well. Figure 3.12 exemplifies the change in only one parameter, namely the IPTG concentration, while performing the induction at room temperature, overnight.
Figure 3.12 Protein over-expression at room temperature. Induction at room temperature, overnight with varying IPTG concentrations. It was observed that the induced protein localizes into inclusion bodies.
3.4.2 Solubilization of inclusion bodies and refolding

Harvested inclusion bodies from an induced culture were washed several times and then solubilized under mildly denaturing conditions at pH 11.0 maintained by CAPS zwitterionic buffer. N-lauroyl sarcosine detergent was then added in order to disrupt hydrophobic aggregation, a key aspect of inclusion bodies. A reducing agent such as DTT was added in order to disrupt intermolecular disulfide bridges. At this point, the protein was collected and visualized with the use of Coomassie stain on a 10% polyacrylamide gel.

Due to the large number of proteins present in the newly solubilised fraction, further purification was required. The GST-TgAgo protein that was obtained was then succumbed to dialysis in order to remove the N-lauroyl sarcosine detergent, as it might interfere with the binding between the glutathione resin and the GST tag in subsequent purification steps. Dialysis was performed 3 times in a buffer containing 10 mM Tris and 150 mM NaCl of pH 7.4. The results of this are displayed in Figure 3.13. Further purification was attempted through the GST tag, however no binding between the resin and the tagged protein occurred. This may be due to misfolding of the tag, and a GST-Tag assay was performed in order to check the quality of the tag. The substrate used in this particular assay was 1-chloro-2,4-dinitronbenzene (CDNB), the reaction was monitored at 340 nm and the change in absorbency was directly proportional to the amount of active GST tag in the sample. Upon completion of this assay, it was determined that the GST tag on the fusion protein was indeed damaged or misfolded, since no relative change in absorbance occurred.
Figure 3.13 Solubilization of Inclusion Bodies. The process of solubilisation of inclusion bodies was successful and performed with minimal loss of protein during the wash steps.
3.4.3 His-TgAgo solubilisation from inclusion bodies and protein refolding

Although previous experiments have also shown that the His version of TgAgo is associated with inclusion bodies purification of HisTgAgo became more attractive due to the relatively small size of the tag and the availability of a reduced and oxidized glutathione pairs which can be used in the protein refolding step. The bacterial culture was grown to an OD of 0.4, after which it was induced with 5 μM IPTG and the bacterial pellet was collected through centrifugation. Cellular lysis was performed using the French Press, and after several washing steps using the CAPS buffer discussed previously, the inclusion bodies were harvested. The inclusion bodies were then solubilised via the N-lauroyl sarcosine method, after which two dialysis steps were performed in order to remove the detergent used. Lastly, three additional dialysis steps were performed using the reduced and oxidized glutathione pair in order to promote intramolecular disulfide bridge formations and to promote the isolated proteins to fold back to their native conformation. A Bradford assay was conducted in order to determine the concentration of the proteins obtained from the inclusion bodies. A volume of 10 mL was isolated, with a concentration of 0.4325 μg/μL for a total of 4.325 mg of protein. A Coomassie stain was then used to assess the purity of the sample, which can be seen below (Figure 3.14). Also, the isolated protein was subjected to Western blot analysis with a primary antibody specific to the His sequence and the results show that a protein is recognized at the expected molecular weight of 62.5 kDa (Figure 3.14). Mass spectrometry was then used to identify the protein that emerged during the western blot analysis and it was concluded that the protein isolated was indeed His-TgAgo.
Figure 3.14 Detection of His tag via anti-His antibody. Panel A depicts the successful solubilisation of His-TgAgo visualized on a Coomassie stained gel. The purity of this was assessed at 90%. Panel B represents the Western Blot analysis of the isolated protein which was tested against anti-His antibody.
3.4.4 RNA binding capacity of TgAgo

Although the TgAgo protein was isolated and purified from inclusion bodies, the protein refolding protocol used should have, in theory, promoted the formation of disulfide bridges and in turn the folding of the protein into a native-like structure. To test the binding capacity of HisTgAgo to RNA species, an RNase A protection assay was performed.

This assay relies on the ability of a protein to bind target RNA, thus protecting it from subsequent RNase A addition through its binding interaction. Two unrelated proteins were used for this particular experiment, namely PDI and BSA. From Figure 3.15 it can be seen that HisTgAgo does not display any binding affinity towards the full length ssRNA substrate, while PDI and BSA show some affinity towards the substrate. Interestingly enough, although the protection did not take place for the full length UPRT ssRNA, however it was clear that the purified Argonaute protein offered some protection towards smaller fragments as it can be seen in Figure 3.15. Moreover, by incubating the recombinant protein with RNA alone (lane 3), it becomes clear that the degradation is caused by the addition of RNase A and that the HisTgAgo protein is not the culprit.
**Figure 3.15 RNase A protection Assay.** Proteins were pre-incubated with 5 pmol UPRT ssRNA for 30 minutes, followed by the addition of 1μL RNase A (0.003μg/mL). Lane 5 indicates that the isolated protein does not protect the full length ssRNA specie from degradation, however it does offer some protection to cleaved portions of the substrate as indicated by the asterix symbol. Furthermore, HisTgAgo is not responsible for this observed cleavage (lane 3).

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<td>RNA + Buffer</td>
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<td>5 μg BSA + RNA + RNase A</td>
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<td>5 μg HisTgAgo + RNA</td>
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<tr>
<td>6</td>
<td>2.5 μg PDI + 2.5 μg HisTgAgo +RNA + RNase A</td>
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3.4.5 Ribonuclease activity of TgAgo

Having observed that the recombinant protein is unable to protect target RNA from degradation by RNase A, another experiment was performed in order to determine whether it has any sort of binding affinity to RNA. To do this, a gel shift assay was performed in which varying concentrations of Argonaute protein (0.5 to 10 μg) were incubated with 3 pmols of ssRNA respectively. The different aliquots were ran on a non-denaturing polyacrylamide gel, with the results shown in Figure 3.16.

As displayed in Figure 3.15 there does not seem to be any binding interaction between the purified recombinant protein and ssRNA due to the first lane (control) running at the same size with the other ones.
Figure 3.16 Gel shift assay. Varying concentrations of purified TgAgo protein was incubated with UPRT ssRNA (5 pmol/well). The mixture was allowed to incubate on ice for 30 minutes. No significant changes are observed between the control lanes (1 and 9) and the rest of the gel.
4.1 Bioinformatic studies of RNAi components in *T. gondii*

Through bioinformatic studies, several possible components responsible for the RNAi activity in *T. gondii* have been identified. Well annotated genes from the *C. elegans* RNAi pathway were used in order to determine the presence of homologs in *T. gondii*. The NCBI database was scanned for the known protein sequences and fed back into ToxoDB using the blastp feature. This feature searches for similarities between the known sequences and the *Toxoplasma* genome. Several genes of interest surfaced through these searches, the most important of which were determined to be an RNA-dependent RNA polymerase (TGME49_017190), a PIWI-PAZ domain containing protein (TGME49_110160) as well as four possible candidates for Dicer (TGME49_064840, TGME49_106080, TGME49_116300 and TGME49_067030).

The RNA-dependent RNA polymerase was obtained through independent searches using sequences of known RdRps from *C. elegans*, namely rrf-1 and rrf-3. The same protein surfaced when ego-1, a protein associated with regulation of spermatogenesis, was used. The highest confidence score was obtained using the rrf-3 protein sequence, which has a role in somatic RNAi in *C. elegans*. The finding of a RNA-dependent RNA polymerase suggests that *T. gondii* may have the capability to create double stranded RNAs from single stranded RNAs (generally antisense) to provide the dsRNA substrate for the dicer protein. Moreover, in the well defined *C. elegans* RNAi response, these proteins have been associated with signal amplification, and the
substrates produced by these are generally associated with secondary Argonaute proteins in the RNAi cascade (Grishok, 2005).

The PIWI-PAZ domain containing protein that was identified in this study was obtained through several searches of related proteins in *C. elegans*. These proteins included alg-1, alg-2, ppw-1, ppw-2 and rde-1. The highest homology of the *Toxoplasma* Argonaute protein was to the alg-1 protein, although the alg-2 is also closely related. It has been previously shown that some important metabolic enzymes in *Toxoplasma gondii* are closely related to plant homologs, and the fact that the Argonaute protein obtained here is also linked to the Argonaute (plant) like 1 and 2 of *C. elegans* suggests a strong link to its plant lineage.

The Argonaute protein obtained through the blastp feature of ToxoDB has been previously identified and partially characterized by one of our lab members, indicating that the protein itself lacks the PAZ domain when transcribed (Al Riyahi *et al*., 2006). The PAZ domain is not a requirement for slicer activity, as was shown in studies carried out with *Drosophila* mutants (Miyoshi *et al*., 2005). Furthermore, ToxoDB predicts that the identified Argonaute protein will interact with double stranded RNA, suggesting that it may be functional.

The search for a RNase III domain ribonuclease involved the use of protein sequences from *C. elegans*, namely der-1 and drh-1, as well as the dicer protein sequence from *N. crassa*, qde-1. Using the first two protein sequences, a putative helicase protein emerged as a possible candidate, and upon closer inspection, it was determined that it carries a DEAD/H box helicase domain, a key feature of Class 3 RNase III proteins. Furthermore, the three dicer candidates obtained using the *N. crassa* dicer sequence all carried the signature DExD/H box helicase motif documented in Class 3 RNase III
proteins. Interestingly enough, studies in *Giardia* revealed that this lower eukaryote lacks the DExD/H box helicase feature (Macrae *et al.*, 2006), which makes these findings even more intriguing due to the evolutionary proximity between *Toxoplasma* and *Giardia*. It should be noted that a putative ATP-dependent DNA helicase also surfaced when the qde-3 protein sequence was used, however it is unlikely that this protein is a *T. gondii* dicer candidate since the well preserved motif is not present, it may however play a role in transcriptional regulation.

Another important feature of RNAi was identified through this work, namely the tudor staphylococcal nuclease domain-containing protein (TGME49_038050). This protein was identified using the protein sequence of tsn-1 of *C. elegans*, which has been described to associate with the RISC complex and to regulate the activity of let-7 miRNAs. Since several components of the classical RISC complex have been identified, it is likely that the *T. gondii* RNAi mechanism also involves a gene silencing complex containing both the tudor protein and the Argonaute protein described above for its activity.

Other important proteins found in this study include two putative ATP-dependent RNA polymerases (TGME49_026250 and TGME49_112280) found by using the *C. elegans* protein sequences of mut-14 and rha-1, which are involved in transgene silencing and regulation of transcription respectively. The second putative protein carries a well defined ATP-dependent DEAD/H box RNA helicase domain and it is predicted to bind nucleic acids and ATP and also carry out helicase activity in an ATP dependent manner. Using this approach, a homolog for smg-2 was also identified in *T. gondii*. The *C. elegans* smg-2 protein is responsible for mRNA surveillance, and interestingly enough, the top hit obtained through this homology search was a putative regulator of nonsense
transcripts UPF1 (TGME49_091820). A protein which may play an active role in chromatin repression and transgene monitoring was also identified, namely the SET-domain containing protein (TGME49_057770). This protein was identified using the mes-2 and mes-4 C. elegans protein sequences which perform similar roles to the ones described above.

4.2 dsRNA-directed ribonuclease activity in T. gondii lysates

4.2.1 dsRNA-directed ribonuclease activity in Ui, RH, and HFF lysates

The Argonaute protein has been identified as a major component of RISC, and without it, the organism loses its ability to perform siRNA mediated mRNA degradation (Liu et al., 2004); (Meister et al., 2004). The bioinformatic evidence coupled with our preliminary data prompted the investigation of the RNAi mechanism and the Argonaute protein in T. gondii.

In vitro assays were then conducted to show the specificity of the RNAi activity in T. gondii with respect to the initiator, target substrate and the role of metal ions. A primary experiment in which lysates from the Ui and RH parental strains were tested against their ability to promote the degradation of sense strand RNA derived from the ORF of the UPRT gene. The Ui parasite line is able to constitutively express dsRNA homologous to the UPRT gene, while the RH parasite lacks this feature. If active RNAi machinery is present in T. gondii, the UPRT dsRNA expressed in the Ui line will be converted into siRNAs by a Dicer-like protein resulting in the degradation of the target substrate, UPRT ssRNA. This method of testing for RNAi activity is very advantageous because it utilizes lysates obtained from freshly isolated parasites and since the buffer used was at physiological pH, it is unlikely that the native protein structures and their
interactions are disrupted. The outcomes of this experiment confirmed the prediction that degradation of target UPRT ssRNA would be witnessed only in the lysates obtained from the Ui line and not in the RH ones (Figure 3.2). From this figure, rapid cleavage of the substrate is observed, with a rate of 71.8 \( \mu \text{M.min}^{-1}.\text{mg}^{-1} \) over the first 15 minutes. Over the course of the experiment, the rate plateaus to 20.9 \( \mu \text{M.min}^{-1}.\text{mg}^{-1} \). Additionally, the target RNA was also incubated for the duration of the experiment in assay buffer and loading dye to determine if degradation is caused by these two experimental components however it was found that the degradation observed is a result of the lysate itself.

Since \textit{T. gondii} is an intracellular parasite that is cultured in human cells, there is the possibility of cross contamination between host proteins and parasitic proteins upon lysis. Human cells, like many other mammalian cells have an active RNAi response (Liu \textit{et al.}, 2004). In order to show that the RNAi-like specific degradation of target substrate stems from \textit{T. gondii} lysates alone, comparative analysis between Ui lysates, HFF lysates and HFF lysates that were supplemented with UPRT dsRNA was performed. As expected, the dsRNA induced degradation of target substrate was observed only in the Ui lysates and the HFF lysates supplemented with UPRT dsRNA, the latter of which showed negligible degradation rates by comparison to the parasitic lysates. HFF lysates that were not supplemented with UPRT dsRNA show virtually no degradation in target UPRT ssRNA (Figure 3.3). Kinetically, the observed rate of UPRT ssRNA cleavage for the Ui lysates was calculated to be 48.8 \( \mu \text{M.min}^{-1}.\text{mg}^{-1} \) with respect to the primed HFF lysates in which the observed rate was 16.8 \( \mu \text{M.min}^{-1}.\text{mg}^{-1} \). Although the degradation rate of target UPRT ssRNA substrate in primed HFF lysates is significantly reduced, it may alter the degradation profile slightly if contamination occurs. To avoid this, parasites should be
needle passed, therefore preventing cellular debris from being included in subsequent lysis steps.

4.2.2 The requirements of dsRNA-directed ribonuclease activity

Studies in other organisms showed that the presence of divalent metal ions promotes the dsRNA induced target RNA degradation, while the lack of these ions prevents target degradation all together. An experiment was thus designed to test the degradation capacity in Ui lysates in the absence or presence of such divalent metal ions. Figure 3.4 in the results section displays the behaviour of magnesium dependency for the RNAi-like mechanism observed in *T. gondii*. It was observed that a lack in magnesium is linked to non-degradation of target substrate, while the addition of magnesium sparked more aggressive degradation on the target UPRT ssRNA substrate. From a classical RNAi point of view however, this experiment also shows that magnesium dependency is most likely linked to the second part of the mechanism in which siRNA directed degradation of ssRNA is observed (Figure 3.4). The observed rate for the cleavage of ssRNA in the Ui lysate was calculated to be 20 μM.min⁻¹.mg⁻¹ in comparison to the 33.6 μM.min⁻¹.mg⁻¹ rate obtained by the addition of divalent metal ions. The average rate obtained for the magnesium enhanced reactions was found to be 49.53 ± 24.29 μM.min⁻¹.mg⁻¹. This significant deviation suggests the lack of accuracy in the method used to quantify the relative fluorescence units. It is likely that magnesium is also a requirement for the production of siRNAs, which in this case occurs in vivo however magnesium removal in live parasites was not attempted. An alternative to this would be the removal of divalent metal ions in RH lysates, followed by the addition of dsRNA to induce siRNA formation and ssRNA target to assess the degradation profile, if present.
Another key feature of the classical RNAi mechanism was tested through the specificity of the RNA target. The siRNAs that recruit the formation of the RISC complex are subsequently cleaved by the slicer enzyme, Argonaute, with the passenger strand being expelled, and the guide strand promoting the binding to the mRNA target (Preall et al., 2006). In order to show that the RNAi-like mechanism in *T. gondii* displays the same discrimination between the passenger and guide strands, Ui lysates were incubated with UPRT ssRNA as well as UPRT asRNA and their respective degradation profiles were visualized. Figure 3.5 displays the selectivity in degradation between the UPRT ssRNA and the UPRT asRNA species. This result implies that the RNAi mechanism in *T. gondii* resembles the classical mechanism with respect to the antisense siRNA or the guide strand incorporation in the degradation complex that may form.

### 4.2.3 Inducing dsRNA-directed ribonuclease activity in RH lysates

Previous observations from the HFF experiment suggest that if the initiator dsRNA is present, dsRNA-directed ribonuclease activity is observed. RH lysates generally lack the capability to undergo dsRNA-directed ribonuclease activity towards the target ssRNA due to the fact that these lysates lack the dsRNA initiator. RH lysates were thus primed with UPRT dsRNA and tested against the parasite line that does exhibit ribonuclease activity, Ui. As it can be observed from Figure 3.6, both lysates behave similarly. The overall experimental rate averages for the primed RH lysates were calculated to be $41.33 \pm 8.67 \mu M.min^{-1}\cdot mg^{-1}$, while the Ui rate was $44.09 \pm 8.06 \mu M.min^{-1}\cdot mg^{-1}$. These averages were obtained by calculating triplicate rates for three different experiments. The variation between the two rates is largely insignificant, suggesting that the cleavage of UPRT ssRNA is due to the production of siRNAs from a UPRT dsRNA.
precursor. Since the rates are virtually identical, the same set of proteins may participate in the dsRNA-directed ribonuclease activity of UPRT ssRNA substrate.

Since target RNA recognition takes place, we postulated that the inducer molecule is also essential. To show this, RH lysates were pre-treated with HX dsRNA and UPRT dsRNA initiators, followed by the addition of HX ssRNA substrate. The outcome, as suggested by Figure 3.7 in the results section, is that specific target degradation takes place in a homologous fashion, with HX ssRNA target degradation observed only in the reaction initiated with HX dsRNA. This is a significant result due to the fact that it illustrates another key feature of the classical RNAi mechanism described in higher eukaryotes. This feature is the specific degradation of target ssRNA based on the homology of the induced dsRNA sequence.

To demonstrate this outcome further, HX dsRNA primed RH lysates were tested in the presence of UPRT ssRNA as well as UPRT asRNA. As Figure 3.8 suggests, the mismatch between the RNAi inducing specie HX dsRNA lacks homology with both substrates, therefore no degradation is detected.

4.3 dsRNA specific ribonuclease activity in \textit{T. gondii}

The hallmark of the classical RNAi mechanism is the production of siRNAs which vary in length from 21-23 base pairs in higher eukaryotes to 26 base pairs in plants. In terms of evolution, \textit{T. gondii} has been shown to harbour genes closely related to those of plants, especially in the metabolic pathway. If this holds true to the RNAi pathway present in the parasite, the expected length for the siRNAs produced should be closer in length to their plant counterparts. Experimentally, this was successfully shown that a accumulation of product within the region of 20-39 base pairs which may be attributed to
the Dicer enzyme in *T. gondii*. Although a smear is present in Figure 3.9 panel A in the results section, it does accumulate in a time dependent fashion reaching the highest level after 18 hours. Similarly, in panel B of the same figure, a intermediate product may be observed, suggesting that the lysate does perform cleavage onto the UPRT dsRNA used. An accumulation of this intermediate RNA specie is observed in a time dependent manner as well, however no accumulation of siRNAs was observed. To paint a clearer picture, a higher percentage polyacrylamide gel may be used to discern between the bands present. Further characterization still needs to be performed in order to determine the divalent cation and ATP dependency of the Dicer protein. It should also be assessed whether Dicer actively cleaves dsRNA precursors only or if hairpin-RNA undergoes degradation in lysates as well. Here it was shown that specific degradation of RNA dsRNA takes place through the incubation with RH lysates producing an unmistakable degradation product in the expected siRNA size frame.

### 4.4 Protein expression and purification

#### 4.4.1 Induction optimization

Purified TgAgo in its native form would open the door to a myriad of questions and answers. Generally, a soluble protein retains its native-like structure and consequently its function however throughout the induction experiments it was found that BL21 induced *E. coli* produced the protein in inclusion bodies (IBs). Inclusion bodies generally contain proteins that are misfolded and in turn inactive, although some reports have indicated that inclusion bodies may harbour some active proteins (Panda, 2003).

The parameters that affect the solubility of a protein upon induction are the concentration of IPTG inducer, the temperature at which the induction is taking place, the
OD of *E. coli* when induction is started and lastly the time allowed for induction. Systematically, these parameters were altered for both the GST and the His tagged versions of the recombinant protein. The inducer concentration was lowered to 5 μM IPTG, however upon the separation of the supernatant and insoluble fraction it was observed that the protein of interest is still predominantly in the pellet (Figure 3.10). The second parameter that was altered was optical density. By inducing at earlier bacterial growth phases, the accumulation of the recombinant protein is rather slow in culture. This is an important feature because it allows for greater soluble protein production before triggering the formation of inclusion bodies. A proposed mechanism of inclusion body formation is that cells trigger the formation of IBs upon recognition of toxic cellular levels of recombinant protein. Based on this phenomenon, induction was attempted at mid-exponential phase (OD$_{600}$ = 0.4). TgAgo ever-expression was successful, however it was found in the insoluble fraction. The soluble induced fraction was almost indistinguishable to the uninduced sample that serves as a induction control as observed in Figure 3.11. It was also observed that induction at later phases of bacterial growth was accompanied by a reduction in the concentration of recombinant protein. This is due to the accumulation of β-lactamase secreted during bacterial fermentation which accelerates the degradation of ampicillin, limiting transformant growth in the culture in the case of the HisTgAgo transformants. In the case of GST-TgAgo the selectable antibiotic was kanamycin, and the reduction in protein expression in this case could be attributed to the lack of nutrients in the media based on an elevated number of divisions of the *E. coli* cells.

The parameter of temperature was also addressed in which *E. coli* cultures were induced at an optical density of 0.4 with minimal concentration of inducer and grown at
room temperature and 37°C respectively, for a period of 3 hours. This approach failed to produce an increase in the amount of GST-TgAgo since the uninduced sample closely resembles the supernatant profile from the room temperature induced species (Figure 3.11). In a last effort to obtain soluble GST-TgAgo, the induction time was increased to overnight, while several IPTG concentrations were tested alongside the induction temperature. The results of this experiment are contained in Figure 3.12 in the results section. It was observed that even in this attempt, the recombinant protein was packaged into inclusion bodies rendering it potentially inactive.

4.4.2 Solubilization of inclusion bodies and refolding

Although the presence of a GST tag on a recombinant protein generally increases its solubility, in the case of GST-TgAgo it was found to not be effective at producing soluble proteins. For this reason, solubilisation of the inclusion bodies was attempted. GST tagged recombinant protein was therefore solubilised with the addition of N-lauroyl sarcosine with minimal protein loss in the washing steps, however contaminants were present alongside a well defined GST-TgAgo band, as seen in Figure 3.13. Further purification is thus necessary however it was observed that there was no binding between the resin and the GST-TgAgo, an outcome of a perhaps misfolded GST tag on the recombinant protein. A GST colorimetric assay was performed and it was determined that the GST tag is not active, explaining the lack of binding to the resin.

Due to the small size of the His tag and the availability of reduced and oxidized glutathione for protein refolding, HisTgAgo was used. It was previously shown that the His tagged version of the protein also localizes in inclusion bodies, however through protein refolding techniques, it may be rescued. HisTgAgo was also isolated from
inclusion bodies via the same method used in the GST scenario, and refolding was attempted. Confirmation of HisTgAgo isolation was done through mass spectrometry and western blot techniques. Figure 3.14 shows the detection of a His tagged protein by an anti-6xHis antibody at the expected molecular weight of 62.5 kDa confirming that the protein isolated is recombinant TgAgo. Moreover, the detection of the His tag does not verify that the isolated protein is active.

4.4.3 Argonaute activity

To determine the activity of the isolated protein, several experiments were performed in which the binding capacity was tested. It was found that through the RNase A assay that HisTgAgo cannot protect the full length 735 base pair UPRT ssRNA substrate from degradation, however it was able to prevent the degradation of slightly smaller fragments as shown in Figure 3.15. Moreover, by setting Argonaute and the substrate alone, it conclusively proved that the purified protein is not responsible for the observed degradation (Figure 3.15).

The experiment that aimed to elucidate whether or not there is any interaction between the purified protein and sense strand RNA was the gel mobility shift assay. The results shown in Figure 3.16 show that there is no relative shift between the lanes containing ssRNA (lane 2-8) and protein and the lanes containing RNA alone (Lane 1 and 9). This finding suggests that perhaps the Argonaute protein isolated from inclusion bodies is not active due to severe misfolding. It is also possible that in order for binding to occur an interacting partner is needed. This may be the case as the Argonaute protein in T. gondii lacks a PAZ domain which in other organisms generally facilitates binding to target mRNA. Studies in Drosophila have indicated that the PAZ domain is not a
requirement for slicing activity, however the study did not address the binding of an Argonaute protein lacking a PAZ domain to its substrate RNA (Miyoshi et al., 2005).

Another possible explanation for the lack of interaction between the Argonaute protein and the target RNA is that perhaps a siRNA molecule facilitates the binding between the two. It has been often been reported that the presence of siRNAs promotes the formation of the RISC complex, which in turn binds target mRNA (Elbashir et al., 2001). Similarly, the Argonaute protein in *T. gondii* may not act alone, perhaps relying on a primitive RISC like complex for the stabilization of target RNA.

To determine if the Argonaute protein has any interacting partners, an immunoprecipitation assay should be performed in which modified siRNAs are incubated with fresh *Toxoplasma* lysates in order to pull down the hypothetical RISC complex in this parasite.

### 4.5 Conclusions

Through bioinformatic studies, several putative key components of the RNAi mechanism of *T. gondii* have been identified. These include a PIWI-PAZ domain containing protein, several RNase III ribonuclease candidates, a RNA-dependent RNA polymerase, a tudor staphylococcal nuclease domain containing protein and several other proteins which may serve as transcriptional regulators. This suggests that *T. gondii* has the necessary components to perform RNA induced gene silencing.

The ability of *T. gondii* to amount an RNAi response was tested *in vitro*. Through the course of the experiments the specificity and requirements of target RNA cleavage became clear. More importantly, it was determined that the introduction of UPRT dsRNA causes a time wise degradation in UPRT ssRNA but not UPRT asRNA.
Moreover, the specificity between the initiator and target RNA was assessed and it was found that degradation of the substrate occurs only when the initiator is homologous to the target. It was also recognized that divalent metal ions such as magnesium play a crucial role in the RNAi-like mechanism in *T. gondii*, since removal of magnesium prevented the degradation of the substrate RNA.

A poly-histadine tagged construct of *T. gondii* Argonaute was used to successfully express it and isolate it from the inclusion bodies the BL21 *E. coli* cells produced. The protein was successfully identified through mass spectrometry as well as western blot technique with an antibody that recognizes the poly-histadine tag. A refolding technique was used however this method seems to have been inefficient due to a lack of binding between TgAgo and various RNA species under non denaturing conditions. Furthermore, pre-incubated substrate RNA with TgAgo offered no protection from the degradation effects of RNAses A towards the full length UPRT ssRNA substrate, showing that the interaction between the isolated protein and RNA is limited. One important find is that partial protection was observed, suggesting that the purified protein still maintains some RNA binding capacity, although the gel shift assay proved otherwise.

New purification methods and induction methods should be performed in order to obtain TgAgo in its native form, which would elucidate its role in the RNAi-like mechanism of this parasite. X-ray crystallography of purified native TgAgo would shed some light on the organization of the PIWI domain and it would assess the possibility of it being the silencer observed in these experiments. Furthermore, a purified native protein can be used in the production of antibodies which may be used in pull down assays to determine the interacting partners of TgAgo.
REFERENCES


APPENDIX A

UPRT OPEN READING FRAME

ATGGCGCAGGTCCAGCGACGCAAGAGCTCTTTGTGATCATCCCCGATATCCGAAATAAGAGGACG
AGGAAAGAAGCATTTCTCCAGGACATACATCAACAGAGTTTCCAAATGTGTGGTCTCATGAACGAG
AGGGCTCAGCTTTGACAGATGACCATATTCTGAGATAAAGAAGACACGGAGAAAGAATT
CGTCTGCTACGAGCAGCGCGCAGGGTTTGCTCCTCTACTGGAAGACCTTTGGTAAACTGCGAT
TGAAAGGAAATCCCTTGCACTTGTCTCTATGGACCTTCAACATGGCTCAGCGG

HXGPRT OPEN READING FRAME

ATGAGCGGCTCCGACGGCAAGGGCAAGGTAATTGAGCCATGTATATCCCGACAACACTCTGAGATTTGGTCTCATGGAACGAG
AGTATGGCAGCTCGCATTTGAGGTTGAAAGACGAGCTTTGCTCCTCTCTGACATCTGCTGCTGTTGACATCTGCTGCTGACTTC
AGCATCTCGAAGCGTCGAGCGAGACGTGATTGCAAGGACAGAGTTGAGAAGTTGGCGTATG

Figure A1 UPRT and HXGPRT open reading frames - Plasmids carrying the ORF for HXGPRT and UPRT respectively were used with the appropriate primer pairs in order to obtain the desired PCR product. The PCR products were purified and used for generation of RNA species as described in the Materials and Methods section.
**APPENDIX B**

**pET28bHisAgoORF PLASMID**

**TgAgo OPEN READING FRAME**

ATGGGCAGCAGCAGCCATCATCATCATCATCAACACCACGGGCCGCTTGTCGCCGCGCGCGAGCAT
TGCTAGCATGACTGTTGAGCAAGCAAATGGAAGTCTGCTTCTGGGCCACAGGACAT
TCGCCCGGAAGGTCTGCTTCTGGTGAATTATGGCAGCCTGTAAGGCAAGAACAT
GGAGGAGGTCTTTGGAATTTCCCTTTATTCAATTACCCGGAATGACGGGAGGCCCTG
CGGCATTTTGTGCACTGCCAACAAACAGGGTGAAATCCGGAATATTCTGCAAAAC
GGTCTCCTGACATCTCACCAGAAGGTCAAATTTGTGAGCTCGAGCTACAG
TGTCTGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA

**Figure B1** *T. gondii* Argonaute open reading frame
Figure C1 Mass Spectrometry evidence of TgAgo isolation
APPENDIX D

QUANTIFICATION OF ISOLATED HisTgAgo

Bradford assays were read at 595 nm using a UV-Vis Spectrophotometer. A standard curve was created using known concentrations of Bovine Serum Albumin (BSA). The standard curve is depicted below.

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<th>Sample</th>
<th>Absorbance @ 595 nm</th>
<th>Concentration (μg/mL)</th>
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<td>0</td>
<td>0</td>
</tr>
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<td>BSA</td>
<td>0.049</td>
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<td>HisTgAgo</td>
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Figure D1 Quantification of isolated protein through Bradford assay
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EDUCATION

2006 – Present   University of Windsor (ON, Canada)
M.Sc., Biochemistry

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