zinc finger　GARP

(1/2)

94　5　31

zinc finger   GARP

Role of zinc finger and GARP families in transcriptional regulation and encystation of *Giardia lamblia*

Abstract

In this project, we characterized several giardial proteins, the previously described GARP-like protein 1 (GLP1), its related GLP2, and zinc finger protein 1 (ZFP1) and ZFP2. In the previous study, we showed that *glp1* was up-regulated during encystation. However, *glp2* and *glp3* were down-regulated during encystation. GLP1 could bind to the 32-bp *ran* promoter element and the *cwp1* promoter in the double-stranded configurations in electrophoretic mobility shift assays. Using mutated plasmid and transfection assays we demonstrated that the GLP1 binding sites are positive *cis*-acting elements of the *cwp1* and *ran* gene promoters in both trophozoites and encysting cells. The results indicate that GLP1 could function as a transcriptional activator. In this project, we found that GLP2 could also bind to the same sequence in the *cwp1* and *ran* gene promoters, suggesting that GLP2 could also function as a transcriptional activator. We also identified another gene (*glp4*) by *in silico* screening of the *Giardia lamblia* genome data base. The *glp4* gene was down-regulated during encystation. The *zfp1* and *zfp2* genes were found previously by *in silico* screening of the *G. lamblia* genome data base. In the previous study, we showed that ZFP1 localized to the two nuclei in both vegetative and encysting trophozoites. In this project, we found that both *zfp1* and *zfp2* mRNA levels increased during encystation. We also found that the C-terminal region of ZFP1 containing the glutamine rich domain functioned as a transactivation domain in *Giardia* when combined with the DNA-binding domain of yeast GAL4. ZFP1 promoted expression of the reporter gene driven by the *cwp1* promoter in *Giardia* in cotransfection assays. The result suggests that ZFP1 could function as a transcriptional activator. On the other hand, we observed that the luciferase activity was increased ~25 or ~269-fold when cotransfected with pRANneo or 5'Δ5N-Pac plasmid, respectively. The increased expression of luciferase gene could occur at translation level but not transcription level. We are currently investigating the potential function of the GLPs and ZFPs in transcriptional regulation in *G. lamblia*.

GARP-like protein 1 (GLP1)   GLP2   zinc finger protein 1 (ZFP1)
ZFP2   *glp1*
   glp2   *glp3*

GLP1   32-bp *ran*   *cwp1*

                                ran   *cwp1*   GLP1

     vegetative   encysting trophozoite   GLP1

                                        GLP2   *cwp1*

      ran   GLP2

                                glp4   *glp4*

                                                        zfp1

zfp2   ZFP1   vegetative   encysting trophozoites

                zfp1   *zfp2*   mRNA

          ZFP1 C   Glutamine   GAL4 DNA

                                        ZFP1   *cwp1*

                        ZFP1

          pRANneo   5' Δ5N-Pac   luciferase   ~25   ~269

                                                                GLP

ZFP

Introduction

   G. lamblia is an important human intestinal pathogen that causes outbreaks of waterborne diarrhea (Adam, 2001; Wolfe, 1992). *Giardia* belongs to one of the earliest diverging eukaryotic lineages (Cavalier-Smith, 1991; Hashimoto *et al.*, 1994; 1995; Sogin *et al.*, 1989). There is little understanding of the biology of the origins of eukaryotes, particularly of transcriptional regulation during *Giardia* encystation. The rapid progress in analyses of the *Giardia* genome greatly helps us to find certain candidates in studies (McArthur *et al.*, 2000; www.mbl.edu/*Giardia*).

   During encystation, *G. lamblia* cyst wall is formed and both nuclei divide simultaneously, resulting in a cyst with four nuclei (Reiner *et al.*, 1989; Lujan *et al.*, 1997). Synthesis and secretion of certain components, such as cyst wall protein, are required for the formation of a protective cyst wall (Lujan *et al.*, 1995; Mowatt *et al.*, 1995). The life cycle of *G. lamblia* may provide perspectives on the cell differentiation in response to different environments. We have learned to complete the life cycle of *G. lamblia in vitro*, making possible study of the cellular and molecular biology of differentiation (Eichinger, 2001; Gillin *et al.*, 1987; Gillin and Reiner, 1996; Lujan *et al.*, 1997).

   The lack of clear giardial homologs to many of basal transcription proteins

suggests that *Giardia* may have diverged early and represents a transition during the evolution of eukaryatic transcription systems (Best *et al*, 2004). Many giardial transcription factors diverge at a higher rate than those of crown group eukaryotes (Best *et al*, 2004). *G. lamblia* has many unusual features with regard to transcription. No consensus TATA boxes or other *cis*-acting elements identified in higher eukaryotic promoters have been observed in the promoters of many protein-coding genes (Elmendorf *et al.*, 2001; Holberton and Marshall, 1995; Knodler *et al.*, 1999; Lujan *et al.*, 1995; Mowatt *et al.*, 1995; Sun and Tai, 1999; Yee *et al.*, 2000). *Giardia* has a highly divergent TBP and lacks most of the canonical RNA Polymerase II tranascription factors (Best *et al*, 2004). AT-rich sequences have been found around the transcription start sites of many genes (Elmendorf *et al.*, 2001; Holberton and Marshall, 1995; Knodler *et al.*, 1999; Lujan *et al.*, 1995; Mowatt *et al.*, 1995; Sun and Tai, 1999; Yee *et al.*, 2000). The AT-rich sequences are essential for promoter activity and play a predominant role in the determining the positions of the transcription start sites, functionally similar to the initiator element in higher eukaryotes (Elmendorf *et al.*, 2001; Sun and Tai, 1999; Yee *et al.*, 2000). The promoter regulatory mechanism could be unusual because very short 5'-flanking regions (<65 bp) are sufficient for the expression of some genes (Elmendorf *et al.*, 2001; Sun and Tai, 1999; Yee *et al.*, 2000).

Despite the importance of cyst wall biogenesis during *Giardia* encystation, the molecular mechanisms governing transcriptional regulation remain poorly understood. Expression of genes encode three cyst wall structural proteins (CWP1, 2, and 3; Lujan *et al.*, 1995; Mowatt *et al.*, 1995; Sun *et al.*, 2003) and an enzyme in the cyst wall polysaccharide biosynthetic pathway (glucosamine-6-phosphate isomerase-B, G6PI-B) (Knodler *et al.*, 1999; Van Keulen *et al.*, 1998) increases with similar kinetics (Knodler *et al.*, 1999; Lujan *et al.*, 1995; Mowatt *et al.*, 1995; Van Keulen *et al.*, 1998), suggesting the importance of regulation at transcriptional level. We also identified a *gmyb2* gene whose expression increases during encystation (Sun *et al.*, 2002). gMyb2 binding sites are present in the promoter regions of all of the key encystation-induced genes, *cwp1*, *cwp2*, *cwp3*, *g6pi-b*, and *gmyb2* itself and are known to mediate induction of gene expression during encystation.

We hope to study the differential regulation of gene expression as well as transcriptional mechanisms in *G. lamblia*. When our previous grant was funded, we started to clone and identify transcription factors that might be involved in encystation-induced genes or that might interact with gMyb2 by querying the *Giardia* Genome Project Database (NSC92-2314-B-002-354). We found several proteins containing zinc-finger motifs and GARP domains which function as parts of DNA-binding in plants and higher eukaryotes. Since many of the zinc-finger and GARP proteins have been implicated in important biological processes in plants and higher eukaryotes (Makino *et al.*, 2000; Imamura, 1999; Sakai *et al.*, 2001; Kerstetter

et al., 2001; Takatsuji, 1998; Krempler and Brenig, 1999), these proteins could be involved in the regulation of important biological processes that are unique to *G. lamblia*, such as encystation, excystation, and host-parasite interaction. It is also possible that *G. lamblia* might have some novel classes of zinc fingers and GARP domains to adapt them to specific developmental processes.

In this study, we characterized several possible giardial transcription factors, the previously described GARP-like protein 1 (GLP1), its related GLP2, and zinc finger protein 1 (ZFP1) and ZFP2. We also observed that the luciferase activity was highly increased when cotransfected with pRANneo or 5'Δ5n.pac plasmid. We further investigated the mechanism responsible for this phenomemon.

Experimental procedures

Giardia **culture.** Trophozoites of *G. lamblia* isolate WB (ATCC 30957), clone C6, were cultured in modified TYI-S33 medium (Keister, 1983). Encystation was induced according to our published procedure (Sun *et al.*, 2003).

Plasmid construction. All *glps*, *zfps*, and *cwp1* fragments were amplified from genomic DNA by PCR. Oligonucleotide sequences are available upon request. All constructs were verified by DNA sequencing (Applied Biosystems). Plasmid 5'Δ5N-Pac was a gift from Dr Steven Singer and Dr Theodore Nash (Singer *et al.*, 1998). Plasmid pRANneo, pNG, pNGI, -409/-1, GAL4ran32, and pNGAL4 have been described before (Sun *et al.*, 1998; report for NSC grant). For constructing pNG5, the 5'-flanking region of the *glp1* gene was amplified with oligonucleotides G5NF and G5BR, digested with *Nhe*I/*Bsp*HI, and ligated in place of the *Nhe*I/*Nco*I-excised 32-bp *ran* promoter and two copies of a 19-bp *tet* operator sequence in pNLop2-1 (Sun *et al.*, 2002). The resulting plasmid, pNG5, contained the luciferase gene under the control of the *glp1* promoter. For constructing pNG, the *Nhe*I/*Bsp*HI digested 5'-flanking region of the *glp1* gene described above was ligated in place of the *Nhe*I/*Nco*I-excised 32-bp *ran* promoter and two copies of a 19-bp *tet* operator sequence in pNGI, resulting in pG5T. Then the 3'-flanking region of the *glp1* gene was amplified with oligonucleotides G3EF and G3SR, digested with *Eco*RI/*Sal*I, and ligated in place of the *Eco*RI/*Sal*I-excised 3'-flanking region of the *ran* gene in pNG5T, resulting in pNG. For constructing pNTG, the *glp1* gene coding region was amplified with oligonucleotides GNF and GAUER, digested with *Nco*I/*Eco*RI, and ligated in place of the *Nco*I/*Eco*RI-excised luciferase gene in pNT5 (Sun *et al.*, 2002). The resulting plasmid, pNTG contained the *glp1* gene controlled by the α2-tubulin promoter with an AU1 tag fused at its C terminus. For constructing pNGm1 (mutation of region 1, residues 7-8), a PCR product generated with oligonucleotides Gm1F and

GAUER was digested with *Bam*HI and *Eco*RI. Another PCR product generated with oligonucleotides GNF and Gm1R was digested with *Nco*I and *Bam*HI and cloned into *Nco*I/*Eco*RI digested pNGI with the *Bam*HI/*Eco*RI fragment. Similar strategy was used to clone pNGm2 (mutation of region 2, residues 153-155) and pNGm3 (mutation of region 3, residues 33, 36, and 38). For constructing pNMybGLPC, a PCR product for the 5'-flanking region of the *gmyb2* gene and gMyb2 N-terminal region was generated with oligonucleotides Myb2NF and MD3BR and then digested with *Nhe*I and *Bgl*II. Another PCR product for C-terminal region of GLP1 was generated with oligonucleotides GD1BF and GAUER, digested with *Bgl*II and *Eco*RI, and cloned into *Nhe*I/*Eco*RI digested pNT5 (Sun *et al.*, 2002) with the *Nhe*I/*Bgl*II fragment. The resulting plasmid, pNMybGLPC, contains a fusion of N-terminal region of gMyb2 and C-terminal region of GLP1 under the control of the *gmyb2* promoter. For constructing pNZFP1, the *zfp1* gene and its 5'-flanking region was amplified from genomic DNA with oligonucleotides Z5NF and ZAUER, digested with *Nhe*I/*Eco*R*I, and ligated in place of the *Nhe*I/*Eco*RI-excised luciferase gene, 32-bp *ran* promoter and two copies of a 19-bp *tet* operator sequence in pNLop2-1 (Sun *et al.*, 2002). The resulting plasmid, pNZFP1 contained the *zfp1* gene controlled by its own promoter with an AU1 tag fused at its C-terminus. For constructing pNGAL4-ZFP1C, N, or FL, a PCR product for C-terminal, N-terminal, or full-length portions of the ZFP1 coding region generated with specific oligonucleotides was digested with *Eco*RI and cloned into *Eco*RI digested and phosphatase treated pNGAL4. During the cloning process of pPW156/51m (not shown), the PCR resulted in two nucleotide changes of the luciferase gene, in which Leu 411 was mutated to a termination codon and Asp 500 was mutated to Asn. We also constructed pPW1m by replacing the luciferase gene of the pPW1 to the mutant with *Nco*I digestion. For constructing pPW1s, the partial luciferase gene was amplified with oligonucleotides cwp15BF and lucXR on pPW1m, digested with *Sph*I/*Xba*I, and ligated in place of the *Sph*I/*Xba*I-excised partial luciferase gene in pPW1m. The resulting plasmid, pPW1s, contained the truncated luciferase gene with a termination codon at aa 410. For constructing pNW1m, the luciferase gene with flanking region in pPW1m was excised with *Kpn*I/*Cla*I and ligated in place of the *Kpn*I/*Cla*I-digested pRANneo.

Transfection and luciferase assay. Cells transfected with pN series plasmids were selected with G418 as described (Sun *et al.*, 1998). Stable transfectants were maintained at 150 μg/ml G418. Cells transfected with other plasmids containg *pac* gene were selected and maintained with 54 μg/ml puromycin. The luciferase activity was determined as described (Knodler *et al.*, 1999). Luciferase activity was measured with an Optocomp I luminometer (MGM Instruments). Western blots were probed with anti-AU1 monoclonal antibody (BAbCO,1/5000 in blocking buffer) and detected with Peroxidase conjugated goat anti-mouse IgG (Pierce, 1/5000) and enhanced

chemiluminescence (Amersham).

Immunofluorescence assay. The stable transfectants were cultured in growth specific medium under G418 selection. Cells were harvested after 0 hr or 24 hr encystation, washed in PBS, and attached to glass coverslips (2×10^6 cells/coverslip) then fixed and stained (Abel *et al.*, 2001). Cells were reacted with anti-AU1 monoclonal antibody (BAbCO, 1/300 in blocking buffer), and anti-mouse ALEXA 568 (Molecular Probes, 1/500 in blocking buffer) as the detector. GLP1 was localized on a Zeiss LSM 510 laser scanning confocal microscope.

Expression and purification of recombinant GLP2 protein. The genomic *glp2* gene was amplified using oligonucleotides G2F and G2R and the product was cloned into the expression vector pCRT7/CT-TOPO (Invitrogen) to generate plasmid pG2. The pG2 plasmid was freshly transformed into *Escherichia coli* BL21(DE3)pLysS (QIAexpressionist, Qiagen). An overnight pre-culture was used to start a 250-ml culture. *E. coli* cells were grown to an A_{600} of 0.6, and then induced with 1 mM isopropyl-D-thiogalactopyranoside (Promega) for 6 hr. Bacteria were harvested by centrifugation and sonicated in 10 ml of buffer A (50 mM sodium phosphate, pH8.0, 300 mM NaCl) containing 10 mM imidazole and complete protease inhibitor cocktail (Roche). The samples were centrifuged and the supernatant was mixed with 1 ml of a 50% slurry of Ni-NTA superflow (Qiagen). The resin was washed with buffer A containing 20 mM imidazole and eluted with buffer A containing 250 mM imidazole. Fractions containing GLP2 were pooled, dialyzed in 25 mM HEPES pH 7.9, 40 mM KCl, 0.1 mM EDTA, and 15 % glycerol, and stored at -70°C. Protein purity and concentration were estimated by Coomassie Blue and silver staining compared with bovine serum albumin. GLP2 was purified to apparent homogeneity (>95%).

Electrophoretic mobility shift assay. Double-stranded oligonucleotides specified in the text were 5'-end-labeled as described (Sun and Tai, 1999). Binding reaction mixtures contained the components as described (Sun and Tai, 1999), except that Tris buffer was replaced by 10 mM HEPES buffer (pH 7.9). Labeled probe (0.02 pmol) was incubated for 15 min at 25°C with 5 ng of purified GLP2 protein in a 20 μl volume supplemented with 0.5 μg of poly (dI-dC) (Sigma). Competition reactions contained 200-fold molar excess of cold oligonucleotides. In an antibody supershift assay, 0.8 μg of an anti-V5-HRP (Invitrogen) antibody was added to the binding reaction mixtures.

Results

Identification of the *glp* genes.

In the previous study, we identified three proteins containing the putative GARP

domain, we named GLP1, 2, and 3 (GenBank accession number AY822599, AY822600, and AY822601, respectively). To identify more *glp* genes from *Giardia*, we searched the *G. lamblia* genome database (http://www.mbl.edu/Giardia, McArthur *et al.*, 2000) with the GARP domain from *Arabidopsis* ARR1 as a query sequence (GenBank accession no. AY056099). Amino acid sequences with similarity to the GARP domain were found in one protein we named GLP4 (GenBank accession number AY822602). We found that the *glp1* gene was slightly up-regulated, but the *glp2, glp3,* and *glp4* genes were down-regulated during encystation (Fig. 1A).

To determine the expression of GLP1 protein, we prepared construct pNG in which the *glp1* gene is controlled by its own promoter and contains an AU1 epitope tag at its C terminus (Fig. 1B) and stably transfected it into *Giardia* . The GLP1 protein was not detected in either vegetative or encysting cells in Western blots (data not shown), suggesting that the expression of the *glp1* gene under its own promoter may be very low. We therefore prepared construct pNGI in which the *glp1* gene is controlled by a tetracycline regulatable 32-bp *ran* promoter and contains an AU1 epitope tag at its C terminus (Fig. 2B). A ~30-kDa protein, slightly larger than the predicted ~26.3 kDa molecular mass of GLP1 with the AU1 tag (~0.8 kDa), was detected with anti-AU1 antibody after tetracycline induction (Fig. 1C). The pNGI transfectants showed no phenotypic difference in the presence or absence of tetracycline, suggesting that expressed GLP1 is not toxic. The *glp1* mRNA was detected at low and high levels in the absence and presence of tetracycline (Fig. 1C). We also prepared a pNTG construct in which the *glp1* gene is controlled by the well-known strong α2-tubulin promoter and contains an AU1 epitope tag at its C-terminus. However, the GLP1 protein was not detected in both vegetative and encysting cells in Western blot or immunofluorescence assays (data not shown). The *glp1* mRNA expressed from the pNTG construct was much lower than that from pNGI in the absence of tetracycline (Fig. 1C).

Localization of the GLP1 protein.
The cellular locations of GLP1 were examined using tagged fusion proteins in the pNGI transfectants. As shown in Fig 2A, the AU1-tagged GLP1 could be detected exclusively in the nucleus in tetracycline induced cells (Fig. 2A, right panel), indicating that GLP1 is a nuclear protein in *Giardia*. Expression was ~1% and ~50% positive in tetracycline noninduced and induced cells, respectively. There was also a weak cytosolic staining in the 1% of stained noninduced cells (Fig. 2A, left panel) but not in the induced cells. We also found that the GLP1 expressed by the pNG construct in which the *glp1* gene is controlled by its own promoter localized to the nuclei and weakly in cytosol and the proportion of stained cells increased (5% vs 50%) during encystation (data not shown). The staining intensity of the non-regulated pNG transfectants is much less than that of the tet-inducible pNGI transfectants. We further

used the pNGI transfectants to identify the portion of GLP1 that is sufficient to direct the protein to the nuclei.

No typical nuclear localization signal was predicted in GLP1 using the PSORT software (http://psort.nibb.ac.jp/, Nakai and Kanehisa, 1992). Mutation of the N-terminal basic amino acids (region 1, residues 7-8, pNGm1). or a stretch of basic amino acids in the GARP-like domain (region 2, residues 153-155, pNGm2) did not affect the nuclear localization (data not shown). Mutation of the three lysine residues near the N-terminus (region 3, residues 33, 36, and 38, pNGm3) resulted in protein targeting to the nuclei and more targeting to the cytosol, including faint staining of an axonemal structure in the presence of tetracycline (Fig. 2B), suggesting that these three lysine residues may play a role in the exclusively nuclear localization. Deletion of the C-terminal region containing the GARP-like domain (residues 143-232, pNGΔC) or deletion of the N-terminal region (residues 2-141, pNGΔN) led to an absence of immunofluorescence (data not shown), indicating that these GLP1 deletion mutants were expressed at very low levels or were unstable proteins. We wanted to know if the C-terminal GARP-like domain of GLP1 could also help the nuclear localization of another protein. Previously, we showed that the gMyb2 transcription factor with a deletion of its C-terminal Myb repeats could not target to the nuclei but was unevenly distributed in the cytosol (pNMybΔC, Sun *et al*., 2002). Therefore, we constructed a pNMybGLPC plasmid which contains a fusion of the N-terminal region of gMyb2 and the C-terminal region of GLP1 under the control of the *gmyb2* promoter, which is upregulated in encystation. The chimeric protein was localized exclusively in the cytosol during encystation (Fig. 3C), indicating that the C-terminal region of GLP1 which contains the GARP-like domain does not contain a functional NLS.

Identification of the GLP2 binding sites.

We have shown that GLP1 binds to the 32-bp *ran* promoter element and the *cwp1* promoter in the double-stranded configurations in electrophoretic mobility shift assays. The 32-bp *ran* promoter element was previously shown to play a predominant role in determining promoter activity and positions of the transcription start sites (Sun and Tai, 1999), indicating that GLP1 may be involved in transcriptional regulation. Mutation analysis revealed that (A/G)ATCN sequences were required for the binding of GLP1.

To understand the potential function of GLP2 in *G. lamblia*, we further investigated the ability of the GLP2 to bind to DNA. We expressed GLP2 with a C-terminal V5 tag in *E. coli* and purified it to >95% homogeneity as assessed in silver stained gels (data not shown). To determine whether purified GLP2 binds DNA, we performed electrophoretic mobility shift assays with double-stranded DNA sequences from the 5'-flanking region of an encystation-induced gene, *cwp1*, and a constitutive gene, *ran*.

Incubation of a labeled double-stranded DNA probe cwp1 -45/-1 and -90/-46 (Table 1) with GLP2 resulted in the formation of retarded bands (Fig. 3A). GLP2 did not bind to either single strand of the cwp1 -90/-46 probe (data not shown). Within this region, GLP2 bound to the 3' region, cwp -68/-46, but not to cwp1 -90/-69 (Table 1). GLP2 also bound to cwp1 -45/-1 and to a well-characterized constitutive core promoter, ran -51/-20, but not to ran -30/-1 (Table 1).

The binding specificity was confirmed by competition and supershift assays (Fig. 3A, additional data not shown). The formation of the retarded cwp1 -45/-1 bands was almost totally competed by a 200-fold molar excess of unlabeled cwp1 -90/-46, but not by the same excess of a nonspecific competitor (NS), ran -30/-1 (data not shown). The bound form on cwp1 -90/-46 could also be supershifted by an anti-V5-HRP antibody (Fig. 3A).

The cwp1 -68/-46 probe contains the sequence AATCT, which is the consensus binding sites of GLP1. Mutation analysis of the cwp1 -68/-46 probe revealed that the short AATCT sequence was required for binding (Table 1, cwp1 -68/-46m). Mutation analysis of the cwp1 -45/-1 probe (Table 1, cwp1 -45/-1m) and ran -51/-20 (Table 1, ran -51/-20m), revealed that the GATCA and AATCG sequences were required for binding, respectively (Fig. 3B). These results indicate that the (A/G)ATC(A/G/T) sequence could be the GLP2 binding sites. The other probes tested, cwp1 -90/-69, g6pi-b -30/-1, g6pi-b -60/-31, ran -30/-1, and ran -81/-52, neither contained the (A/G)ATC(A/G/T) sequence nor bound to GLP2 (Table 1, data not shown).

Scanning mutagenesis of the 5-bp AATCT sequence in the cwp1 -68/-46 probe showed that any single substitution within the AATC sequence abolished the DNA-protein interaction (data not shown), but mutations of the fifth T to A or G did not completely eliminate binding (data not shown). Mutations of the fifth T to C abolished binding (data not shown). In summary, GLP2 can bind to (A/G)ATC(A/G/T). This is similar to the sequence of GLP1 binding sites, (A/G)ATCN.

Identification and characterization of *zfp1* and *zfp2*.

The *zfp1* and *zfp2* genes were found previously by *in silico* screening of the *G. lamblia* genome data base. In the previous study, we showed that ZFP1 localized to the two nuclei in both vegetative and encysting trophozoites. In this project, we found that both *zfp1* and *zfp2* mRNA levels increased during encystation (Fig. 4). ZFP1 promoted expression of the reporter gene driven by the *cwp1* promoter in *Giardia* in cotransfection assays, indicating that the gMyb2 can transactivate the *cwp1* promoter (Fig 5). We also used the GAL4-based system to determine the transactivation domain of ZFP1. We found that transactivation by ~4.2-fold occurred when the effector contained the C-terminal region of ZFP1 containing glutamine rich domain (Fig. 6). The full-length and N-terminal portions of GLP1 showed no transactivation ability.

Effect of G418 and puromycin on G. lamblia.

Despite extensive usage of G418 and puromycin for selection of stable transfectants in the lab, very little is known about what causes toxicity of these antibiotics in *Giardia* stable transfectants. To understand the effect of these drugs on *G. lamblia* transcription or translation, we made two kinds of luciferase mutant, a truncated luciferase comprised of only 1-410 amino acids (luc+s) and a mutated luciferase gene (luc+m). The luc+m differed from the normal one at two positions, in which Leu 411 was mutated to a termination codon and Asp 500 was mutated to Asn. Trophozoites transfected with a pPW1s construct containing the luc+s gene under the control of the *cwp1* promoter displayed only a background level of activity, indicating that the truncated luciferase lacks enzymic activity (Fig. 7). However, trophozoites transfected with a pPW1m construct containing the luc+m gene under the control of the *cwp1* promoter exhibited ~200-fold of background activity (Fig. 7). The levels of luc+m were about five orders of magnitude reduction than wild type luc+ in luciferase activity. The results indicate that the nonsense mutation in the luc+m was supressed and the lucifease activity was restored in G. lambila trophozoites.

To understand whether pRANneo influences luciferase expression, pPW1m was stably cotransfected with pRANneo. The luciferase activity in the cotransfectants increased by ~25-fold than pPW1 transfectants, indicating that pRANneo could suppress nonsense mutation. However, cotransfection of pRANneo transiently to pPW1 transfectants without of addition of G418 selection did not increase luciferase activity (data not shown). Since the aminoglycoside gentamicin G418 was used to select and maintain stable transfectants, this effect could be from G418 treatment. We further tested the effect of G418 by adding of G418 to pPW1m transfectants. As shown in Fig. 7, G418 treatment significantly increased luciferase activity by ~63-fold. However, treatment of tetracycline to pPW1 transfectants did not influence luciferase activity (data not shown).

To understand whether 5'Δ5N-Pac also influences luciferase expression, we made a pNW1m constuct in which the pac gene was used instead of neo gene. The NW1 stable transfectants displayed ~2-fold of luciferase activity than pPW1 (Fig. 7). Cotransfection of pNW1 with 5'Δ5N-Pac increased luciferase activity by ~269-fold than pNW1m transfectants, indicating that 5'Δ5N-Pac could also suppress nonsense mutation (Fig. 7). We did not success in testing the effect of puromycin because the treatment of puromycin resulted cell death.

To understand whether the increase of luciferase activity was due to the increase of mRNA level, we further detected the luciferase mRNA of different transfectants by Northern blot analysis. As shown in Fig. 8A, similar high levels of luciferase mRNA were found in the pPW1m and pPW1s transfectants. This indicates that the lack of activity of luc+s was not due to the low mRNA levels. Similar high levels of

luciferase mRNA were also found in the pPW1m transfectants and its pRANneo cotransfectants (Fig. 8B). In addition, similar high levels of luciferase mRNA were also found in the pNW1m transfectants and its 5'Δ5N-Pac cotransfectants (Fig. 8B). The results indicate that the higher activity in the pRANneo or 5'Δ5N-Pac cotransfectants was not due to higher mRNA levels.

Discussion

Identification and characterization of the *glp* genes.

   The constitutive and encystation-induced giardial promoter sequences defined to date contain A/T-rich Inr-like elements and are short (Holberton and Marshall, 1995; Lujan *et al.*, 1995; Mowatt *et al.*, 1995; Elmendorf *et al.*, 2001; Sun and Tai, 1999; Knodler *et al.*, 1999; Yee *et al.*, 2000; Davis-Hayman *et al.*, 2003). In earlier studies, unidentified nuclear extract proteins bound only to single-stranded oligonucleotide targets, suggesting a fundamental divergence from transcription mechanisms in other eukaryotes (Sun and Tai, 1999; Yee *et al.*, 2000). Our previously identified gmyb2 gene whose expression increased during encystation could be important for transcriptional activation of encystation genes, and may help co-ordinate synthesis of cyst wall proteins and polysaccharide (Sun *et al.*, 2002; Sun *et al.*, unpublished data). In this study, we have identified a transcription factor GLP1/2 which binds specific sequences in double-stranded configuration. We found that the core promoter region of a constitutive *ran* gene and the proximal upstream region of an encystation-induced *cwp1* gene contained GLP1/2 binding sites.

   The GARP homologues constitute a large family of plant DNA-binding proteins that perform a variety of cellular functions. These GARP domains may have similar sequence specificities for DNA binding, because DNA bound to ARR1 and CCA1 contains GGATT and AGATT sequences, respectively (Sakai *et al.*, 2000; Wang *et al.*, 1997). Although divergent from plant GARP proteins, GLP1 still maintains the ability to bind DNA in a sequence-specific manner. The GLP1 binding sites (A/G)ATCN and the GLP2 binding sites (A/G)ATC(A/G/T) are similar to that of *Arabidopsis* ARRs binding sites (A/G)ATC(T/C), although ARR is on the noncoding strand (Sakai *et al.*, 2000; Hosoda *et al.*, 2002). This indicates that the binding sites of the members of GARP family have been conserved in evolution. ARR1 has a strong preference for a C or T at position 5; GLP1 tolerates either A, T, C, or G at position 5; GLP2 tolerates either A, T, or G at position 5. The key residues contacting position 5 are E and A of ENVA in *Arabidopsis* ARRs (Hosoda *et al.*, 2002). However, they are predicted to be Q and E of QNVE in GLP1; K and Q of KHVQ in GLP2 (data not shown). The non-conservative substitutions might explain why GLP1/2 tolerates more nucleotides at position 5. The best GLP1/2 target sequennces were identified as AATCT, GATCA, and AATCG. The ability of binding activity of GLP1/2 to bind GATCA could be

attributed to the creation of another ATC sequence in the complementary strand as a result of the replacement of A at the first position with G. As the specificity determined by a 4 or 5 bp sequence does not seem high enough for choosing target genes on the giardial genome, it is likely that additional transcription factors interacting with GLP1/2 help increase the specificity for transcriptional regulation. Unlike *Arabidopsis* ARR1 and other GARP proteins, which contain central GARP domains, the GARP-like domains of giardial GLPs are located near C terminus. The purified C-terminal region containing the GARP-like domain of GLP1 contained the DNA-binding activity of GLP1 (report for grant). It is expected that other members in *Giardia* containing the GARP-like domain, such as GLP3 and GLP4, may also able to bind DNA with similar sequences. Since the resemblance of GLP1-4 does not extend beyond this domain region, the GARP-like domain may be a functional unit for DNA binding in the giardial GARP family of proteins.

GARP domains have been found to confer the ability to direct nuclear localization in *Arabidopsis* (Hosoda *et al.*, 2002). However, the GARP-like domain and its C-terminal sequence of GLP1 did not direct the truncated gMyb2 protein to nuclei. No probable nuclear localization signals except some basic amino acids are present in GLP1. The basic sequences may be functionally redundant in terms of the nuclear targeting. This is consistent with our results which showed that mutation of two stretches of basic amino acids (regions 1 and 2, residues 7-8 and residues 153-155) did not affect the nuclear localization. It is also possible that regions 1 and 2 are not responsible for nuclear localization. Mutation of three lysine residues near N-terminal (region 3) resulted in slight protein targeting to the cytosol and axonemes, as well as the nuclei. Although the specific signal sequence by which GLP1 localize to nuclei was not determined completely, our identified region 3 (residues 33, 36, and 38), presumably contributes at least in part to nuclear localization.

Although many plant GARP proteins have been classified as type B two-component response regulators (Sakai *et al.*, 1998; Sakai *et al.*, 2000; Imamura, 1999) and pseudo-response regulators (Makino *et al.*, 2000; Makino *et al.*, 2001). Giardial GLPs do not contain the amino-terminal receiver domain conserved in this class of proteins. A number of GARP proteins in plants can function as transcriptional activators and are involved in the adaptive response and differentiation processes (Makino *et al.*, 2000; Imamura, 1999; Sakai *et al.*, 2001; Kerstetter *et al.*, 2001). Since *Giardia* colonizes the intestinal tract, it must constantly react to changing signals from the host environment (Adam, 2001). GLP1/2 binding sites are present in the proximal 5'-flanking regions of some encystation-induced genes such as *cwp1* and other genes which are not induced during encystation. This suggests that GLP1/2 may be involved in transcriptional regulation of many different genes. The *ran* and *cwp1* gene promoters were activated by GLP1/2, suggesting that GLP1/2 is a transactivator *in vivo*. Other genes whose promoter regions contain the GLP1/2 recognition sequences

are possibly transactivated by GLP1/2. An as yet unidentified signal may modulate the capability for transactivation and/or DNA binding.

The *garp* genes have been found only in plant genomes to date. Although it is divergent, GLP1/2 is clearly recognizable as a member of the GARP family. This suggests that members of the GARP family evolved before divergence of *Giardia* from the main eukaryotic line of descent. GARP domains may be important in regulating gene expression in the earliest eukaryotes. The similarity in the binding targets of GLP1/2 and *Arabidopsis* ARR1 may be co-incidental or may reflect similar structure or function. Further understanding awaits the exploration of other GARP proteins in other deep-branching protist, such as *Trichomonas vaginalis* and *Entamoeba histolytica*.

Identification and characterization of *zfp1* and *zfp2*.

Our results showed that cotransfected ZFP1 could increase the expression of *cwp1* gene promoter by ~8-fold in vegetative trophozoites. The results indicate that ZFP1 could function as a transcriptional activator. We are trying to understand if other gene promoters can also be upregulated by the overexpressed ZFP1. We also found that the domain responsible for *in vivo* transactivation of ZFP1 was localized within the C-terminal region of ZFP1. This indicates that the a stretch of glutamine-rich amino acids may have transactivation ability. Glutamine-rich characteristics are frequently associated with the transactivation domain of eukaryotic transcription factors. We are analyzing the biological function of *zfp1/2* gene which is up-regulated during encystation.

Effect of G418 and puromycin on *G. lamblia*.

The firefly luciferase molecule is folded into two compact domains (Conti *et al*., 1996). The large N-terminal domain, comprising residues 4-436, consists of a distorted antiparallel beta-barrel and two beta-sheets. The C terminus of the protein (440-544) forms a distinct domain, which is separated from the N-terminal domain by a wide cleft. In our study, the luciferase deletion mutant (luc+s) that lacks the C-terminal residues 411-544 did not display any activity, suggesting that the C terminus of luciferase is important for enzymic activity. However, transfection of a luciferase mutant (luc+m) in which Leu 411 was mutated to a termination codon and Asp 500 was mutated to Asn exhibited ~200-fold of background levels. The luciferase activity in this mutant was much lower than the wild type activity (~10^{-5} of the wild type activity). The results indicate that the nonsense mutation in the luc+m was

supressed in G. lambila trophozoites. We also found that stably cotransfection of luc+m containing construct with pRANneo highly increased luciferase activity. However, addition of the aminoglycoside G418 but not transient transfection of pRANneo significantly increased in luciferase activity, suggesting that the G418 treatment induced suppression of premature stop mutation of the luc+m. In addition, mRNA levels in the luc+m stable transfectants and their pRANneo cotranfectants were similar in Northern blot analysis. These results indicate that G418 can suppress the premature stop mutation. We also found that puromycin, a protein synthesis inhibitor, can also suppress the premature stop mutation to a significant extent.

Aminoglycosides bind to ribosomal RNA and cause a conformational change in ribosomal RNA (Eustice and Wilhelm, 1984; Schroeder *et al.*, 2000). They disturb codon-anticodon recognition at the aminoacyl-tRNA acceptor site, dissrupting high-fidelity reading of the genetic code. Aminoglycosides cause the ribosome to introduce missense mutations and translates through termination codons in both bacterial and eukaryotic cells. Growth of mammalian cells in the presence of an aminoglycoside antibiotic can promote translation read-through of the nonsense codon (Burke, J.F., and Mogg, A.E. 1985.). Our results also correlated the potentiation of misreading of the Gentamicin G418 in *Giardia*.

Puromycin is an analog of the aminoacyl terminus of an aminoacyl-tRNA. In higher eukayotes, its amino group linked non-specifically to the carbonyl group of growing polypeptide chains to form an adduct that dissociates from the ribosome, thereby causing premature termination of translation (Nathans *et al.*, 1964; Kirillov *et al.*, 1997). Our results showed that puromycin can suppress the premature stop mutation in *G. lamblia*. This is quite different from the effect of puromycin in higher eukaryotes. We are currently working on analyzing the potential mechanism of puromycin in *G. lamblia*.

References

Abel, E.S., Davids, B.J., Robles, L.D., Loflin, C.E., Gillin, F.D., and Chakrabarti, R. (2001) Possible roles of protein kinase A in cell motility and excystation of the early diverging eukaryote *Giardia lamblia*. *J Biol Chem* **276:** 10320-10329.
Adam R.D. (2001) Biology of *Giardia lamblia*. *Clin Microbiol Rev* **14:** 447-475.
Best, A.A., Morrison, H.G., McArthur, A.G., Sogin, M.L., and Olsen, G.J. (2004) Evolution of eukaryotic transcription: insights from the genome of *Giardia lamblia*. *Genome Res* **14:**1537-1547.
Burke JF, Mogg AE. Suppression of a nonsense mutation in mammalian cells in vivo by the aminoglycoside antibiotics G-418 and paromomycin. Nucleic Acids Res. 1985 Sep 11;13(17):6265-72.
Cavalier-Smith, T. (1991) The evolution of cells. In Evolution of Life, Fossils: Fossils,

Molecules, and Culture. Eds. S. Osawa, T. Honjo, p. 271-304, Tokyo: Springer-Verlag.

Conti, E., Franks, N.P., and Brick, P. Structure. (1996) Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes. *Structure* **4:** 287-298.

Eichinger, D. (2001) Encystation in parasitic protozoa. *Curr Opin Microbiol* **4:** 421-426.

Davis-Hayman SR, Hayman JR, Nash TE. Encystation-specific regulation of the cyst wall protein 2 gene in *Giardia lamblia* by multiple cis-acting elements. Int J Parasitol. 2003 Sep 15;33(10):1005-12.

Elmendorf, H.G., Singer, S.M., Pierce, J., Cowan, J., and Nash, T.E. (2001) Initiator and upstream elements in the α2-tubulin promoter of *Giardia lamblia. Mol Biochem Parasitol* **113:** 157-169.

Eustice DC, Wilhelm JM. Fidelity of the eukaryotic codon-anticodon interaction: interference by aminoglycoside antibiotics. Biochemistry. 1984 Mar 27;23(7):1462-7.

Gillin, F.D. and Reiner, D.S. (1996) Cell biology of the primitive eukaryote *Giardia lamblia. Annu Rev Microbiol* **50:** 679-705.

Gillin, F.D., Reiner, D.S., Gault, M.J., Douglas, H., Das, S., Wunderlich, A., and Sauch, J.F. (1987) Encystation and expression of cyst antigens by *Giardia lamblia in vitro. Science* **235:** 1040-1043.

Hashimoto, T., Nakamura, Y., Nakamura, F., Shirakura, T., Adachi, J., Goto, N., Okamoto, K., and Hasegawa, M. (1994) Protein phylogeny gives a robust estimation for early divergences of eukaryotes: phylogenetic place of a mitochondria-lacking protozoan, *Giardia lamblia.* Mol. Biol. Evol. **11:** 65-71.

Hashimoto, T., Nakamura, Y., Kamaishi, T., Nakamura, F., Adachi, J., Okamoto, K., and Hasegawa, M. (1995) Phylogenetic place of mitochondrion-lacking protozoan, *Giardia lamblia,* inferred from amino acid sequences of elongation factor 2. Mol. Biol. Evol. **12:** 782-93.

Holberton, D.V., and Marshall, J. (1995) Analysis of consensus sequence patterns in *Giardia* cytoskeleton gene promoters. *Nucleic Acids Res* **23:** 2945-2953.

Hosoda, K., Imamura, A., Katoh, E., Hatta, T., Tachiki, M., Yamada, H., et al. (2002) Molecular structure of the GARP family of plant Myb-related DNA binding motifs of the *Arabidopsis* response regulators. *Plant Cell***14:**2015-2029.

Imamura, A., Hanaki, N., Nakamura, A., Suzuki, T., Taniguchi, M., Kiba, T., et al. (1999) Compilation and characterization of *Arabidopsis thaliana* response regulators implicated in His-Asp phosphorelay signal transduction. *Plant Cell Physiol* **40:** 733-742.

Keister, D.B. (1983) Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. *Trans R. Soc Trop Med Hyg* **77:** 487-488.

Kerstetter, R.A., Bollman, K., Taylor, R.A., Bomblies, K., and Poethig, R.S. (2001)

KANADI regulates organ polarity in *Arabidopsis*. *Nature* **411:** 706-9.

Knodler, L.A., Svard, S.G., Silberman, J.D., Davids, B.J., and Gillin, F.D. (1999) Developmental gene regulation in *Giardia lamblia*: first evidence for an encystation-specific promoter and differential 5' mRNA processing. *Mol Microbiol* **34:** 327-340.

Kirillov S, Porse BT, Vester B, Woolley P, Garrett RA. Movement of the 3'-end of tRNA through the peptidyl transferase centre and its inhibition by antibiotics. FEBS Lett. 1997 Apr 14;406(3):223-33.

Krempler, A., and Brenig, B. (1999) Zinc finger proteins: watchdogs in muscle development. Mol. Gen. Genet.. **261:** 209-215.

Lujan, H.D., Mowatt, M.R., Conrad, J.T., Bowers, B., and Nash, T.E. (1995) Identification of a novel *Giardia lamblia* cyst wall protein with leucine-rich repeats. Implications for secretory granule formation and protein assembly into the cyst wall. *J Biol Chem* **270:** 29307-29313.

Lujan, H.D., Mowatt, M.R., Nash, T.E. (1997) Mechanisms of *Giardia lamblia* differentiation into cysts. *Microbiol Mol Biol Rev* **61:** 294-304.

Makino, S., Kiba, T., Imamura, A., Hanaki, N., Nakamura, A., Suzuki, T., et al. (2000) Genes encoding pseudo-response regulators: insight into His-to-Asp phosphorelay and circadian rhythm in *Arabidopsis thaliana*. *Plant Cell Physiol***41:** 791-803.

Makino, S., Matsushika, A., Kojima, M., Oda, Y., and Mizuno, T. (2001) Light response of the circadian waves of the APRR1/TOC1 quintet: when does the quintet start singing rhythmically in *Arabidopsis*? *Plant Cell Physiol* **42:** 334-339.

McArthur, A.G., Morrison, H.G., Nixon, J.E., Passamaneck, N.Q., Kim, U., Hinkle, G., et al. (2000) The *Giardia* genome project database. *FEMS Microbiol Lett* **189:** 271-273.

Mowatt, M.R., Lujan, H.D., Cotton, D.B., Bower, B., Yee, J., Nash, T.E., and Stibbs, H.H. (1995) Developmentally regulated expression of a *Giardia lamblia* cyst wall protein gene. *Mol Microb* **15:** 955-963.

Nakai, K. and Kanehisa, M. (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* **14:** 897-911.

NATHANS D. PUROMYCIN INHIBITION OF PROTEIN SYNTHESIS: INCORPORATION OF PUROMYCIN INTO PEPTIDE CHAINS. Proc Natl Acad Sci U S A. 1964 Apr;51:585-92.

Reiner, D.S., Douglas, D., and Gillin, F.D. (1989) Identification and localization of cyst-specific antigens of *Giardia lamblia*. *Infect Immun* **57:** 963-968.

Sakai, H., Aoyama, T., and Oka, A. (2000) *Arabidopsis* ARR1 and ARR2 response regulators operate as transcriptional activators. *Plant J.* **24:** 703-711.

Sakai, H., Aoyama, T., Bono, H., and Oka, A. (1998) Two-component response regulators from *Arabidopsis thaliana* contain a putative DNA-binding motif. *Plant Cell Physiol* **39:** 1232-1239.

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., and Oka, A. (2001) ARR1, a transcription factor for genes immediately responsive to cytokinins. *Science* **294:** 1519-1521.

Singer SM, Yee J, Nash TE. Episomal and integrated maintenance of foreign DNA in *Giardia lamblia*. Mol Biochem Parasitol. 1998 Apr 1;92(1):59-69.

Schroeder R, Waldsich C, Wank H. Modulation of RNA function by aminoglycoside antibiotics. EMBO J. 2000 Jan 4;19(1):1-9.

Sogin, M.L., Gunderson, J.H., Elwood, H.J., Alonso, R.A., and Peattie, D.A. (1989) Phylogenetic meaning of the kingdom concept: an unusual ribosomal RNA from *Giardia lamblia*. *Science* **243:** 75-77.

Sun, C.H., and Tai, J.H. (1999) Identification and characterization of a *ran* gene promoter in the primitive protozoan pathogen *Giardia lamblia*. *J Biol Chem* **274:** 19699-19706.

Sun, C.H., Chou, C.F., and Tai, J.H. (1998) Stable DNA transfection of the primitive protozoan pathogen *Giardia lamblia*. *Mol Biochem Parasitol* **92:** 123-132.

Sun, C.H., McCaffery, J.M., Reiner, D.S., and Gillin, F.D. (2003) Mining the *Giardia lamblia* genome for new cyst wall proteins. *J Biol Chem* **278:** 21701-21708.

Sun, C.H., Palm, D., McArthur, A.G., Svard, S.G., and Gillin, F.D. (2002) A novel Myb-related protein involved in transcriptional activation of encystation genes in *Giardia lamblia*. *Mol Microbiol* **46:** 971-984.

Takatsuji, H. Zinc-finger transcription factors in plants. (1998) Cell Mol. Life Sci. **54:** 582-596.

Van Keulen, H., Steimle, P. A., Bulik, D. A., Borowiak, R. K., and Jarroll, E. L. (1998) Cloning of two putative *Giardia lamblia* glucosamine 6-phosphate isomerase genes only one of which is transcriptionally activated during encystment. J. Eukaryot. Microbiol. **45:** 637-642.

Wang, Z.Y., Kenigsbuch, D., Sun, L., Harel, E., Ong, M.S., and Tobin, E.M. (1997) A Myb-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis* Lhcb gene. *Plant Cell* **9:** 491-507.

Wolfe, M.S. (1992) Giardiasis. *Clin. Microbiol. Rev.* **5:** 93-100.

Yee, J., Mowatt, M.R., Dennis, P.P., and Nash, T.E. (2000) Transcriptional analysis of the glutamate dehydrogenase gene in the primitive eukaryote, *Giardia lamblia*. Identification of a primordial gene promoter. *J Biol Chem* **275:** 11432-11439.
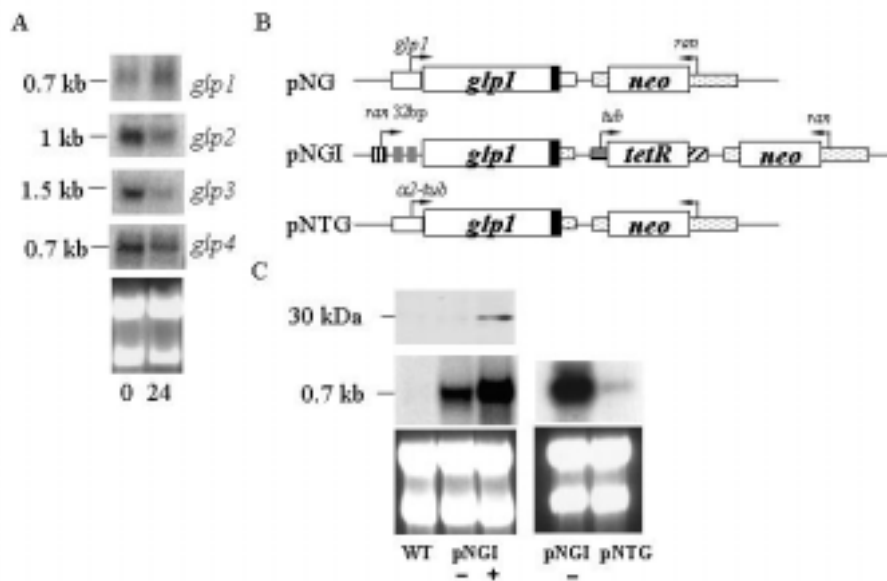
Figures and legends

Fig. 1. Analysis of *glp1* gene expression. (A) Northern blot analysis of *glps* transcription using specific probes. *G. lamblia* cells were cultured in growth (0 hr) or 24hr in encystation medium (upper panel). A ribosomal RNA loading control is in the lower panel. (B) Diagrams of the pNG, pNGI, and pNTG plasmids. A *neo* gene (open box) is under the control of the 5'- and 3'-flanking regions of the *ran* gene (dotted box). The *glp1* gene (open boxes) is under the control of its own 5'- and 3'-flanking regions (open boxes) in pNG or it is under the control of the 32-bp *ran* promoter (striped box) and the 3'-flanking of the *ran* gene (dotted box) in pNGI. Two *tet* operators (gray box) were inserted into the promoter context in the pNGI plasmid. The filled box indicates the coding sequence of the AU1 epitope tag. In pNTG, the *glp1* gene (open boxes) is under the control of the 5'-flanking regions (open boxes) of the α2-tubulin gene the 3'-flanking of the *ran* gene (dotted box) in pNGI. The *tet* repressor (*tetR*) is flanked by α2-tubulin promoter (open box) and the 3'-flanking region of the *ran* gene (dotted box). The arrows show the directions of gene transcription. (C) GLP1 protein expression in pNGI stable transfectants. The pNGI transfectants were cultured in growth medium with (+) or without tetracycline (-) and harvested at 24 hr. AU1 tagged GLP1 protein was detected by anti-AU1 antibody. WT means nontransfected wild type cells. Northern blot analysis of *glp1* transcription in pNGI and pNTG stable transfectants are shown in the middle panel. Ribosomal RNA loading controls are in the lower panel.
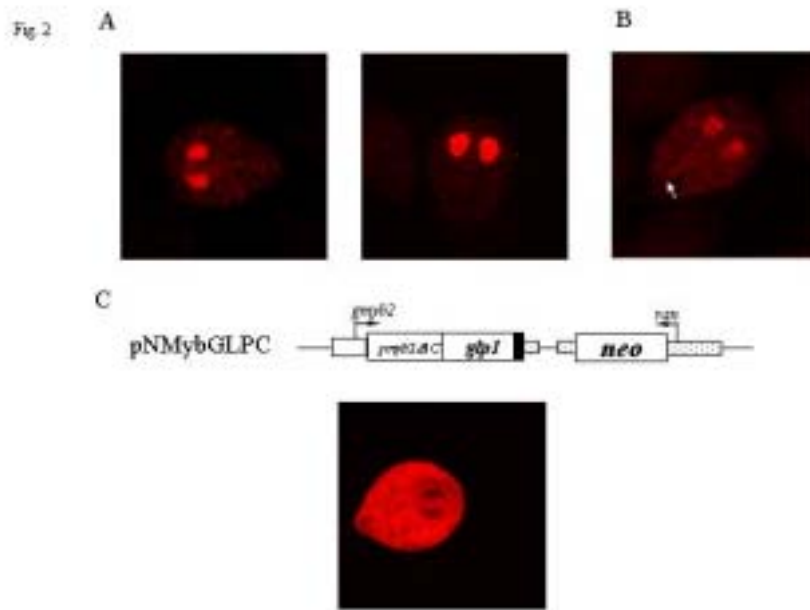
Fig. 2. (A) Nuclear localization of GLP1. The pNGI transfectants were cultured in growth medium with (right panel) or without (left panel) tetracycline for 24 hr and then subjected to immunofluorescence analysis, using anti-AU1 antibody for detection. (B) Nuclear localization of GLP1 mutant protein in the pNGm3 transfectants. It shows that the product of pNGm3 localizes to nuclei, cytosol, and axonemes (arrow) in tetracycline induced cells. (C) Nuclear localization of GLP1 mutant proteins in the pNMybGLPC transfectants. To construct pNMybGLPC, we fused the C-terminal region of GLP1 containing the GARP-like domain (residues 143-232) to the C terminus of the truncated version of the gMyb2 which lacked the Myb repeats and was directed by the *gmyb2* promoter. The AU1 tag was also fused to the C terminus of the protein. It shows that the product of pNMybGLPC localizes in cytosol during encystation.
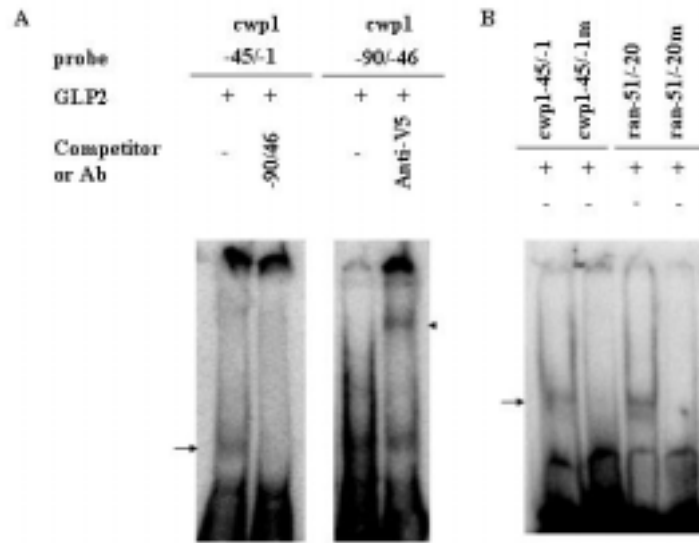
Fig. 3. DNA-binding ability of GLP2 revealed by electrophoretic mobility shift assays. (A) Detection of GLP2 binding sites. Electrophoretic mobility shift assays were performed using purified GLP2 and various ^{32}P-end-labeled oligonucleotide probes (see Table 1). Components in the binding reaction mixtures are indicated above the lanes.
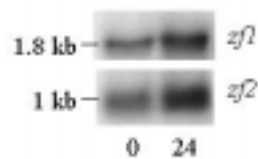

Fig. 4. Northern blot analysis of *zfp1* and *zfp2* transcription using specific probes. *G. lamblia* cells were cultured in growth (0 hr) or 24hr in encystation medium (upper panel). A ribosomal RNA loading control is in the lower panel.
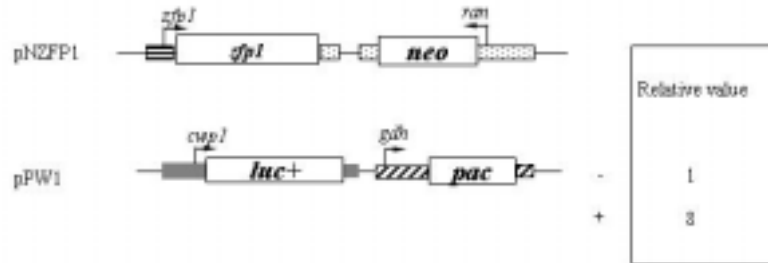
Fig. 5. Transactivation by ZFP1 in the cotranfection system. After stable transfection with these constructs, luciferase activity was measured in vegetative cells. The luciferase activity was expressed as relative light units (RLU) per μg protein. At least three independent experiments were performed for each construct. The activity of cotransfectants (+) relative to single transfection (-) was presented.
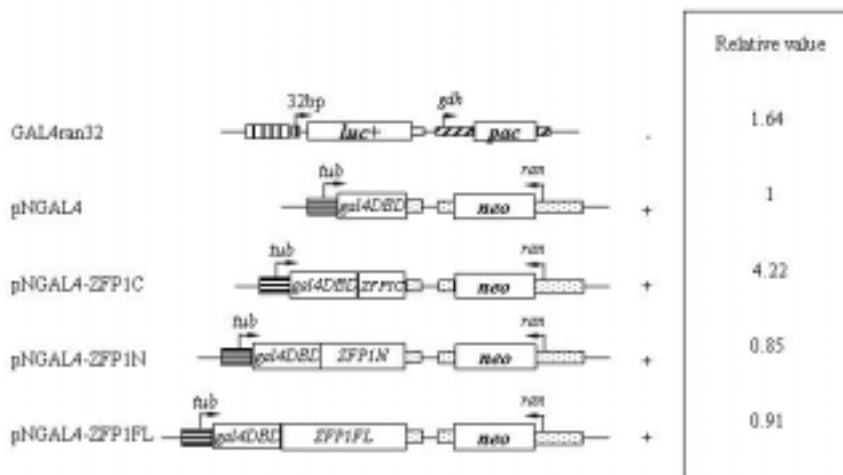

Fig. 6. Analysis of transcription acivity by a GAL4 system. The luciferase reporter gene (*luc +*) is under the control of the 32-bp *ran* promoter as shown. Five GAL4 binding sequences (open boxes) were inserted upstream of the promoter in

GAL4ran32 reporter plasmid. The *pac* gene is under the control of 5'- and 3'-flanking region of the *gdh* gene as shown. The C-terminal, N-terminal, or full-length portions of ZFP1 were fused to the DNA-binding domain of yeast GAL4 which is driven by the α2-tubulin promoter. Single reporer plasmid was transfected to test the promoter activity (-). Transactivation was examined by stable transfection of a reporter plasmid GAL4ran32 first and then stable transfection of an effector plasmid (+). Luciferase activity was measured in vegetative cells. Results are expressed as the mean +/- SD of at least three separate experiments. The basal level of luciferase activity of GAL4ran32 and pNGAL4 cotransfectants was arbitrarily set to 1.
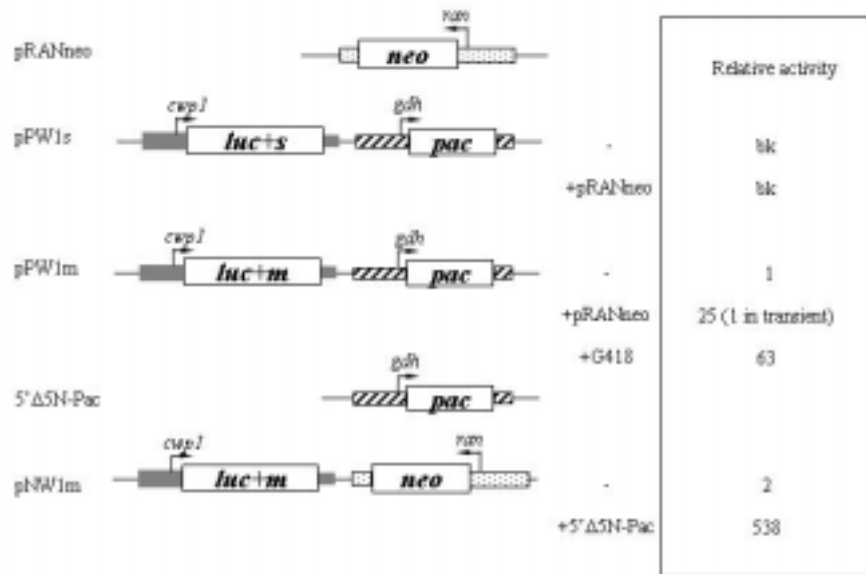

Fig. 7. Induction of luciferase activity by pRANneo and 5' Δ5N-Pac in the cotranfection system. After stable transfection with these constructs, luciferase activity was measured in vegetative cells. The luciferase activity was expressed as relative light units (RLU) per μg protein. At least three independent experiments were performed for each construct. The activity of cotransfectants (+) relative to pPW1m transfection (-) was presented.
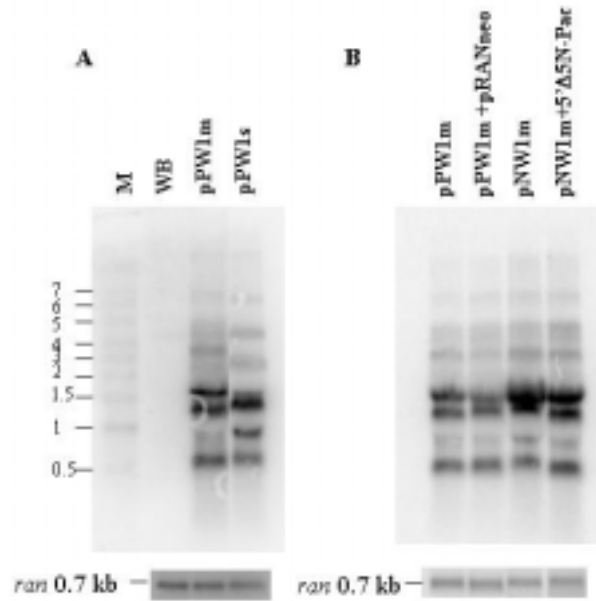
Fig. 8. Northern blot analysis of luciferase transcription. Specific *G. lamblia* transfectants were cultured in growth medium and detected using luciferase probe (upper panel). Ribosomal RNA loading controls are in the lower panel.

Table 1. Oligonucleotides and electrophoretic mobility shifts.

Name	Sequence (5' to 3')	Binding Activity[a]
cwp1 -45/-1	GTTTACAACTCTCA<u>GATCA</u>AGAAACACAGAAATAAAATATCAGGG	+
cwp1 -45/-1m	GTTTACAACTCTCA**CTAGT**AGAAACACAGAAATAAAATATCAGG	-
cwp1 -90/-46	CTGGCTAACAGTCTACAGTCTACAATTTACAGTATAC<u>AATCT</u>ACA	+
cwp1 -90/-69	CTGGCTAACAGTCTACAGTCTA	-
cwp1 -68/-46	CAATTTACAGTATAC<u>AATCT</u>ACA	+
cwp1 -68/-46m	CAATTTACAGTATAC**TTAGA**ACA	-
g6pi-b -30/-1	TATATATGTAACTGCCTCGCCAAAATAAAA	-
g6pi-b -60/-31	TCCTGTAAGGGTTTACTACAGCAGTAGCAG	-
ran -30/-1	TAAAACTTTAACCCAGGAACCGAGAGTAGC	-
ran -51/-20	TAAAATAAATTA<u>AATCG</u>AAATTAAAACTTTAA	+

ran -51/-20m	TAAAATAAATTA**TTAGC**AAATTAAAACTTTAA	-
ran -81/-52	CAGAAAAAGTTGTCAAGGAGGTGATAGCCG	-

[a] The ability of oligonucleotides to bind to the GLP2 protein. "+" and "-" represent binding and no binding, respectively. The putative GLP2 binding sequences are underlined. Base changes in the mutants tested are indicated in boldface.

In this study, we cloned and identified other putuative transcription factors that may be important for *Giardia* gene expression. We also characterized previously identified transfection factors GLP1 and ZFP1 and found that they functioned as transactivators. The studies in this proposal may reveal basic insights into formation and function of the *G. lamblia* encystation. Since cystic stages are key to transmission of many parasites, this differentiation may be a target for interruption of the life cycle. The difference of amino acid sequence and function of transcription factors in *G. lamblia* and higher eukaryotes will provide an indication of the degree of evolutionary variation. We are wring two papers for the data from this study. This study also provided the master students to learn how to do research work on parasites and learn cellular and molecular biology techniques.