Regulation of Cyst Wall Protein Promoters by Myb2 in *Giardia lamblia*

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Myb family transcription factors are important in regulating cell proliferation, differentiation, and cell cycle progression. *Giardia lamblia* differentiates into infectious cysts to survive outside of the host. During encystation, genes encoding cyst wall proteins (CWPs) are coordinately induced. We have identified an encystation-induced Myb2 protein, which binds to the promoter regions of the cwp genes and myb2 itself in vitro. To elucidate the role of Myb2 in *G. lamblia*, we tested the hypothesis that Myb2 can activate encystation-induced genes. We found that overexpression of Myb2 resulted in an increase of expression of CWP1 at both protein and mRNA levels. Interestingly, the Myb2-overexpressing trophozoites had increased capability to differentiate into cysts. In cotransfection assays, Myb2 was able to transactivate the cwp promoters and its own promoter in vivo, suggesting that its gene can be positively autoregulated. Moreover, deletion of the N- or C-terminal domain resulted in a decrease of transactivation and autoregulation function of Myb2. We also found that the promoter of a newly identified encystation-induced gene, the giardial myeloid leukemia factor-like gene, has the Myb2 binding sites and that its mRNA levels were increased by Myb2 overexpression. Chromatin immunoprecipitation assays confirmed that Myb2 was bound to the promoters with its binding sites. Transfection of the myb2 antisense construct reduced the levels of the cwp1 transcripts and cyst formation. Our results suggest that Myb2 is a potent transactivator of the cwp genes and other endogenous genes and plays an important role in *G. lamblia* differentiation into cysts.

Numerous parasitic protozoa have developmental stages in their life cycle that are essential for disease transmission (1). We used *Giardia lamblia*, which has a relatively simple two-stage life cycle that can be completed in vitro as a model for studying the evolution of cellular differentiation (2, 3). *G. lamblia* is an important human pathogen that causes outbreaks of water-borne diarrhea (4, 5). During encystation, *G. lamblia* synthesizes a resistant extracellular wall, which is composed of proteins and polysaccharide (2, 3), protecting the parasite from hypotonic lysis by fresh water and from gastric acid during infection of the new host.

Despite the importance of cyst wall biogenesis during giardial encystation, the molecular mechanisms governing transcriptional regulation remain poorly understood. Expression of genes encoding three cyst wall structural proteins (Cyst Wall Protein 1 (CWP1), CWP2, and CWP3) (6–8) and an enzyme in the cyst wall polysaccharide biosynthetic pathway (glucosamine-6-phosphate isomerase-B, G6PI-B) increases with similar kinetics (9–10), suggesting the importance of regulation at transcriptional level.

In addition to its medical importance, *G. lamblia* is of biological interest in understanding the mechanisms of eukaryotic evolution (11–13). It has fewer cellular components for DNA synthesis, transcription, and RNA processing (13). The lack of clear giardial homologs to these proteins suggests their divergence or their functional redundancy with other proteins in some pathways. *G. lamblia* has a highly divergent TATA-binding protein and lacks eight of the twelve general transcription initiation factors (14, 15). It also has many unusual features with regard to transcription. Unusually short 5′-flanking regions (<65 bp) with no consensus TATA boxes or other cis-acting elements identified in late-branching eukaryotic promoters are sufficient for the expression of some genes (6, 7, 10, 16–19). Instead, AT-rich sequences have been found around the transcription start sites of many genes, functionally similar to the initiator element in late-branching eukaryotes (6, 7, 10, 16–19).

Few transcription factors that have been characterized to date are involved in cwp gene regulation (20–22). The GARP-like protein 1 (named from the maize GOLDEN2, *Arabidopsis* response-regulator proteins and the *Chlamydomonas* *Pst*1 protein) and the AT-rich interaction domain-family transcription factors may be involved in transcriptional regulation of many different genes including the encystation-induced cwp1 gene (21, 22). Interestingly, we have identified an encystation-induced Myb2 protein, which binds to the promoters of four key encystation-induced genes, *cwp1*, *cwp2*, *cwp3*, *g6pi-b*, and *myb2* itself, suggesting that Myb2 may be involved in co-ordinating their differential expression (20).

In late-branching eukaryotes, Myb proteins are DNA-binding transcription factors that regulate specific gene expression.
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in differentiation of different cell types (23). Myb family transcription factors are important in regulating physiological processes in organisms as diverse as fungi, plants, and mammals (24–29). Myb proteins function as transcriptional activators or repressors via association with some cofactors on the promoter context of specific target genes to regulate development, cell differentiation, cell cycle, apoptosis, and cancer (26, 30–34). For example, mammalian c-Myb is required for development of mature B and T cells (31). Several c-Myb target genes have been identified, such as promyelocytic-specific min-1, T cell receptor gamma and delta chains, and c-myb itself (35–42).

In the present study, we have identified an encystation-induced Myb2 protein, which binds to the promoters of the cwp genes in vitro (20). In this study, we found that the constitutively overexpressed Myb2 can increase the expression of endogenous CWP1 at both protein and mRNA levels. Interestingly, the Myb2 overexpressing trophozoites had increased capability to differentiate into cysts. We also found that the cwp1, cwp3, myb2, and g6pi-b promoters can be activated to differing degrees by Myb2, suggesting that Myb2 can transactivate these genes and that the myb2 gene can be positively auto-regulated. Deletion of the N- or C-terminal domain resulted in a decrease of transactivation and autoregulation function of Myb2. Transfection of the myb2 antisense construct reduced the levels of the cwp1 transcripts and cyst formation. In the previous studies, we have found that stable transfection systems may trigger an encystation-like physiological response in G. lamblia trophozoites (43). Interestingly, we have found that the expression of the gene encoding orf 16424 can increase the levels of cwp1-3 transcripts, CWP2 protein and cwp3 transcripts and cyst formation. In the present study, we found that stable transfection systems can increase the levels of cwp1-3 transcripts, CWP2 protein and cyst formation during vegetative growth, indicating that stable transfection systems may trigger an encystation-like physiological response in G. lamblia trophozoites (43).

Experimental Procedures

G. lamblia Culture—Trophozoites of G. lamblia WB (ATCC 30957), clone C6, were cultured in modified TYI-S33 medium (44) and encysted as previously described (8). Cyst count was performed on vegetative cultures as previously described (43).

RNA Extraction and Northern Blot Analysis—Total RNA was extracted from G. lamblia clones C6 at the indicated differentiation stages in the legends of Figs. 1, 5B, and 8C using TRIzol reagent (Invitrogen). For Northern blot analysis, 10 µg of total RNA was fractionated and transferred to charged nylon membranes (Biodyne B membrane, Pall). Full-length coding region probes of myb2 (GenBank™ accession number AY082882), cwp1 (GenBank™ accession number U09330), cwp2 (GenBank™ accession number U28965), ran (GenBank™ accession number U02589), mlfl (orf 16424, GenBank™ accession number for genomic DNA: XM_764168; orf 16424 in G. lamblia genome data base)(13), phosphoglycerate kinase (pgk, GenBank™ accession number for genomic DNA: XM_762975), and bip (GenBank™ accession number for genomic DNA: XM_766560, for protein: XP_771653) genes were prepared by PCR amplification of genomic DNA using primers: myb2F (ATGTACCAGGTATCCTTCTC) and myb2R (TCAGGTTAGCTTCCTCACG), cwp1F (ATG-ATGCTCGCTTCTCCT) and cwp1R (TCAAGGCGGGGTGAAG), cwp2F (ATGATCGACGCGCTTTGTT) and cwp2R (TCACCTCTGCGCAAAAT), ranF (ATGTCTGACCAAAT-CAGC) and ranR (TCAATCTGCTGCGGAAAT), mlflF (CACC-ATGAGTGAAGCGCCAAA) and mlflR (GTAGCCAGTTACCGG), pgkF (ATGTCTTAGCCAGACTTCC) and pgkR (CTTCTTGTCACAGCTGTAG), and bipF (ATGACGCTT-AGTCAGCTTAA) and bipR (GAGTTCATCTTTCAGCTC), respectively. Radiolabeled probes were prepared using the Rediprime II kit (Amersham Biosciences). The membranes were hybridized and washed as previously described (10). Equal loading was confirmed by reprobing the Northern blots with radiolabeled ribosomal RNA. The ribosomal DNA fragment for large subunit ribosomal RNA (GenBank™ accession number X05397) was amplified by PCR using primers RiboF (GGCCCTGCCCCTCGCCGCGC) and RiboR (CCCCCTAGCTTCCTCCGGG) and a genomic DNA template. Radiolabeled ribosomal DNA probes were prepared as described above. Hybridization signals were imaged and quantified using a Storm system (Molecular Dynamics). Two independently generated stably transfected lines were made from each construct and each of these lines was assayed three separate times. The results are expressed as relative expression level over control. Student’s t-tests were used to determine statistical significance of differences between samples.

Plasmid Construction—All constructs were verified by DNA sequencing with BigDye Terminator 3.1 DNA Sequencing kit and an ABI 3100 DNA Analyzer (Applied Biosystems). Plasmid 5’-Δ5N-Pac was a gift from Dr. Steven Singer and Dr. Theodore Nash (45). Plasmid pRANneo, pPW1, pNM5, 4s/5N-Pac, and two copies of a 19-bp operator sequence from pPop2N (47) were replaced by the NheI/ClaI-excised luciferase gene and 2-tubulin promoter sequence from pTM42/3 (48) to generate pNT5 (20). The resulting plasmid, pNTMyb2, contained the myb2 gene was amplified with oligonucleotides Myb2BF (GGCGTCATGATACCGGTAC CTTCTCAGCCA) and Myb25m1NR (GGCGC-CTTCTGTCAGACAGTCTGAT), and bipR (GAGTTCATCTTTCAGCTC), respectively. Radiolabeled probes were prepared as described above. Hybridization signals were imaged and quantified using a Storm system (Molecular Dynamics). Two independently generated stably transfected lines were made from each construct and each of these lines was assayed three separate times. The results are expressed as relative expression level over control. Student’s t-tests were used to determine statistical significance of differences between samples.
CATGGTAGACGTACAGTAGTATTATTATTTTAGTAA-GGACATCAAGAGAAAAATACAT, mutated nucleotides are underlined), digested with Nhel/IcoI, and ligated in place of the Nhel/IcoI-excised 32-bp ran promoter and two copies of a 19-bp tet operator sequence in pNLOp2–1(20). The resulting plasmid, pNM5m, contained the luciferase gene under the control of the myb2 promoter with a mutation on the Myb2 binding site. For constructing pNMybΔ2, a PCR with oligonucleotide Myb2D2F (GGCGCTGCAGCAGTACTAGGAGA-GTACTACCAT) and Myb2AUER generated a 0.7-kb PCR product that was digested with PstI and EcoRI. Another PCR with primers Myb2D2R (GGCGCTGCAGCTCCATGGGATGTGAGTAATAAC) and Myb25NF generated a 1-kb PCR product that was digested with PstI and Nhel and cloned into Nhel/EcoRI-digested pNLOp2–1 (20) with the 0.7-kb PstI/EcoRI fragment. The resulting pNMybΔ2 contains a myb2 gene lacking a region with some similarity to Someponas putida dehRI protein (residues 157–212) (Fig. 4). For constructing pNMMybΔN, a PCR with oligonucleotide Myb2D4F (GGCGCTGCAGATGCA-CTGGGCCAGCGAAGAGAC) and Myb2AUER generated a 0.4-kb product that was digested with PstI and EcoRI. Another PCR with primers Myb2D4R (GGCGCTGCAGTA-CAGTAATATTATTATTTGTAACG) and Myb25NF generated a 0.3-kb PCR product that was digested with PstI and Nhel and cloned into Nhel/EcoRI-digested pNLOp2–1 (20) with the 0.4-kb PstI/EcoRI fragment. The resulting pNMMybΔN contains a myb2 gene lacking the N-terminal 343 amino acids (residues 2–410) and leaves both Myb repeat and the C-ter-minal 10 amino acids (residues 410–530)(Fig. 4, A and B). For constructing pNMyb2as, a PCR with primers Myb2BF (GGC-GTCATGATCAGGGTAGCTCTCAGGGGAAG) and Myb2EF (GGCGGAAATTCGTATGTTACCCCGTTCTT-CTAGC) generated a 1590-bp PCR product that was digested with BspHI and EcoRI and ligated in place of the NcoI/EcoRI-ex-cised luciferase gene in pNM5 (20).

Transfection, Luciferase Assay, and Western Blot Analysis—Cells transfected with pN series plasmid were selected with G418 as previously described (46). Stable transfec-tants were maintained at 150 μg/ml G418. Cells transfected with pP series plasmid containing the pac gene were selected and maintained with 54 μg/ml puromycin. For co-transfection assays (see Fig. 3), G. lamblia cells were first transfected with pP series plasmids and selected in 54 μg/ml puromycin. The stable transfections were transfected with pN series plasmids, and then the cells were doubly selected in both 150 μg/ml G418 and 54 μg/ml puromycin. After stable transfection with specific con-structs, luciferase activity was determined in vegetative cells at late log/stationary phase (1.5 × 10^7 cells/ml) or in 24 h encysting cells as described (10) and was measured with an Optocomp I luminometer (MGM Instruments). Two independently generated stably transfected lines were made from each construct and each of these lines was assayed three separate times. Western blots were probed with anti-AU1 monoclonal antibody (Covance, Princeton, 1/5000 in blocking buffer), and detected with peroxidase-conjugated goat anti-mouse IgG (Pierce, 1/5000) and enhanced chemiluminescence (GE Healthcare).

Generation of anti-CWP1 Antibody—The genomic cwp1 gene was amplified using oligonucleotides W1F (ATGATGC-TGCCTCTCCTGTCTTGT) and W1R (AGGCGGGGTGA-GGCGATGACTCTCCTGCG). The product was cloned into the expression vector pCRT7/CT-TOPO (Invitrogen) in-frame with the C-terminal His and V5 tags to generate plasmid pCW1. The pCW1 plasmid was freshly transformed into Escherichia coli BL21(DE3)pLysS (QIAexpressionist, Qiagen). An overnight preculture was used to start a 250-ml culture. E. coli cells were grown to an A600 of 0.5, and then induced with 1 mM isopropyl-D-thiogalactopyranoside (Promega) for 2 h. Bacteria were harvested by centrifugation and sonicated in 10 ml of buffer A (100 mM sodium phosphate, 10 mM Tris-Cl, 6 mM guanidine hydrochloride, pH 8.0) containing 10 mM imidazole and complete protease inhibitor mixture (Roche Applied Sci-ence). 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 B (100 mM sodium phosphate, 10 mM Tris-Cl, 8 mM urea, pH 8.0) and buffer C (100 mM sodium phosphate, 10 mM Tris-Cl, 8 mM urea, pH 6.3) and eluted with buffer E (100 mM sodium phosphate, 10 mM Tris-Cl, 8 mM urea, pH 4.5). Fractions containing CW1 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 concen-tration were estimated by Coomassie Blue and silver staining compared with bovine serum albumin. CW1 was purified to apparent homogeneity (>95%). Purified CW1 protein was used to generate rabbit polyclonal antibodies through a commercial vendor (Angene, Taipei, Taiwan).

Immunofluorescence Assay—Stably transfected cells were harvested from growth medium under drug selection, were washed in PBS and attached to glass coverslips (2 × 10^6 cells/cover-slip), then fixed as previously described (10). Cells were reacted with anti-AU1 monoclonal antibody (Babco, 1/300 in blocking buffer) and anti-CWP1 (1/300 in blocking buffer). Anti-mouse ALEXA 568 (Molecular Probes, 1/500 in blocking buffer) and anti-rabbit ALEXA 488 (Molecular Probes, 1/500 in blocking buffer) was used as the detector. Detected proteins were visualized using a Leica TCS SP2 Spectral Confocal System.

ChIP Assay—The pNM stable cell line and non-transfected WB cells were inoculated into encystation medium (5 × 10^7 cells in 45 ml of medium) and harvested after 24 h in encys-tation medium under drug selection and washed in PBS. Formaldehyde was then added to the cells in PBS at a final concentra-tion of 1%. Cells were incubated at room temperature for 15 min and reactions were stopped by incubation in 125 mM gly-cine for 5 min. After PBS washes, cells were lysed in luciferase lysis buffer (Promega) and protease inhibitor (Sigma) and then vortexed with glass beads. The cell lysate was sonicated on ice and then centrifuged. Chromatin extract was incubated with anti-AU1 antibody conjugated to beads (Bethyl Laboratories Inc.). The beads were washed twice with luciferase lysis buffer and twice with PBS. The beads were resuspended in elution buffer containing 50 mM Tris-HCl, pH 8.0, 1% SDS and 10 mM EDTA at 65 °C for 4 h. To prepare DNA representing input DNA, 2.5% of chromatin extract without incubation with anti-AU1 beads was combined with elution buffer. Eluted DNA was purified by the QIAquick PCR purification kit (Qiagen). Purified DNA was subjected to PCR followed by agarose gel electrophoresis. The following primers were used to amplify
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Overexpression of Myb2 Induced the Expression of cwp1/2 Genes—To study the role of Myb2 in G. lamblia, we expressed myb2 constitutively under the control of the α2-tubulin gene promoter (pPTMyb2, Fig. 1A) and observed its gene expression in vegetative cells. Northern blot analysis showed that the mRNA levels of the endogenous myb2 plus vector expressed myb2 in the Myb2-overexpressing cell line increased by ~2.7-fold (p < 0.05) relative to those in the control cell line which expressed only the puromycin selection marker (5′Δ5N-Pac, Fig. 1B). The endogenous cwp1 and cwp2 genes were up-regulated by ~3.6–3.8-fold (p < 0.05) in the Myb2 overexpressing cell line (Fig. 1B), suggesting that the overexpressed Myb2 can transactivate the cwp1 and cwp2 genes. The levels of endogenous ran mRNA decreased by ~70% (p < 0.05) in the Myb2-overexpressing cell line compared with those in the control cell line (Fig. 1B).

In previous studies, we have found that the expression of the cwp1, cwp2, and myb2 genes were up-regulated in stable cell line with drug selection (43). Interestingly, we have found that the expression of the gene encoding orf 16425 was also up-regulated by ~1.4–4.8-fold in both encysting cells and stably transfected cells (43). The orf 16425 is a myeloid leukemia factor-like (MLF1) protein, and its function is unknown. We have also found that the expression of the phosphoglycerate kinase (pgk) and bip genes was up-regulated in stably transfected cells but not changed in encysting cells (43). We wished to understand the importance of Myb2 for expression of these genes. We found that the mRNA levels of the mlfl gene also increased ~1.4-fold (p < 0.05) in the Myb2-overexpressing cell line and that the mRNA levels of the pgk and bip gene did not change in the Myb2-overexpressing cell line (Fig. 1B).

Overexpression of Myb2 Increased the Levels of Cyst Wall Protein 1 and Cyst Formation—We further investigated the effect of giardial Myb2 on cyst formation. In previous studies, we have found that some G. lamblia trophozoites may undergo spontaneous differentiation (43). We obtained consistent cyst count data for vegetative G. lamblia cultures growing to stationary phase (~4800 cysts/ml for 5′Δ5N-Pac cell line) (43). In this study, we found that the cyst number in the Myb2 overexpressing cell line increased ~3.2-fold (p < 0.05) relative to the control levels in the 5′Δ5N-Pac cell line (Fig. 2A), indicating that the overexpressed Myb2 can increase the cyst formation.

RESULTS

We next asked whether the levels of cyst wall protein 1 increased with the increase of the cwp1 transcripts in the Myb2-overexpressing cell line. The CWP1 protein was not detected in Western blots, which could be due to its low expression during vegetative growth (43). In immunofluorescence assays, the AU1-tagged Myb2 was detected in the nuclei and slightly in the cytoplasm during vegetative growth (Fig. 2, C and D). This finding was similar to that in our previous study (20). Expression was ~8% positive in vegetative cells. The endogenous CWP1 protein was stained in encystation-secretory vesicles (ESVs) in ~2% of the control cell line 5′Δ5N-Pac (data not shown) (43). A ~10-fold increase in numbers (~20%) of cells positively stained for CWP1 protein was found in the Myb2-overexpressing cell line (Fig. 2B). Interestingly, the CWP1 protein was stained in the ESVs of all of Myb2-AU1 positive stained cells (Fig. 2, C and D and data not shown). The appearance of discrete CWP1-
localized ESVs could be due to the presence of the constitutively overexpressed Myb2 in the trophozoites. The results suggest that Myb2 may function in inducing the ESV/cyst formation and in activation of the cwp1 gene.

Myb2 Can Transactivate the cwp Genes Through Its Target Sequence—To determine if Myb2 has the capacity to activate transcription, we co-transfected the Myb2 expression plasmid pNTMyb2 together with constructs in which the luciferase reporter gene is under the control of the cwp1, cwp3, myb2, or g6pi-b synthetic promoter with four Myb2 binding sites (Fig. 3, pPW1, pPC3, pPM5, or 4s/−42/+3). The Myb2-AU1 protein was expressed in the pNTMyb2 cell line as detected in immunofluorescence assays (data not shown). Cotransfection of the pPW1 reporter construct with pNTMyb2 resulted in a ~3.2-fold enhancement of activity in vegetative cells relative to the control cell line (pPW1 + pRANneo, Fig. 3), indicating that Myb2 can transactivate the cwp1 promoter. We also tested other promoter-luciferase constructs and found that the cwp3 and myb2 promoter, and g6pi-b synthetic gene promoter with four Myb2 binding sites can be activated ~2.1- to ~4.3-fold by Myb2 (Fig. 3). The results indicate that Myb2 can transactivate the promoters containing its binding sites and it can autoregulate its own promoter. We also found that deletion of the Myb2 binding site in the g6pi-b promoter abolishes transactivation by Myb2 (~42/+3, Fig. 3), indicating that Myb2 can transactivate the g6pi-b promoter through its binding sites.

Mapping of the Myb2 Transactivation Domain—To identify the transactivation domain of the Myb2 protein, we established a pNMyc cell line expressing the myb2 gene controlled by its own promoter with sequences encoding an AU1 tag at its C terminus (Fig. 4A) (20). Using this parental system, we constructed four deletion mutants of Myb2: MybΔ1 that deletes the first Myb repeat (residues 410–468), MybΔ2 that deletes a region with some similarity to P. putida dehRI protein (residues 157–212), MybΔC that deletes both Myb repeats and the C-terminal 10 amino acids (residues 410–530), and MybΔN that deletes the N-terminal 343 amino acids (residues 2–410) and leaves both Myb repeats and the C-terminal 10 amino acids (residues 410–530) (Fig. 4, A and B). MybΔN that deletes the N-terminal 343 amino acids (residues 2–410) also lacks the region with some similarity to Saccharomyces cerevisiae Rlr1p (residues 340–403) (Fig. 4, A and B). These constructs were transfected into G. lamblia and the cellular locations of Myb2 in the transfectants were also examined by immunofluorescence assays. The wild-type Myb2, MybΔ1, and MybΔ2 were
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**A**

![Diagram](image)

**B**

![Diagram](image)

**FIGURE 4. Localization of Myb2 and its deletion derivatives.** A, diagrams of the plasmids for Myb2 deletion mapping. Each plasmid contains a neo gene (open box) under the control of the 5′- and 3′-flanking regions of the ran gene (dotted box). Plasmid pNMMyb contains the myb2 gene (open boxes) under the control of the myb2 gene promoter and the 3′-flanking region of the ran gene. The filled box indicates the coding sequence of the AU1 epitope tag. The Myb repeats are indicated as open boxes. B, alignment of the amino acid sequences of the giardial Myb2 and Myb2 genes from the P. putida and S. cerevisiae genomes. Specific sequence similarity search was performed against the GenBankTM database. Numbers indicate positions of the residues relative to the first amino acid. Plus (+) and hyphens indicate conserved amino acids and gaps in the respective proteins, respectively.

localized to nuclei, but MybΔC was localized to cytosol in both vegetative and encysting cells (Fig. 4A, additional data not shown) (20). The MybΔN protein was not detected in vegetative cells, but it was detected in nuclei during encystation (Fig. 4A, additional data not shown).

We further asked whether the protein levels of the Myb2 deletions were changed. As shown by Western blot analysis, the levels of MybΔ1 or MybΔ2 increased significantly compared with those of wild-type Myb2 during encystation (Fig. 5A). However, the levels of MybΔC or MybΔN decreased significantly (Fig. 5A). To understand the effect of different Myb2 deletion mutants, we observed the expression of endogenous cwp1 gene in encysting cells. We found that the cwp1 gene was up-regulated by ~2–3-fold (p < 0.05) in the pNMMybΔ1 and pNMMybΔ2 cell lines relative to the pNMMyb cell line (Fig. 5B). Higher levels of the MybΔ1 or MybΔ2 protein might account for the higher transactivation activity observed in the pNMMybΔ1 and pNMMybΔ2 cell lines (Fig. 5A). However, the cwp1 gene was down-regulated by ~2-fold (p < 0.05) in the pNMMybΔC or pNMMybΔN cell line relative to the pNMMyb cell line (Fig. 5B). The results suggest that the lower levels of the MybΔC or MybΔN protein may be correlated with loss of transactivation. In addition, the inactivity of the MybΔC protein could be due to its inability to enter nucleus (Fig. 4A). The inactivity of the MybΔN protein could be due to the lack of transactivation domain as the MybΔN protein cannot enter nuclei (Fig. 4A).

**Myb2 Gene May Be Autoregulated**—We further asked whether the mRNA levels of the Myb2 deletions were changed. As shown by Northern blot analysis, the levels of endogenous myb2 plus mybΔ1 or mybΔ2 mRNA increased by 2–5-fold (p < 0.05) in the pNMMybΔ1 or pNMMybΔ2 cell line relative to the pNMMyb cell line (Fig. 5B). This result suggests that the increase in the protein levels of the pNMMybΔ1 or pNMMybΔ2 could be due to the increased mRNA levels. However, the levels of endogenous myb2, mybΔC, or mybΔN mRNA decreased significantly in the pNMMybΔC or pNMMybΔN cell line relative to the pNMMyb cell line (Fig. 5B). The results suggest that deletion of the N- or C-terminal domain may result in a decrease of the transactivation and autoregulation function of Myb2.

Because the myb2 promoter contains a Myb2 binding site (20), we would like to know whether this Myb2 binding site is required for the myb2 promoter function. To address this, we constructed a myb2 promoter mutant construct (pNM5m, Fig. 6) with an altered Myb2 binding site in the myb2 promoter-reporter construct pNM5 (20). We found that this mutation decreased promoter activity to ~72 and ~15% of the wild-type promoter activity during vegetative growth and encystment, respectively (Fig. 6). The induction ratio (luciferase expression in encysting cells relative to that in vegetative cells) of the myb2 promoter mutant construct was ~2.6. This is much lower than that of the wild-type myb2 promoter construct (~12.4), indicating that the Myb2 binding site is important for the myb2 promoter activity and that the myb2 gene could be positively autoregulated.
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In the previous studies, we have identified a myb2 gene whose expression increased during encystation (20). The presence of the Myb2 binding sites in the proximal 5'-flanking regions of key encystation-induced genes, cwp1, cwp2, cwp3, g6pi-b, and myb2 itself suggests that Myb2 may be involved in co-ordinating their differential expression. To gain insight into the function of Myb2 in cell differentiation, we tested the hypothesis that Myb2 can activate transcription of the endogenous encystation-induced genes. Our results showed that the constitutively overexpressed Myb2 increased the levels of the cwp1 and cwp2 mRNA in vegetative trophozoites. The levels of the CWP1 protein and cyst formation also increased in the Myb2-overexpressing cell line. The overexpressed Myb2 also can transactivate the cwp1, cwp3, myb2, and g6pi-b promoters that contain Myb2 binding sites in vivo. We also found that deletion of the Myb2 binding site abolished transactivation by Myb2, indicating that Myb2 can transactivate the encystation-induced genes through its target sequence. In addition, deletion of the N- or C-terminal domain resulted in a decrease of transactivation function of Myb2. Transfection of the myb2 antisense construct reduced the levels of the cwp1 transcripts and cyst formation. The results suggest that Myb2 may play an important role in induction of encystation.

Many important transcription factors involved in developmental regulation have an autoregulation mechanism, including mammalian c-Myb (36, 48). The presence of a Myb2 binding site upstream of myb2 itself raises the possibility of myb2-positive autoregulation in G. lamblia. We also addressed this by mutation of this Myb2 binding site and found a significant decrease of Myb2 promoter activity. The overexpressed Myb2 also can transactivate myb2 its own promoter. In addition, we also found that deletion of the N- or C-terminal domain resulted in a decrease of autoregulation function of Myb2. ChIP assays confirmed the association of Myb2 with its own promoter. Our results suggest that the myb2 gene may be positively autoregulated, and this may help attain a higher level of Myb2 for induction of cwp genes during encystation.

**DISCUSSION**

Transfection of the myb2 Antisense Construct Reduced the Levels of the cwp1 Transcripts and Cyst Formation—We next asked whether Myb2 is important for encystation. We found that, when a myb2 antisense-expressing construct (pNMyb2as) was transfected into G. lamblia, the levels of cyst formation decreased significantly in comparison to that in cells transfected with luciferase expression construct (pNM5) or the non-transfected WB C6 cells (Fig. 8, A and B). Northern blot analysis showed that the levels of the myb2 mRNA decreased by ~60% (p < 0.05) in the pNMyb2as transfectants relative to those in the WB C6 control (Fig. 8C). The levels of the cwp1 mRNA decreased by ~70% (p < 0.05) in the pNMyb2as transfectants relative to those in the WB C6 control (Fig. 8C). The levels of the raw mRNA increased ~1.3-fold (p < 0.05) in the pNMyb2as transfectants relative to those in the WB C6 control (Fig. 8C). Similar mRNA levels of the bip gene were found in the pNMyb2 transfectants compared with those in the WB C6 control (Fig. 8C). The results suggest an important role of Myb2 during encystation. In the previous studies, we have found that the cyst formation and the expression of the cwp1 and myb2 genes increased significantly in stable cell lines with drug selection (43). The increased cyst numbers and expression of the cwp1 and myb2 genes were also found in the pN5 stable transfectants relative to those in the non-transfected WB C6 cells (Fig. 8, B and C).

**FIGURE 5. Mapping of the Myb2 transactivation domain.** A, analysis of Myb2 deletion mutants. The specific transfectants were cultured in encystation medium for 24 h (Enc) and then subjected to Western blot analysis, using anti-AU1 antibody for detection. Coomassie Blue-stained total protein loading control is shown below. B, analysis of cwp1 gene expression. Total RNA was harvested from specific transfectants cultured in encystation medium for 24 h. Northern blots were hybridized with the cwp1 gene (shown by arrows) and deletion mutant transcripts (shown by arrowheads) were detected in the pNMybΔC and pNMybΔN transfectants. Only the relative activity of the full-length myb2 transcripts (shown by arrows) was shown for the pNMybΔC and pNMybΔN transfectants.

**FIGURE 6. Mutation analysis of the Myb2 binding site in the myb2 promoter region.** In the pNM5 construct, the luciferase gene (luc+, open box) is under the control of the myb2 promoter and 3’-flanking region of the ran gene (dotted box). The neo gene is under the control of the 5’- and 3’-flanking regions of the ran gene (dotted box). In the pNM5m construct, the mutated Myb2 binding site is indicated by a filled box. After stable transfection with these constructs, luciferase activity was measured in vegetative cells and 24 h encysting cells as described under “Experimental Procedures.” Values are shown as means ± S.E. in the right panel. The induction ratio was obtained by dividing the activity in the encysting cells by the activity in the vegetative cells of each construct.
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The Myb2-overexpressing system could be used to increase some possible encystation-induced genes in vegetative cells. In the previous studies, we have found that the expression of the cwp1, cwp2, myb2, and mlfl (orf 16424) genes were up-regulated in both encysting cells and stably transfected cells (43). In the present study, we found that the levels of mlfl mRNA, like those of cwp1 mRNA, also increased in the Myb2-overexpressing cell line. It is interesting that 4–6 Myb2 binding sequences are present in the 500-bp 5′-flanking regions of the myb2, cwp1, or cwp2 genes. One to two Myb2 binding sequences are present in the 500-bp 5′-flanking regions of the mlfl, or ran genes. Interestingly, ChIP assays confirmed the association of Myb2 with these promoters containing the Myb2 binding sites. Therefore, the ability of Myb2 to transactivate the encystation-induced cwp or mlfl genes may require the binding of Myb2 to its binding sequences. In the previous studies, we have also found that the expression of the phosphoglycerate kinase (pgk) and bip genes was up-regulated in stably transfected cells but not changed in encysting cells (43). In this study, we found that the levels of pgk and bip mRNA did not change in the Myb2-overexpressing cell line. In addition, these two promoters have no Myb2 binding site (−500/−1 of the 5′-flanking region, Fig. 7) and Myb2 was not associated with them. These findings suggest that there might be different signal transduction pathways or different transcriptional mechanisms regulating the expression of the pgk and bip genes in stably transfected cells and Myb2-overexpressing cells.

Unlike Myb proteins in many other organisms whose Myb domains are N-terminal (24–26, 49), the Myb domain in giardial Myb2 is near the C terminus. Myb2 contains only two repeats that appear to function like the R2R3 domains in Myb proteins of other organisms. In this study, we found that the first Myb repeat may not be important for nuclear localization and transactivation. Deletion of the first Myb repeat (MybΔ1) or a region with some similarity to P. putida dehR1 protein (MybΔ2, residues 157–212) retained higher levels of nuclear Myb2 proteins with transactivation activity. This indicates that these two coding regions may be negative regulatory regions for repression of transcription or mRNA stability. Myb proteins with one repeat have been identified in plants (25). The one repeat Myb protein can bind to DNA as a homodimer (51, 52). Therefore, the one-Myb-repeat MybΔ1 that can enter the nucleus may still be functional. On the other hand, the levels of the myb2 mRNA from the N- or C-terminal deletion mutants (MybΔN or MybΔC) were very low, indicating that both N- and C-terminal coding regions were required for myb2 transcription or mRNA stability. Interestingly, the levels of endogenous myb2 and cwp1 mRNA were also lower in the MybΔN- or MybΔC-overexpressing cell line, indicating that removal of the N- or C-terminal region results in a significant decrease of transactivation and autoregulation ability. The lower transactivation ability of MybΔC protein could be due to its lower levels or its inability to enter nucleus. However, because the MybΔN protein can enter nuclei, the inactivity of the MybΔN protein could be due to its lower levels or the lack of the transactivation domain. This indicates that the N-terminal 343 amino acids (residues 2–410) containing a region with some similarity to
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S. cerevisiae Rlr1p (residues 340–403) or a stretch of acidic amino acid (residues 225–232, Fig. 1A) has a transactivation ability. It is known that acidic characteristics are associated with the eukaryotic transactivation domains. The existence of distinct Myb transactivation domains may interact with other transcription factors to modulate transcription in vivo.

Our results showed that constitutively expressed Myb2 increased the expression of cwp1 and cwp2 genes by ~3.6–3.8-fold in vegetative trophozoites. However, the cwp1 promoter could be increased by ~47-fold during encystation (21). Although Myb2 can also function as a transactivator in vegetative cells, it may still need to cooperate with some other transcription factors that are induced during encystation to transactivate these cyst wall protein genes. In late-branching eukaryotes, Myb proteins regulate specific target genes by interacting with other classes of DNA binding proteins that occupy directly adjacent binding sites within the target promoter region. For example, c-Myb can cooperate with NF-M/c-EBP (b-ZIP family) or Ets-2 (helix-turn-helix) to activate the promyelocytic-specific mim-1 gene (38, 53). c-Myb and AML1/CBF (Runt family) activate T-cell receptor delta enhancer (39, 40). Therefore, it is possible that giardial Myb2 functions as an activator via association with some encystation-specific cofactors on the promoter context of encystation-induced genes.

Our study provides evidence for the involvement of Myb2 in the differentiation of G. lamblia trophozoites into cysts. Our findings provide new insight into distinct functional domains of Myb2 and suggest testable transcriptional mechanisms in the protozoan G. lamblia.

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