Activation of Multifarious Transcription of an Adhesion Protein *ap65-1 Gene by a Novel Myb2 Protein in the Protozoan Parasite *Trichomonas vaginalis*

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Multifarious transcription of the adhesion protein *ap65-1* gene in the human pathogen, *Trichomonas vaginalis*, is critically regulated by the coordination of two similar but opposite oriented DNA regulatory regions, MRE-1/MRE-2r and MRE-2f, both of which are binding sites for multiple Myb-like proteins. In the present study, MRE-1/MRE-2r was demonstrated to be composed of multiple overlapping promoter elements, among which the entire region is required for growth-related *ap65-1* transcription, and the 5′-MRE-1 antagonizes the suppressive activity of the 3′-MRE-2r in iron-inducible transcription. The recombinant Myb2 protein derived from a previously identified *myb2* gene was demonstrated to recognize distinct sequence contexts in MRE-2r and MRE-2f, whereas Myb2 in the nuclear lysate preferentially binds to MRE-2f to MRE-2r. Iron repletion resulted in persistent repression of the *myb2* gene, and temporal activation/deactivation of Myb2 promoter entry, which was also activated by prolonged iron depletion. The hemagglutinin-tagged Myb2 when overexpressed during iron-depleted conditions facilitated basal and growth-related *ap65-1* transcription to a level that was achieved in iron-replete cells, whereas iron-inducible *ap65-1* transcription was abolished with knockdown of Myb2. These findings demonstrated that Myb2 is involved in activation of growth-related and iron-inducible transcription of the *ap65-1* gene, possibly through differential promoter selection in competition with other Myb proteins.

*Trichomonas vaginalis* is a protozoan parasite that causes the most common sexually transmitted disease of nonviral origin in humans. The disease poses an imminent threat to public health as revealed by recent findings that transmission of the human immunodeficiency virus increases in patients with trichomoni-asis (1). The parasite persistently inhabits the human urogenital tract without an alternating life stage outside of the host. Cytoadherence, which is crucial for *T. vaginalis* to establish an infection, has been shown to involve multiple surface adhesion proteins and lipophosphoglycans (2–4). The iron supply, which undergoes periodic fluctuations in the human vagina, is one of the principle determinants modulating cytoadherence of the parasite toward human vaginal epithelial cells (5, 6), possibly through transcriptional regulation of some of the adhesion protein (ap) genes, especially those in the *ap65* family (7, 8), which encode proteins identical to malic enzymes (9, 10). Iron has also been implicated in modulating phenotypic variation of the parasite as well as its resistance to complement lysis (11, 12). These observations underscore the importance of iron in modulating expression of parasite virulence.

Gene transcription in *T. vaginalis* is monocistronic with only a few intron-containing genes capable of undergoing RNA splicing (13). Transcription initiation by RNA polymerase II is thus a key step in controlling expression of the protein coding genes in the parasite. Using transcription of the *ap65-1* gene as a model system, we have been studying transcription machinery that controls parasitic gene expression in coping with rapid changes in the growth environment (14–16). The *ap65-1* promoter was demonstrated to comprise a simple core promoter that only contains a ubiquitous initiator element spanning the transcription initiation site (+1) (14, 17), a proximal promoter (−132 to −37) that controls iron-inducible as well as growth-related promoter activities (14, 15), and a distal regulatory region (16). The proximal promoter region contains eight closely spaced promoter elements (15), among which three Myb recognition elements (MRE), MRE-1/MRE-2r which overlap, and MRE-2f, are the binding sites for several Myb-like DNA binding transcription factors (14–16). The promoter distal region, which is essential for optimal promoter activity, also contains two additional clusters of MRE-1/MRE-2r and MRE-2f-like DNA sequences (16).

The MRE-1/MRE-2r and MRE-2f regions share similar but opposite oriented DNA sequences, ATACGATA and TATCGTC, respectively, each of which is also the binding site for multiple nuclear DNA-binding proteins (15). Both DNA regions are required for optimal growth-related transcription, but MRE-1/MRE-2r counteracts MRE-2f positive action on iron-inducible transcription (16). Southwestern screening of a

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2 The abbreviations used are: ap, adhesion protein; CHIIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; IFA, immunofluorescence assay; MRE, Myb-recognition element; MRE-1/BP, MRE-1-binding protein; MRE-2/BP, MRE-2-binding protein; RT-PCR, reverse transcriptase-polymerase chain reaction; utr, untranslated region.

3 J. H. Tai, unpublished observations.
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DNA Transfection and Selection for Stable Transfectants—Plasmid DNA was electroporated into T. vaginalis for paromomycin selection of stable transfectants as previously described (15).

Promoter Assay—Stable cell lines harboring the mutated reporter plasmid, pAPm(MRE-1) or pAPm(MRE-2r) (see below), are referred to as m(MRE-1) or m(MRE-2r), respectively. Luciferase activity in stable cells conferred by the expression of the luc+ reporter gene was measured as previously described (14). In the promoter assay, relative amounts of respective plasmids in cells from individual cell lines were determined by dot hybridization as previously described (15), and their promoter activities were normalized accordingly.

Oligonucleotides—Sequences of the oligonucleotides used in the present study are either listed in Table 1 or were reported in a previous study (16).

Cloning of the Genomic myb2 Gene—A T. vaginalis T1 genomic DNA library and a partial cDNA sequence of the myb2 gene were obtained from a previous study (16). The sequence flanking 5'-end of the myb2 gene was amplified from the genomic DNA library by a polymerase chain reaction (PCR) using the primer pair, T3 and myb2-3'-2. The amplified DNA was then cloned into pGEM_Teasy for DNA sequencing as described by the supplier (Promega).

Construction of Plasmids—The plasmid, pAP65-1luc+/TUBneo (Fig. 1A), was obtained from a previous study (15). A 5'-PCR product was amplified from pAP65-1luc+/TUBneo using tub90f as the 5'-primer and a 3'-antisense primer, m(95)/94-3' or m(89)/88-3', at the target site to create mutations in MRE-1 or MRE-2r, respectively. A 3'-PCR product was amplified from pAP65-1luc+/TUBneo using a 5'-primer, m(95)/94-5' or m(89)/88-5', at the target site and luc344r as the 3'-primer. The 5' and 3' PCR products were purified and mixed as templates for second round of the

**Table 1** Sequences of oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' to 3')</th>
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<tbody>
<tr>
<td>For RT-PCR</td>
<td></td>
</tr>
<tr>
<td>myb2-173f</td>
<td>AAATTGCTCGAGCGCTTCTGCGCTTC</td>
</tr>
<tr>
<td>myb2-3'utr</td>
<td>TTTTCTTAAAGACTCCAAAAAATACAOGT</td>
</tr>
<tr>
<td>For plasmid construction*</td>
<td></td>
</tr>
<tr>
<td>tub-90f</td>
<td>GATACCGCGCTCCGTTAAGAC</td>
</tr>
<tr>
<td>m(95)/94-5'</td>
<td>CCGTTTTTGAAGGAGACGCGATATTTAAAGG</td>
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<tr>
<td>m(95)/94-3'</td>
<td>TCTTTTAAATATCGCTTCTCCCTTAAGAAATAGG</td>
</tr>
<tr>
<td>m(89)/88-5'</td>
<td>CAATTACTCTTTTTAAAGACCTCGTTACTCCCT</td>
</tr>
<tr>
<td>m(89)/88-3'</td>
<td>ACCAGGGGACTGTAATCTCATGAGCAGC</td>
</tr>
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<tr>
<td>lic-myb2-3'</td>
<td>TTTTCTTAAAGACTCCAAAAAATACAOGT</td>
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</table>

*The sequence of the restriction enzyme site as indicated in the name is underlined.

**Experimental Procedures**

Cultures—T. vaginalis T1 cells were maintained as previously described (14). Iron repletion or depletion was achieved with the addition of 250 μM ferrous ammonium sulfate or 50 μM of an iron-chelator, 2,2'-dipyridyl, respectively, in growth medium. A WT-13 cell line harboring a reporter plasmid, pAP65-1luc+/TUBneo, for the activity assay of the ap65-1 promoter was obtained from a previous study (15).
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PCR using the primer pair tub90f and luc344r. The mutated plasmid, pAPm(MRE-1) or pAPm(MRE-2r), was obtained by replacing the SacII/HindIII fragment in pAP65-1luc+/TUBneo with the final PCR product digested with SacII and HindIII.

To construct a gene overexpression system, a DNA fragment spanning the coding region of the myb2 gene was amplified from genomic DNA by PCR using the primer pair ha-myb2-5’nde1 and myb2-3’bgl2, and was then cloned into pGEM_Teasy to generate pTA-ha-myb2. A DNA fragment spanning the 3’-untranslated region of the ap65-1 gene was amplified by PCR from genomic DNA using the primer pair, ap65-1-3’utr-blg2 and ap65-1-3’utr-nsi1. The DNA fragment was cloned into pGEM_Teasy to generate pTA-AP65-1–3’utr. The SacII/NdeI fragment from pTA-AP65-2.1, the NdeI/BglII fragment from pTA-ha-myb2, and the BglII/NSiI fragment from pTA-AP65-1–3’utr were cloned into pAP65-1luc+/TUBneo digested with SacII and NSiI to generate the HA-Myb2 expression plasmid, pAP65-2.1-ha-myb2/TUBneo (Fig. 5A).

To construct an antisense gene knockdown system, a DNA fragment spanning the coding region of the myb2 gene was amplified from genomic DNA by PCR using a forward primer, as-myb2-5’nde1 and a reverse primer, as-myb2-3’bgl2, and was then cloned into pGEM_Teasy to generate pTA-Asss-myb2. The plasmid, pAP65-2.1-as-myb2/TUBneo, was generated by replacing the NdeI/BglII fragment in pAP65-2.1-ha-myb2/TUBneo with the NdeI/BglII fragment from pTAAs-myb2 (Fig. 6A).

To express recombinant protein, the coding region of the myb2 gene was amplified from genomic DNA by PCR using the primer pair lic-myb2-5’ and lic-myb2-3’. The plasmid, pET30/Myb2, was generated by ligation of the PCR product with pET30 using a pET30EK/LIC vector kit as suggested by the supplier (Novagen).

Northern Hybridization—Cellular RNA was extracted from T. vaginalis using the TRIzol reagent (Invitrogen), and mRNA was purified using oligo(dT) cellulose chromatography. Probe labeling and Northern hybridization were performed as previously described (16). The [(α-32P)dCTP]labeled myb2 DNA probe was synthesized from a pTAha-myb2 template.

Reverse Transcriptase-PCR (RT-PCR)—A semiquantitative RT-PCR assay was performed to examine expression levels of ap65-1, β-tubulin, and myb2 transcripts in total RNA as previously described (16). The myb2 cDNA was amplified using the primer pair myb2-173f and myb2-3’utr, and was annealed at 55 °C.

Expression of the Recombinant Myb2 Protein (rMyb2)—The rMyb2 protein expression vector, pET30/Myb2, was transformed into the Escherichia coli BL21-Codon Plus DE3-RIL strain (Stratagene) for the production of rMyb2. E. coli transformed with pET30/Myb2 in shaking cultures was incubated at 37 °C until the A600 reached 0.6. The induction was performed with the addition of 1 mM isopropyl-thio-β-D-galactoside for 2 h. Under these conditions, the majority of rMyb2 was determined to be in the inclusion bodies (see Fig. 4A). Soluble rMyb2 was purified using a His-bind nickel column as described by the supplier (Novagen).

Antibody Production—Purified rMyb2 was used for rabbit immunization by a standard protocol (18), and antiserum was purified by protein A affinity chromatography as described by the supplier (Sigma).

Western Blotting—Cytoplasmic and nuclear fractions of T. vaginalis total lysate were prepared for the Western blotting and DNA binding assay described below using a cellular fractionation kit, NE-PERTM, as described by the supplier (Pierce). In some of the experiments, a semiquantitative Western blot assay using serially diluted protein samples from lysate equivalent to 105 ~ 106 cells was performed as previously described (16). The ECL system was used for signal detection as instructed by the supplier (Pierce). Reaction conditions for antibodies from commercial sources, including the mouse monoclonal anti-α-tubulin antibody (5,000×) (DM1A, Sigma), rat monoclonal anti-HA antibody (2,000×) (3F10, Roche Applied Science), and Hisα monoclonal antibody (10,000×) (Clontech), were as described by the supplier. The Myb2 and AP65 proteins were detected using a rabbit anti-Myb2 antibody (2,000×) and mouse monoclonal anti-malic enzyme antibody 15D7 (19) (10,000×), respectively.

Immunofluorescence Assay (IFA)—Subcellular localization of HA-Myb2 or the NEO selective marker was performed by IFA using a mouse anti-HA monoclonal antibody (200×) (HA-7, Sigma) or rabbit anti-NPT-II antibody (800×) (Upstate) as previously described (16).

Electrophoretic Mobility Shift Assay (EMSA)—Probe labeling and EMSA were performed as previously described (14), except that in some of the binding reactions, the serially diluted anti-Myb2 antibody or normal rabbit serum was included. Signal intensity of the 32P isotope was measured using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences).

Chromatin Immunoprecipitation Assay (ChIP)—A ChIP assay was performed as previously described (16, 20). In some of the reactions, an aliquot of supernatant recovered from the DNA shearing step was reacted with 20 μl of the anti-Myb2 antibody or normal rabbit serum followed by immunoprecipitation with protein A-agarose (Sigma). The DNA fragment spanning region I, II, or III of the β-tubulin promoter was amplified by PCR using primer pairs tub-1f and tub-1r, tub-2f and tub-2r, or tub-3f and tub-3r, respectively.

RESULTS

Overlapping DNA Elements in MRE-1/MRE-2r—The DNA sequence, ATAACGATA, spanning the MRE-1/MRE-2r overlap was found to be composed of three distinct nuclear protein-binding sites, ANAACGAT for Myb1 (16), and TAACGA (MRE-1) and CGATA (MRE-2r) for the reputed MRE-1-binding protein (MRE-1-BP) and MRE-2r-binding protein, respectively (15). To study whether MRE-1/MRE-2r comprises multiple, functionally distinct promoter elements in vivo, site-directed mutagenesis of the ap65-1 promoter was conducted using a reporter plasmid, pAP65-1luc+/TUBneo, and two related mutant plasmids (Fig. 1). The mutation of MRE-1 that retains intact MRE-2r resulted in diminished
eight base-contacting amino acid residues identified in the mammalian cMyb (21) were identical between the protein sequences of Myb1 and Myb2 (Fig. 2B).

The myb2 gene was expressed as a 0.6-kb mRNA species in T. vaginalis as revealed by Northern hybridization (Fig. 3A). The expression level of myb2 mRNA under iron-replete conditions was 2-fold lower than that under iron-depleted conditions as examined by semiquantitative RT-PCR (Fig. 3B). The expression level slightly varied in an 18-h period. The expression level of β-tubulin mRNA remained constant under all test conditions.

A major 27-kDa band and several faster migrating minor bands with sizes between 21 and 25 kDa were detected in cell lysate from T. vaginalis by Western blotting using the anti-Myb2 antibody (Fig. 3, C and D). The 27-kDa band was distributed in both the cytoplasmic and nuclear fractions to similar extents, but those faster migrating ones were only detected in the cytoplasmic fractions even when samples were overloaded to increase detection sensitivity (Fig. 3C). The purity of these cellular fractions was examined using an antibody against a cytosolic malic enzyme (22) or α-tubulin that detected a 50-kDa band only in the cytoplasmic fractions or a 55-kDa band only in the nuclear fractions, respectively. None of these protein bands was detected on a duplicate blot either using preimmune serum or the anti-Myb2 antibody that had been pre-adsorbed with purified rMyb2 (data not shown). The cellular distribution of Myb2 as examined by semiquantitative Western blotting only slightly varied under our test conditions (Fig. 3D). Consistent with the RNA analysis (Fig. 3B), the signal intensity of the 27-kDa protein in samples from iron-replete cells was also 2-fold lower than that in samples from iron-depleted cells.

**DNA Binding Specificity of Myb2**—The rMyb2 protein was purified (Fig. 4A) for use in EMSA. rMyb2 at as little as 2.5 ng was sufficient to form a major complex with the MRE-1/MRE-2r-containing [32P]IR probe (Fig. 4B, left panel), and two discernible DNA-protein complexes with the MRE-2f-containing [32P]IR3 probe (Fig. 4B, right panel), with similar activities. The DNA binding specificity of rMyb2 against [32P]IR was then tested in competition assays using a 250× molar excess of the cold IR or mutated sequences of the miR series (Fig. 4C) as previously described (15). The DNA-protein complex was incompletely competed to various degrees with various mutant competitors. Similar results were observed in the reactions with [32P]IR3 in the competition assays (Fig. 4D). The signal intensity of the DNA-protein complexes in individual reactions was measured, revealing that CGATA, which resembles the target site of the reputed MRE-2r-binding proteins (15), and tATCGTc spanning MRE-2f are the primary binding sites (upper and lowercase letters indicate strong or weak contact sites, respectively) of rMyb2.

The DNA binding activity of Myb2 in the nuclear lysate was then examined. Two DNA-protein complexes (I and II) were detected in the binding reactions including 10 μg of nuclear proteins and either [32P]IR or [32P]IR3′ (Fig. 4, E and F, respectively). Co-incubation with the serially diluted anti-Myb2 antibody only resulted in disruption of complex I in each binding reaction to a level dependent on the serum concentration, indicating that Myb2 is only one of the nuclear proteins targeting
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**FIGURE 2. Sequence conservation of the Myb2 protein.** A, protein sequences of *T. vaginalis* Myb1 (AY948338) and Myb2 (AY948337) were aligned, and the conserved amino acid residues are highlighted. B, alignment of putative strong (circles) or weak (down-pointing triangles) base-contacting amino acid residues relative to those of vertebrate cMyb (CAFO4477) (21) or plant Myb2.2 (86253_m00007) from *T. vaginalis* Myb1, Myb2, or two Myb2-like proteins, referred to as Myb2.1 (83589_m00056) or Myb2.2 (86253_m00007) from *T. vaginalis* genome data base.

**FIGURE 3. Expression of the myb2 gene in *T. vaginalis*.** A, 10 μg of mRNA purified from *T. vaginalis* was examined by Northern hybridization using an [α-32P]dCTP-labeled DNA probe derived from pTAha-myb2. B, 10 μg of cellular RNA from *T. vaginalis* under iron-replete (+) (lanes 1 and 2) or -depleted conditions (−) (lanes 2 and 4) for 8 (lanes 1 and 2) or 18 h (lanes 3 and 4) was assayed for myb2 or β-tubulin (β-Tub) mRNA by semiquantitative RT-PCR (top panels). The average signal intensity of myb2 versus β-Tub from three separate experiments is depicted (bottom panel). C, Myb2 in total lysate (T), or nuclear (N), or cytoplasmic (C) fractions from 10^6 *T. vaginalis* cells was assayed by Western blotting using the antibody against MYB2, cytosolic malic enzyme (cME) or α-tubulin (α-Tub). D, expression of Myb2 in *T. vaginalis* under iron-replete (+) (lanes 1–3 and 7–9) or -depleted (−) conditions (lanes 4–6 and 10–12) for 8 (lanes 1–6) or 18 h (lanes 7–12) was assayed by semiquantitative Western blotting using the antibody against Myb2, cME, or α-Tub. Data shown herein were from 2 × 10^6 of *T. vaginalis* cells. The average signal intensity of Myb2 versus α-Tub from three separate experiments is depicted below panel D.

With divergent promoter sequences in six members of the *ap65* gene family (16), the effect of Myb2 overexpression on *ap65-1* transcription was studied by semiquantitative RT-PCR (Fig. 5E). With iron depletion for 8 h, the signal intensity of *ap65-1* mRNA was 4-fold higher in samples from transfected cells than from non-transfected cells under iron-depleted conditions for 8 h, and a 2-fold growth-related increase was only detected in samples from non-transfected cells. Iron repletion for the same periods that facilitated a 2-fold increase in *ap65* expression in non-transfected cells had little effect on transfected cells. The signal intensity of α-tubulin in these samples only slightly varied.

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MRE-1/MRE-2r or MRE-2f. This interference effect was not observed with co-incubation of serially diluted normal rabbit serum. The signal intensity of the Myb2-DNA complex in the binding reactions revealed that nuclear Myb2 bound 6-fold as much [32P]IR as [32P]IR3 (Fig. 4G), suggesting that nuclear Myb2 preferentially binds MRE-2f over MRE-2r.

**HA-Myb2 Overexpression**—The HA-Myb2 expression plasmid, pAP65-2.1ha-myb2/TUBneo (Fig. 5A), was used to overexpress a HA-tagged Myb2 protein in *T. vaginalis*. The HA signal was primarily detected in nuclei of more than 95% of transfected cells, but in none of the non-transfected cells, by IFA using the mouse anti-HA monoclonal antibody (Fig. 5B). A major ~29-kDa band and a minor ~27-kDa one were only detected in samples from transfected cells by Western blotting using the rat anti-HA monoclonal antibody (Fig. 5C), and only the major band was detected in the nuclear fraction. Both bands were also detected by the anti-Myb2 antibody, suggesting that the addition of the HA tag changes the mobility of the overexpressed Myb2 in SDS-PAGE. The signal intensity of the 29-kDa HA-Myb2 as detected by the anti-Myb2 antibody was equivalent to that of the 27-kDa Myb2 in samples from non-transfected cells, indicating that HA-Myb2 was overexpressed to a level similar to that of endogenous Myb2. Similar levels of overall Myb2 overexpression were detected in transfected cells under our test conditions (Fig. 5D). On the other hand, the signal intensity of the AP65 protein was 2.5-fold higher in samples from transfected cells than from non-transfected cells under iron-depleted conditions for 8 h, and a 2-fold growth-related increase was only detected in samples from non-transfected cells. Iron repletion for the same periods that facilitated a 2-fold increase in *ap65* expression in non-transfected cells had little effect on transfected cells. The signal intensity of α-tubulin in these samples only slightly varied.
These results suggest that when overexpressed, Myb2 can

be detected in total RNA when the reverse transcriptase was
omitted from the RT-PCR amplifications (data not shown).

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FIGURE 4. DNA binding specificity of the Myb2 protein. A, soluble (lanes 1 and 3) or insoluble (lanes 2 and 4) fractions of lysate from Myb2 expressing E. coli, and purified Myb2 (lane 5), were separated by SDS-PAGE in 12% gel and stained with Coomassie Blue (lanes 1, 2, and 5) or examined by Western blotting using the anti-His antibody (lanes 3 and 4). B, 1.25 ng (lanes 1 and 4), 2.5 ng (lanes 2 and 5), or 5 ng (lanes 3 and 6) of rMyb2 was incubated with [γ-32P]IR (lanes 1–3) or [γ-32P]IR3′ (lanes 4–6). C–F, 2.5 ng of rMyb2 (C, lanes 2–15; D, lanes 2–12) or 10 μg of nuclear lysate (E and F, lanes 2–9) was incubated with [γ-32P]IR (C, lanes 1–15; E, lanes 1–9) or [γ-32P]IR3′ (D, lanes 1–12; F, lanes 1–9), respectively. The DNA sequences of IR (C and F) and IR3′ (D) are shown at the top of each panel in uppercase letters, with MRE-1/MRE-2 (C) and MRE-2 (D) underlined. A 250× molar excess of the cold competitor (C–F, lane 3) or a series of mutant competitors (C and D), each with a single point mutation as indicated by a lowercase letter at the top of each lane, was included in the binding reactions (lanes 4–15 in C; lanes 4–12 in D). Serially diluted (8×, 16×, 32×) normal rabbit serum (E and F; lanes 4–6) or the anti-Myb2 antibody (E and F, lanes 7–9) was included in the binding reactions. The reaction mixtures were separated on 10% polyacrylamide gels by electrophoresis. G, 5 ng of rMyb2 (lanes 1 and 3) or 10 μg of the nuclear proteins (lanes 2 and 4) were incubated with [γ-32P]IR (lanes 1 and 2) or [γ-32P]IR3′ (lanes 3 and 4) in the binding reactions. The DNA-protein complexes were detected using autoradiogram. Results from three separate experiments were averaged as shown at the bottom panel (C, D, and G). The signal intensity of the Myb2–γ-32P-IR or Myb2–γ-32P-IR3′ complex in G (arrows) was normalized by the signal intensity of rMyb2–γ-32P-IR or rMyb2–γ-32P-IR3′ complex (arrowheads), respectively.

FIGURE 5. Overexpression of the HA-Myb2 protein in T. vaginalis. A, plasmid, pAP65-2.1-ha-myb2/TUBneo, was used to overexpress HA-Myb2 in T. vaginalis. In this plasmid, the ap65-2.1 promoter (AP65-2.1) drives a HA-tagged myb2 gene, and the β-tubulin (TUB) promoter drives a selective marker, the neo gene. B, HA-Myb2 in non-transfected cells (top row) or transfected cells (bottom row) was detected by IFA using the anti-HA antibody. Images of cells depicted as DAPI, FITC, and phase contrast were recorded using confocal microscopy. C, total lysates from non-transfected (lanes 1 and 3) or transfected cells (lanes 2 and 4), and nuclear (N) (lane 5) or cytoplasmic lysates (C) (lane 6) from transfected cells, with the amount from 10^7 T. vaginalis cells, were examined by Western blotting using the antibody against Myb2 (lanes 1 and 2) or HA tag (lanes 3–6). D and E, total lysate from 2 × 10^6 cells (D) or 10 μg of cellular RNA (E) from non-transfected (lanes 1, 2, 5, and 6) or transfected cells (lanes 3, 4, 7, and 8) under iron-depleted (−) (lanes 1, 3, 5, and 7) or replete (+) (lanes 2, 4, 6, and 8) for 8 (lanes 1–4) or 18 h (lanes 5–8) was examined by semiquantitative Western blotting (D) or RT-PCR (E), respectively. The average signal intensity of AP65 versus β-tubulin (D) or ap65-1 mRNA versus β-tubulin mRNA (E) from three separate experiments is depicted below the panels.
increase basal and growth-related, but not iron-inducible, ap65-1 transcription.

Myb2 Knockdown—To further study the role of Myb2 in iron-inducible transcription, the antisense knockdown strategy, which had been explored to successfully knockdown expression of the ap65 genes (7), was employed as described below.

T. vaginalis was transfected with the plasmid, pAP65-2.1-as-myb2/TUBneo (Fig. 6A), which overexpresses the antisense myb2 transcript in the transgenic parasite. More than 95% of transfected cells expressed the NEO protein as detected by IFA using the anti-NPT-II antibody (Fig. 6B). Protein expression in transfected cells was then assayed by semiquantitative Western blotting (Fig. 6C). At both 8 and 18 h of iron depletion or repletion, the signal intensity of Myb2 as detected by the anti-Myb2 antibody was 2-fold lower in samples from transfected cells than from non-transfected cells. With iron repletion, the signal intensity of AP65 was 2-fold lower in samples from transfected cells than from non-transfected cells. Iron depletion that repressed AP65 expression by 2-fold in non-transfected cells had only a slight repressive effect on transfected cells. The signal intensity of α-tubulin in these samples only slightly varied.

Transcription of the ap65-1 gene in the knockdown parasite was examined by semiquantitative RT-PCR (Fig. 6D). Under iron-depleted conditions for 8 h, the signal intensity of ap65-1 mRNA in samples from transfected cells was similar to that in samples from non-transfected cells. Iron repletion for the same period that increased ap65-1 transcription by 4-fold in non-transfected cells had little effect on transfected cells. Similar results were obtained with prolonged treatments for 18 h. The signal intensity of β-tubulin mRNA in samples from transfected and non-transfected cells varied little under the test conditions. No signal was detected in total RNA when the reverse transcriptase was omitted from the RT-PCR amplifications (data not shown). These results suggest that when knocked down, Myb2 can repress iron-inducible ap65-1 transcription, also with a slight repression of basal and growth-related transcription.

Differential Promoter Selection by Myb2—The promoter region of the ap65-1 gene contains multiple potential entry sites for Myb2 (Fig. 7A). Promoter entry by the endogenous Myb2 protein in T. vaginalis was examined by exploring the efficacy of the anti-Myb2 antibody in ChIP (Fig. 7). In samples pulled down using the anti-Myb2 antibody, the PCR product amplified from region I, II, or III of the ap65-1 promoter was to 3- or 4-fold higher in samples from iron-replete than from iron-depleted conditions for 8 h, or in samples from iron-depleted conditions for 18 h compared with those for 8 h, respectively (Fig. 7B). By 18 h, the association of Myb2 with the ap65-1 promoter was 2-fold lower in samples from iron-replete than those from iron-depleted conditions. No PCR product was obtained from region IV in these samples or from three discrete promoter regions of the β-tubulin gene that contains a potential Myb2-binding site in region I (Fig. 7A). Similar results were obtained with ChIP using the anti-HA antibody to study the association of HA-Myb2 with the ap65-1 promoter in transfected cells overexpressing HA-Myb2 (Fig. 7C). None of the DNA fragments described above was amplified from samples pulled down by normal rabbit serum, indicating that Myb2 differentially enters the defined promoter sites when the iron supply or cell growth stage changes.

DISCUSSION

We demonstrate herein that the MRE-1/MRE-2r region in the ap65-1 promoter is composed of multiple overlapping promoter elements (Fig. 1). In conjunction with an earlier study
Transcriptional Regulation by Myb2 in *T. vaginalis*

**TABLE 2**
Promoter activities of the Myb recognition elements in the *ap65-1* promoter

<table>
<thead>
<tr>
<th>Promoter element</th>
<th>Basal transcription</th>
<th>Growth-related transcription</th>
<th>Iron-inducible transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRE-1/MRE-2r</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>MRE-1</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>MRE-2r</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>MRE-2f</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

*The level of basal, growth-related, and iron-inducible transcription is defined as in the legend to Fig. 1.*

*Either MRE-1/MRE-2r or MRE-2f is sufficient to suppress transcription to the basal level (15).*

**TABLE 3**
Biochemical properties and functional roles of Myb1 and Myb2

<table>
<thead>
<tr>
<th>Role in <em>ap65</em> transcription</th>
<th>Myb1</th>
<th>Myb2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>↓</td>
<td>←</td>
</tr>
<tr>
<td>Iron-inducible</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Growth-related</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

*Increases or decreases at early or late stages of cell growth.*

(16), we found that the growth-related promoter activity requires both the entire MRE-1/MRE-2r region and downstream MRE-2f, but it is down-regulated by either MRE-1 or MRE-2r. The MRE-1 also acts in synergy with MRE-2f to antagonize the suppressive role of MRE-2r on iron-inducible transcription. Moreover, either MRE-1/MRE-2r or MRE-2f is sufficient to repress transcription to the basal level, while MRE-1 alone further suppresses basal transcription. The roles of respective promoter elements described herein are summarized in Table 2. This intricate gene regulation and the binding of multiple Myb proteins to each of these DNA elements (Refs. 15 and 16, Fig. 4, E, and F) suggest that regulation of *ap65-1* transcription involves competition or coordination among multiple Myb proteins to gain access to the overlapping promoter sites. This postulation is supported by the biochemical characteristics and functional roles of Myb2 versus Myb1 (16, Table 3). Competition or coordination for access to the overlapping promoter sites by two distinct DNA binding transcription factors is not unusual as also observed in other eukaryotic gene expression systems (23–27); however, the Myb proteins in *T. vaginalis* seem to act differently from animal A-Myb, B-Myb, and c-Myb, which exhibit similar DNA binding specificities but transactivate distinct sets of genes (28, 29).

DNA binding specificity is only one of the principle determinants for a DNA binding transcription factor to select its target genes. In this regard, rMyb2 was found to possess dual DNA binding specificity toward two distinct sequence contexts (Fig. 4, C and D), which deviate from those recognized by rMyb1 (16), in the MRE-1/MRE-2r and MRE-2f regions. The distinction may derive from the divergence of the protein sequences of the reputed base-contacting amino acids (Fig. 2A). Intriguingly, Myb2 is only one of the nuclear proteins targeting MRE-2r or MRE-2f (Fig. 4, E and F). Two *myb2*-like genes, which share six or seven of the eight reputed base-contacting amino acids in the
Transcriptional Regulation by Myb2 in T. vaginalis

FIGURE 8. Transcriptional regulation of the ap65-1 gene. In this model, ap65-1 transcription is regulated by coordination or competition of multiple Myb proteins to gain access to the promoter sites, MRE-1 (ovals), MRE-2r (left-pointing triangles), MRE-2f (right-pointing triangles), and MRE-1/MRE-2r. N indicates the nucleus, and C indicates the cytoplasm. Activators or repressors that bind to the positive or negative promoter elements are depicted in open or closed symbols, respectively. Free Mybs are indicated by gray symbols. The height of a bent arrow, which symbolizes the transcription start site, indicates transcription efficiency under a specific condition. Under iron depletion for 8 h, an inhibitory MRE-2-BP may preferentially occupy MRE-2f, whereas MRE-2-BP and MRE-1-BP may either compete or coordinate for access to MRE-1/MRE-2r to fine-tune basal transcription. Myb2 may be modified at a specific site (indicated as PTM-1) under prolonged iron depletion (18 h) to accelerate Myb2 promoter entry into MRE-2f replacing the original MRE-2-BP. Excessive Myb2 may be redirected to MRE-2r replacing the original MRE-2-BP on MRE-1/MRE-2r to facilitate transcription in synergy with a low level of MRE-1-BP. The majority of Myb1 is in the cytoplasm under these conditions. Under iron repletion for 8 h, a second site on Myb2 may be modified (indicated as PTM-2) to facilitate Myb2 promoter entry into MRE-2f. Increasing Myb2 on MRE-2f acts in synergy with a low level of MRE-1-BP on MRE-1 to activate the initial phase of iron-inducible transcription. Under prolonged iron repletion, increasing amount (overlapping images) of MRE-1-BP may enter MRE-1 to corroborate Myb2 action in iron-inducible transcription. Myb1 is likely modified at a specific site (indicated as PTM) for nuclear import upon iron repletion. The nuclear Myb1 may then compete with MRE-1-BP for promoter entry to repress iron-inducible transcription.

R2R3 domain with the myb2 gene in the protein sequences (Fig. 2B), are candidates for testing whether it also encodes a Myb protein that compete with Myb2 in binding MRE-2r or MRE-2f. DNA binding specificity of Myb2 would only provide the recognition code for its promoter selection as suggested by the inability of Myb2 to enter the β-tubulin promoter (Fig. 7). Timely and gene-specific promoter entry by Myb2 requires additional controls imposed at multiple cellular levels as discussed below.

Nuclear translocation is likely another critical step for the parasite to modulate the function of Myb2. Like Myb1 (16), Myb2 is expressed into multiple sizes in T. vaginalis with only the largest one capable of entering the nucleus (Fig. 3C). In general, small proteins such as Myb2 can passively diffuse through nuclear pores (30). T. vaginalis is no exception to this rule as suggested by a functional study of a 23-kDa bacterial Tet repressor overexpressed in the parasite (31). Exclusion of premature Myb2 from the nucleus implies that Myb2 may either self-oligomerize or interact with a protein inhibitor to form a larger protein complex for cytoplasmic retention, and that its nuclear import is likely to be activated by post-translational modifications, such as phosphorylation or sumoylation, as in other eukaryotic systems (32–34). Unlike iron-activated ap65-1 transcription. This speculation is supported by synergistic actions of MRE-1 and MRE-2f in iron-inducible transcription in conjunction with preferential binding of the nuclear Myb2 to MRE-2f over MRE-2r (Table 2 and Fig. 4G). On the other hand, the Myb2 positive role in basal transcription as defined herein does not comply with our previous findings that either MRE-1/MRE-2r or MRE-2f is sufficient to suppress transcription to the basal level (16), indicating that another MRE-2-binding protein(s) (MRE-2-BP) (Fig. 4, E and F) may compete with Myb2 for promoter entry to repress transcription in the early phase of iron depletion. Intriguingly, the level of ap65-1 transcription correlates with the level of Myb2 promoter entry only in growth-related transcription and early phase of iron-inducible transcription, whereas the late phase of iron-inducible transcription is inversely related to the level of Myb2 promoter entry (Fig. 7B). The significance is postulated below. These findings support the modulation of Myb2 promoter entry likely being a far more crucial step than modulation of Myb2 expression in transcriptional regulation of the ap65-1 gene. Information derived from these observations will be useful for investigation on the signaling pathways leading to Myb2 differential promoter selection. Moreover, with the roles of Myb2 in overall expression of the AP65 proteins (Figs. 5 and 6),

nuclear localization of Myb1 (16), cellular distribution of Myb2 only slightly varied under our test conditions (Fig. 3D), indicating that the nuclear imports of Myb1 and Myb2 are likely activated by different signaling pathways.

Iron exerted dual effects on Myb2 activity by persistent repression of Myb2 expression and temporal activation/deactivation of Myb2 promoter entry that may have variable impacts on multifarious ap65-1 transcription (Figs. 3 and 7). The level of ap65-1 transcription is partially attributable to Myb2 expression level as suggested by the transgenic assays, in which overexpression of HA-Myb2 was correlated with increased transcription under iron-depleted conditions (Fig. 5E), while knockdown of Myb2 was correlated with repression of iron-inducible transcription (Fig. 6D). Overexpression of HA-Myb2 under iron repletion did not further improve the transcription level of the ap65-1 gene in the transgenic parasite beyond that already achieved under iron-depleted conditions (Fig. 5E), implying that an additional rate-limiting factor or factors, such as that which binds MRE-1, is needed to corroborate Myb2 activity in iron-inducible
the transgenic cell lines generated from this study will also be useful for testing the role of Myb2 in controlling cytoadherence of the parasite.

Based on this and earlier studies (15, 16), we propose that transcription of the *ap65-1* gene is regulated by coordination or competition of multiple Myb proteins to gain access to the MRE-1/MRE-2r and MRE-2f sites (Fig. 8). In this model, only a negligible fraction of Myb1 is available in the nucleus when *T. vaginalis* is exposed to limited iron (15). In the initial phase of iron depletion, a low level of MRE-1-BP may either compete or coordinate with MRE-2-BP to enter the MRE-1/MRE-2r site in competition with Myb2 (Fig. 4F). Upon prolonged iron depletion, Myb2 may be modified at a specific site to facilitate promoter entry replacing the original MRE-2-BP on MRE-2f. Excessive Myb2 upon entering the *ap65-1* promoter may then be redirected to MRE-2r to increase transcription in synergy with MRE-1-BP. When cells are initially exposed to ample iron supply, Myb2 may be modified at a different site to facilitate entry into MRE-2f, and then acts in synergy with MRE-1-BP to facilitate *ap65-1* transcription. Increasing level of MRE-1-BP may enter the MRE-1 site upon prolonged iron repletion to further corroborate the action of Myb2 on MRE-2f. Iron may also activate nuclear import of Myb1 (15), which may compete with MRE-1-BP for access to MRE-1/MRE-2r to repress iron-inducible transcription. The model provides a working hypothesis for further study on the mechanism underlying iron-inducible *ap65-1* transcription. It will also be useful for testing whether some of the components in this scheme control the global gene expression of the parasite in response to changes in iron supply and some unidentified growth-derived factors.

In summary, our results suggest that Myb2 plays an active role in various aspects of *ap65-1* transcription, and that the Myb2 function is largely modulated at the level of promoter selection by iron and some growth-derived factor(s).

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