Characterization of an Iron-responsive Promoter in the Protozoan Pathogen *Trichomonas vaginalis*

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Iron has been shown to regulate transcription in the protozoan pathogen *Trichomonas vaginalis*. In this study, a DNA transfection system was developed to monitor ap65-1 promoter activity in response to changing iron supply. In conjunction with electrophoretic mobility shift assay, iron-induced transcription of the ap65-1 gene was shown to be regulated by multiple closely spaced DNA elements spanning an iron-responsive region (−110/−54), including an iron-responsive DNA element (−98AGATAACGA−99), which overlaps with a 3'-MYB-like protein binding sequence (−95TACCGATAT−87), and three nearby T-rich sequences (−110ATTTT−105, −78ATATT−72, and −59ATTTT−54). 5'- and 3'-flanking sequences of the iron-responsive region were shown to regulate basal transcription. A distal DNA regulatory region was shown to enhance both basal and iron-induced transcription. These findings delineate the DNA regulatory elements and nuclear proteins involving in iron-induced transcription of the ap65-1 gene, which provide useful tools for the future study of transcriptional regulation in *T. vaginalis*.

Human infection by the protozoan pathogen *Trichomonas vaginalis* causes one of the most common sexually transmitted diseases throughout the world (1). Although this protozoan infection usually manifests itself as self-limiting in males, it can impose serious health problems for female patients especially during pregnancy, and it is also implicated as a risk factor for cervical cancer and as a predisposition to human immunodeficiency virus contagion (2–3). As one of the deepest branches of the eukaryotic lineage, this organism exhibits interesting features that deviate from higher eukaryotes and may deviate significantly from the well known machinery operating in higher eukaryotes.

In this study, the ap65-1 gene, which encodes a 65-kDa protein reputed to be one of the surface adhesin proteins (12), was selected as a model system to study iron-induced gene expression in *T. vaginalis*. The DNA regulatory elements in the ap65-1 promoter were characterized by promoter analysis in vitro in conjunction with DNA-protein interaction assays in vivo. The DNA regulatory elements distributed within −110/−54 were found to regulate iron-induced gene expression, whereas those flanking this region were found to regulate basal transcription. A distal region was found to activate both basal and iron-induced transcriptional activities of the ap65-1 promoter. These findings provide a useful model system for future investigations of basal as well as iron-induced transcriptional regulation in *T. vaginalis*.

EXPERIMENTAL PROCEDURES

Culture—*T. vaginalis* axenic cultures were maintained at 37 °C in TYI-S33 medium as described previously (23). The medium was supplemented with 10% heat-inactivated bovine calf serum without iron.

* The abbreviations used are: α-scs, α-succinyl Co-A synthetase gene; ap, adhesin protein gene; luc, luciferase gene; ANOVA, analysis of variance.

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fortification (Hyclone), and the iron concentration in this medium was estimated to be 1 μM. Ferrous ammonium sulfate was added to the desired concentrations as described previously (8).

**Molecular Cloning of the DNA Sequences Flanking the ap65-1 Gene**—Sequences of the oligonucleotides used to clone the flanking regions of the ap65-1 genes and to construct the DNA transfection vectors are listed in Table I. An automatic DNA-sequencing method as described by the supplier (ABI) was used to verify the DNA sequences.

A Sau3AI genomic DNA library derived from *T. vaginalis* JH32A#4 was constructed in pBluescriptIIKS (+) (Stratagene). The sequence flanking the 5' of the ap65-1 gene was amplified from the library using a gene-specific 3'-primer ap1 and either T7 or T3 on the vector as 5' primer by PCR amplification. The PCR products were then cloned into a pGEM-T vector (Promega), and a positive clone pAP5' with a 0.25-kb DNA sequence spanning the −235/+35 region of the ap65-1 gene was obtained (Fig. 1A). An overlap DNA sequence spanning the −217/+217 region was amplified from *T. vaginalis* T1 genomic DNA by a second PCR amplification using primers p−217 and ap2 and cloned into pGEM-T to produce pAP−217/ap2. The 3'-untranslated region of the ap65-1 gene was amplified by PCR using the primer pair ap3 and ap4 from genomic DNA and cloned into pGEM-T to produce pAP3'/ap4.

**Primer Extension**—Cellular RNA was extracted using the UltraSpec RNA reagent (Biotek). Primer extension was performed as described previously (24), with the exception that the γ-32P-labeled oligonucleotide was purified using a NAP-5 gel filtration column (Amersham Biosciences, Inc.) and that primer extension was performed at 52 °C by Moloney murine leukemia virus reverse transcriptase (SuperScriptII (Invitrogen). Oligonucleotides ap5 and tub3 were used to prime ap65-1 messenger RNA and tubulin messenger RNA, respectively (Table I).

**Plasmid Construction**—pAP5' was digested by BglII/SalI and fused together with a 1.7-kb luc + fragment excised from pAPLuc+ (Promega) by BglII/XhoI digestion, resulting in pAPLuc+.5Δ. The insert from pAP3' was excised by EcoRI/Ndel digestion and cloned into EcoRI/Ndel-digested pAPLuc+.5Δ to produce pAPLuc+.5Δ (Fig. 2A). The sequence spanning the −234/+399 region of the β-tubulin gene was amplified from genomic DNA by PCR amplification using primers tub1 and tub3 (25) and cloned into pGEM-T to produce pTUB5'. The region spanning the −234/+32 region of pTUB5' was then amplified by primers tub1 and tub 2 and cloned into pGEM-T. The insert was excised by digestion with SacII and BglII, and the resulting DNA fragment was cloned into SacII/BglII-digested pAPLuc+.5Δ to produce pTUBLuc+.5Δ (Fig. 2B).

5' deletion mutants with the exception of p−114 were constructed by amplifying DNA from pAPLuc+ using one of the 5' primers at the defined site (Table I) and a 3' primer luc344R derived from the luc + gene (24). The PCR products were cloned into pGEM-T. The inserts were then excised by SacII/NruI and cloned back into pAPLuc+ to replace the original SacII/NruI site. The SacII/EcoRV fragment was removed from pAPLuc+ by restriction enzyme digestion, and the resulting DNA was treated with Klenow DNA polymerase before ligation to produce p−114. All deletion constructs are named according to the location of the 5' end relative to the transcription start site (Fig. 3).

Targeted mutagenesis was performed by PCR to create mutations in pAPLuc+. A restriction enzyme site was designed on oligonucleotides for each region to be mutated (Table II). To create mutations within the −230/+192 region, a PCR product was amplified from pAPLuc+ using a 5' primer with clustered mutations at the target site and luc344R as the 3' primer. To create two point mutations in the initiator region, a PCR product was amplified from pAPLuc+ using ap5' as the 5' primer and an antisense primer luc344R and ligated with the −187/+3 region with the exception of the −109/+102 and −101/+96 regions, a 5' PCR product was amplified from pAPLuc+ using ap5' as the 5' primer and an antisense primer at the target site, and a 3' PCR product was amplified using a 5' primer at the target site and luc344R as the 3' primer. The PCR products were cloned into pGEM-T. The inserts were excised by EcoRV and NruI and ligated with EcoRV/NruI-digested pAPLuc+ to produce a series of mutant constructs (see Fig. 4).

**DNA Transfection and Luciferase Assay**—*T. vaginalis* T1 cells grown to 1.5 × 10⁶ trophozoites ml⁻¹ were diluted 10-fold with fresh medium and incubated overnight until cell density reached 1.5 × 10⁶ trophozoites ml⁻¹. Cells were harvested from cultures by centrifugation at 900 × g for 10 min (GPR centrifuge, Beckman) and resuspended in fresh medium containing 10⁶ trophozoites ml⁻¹. The cells were then passed through a 23-gauge needle gently four times using a 5-ml syringe to disperse cell clumps. An aliquot of 300 μl of cell suspension was mixed with 60 μg of plasmid DNA in a 0.4-cm gap ice-cold electroporation cuvette (Invitrogen). Electroporation was performed at 300 V, 1000 microfarads, and 720 ohms using a BTX Electro Cell Manipulator 600 (BTX). Cells were kept on ice for 15 min after electroporation and divided into two tubes with fresh medium. A preliminary study using pTUBLuc+ (see below) to transfected cells from various *T. vaginalis* isolates revealed that transfection efficiency of cells from the T1 isolate was at least 100-fold higher than cells from JH32A#4, NIH-C1, or T068II isolates under our experimental conditions (8). The *T. vaginalis* T1 isolate was therefore selected for the transfection experiments performed in this report. Luciferase reporter activities of transfected cells was performed as described previously (24), with the exception that 200 μg ml⁻¹ N⁴-p-tosyl-L-lysine chloromethyl ketone was used as protease inhibitor to replace aprotinin and that the cell lysate was directly assayed without pretreatment at −70 °C. The data were analyzed by one-way analysis of variance (ANOVA) in SPSS software version 8.0 (1997).

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay**—Nuclear extract was prepared as described previously (24), with the exception that 200 μg ml⁻¹ N⁴-p-tosyl-L-lysine chloromethyl ketone was used as protease inhibitor instead of aprotinin and that a Dounce-type homogenizer (Wheaton) was used to homogenize cells. A BCA protein quantification kit was used to determine protein concentration in nuclear lysate as described by the supplier (Pierce). Probe labeling and electrophoretic mobility shift assay were performed as described previously (24).

**RESULTS**

**Mapping the Start Site of ap65-1 Messenger RNA**—The 5'-flanking sequence of the ap65-1 gene was first cloned from the *T. vaginalis* JH32A#4 and T1 isolates by two separate PCR amplifications, and an identical 0.25-kilobase pair DNA sequence was obtained (Fig. 1A) (GenBank accession number AF364546), indicating that the ap65-1 gene is conserved between the two isolates. The transcription start site of ap65-1 messenger RNA was then mapped by primer extension using RNA extracted from *T. vaginalis* T1 cells grown in 12 μl iron. A major extension product of 71 nucleotides and a minor extension product of 72 nucleotides were consistently produced in reactions priming 50 μg of cellular RNA with γ-32P-labeled ap5 (Fig. 1B, lane 1). The major extension product is mapped to an adenosine 14 upstream of the translation start site, indicating that the initiator-like sequence most proximal to the translation start site serves as the initiator element of the ap65-1 promoter. The adenosine residue in this initiator element is defined as +1 in the text.

**Table I**

| Name | Sequence (5' → 3') |
|------|------------------|
| For molecular cloning and primer extension of the ap65-1 gene | ap217 - 359/1719 CCAATTCGAGCTGGAGGAATAC |
| ap3 - 159/1615 | CCGGATCCCTGCTGCTGGAGG |
| ap4 - 1759/1719 | TGGGAGAGCCTCCTGGGAGGGG |
| tub3 - 399/380 | CTCTTCTTTGAGGAGATG |
| tub1 - 234/2111 | GATCCACCATATATGCTCA |
| tub2 - 33/14 | AGACGGCTGGAATTTTCTG |
| tub3 - 57/34 | GTTCATCCGGCTGCCCAGG |

* Restriction enzyme sites are underlined. AGATCT, BglII; CCGG, ScaI; and GGTCCTCT, EcoRI.
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**TABLE II**

| Name | Sequence (5' → 3')a |
|------|---------------------|
| mi-230/-224 | GATCCGGCGCGCGTTTTTCAATTTTTCAAACCTTTTTGAAATT |
| mi-222/-216 | GATGGTTTTGTTTTTTCGCGCGCGGTTTTTTTATAAAAAATT |
| mi-215/-208 | GATCGTTTGTTCGTTCAACAAAATTTTGCAATTTTTAGAAT |
| mi-207/-200 | GATCCGTTTTGTTTTTCGCGCGGCTATGAATTTTTGGCGGAAAT |
| mi-199/-192 | GATCCGTTTTGTTTTGTTTTTTCACTTTTTGAAATT |
| mi-187/-179/-3 | GAATTTCTCAGAGCGGAGAT |
| mi-187/-170/-3 | GAATTTCTCAGAGCGGGAAAT |
| mi-177/-167/-3 | GATCGTTTGCAGCATGGAAT |
| mi-177/-167/-3 | GATCGTTTGCAGCATGGAAT |
| mi-166/-156/-5 | GAATTTCTCAGAGCGGGAAAT |
| mi-166/-156/-5 | GAATTTCTCAGAGCGGGAAAT |
| mi-155/-140/-5 | GAATTTCTCAGAGCGGGAAAT |
| mi-155/-140/-3 | GATCGTTTGCAGCATGGAAT |
| mi-139/-134/-5 | TCAGCGTACGAGTAAAGTAGATGATACCTATTGTAAGG |
| mi-139/-134/-3 | TCGAGATTAGATATATTTGCGGAAATGGCGCTAGT |
| mi-133/-128/-5 | TCGAGAAGATGTAAGATGATACCTATTGTAAGGAGGAT |
| mi-133/-128/-5 | TCGAGAAGATGTAAGATGATACCTATTGTAAGGAGGAT |
| mi-127/-122/-5 | TCGAGAACTGGGAATCTAATTGATATACCTATTGGAAGGAGT |
| mi-127/-122/-5 | TCGAGAACTGGGAATCTAATTGATATACCTATTGGAAGGAGT |
| mi-121/-118/-5 | TCGAGATACATCTTTGGAAGGAGTAAAGCAGATTAAAGGAT |
| mi-119/-118/-5 | TCGAGACTCTCAGCAGGAAATCTAATGATATACCTATTGGAAGGAGT |
| mi-117/-114/-5 | TCGAGATACATCTTTGGAAGGAGTAAAGCAGATTAAAGGAT |
| mi-114/-111/-3 | TCGAGATACATCTTTGGAAGGAGTAAAGCAGATTAAAGGAT |
| mi-109/-102/-5 | GATAATCCGCGCGCGAGAATAGATGATACCTATTGGAAGGAGT |
| mi-101/-96/-3 | GATAATCCGCGCGCGAGAATAGATGATACCTATTGGAAGGAGT |
| mi-95/-92/-5 | GCGGCCGCAGGAAATGACGACACTATC |
| mi-95/-92/-3 | GCGGCCGCAGGAAATGACGACACTATC |
| mi-80/-66/-5 | GCGGCCGTTCATTTACATGTTACAT |
| mi-80/-66/-3 | GCGGCCGTTCATTTACATGTTACAT |
| mi-61/-56/-5 | CCGCGAATTAGACCTTTGGAAGGAGGAT |
| mi-61/-56/-3 | CCGCGAATTAGACCTTTGGAAGGAGGAT |
| mi-52/-48/-3 | TCGCGAAATTTGATAATCGTTGCTCAGG |
| mi-44/-39/-5 | CCGGAATCCGTTCTAGCAGCAGCGT |
| mi-44/-39/-3 | CCGGAATCCGTTCTAGCAGCAGCGT |
| mi-37/-33/-5 | AAGCTCCGATATCTCGATGATAGGAAG |
| mi-37/-33/-3 | AAGCTCCGATATCTCGATGATAGGAAG |
| mi-29/-24/-5 | TCGCGAGTTAGATATATGATCCCTACT |
| mi-29/-24/-3 | TCGCGAGTTAGATATATGATCCCTACT |
| mi-22/-17/-5 | TCGCGATATGGCTCCTAATAT |
| mi-22/-17/-3 | TCGCGATATGGCTCCTAATAT |
| mi-17/-12/-5 | GATCGCGCGTTCTCATTGTTGAT |
| mi-17/-12/-3 | GATCGCGCGTTCTCATTGTTGAT |
| mi-11/-3/-3 | acATCGATGCTATTGTTGATGAGAT |
| mi-11/-3/-3 | acATCGATGCTATTGTTGATGAGAT |

* Restriction enzyme sites are underlined. GGATCC, BamHI; ATCGAT, ClaI; GAATCC, EcoRI; AAGCTT, HindIII; GCGGCCGC, NotI; TCGCGA, NruI; GCGGCCG, SacII. The upper case letter represents the sequence of ap65-1 promoter, and the italics are mutated sequences. The lower case letter represents irrelevant sequence.

Primer extension reactions were then performed to analyze transcriptional regulation of the ap65-1 gene by iron using RNA samples from a cloned T. vaginalis T1 cell line. In each of these reactions, 50 μg of cellular RNA was primed with γ-32P-labeled ap5 or tub3. Overall extension signals of ap65-1 messenger RNA from cells treated with 250 μM iron were 3-fold higher than those from cells treated with 1 μM iron (Fig. 1B, lanes 2 and 3, respectively). Consistent with previous findings (25), the primer extension of β-tubulin messenger RNA resulted in a major product of 58 nucleotides, and the intensity of this band only changed slightly in response to changing iron supply (Fig. 1B, lanes 4 and 5).

**Primer Extension**—Two luciferase expression plasmids, pAPLuc + and pTUBLuc + (Fig. 2), were used to monitor transcriptional activities of the ap65-1 and β-tubulin promoters, respectively, in T. vaginalis T1 cells. In these experiments, luciferase activity in cells transfected with pSPLuc + was taken as background. A low level of luciferase activity (~80-fold above background) was first detected in pAPLuc + -transfected cells at 11 h post-transfection. The activity increased steadily until reaching an optimal level (~750-fold above background) at 32 h post-transfection and declined slowly as the stationary phase of cell growth was reached. Luciferase activity of pAPLuc + -transfected cells measured at 30 h post-transfection exhibited an iron-dependent increase from 1 to 500 μM iron and leveled off at a higher concentration (Fig. 2A). Iron concentration below 1 μM was not tested, because it requires the addition of the iron-chelator 2-2’-dipyridyl, which retards the growth of transfected cells in our experimental conditions. Luciferase expression in pTUBLuc + -transfected cells was 370-fold and nearly 30,000-fold above background at 13 and 28 h post-transfection, respectively. However, luciferase activity in pTUBLuc + -transfected cells was independent of iron concentration (Fig. 2B). Whereas transcriptional activity of the ap65-1 promoter was enhanced by 15-fold in the presence of 250 μM Fe2+, it was rather insensitive to other divalent metal ions such as Ca2+, Co2+, Cu2+, Mg2+, Mn2+, and Zn2+ at a similar concentration (Fig. 2C), indicating that this promoter is specifically responsive to ferrous iron.

Mapping the Regulatory Regions of the ap65-1 Promoter—In subsequent experiments, luciferase activities in cells grown in 1 and 250 μM iron measured at 30 h post-transfection were
taken as basal and inducible transcriptional activities, respectively. Luciferase activity measured in pAP$luc$/H11001-transfected cells from low iron cultures was taken as the original activity (100%).

The regulatory regions in the ap65-1 promoter were first mapped by testing the transcription efficiency of a series of pAP$luc$/H11001/H11032 deletion mutants in transfected cells (Fig. 3). In these experiments, pAP$luc$-transfected cells exhibited an average ~16-fold induction with iron treatment. Basal luciferase activity was reduced to ~35%, ~24%, and ~18% original level

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**A**

\[
\begin{array}{cccccccccccc}
-235 & GATCGTTTTT & TTGGTTTCCA & AATTTTCAA & CTTTTTTATG & AAATTATTT & -186 \\
TGGGCGAAAT & CACGGAATTC & GAGCCATTG & CGTAATAT & ACTATTAGAT & -136 \\
TTTCAGGTGA & ATGTATGAGA & TATCCATTTT & TGAAGGAAGA & TAAGATATT & -86 \\
TAAAAAGATT & ATTAGACAC & TTATCAATT & TTTGAGAGCT & GTATCGTCTT & -36 \\
GACTGACAAT & TGCGTAGTTA & TAAGTCTT & CTTCCATT & TGATTTAAG & +15 \\
TGCTTACCATCTTCAGTCTCT &
\end{array}
\]

**B**

| Primer | ap5 | ap5 | tub3 |
|--------|-----|-----|------|
| Iron   | 12 μM | 250 μM | 1 μM |
|        | 250 μM | 1 μM | 1 μM |

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**Fig. 1. Start site of ap65-1 messenger RNA and its expression level in response to changing iron supply.** A, the DNA sequence flanking 5' of the ap65-1 gene. The consensus initiator-like sequences are underlined. The major transcription start site is indicated by a dot above the sequence and is defined as +1. The translation start site is boxed. B, lane 1, the start sites of ap65-1 messenger RNA were mapped by priming 50 μg of cellular RNA extracted from T1 cells grown in 12 μM iron with γ-32P-labeled ap5 in a primer extension reaction. Dideoxy sequencing (lanes T, A, C, and G) of pAPL−217/+/217 was used as the size markers. B, in another experiment, 50 μg of cellular RNA (lanes 2–5) extracted from T1 cells pretreated with 250 μM (lanes 2 and 4) or 1 μM (lanes 3 and 5) iron for 30 h was primed with γ-32P-labeled ap6 (lanes 1–3) and tub3 (lanes 4 and 5) in primer extension reactions. Dideoxy sequencing of pTUB5' was used as the size markers.

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with deletions to −217, −204, and −184, respectively, and iron-induced luciferase expression was reduced to −5.5, −3.3, and −3-fold, respectively. Basal luciferase activity remained at −18% original level with deletions to −133, −114, and −80, but the induction level with iron treatment reduced to 2.2, 1.3, and 0.9-fold, respectively. Basal luciferase activity dropped to 11 and 7% original level with deletions to −40 and −12, respectively, without obvious iron-induced gene expression. With the deletion of the 3'-untranslated region (pAP luc+3Δ), luciferase activity decreased to 10% original level, but the induction level remained at 14-fold with iron treatment. These findings suggest that iron-inducible gene expression is primarily regulated by the DNA element(s) distal to the transcription start sites.

The 5'-flanking sequence in pAPlec+ was further studied by scanning mutagenesis (Fig. 4). Significant reduction in basal luciferase expression was observed in mutants with clustered mutations spanning the −121/−102 (Fig. 4, pm−121/−118, pm−114/−111, and pm−109/−102) and −52/−39 (Fig. 4, pm−52/−48 and pm−44/−39) regions. Their activities were reduced to −30% original level. The most severe reduction in basal transcription was seen in a mutant with two point mutations in the reputed initiator region (Fig. 4, pm+1/+3), which was only −2% original level. On the other hand, a significant reduction in iron-induced luciferase expression was seen in mutants with clustered mutations spanning the −109/−56 region (Fig. 4, pm−109/−102, pm−101/−96, pm−95/−81, pm−80/−66, and pm−61/−56). These findings suggest that the basal transcription of the 65-1 gene is regulated by the DNA elements spanning the −121/−102 and −52/−39 regions in concert with the proximal initiator sequence, and iron-induced gene expression is regulated by the DNA element(s) spanning the −109/−56 region. In conjunction with the deletion mapping experiments (Fig. 3), these results also suggest that the −230/−184 region may contain DNA regulatory elements essential for optimal transcriptional activity of the ap65-1 promoter.

Binding of Nuclear Proteins to the Iron-responsive Region—
Nuclear proteins interacting with the DNA regulatory elements in the iron-responsive region of the ap65-1 promoter were then studied by electrophoretic mobility shift assays.

A major DNA-protein complex was detected in 8% polyacrylamide gel testing for binding of nuclear proteins to 32P-labeled −68/−45 (Fig. 5). Similar banding patterns were observed in reactions using nuclear lysate from cells treated with either
The results showed that the activity is shown as percentage of activity above background). Basal transcriptional activity is taken as the original activity (Fig. 5A). Further competition assays were performed using 1000× molar excess of mutated sequences m(−68/−45) series, each with 3-bp mutation within the −68/−45 region (Fig. 5B). The results showed that −5′ATTTTT−54 is a nuclear protein binding site. Mutation of the adenosine residue to a guanosine or cytosine residue in this binding site resulted in less efficient competition (Fig. 5C), indicating that a sequence with five contiguous thymine residues is a potential nuclear protein binding site and that the adenosine residue preceding the thymine residues is preferred for optimal DNA-protein interaction. A similar protein-DNA complex was also formed in reactions using 32P-labeled m7(−68/−45) probe in which the 3′ moiety of the tvMYBl binding site is mutated (see sequence in Fig. 6B). As expected, nuclear protein-DNA complexes targeting to the tvMYBl binding site were abolished in binding reactions using nuclear proteins from cells without iron treatment (Fig. 7A, lane 1). By contrast, two major DNA-protein complexes, which migrated slower than the complexes targeting to the tvMYBl binding site, formed in binding reactions using nuclear proteins from iron-treated cells (Fig. 7A, lane 2). These complexes were displaced efficiently by 50× molar excess of m7(−107/−85) (Fig. 7A, lanes 3–5) but not by up to 500× molar excess of (−133/−110) (Fig. 7A, lanes 6–8). Further competition assays were performed using 200× molar excess of (−107/−85) and the mutated sequences m(−107/−85) series. The DNA-protein complexes were completely displaced by m7(−107/−85) (Fig. 7B, lane 10). They were not displaced by m5(−107/−85) and m6(−107/−85) but were displaced to a lesser extent by m4(−107/−85) than by any other mutated sequences or (−107/−85) (Fig. 7B). These observations indicate that the DNA sequence centered at −98AGATAACGA−90 contains a targeting site for iron-induced nuclear proteins, and its flanking sequences may also contribute to binding affinity. This possibility remains to be investigated. The DNA sequence −98AGATAACGA−90 is referred to as the iron-responsive DNA element. In conjunction with Fig. 6, these observations indicate that the 3′ moiety of the iron-responsive DNA element overlaps with 5′ moiety of the tvMYBl binding site.

**DISCUSSION**

Transcriptional regulation has been implicated as one of the major regulatory mechanisms in modulating expression of certain *T. vaginalis* virulence phenotypes in response to changing iron supply (8). In this study, the ap65-1 gene was selected as a model system to investigate iron-mediated transcriptional regulation in *T. vaginalis*. Using primer extension and transient luciferase expression assays (Figs. 1B and 2), we found that steady-state ap65-1 messenger RNA as well as transcriptional activity of the episomal ap65-1 promoter in *T. vaginalis* T1 cells are positively regulated by iron (Fig. 2A). We also found that the transcriptional activity of the ap65-1 promoter

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3 H.-W. Liu and J.-H. Tai, unpublished observations.
FIG. 4. Scanning mutagenesis of the \textit{ap65-1} promoter. Clustered mutations were introduced into the -235/+3 region of pAP\textsubscript{Luc}+. Luciferase activity in transfected cells was assayed at 30 h post-transfection. The mutated sequence in each mutant construct is shown in \textit{capital letters}, and the original sequence is shown in \textit{hyphens}. The results are the average ± S.E. of duplicate samples from three separate experiments. Luciferase activity measured in pAP\textsubscript{Luc}+-transfected cells without iron treatment was taken as the original activity (750-fold above background). The data in each set of experiments were analyzed by ANOVA. Significant reduction in basal (\(p < 0.1\)) or iron-induced (\(p < 0.05\)) luciferase expression of a mutant construct is indicated by \textit{asterisk}. 

| Mutant | % Activity | Induction fold |
|--------|------------|----------------|
| pAP\textsubscript{Luc}+ | GATCCTTTTTTTGCTTTTCACTTTTTTCAACTTTTTAGAATTTAT/ | 100 | 17.9±6.3 |
| pm-230/-224 | -GCGCCGC- | 53±19 | 8.8±0.9 |
| pm-222/-216 | -GCGCCGC- | 69±18 | 6.32±0.6 |
| pm-215/-208 | -GCGCCGC- | 51±24 | 5.7±1.3 |
| pm-207/-200 | -GCGCCGC- | 80±27 | 12.6±6.0 |
| pm-199/-192 | -GCG-CGC- | 56±5 | 7.1±1.6 |

| Mutant | % Activity | Induction fold |
|--------|------------|----------------|
| pAP\textsubscript{Luc}+ | --/TTTGGCAGGAAATCAGGAAATCAGGAGATCCG/ | 88±15 | 5.6±1.4 |
| pm-187/-178 | --/GAATTCCTCC- | 84±11 | 6.9±1.4 |
| pm-177/-167 | --/CGAATTCCG- | 57±18 | 14.6±3.0 |
| pm-166/-156 | --/TTGAATTCGA- | 99±15 | 10.6±1.4 |

| Mutant | % Activity | Induction fold |
|--------|------------|----------------|
| pAP\textsubscript{Luc}+ | --/AGATTCGCAGTGAAATGGATGAGATACCATTCTCAATTATTTAGAAAGAAGA/ | 60±9 | 17.3±5.4 |
| pm-139/-134 | --/TCGGCA- | 43±12 | 6.9±0.9 |
| pm-133/-128 | --/TCGG- | 52±5 | 7.9±0.7 |
| pm-127/-122 | --/TCGGCA- | 31±6 | 3.6±0.5 |
| pm-121/-116 | --/TCGGCA- | 28±9 | 5.2±0.6 |
| pm-114/-111 | --/CGG- | 32±10 | 2.4±0.4 |
| pm-109/-102 | --/TCGGCA- | 32±10 | 2.4±0.4 |
| pm-101/-96 | --/AGCTT- | 46±20 | 1±0.2 |

| Mutant | % Activity | Induction fold |
|--------|------------|----------------|
| pAP\textsubscript{Luc}+ | --/TAACGATATTAAAGAATATTGAGAGAATTTTGGGAGAGG/ | 90±10 | 1.4±0.2 |
| pm-95/-91 | --/GGCCACCCGCACCGGCC- | 102±26 | 1.9±0.3 |
| pm-80/-66 | --/AGCCGCACCAACCC- | 66±18 | 2.5±0.4 |
| pm-61/-56 | --/TCGGCA- | 32±11 | 4.3±0.4 |

| Mutant | % Activity | Induction fold |
|--------|------------|----------------|
| pAP\textsubscript{Luc}+ | --/CTGTATCCTGACTGACATAGTATATAAGGTTTCTCTCTCTTT/ | 36±8 | 6.3±0.7 |
| pm-44/-39 | --/-GGATCC- | 67±37 | 8.9±2.6 |
| pm-37/-33 | --/-AA-CT- | 84±10 | 9.5±0.3 |
| pm-29/-24 | --/-TCGGCA- | 87±29 | 7.3±1.5 |
| pm-17/-12 | --/-GATCC- | 99±24 | 4.7±0.9 |
| pm-11/-3 | --/-ACATCGATG- | 46±17 | 6.3±1.8 |
| pm1/+3 | --/-G-G- | 2.4±0.7 | 6.4±2.9 |

The data in each set of experiments were analyzed by ANOVA. Significant reduction in basal (\(p < 0.1\)) or iron-induced (\(p < 0.05\)) luciferase expression of a mutant construct is indicated by \textit{asterisk}.
is insensitive to other divalent metal ions (Fig. 2C). On the other hand, \(\beta\)-tubulin messenger RNA and transcriptional activity of the episomal \(\beta\)-tubulin promoter are independent of changing iron supply (Fig. 2B). These findings are consistent with previous results describing the expression features of the \(\alpha\)-65 gene family in other \(T.\) \(vaginalis\) isolates (8, 12, 15) and show that our experimental system is suitable for the study of iron-regulated expression of the \(\alpha\)-65 gene.

Primer extension experiments and mutational analysis of the \(\alpha\)-65 promoter in \(p\)AP\(\text{pluc}\) revealed that the basal transcription of the \(\alpha\)-65 gene in \(T.\) \(vaginalis\) T1 cells is primarily regulated by a conserved initiator element closest to the translation start site (Figs. 1B and 4). In good agreement with the properties of the metazoan initiators (30, 31), this initiator is both essential and sufficient to confer nearly 7% basal transcriptional activity (Figs. 3 and 4). This minimal transcriptional activity can be activated nearly up to 15- and 250-fold in low and high iron environments, respectively, in concert with distinct sets of distal DNA elements grouped into overlapping basal and iron-responsive regions at the proximal site and a discrete activation region at the distal site (Figs. 3 and 4).

Unlike the distal DNA regulatory elements identified in the \(\alpha\)-scs promoter (22), the mutation of any one of these distal DNA regulatory elements only resulted in at most 3-fold reduction in basal transcriptional activity (Fig. 4). Common DNA regulatory elements used by the \(\alpha\)-scs and \(\alpha\)-65 promoters...
were not found other than the conserved initiator elements.

The most intriguing feature of the ap65-1 promoter is the presence of multiple closely spaced DNA regulatory elements spanning −110/−54 to regulate iron-induced transcription. These DNA elements include an iron-responsive DNA element overlapping with a tvMYBl binding site and three flanking

were not found other than the conserved initiator elements.

The most intriguing feature of the ap65-1 promoter is the presence of multiple closely spaced DNA regulatory elements
T-boxes as summarized in Fig. 7C. It appears that the inaccessibility of the iron-responsive DNA element to iron-induced nuclear proteins in electrophoretic mobility shift assays is resulting from preoccupation of the site with constitutively expressed MYB-like nuclear proteins (Figs. 6 and 7), and these two distinct types of nuclear DNA-binding proteins may compete for same binding site in vivo to fine tune the transcription of the ap65-1 gene in response to environmental stimuli. This possibility remains to be examined. It is tempting to speculate that the constitutively expressed nuclear proteins targeting to the flanking T-boxes may actively participate in the formation of iron-induced transcriptional complex surrounding the iron-responsive DNA element, because mutation of any one of these three T-boxes resulted in a significant loss of iron-responsive-ness (Fig. 4). Whether iron induces de novo biosynthesis of certain transcription factor(s) or modifies certain existing transcription factor(s) to interact with the iron-responsive DNA element has yet to be determined. The iron responsive region alone is also insufficient to confer iron-inducible gene expression without the distal activation region (Fig. 3), indicating close interactions of potential transcription factors targeting to each DNA regulatory element in these regions. It is clear that iron-mediated transcriptional regulation in T. vaginalis is distinct from other iron-mediated transcriptional regulations of iron acquisition, storage, or detoxification in prokaryotes (32–34), yeasts (35, 36), fungi (37), and plants (38). Because the sequences in the 5′ and 3′-untranslated region of ap65-1 messenger RNA are not involved in the iron-induced transcriptional activity of pAPlac+ (Fig. 3), the iron-regulated ap65-1 gene expression is unlikely to occur at a post-transcriptional or translation level similar to the iron-induced gene expression in more complex organisms (39).

In summary, our study of the ap65-1 promoter provides an excellent model system for future investigations on basal transcription as well as iron-induced transcription in T. vaginalis, one of the earliest diverging eukaryotic single cells.

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