Transcriptional Analysis of the EhPgp5 Promoter of Entamoeba histolytica Multidrug-resistant Mutant*

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We report here the cloning and transcriptional characterization of the EhPgp5 multidrug resistance gene promoter isolated from the drug-resistant clone C2 of Entamoeba histolytica. The EhPgp5 promoter has the TATA-like motif at −31 base pairs; transcription initiation occurs near the ATG in trophozoites grown in 225 μM emetine (clone C2(225)), whereas in those grown without the drug (clone C2) a product with no open reading frame was detected. The promoter was active in transfected clone C2 trophozoites, its activity increased when trophozoites were cultured in 40 μM emetine, while it was turned off in the drug-sensitive clone A. The first −235 base pair kept full promoter activity, suggesting that it has important drug responsive elements. Gel shift assays detected the complex Ib in clone C2, which was augmented in clone C2(225). Competition experiments suggested that complex Ib may be constituted by HOX and AP-1 like factors in clone C2, whereas in clone C2(225), complex Ib was only competed by the HOX sequence. Complexes Ie, detected in clones A and C2 but not in C2(225), and Ia, present in all clones, were competed by the TATA box oligonucleotide. Our results suggest that proteins forming complexes Ib and Ie may be participating in the regulation of the EhPgp5 gene expression.

The MDR1 phenotype in Entamoeba histolytica seems to be a consequence of an increased expression of the P-glycoprotein, although the protein has not been detected (1). However, E. histolytica drug-resistant mutants overexpress mdr-like genes (EhPgp) (2, 3) and common characteristics are shared among the MDR phenotype of this parasite, Plasmodium falciparum, Leishmania tarentolae, and mammalian cells (Refs. 4—6 and reviewed in Ref. 7). As in some mammalian transformed cells, where only certain mdr genes are expressed, three out of the four EhPgp genes are transcribed in the drug-resistant trophozoites (clone C2) (3, 4). While the EhPgp1 and EhPgp6 genes are transcribed in clone C2 grown in the absence of the drug, the amount of EhPgp5 transcript increases according to the emetine concentration (3, 4).

The overproduction of the P-glycoprotein has been proposed to be mediated mainly by transcription, gene amplification, or both (8). However, the amplification alone may not be sufficient to activate the expression of a gene that is normally turned off or transcribed at very low levels (9). Increased mdr gene transcription precedes gene amplification in several mouse cell lines (10), and in human breast cancer and neuroblastoma cell lines (11, 12), supporting that the regulation of the mdr genes is principally controlled at the transcriptional level.

As occurs in the EhPgp5 gene, the expression of the mouse mdr1b gene is induced by the presence of the drug in the medium. The functional analysis of the mdr1b promoter demonstrated that three nuclear protein binding sequences from −82 to −59, from −123 to −101, and from −272 to −249, participate in the mdr1b gene regulation (13). In addition, DNase I footprinting experiments showed that the site from −272 to −249 is recognized by the human AP-1 transcription factor, suggesting that this factor plays a role in the transcriptional activation of the mdr1b gene. On the other hand, deletion and mutation studies of the pgp5 promoter in ovarian hamster cells showed that the AP-1 binding sequence, located near the transcription initiation site, is required for full promoter activity (14). The nuclear proteins and sequences involved in transcription regulation of E. histolytica genes have not been identified as yet. However, due to the high conservation of these proteins through evolution, it is possible to find similarities between E. histolytica and mammalian transcription factors. In fact, the TBP, the only transcription factor component of the EhPgp5 promoter isolated from the drug-resistant clone C2 is active in this mutant, and its activity increases in trophozoites grown in the presence of emetine, while it is turned off in the drug-sensitive clone A. Gel shift assays exhibited interesting differences in DNA-protein interactions of the EhPgp5 promoter with nuclear proteins from clone C2 grown in the presence and absence of the drug. Our results suggest that different transcriptional
Cloning and Sequencing of the EhPgp5 Promoter—

**EXPERIMENTAL PROCEDURES**

**E. histolytica** Cultures—Trophozoites from clones A and C2 (strain HM1:IMSS) (16) were axenically cultured in TYI-S-33 medium (17). Clones C2 was drug step selected to 40 (C2(40)) and 225 μM (C2(225)) emetine.

Cloning and Sequencing of the EhPgp5 Promoter—The EhPgp5 promoter of clone C2 was obtained from the p12 recombinant pBluescript plasmid (pBS) (Stratagene, CA) containing the first 3300 bp of the EhPgp5 ORF and 1087 bp upstream of the ATG. A TATA box and ATF-binding sites. The entire sequence for the resistant clone C2 promoter is shown. For the sensitive clone A promoter, the identical sequence is numbered relative to the transcription initiation site at position +1 (arrow).

The TATA box (TATTTAAA) consensus sequence is boxed and the palindromic and repeated sequences are underlined. The sequence is numbered relative to the transcription initiation site at position +1 (arrow).

**FIG. 1.** Nucleotide sequence of the EhPgp5 promoter of *E. histolytica* clones C2 and A. A, schematic representation of the 4387-bp insert from the p12 plasmid containing 3300 bp of the EhPgp5 ORF and 1087 bp upstream of the ATG. ATP-binding sites. Arrow, marks the transcription initiation site (+1). B, nucleotide sequence of 1087 bp upstream the ATG. The entire sequence for the resistant clone C2 promoter is shown. For the sensitive clone A promoter, the identical nucleotides are marked with a dash (—). The nucleotide changes in the sequence are shown by italic bold letters. A single gap in the clone A promoter is represented by a slash (/). The translation initiation codon is in bold. The TATA box (TATTTAAAgg) consensus sequence is boxed and the palindromic and repeated sequences are underlined. The sequence is numbered relative to the transcription initiation site at position +1 (arrow).

**FIG. 2.** Transcription initiation site of the EhPgp5 gene. Primer extension products were analyzed by electrophoresis alongside sequencing ladder extended with the same 18-bp primer (see “Experimental Procedures”). The two lanes at the left show the products from the drug-resistant clones, C2(225) (lane 1) and C2 (lane 2). The transcription start site at the consensus sequence (ATTTCG) is indicated by a solid double arrow, a minor primer extension product is indicated by a dashed single arrow. Met indicates the ATG start codon.

Sequenase version 2.0 DNA polymerase (U. S. Biochemical Corp.). Comparison of sequence data was carried out by using the Fasta algorithm (19) in the EMBL and GeneBank data bases. Identification of putative consensus sequences for eukaryotic transcription factors were done using the software package of the University of Wisconsin Genetics Computer Group (GCG) (20).

**Primer Extension**—Assays were carried out using a reverse transcriptase sequencing kit (Promega, Madison, WI) (21) and an oligonucleotide (5’-CACTAACCTTCTCTTCGT-3’) complementary to nucleotides +62 to +80. Ten μg of total RNA from clones C2 and C2(225) were hybridized to the γ-end-labeled oligonucleotide (5 × 10⁶ cpm). Annealing was carried out at 47 °C for 25 min and extension at 42 °C for 30 min with 15 units of avian myeloblastosis virus reverse transcriptase. The products were separated on denaturing 8% urea-polyacrylamide gels together with the sequence reaction using the same primer.

**Plasmid Constructions**—To assay the EhPgp5 promoter activity, two constructs were generated using the promoter-less pBSCAT-ACT reporter vector (46). The 1108- and 259-bp fragments containing 1084 and 235 bp of the EhPgp5 promoter, respectively, and 24 bp downstream from the transcription initiation site, were PCR amplified using the p12 plasmid as template and the EhPgp5-S30 (5’-TTACTGATGGCTTTCGTCATGA-3’) and EhPgp5-AS36 (5’-CCACCATCATGCTTTCGTCAT-3’) oligonucleotides, respectively. The 1108-bp fragment was cloned in PstI and HindIII sites (p1108Pgp5) and the 259-bp fragment in the Small and HindIII sites (p259Pgp5) into the pBSCAT-ACT vector (see Fig. 3). As a positive control we used the pA5’-CAT vector containing the actin promoter (22). The orientation and sequence of each construct were confirmed by DNA sequence analysis (18).

**Transfection and CAT Assays**—Transfection assays were carried out by the electroporation method as described previously (22). Electroporated trophozoites were transferred into plastic flasks (Nunc) containing 30 ml of TYI-S-33 medium and then, incubated at 37 °C for 48 h. CAT activity was determined by the two-phase diffusion CAT assay as described (23) after 16 h incubation of trophozoite extracts (50 μg) with 80. Ten μg of total RNA from clones C2 and C2(225) were hybridized to the γ-end-labeled oligonucleotide (5 × 10⁶ cpm). Annealing was carried out at 47 °C for 25 min and extension at 42 °C for 30 min with 15 units of avian myeloblastosis virus reverse transcriptase. The products were separated on denaturing 8% urea-polyacrylamide gels together with the sequence reaction using the same primer.

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nucleotides, 50 ng of the p12 plasmid, and 0.5 unit of Deep Vent DNA polymerase (New England Biolabs, Beverly, MA), during 28 cycles (94 °C, 30 s; 42 °C, 30 s; and 72 °C, 35 s) in a Perkin Elmer 9600 Thermal Cycler. The oligonucleotides used as primers for each one of the fragments were: Is (5'-CTCAAACTTTCTAAATTC-3') and Ias (5'-TGCTGGTTCACTTGTCAT-3') for fragment I, IIs (5'-ATAGTA-AATATAAATA-3') and IIas (5'-TACGGAGTTTGAAAG-3') for fragment II, and IIIs (5'-GAATGAAAGAGAAA-3') and IIIas (5'-AGTATTATCATTTAT-3') for fragment III. Nuclear extracts (NE) from clones C2(225), C2, and A and gel shift assays were performed as described (46). Competition assays were done using a 150-fold excess of the same unlabeled fragments or unlabeled double stranded oligonucleotides containing putative consensus sequences for the following transcription factors: AP-1 (5'-GGGCGTGAGTCATGGGCG-3'), HOX (5'-GGTCGTTCACTTGTCAT-3'), OCT (5'-AGCAGTTATTTAGAT-3'), C/EBP (5'-ATTCAATTGGGAAAT-3'), C/EBP (5'-ATTCAGCAATAGAGGAAAT-3'), CF-1 (5'-AGTCAGCTTGAGTGC-3'), and TATA box (5'-AGTCAGCTTGAGTGC-3') or 1.5 µg of poly[d(I-C)] as nonspecific competitor (350-fold molar excess).

RESULTS

Sequence Analysis of the EhPgp5 Promoter—To analyze the upstream region of the EhPgp5 gene isolated from clones C2 and A, we selected the p12 plasmid which includes the first 3300 bp of the ORF and 1087 bp upstream from the ATG start codon and the pBS plasmid containing an amplified fragment obtained from total DNA of clone A, respectively (Fig. 1A). Sequence data showed that the EhPgp5 promoters isolated from both clones were 99.6% identical, showing only five changes at 23, 225, 235, 250, and 1073 bases (Fig. 1B). The PCR reaction made with the pBS plasmid was not amplified any fragment from clone A. The 1087 bp upstream region of the EhPgp5 gene isolated from clones C2 and A, we selected the p12 plasmid which includes the first 3300 bp of the ORF and 1087 bp upstream from the ATG start codon and the pBS plasmid containing an amplified fragment obtained from total DNA of clone A, respectively (Fig. 1A). Sequence data showed that the EhPgp5 promoters isolated from both clones were 99.6% identical, showing only five changes at 23, 225, 235, 250, and 1073 bases (Fig. 1B). The PCR reaction made with the pBS plasmid was not amplified any fragment from clone A. The 1087 bp upstream

FIG. 3. Transient transfection of the EhPgp5 promoter from clone C2. Left, schematic representation of the relevant features in p1108Pgp5, p259Pgp5, and pA5 A3 CAT plasmids. The p1108Pgp5 and p259Pgp5 contain +24 bp downstream and −1084 and −235 bp upstream from the transcription initiation site of the EhPgp5 gene, respectively. The pA5 A3 CAT contains −480 bp of the actin promoter. All plasmids carry the CAT reporter gene and the 5'-flanking actin region (5'-ACT). Restriction sites are: B, BamHI; E, EcoRI; K, KpnI; H, HindIII; P, PstI; S, SalI; X, XhoI. Right, bars show CAT activities (cpm) obtained by the two-phase diffusion assays, after 16 h incubation of the CAT substrate and extracts from trophozoites transfected with p1108Pgp5, p259Pgp5, or pA5 A3 CAT (positive control) plasmids. Each bar corresponds to the average of CAT activities ± S.D., representative of three independent experiments performed in duplicate. The background given by the trophozoites transfected with the pBS-CAT-ACT was subtracted in all experiments.

FIG. 4. Structure of the EhPgp5 promoter and nuclear protein binding to fragments I, II, and III. A, schematic representation of the first 235 bp of the EhPgp5 promoter and the putative consensus binding sequences (boxes). Arrow indicates the transcription initiation site. Inverted short arrows indicate sense and antisense primers used for the PCR of fragments I, II, and III. B-D, gel shift assays performed with 15 µg of NE from clones A, C2, and C2(225) and 1 ng of α-32P-labeled fragments I, II, and III as described under “Experimental Procedures.” B, fragment I; C, fragment II; and D, fragment III. Lane 1, free probe; lane 2, no competitor; lane 3, specific competitor (Sc) (150-fold excess of the homologous cold fragments); lane 4, unspecific competitor (Uc) (350-fold excess of poly[d(I-C)]). The DNA-protein complexes are indicated by lowercase letters.
the ATG start codon are 78% A/T-rich, with different sized repeats and palindromic regions (underlined in Fig. 1B). The region from −472 to −700 bp has 61% identity to the −325 to −33 region of the E. histolytica EhPgp1 promoter (46) and 59% identity to the −119 to −265 region of the dyein gene promoter from Dictyostelium discoideum (25). Comparison of the 5′-flanking sequence with other E. histolytica genes revealed an 8-bp TATA box-like motif (TATTTTAAA) (26) at −31 nucleotides upstream the transcription initiation site, which was determined later in this paper (Fig. 1B, boxed sequence).

**Transcription Initiation Site of the EhPgp5 Gene**—To find the 5′ end of the EhPgp5 mRNA of clone C2, we determined the transcription initiation site by primer extension assays in trophozoites grown in the presence and absence of the drug. A single product mapping at the ATTCG sequence, three bases upstream from ATG, was detected in mRNA from clone C2(225) (Fig. 2, lane 1). Whereas this product was not detected in mRNA from trophozoites grown without the drug, instead, we found a minor primer extension product at 16 bases downstream the ATG, which has no ORF (Fig. 2, lane 2). These results suggest that EhPgp5 gene expression could be associated with the accurate selection of the transcription initiation site.

**Transient Expression Analysis of the EhPgp5 Promoter**—Two different plasmids (p1108Pgp5 and p259Pgp5) carrying 1084 and 235 bp of the EhPgp5 promoter, respectively, and 24 bp downstream from the transcription initiation site, were constructed to analyze their functional activity in clones C2(40), C2, and A (Fig. 3). We used clone C2(40) for these experiments because emetine-resistant trophozoites grown at drug concentrations higher than 40 μM are very fragile and most of them died after electroporation. The CAT activity was measured after 16 h incubation of the trophozoite extract with the substrate, because when CAT assays were done after a 2-h incubation, butyrylated chloramphenicol forms were poorly detected. In contrast, trophozoites transfected with the p964Pgp1 and p268Pgp1 plasmids, containing the EhPgp1 promoter of clone C2, expressed enough CAT enzyme to be detected in the first 2-h incubation (46). The p1108Pgp5 and p259Pgp5 plasmids were able to drive the expression of the CAT reporter gene in C2 and C2(40) trophozoites, while no activity was found when these plasmids were transfected into drug-sensitive clone A (Fig. 3). Both plasmids showed a higher CAT activity in C2(40) than in C2 (from 2084 to 3215 and from 2045 to 3854 cpm, respectively) (Fig. 3). In all experiments, the p259Pgp5 plasmid presented similar activity to the p1108Pgp5 construction, strongly suggesting that the full promoter activity is found in the first −235 bp. The positive control carrying the actin promoter (22) showed similar CAT activity in the three clones, independently if it was measured at 16 h (Fig. 3) or 2 h (data not shown) incubation. Thus, we identified a functional and inducible promoter within the p235Pgp5 plasmid which may control the expression of the EhPgp5 gene in a drug dependent fashion.

**DNA-Protein Interactions on the Proximal −235 bp of the**

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**Table 1**

| Factor| Position | Ref. |
|-------|----------|------|
| AP-1  | −9 to −2 | 27 |
| HOX   | −16 to −10 | 28 |
| C/E/BP| −76 to −64 | 29 |
| OCT-1 | −179 to −174 | 30 |
| PIT-1 | −704 to −698 | 31 |
| OCT-6 | −837 to −829 | 32 |
| CF-1  | −208 to −203 | 33 |
| MYC   | −255 to −248 | 34 |

*Consensus sequences are given between parentheses.*

**Fig. 5.** Gel shift competition assays of the DNA-protein complexes in fragment I. A, gel shift assays were performed as described in the legend to Fig. 4 in the presence of different unlabeled competitors: AP-1 and HOX oligonucleotides (150-fold excess); Uc, unspecific competitor (350-fold excess). Complexes formed are marked with lowercase letters. Arrowheads show the complexes competed. B, competition experiments performed with 15 μg of NE from clone C2 using 150 (+) and 250 (+) fold excess of the unlabeled oligonucleotide containing the TATA binding sequence (TATTTTAAA) and 150-fold excess of the Uc. Arrowheads show the complexes competed. C, schematic representation of fragment 1 with the putative consensus binding sequences HOX, AP-1, and TATTTTAAA box. Numbers indicate the base pairs at the 5′ and 3′ ends of the fragment. D, HOX, AP-1, and TATA, EhPgp5, represents the corresponding sequences found in the fragment; below them are the oligonucleotides used as competitors. Consensus means the reported consensus sequence for other organisms. Bold letters in the sequences indicate the identical bases shared by the oligonucleotides.
EhPgp5 Promoter—As the first 235 bp of the promoter seem to contain the core promoter, we focussed our studies on the structural analysis of this region (Fig. 4). Overlapping DNA fragments of approximately 100 bp each (fragments I, II, and III) were PCR amplified using specific oligonucleotides (Fig. 4A) and DNA-protein interactions were studied by gel shift assays using NE from clones C2(C225), C2, and A. Four main complexes (Ia, Ib, Ic, and Id) appeared on fragment I incubated with NE from clone C2(C225) (Fig. 4B). Complexes Ia, Ic, and Id were also formed with NE from clones C2 and A, although the intensity of complexes varied among them. Interestingly, complex Ib was strongly detected in clone C2(C225), it was fainter in clone C2 and not detected in clone A, whereas complex Ie was strongly detected in clone A, diminished in clone C2, and very faint in clone C2(C225). Fragment II formed four complexes with NE from clones C2(C225), C2, and A (IIa, IIb, IIc, and IId). NE from clones C2 and A produced complex Ile which varied in intensity from experiment to experiment and was very faint or absent in clone C2(C225) (Fig. 4C). There were no qualitative differences in the complex formed with NE from clones C2(C225), C2, and A on fragment III; although complexes IIb and IIIc were reproducibly detected in lower amounts in clone A (Fig. 4D). All complexes were specifically inhibited or diminished by a 150-fold molar excess of the same unlabeled fragments, but were not affected by a 350-fold molar excess of unspecific DNA. In summary, complex Ib is a candidate to be involved in the EhPgp5 promoter activation, while complex Ie may be participating in its repression. The functional role of these complexes is currently under study by mutation and transfection experiments.

Competitive Binding Analysis of the Complexes Formed on the EhPgp5 Promoter—Several sequences described in higher eukaryotes as consensus for transcription factors were identified in the EhPgp5 gene promoter isolated from clone C2, although their function is still unknown (Fig. 4A and Table I). Experiments were done to ascertain if the putative binding sequences identified in the first 235 bp of the EhPgp5 promoter could be associated with the complexes formed with NE from clones C2(C225), C2, and A. Double stranded oligonucleotides containing reported consensus sequences for specific transcription factors were used as competitors (Figs. 5–7). The competition experiments of fragment I were done with an AP-1 oligonucleotide (Fig. 5, C and D), which competed the complex Ib in clone C2, but not in clone C2(C225). In contrast, the HOX sequence inhibited complexes Ia and Ib in clones C2 and C2(C225). We also observed that in clone A, complex Ia diminished when it was competed by HOX and AP-1 oligonucleotides (Fig. 5A). Fragment I has two putative HOX sequences, one is close to the AP-1 site, while the other overlaps with the TATA box at −31 bp (Fig. 5, C and D), whose sequence differs only in two bases from the consensus HOX oligonucleotides used for these experiments. To investigate which of the complexes were formed by an HOX-like protein and which by other factors interacting with the TATTTAAA box sequence, as could be the TBP, we carried out competition experiments with 150- and 250-fold molar excess of the TATTTAAA oligonucleotide using NE from clone C2 (Fig. 5B). The oligonucleotide competed the complexes Ia and Ie, but not the complex Ib, which was competed by the HOX sequence, suggesting that complex Ib is formed by an HOX-like protein, while complex Ia and Ie may be formed by a protein related to TBP. The results from competition experiments showed that complex Ib could be formed by an associated HOX and API-like factors in clone C2, reflecting the fact that both sequences are very close in fragment I (Table I, Fig. 5C). Whereas in clone C2(C225) qualitative or quantitative differences in the factors forming complex Ib could exist.

In fragment II, we detected two putative binding sites for a C/EBP factor, a sequence represented several times in the E. histolytica promoters (Fig. 6, B and C). However, oligonucleotides containing the C/EBP\(^\text{a}\) consensus sequences did not eliminate any complex (Fig. 6A). Fragment III has putative consensus sequences for C/EBP, HOX, OCT, and CF-1 transcription factors (Fig. 7, B and C), but oligonucleotides containing C/EBP\(^\text{b}\), OCT\(^\text{a}\), and CF-1 sequences failed to eliminate any of the complexes (Fig. 7A), although in clone C2 a lower amount of complex IIIc was detected when we used these oligonucleotides. The HOX oligonucleotide competed the complex IIIc in experiments carried out with NE from clones A and C2, whereas the competence of this sequence with NE from clone C2(C225) was lower (Fig. 7A), suggesting that there are qualitative or quantitative differences in the factors forming some of the complex detected here, especially in complex IIIc formed with NE from clone C2(C225).

**DISCUSSION**

Factors involved in the EhPgp5 gene expression are important for understanding MDR in *E. histolytica*. Transcriptional analysis of the EhPgp5 promoter of clone C2 was undertaken in this paper to study elements that may play a role in the differential expression of this gene. The EhPgp5 promoter was 99.6% identical in the sensitive clone A and in the drug-resistant clone C2, and although one of the changes (A-G) was at the first base of the consensus sequence of the transcription initiation site, it could be not relevant in the promoter activity, because the presence of a G has been reported in the same position for the transcription initiation site motif in other *E. histolytica*.
genes (35). In contrast to the constitutively expressed EhPgp1 gene, which does not contain a TATA box and has several transcription initiation sites (46), the EhPgp5 gene expression is induced by the presence of emetine in the medium, and it has the E. histolytica reported TATA-box like motif at 231 bp (TATTTAAA) which may confer tighter regulation on the selection of the transcription initiation at the ATTCG motif. Primer extension showed that although no transcripts were detected in clone C2 by Northern blot assays (3, 4), a minor product which has no ORF was evident, suggesting that the EhPgp5 gene could be transcribed in an unfunctional product at a low amount in the absence of drug. The presence of functional transcripts with a unique initiation site mapping at sequence ATTCG correlates with EhPgp5 gene overexpression in trophozoites grown in 225 μM emetine. The p1108Pgp5 and p259Pgp5 plasmids, containing two different fragments of the EhPgp5 promoter, drove the CAT gene expression in trophozoites of clones C2 and C2(40), while no activity was detected in clone A. Full promoter activity was kept in the p259Pgp5 plasmid that was higher in the presence of emetine in the culture medium, strongly suggesting that the minimal promoter and important drug responsive elements could be located in this region. Similar CAT activity in the sensitive- and drug-resistant clones was observed in extracts from trophozoites transfected with the plasmid containing the actin promoter, showing that the drug response was specific for the EhPgp5 promoter. Interestingly, at a low drug concentrations, the EhPgp1 promoter activity was stronger (46) than that presented by the EhPgp5 promoter, evidencing the differences between the constitutively expressed actin and EhPgp1 genes with the inducible expressed EhPgp5 gene. Our previous results indicated that the amount of the EhPgp5 transcript is much lower in trophozoites grown in 40 μM emetine than in those grown in 225 μM (3, 4). Thus, we could expect a much stronger activity when we will be able to efficiently transfect the trophozoites of clone C2(225). The high identity between the EhPgp5 promoters of clones C2 and A together with the transfection and gel shift experiments strongly suggest that the expression of the EhPgp5 gene in clone C2(225) is regulated by transcriptional factors induced by the presence of emetine. This assumption is supported by the fact that trophozoites of clone C2(225) revert to the phenotype of clone C2 when they are cultured for a short period in the absence of emetine, conditions in which the EhPgp5 gene is poorly expressed, and its high resistance is recovered when trophozoites are step cultured in increasing concentrations of the drug. Additionally, the EhPgp5 promoter isolated from clone C2 was unable to drive CAT activity in the sensitive clone A.

Transcriptional regulation of mdr gene promoters in mammalian cells is mediated by the interaction between specific nuclear factors and regulatory sequences present in the promoter region (36–40). In vitro studies with the human MDRI promoter revealed that sequences located a few hundred base pairs relative to the transcription initiation site influence mdr gene transcription (41–43) and that the AP-1 factor plays an important role in the positive regulation of the hamster pgp1 gene (14). In the DNA-protein interactions on the first 249 bp, complex Ib was competed by AP-1 and HOX oligonucleotides in clone C2, suggesting that an associated protein complex constituted by HOX and AP-1-like factors could be interacting with...
the DNA. In contrast, it is possible that in clone C2(225), the AP-1-like factor could be synthesized in a higher amount, and therefore it could not be competed by the concentration of the AP-1 oligonucleotide used in our experiments. It is also possible that another unidentified factor could be forming complex Ib in clone C2(225).

Additional assays are in progress to precisely identify and characterize factors forming complex Ib in clones C2 and C2(225). We postulate that AP-1-like factor is present in the NE of *E. histolytica*, because heterologous mammalian antibodies against c-Jun and c-Fos recognized two 39- and 55-kDa bands in Western blot experiments of *E. histolytica* proteins (data not shown).

Based on the functional assays and DNA-protein interactions, we propose a working model to explain a possible mechanism for the *EhPgp5* induced expression (Fig. 8): (i) complex Ib, which seems to be formed by HOX and AP-1-like factors in clone C2 and by HOX and an unidentified factor in clone C2(225), may be involved in the up-regulation of the promoter activity. An AP-1 motif located near the transcription initiation site in the human MDRI, mouse mdr1a, and hamster pgp1 gene promoters, is involved in up-regulation of these genes (36). On the other hand, an HOX-like protein, which recognized the ATATTAA motif has been implicated in the developmental-specific activation of the γ-globin gene by promoting specific protein-protein interactions between factors bound to the promoter region (44). (ii) Complexes IIIb and IIIc are augmented in the trophozoites of clone C2(225) and could also be proposed as candidates for the positive regulation of *EhPgp5* gene expression. These complexes were always detected in a lower amount in clones A and C2 grown without the drug. It is possible that drug pressure provokes the overproduction of certain transcription factors in clone C2(225) or the modification of pre-existing proteins, or both, facilitating their DNA binding. It has been postulated in the transcriptional regulation of the mouse *mdr1b* gene that certain factors present in the drug-sensitive cells are activated or modified by the drug pressure (13). (iii) An unidentified factor forming complex Ie in clones A and C2 could be acting as a negative regulator of the *EhPgp5* gene transcription. Complex Ie was competed (together with the complex Ia) by the TATA box oligonucleotide, thus, it is possible that factors forming this complex may interfere directly or indirectly with the TBP interaction at the TATTAA sequence, provoking loss of promoter activity in clone A and the wrong selection of the initiation site in clone C2, in comparison with clone C2(225) in which complex Ie was poorly detected. This is supported by the null or very low CAT activities showed by transfected trophozoites of clones A, C2, and also by the primer extension experiments, which detected an *EhPgp5* transcript with no ORF in the clone C2. However, more experiments are necessary to elucidate the biological significance of these interactions and factors involved in the complex formation. The expression of *mdr* genes in rat is negatively controlled by an unidentified transcriptional repressor, present in the sensitive cells (45). We do not know yet the identity of factors forming complexes Ie, IIIb, and IIIc and our model does not discard the participation of other unidentified factors in the regulation of *EhPgp5* gene expression. However, the findings presented in this paper provide the first basis for the identification and characterization of trans-acting factors and cis-acting elements mediating the *EhPgp5* gene regulation. Further functional and biochemical studies are currently in progress to define the role of these regions and their corresponding factors.

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