THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 13, Issue of March 27, pp. 7277–7284, 1998
Printed in U.S.A.

Transcriptional Analysis of the *EhPgp1* Promoter of *Entamoeba histolytica* Multidrug-resistant Mutant*

(Received for publication, July 16, 1997, and in revised form, December 10, 1997)

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We present here the cloning and characterization of the *EhPgp1* multidrug resistance gene promoter isolated from the *Entamoeba histolytica* drug-resistant mutant clone C2. The *EhPgp1* promoter lacks the typical TATA box and the transcriptional initiation sequences described for other *E. histolytica* promoters. The major transcription initiation site of the *EhPgp1* gene was located at the ATG start codon. The *EhPgp1* core promoter located within the first 244 base pairs showed a higher chloramphenicol acetyltransferase expression in the transfected trophozoites of clone C2 than in those of the sensitive clone A. Gel shift assays revealed three specific DNA-protein complexes (Ia, IIa, and IIIc) using nuclear extracts from clone C2, whereas three main complexes (If, IIf, and IIg) were limited to clone A. Competition assays suggested the presence of C/EBP-like and OCT-like proteins in complexes Ia and IIa, respectively, probably involved in the expression of the *EhPgp1* gene, whereas complex IIIc was competed by GATA-1, C/EBP, OCT, and HOX oligonucleotides. Thus, differential DNA-protein complexes may be formed by transcriptional factors involved in the regulation of the *EhPgp1* gene expression.

Entamoeba histolytica is the protozoan responsible for human amoebiasis. Like other parasitic infections, amoebiasis is primarily controlled by drug treatment of symptomatic individuals using drugs such as metronidazole or emetine (1, 2). Differences in drug susceptibility have been found in several *E. histolytica* strains (3, 4) and clones (5). Case reports of failed drug treatments (6, 7) suggest that drug resistance can occur in this parasite. The multidrug resistance (MDR)[1] phenotype first described in mammalian cells (reviewed in Ref. 8) has also been described for parasites, including *Plasmodium falciparum*, *Leishmania tarentolae*, and *E. histolytica* (9–11). *E. histolytica* emetine-resistant mutants (clone C2) (5) (i) present cross-resistance to several drugs, (ii) present increased efflux and decreased accumulation of radiolabeled emetine, (iii) present resistance reversion by calcium channel blockers, and (iv) overexpress a 4.0-kilobase mRNA transcript (11, 12). The transfection with the *EhPgp1* gene, cloned in front of the *actin* promoter, also confers emetine resistance to sensitive trophozoites (13).

A membrane glycoprotein (Pgp), encoded by *mdr* genes, functions as an energy-dependent drug efflux pump, maintaining the intracellular drug concentration below cytotoxic levels (14). *mdr* genes are amplified or overexpressed in resistant cell lines exposed to drugs, hormones, or metals (15). In cell lines and cancers, the increased expression of the Pgp may be caused by transcriptional regulation alterations, gene amplification, promoter mutations, DNA rearrangements, or mRNA stability (16). The multigenic *mdr* families consist of three *mdr* genes in rodents and two in humans (17), whereas there are two *mdr* genes in *P. falciparum* (9) and three in *L. tarentolae* (10). In *E. histolytica*, four *mdr* genes (*EhPgp1*, *EhPgp2*, *EhPgp5*, and *EhPgp6*) have been cloned and sequenced (18, 19). They have between 61 and 67% homology among them and 41% homology with the human *MDR1* gene (18). In the drug-resistant clone C2, the *EhPgp1* gene is constitutively transcribed independently of drug concentration; the *EhPgp2* transcript has not been detected but the *EhPgp5* gene is transcribed at high emetine concentrations (19, 20), suggesting that their expression is regulated at the transcriptional level. Like the *EhPgp1* gene, the human *MDR1* gene is constitutively expressed. *MDR1* mRNA has been detected in several normal tissues, suggesting a role for its encoded protein in toxin or steroid transport (21). Several tumors, including leukemia and lymphoma, express high levels of *MDR1*. Thus, tissue-specific factors appear to be important in the regulation of the Pgp expression in normal and transformed tissues (22).

Transcriptional regulation in *E. histolytica* and the underlying mechanisms for the *EhPgp* genes activation are poorly understood. This study presents the functional and structural analysis of the *EhPgp1* promoter isolated from clone C2 as a step toward elucidating the mechanisms involved in the control of its constitutive expression. Nucleotides −244 to +24 of the *EhPgp1* promoter efficiently directed the expression of the chloramphenicol acetyltransferase (CAT) reporter gene in clone C2, but reduced activity was detected in the drug-sensitive clone A. Gel shift analysis showed interesting differences between nuclear factors from clones C2 and A bound to the first −244 bp of the *EhPgp1* promoter. This suggests that specific transcriptional regulators may be involved in the constitutive expression of the *EhPgp1* gene in clone C2.

EXPERIMENTAL PROCEDURES

E. histolytica Cultures—Trophozoites of clones A and C2 (strain HM1:IMSS) (5) were axenically cultured in TY1-S-33 medium (23).

* This work was supported in part by Consejo Nacional de Ciencia y Tecnologia (CONACyT) (México). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF010402.

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[1] The abbreviations used are: MDR, multidrug resistance; Pgp, P-glycoprotein; CAT, chloramphenicol acetyltransferase; pBS, pBluescript; bp, base pair(s); PCR, polymerase chain reaction; NE, nuclear extract; Inr, initiator.

Cloning and Sequencing of the EhPgp1 Promoter—The *EhPgp1* promoter of clone C2 was obtained from a recombinant pBluescript (pBS) (Stratagene, CA) plasmid (p7) containing 2230 bp of the *EhPgp1* coding region and 1770 bp upstream from the ATG start codon. This plasmid was previously isolated from a genomic DNA library constructed in Lambda Zap II vector with DNA from clone C2 (18). The *EhPgp1* promoter of clone A was isolated by PCR of total DNA using the primers EhPgp1-S28 and EhPgp1-AS33 described below, and the PCR product was cloned in pBS. As a negative control, we used the EhPgp1-AS33 primer and the reverse primer from pBS. Sequence was done with overlapping oligonucleotides by the dideoxynucleotide chain-termination method (24) using Sequenase, version 2.0, DNA polymerase (U. S. Biochemical Corp.). Sequence data analysis and sequence alignments were done with Fasta algorithm (25) in the EMBL and GenBank data bases. The localization of consensus sequences was performed with the software package of the University of Wisconsin Genetics Computer Group (26).

Primer Extension—Assays were done using a reverse transcriptase sequencing kit (Promega, Madison, WI) (27). Ten μg of total RNA from clones A and C2 were hybridized to a γ-end-labeled 18-bp primer (5′TACTCCTGCATACTGAAA3′) (5×10^5 cpm) complementary to nucleotides +110 to +128 of the *EhPgp1* gene. Annealing was carried out at 45 °C for 25 min, and the extension reaction was performed at 42 °C for 30 min with 15 units of avian myeloblastosis virus reverse transcriptase (Promega). Nucleic acids were phenol-chloroform extracted, ethanol precipitated, and separated by electrophoresis on 8% urea-polyacrylamide gels. The product length was determined by comparison with the corresponding DNA sequence obtained with the same primer.

Plasmid Constructions—For transfection experiments several plasmids were constructed using PCR-amplified DNA fragments, inserted into the multiple cloning site of the pBS plasmid. The promoterless pBSCAT-ACT plasmid was constructed after PCR amplification of the bacterial CAT reporter gene (659 bp) and the 3′-flanking sequence of the *actin* gene (600 bp) from the pA5′A3′CAT vector (28). The CAT-ACT fragment was amplified using the sense CAT-S26 (5′-CCCAAGCTTATGGAGAAAAAAATCAC-3′) and the antisense oligonucleotides Eh-Ac3′-AS29 (5′-CCGCTCGAGTTCTCTCTCCTGTGTACACC-3′) (28), cloned into the *Hin*dIII and *Xho*I sites of the pBS vector. The 964-bp fragment was PCR-amplified using the p7 plasmid as template and the sense EhPgp1-S28 (5′-AAAACTGCAGTGAAGTGTCAGCACTTAA-3′) and antisense EhPgp1-AS33 (5′-CCCAAGCTTAAACTCACTTTCAGTTATATCCAT-3′) oligonucleotides. For the 268-bp fragment, the sense IIIs (5′-TAAATGAACTAAAAAATA-3′) and the antisense EhPgp1-AS33 oligonucleotides were used. These fragments contained 940 and 244 bp of the *EhPgp1* promoter, respectively, and 24 bp of its coding region. The 964-bp fragment was cloned into the *Pst*I and *Hin*dIII sites (p964Pgp1), and the 268-bp fragment was cloned into the *Sma*I and *Hin*dIII sites (p268Pgp1) in front of the CAT gene, into the pBSCAT-ACT vector (see Fig. 3*A*). The orientation and sequence of constructions were confirmed by DNA sequencing (24).

Transfection and CAT Assays—Transfection was carried out by electroporation as described previously (28). Briefly, 10^6 trophozoites were transfected with 100 μg of the p964Pgp1, p268Pgp1, pA5′A3′CAT, or pBSCAT-ACT plasmids. Electroporated trophozoites were transferred into plastic flasks (Nalgene, Rochester, NY) containing 30 ml of TYI-S-33 medium and incubated for 48 h at 37 °C. CAT activity was analyzed by thin layer chromatography (29) using 150 μg of trophozoite extracts, 0.5 mM acetyl coenzyme A, and 1 μCi (37 kBq) of [^{14}C]chloramphenicol (50–60 mCi/mmol) incubated for 16 h at 37 °C. In other experiments, CAT activity was determinated by the two-phase diffusion assay (30) using 5 μg of trophozoite extracts and 200 μl of chloramphenicol (1.25 mM), which were incubated with [^{14}C]butyryl-CoA (NEN Life Science Products) for 2 h. Protein concentration was determined by the Bradford method (31). CAT activities were expressed as cpm of the butyrylated derivatives. The background given by the pBSCAT-ACT plasmid transfected into the trophozoites was substracted from the positive results obtained with the other plasmids. CAT activity was determined in the linear range of the assay.

Nuclear Extracts—NEs were prepared from trophozoites of clones A and C2 by a modified Schreiber's protocol (32). Briefly, 10^7 trophozoites were harvested, washed twice with cold phosphate-buffered saline, pH 6.8, resuspended in 4 volumes of Buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and incubated 20 min at 4 °C. The trophozoites were centrifuged at 8600 rpm in a JA-20 Beckman rotor and resuspended in 5 volumes of Buffer A supplemented with a protease inhibitor mixture (0.5 mM phenylmethylsulfonyl fluoride; 2 mM benzamidine; 5 μg/ml of each aprotinin, pepstatin A, leupeptin, and E-64). The trophozoites were homogenized with 25 strokes in an all-glass Dounce homogenizer using a pestle. Integrity of the nuclei was monitored by phase-contrast microscopy. The nuclei were lysed by incubation for 40 min at 4 °C in 100 μl of Buffer C (20 mM Hepes, pH 7.9, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol) in the presence of the protease inhibitor mixture. After incubation, NEs were microcentrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant was aliquoted and stored at −70 °C. Protein concentration was determined by the Bradford method (31). Reagents were purchased from Sigma.

Gel Shift Assays—Gel shift assays were performed as described previously (33) with some modifications. Briefly, three different overlapping ~100-bp fragments, corresponding to the first 244 bp upstream from the ATG start codon, were amplified and labeled by PCR. The PCR mixture contained [α-^{32}P]dATP, 2 mM cold nucleotides, 50 ng of template DNA and 0.5 units of Deep Vent DNA polymerase (New England Biolabs). The reaction was carried out during 28 cycles (94 °C for 30 s, 42 °C for 30 s, and 72 °C for 35 s) in a Perkin-Elmer 9600 Thermal Cycler. The oligonucleotides used as primers for each fragment were as follows: Is (5′-TTTTAGATTTAATGTGTT-3′) and Ias (5′-CACTTTCAGTTATATCCA-3′) for fragment I, IIs (5′-TAACAAAGGAGAGAAAAT-3′) and IIas (5′-ACCAAACACTAACACATT-3′) for fragment II, and IIIs and IIIas (5′-CTTATTATTTTCTCTCCT-3′) for fragment III. The labeled fragments were separated on 12% nondenaturing polyacrylamide gels and purified after elution. DNA fragments (0.5–1 ng) were incubated with 15 μg of NEs from clones A or C2, 1 μg of poly[d(I·C)] (Pharmacia Biotech Inc.) and 10% glycerol in DNA-protein binding buffer (12 mM Hepes, pH 7.9, 60 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 4 mM Tris-HCl, pH 7.9, 1 mM spermidine, 1 mM $MgCl_2$) for 10 min at 4 °C. The bound and unbound complexes were separated on 6% nondenaturing polyacrylamide gels in 0.5× TBE (44.5 mM Tris-HCl, pH 7.9, 44.5 mM boric acid, 1 mM EDTA) at 25 °C and 100 V for 4 h and visualized by autoradiography. Competition assays were performed using a 150-fold excess of the same unlabeled fragments or unlabeled double-stranded oligonucleotides containing consensus sequence for the following transcription factors: C/EBP$^{\alpha}$ from rat albumin (5′-GGTATGATTTTGTAATGGGGTAGG-3′) (34), a putative C/EBP$^{\beta}$-like sequence represented several times in different *E. histolytica* promoters (5′-ATTCAATTGGGCAATCA-3′), GATA-1 (5′-GTTGCAGATAAACATT-3′), HOX (5′-GTAAGAGTTATTATTGAT-3′), OCT$^{\alpha}$ (5′-ACATAGTTTATGCAACCGAAA-3′) and OCT$^{\beta}$ (5′-AGCTAATTGCATACTTGGCTTGTAC-3′) oligonucleotides or 1.5 μg of poly[d(I·C)] as a nonspecific competitor (350-fold excess).

RESULTS

Sequence Analysis of the EhPgp1 Promoter—The 942 bp upstream from the ATG start codon from EhPgp1 gene were sequenced using as template the p7 plasmid (18) or the pBluescript plasmid containing a PCR-amplified fragment obtained from total DNA of clone A (Fig. 1). The *EhPgp1* promoters isolated from both clones were 99.7% identical, except for three changes at the position −501, −928, and −930. The PCR made with the pBS reverse primer did not amplify any fragment from clone A. This region was 75% A/T rich with different-sized repeated and palindromic sequences (Fig. 1*B, underlined*). The −325 to −33-bp region presented 61% identity with the −472 to −700-bp region of the *EhPgp5* promoter (58). Homology of 53–66% with promoter regions of *discoidin* (−448 to −1046 bp), *D19* (−820 to −1164 bp), *dynein* (−360 to −886 bp) and *ecm A* (−1530 to −1783 bp) genes from *Dictyostelium discoideum* (35–38) was found. The TATA box-like motif (TATTTAAA) described for other *E. histolytica* promoters (39) was not detected, but two putative initiator (Inr) elements at positions −18 and −67 (Fig. 1*B, boxes*) were found. The Inr element found at −18 bp (GAACTAA) contains the conserved sequence CE2 (GAAC) recently reported for several 5′-flanking regions in *E. histolytica* genes (40). Interestingly, the 5′-flanking region of the *APorC* gene of *E. histolytica* (40) also has the GAACTAA sequence localized at −18 bp, whereas the putative Inr element (TTAGATT) is identical to that described in mammalian cells (41). In other gene promoters, mainly in the TATA-less promoters, Inr elements interact with transcriptional factors to influence accurate transcription (42).

Transcription Initiation Sites of the EhPgp1 Gene—Primer

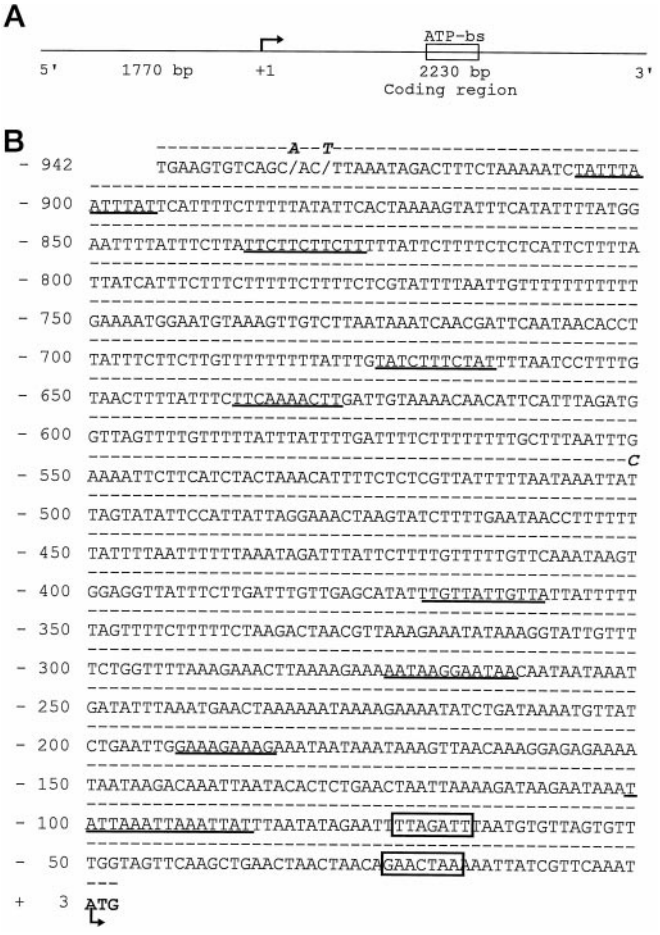

FIG. 1. **Nucleotide sequence of the *EhPgp1* promoter of *E. histolytica* clones C2 and A.** *A*, schematic representation of the 4000-bp insert from the p7 plasmid containing 2230 bp of the *EhPgp1* gene and 1770 bp of the promoter. *ATP-bs*, ATP binding site. The *arrow* marks the transcription initiation site (+1). *B*, nucleotide sequence of the 942 bp upstream from 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 *dashes*, and the nucleotide changes in the sequence are shown by *boldface italic letters*. Two gaps in the C2 promoter are represented by *slashes*. The initiation site and transcription direction are marked by *arrows*. Translation initiation codon is shown in *boldface type*. Putative Inr sequences are *boxed*. Palindromic and repeated sequences are *underlined*.

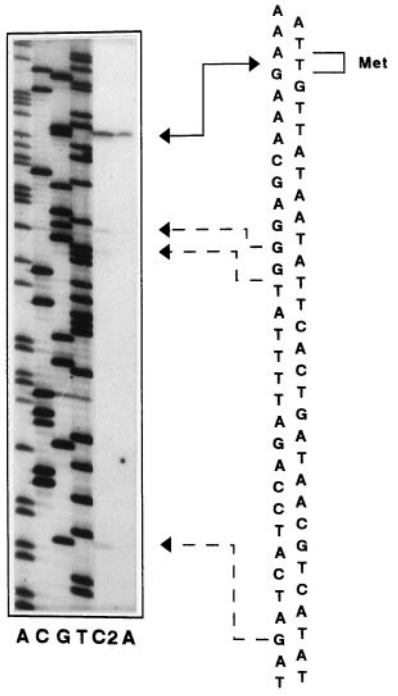

FIG. 2. **Transcriptional initiation sites of the *EhPgp1* gene.** Primer extension products were analyzed alongside sequencing ladder extended with the same 18-bp primer (see "Experimental Procedures"). The two lanes at the *right* show the products from clones C2 (*lane C2*) and A (*lane A*). The major transcription start site in both clones and their corresponding positions in sequence are shown by a *solid double arrow*; minor primer extension products are indicated by *discontinuous single arrows*. *Met* shows the ATG start codon.

extension experiments with total RNA were used to identify the 5′ end of the *EhPgp1* transcript. The major extension product of the *EhPgp1* gene from clone C2 initiated at the base A of the ATG start codon, although other, fainter bands were visible at positions +16, +19, and +55 (Fig. 2, *lane C2*), but no open reading frame was found. In clone A, the major extension product also mapped at the ATG start codon; however, the amount of the main product was less than that in clone C2 (Fig. 2, *lane A, right*), indicating that the *EhPgp1* gene is also transcribed in clone A, even though transcripts were not detected by Northern blot assays (19). These results suggest that the differential amount of the *EhPgp1* transcript in sensitive and resistant clones may be the regulating mechanism of the MDR phenotype in *E. histolytica*. Results were highly reproducible, supporting the specific binding of the primer.

Transient Expression Analysis of the EhPgp1 Promoter—Transfection assays were done to examine whether the region upstream from the *EhPgp1* gene had a functional promoter. The p964Pgp1 and p268Pgp1 plasmids, which contain 24 bp of the open reading frame and −940 and −244 bp of the *EhPgp1* promoter, respectively (Fig. 3*A*), were transfected into clones A and C2. Both plasmids drove the CAT expression in the resistant and sensitive clones. However, after 2 h of incubation of the trophozoite extracts with the substrate, CAT activities were significantly higher in trophozoites of clone C2 compared with clone A (Fig. 3*A*), suggesting the presence of positive transcriptional regulators in clone C2, which may be absent, diminished, or modified in clone A. To define a shorter region with promoter activity, the p268Pgp1 plasmid was transfected into trophozoites of clones C2 and A. Interestingly, no differences in CAT expression were detected in trophozoites of clone A transfected with p964Pgp1 or p268Pgp1 plasmids, whereas when clone C2 was transfected with p268Pgp1, it had more CAT activity than when it was transfected with the p964Pgp1 plasmid (Fig. 3*A*). The latter proved that the *EhPgp1* core promoter is located within the first 244 bp. In contrast to the strong *EhPgp1* promoter activity, the *EhPgp5* promoter presented little activity in clone C2 grown without drug (Fig. 3*B* and Ref. 58). CAT activity of the plasmid carrying the *actin* promoter was similar for clones A and C2 but less than that obtained with the p964Pgp1 and p268Pgp1 plasmids when activity was measured after 2 (Fig. 3*A*) or 16 (Fig. 3*B*) h of incubation. In both experiments the negative control, the promoterless pBSCAT-ACT plasmid and the cpm obtained from the trophozoites transfected with this plasmid (basal activity) were substracted from the activity obtained with the p964Pgp1, p268Pgp1, and pA5′A3′CAT plasmids (Fig. 3).

DNA-Protein Interactions on the Proximal −244 bp of the EhPgp1 Promoter—The structural analysis of the *EhPgp1* promoter was done using the 244 bp upstream from the ATG start codon of the *EhPgp1* gene because transfection experiments indicated that the core promoter was located in this region. Overlapping DNA fragments of approximately 100 bp each (fragments I, II, and III), covering bp −244 to +19 were PCR-amplified using specific oligonucleotides (Fig. 4*A, bottom*) and

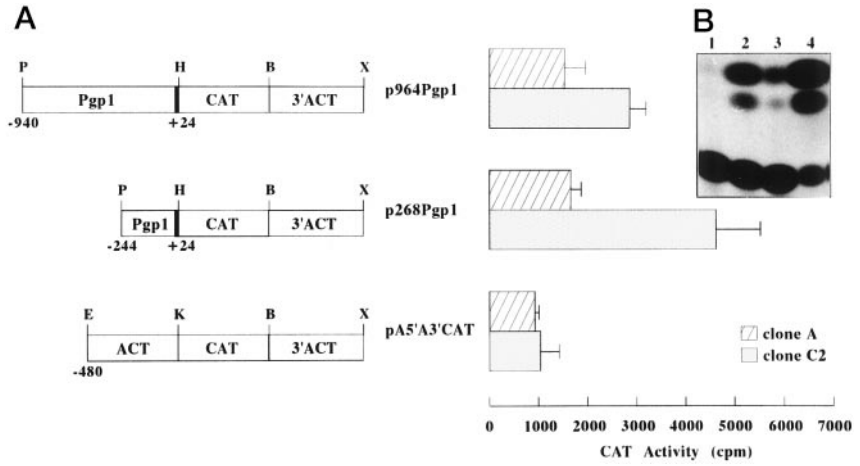

FIG. 3. **Transient transfection of the *EhPgp1* promoter from clone C2.** *A, left,* schematic representation of the relevant features of the p964Pgp1, p268Pgp1, and pA5′A3′CAT plasmids. The p964Pgp1 and p268Pgp1 plasmids contain +24 bp downstream from the ATG and −940 or −244 bp upstream from the *EhPgp1* gene, respectively. Plasmid pA5′A3′CAT contains −480-bp fragment from the *actin* promoter. All of the plasmids have the CAT reporter gene and the 3′-flanking *actin* region (*3′ACT*). *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *K*, *Kpn*I; *P*, *Pst*I; *X*, *Xho*I. *Right*, *bars* show the CAT activity (cpm) obtained by the two-phase diffusion assays after a 2-h incubation of the CAT substrate and extracts from trophozoites transfected with p964Pgp1, p268Pgp1, 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 pBSCAT-ACT was substracted in all experiments. *B*, representative CAT chromatogram obtained after a 16-h incubation of trophozoite extracts with the CAT substrate. *Lane 1*, pBSCAT-ACT promoterless construct; *lane 2*, pA5′A3′CAT construct; *lane 3*, p1108Pgp5 construct; *lane 4*, p964Pgp1 construct.

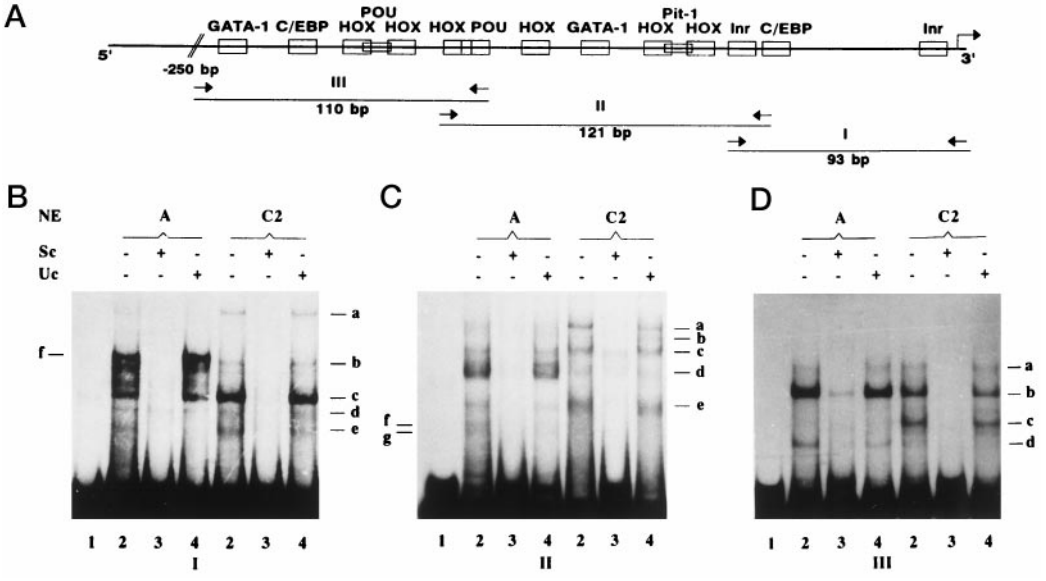

FIG. 4. **Structure of the *EhPgp1* promoter and nuclear protein binding to fragments I, II, and III.** *A*, schematic representation of the first 250 bp of the *EhPgp1* promoter and the putative consensus binding sequences (*boxes*); *arrow* at *right* indicates the transcription initiation site. At the *bottom* of the scheme are the DNA fragments (I, II, and III) used for gel shift assays. *Arrows* indicate sense and antisense primers used for PCR. *B, C,* and *D*, gel shift assays were performed with 15 μg of NEs from drug-sensitive clone A or drug-resistant clone C2 trophozoites and 1 ng of α-^{32}P-labeled fragments I, II and III, as described under "Experimental Procedures." *B*, fragment I; *C*, fragment II; *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 shown by *lowercase letters*.

their interactions with NEs from clones C2 and A were studied by gel shift assays. NEs from clone C2 incubated with fragment I (−74 to +19 bp) formed five main complexes (Ia, Ib, Ic, Id, and Ie). Interestingly, complex Ia was not detected in experiments carried out with NEs from clone A (Fig. 4*B*). Additionally, NEs from clone A formed complex If, which was not detected in clone C2 (Fig. 4*B*). Fragment II (−167 to −47 bp) formed five complexes with NEs from clone C2 (IIa, IIb, IIc, IId, and IIe) (Fig. 4*C*). Except for complex IId, all complexes were more abundant in clone C2 than in clone A; this was particularly clear for complex IIa, which was very strong in clone C2 and very faint in clone A. In contrast, the doublet IIf and IIg, present in clone A, was not detected in clone C2 (Fig. 4*C*). Fragment III (−244 to −144 bp) (Fig. 4*D*) formed four complexes (IIIa, IIIb, IIIc, and IIId) with NEs from clone C2, one of which (IIIc) was not detected in clone A. All of the complexes were specifically competed by the same cold fragments and were maintained in the presence of poly[d(I·C)], used as nonspecific competitor. In summary, from gel shift assays, complexes Ia, IIa, and IIIc, detected mainly with NEs from clone C2, were identified. These complexes may be involved in the constitutive *EhPgp1* gene expression. On the other hand, complexes If, IIf, and IIg, which were reproducibly detected only with NEs from clone A, may be acting as negative regulators and may be responsible for suppressing the *EhPgp1* expression. Their specific function is currently under study by mutation and transfection experiments.

TABLE I
Putative consensus sequences for transcriptional factors in the EhPgp1 promoter

Factor[a]		Position	Ref.
		bp	
C/EBP	(TGTTTGGTAGTT, ATTGG)	−54 to −43	43
		−196 to −192	
HOX	(TATTAT, TATTTA)	−84 to −79	44
		−87 to −82	
		−100 to −95	
		−137 to −132	
		−176 to −171	
		−247 to −242	
OCT	(TATTTAAT)	−92 to −85	45
		−163 to −157	
GATA-1	(AGATAA)	−113 to −108	46
		−213 to −208	
		−799 to −794	
TCF-1	(AAAAG)	−116 to −112	47
		−226 to −222	
HMG-1	(TCTTTTTC)	−353 to −346	48
MEF-2	(TTATTTTTAA)	−527 to −548	49
CF-1	(AAATGG)	−604 to −599	50
		−747 to −742	

[a] Consensus sequences are given in parentheses.

Competitive Binding Analysis of the Complexes Formed on Fragments I, II, and III of the EhPgp1 Promoter—Promoters share common structural features, reflecting similar interaction with RNA polymerase. This fact has allowed the identification of DNA consensus sequences for different transcriptional factors. The *EhPgp1* promoter was scanned for potential consensus sequences candidates that might participate in the binding of transcriptional regulatory factors (Table I and Fig. 4*A*). To investigate the identity of some of the sequences and complexes formed by fragments I, II, and III and the NEs from clones A and C2, we carried out competition experiments using double-stranded oligonucleotides containing consensus sequences for transcription factors present in these fragments (Table I and Fig. 4*A*). Two different putative C/EBP binding sequences were used to compete the complexes formed on fragment I: (i) the C/EBP$^{\alpha}$ binding sequence from the rat albumin gene (34), and (ii) a C/EBP$^{\beta}$-like, putative sequence represented several times in some *E. histolytica* sequences upstream from the ATG start codon. The C/EBP$^{\alpha}$ oligonucleotide competed complex Ia formed exclusively with NEs from clone C2, whereas the C/EBP$^{\beta}$ oligonucleotide did not compete any complex (Fig. 5*A*). The C/EBP$^{\alpha}$ sequence shares 9 of 12 bases of the putative C/EBP sequence found between −43 and −54 bp in the *EhPgp1* promoter (Fig. 5, *B* and *C*), whereas the C/EBP$^{\beta}$-like sequence shares only 5 bases (Fig. 5*C*). These results suggest the presence of a C/EBP-like transcription factor interacting with the *EhPgp1* promoter that could be involved in the expression of the *EhPgp1* gene in clone C2 but not in clone A.

Double-stranded oligonucleotides containing the consensus sequences for OCT$^{\alpha}$, OCT$^{\beta}$, HOX, and GATA-1 were used as competitors of complexes formed with fragment II (Fig. 6*A*). Oligonucleotides for HOX, GATA-1, OCT$^{\beta}$, and C/EBP$^{\beta}$ factors (the latter was used as a negative control because no identifiable binding sequence were present in this fragment) failed to compete the complexes formed with fragment II and NEs from clone C2 (Fig. 6*A*); however, the OCT$^{\alpha}$ oligonucleotide competed complexes IIa and IIb. The OCT$^{\alpha}$ oligonucleotide shares 6 of 8 bases of the Pit-1 putative consensus sequences identified between positions −81 and −88 in the *EhPgp1* promoter, whereas the OCT$^{\beta}$ oligonucleotide shares 4 of 7 bases of the POU putative consensus sequence present between positions −145 and −151 of the promoter (Fig. 6*C*). Complex IIe, formed with NEs from both clones, was competed exclusively in clone A by the HOX oligonucleotide, whereas complexes IIf and IIg, exclusive of clone A, were competed by the OCT$^{\beta}$ oligonucleotide (Fig. 6*A*). This fragment has putative consensus sequences for HOX, POU, GATA-1, and Pit-1 transcription factors (Fig. 6*B*). The HOX sequence overlaps at −83 and −88 bp with Pit-1 and at −146 and −151 bp with POU sequences (Fig. 6*B* and Table I).

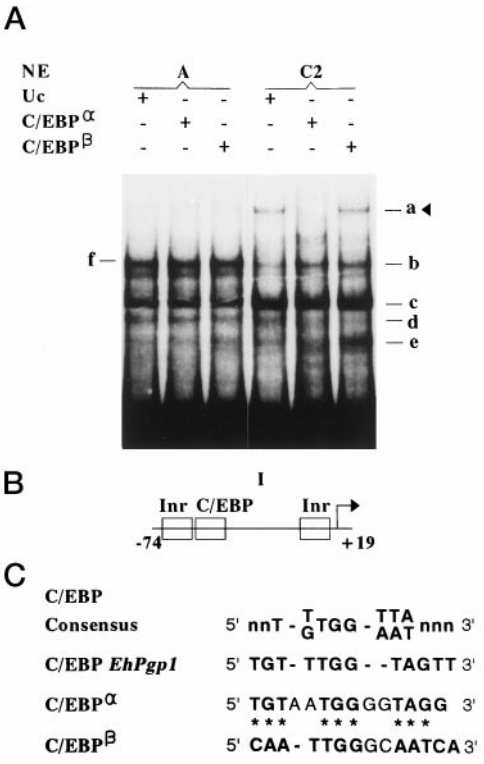

FIG. 5. **Gel shift competition assays of the DNA-protein complexes in fragment I.** *A*, gel shift assays were performed as described in the legend to Fig. 4 in the presence of different unlabeled competitors: *Uc*, unspecific competitor (350-fold excess); *C/EBP*$^{\alpha}$, oligonucleotide sequence obtained from rat albumin; *C/EBP*$^{\beta}$, oligonucleotide sequence represented several times in different promoters of *E. histolytica* (150-fold excess). *Arrowhead* shows the complex competed by C/EBP$^{\alpha}$. *B*, schematic representation of fragment I with the putative consensus binding sequences C/EBP and Inr. *C*, sequence of the C/EBP$^{\alpha}$ and C/EBP$^{\beta}$ oligonucleotides used as competitors compared with the C/EBP consensus sequence (*Consensus*) found in other organisms and with the C/EBP *EhPgp1* sequence found in the *EhPgp1* promoter. *Boldface letters* in sequence show the identical bases shared by the oligonucleotides, and *n* represents any nucleotide. *Asterisks* emphasize bases that are identical in the C/EBP *EhPgp1* and C/EBP$^{\alpha}$. *Numbers* indicate the base pairs at the 5′ and 3′ ends of the fragment.

Competition experiments of complexes formed on fragment III were done with C/EBP$^{\beta}$, GATA-1, OCT$^{\alpha}$, OCT$^{\beta}$, and HOX oligonucleotides (Fig. 7*A*). The formation of complex IIIc with NEs from clone C2 was competed by all oligonucleotides used, except for OCT$^{\alpha}$. The nonspecific competitor and the OCT$^{\alpha}$ oligonucleotide maintained complex IIIc in clone C2, suggesting that competition with the other oligonucleotides used here was specific. These results suggest that complex IIIc, formed with NEs from clone C2, may be constituted by more than one protein because it was competed by four different oligonucleotides. In experiments done with clone A, only the HOX oligonucleotide competed the formation of complex IIId (Fig. 7*A*).

From gel shift assays and competition experiments analysis, the presence of nuclear proteins in clone C2 that could correspond to C/EBP-, OCT-, GATA-, and HOX-like factors interacting with the *EhPgp1* promoter suggests that they could be involved in the positive regulation of the *EhPgp1* gene expression.

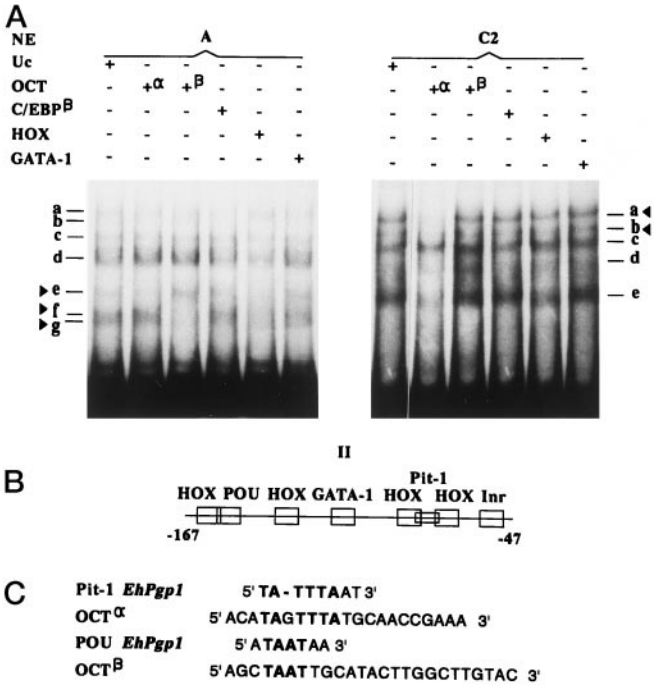

FIG. 6. **Gel shift competition assays of the DNA-protein complexes in fragment II.** *A*, gel shift assays were performed as described in the legend to Fig. 4, using different unlabeled competitors: *Uc*, unspecific competitor (350-fold excess) and OCT$^{\alpha}$, OCT$^{\beta}$, C/EBP$^{\beta}$, HOX, and GATA-1 oligonucleotides (150-fold excess). *Arrowheads* show the complexes competed. *B*, schematic representation of fragment II with the putative consensus binding sequences. *C*, sequences of the OCT$^{\alpha}$ and OCT$^{\beta}$ oligonucleotides used as specific competitors compared with the Pit-1 and POU putative binding sequences found in the *EhPgp1* promoter. *Boldface letters* in the sequences show the identical bases shared by the oligonucleotides. *Numbers* indicate the base pairs at the 5′ and 3′ ends of the fragment.

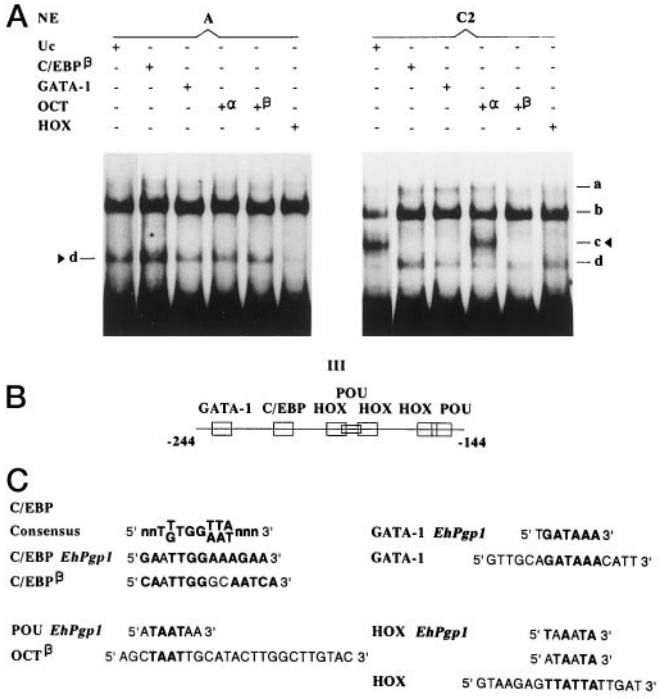

FIG. 7. **Gel shift competition assays of the DNA-protein complexes in fragment III.** *A*, gel shift assays were performed as described in the legend to Fig. 4, using different unlabeled competitors: *Uc*, unspecific competitor (350-fold excess) and C/EBP$^{\beta}$, GATA-1, OCT$^{\alpha}$ and OCT$^{\beta}$, and HOX oligonucleotides (150-fold excess). *Arrowheads* show the complexes competed. *B*, schematic representation of fragment III with the putative consensus binding sequences. *C*, C/EBP$^{\beta}$, GATA-1, POU, and HOX *EhPgp1* represent the corresponding sequences found in the fragment. The oligonucleotides used as competitors are shown below. *Consensus* indicates the reported consensus sequence for other organisms. *Boldface letters* in the sequences indicate the identical bases shared by the oligonucleotides. *n* represents any nucleotide. *Numbers* indicate the base pairs at the 5′ and 3′ ends of the fragment.

DISCUSSION

The structural and functional characterization of the *EhPgp1* promoter isolated from clone C2 was analyzed. *EhPgp1*, one of the genes responsible for the MDR phenotype in *E. histolytica*, is differentially transcribed in the drug-sensitive clone A and in the drug-resistant clone C2. This work and the accompanying paper (58) represent one of the first attempts to study the concerted interplay of protein transcription factors and promoters on the regulatory regions of *E. histolytica* genes. The results suggest that the expression of the *EhPgp1* gene involved in the MDR phenotype of clone C2 is finely regulated at transcriptional level.

The *EhPgp1* promoters from the drug-sensitive and drug-resistant clones were 99.7% identical in the 942 bp sequenced. Sequence analysis revealed that the *EhPgp1* promoter lacks a TATA box motif found in other *E. histolytica* genes (39). Instead, it has two putative Inr sequences that have been described as being involved in the same functions as TATA boxes (41, 42). In fact, the putative Inr sequence found at −18 bp is necessary for the transcription of the *hgl5* gene of *E. histolytica* (40). The human *MDR1* gene, which is constitutively expressed in several tissues, also lacks a TATA box and has an Inr element (22). By analogy, a similar regulatory mechanism may exist for the *EhPgp1* and *MDR1* promoters. The function of these putative Inr sequences is being investigated. The *EhPgp1* promoter has several palindromic and repeated sequences that may be also involved in transcriptional regulation, as has been reported for *Dictyostelium*, *Drosophila*, and yeast genes (35, 51, 52).

In contrast to other *E. histolytica* genes, which start their transcription at the ATTCA or ATCA motifs, located near the ATG start codon (39, 40), the major *EhPgp1* mRNA initiates just at the ATG start codon, whereas other minor products initiate downstream from the ATG. In other systems, differences in the 5′ end of mRNA influence the translation efficiency through the creation or elimination of binding sites for trans-acting factors or through the formation of stable secondary structures that can modulate the overall translation efficiency (53). Additionally, variations in the 5′ upstream sequences may influence steady state mRNA levels (54). However, any of these mechanisms appear to act on the *EhPgp1* promoter because the minor products lack open reading frames. In the *EhPgp1* gene activation, the correct selection of the transcription initiation site and the amount of the transcript may function as regulatory mechanisms.

We demonstrated the functionality of the *EhPgp1* promoter isolated from clone C2 by transfection assays. The p964Pgp1 and p268Pgp1 plasmids carrying 940 and 244 bp upstream and +24 bp downstream from the ATG, respectively, were able to drive the transcription of the CAT gene in clones C2 and A. However, CAT activity was higher in the resistant clone C2, suggesting that different factors may be interacting with regulatory sequences in this promoter to modulate the transcriptional repression or activation of the *EhPgp1* gene. Because the sequences of the core promoters in the sensitive clone A and the resistant clone C2 were identical, we suggest that clone A, in contrast to clone C2, does not have the elements to efficiently enhance the *EhPgp1* gene expression. Transfection experiments using the promoter from the resistant clone C2 and gel shift assays support this assumption. The p268Pgp1 plasmid contains all the necessary elements to efficiently drive the CAT

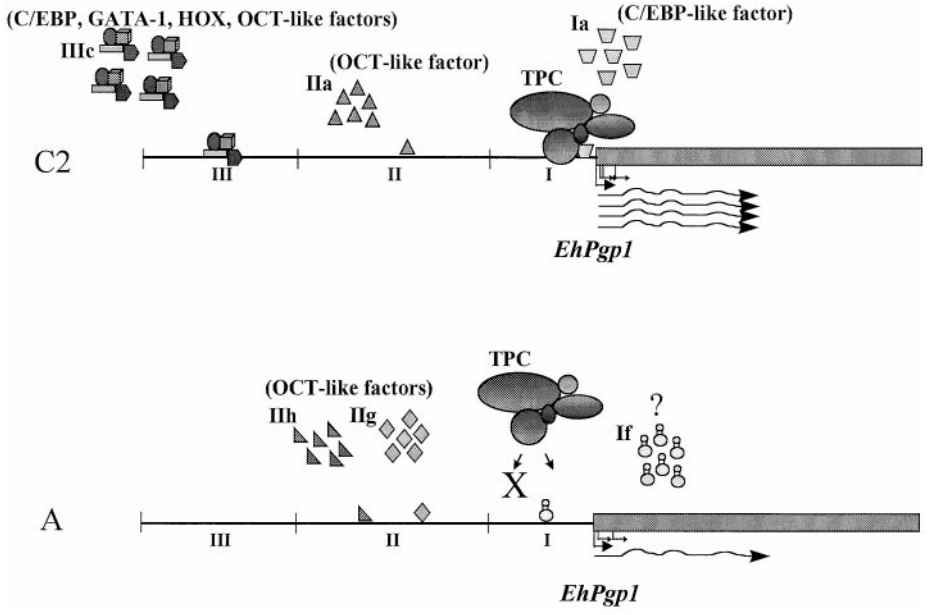

FIG. 8. **Schematic representation of the main transcription factors regulating the *EhPgp1* gene expression.** The nuclear factors of complexes Ia, IIa, and IIIc may be in abundance and be recruited to the *EhPgp1* promoter in clone C2. These factors could recognize the promoter or basal factors helping the binding of the transcription preinitiation complex (*TPC*) and mediating the activation of this promoter, whereas in clone A, the presence of negative regulators that could be part of complexes If, IIg, and IIh (which are formed exclusively in clone A) could provoke diminishing of transcription activation. The putative activators could be modified or diminished or may not be synthesized in this clone. *Wavy arrows* represent the *EhPgp1* transcript in both clones. *Small arrows* represent the transcription initiation sites detected by primer extension.

expression in clone C2, even better than the p964Pgp1 construct, suggesting the presence of sequences acting as silencer regulatory elements between nucleotides −244 and −940. Both plasmids showed a higher activity than the pA5′A3′CAT plasmid, which contains the *actin* promoter, probably due to the presence of the +24 bp downstream from the ATG in the *EhPgp1* promoter plasmids. It has been suggested that a small sequence downstream from the ATG enhances the transcription activity of other *E. histolytica* promoters (55).

By gel shift assays, three complexes (Ia, IIa, and IIIc) were found on fragments containing the first 244 bp of the *EhPgp1* promoter with NEs from clone C2. Complex Ia was competed by the C/EBP$^{\alpha}$ oligonucleotide, suggesting the presence of a putative C/EBP-like protein in *E. histolytica* that could be binding to the sequence found at −43 to −54 bp. We consider that a C/EBP-like nuclear protein may be a good candidate with a positive role in the *EhPgp1* gene expression in clone C2 because (i) complex Ia, competed by C/EBP$^{\alpha}$, was poorly detected in clone A; (ii) a putative C/EBP binding sequence is close to the transcription initiation site; (iii) in Western blot assays, antibodies against the human C/EBP protein recognized a ~20-kDa band in NEs of *E. histolytica* (data not shown), supporting the presence of a C/EBP-like factor in this parasite; (iv) the C/EBP protein has been described as an activator factor in mammalian genes; and (v) C/EBP binding sequences have been found also in the human *MDR3* and in the mouse *mdr1b* promoters, probably acting as cis-elements regulating their transcriptional activity (43, 56).

Complex IIa, also specific for clone C2, was competed by an OCT$^{\alpha}$ oligonucleotide, even though no OCT binding sequences in fragment II were detected. However, POU and Pit-1 binding sequences are found, and POU and Pit-1 proteins share the DNA binding domains with the OCT family proteins (45). OCT factors participate in the regulation of the expression of housekeeping genes. The *EhPgp1* promoter shares some characteristics with housekeeping promoters, such as the presence of putative Inr elements and its constitutive expression in clone C2.

Complex IIIc, which is also specific for clone C2, was competed by GATA-1, C/EBP$^{\beta}$, OCT$^{\alpha}$, and HOX binding sequences but not by the OCT$^{\beta}$ oligonucleotide. GATA-1, C/EBP$^{\beta}$, Pit-1, and HOX sequences were located in fragment III in a region of 37 bp, suggesting that some factors could be interacting each other to form a multiprotein complex, which is probably required for transcription regulation of the gene. This assumption is supported by the fact that binding sequences for these four putative transcription factors are very close. It has been demonstrated that many transcription factors contain domains that mediate the formation of homo- and heterodimers, forming multiprotein complexes that could bind to the DNA and that may be involved in transcriptional regulation (57). On the other hand, some of the complexes found exclusively in clone A (If, IIf, and IIg) may be candidates for negative regulation of *EhPgp1* gene transcription. Complexes IIf and IIg were competed by an OCT$^{\beta}$ oligonucleotide using the NEs from sensitive clone A, suggesting that the presence of a putative OCT transcription factor could be a repressor when it binds to fragment II in clone A. When the OCT$^{\beta}$ oligonucleotide competes complex IIIe formed with NEs from clone C2, the corresponding transcription factor could be acting as an activator. Another possibility is that two different members of the OCT family may be acting in different regions with different sequences.

A combination of positive and negative control regulatory mechanisms is frequently responsible for the inducible expression of certain genes. Based on the results obtained, we propose a working model to explain the regulation of the *EhPgp1* gene expression in the resistant clone C2, which may be mediated by the interaction of transcription factors with regulatory elements present in the *EhPgp1* promoter (Fig. 8). We postulate the presence of (i) activators in clone C2, which may be absent, modified or diminished in clone A. These factors may be related to complexes Ia (C/EBP-like protein) and IIa (OCT-like protein). (ii) The other possible activator could be a multiprotein complex (IIIc), which was competed by GATA-1, C/EBP$^{\beta}$, OCT$^{\alpha}$, and HOX sequences. (iii) Repressors in clone A may be absent, modified or diminished in clone C2. These proteins could be related to complexes If, IIf, and IIg, which are formed mainly with NEs from clone A. The existence of other factors participating in the positive or negative regulation of the *EhPgp1* gene cannot be discarded by the proposed working model. Additionally, detection of a viable product in clone A by primer extension assays and the low promoter activity in transfection assays suggest that basal expression level of the *EhPgp1* gene is mediated by nuclear factors that may be present in different amount in drug-sensitive and drug-resistant trophozoites. Mutations analysis of the binding sequences

found are currently carried out to define the precise role of these DNA regions in the *EhPgp1* promoter. Furthermore, the identification of the nuclear proteins involved and the knowledge of their expression pattern in sensitive and resistant clones will allow a better understanding of the regulation of the *EhPgp* genes.

Acknowledgments—We thank Dr. García Carranca for providing the oligonucleotides containing the consensus sequences for the transcription factors. We thank Drs. Joseph DiPaolo, Rima Gharaibeh, and Patricio Gariglio for critical readings of the manuscript. We also thank Francisco Paz for technical assistance.

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Transcriptional Analysis of the *EhPgp1* Promoter of *Entamoeba histolytica* Multidrug-resistant Mutant

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J. Biol. Chem. 1998, 273:7277-7284.
doi: 10.1074/jbc.273.13.7277