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Entamoeba histolytica: Functional characterization of the −234 to −196 bp promoter region of the multidrug resistance *EhPgp1* gene

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Abstract

The multidrug resistance *EhPgp1* gene is constitutively expressed in drug resistant trophozoites from *Entamoeba histolytica*. It has been demonstrated that two CCAAT/enhancer binding sites located in the *EhPgp1* gene promoter control its transcriptional activation. However, functional assays of the 5′ end of its promoter showed that region from −234 to −196 bp (38 bp) is also important for the *EhPgp1* gene transcription. Here, we demonstrated that in the 38 bp region putative *cis*-activator sequences are located. In silico analysis showed the presence of GATA1, Gal4, Nit-2, and C/EBP consensus sequences. Additionally, we identified three specific DNA–protein complexes, which were competed by a C/EBP, GATA1, and HOX oligonucleotides. Finally, we partially purified three proteins of 64.4, 56.7, and 27.4 kDa. Further investigations are currently in progress to determine the identity of these nuclear factors and how they are interacting with the *EhPgp1* gene promoter.

Index Descriptors and Abbreviations: CAT, chloramphenicol acetyltransferase; ATG, translation initiation codon; bp, base pair(s)

Keywords: EhPgp1 gene; Transcription; MDR phenotype; Drug resistance; Promoter; Amoebiasis

1. Introduction

Entamoeba histolytica is the protozoan parasite causative of human amoebiasis. The disease is primarily controlled by drug treatment; however drug resistant strains have been isolated from patients with amoebiasis (Hanna et al., 2000), even more drug resistant clones have been generated in the laboratory (Orozco et al., 1985; Prabhu et al., 2000) and different drug resistant mechanisms have been identified in this parasite (Descoteaux et al., 1995). These findings strongly suggest that drug resistance could be occurring in the natural population of the parasite. The multidrug resistance phenotype (MDR) has been identified in *E. histolytica*. It is associated with the overexpression of the *EhPgp1* and *EhPgp5* genes (Orozco et al., 2002), and with the overproduction of a P-glycoprotein (Pgp) encoded by these genes (Bañuelos et al., 2002; Delgadillo et al., 2002). In drug resistant mutants the *EhPgp1* gene is constitutively expressed, while in drug sensitive trophozoites the transcript has not been detected (Descoteaux et al., 1995), suggesting that their expression is regulated at transcriptional level.

Structural and molecular characterization of the *EhPgp1* promoter showed that the core promoter is located at the first −300 bp upstream the transcription initiation site. Additionally, differences in the DNA–protein complexes formation were detected between the nuclear extracts from sensitive and resistant trophozoites,

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suggesting the presence of specific transcription factors involved in the *EhPgp1* gene expression (Gómez et al., 1998). Recently, two functional CCAAT/enhancer binding elements and the proteins interacting with these sites (C/EBP) were identified at −54 to −43 and −198 to −186 bp upstream the transcription initiation site. The results showed that these CCAAT elements participate in the transcriptional activation of *EhPgp1* gene promoter (Marchat et al., 2002). In other organisms gene transcription is regulated by the concerted action of different transcription factors that recognize specific consensus sequences at the promoter and more than one sequence is involved in the transcriptional regulation of a specific gene. A similar mechanism seems to be occurring in the *EhPgp1* gene transcription. In this study, we reported the functional role of a 38 bp promoter region located at −234 to −196 bp that is necessary for the *EhPgp1* gene expression. Our results revealed the formation of three specific DNA–protein complexes within the 38 bp region. Competition assays using C/EBP, GATA1, and HOX specific sequences suggest that factors forming the DNA–protein complexes could have similar binding domains to C/EBP and GATA1 transcription factors. Three interacting transcription factors of 64.4, 56.7, and 27.4 kDa were purified. The identity and biochemical characterization of these proteins are currently under study. The findings obtained in this paper provide new insights into the *EhPgp1* gene transcriptional regulation.

2. Materials and methods

2.1. Entamoeba histolytica cultures

Trophozoites of clone C2 (strain HM1:IMSS) were axenically cultured in TY1-S-33 medium (Diamond et al., 1978).

2.2. Transfection and CAT assays

Transfection assays were carried out by electroporation as described previously (Nickel and Tannich, 1994). Briefly, trophozoites were transfected using 100 μg of the p268Pgp1 (*EhPgp1* core promoter); p206Pgp1 (lacking 53 bp at the 5′-end of the *EhPgp1* core promoter), pBSCATACT (without promoter, as negative control) or pA5′A3′CAT (as positive control) plasmids (Gómez et al., 1998; Marchat et al., 2002). Then, CAT activity was measured by the two phase diffusion assay (Buß et al., 1995) using 100 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. CAT activity was expressed as cpm of the butyrylated derivatives. The background obtained from the trophozoites transfected with the pBSCATACT plasmid was subtracted from the results given by the plasmids containing the different promoter constructions. CAT activity was determined in the linear range of the assay. The efficiency of the transfection experiments was monitored by the results given by the pA5′A3′CAT plasmid.

2.3. Nuclear extracts

Nuclear extracts (NE) were prepared from resistant trophozoites (clone C2) by the Schreiber's protocol (Schreiber et al., 1989) modified by Gómez et al., 1998. Protein concentration was determined by the Bradford method (Bradford, 1976).

2.4. Gel shift assays

Double-stranded oligonucleotides corresponding to the region from −234 to −196 bp of the *EhPgp1* gene core promoter (5′-TATCTGATAAAAATGTTATCTG AAAAAATGTTATCTGA-3′) were annealed by heating single-stranded 5′ and 3′ oligonucleotides in a boiling water bath and gradually cooling to room temperature. The 10 ng of double-stranded oligonucleotides were 5′ end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Gibco-BRL). Specific activity was determined by scintillation counting. Gel shift assays were performed as described previously (Gómez et al., 1998). Briefly, 15 μg of NE were incubated with the end-labelled oligonucleotides (20,000 cpm), 1 μg of poly[d(I–C)] (Amersham-Pharmacia Biotech) and 10% glycerol in DNA–protein binding buffer for 10 min at 4 °C. For cold competition, NE were incubated with a 150-fold molar excess of unlabelled oligonucleotides for 10 min at 4 °C prior to incubation with the labelled probe. As competitors we used a single- and double-stranded oligonucleotides corresponding to the region from −234 to −196 bp of the *EhPgp1* gene core promoter, and a double-stranded oligonucleotides corresponding to the consensus sequences for the GATA1 (5′-GTTGCAGAT AAACATT-3′), HOX (5′-GTAAGAGTTATTAT TGAT-3′), and C/EBP (5′-CTGATGAATTGGAA AAGAAAGA-3′) transcription factors.

2.5. Purification of proteins that bind to −234 to −196 bp region of the EhPgp1 gene core promoter

Entamoeba histolytica nuclear proteins that bind to −234 to −196 bp region of the *EhPgp1* gene core promoter were partially purified under non-denaturing conditions using a DNA binding protein purification kit (Roche Molecular Biochemical) and NE from clone C2 trophozoites. Concatameric polynucleotides were prepared by self-primed PCR technique using double-stranded oligonucleotides and Deep Vent polymerase. The oligomer was coupled to magnetic particles coated

with streptavidin as described by the manufacturer. NE were desalted and concentrated with centricon YM-10 (Millipore Amicon Bioseparations). Fifty micrograms of these NE were mixed with the magnetic particles in the protein binding buffer, poly[d(I–C)] (6.5 μg) and poly-L-lysine (0.65 μg), then were incubated at 4 °C for 60 min. After three washes, the DNA binding proteins were eluted with buffer containing KCl 2.5 M. The fractions were analyzed by 10% SDS–PAGE (Laemmli, 1970). Gels were silver stained.

3. Results and discussion

3.1. Participation of the 5′-end region of the EhPgp1 core promoter in the gene transcriptional activation

To determine the functional role of the region from −234 to −196 bp of the *EhPgp1* promoter in its transcriptional activation, we transfected trophozoites of clone C2 with the p268Pgp1 plasmid, containing the core promoter and with the p206Pgp1 construct lacking the −234 to −196 bp region. The results showed an 88% decrement of the CAT activity when the p206Pgp1 construct was used in comparison to the CAT activity showed by the transfected trophozoites with p268Pgp1 plasmid (Fig. 1). The results suggest that this region contains sequences involved in the transcriptional activation of the *EhPgp1* gene, besides of the two CCAAT enhancer sequences previously reported by our group (Marchat et al., 2002). It has been reported in several genes from different organisms that their transcriptional regulation depends on the combinatorial and synergistic function of *cis*-elements in their promoters, accompanied by the combinatorial mode of action of the *trans*-acting factors that bind to those sites (Payton et al., 2005; Takeshita et al., 2004). A similar regulation mechanism could be occurring in the *EhPgp1* gene expression and could converge through different *cis*-acting elements on the promoter to coordinately regulate the *EhPgp1* gene activation.

The structural analysis of the region from −234 to −196 bp reveals putative consensus sequences for GATA1, Gal4, Nit-2, and C/EBPβ transcription factors that might be participating in the recruitment of transcriptional *EhPgp1* gene activators. These transcription factors have been described as activators in globin, GAL, nitrate reductase, and IL-6 gene promoters from different organisms (Chiang and Marzluf, 1994; Ghirlando and Trainor, 2003; Kang et al., 1993; Xiao et al., 2004). However, in *E. histolytica* we do not know yet if some of them exist in its genome.

3.2. Formation of DNA–protein complexes in the −234 to −196 bp region of the EhPgp1 core promoter with NE of clone C2

The functional assays demonstrated that the region from −234 to −196 bp of the *EhPgp1* core promoter is essential for the *EhPgp1* transcriptional gene activation in *E. histolytica*, independently of the CCAAT/enhancer sequences located at −54 to −43 bp and −198 to −186 bp also involved in the promoter activity (Fig. 1) (Marchat et al., 2002). To identify and characterize the putative transcription factors interacting with this region, we performed gel shift and competition assays using a DNA probe containing 38 bp (−234 to −196 bp) from the *EhPgp1* promoter and NE from the drug resistant trophozoites. Fig. 2 shows the formation of three specific DNA–protein complexes, which were competed by a 150-fold molar excess of unlabelled probe (double- and single-stranded oligonucleotides) (lanes 4–6, respectively), but were not competed by the unspecific poly[d(I–C)] competitor (lane 3). Additionally, the three DNA–protein complexes were also competed with a 150 molar excess of C/EBP double-stranded oligonucleotides (lane 9), suggesting that a C/EBP-like protein has affinity for a specific sequence located within this region.

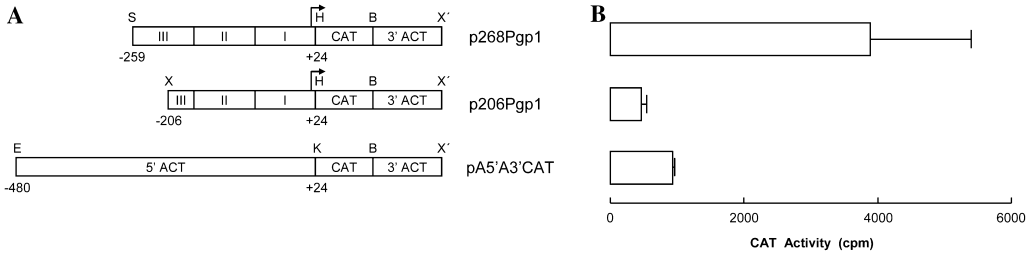
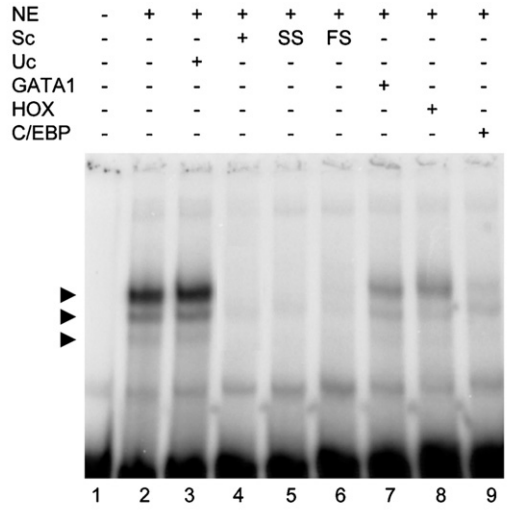


Fig. 1. Functional analysis of the 5′-end region (−234 to −196 bp) of the *EhPgp1* gene core promoter. (A) Schematic representation of the constructs used in the transfection assays. The p268Pgp1 and p206Pgp1 plasmids contain the *EhPgp1* gene core promoter and a deletion of the first 53 bp of the 5′end of this promoter, respectively. The pA5′A3′CAT plasmid contains the *actin* gene promoter. All the plasmids contain the CAT reporter gene and the 3′-flanking *actin* region (3′ACT). Arrows, transcription initiation sites. S, *Sma*I; H, *Hin*dIII; B, *Bam*HI; X′, *Xho*I; X, *Xba*I; E, *Eco*RI; K, *Kpn*I. (B) Bars show the average of CAT activity (cpm) of the transfected plasmids obtained by the two phase diffusion assays ± SD representative of three independent experiments performed in duplicate. The background given by the trophozoites transfected with the pBSCATACT plasmid was subtracted in all experiments. The efficiency of transfection assays was monitored by the results given by the pA5′A3′CAT plasmid.



38 bp oligonucleotide	5′ TATCTGATAAAAATGTTATCTGAAAAAATGTTATCTGA 3′
GATA1	5′ GTTGCAGATAAACATT 3′
HOX	5′ GTAAGAGTTATTATTGAT 3′
C/EBP	5′ CTGATGAATTGGAAAAGAAAGA 3′

Fig. 2. Nuclear protein binding in the region from −234 to −196 bp of the *EhPgp1* gene core promoter. Gel shift assays were performed with 15 μg of NE from drug resistant clone C2 trophozoites, 1 ng of [γ-^{32}P]-radiolabelled double-stranded fragment and different unlabelled competitors (150-fold molar excess). Lane 1, free probe; lane 2, no competitor; lane 3, unspecific competitor (Uc, 350-fold molar excess of poly[d(I–C)]); lane 4, specific competitor (Sc); lanes 5 and 6, unlabelled straight (SS) and forward (FS) single strand specific competitor, respectively; and in lanes 7–9, the double-stranded oligonucleotides for GATA1, HOX, and C/EBP transcription factors were used. Arrowheads, specific complexes formed with NE from clone C2 trophozoites. In the bottom, we show the sequence of the 38 bp oligonucleotide (from −234 to −196 bp region) and the sequence of the GATA1, HOX, and C/EBP oligonucleotides used as competitors.

Complexes formation was also diminished by GATA1 and HOX oligonucleotides (lanes 7 and 8, respectively). HOX oligonucleotide was used as competitor because although we do not detect a HOX consensus sequence in the 38 bp (−234 to −196 bp) of the *EhPgp1* promoter, we previously reported the participation of a HOX-like factor in the formation of a multiprotein complex at the region from −144 to −250 bp of the *EhPgp1* promoter (Gómez et al., 1998). This complex is probably required for the transcriptional regulation of the gene. The results obtained from competition experiments performed in this work support the idea that C/EBP, GATA, and HOX-like proteins would constitute the multiprotein complex formed within this region.

3.3. Partial affinity chromatography purification of nuclear factors that bind to −234 to −196 bp of the EhPgp1 promoter region

To initiate the characterization of factors interacting within the −234 to −196 bp region, we performed an affinity partial purification using a DNA binding protein purification kit (Roche Molecular Biochemical), NE from trophozoites of clone C2 and a concatamerized oligonucleotide containing this region. The purification showed in silver stained gels, three enrichment protein bands of 64.4, 56.7, and 27.4 kDa (Fig. 3, lane 3) that could correspond to three distinct nuclear

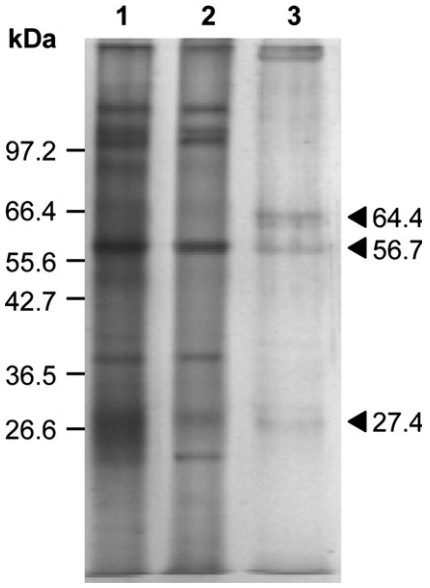


Fig. 3. Purification of the nuclear proteins that bind to the region from −234 to −196 bp. Ten percentage SDS–PAGE of protein fractions obtained from the purification of the nuclear proteins that bind to −234 to −196 bp region. Lane 1, NE (5 μg); lane 2, not retained fraction, and lane 3, purified fraction (pooled from two purifications processes). Arrowheads, silver stained polypeptides.

factors involved in the *EhPgp1* gene transcription. Other minor bands were also detected in some experiments. According to our previous report that suggests

the formation of a multiprotein complex in the *EhPgp1* core promoter, results presented here support this hypothesis due to at least three different nuclear factors interacting within a 38 bp sequence from −234 to −196 bp of *EhPgp1* promoter region. The transcription factors are characterized by highly conserved DNA binding, dimerization, transactivation, and repression motifs, however the molecular weight of these transcription factors identified in different organisms is highly variable. For example, the family of GATA transcription factors includes at least 93 proteins showing a molecular weight range between 109.3 and 30.8 kDa (Lowry and Atchley, 2000). In this work two of the three detected proteins have molecular weights in the range of GATA transcription factors, suggesting in junction with the competition assays that it could be a GATA1-like factor. GATA1 transcription factor has been described as activator of the globin genes in red blood cells (Talbot and Grosveld, 1991). On the other hand competition assays suggested the participation of a C/EBP transcription factor. The molecular weights of C/EBPs from different organisms are in the range of 48–20 kDa (Hurst, 1996). These proteins participate in the transcriptional regulation of genes involved in cellular proliferation and differentiation pathways of hepatocytes and adipocytes (Hurst, 1996; Umek et al., 1991). One of the nuclear proteins purified in this work has a molecular weight of 27.4 kDa, suggesting that it could be a putative C/EBPβ transcription factor. However, further biochemical characterization of these purified proteins and the identification of the precise binding sequences are currently in progress to determine the concerted interplay between the *EhPgp1* DNA binding sequences, their transcription factors, and the protein–protein interactions that allow the *EhPgp1* gene transcription.

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