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Possible Role of the CCAAT/Enhancer Binding Protein in the Expression Regulation of the *EhPgp1* Multidrug Resistance Gene in *Entamoeba histolytica*

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Introduction

Several potential binding sites for transcription factors participating in gene expression regulation have been identified in the 5′-flanking region of the *EhPgp1* gene involved in the multidrug resistance (MDR) phenotype of the emetine-resistant mutant clone C2. To begin the fine characterization of the *cis*-regulatory sequences of the *EhPgp1* gene core promoter, we focused our attention on two highly conserved CCAAT/enhancer binding protein (C/EBP) motifs located at −54 and −196 bp positions at the *EhPgp1* gene promoter. According to the results obtained from electrophoretic mobility shift assays (EMSA), these sites seem to be involved in the expression of this gene (1). The C/EBP family members are important in regulating many eukaryotic genes during differentiation processes. These nuclear proteins have between 150 and 360 amino acids. They present a bipartite DNA-binding domain composed of a positively charged region that contacts the DNA, an amphipathic helix at the conserved carboxy-terminal end that mediates dimerization through the formation of a leucine zipper, and a less well-conserved amino-terminal region containing regulatory and transactivation domains. All C/EBPs share homology in their basic domain and, as a result, recognize the same DNA motif ($T^{T}/_{G}TGG^{T}/_{A}{}^{T}/_{A}{}^{A}/_{T}$), forming homo- or heterodimers with each other (2).

In this report, we present the location and alignment of consensus C/EBP sequences in *mdr* gene promoters of mammals and protozoan parasites. By EMSA, we detected a nuclear factor able to bind the C/EBP sites in *E. histolytica* and propose it as a possible *trans*-regulatory element in MDR phenotype regulation, as described in mammals.

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Materials and Methods

Computer analysis. The 5′ flanking region of *mdr* genes of protozoan parasites were obtained from the GenBank using the following accession numbers: AF010402 (*E. histolytica EhPgp1*); AF010401 (*E. histolytica EhPgp5*); U01056 (*E. histolytica EhPgp6*); PFPFMDR1 (*Plasmodium falciparum pfmdr1*); PFU04640 (*P. falciparum pfmdr2*); LTHCPG (*Leishmania tarentolae ltpgpA*); and LEILDMDR1A (*L. donovani ldmdr1*). C/EBP sites of human *mdr1*, mouse *mdr1a* and *1b*, and hamster *pgp1* were from Combates et al. (3). The location and alignment of C/EBP sequences were performed in silico with the MatInspector and DiAlign programs at http://genomatix.gsf.de (4).

Electrophoretic mobility shift assays. EMSA were performed (1) using nuclear extracts (NE) from emetine-sensitive clone A and emetine-resistant clone C2 trophozoites (strain HM-1:IMSS) axenically cultured in TYI-S-33 medium, and radiolabeled oligonucleotides C/EBPI and C/EBPIII, corresponding to C/EBP binding sites identified in *EhPgp1* gene promoter. As specific competitor, we used 150-fold molar excess of unlabeled probe. Poly[d(I-C)] was used as an unspecific competitor. Mixtures were separated by electrophoresis through nondenaturing 6% polyacrylamide gels and analyzed by autoradiography.

Results and Discussion

The C/EBPI and C/EBPIII binding sites located in the *EhPgp1* gene promoter (Figure 1A) were previously proposed to be involved in the regulation of the MDR phenotype in *E. histolytica.* The C/EBPI site differs from the mammalian consensus sequence by one gap, while the C/EBPIII site differs in one nucleotide (Figure 1B). The analysis of several *mdr* gene promoters from mammals and protozoan parasites for consensus C/EBP binding sites showed that most *mdr* promoters have C/EBP sequences located in a

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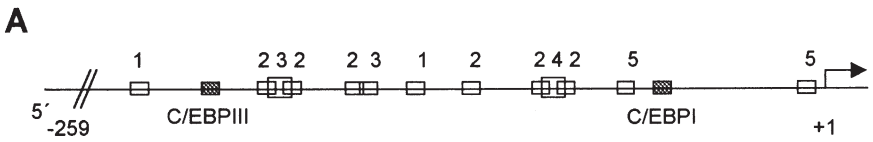

B

C/EBP

Consensus 5′ $T^{T}_{G}TGG-^{TTA}_{AAT}$ 3′

		1 8
EhPgp1	-198	Ga A**TTGG-AAA** GAAa
	-54	TG **TTTGG-TA**G TTc
EhPgp5	-162	TT **TTTGG-AAT** Ca
	-82	aaTT **TTTGG-AAT** Ac
EhPgp6	-51	aG A**TTGG-AT**G TGGG
pfmdr1	-75	TG **TTTG**T-**TA**C AAAt
	-21	TA **TTTG**A-**TTT** TGt
pfmdr2	-104	TT A**TTGG TT**C ATCC
ldmdr1	-268	TC **TTTGG-TGT** GTG
Ha *pgp1*	-94	CCTG **TTTCGCAAT** TGCTC
Hu MDR1	-157	CCTG **TTTCGCAGT** TTCTC
Mu *mdr1a*	-160	TG **TTTCGCAAT** TT
Mu *mdr1b*	-123	TG **TTTCGCAAC** cg
		** *

Figure 1. Identification of C/EBP consensus binding sites in *mdr* gene promoters. (A) Map of the *EhPgp1* gene promoter. Open boxes mark the putative consensus binding sequences for GATA-1 (1), HOX (2), POU (3), PIT-1 (5), and Inr (5). Filled boxes correspond to C/EBP sites. The arrow indicates the transcription initiation site. (B) Alignment of the consensus C/EBP binding site with mammalian and protozoan *mdr* gene promoters. Identical matches with the consensus sequence are in bold. Stars show the conserved nucleotides in all sites.

region spanning about 250 bp upstream from the ATG. All sites present high homology (from 75–100%) with the consensus C/EBP sequence and with the C/EBP sites identified in the *EhPgp1* gene promoter. Nucleotides T, T, and A/T in respective positions 2, 3, and 6 of the consensus sequence are conserved in all sites. The base pair GA (positions 5–6), important for DNA–protein interaction (3), is maintained in almost all C/EBP sequence (Figure 1B). The presence of the C/EBP site in the *mdr* promoters suggests that the regulation of these genes should involve a common mechanism of specific protein interaction with this *cis*-regulatory element.

The nuclear protein that interacts with the C/EBP binding site of the human *mdr1* gene promoter and activates its transcription has been identified as C/EBPβ (or NF-IL6) (3). The C/EBP participates in a multiprotein activator complex binding the C/EBP sequence located in the mouse *mdr1b* promoter (5). We searched for a C/EBP-like protein in *E. histolytica* participating in *EhPgp1* gene regulation. EMSA, using C/EBPI and C/EBPIII probes, showed the formation of specific retarded complexes with NE from sensitive and resistant clones of *E. histolytica* trophozoites. Crosscompetition experiments inhibited its formation, indicating that the same factor bound both sites. This factor was identified as a C/EBP-like protein by supershift assays, because DNA–protein interaction was blocked when a rabbit polyclonal antibody against human C/EBPβ was added. In Western blot experiments, the same antibody recognized a 65-kDa protein in NE from both clones of *E. histolytica.* The similar expression pattern of the C/EBP-like factor in sensitive and resistant clones of *E. histolytica* suggests that the *EhPgp1* gene control probably involves a coordination of the C/EBP-like protein with other differential transcription

factors to form a multiprotein regulator complex. Transfection assays using plasmids containing *EhPgp1* promoter with mutated C/EBP sites showed a significant reduction in promoter activity, indicating the importance of these *cis*-regulatory elements in the MDR phenotype control in *E. histolytica.*

In conclusion, we report here the presence of a C/EBP-like protein in *E. histolytica*, and propose its participation as a *trans*-regulatory factor in MDR phenotype regulation.

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