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Experimental Parasitology 110 (2005) 238–243
Experimental Parasitology
www.elsevier.com/locate/yexpr

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

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Received 18 January 2005; received in revised form 8 March 2005; accepted 9 March 2005
Available online 14 April 2005

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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0014-4894/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2005.03.011

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