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Entamoeba histolytica EhPgp5 transcriptional activation depends on putative emetine response elements

Alma Nieto [a], D. Guillermo Pérez [a], Esther Orozco [b], Francisco Paz [a], Consuelo Gómez [a,*]

[a] *Programa Institucional de Biomedicina Molecular, ENMyH-IPN, Guillermo Massieu Helguera No. 239. Fracc. La Escalera. Ticomán, CP 07320, Mexico, D.F., Mexico*

[b] *Departamento de Patología Experimental, Centro de Investigación y de Estudios Avanzados del IPN, AP 14-740, Mexico, D.F., Mexico*

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Abstract

The multidrug resistance *EhPgp5* gene promoter is active in drug resistant clone C2 trophozoites and its activity increases when trophozoites are cultured in the presence of emetine, suggesting that the *EhPgp5* gene shows an inducible drug dependent mechanism. We analyzed different promoter fragments to detect those regions that activate transcription in the presence of emetine. Trophozoites were transfected with p375Pgp5, p259Pgp5, p187Pgp5, and p76Pgp5 plasmids and incubated with different emetine concentrations. p375Pgp5 and p259Pgp5 plasmids were able to drive CAT expression in A and C2 trophozoites only in the presence of emetine. CAT activity was turned off in the absence of drug. Interestingly, no CAT activity was detected in the presence or in the absence of emetine with p187Pgp5 plasmid in which 59 bp were deleted at the 5′ end of the *EhPgp5* minimal promoter (p259Pgp5). These results suggest that the overexpression of the *EhPgp5* gene is a consequence of transcriptional activation of the gene promoter by putative drug responsive elements, located within the −111 to −170 bp of the transcription initiation site.

Index Descriptors and Abbreviations: Transcription; MDR, multidrug resistance phenotype; CAT, chloramphenicol acetyltransferase

1. Introduction

Drug resistance has emerged as a problem in the treatment and control of many infectious diseases produced by protozoan parasites (Cowman et al., 1994). Due to the health impact of these diseases, especially in developing countries, the interest in the identification of mechanisms involved in drug resistance, and the design of new antiparasitic drugs has been growing (Ullman, 1995). In *Entamoeba histolytica* the multidrug resistance (MDR) phenotype shows similar characteristics to the MDR phenotypes identified in mammalian cells, and other parasites such as *Plasmodium falciparum* and *Leishmania* (Cortes-Selva et al., 2004; Rajagopal and Simon, 2003; Rubio and Cowman, 1996). The MDR phenotype in amoeba is a consequence of an increased expression of P-glycoprotein, due mainly to the overexpression of *EhPgp1* and *EhPgp5* genes (Descoteaux et al., 1995). The *EhPgp1* gene is constitutively expressed in drug resistant trophozoites of clone C2, while the *EhPgp5* gene is overexpressed in C2 trophozoites grown in the presence of emetine. The differential expression pattern of both genes suggest that exist a finely regulated mechanisms mainly controlled at transcriptional level.

However, recent work reported that the *EhPgp5* mRNA stability is also increased in the trophozoites growing at high emetine concentrations, indicating that, although the transcriptional regulation seems to be the major control point, other mechanisms such as the mRNA stability or the DNA amplification are also regulating the MDR phenotype (López-Camarillo et al., 2003; López et al., 1997).

* Corresponding author. Fax: +52 55 5729 6300x55532.
E-mail address: cgomezg@ipn.mx (C. Gómez).

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The *EhPgp5* gene is only transcribed in the presence of emetine in drug resistant trophozoites (clone C2) and the transcript level increases according to the emetine concentration in the culture medium (Descoteaux et al., 1995). These findings show that the *EhPgp5* gene presents an emetine inducible gene expression pattern. Transcriptional analysis of the *EhPgp5* promoter demonstrated that minimal promoter activity was kept by the −235 bp upstream the transcription initiation site. The results also showed that this activity was higher in C2(40) (trophozoites growing for several months in the presence of 40 μM of emetine) than in C2 trophozoites, suggesting that drug responsive elements are located within this region (Pérez et al., 1998).

In this paper, we detected functional drug responsive elements between −111 to −170 bp of the *EhPgp5* transcription start site. Experiments currently in progress will allow us to identify the precise sequence of these elements as well as the biochemical characterization of transcriptional factors interacting with it.

2. Materials and methods

2.1. Entamoeba histolytica cultures

Trophozoites of emetine sensitive (A) and resistant (C2) clones were axenically cultured in TYI-S-33 medium supplemented with 15% fetal bovine serum (Biofluids), 6% vitamins (JRH, Biosciences), and antibiotics (Lakeside) (Diamond et al., 1978). Exponential trophozoites cultures were harvested by ice-chilling and centrifugation at 150*g* during 5 min at 4 °C.

2.2. Plasmid constructions

Three serial deletions of the minimal promoter were done by PCR DNA amplification of different fragments (360, 140, and 79 bp) using the p1108Pgp5 plasmid as template (Pérez et al., 1998). As primers we used sense EhPgp375-S31 (5′-AAAACTGCAGATAAAATGAT GATAAAGAATA-3′), EhPgp187-S18 (5′-ATGAAGA AAATGGATAAT-3′), and EhPgp76-S18 (5′-CTCA AACTTTCTAAATTC5 3′) oligonucleotides. The antisense *EhPgp5*-AS36 (5′-CCCAAGCTTGTATGCT GGTTCACTTGTCATGCGAAT-3′) oligonucleotide was used for all constructs. These fragments contain 330, 110, and 49 bp of the *EhPgp5* promoter and 30 bp of the *EhPgp5* coding region. The 360 bp fragment was cloned into *Pst*I and *Hin*dIII sites (p375Pgp5), while the 140 and 79 bp fragments were cloned into the *Sma*I and *Hin*dIII sites (p187Pgp5 and p76Pgp5, respectively) in front of the CAT reporter gene of pBSCATACT vector (Gómez et al., 1998). p259Pgp5 (Pérez et al., 1998) and pA5′A3′CAT (Nickel and Tannich, 1994) vectors contain the minimal promoter of the *EhPgp5* and the *actin* genes, respectively. Both plasmids were used as positive controls in all the experiments. pBSCATACT was used as negative control. The orientation and sequence of each construct was confirmed by DNA sequence analysis (Sanger et al., 1977).

2.3. Transfection and CAT assays

Transfection assays were carried out by the electroporation method as described (Nickel and Tannich, 1994). Briefly, 10^6 trophozoites were transfected with 100 μg of p259Pgp5, p375Pgp5, p187Pgp5, p76Pgp5, pA5′A3′CAT or pBSCATACT constructs. 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.

Two hours after electroporation, we added to the culture medium 8 or 12 μM emetine to trophozoites of clone A and 20 or 40 μM emetine to trophozoites of clone C2. CAT activity was determined by the two phase diffusion assays (Buss et al., 1995) using 100 μg trophozoite extracts and 200 μl 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 (Bradford, 1976). CAT activities were expressed as cpm of the butyrylated derivatives. The background given by pBSCATACT was subtracted from the results obtained with the other constructions. CAT activities were determined in the linear range of the assay, representing three independent experiments performed in duplicate.

2.4. Viability assays of transfected trophozoites

Transfected trophozoites of clones A and C2 were incubated in the presence of 8 or 12 μM and 20 or 40 μM emetine, respectively, for 48 h. As a control, the viability of transfected trophozoites was measured immediately after electroporation. Viability of trophozoites was determined by Trypan blue dye exclusion using an inverted microscopy (Zeiss) and a Neubauer chamber.

3. Results and discussion

3.1. EhPgp5 gene promoter activity is induce by emetine in the wild type clone A and in the resistant clone C2

The multidrug resistance phenotype in *E. histolytica* is produced by the overexpression of the *EhPgp* genes. In contrast to the constitutively expressed *EhPgp1* gene, the *EhPgp5* gene expression is induced by the presence of

emetine in the medium only in the drug resistant clone C2 (Bañuelos et al., 2002; Descoteaux et al., 1995). Transcriptional analysis of the *EhPgp5* gene demonstrated that in both drug sensitive and drug resistant clones, the promoter sequence is similar and the core region is located at −235 bp of the transcriptional initiation site (Pérez et al., 1998). Additionally, these results also showed that the promoter increased its activity in trophozoites grown in the presence of drug (clone C2 cultured with 40 μM emetine), whereas no activity was detected in trophozoites from clone A (Pérez et al., 1998).

The CAT activity in these experiments was measured after 16 h incubation of the trophozoite extracts with the substrate, because when CAT assays were done after 2 h incubation, butyrylated chloramphenicol forms were poorly detected. On the other hand, although the promoter activation was consistent with the *EhPgp5* gene expression, the magnitude of CAT activation does not clearly correlate with the results obtained by the Northern blot and RT-PCR assays (Bañuelos et al., 2002; Descoteaux et al., 1995). To determine the promoter regions involved in the emetine inductor effect and to define the conditions to enhance the promoter activation, we transfected trophozoites from clones A and C2 with the p375Pgp5, p259Pgp5, p187Pgp5 or p76Pgp5 plasmids containing different *EhPgp5* promoter regions. Two hours after transfection, the trophozoites were incubated with 8 or 12 μM, and 20 or 40 μM emetine for clones A and C2, respectively. We used these emetine concentrations, because transfected trophozoites were very fragile in higher drug concentrations and most of them died after emetine incubation. It is important to point out that the conditions used in this paper to evaluate the promoter activity in response to emetine induction differ to those standardized to measure the *EhPgp5* promoter activity (Pérez et al., 1998). Pérez and colleagues evaluated the CAT expression in a resistant clone cultured permanently in the presence of 40 μM emetine, but after electroporation the transfected trophozoites were incubated in the absence of drug. The results showed that in trophozoites of clone A (Fig. 1), the promoter remains inactive in the absence of emetine, independently of the plasmid construction used. However, in the presence of 8 or 12 μM emetine, p375Pgp5 and p259Pgp5 plasmids were able to drive CAT expression. CAT activity displayed by trophozoites transfected with both constructs showed no significant differences after emetine induction. The deletion of 59 bp at the 5′ end from −111 to −170 bp (p187Pgp5) of the *EhPgp5* promoter abolished CAT expression and the promoter responsiveness to drug (Fig. 1). These results suggest that the putative drug responsive elements are located within −111 to −170 bp of the *EhPgp5* minimal promoter. Additionally, we demonstrated that in the trophozoites of the wild type clone A, the promoter is able to turn on the *EhPgp5* gene expression in the presence of 8 or 12 μM emetine.

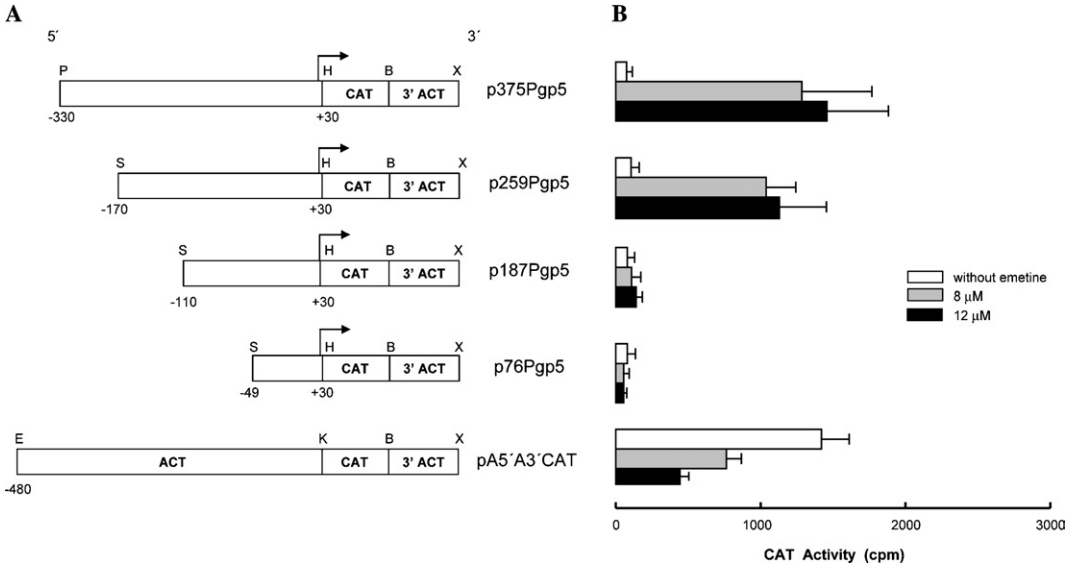

Fig. 1. Functional analysis of different deletions of the *EhPgp5* gene promoter in drug sensitive trophozoites from clone A. (A) Schematic representation of the constructions p375Pgp5, p259Pgp5, p187Pgp5, and p76Pgp5 containing different deletions of the *EhPgp5* gene promoter. All the plasmids contain +30 bp downstream from the ATG of the *EhPgp5* gene, the CAT reporter gene and the 3′-flanking *actin* region (3′ACT). The positive control used was the pA5′A3′CAT plasmid, it contains a 480 bp of the *actin* gene promoter. Restriction sites are: P, *Pst*I; H, *Hin*dIII; B, *Bam*HI; X, *Xho*I; S, *Sma*I; E, *Eco*RI; and K, *Kpn*I. Arrows, transcription initiation site. (B) CAT activity (cpm) of the trophozoites from clone A transfected with the constructs shown in (A). 8 and 12 μM of emetine were added to the culture medium after transfection of the trophozoites. Bars, the average of the CAT activities ± SD representative of three independent experiments performed in duplicate. The background given by the trophozoites transfected with the pBSCATACT was subtracted in all experiments.

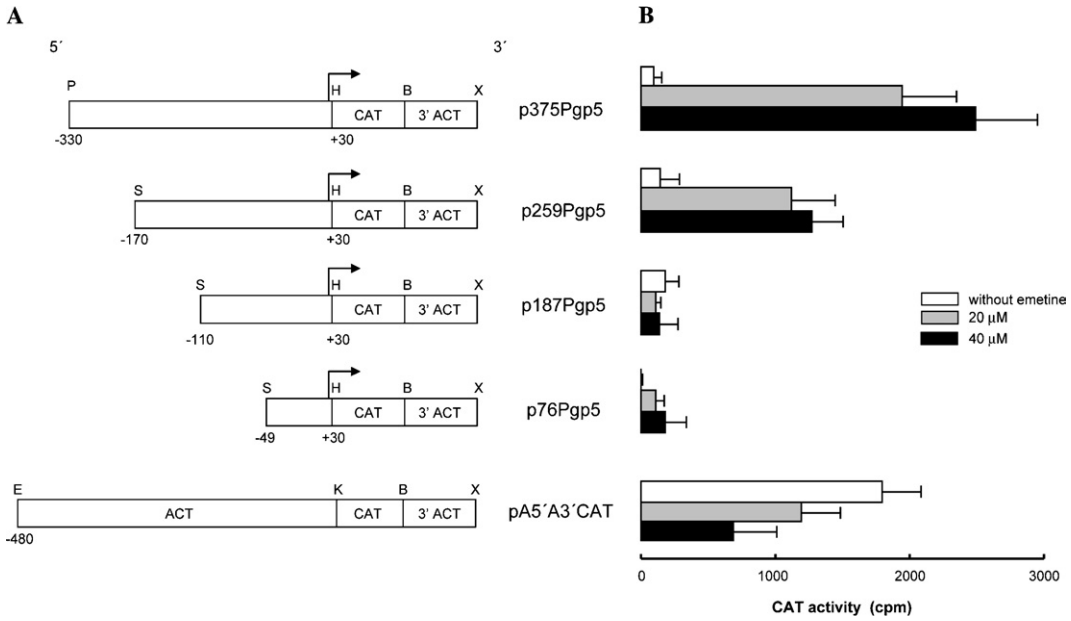

Fig. 2. Functional analysis of different deletions of the *EhPgp5* gene promoter in drug resistant trophozoites from clone C2. (A) Constructs with different deletions in the *EhPgp5* gene promoter as described in Fig. 1 used for transfection assays. Restriction sites are: P, *Pst*I; H, *Hin*dIII; B, *Bam*HI; X, *Xho*I; S, *Sma*I; E, *Eco*RI, and K, *Kpn*I. Arrows, transcription initiation site. (B) CAT activity (cpm) of the trophozoites from clone C2 transfected with the constructs showed in (A). After transfections 20 and 40 μM of emetine were added to the culture medium. Bars, the average of the CAT activities ± SD representative of three independent experiments performed in duplicate. The background given by the trophozoites transfected with the pBSCATACT was subtracted in all experiments.

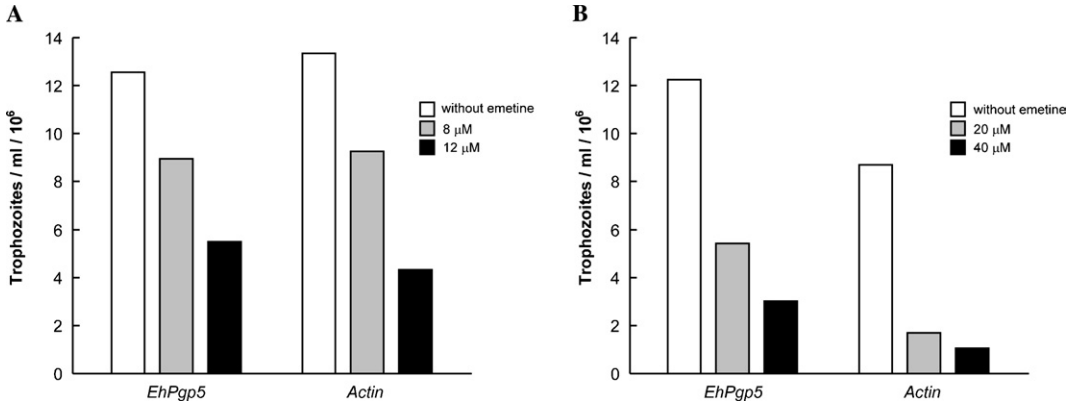

Fig. 3. Viability of the trophozoites transfected with the p259Pgp5 and pA5′A3′CAT plasmids in the presence of emetine. Trophozoites from clones A (A) or C2 (B) were transfected with p259Pgp5 and pA5′A3′CAT plasmids. Then parasites were cultured without or with emetine by 48 h after electroporation, at the end parasite viability was determined by Trypan blue dye exclusion and the trophozoite number was determined.

In the drug resistant clone C2, CAT expression was also induced in trophozoites transfected with p375Pgp5 and p259Pgp5 plasmids only when electroporated trophozoites were cultured in the presence of 20 or 40 μM emetine, while the promoter remains turned off in the absence of drug (Fig. 2). CAT activity diminished 51% (from 2500 to 1200 cpm) when the 159 bp 5′ end region of the p375Pgp5 construct was deleted (p259Pgp5) and no activity was detected when the 220 and 281 bp 5′ end regions were removed (p187Pgp5 and p76Pgp5). No significant CAT activity differences were detected when trophozoites were incubated in the presence of 20 or 40 μM emetine (Fig. 2).

3.2. Viability of the trophozoites transfected with the p259Pgp5 and pA5′A3′CAT plasmids in the presence of emetine

To demonstrate that the inducible effect of emetine is specific for the activation of *EhPgp5* gene promoter, we determine the viability of trophozoites from clones A and C2 transfected with the p259Pgp5 or pA5′A3′CAT plasmids. Figs. 3A and B showed that after electroporation, trophozoites cultured in the presence of 8 or 12 and 20 or 40 μM emetine for clones A and C2, respectively, displayed a similar survival pattern, independently of the plasmid used. These results in conjunction with the CAT

activity presented by *EhPgp5* and *actin* promoters (Figs. 1 and 2), suggest that the drug induction is specific for *EhPgp5* promoter because although the viability of trophozoites transfected with both plasmids was almost the same, the CAT activity generated by the *EhPgp5* promoter increased. While the CAT activity produced by the *actin* promoter tend to reduce, probably because the trophozoites died.

The results presented in this paper strongly suggest that: (i) *EhPgp5* overexpression is mainly a consequence of transcriptional promoter activation induced by the drug; (ii) the overexpression of the P-glycoprotein could occur in *E. histolytica* sensitive clones; (iii) the putative drug responsive elements are located within the −111 to −170 bp of the transcription initiation site; and (iv) a period of at least 48 h of trophozoites incubation in the presence of emetine is sufficient for *EhPgp5* promoter induction. Experiments currently in progress will allow us to define the precise sequence of the emetine response elements. Additionally gel shift, cloning, and expression assays will permit us to identify and characterize transcriptional factors interacting with the drug response elements.

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