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Two CCAAT/enhancer binding protein sites are *cis*-activator elements of the *Entamoeba histolytica EhPgp1* (*mdr*-like) gene expression

Laurence A. Marchat,[1] Consuelo Gómez,[1] D. Guillermo Pérez,[1] Francisco Paz,[1] Leobardo Mendoza[2] and Esther Orozco[2]*

[1]*Programa de Biomedicina Molecular, ENMYH-IPN, Guillermo Massieu Helguera 239 Fracc. 'La Escalera', Ticoman, CP 07320 México D. F.*

[2]*Departamento de Patología Experimental, CINVESTAV IPN, AP 14–470, 07300 México D. F.*

Summary

Here, we show the relevance of promoter regions (–74 to +24, –167 to –75 and –259 to –168 bp) in the transcriptional activation of the multidrug resistance gene *EhPgp1* in *Entamoeba histolytica*, using mutated plasmids and transfection assays. We also demonstrate that both CCAAT/enhancer binding protein sites (–54 to –43 bp and –198 to –186 bp) are *cis*-activating elements of gene expression in the drug-resistant (clone C2) and -sensitive (clone A) trophozoites. Nuclear proteins from trophozoites of both clones and C/EBP sequences of the core promoter formed specific complexes, which were abolished by anti-human C/EBPβ antibodies. UV cross-linking and Western blot assays revealed 25 and 65 kDa bands in urea treated and untreated proteins respectively. The nuclear factors that bind to C/EBP sites were semi-purified by affinity chromatography. They were immunodetected by anti-human C/EBPβ antibodies and formed a specific complex with the C/EBP probe. The antibodies recognized proteins in the cytoplasm, nucleus and EhkO organelles in immunofluorescence and confocal microscopy experiments. Based on our results, we propose that the C/EBP site at –54 bp stabilizes the transcription pre-initiation complex, whereas the other site at –198 bp may be involved in the formation of a multiprotein complex, which provokes DNA folding and promotes the *EhPgp1* gene transcription.

Received 9 February, 2001; revised 18 June, 2002; accepted 18 June, 2002. *For correspondence. E-mail esther@mail.cinvestav.mx; Tel. (+52) 55 5747–3800, ext. 5650; Fax (+52) 55 5747–7108.

Introduction

Entamoeba histolytica, the protozoan responsible for human amoebiasis, presents the multidrug resistance (MDR) phenotype (Ayala *et al*., 1990; Orozco *et al*., 1999) described first in mammalian cells (Dano, 1973; Higgins, 1993) and subsequently in several protozoan parasites (Foote *et al*., 1989; Ouellette and Papadopoulou, 1993; Upcroft and Upcroft, 1993; Cowman *et al*., 1994; Dallagiovanna *et al*., 1994; Johnson *et al*., 1994; Katakura *et al*., 1999). The MDR phenotype is associated with the overproduction of a ≈ 175 kDa surface glycoprotein (Pgp), an energy-dependent pump that maintains intracellular drug concentration below cytotoxic levels (Endicott and Ling, 1989; Loo and Clarke, 1999). *Entamoeba histolytica* has at least four *mdr*-like genes whose predicted products present 11–41% identity to those of mammals, *Plasmodium* and *Leishmania* Pgps (Descoteaux *et al*., 1992; 1995). Genes *EhPgp1*, *EhPgp5* and *EhPgp6* are expressed in drug-resistant mutants, whereas the *EhPgp2* transcript has not been detected. Trophozoites of the emetine-resistant clone C2 constitutively express the *EhPgp1* gene, whereas *EhPgp5* gene transcription is induced by the presence of emetine in the medium (Descoteaux *et al*., 1995; Orozco *et al*., 1995). Structural and functional characterization of promoters indicated that transcriptional regulation plays an important role in the mechanisms underlying the expression of both genes in the drug-resistant cells (Gómez *et al*., 1998; Pérez *et al*., 1998).

Several *cis*-regulatory elements involved in gene expression have been identified in *E. histolytica* genes promoters (Buβ *et al*., 1995; Purdy *et al*., 1996; Hidalgo and Orozco, 1997; Singh and Rogers, 1998). However, very few transcription factors have been characterized in this parasite. The *E. histolytica* TATA-box binding protein (EhTBP) was cloned (Hernández *et al*., 1997; Luna-Arias *et al*., 1999) and antibodies raised against the recombinant protein located the EhTBP in the nucleus and in the kinetoplast-like organelle (EhkO) (Orozco *et al*., 1997) of the trophozoites, suggesting that transcription occurs in both DNA-containing organelles. The EhEBP1 and EhEBP2 proteins, and the URE-3 binding protein, which bind to the URE4 and URE3 sites of the *hgl5* gene pro-

moter, respectively, were recently reported (Gilchrist *et al*., 2001; Schaenman *et al*., 2001).

Gómez *et al*. (1998) showed the presence of putative regulatory domains in the *EhPgp1* gene core promoter (GenBank™/EBI databank accession number AF010402) and proposed that the specific binding of proteins may control gene expression in resistant and sensitive trophozoites. Electromobility shift assays (EMSA) using promoter fragments showed that *E. histolytica* nuclear extracts (NE) bind to two CCAAT/enhancer binding protein (C/EBP) sites of the *EhPgp1* gene core promoter in trophozoites of clone C2 (Gómez *et al*., 1998). C/EBP is a transcriptional activator in mammalian genes (McKnight, 1992) and C/EBP binding sites are also present in the human *MDR1* (Combates *et al*., 1994) and mouse *mdr1b* promoters (Yu *et al*., 1995). Additionally, several *mdr* gene promoters from protozoan parasites have C/EBP sites, suggesting that, as in mammals, their transcription control involves the binding of proteins to C/EBP sites (Marchat *et al*., 2000).

Here, we studied the fine regulation of the *EhPgp1* gene expression, investigating the role of both C/EBP binding sites (C/EBPI site: –54 to –43 bp and C/EBPIII site: –198 to –186 bp) acting as *cis*-activator elements. Using our data and previous studies (Gómez *et al*., 1998), we propose some events to explain the *EhPgp1* gene transcriptional activation.

Results

Regions I, II and III are essential for the EhPgp1 *gene core promoter activity*

To study the relevant segments of the *EhPgp1* gene core promoter (259 bp), we divided it into three regions and constructed several plasmids for transfection assays (Fig. 1A and B). (i) Region I (–74 to +24 bp) has no typical TATA box, it has two putative Inr sequences probably involved in recruiting the transcription pre-initiation complex (TPC), and the C/EBPI consensus binding sequence (–54 to –43 bp). Region I forms a DNA–protein complex only with NE from drug-resistant trophozoites of clone C2, which is competed by unlabelled C/EBP$^{\alpha}$ oligonucleotide (Gómez *et al*., 1998). (ii) Region II (–167 to –75 bp) forms a DNA-protein complex with NE from trophozoites of clone C2, which is competed by an OCT oligonucleotide (Gómez *et al*., 1998). (iii) Region III (–259 to –168 bp) has HOX (–177 to –172 bp and –180 to –176 bp), POU (–181 to –175 bp), C/EBPIII (–198 to –186 bp) and GATA-1 (–228 to –223 bp) consensus sequences and forms a DNA–multiprotein complex, only with NE from trophozoites of clone C2, which is competed by C/EBP$^{\beta}$, GATA-1, OCT and HOX oligonucleotides (Gómez *et al*., 1998).

First, we transfected the trophozoites with the p268Pgp1 plasmid containing the three regions of the *EhPgp1* gene core promoter. CAT activity was determined by the two-phase diffusion assay incubating trophozoites extracts with chloramphenicol and [^{14}C]-butyryl-CoA. In agreement with our previous results, this plasmid drove the chloramphenicol acetyl transferase (CAT) gene expression efficiently in trophozoites of clone C2, as the butyrylated products corresponded to 5255 ± 359 cpm, whereas the trophozoites of clone A showed only 1183 ± 376 cpm (Fig. 1B and C). Then, we used different constructions lacking one or two regions of the promoter to transfect trophozoites of both clones. Trophozoites of clone C2 were transfected with plasmids p167Pgp1 (without region III), p74Pgp1 (containing only region I), ΔII-p268Pgp1 (which lacks region II), ΔI-p268Pgp1 (without region I) and p268Pgp1-IIM (with region II replaced by an irrelevant sequence). All constructions presented a high reduction (88–100%) of CAT activity, in relation to the one showed by the p268Pgp1 plasmid (Fig. 1B and C). Interestingly, CAT activity was maintained in 57% when trophozoites of clone C2 were transfected with the p268Pgp1-IIIR plasmid containing the inverted region III (Fig. 1B and C). Trophozoites of clone A transfected with p167Pgp1, p74Pgp1 and ΔI-p268Pgp1 plasmids also showed a high reduction (80–100%) of its low CAT activity, whereas activity was only 33% reduced with ΔII-p268Pgp1, p268Pgp1-IIM and p268Pgp1-IIIR constructs (Fig. 1B and C). Trophozoites of clones C2 and A gave similar CAT activity when they were transfected with the control pA5′A3′CAT containing the actin promoter. Our results prove that the three regions are important to keep *EhPgp1* gene core promoter activity in trophozoites of both clones. They also suggested that position and orientation of consensus binding sequences forming the previously described DNA–multiprotein complex at region III are relevant for full promoter activity.

C/EBPI and C/EBPIII sites are cis*-activator elements of the* EhPgp1 *gene core promoter*

We studied the role of C/EBPI (–54 to –43 bp) and C/EBPIII (–198 to –186 bp) sites as *cis*-regulatory elements of the *EhPgp1* gene core promoter using constructions with mutations in C/EBPI (p268Pgp1-Im plasmid), C/EBPIII (p268Pgp1-IIIm plasmid) or in both sites (p268Pgp1-Im-IIIm plasmid) (Fig. 2A). No significant CAT activity was detected in trophozoites of clones C2 and A transfected with these plasmids (Fig. 2A and B). To investigate the role of sequences located upstream the C/EBPIII site, we transfected trophozoites with the p206Pgp1 plasmid, which lacks 53 bp at the 5′ end of the promoter, but keeps intact the C/EBPIII site. Results show that this region is also relevant for promoter activity in

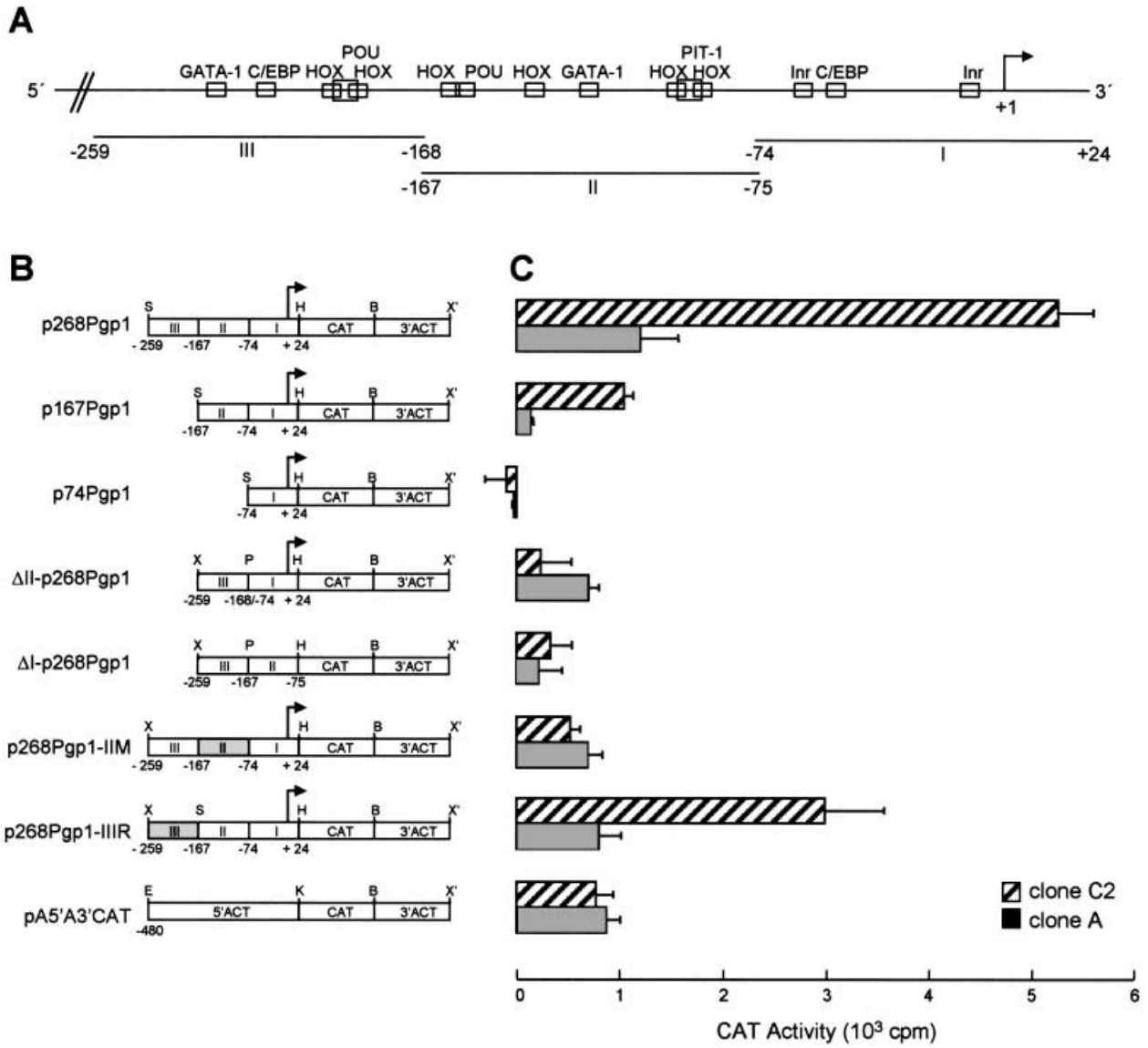

Fig. 1. Functional analysis of regions I, II and III of the *EhPgp1* gene core promoter.
A. Schematic representation of the *EhPgp1* gene core promoter cloned in the p268Pgp1 plasmid. Boxes, putative consensus binding sites. Arrow, transcription initiation site (+1). Regions I, II and III are shown at the bottom. Numbers indicate the bases pairs at the 5′ and 3′ ends of each region.
B. Constructions with different deletions in the *EhPgp1* gene core promoter used for transfection assays. All plasmids contain +24 bp downstream from the ATG of the *EhPgp1* gene (except the pA5′A3′CAT plasmid), the CAT reporter gene and the 3′-flanking *actin* region (3′ACT). The p268Pgp1 plasmid contains the 259 bp of the *EhPgp1* gene core promoter. The pA5′A3′CAT plasmid contains a 480 bp fragment of the *actin* gene promoter. Arrows, transcription initiation sites. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I; X, *Xba*I; X′, *Xho*I.
C. CAT activity (cpm) of the trophozoites transfected with the corresponding plasmids. Bars, average of CAT activities ±S.D. representative of three independent experiments performed in duplicate. The efficiency of transfection assays in both clones was monitored by the results given by the pA5′A3′CAT plasmid. The background given by the trophozoites transfected with the pBSCAT-ACT plasmid was subtracted in all experiments.

trophozoites of both clones, as CAT expression was less than 7% of the activity showed by the p268Pgp1 plasmid (Fig. 2A and B). As expected, trophozoites transfected with plasmids p185Pgp1 (without GATA-1 and C/EBPIII sites), p185Pgp1-Im (without GATA-1 and C/EBPIII sites, and with C/EBPI site mutated) and p206Pgp1-Im (without GATA-1 site and with C/EBPI site mutated) presented no significant CAT activity (Fig. 2A and B). These results show that C/EBPI and C/EBPIII sites are fundamental sequences for the *EhPgp1* gene promoter activity in trophozoites of both clones. They also show that the last 53 bp at the 5′ end, which contain the putative GATA-1 site and other sequences, play a relevant role in the activation of *EhPgp1* gene transcription, probably because

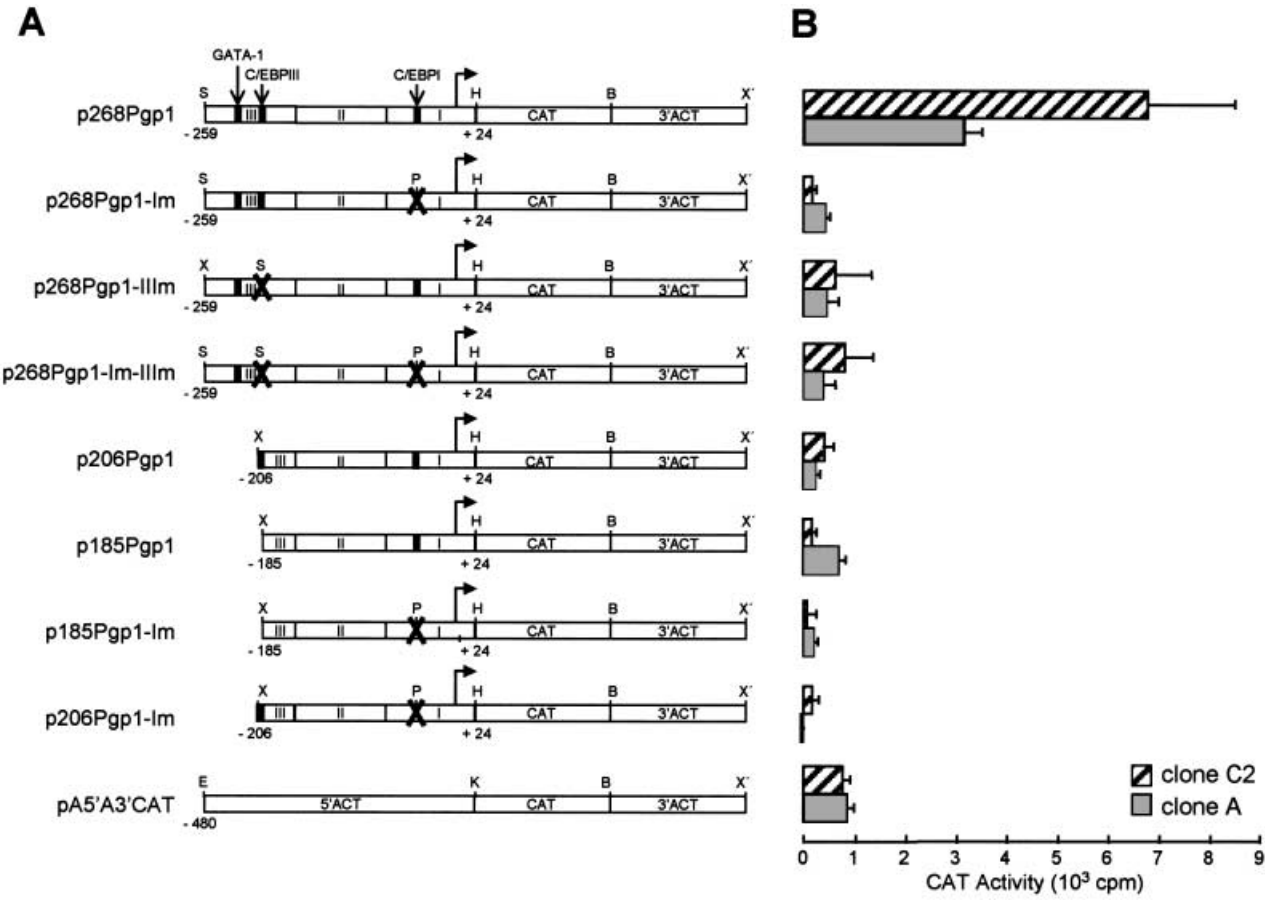

Fig. 2. Functional analysis the 5′ end region and the C/EBP binding sequences of the *EhPgp1* gene core promoter.
A. Constructs with different mutations and deletions in the *EhPgp1* gene core promoter used for transfection assays. X, C/EBP site mutated. Arrows, transcription initiation site. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I; X, *Xba*I; X', *Xho*I.
B. CAT activity (cpm) of the trophozoites transfected with the corresponding plasmids. Bars, average of CAT activities ± S.D. representative of three independent experiments performed in duplicate. The efficiency of transfection assays in both clones was monitored by the results given by the pA5'A3'CAT plasmid. The background given by trophozoites transfected with the pBSCAT-ACT plasmid was subtracted in all experiments.

this region recruits proteins to form the DNA-multiprotein complex previously reported in trophozoites of clone C2 (Gómez *et al*., 1998).

Oligonucleotides containing C/EBPI and C/EBPIII sequences of the EhPgp1 *gene core promoter form specific complexes with trophozoites NE of clones C2 and A*

To demonstrate that both C/EBP sequences of the *EhPgp1* gene core promoter indeed bind to trophozoites nuclear proteins, we carried out EMSA using the 5′-TTAG**TGTTTGGTAGTT**CAAG-3′ and 5′-CTGAT**GAAT TGGAAAGAA**AGA-3′ oligonucleotides, corresponding to the C/EBPI and C/EBPIII sites (bold) respectively (Fig. 3A). C/EBPI oligonucleotide formed a specific DNA–protein complex with NE from trophozoites of clones C2 and A, which was competed by a 150-fold molar excess of unlabelled probe, but not by the unspecific poly[d(I-C)] competitor (Fig. 3B). Complex formation was inhibited by unlabelled C/EBPIII oligonucleotide, suggesting that similar proteins are able to bind to both C/EBP sequences in both clones (Fig. 3B, lanes 4). Complexes were not competed by CBIm and CBIIIm mutated oligonucleotides, in which 6 out of 7, and 7 out of 8 bp of the DNA binding site, were changed respectively (Fig. 3A and C). As reported before (Gómez *et al*., 1998), the region I of the *EhPgp1* gene core promoter formed a specific complex exclusively with NE from trophozoites of clone C2, which was competed by C/EBP$^{\alpha}$ oligonucleotide, whereas TATA-box and NF1 oligonucleotides had no effect. This complex was not detected with NE from clone A trophozoites (Fig. 3D and E). These results pointed out the importance of other detected and undetected DNA–protein interac-

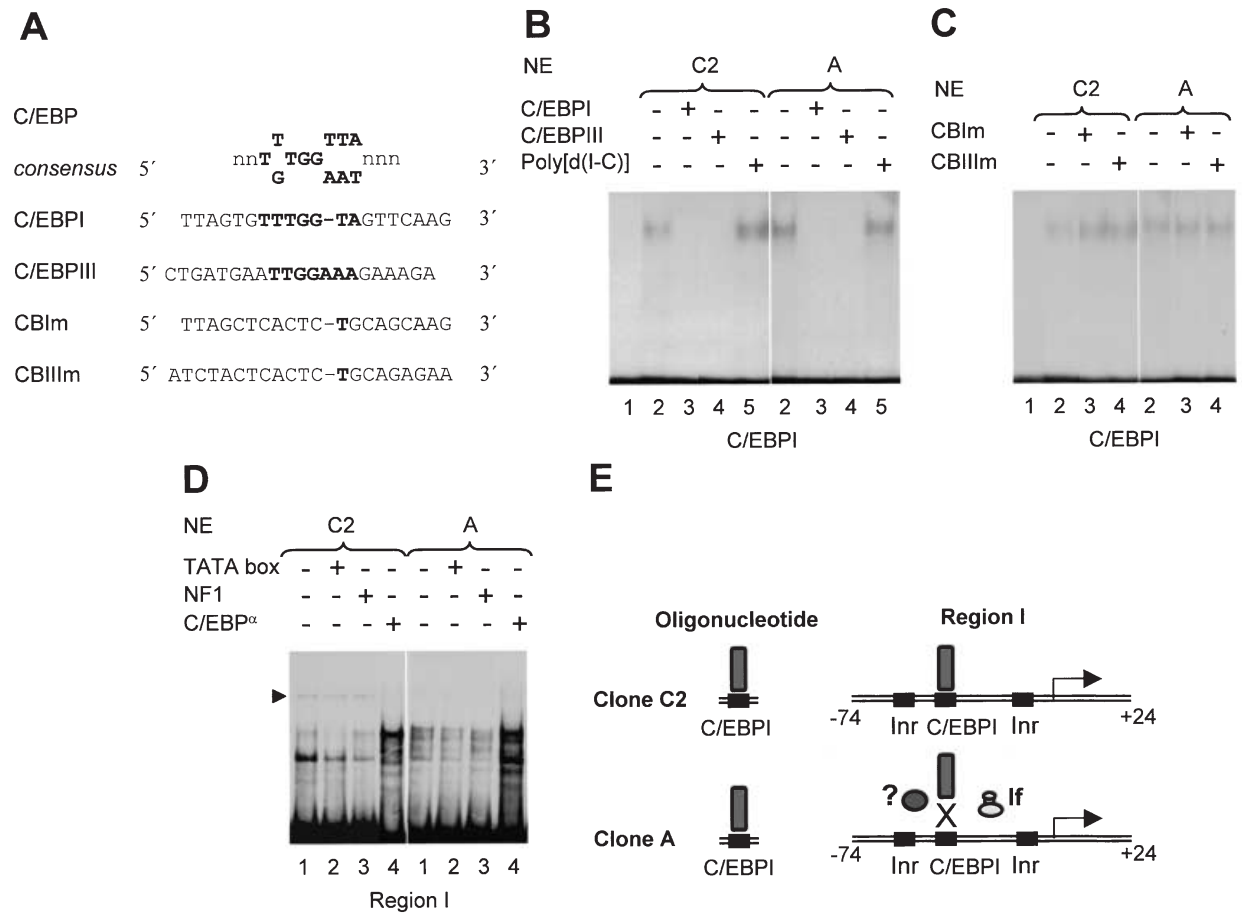

Fig. 3. Nuclear proteins binding to C/EBPI DNA sequence.
A. Sequence of the C/EBPI, C/EBPIII, CBIm and CBIIIm oligonucleotides compared with the mammalian C/EBP sequence (consensus). Boldface, bases that are identical in the oligonucleotides. n, any nucleotide.
B. EMSA were performed with 20 μg of NE from clones C2 and A trophozoites, 1 ng of [γ-^{32}P]-ATP radiolabelled C/EBPI oligonucleotide and different unlabelled competitors (150-fold molar excess). Lane 1, free probe; lanes 2, no competitor; lanes 3, C/EBPI oligonucleotide; lanes 4, C/EBPIII oligonucleotide; lanes 5, poly[d(I-C)].
C. EMSA were performed as described in (B) using the [γ-^{32}P]-ATP-radiolabelled C/EBPI as a probe, and unlabelled mutated C/EBP oligonucleotides as competitors (150-fold molar excess). Lane 1, free probe; lane 2, no competitor; lane 3, CBIm oligonucleotide; lane 4, CBIIIm oligonucleotide.
D. EMSA were performed as described in (B) using 1 ng of [α-^{32}P]-dATP radiolabelled region I as a probe, and different unlabelled competitors (150-fold molar excess). Lane 1, no competitor; lane 2, TATA box oligonucleotide; lane 3, NF1 oligonucleotide; lane 4, C/EBP$^{\alpha}$ oligonucleotide. Arrowhead, specific complex formed with NE from trophozoites of clone C2 competed by C/EBP$^{\alpha}$ oligonucleotide.
E. Schematic representation of the nuclear proteins binding to C/EBPI DNA sequence. In trophozoites of clone C2, a nuclear protein binds to C/EBPI DNA sequence contained in both oligonucleotide and region I. In trophozoites of clone A, a nuclear protein binds to C/EBPI oligonucleotide but unidentified DNA–protein interactions (complexes If) (Gómez *et al*., 1998) may be interfering with its binding to region I.

tions in the promoter allowing the binding of proteins to C/EBPI site in trophozoites of clone C2, and avoiding it in trophozoites of clone A.

C/EBPIII oligonucleotide also formed a specific DNA-protein complex with NE from trophozoites of clones C2 and A (Fig. 4A). Complex formation was inhibited by C/EBPI oligonucleotide (Fig. 4A, lanes 4), supporting the idea that similar nuclear proteins have affinity for C/EBPI and C/EBPIII sequences in both clones. Although complexes formed with C/EBPI and C/EBPIII probes have identical electrophoretic mobility, those formed with C/EBPIII oligonucleotide were more intense, suggesting that these proteins have higher affinity for the C/EBPIII site (Figs 3 and 4). The C/EBPIII–protein complex was not competed by CBIm and CBIIIm mutated oligonucleotides (Fig. 4B). As previously reported by Gómez *et al*. (1998), the region III of the *EhPgp1* gene core promoter formed a specific DNA–multiprotein complex with NE from clone C2 trophozoites, which was competed by C/EBP$^{\beta}$ and GATA-1 oligonucleotides (Fig. 4C and D). The DNA–protein interaction in the core promoter is a concerted interplay that is not necessary repeated when we use a

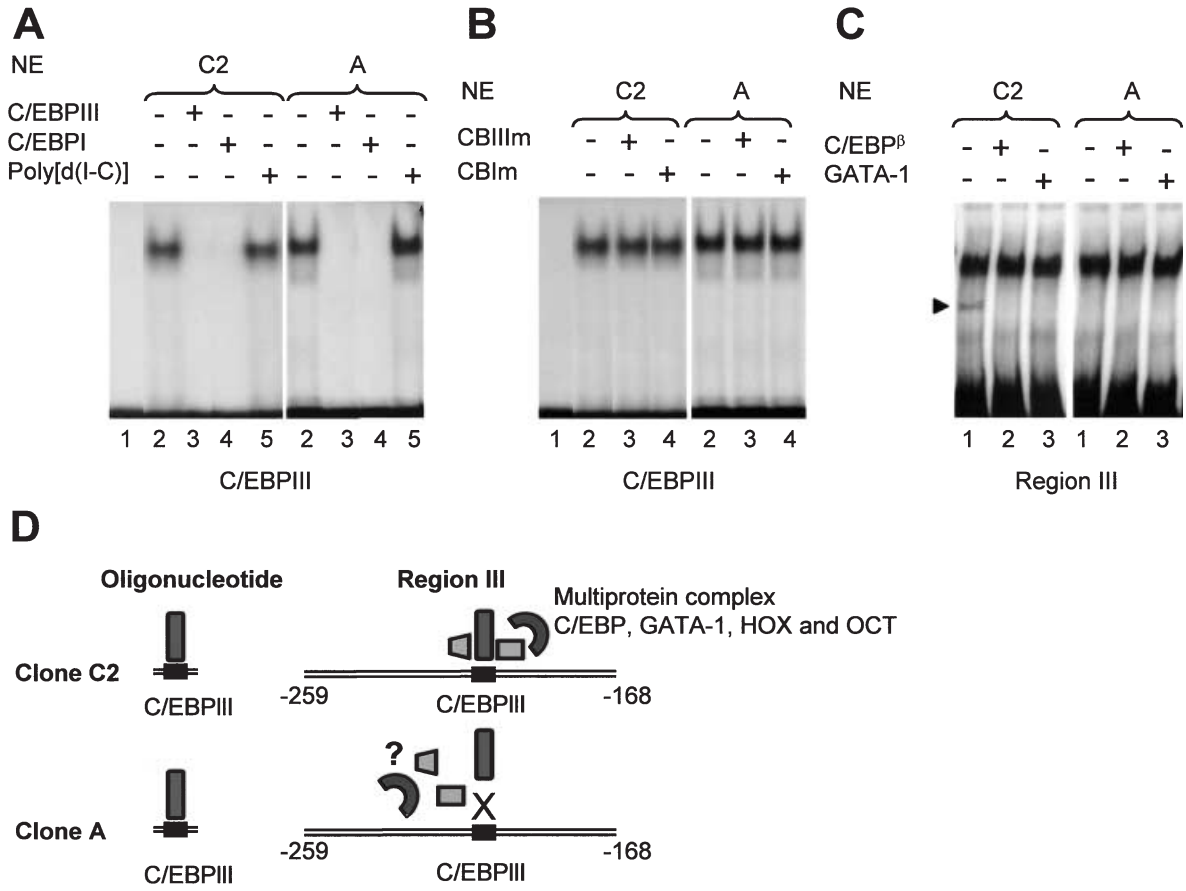

Fig. 4. Nuclear proteins binding to C/EBPIII DNA sequence.
A. EMSA were performed as described in Fig. 3 using the [γ-^{32}P]-ATP radiolabelled C/EBPIII oligonucleotide and different unlabelled competitors (150-fold molar excess). Lane 1, free probe; lane 2, no competitor; lane 3, C/EBPIII oligonucleotide; lane 4, C/EBPI oligonucleotide; lane 5, poly[d(I-C)].
B. EMSA were performed as described in (A) using the [γ-^{32}P]-ATP radiolabelled C/EBPIII oligonucleotide and unlabelled mutated C/EBP oligonucleotides as competitors (150-fold molar excess). Lane 1, free probe; lane 2, no competitor; lane 3, CBIm oligonucleotide; lane 4, CBIIIm oligonucleotide.
C. EMSA were performed as described in (A) using 1 ng of [α-^{32}P]-dATP radiolabelled region III as a probe, and different unlabelled competitors (150-fold molar excess). Lane 1, no competitor; lane 2, C/EBP$^{\beta}$ oligonucleotide; lane 3, GATA-1 oligonucleotide. Arrowhead, specific complex formed with NE from trophozoites of clone C2 competed by GATA-1 and C/EBP$^{\beta}$ oligonucleotides.
D. Schematic representation of the nuclear proteins binding to C/EBPIII DNA sequence. In trophozoites of clone C2, a nuclear protein binds to C/EBPIII oligonucleotide. It also binds to C/EBP site in region III, forming a DNA-multiprotein complex with GATA-1, HOX and OCT-like factors (Gómez *et al.*, 1998). Although a nuclear protein binds to C/EBPIII oligonucleotide in trophozoites of clone A, the DNA-multiprotein complex in region III is not formed.

small oligonucleotide containing only a consensus DNA binding sequence.

E. histolytica *proteins that bind to C/EBP sites of the* EhPgp1 *gene core promoter are recognized by antibodies against the human C/EBPβ*

To initiate the investigation of the identity of *E. histolytica* proteins that bind to C/EBPI and C/EBPIII sites of the EhPgp1 gene core promoter, we performed experiments using a commercial rabbit polyclonal antibody raised against the human C/EBPβ and NE from trophozoites of clone C2. Anti-human C/EBPβ antibodies were used to compete DNA–protein complex formation in EMSA. The complex formation was partially prevented when the binding mixture was incubated with antibodies raised against C/EBP before the addition of radiolabelled C/EBPIII oligonucleotide (Fig. 5A, lane 2). In contrast, antibodies raised against the TFIID factor, used as an internal control, were ineffective to inhibit the complex formation (Fig. 5A, lane 3). In some assays, the complex appeared as a doublet, probably because of the interaction of the antibodies with the DNA-protein complex and the longer electrophoresis running time required for these experiments. The same results were obtained with NE from trophozoites of clone A (data not shown). Thus, at least one of the components

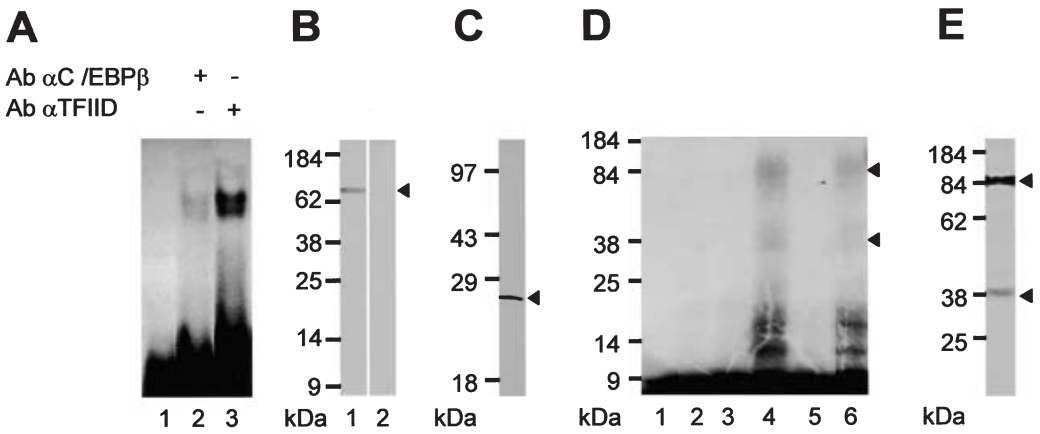

Fig. 5. Immunoanalysis of nuclear proteins that bind to the C/EBP sequence.
A. EMSA were performed as described in Fig. 4 with NE from clone C2 trophozoites and the [γ-^{32}P]-ATP labelled C/EBPIII oligonucleotide. Polyclonal antibodies (6 μg) raised against the human C/EBPβ (lane 2) or TFIID (lane 3) were added to the binding mixture for 1 h at 4°C prior to the addition of the C/EBPIII probe. Lane 1, free probe.
B and C. Western blot analysis using polyclonal antibodies against the human C/EBPβ and NE from clone C2 trophozoites separated by 10% SDS-PAGE in the absence (B, lane 1) and the presence of 8 M urea (C). B, lane 2, negative control using polyclonal antibodies against the human TFIID. Arrowheads, immunopositive polypeptides revealed by the ECL Plus™ system.
D. 100 μg of NE from clone C2 trophozoites were UV cross-linked to 5 ng of the [γ-^{32}P]ATP radiolabelled C/EBPIII oligonucleotide for 10 min at 4°C and DNA-protein complexes were electrophoresed through a 15% SDS-PAGE followed by exposure to X-ray film. Lane 1, free C/EBPIII probe; lane 2, UV treated C/EBPIII probe; lane 3, UV untreated C/EBPIII probe and NE; lane 4, UV treated C/EBPIII probe and NE; lane 5, unlabelled C/EBPIII (150-fold molar excess); lane 6, poly[d(I-C)] (150-fold molar excess). Arrowheads, DNA-protein complexes.
E. Western blot assays of UV cross-linked DNA–protein complexes of lane 4 in D, using polyclonal antibodies against the human C/EBPβ. MW markers are indicated at the left.

of the complex formed by C/EBPIII oligonucleotide and NE from both clones shares epitopes with the human C/EBPβ.

We next investigated if the anti-C/EBPβ antibodies could detect proteins in NE from trophozoites of both clones. Immunoblots assays using NE from clone C2 trophozoites revealed a polypeptide of about 65 kDa (Fig. 5B, lane 1). No bands were detected in identical experiments when we used antibodies against the human TFIID factor (Fig. 5B, lane 2). When proteins were treated with 8 M urea, a single ≈ 25 kDa band was recognized by the anti-C/EBPβ antibodies (Fig. 5C), suggesting that the 65 kDa band may be formed by more than one protein, which were separated by urea. The same results were obtained with NE from clone A trophozoites (data not shown).

To determine the molecular weight (MW) of the proteins bound to the C/EBPIII probe, we performed UV cross-linking assays using NE from trophozoites of clone C2. Proteins were then separated by SDS-PAGE and analysed by autoradiography and Western blot assays. Two major bands of about 84 and 38 kDa were seen in the autoradiography (Fig. 5D, lane 4). Because the MW of C/EBPIII probe (21 bp) was calculated at ≈ 13.65 kDa, the MW of the cross-linked factors to C/EBPIII oligonucleotide were roughly estimated in about 70 and 24 kDa. These bands were not detected when we used a 150-fold molar excess of unlabelled C/EBPIII oligonucleotide in the binding mixture (lane 5), but were unaffected by the unspecific competitor (Fig. 5D, lane 6), indicating the specificity of the complex. Bands of about 12 and 16 kDa at the bottom of the autoradiography corresponded to the oligonucleotide bound to small degraded polypeptides (Fig. 5D). When C/EBPIII oligonucleotide was not UV irradiated (Fig. 5D, lane 1), when C/EBPIII oligonucleotide was irradiated (Fig. 5D, lane 2) or when the DNA–protein mixture was not UV exposed (Fig. 5D, lane 3), no labelled species were found in NE from trophozoites of clone C2. Interestingly, the same bands of about 84 and 38 kDa were detected by the anti-C/EBPβ antibodies in Western blot assays of the UV cross-linked proteins (Fig. 5E). The same results were obtained with NE from trophozoites of clone A (data not shown). The MW of the bands detected in these experiments are close to the MW of polypeptides revealed in Western blot assays of NE. Small differences may be explained by the distinct techniques used.

The nuclear factors that bind to the C/EBPIII site of the EhPgp1 *gene core promoter was semipurified by affinity chromatography*

We partially purified the nuclear factors that bind to C/EBPIII site using the DNA-binding protein purification kit from Boehringer Mannheim, NE from trophozoites of

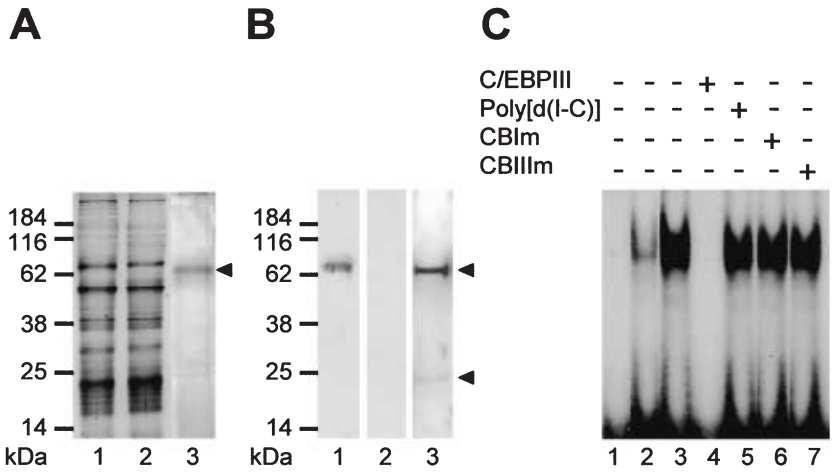

Fig. 6. Purification, immunodetection and functional characterization of the nuclear proteins that bind to the C/EBPIII oligonucleotide.
A. 10% SDS-PAGE of protein fractions obtained from the purification protocol of NE from clone C2 trophozoites. Lane 1, NE (5 μg); lane 2, not retained fraction (5 μg); lane 3, partially purified fraction (pooled from four purification processes).
B. Western blot analysis of fractions obtained from the purification protocol of NE of clone C2 trophozoites. Lane 1, NE; lane 2, not retained fraction; lane 3, partially purified fraction. Arrowhead, silver stained polypeptides detected by the polyclonal antibodies against C/EBPβ. MW markers are indicated at the left.
C. EMSA using the [γ-^{32}P]-ATP radiolabelled C/EBPIII probe, NE (lane 2) or partially purified fraction obtained from clone C2 trophozoites (lanes 3–7) and different unlabelled competitors (150-fold molar excess). Lane 1, free probe: lanes 2 and 3, no competitor; lane 4, C/EBPIII oligonucleotide; lane 5, poly[d(I-C)]; lane 6, CBIm oligonucleotide; lane 7, of CBIIIm oligonucleotide.

clone C2 and the concatamerized C/EBPIII oligonucleotide. In silver stained gels, we could observe the enrichment of a 65 kDa band in the partially purified fraction (Fig. 6A, lane 3). Other minor bands were also present in some experiments. The 65 kDa band was detected by the antibodies against the human C/EBPβ in the semi-purified fraction, and with less intensity in NE (Fig. 6B). The 25 kDa band was also immunodetected in some assays, probably due to the protein concentration during the purification process. Proteins of the semi-purified fraction were able to form a complex with the C/EBPIII probe (Fig. 6C, lane 3). This complex had similar electrophoretic mobility that the one formed with NE (Fig. 6C, lane 2), and its higher intensity corresponded to the extent of enrichment. This complex was competed by unlabelled C/EBPIII oligonucleotide (Fig. 6C, lane 4) but not by unlabelled poly[d(I-C)], CBIm or CBIIIm oligonucleotides (Fig. 6C, lanes 5–7). The same results were obtained with NE from clone A trophozoites (data not shown). These experiments corroborated the presence of an *E. histolytica* nuclear protein, which binds to the C/EBPIII site of the *EhPgp1* gene core promoter and shares epitopes with the human C/EBPβ.

The antibodies raised against the human C/EBPβ recognize proteins in the cytoplasm, nucleus and EhkO organelles of trophozoites

Immunofluorescence and confocal microscopy analysis were done to determine the cellular location of the factors recognized by the rabbit polyclonal antibodies raised against the human C/EBPβ. These factors were co-localized with the propidium iodide stained DNA in the nucleus (Fig. 7A–C) and in EhkO organelles of the trophozoites (Fig. 7D–F), which are also transcriptionally active sites, according to our preliminary results (our unpublished results). Fluorescence was also present in the cytoplasm, where the proteins are synthesized (Fig. 7).

Discussion

The *EhPgp1* gene, one of the genes involved in the MDR phenotype of *E. histolytica*, is differentially transcribed in trophozoites of the emetine-resistant mutant clone C2 and the emetine-sensitive clone A. Previously, the structural and functional characterization of the *EhPgp1* gene core promoter revealed the presence of several *cis*-acting elements to which nuclear proteins specifically bind (Gómez *et al.*, 1998). Here, we demonstrated that the three regions of the core promoter are functionally important for the *EhPgp1* gene transcriptional regulation and that both C/EBP consensus binding sites are *cis*-activating elements. We also show the presence of nuclear proteins, which specifically bind to C/EBP sequences and are recognized by anti-C/EBPβ antibodies.

Deletions in the *EhPgp1* 5′-flanking sequence confirmed that the presence, position and orientation of each region of the *EhPgp1* gene core promoter are essential

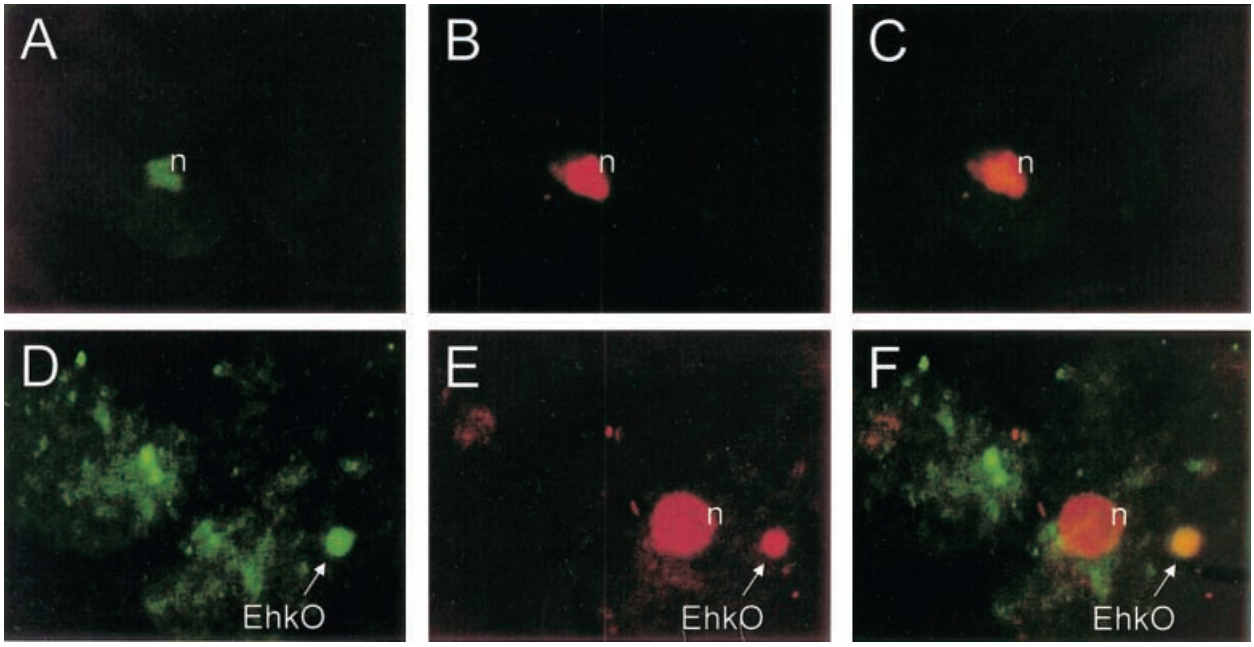

Fig. 7. Confocal immunofluorescence microscopy analysis using antibodies against he human C/EBPβ.
Trophozoites of clone A were incubated with polyclonal antibodies against the human C/EBPβ, treated with a goat anti-rabbit polyclonal antibody and counterstained with propidium iodide (1 μg ml^{-1}). n, nucleus; EhkO, EhkO organelle. A and D, cells observed in the green channel. B and E, cells observed in the red channel. C and F, cells observed simultaneously in both channels.

for its activity. The p167Pgp1 and p74Pgp1 plasmids carrying 167 and 74 bp upstream from the ATG, respectively, were not able to drive the CAT expression in the trophozoites of clone C2. On the other hand, deletion of region I also abolished transcription. Loss of expression might be due to the absence of putative recognition sites for transcription factors, such as the HOX, POU, C/EBP or GATA-1 sites present in the core promoter of the *EhPgp1* gene.

CAT activity was also abolished in trophozoites of clone C2 when region II was deleted (ΔII-p268Pgp1 plasmid). This could be due to the absence of putative DNA binding sites for regulatory factors present in this region (HOX, POU or GATA-1 sites) or to the nearness of region III to the transcription initiation site, interfering with the promoter folding necessary for the correct position of the TPC. However, the insertion of an unrelated spacer fragment between regions I and III (p268Pgp1-IIM plasmid) failed to restore the promoter function, suggesting the presence of unknown DNA–protein interaction in region II favouring the promoter folding and activating transcription. Transfection with a construct carrying inverted region III (p268Pgp1-IIIR construct) gave 57% of CAT activity in trophozoites of clone C2, in relation to the activity showed by the full core promoter. All putative binding sites persist in the reversed region III, but in a different position (GATA-1, C/EBP, POU and HOX sites moved from –228 to –204, –198 to –241, –181 to –252, –177 to –255 and –180 to –251 bp respectively). Reduced transcription might be maintained because proteins still recognizing their binding sites. However, the complex formed with the inverted region may be less stable than the one formed with region III in normal orientation, reducing the transcriptional activity of the *EhPgp1* gene core promoter.

The initiation of the fine characterization of DNA regulatory sequences that control the *EhPgp1* gene core promoter activity showed that both C/EBP sequences, which are located in regions I and III, are necessary for the activation of the promoter in trophozoites of clone C2, as it was shown by transfection assays using plasmids with one or both sites mutated (p268Pgp1-Im, p268Pgp1-IIIm and p268-Pgp1-Im-IIIm plasmids). C/EBPI site is flanked by two putative Inr sequences (–67 and –18 bp) and by their proximity, it is possible that the binding of a protein to the C/EBPI site stabilizes the TPC when it binds to Inr sites. Additionally, the last 53 bp upstream the C/EBPIII site, containing the putative GATA-1 site, are also important for the promoter activation, as their deletion inhibited the CAT expression in trophozoites of clone C2. Mutation of the C/EBPIII site and/or deletion of the 5′ end region including the putative GATA-1 sequence abolished transcription of the *EhPgp1* gene, probably because the DNA-multiprotein complex can not be formed. All these results confirmed the relevance of both C/EBP binding sites as *cis*-activators elements of the *EhPgp1* gene transcriptional regulation. We hypothesized that the DNA-

multiprotein complex could interact with proteins positioned in region I, through proteins bound to C/EBP sites in regions I and III. This could induce the folding of the promoter and stabilize the TPC.

EMSA and UV cross-linking assays revealed that *E. histolytica* possesses nuclear proteins, which interact specifically with C/EBPI and C/EBPIII sequences. Gómez *et al*. (1998) postulated that the activation of *EhPgp1* gene core promoter could be caused by the presence of C/EBP-like factors in trophozoites of clone C2, which may be absent, modified or diminished in clone A. We confirmed these results when we used fragments corresponding to regions I and III in EMSA. However, we did not find differences in both clones when we used oligonucleotides containing only the binding sequences, indicating that proteins that bind to C/EBP sequences are present in trophozoites of both clones. Differences between the EMSA using the promoter regions and the C/EBP oligonucleotides may be explained by the presence of other DNA–protein interactions in these regions, which may modulate the protein binding to the promoter (Figs 3E and 4D).

Antibodies raised against the human C/EBPβ inhibited the DNA-protein complex formation and recognized polypeptides in NE from trophozoites of both clones, suggesting that nuclear proteins that bind to C/EBP sequences share epitopes with the human C/EBPβ. These proteins were located in nucleus and EhkO, strengthening the assumption that transcription is occurring in both organelles.

In conclusion, several lines of evidence showed the relevance of *trans*-activator nuclear proteins bound to both C/EBP sequences for the *EhPgp1* gene transcriptional regulation. (i) Deletions of the C/EBPI and C/EBPIII sites abolished the CAT activity driven by the *EhPgp1* gene core promoter. (ii) NE from trophozoites of resistant and sensitive clones formed specific DNA-protein complexes with C/EBPI and C/EBPIII oligonucleotides. (iii) The DNA–protein interactions were inhibited by antibodies against the human C/EBPβ. These antibodies are raised against the carboxy-terminal domain of the human C/EBPβ, including the leucine zipper, which is a common motif in various eukaryotic transcription factors. Thus, we can not assure that the *E. histolytica* proteins bound to C/EBP sequences are homologous to the human C/EBP factor. The *E. histolytica* nuclear proteins detected here may share sequences of the carboxy-terminal region to which the antibodies are directed. (iv) In Western blot assays, these antibodies recognized bands of 65 and 25 kDa in NE of trophozoites from both clones. (v) UV cross-linking and Western blot assays demonstrated that nuclear proteins of similar MW bind to the C/EBP probe and are recognized by the antibodies. (vi) The partially purified nuclear proteins bound specifically to C/EBP probe and shared epitopes with the human C/EBPβ, as determined by EMSA and Western blot assays. (vii) In immunofluorescence and confocal microscopy assays, the antibodies colocated the proteins with the DNA containing organelles, as expected for a transcription factor.

Proteins showing similarities to C/EBP were not found yet in the data base for the *E. histolytica* genome (http://www.tigr.org/tdb/edb2/htmls). The cloning and characterization of the protein binding to C/EBP sites encoding gene in *E. histolytica*, currently in progress, will allow us to determine if this parasite has indeed C/EBP-like transcription factors.

Experimental procedures

E. histolytica *cultures*

Trophozoites of clones A (emetine-sensitive) and C2 (emetine-resistant) (strain HM1:IMSS) (Orozco *et al*., 1985) were axenically cultured in TYI-S-33 medium (Diamond *et al*., 1978).

Plasmid constructions

To carry out transfection experiments, we constructed several plasmids inserting PCR-amplified *EhPgp1* gene core promoter fragments into the multiple cloning site of the pBSCAT-ACT plasmid, which contains the bacterial CAT reporter gene (659 bp) and the 3′-flanking region of the *actin* gene (600 bp) (Gómez *et al*., 1998). First, we divided the *EhPgp1* gene core promoter into regions I (–74 to +24 bp), II (–167 to –75 bp) and III (–259 to –168 bp) and made plasmid constructions containing one or two of these regions. Regions I and II were PCR amplified together using the EhPgp1-IIS and EhPgp1-AS33 oligonucleotides, to obtain the p167Pgp1 construct. Region I was PCR-amplified using the EhPgp1-IS and EhPgp1-AS33 primers, to obtain the p74Pgp1 construct. Deletion of region II was done by PCR amplification of fragments I (EhPgp1-IS2 and EhPgp1-AS33 primers) and III (EhPgp1-IIIS2 and EhPgp1-IIIAS oligonucleotides), which were successively cloned into the pBSCAT-ACT plasmid to generate the ΔII-p268Pgp1 plasmid. To delete region I, we PCR-amplified regions III and II using the EhPgp1-IIIS2 and EhPgp1-IIAS oligonucleotides to obtain the ΔI-p268Pgp1 plasmid. The p268Pgp1-IIM construct was generated by the insertion of an unrelated blunt-end fragment (98 bp) between regions III and I, into the ΔII-p268Pgp1 plasmid. Finally, to generate the p268Pgp1-IIIR plasmid with inverted region III that was PCR amplified using the EhPgp1-IIIS3 and EhPgp1-IIIAS2 oligonucleotides, and cloned into the p167Pgp1 construct (Table 1 and Fig. 1B). We replaced the 5′-TGTTTGGTAGTT-3′ sequence of the C/EBPI binding site (–54 to –43 bp) by the 5′-CTCACTCTG CAG-3′ sequence, which contains a *Pst*I site (underlined). We first PCR amplified a fragment spanning from –48 to +24 bp using the C/EBPIm-S and EhPgp1-AS33 primers, and cloned it into the pBSCAT-ACT plasmid to obtain the p48Pgp1 plasmid. Next, we amplified the region spanning from –259 to –43 bp using the EhPgp1-IIIS and C/EBPIm-AS oligonucleotides, and cloned it into the p48Pgp1 construct to generate the p268Pgp1-Im plasmid. Mutation of the C/EBPIII site was done by replacing the 5′-GAATTGGAAAGAA-3′ sequence (–198 to –186 bp) by the

Table 1. Oligonucleotides used for plasmid constructions.

Name	Position	Sequence
EhPgp1-IIS	–167	5′-TAACAAAGGAGAGAAAAT-3′
EhPgp1-AS33	+24	5′-CCCAAGCTTAAACTCACTTTCAGTTATATCCAT-3′
EhPgp1-IS	–74	5′-TTTTAGATTTAATGTGTT-3′
EhPgp1-IIIS2	–259	5′-GCTCTAGATAAATGAACTAAAAAATA-3′
EhPgp1-IIIAS	–168	5′-AAACTGCAGACTTTATTTATTATTTCT-3′
EhPgp1-IS2	–74	5′-AAACTGCAGTTTTAGATTTAATGTGTT-3′
EhPgp1-IIAS	–75	5′-CCCAAGCTTTTCTATATTAAATAATTT-3′
EhPgp1-IIIS3	–185	5′-TCCCCCGGGTAAATGAACTAAAAAATA-3′
EhPgp1-IIIAS2	–168	5′-GCTCTAGACTTATTATTTTCTCTCCT-3′
EhPgp1-IIIS	–259	5′-TAAATGAACTAAAAAATA-3′
C/EBPIm-AS	–43	5′-AAAACTGCAGAGTGAGCTAACACATTAAAT-3′
C/EBPIm-S	–48	5′-AAAACTGCAGCAAGCTGAACTAACTAACA-3′
C/EBPIIIm-AS	–193	5′-TCCCCCGGGAGATAACATTTTTTCAG-3′
C/EBPIIIm-S	–198	5′-TCCCCCGGGTCCGATCAGAAATAATAAATAAA-3′
C/EBPIII-RS	–206	5′-GCTCTAGACTGATGAATTGGAAAGAAAGA-3′
EhPgp1-IIIS4	–185	5′-GCTCTAGAAGAAATAATAAATAAG-3′

Restriction sites for *Hin*dIII (EhPgp1-AS33 and EhPgp1-IIAS), *Xba*I (EhPgp1-IIIS2, EhPgp1-IIIAS2, C/EBPIII-RS and EhPgp1-IIIS4), *Pst*I (EhPgp1-IIIAS, EhPgp1-IS2, C/EBPIm-S and C/EBPIm-AS), *Sma*I (EhPgp1-IIIS3) and *Xma*I (C/EBPIIIm-S and C/EBPIIIm-AS) are underlined. *S* and *AS* mean *sens* and *antisens* primers respectively. *Position* refers to the 5′ end of each primer.

5′-CCCGGGTCCGATC-3′ sequence, which contains a *Sma*I site (underlined). We PCR amplified two fragments from –259 to –193 bp, and –198 to +24 bp, using the EhPgp1-IIIS2 and C/EBPIIIm-AS, and the C/EBPIIIm-S and EhPgp1-AS33 primers pairs respectively. Then, we successively cloned them into the pBSCAT-ACT plasmid to obtain the p268Pgp1-IIIm plasmid. The p268Pgp1-Im-IIIm plasmid, with mutations in both C/EBP sites, was prepared amplifying the region spanning from –259 to –43 bp using the EhPgp1-IIIS2 and C/EBPIm-AS primers, and the p268Pgp1-IIIm plasmid. The obtained fragment was subsequently cloned into the p48Pgp1 plasmid. We also prepared the p206Pgp construct, which lacks 53 bp at the 5′-end (containing the putative GATA-1 site), using the C/EBPIII-RS and EhPgp1-AS33 primers. We also generated the p185Pgp1 plasmid, without the GATA-1 and C/EBPIII binding sites, using the EhPgp1-IIIS4 and EhPgp1-AS33 primers. The p185Pgp1-Im plasmid, which lacks the GATA-1 and C/EBPIII sites, and has mutations in the C/EBPI site, was done using the EhPgp1-IIIS4 and C/EBPIm-AS oligonucleotides. The obtained fragment (–185 to –43 bp) was subsequently cloned into the p48Pgp1 plasmid. The p206Pgp1-Im plasmid, which lacks the region upstream the C/EBPIII site and has mutations in the C/EBPI site, was also made, using the C/EBPIII-RS and C/EBPIm-AS primers. The obtained fragment (–206 to –43 bp) was subsequently cloned into the p48Pgp1 plasmid (Table 1 and Fig. 2A).

The promoterless (pBSCAT-ACT) and p268Pgp1 constructions (Gómez *et al*., 1998) were used as negative and positive controls, respectively. The pA5′A3′CAT plasmid (Nickel and Tannich, 1994), which contains a 480-bp fragment from the *actin* gene promoter was used as a control of transfection efficiency (Figs 1B and 2A). The orientation and sequence of all constructs were confirmed by DNA sequencing (Sanger *et al*., 1977).

Transfection and CAT assays

Transfection assays were carried out by electroporation (Nickel and Tannich, 1994) of the trophozoites using the plasmid constructions described above. CAT activity was measured by the two-phase diffusion assay (Buβ *et al*., 1995) using 5 μg of trophozoites 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 pBSCAT-ACT 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 plasmid pA5′A3′CAT plasmid (Nickel and Tannich, 1994).

Nuclear extracts

Nuclear extracts were prepared from trophozoites of clones C2 and A 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).

Electrophoretic mobility shift assays

Regions I and III of the *EhPgp1* gene core promoter were amplified and [α-^{32}P]-dATP labelled by PCR as previously described (López-Bayghen *et al*., 1996; Gómez *et al*., 1998). C/EBPI (5′-TTAG**TGTTTGGTAGTT**CAAG-3′) and C/EBPIII (5′-CTGAT**GAAT TGGAAAGAA**AGA-3′) double-stranded DNA oligonucleotides (Gibco-BRL) corresponding to the C/EBP binding sites of the *EhPgp1* gene core promoter (bolded) were [γ-^{32}P]-ATP labelled using T4 polynucleotide kinase (Gibco-BRL). Specific activity was determined by scintillation counting. EMSA were performed as described (Gómez *et al*., 1998). Briefly, for each reaction we used 0.5–1 ng of labelled probes (10 000 cpm), 1 μg of poly[d(I-C)] (Amersham Pharmacia Biotech), 20 μg of NE from trophozoites, and 10% glycerol in DNA-protein binding buffer. For competition assays, 150-fold molar excess of unlabelled oligonucleotides were incubated at 4°C for 10 min with NE prior to the addition of the radiolabelled probes. As competitors, we used

poly[d(I-C)] and different oligonucleotides, such as C/EBPI, C/EBPIII, CBIm (5′-TTAGCTCACTCTGCAGCAAG-3′), CBIIIm (5′-ATCTACTCACTCTGCAGAGAA-3′), C/EBP$^{\alpha}$ (5′-GGTATGATTTTGTAATGGGGTAGG-3′) and NF1 (5′-TATTTTTGGATTGAAGCCAATATGATAATGA-3′) from rat albumin gene promoter (Cereghini *et al*., 1987), a putative C/EBP$^{\beta}$–like sequence represented several times in different *E. histolytica* promoters (5′-ATTCAATTGGGCAATCA-3′) and GATA-1 (5′-GTTGCAGATAAACATT-3′) (Gómez *et al*., 1998), and TATA box (5′-AATTCTCTATTTAAAGAG-3′) (Luna-Arias *et al*., 1999). In other experiments, we incubated the NE with a rabbit polyclonal antibody raised against the human C/EBPβ (2 μg μ^{-1}l) (Santa Cruz Biotechnology, Santa Cruz, CA) or the same amount of a polyclonal antibody to human TFIID (TBP) (Santa Cruz Biotechnology), at 4°C for 1 h, prior to the addition of the C/EBPIII probe (Ausubel *et al*., 1994). Then, mixtures were incubated for 10 min at 4°C and analysed by electrophoresis through non-denaturing 6% polyacrylamide gels in 0.5× TBE at 25°C and 100 V for 3 h. Gels were dried and analysed by autoradiography.

UV cross-linking

UV cross-linking assays were performed according to Ausubel *et al*. (1994) with some modifications. Standard EMSA mixtures using NE from trophozoites of both clones and the radiolabelled C/EBPIII probe were scaled up fivefold and submitted to irradiation on the surface of a 312 nm UV-transilluminator for 10 min at 4°C, and then, proteins were separated by 15% SDS-PAGE (Laemmli, 1970). Gels were dried and analysed by autoradiography.

Western blot analysis

Western blot assays were performed using the standard procedure (Ausubel *et al*., 1994). NE (20 μg) from trophozoites were separated on 10% SDS-PAGE (Laemmli, 1970) with or without 8 M urea and electrotransferred to nitrocellulose membranes (Hybond^{TM}-C extra, Amersham Pharmacia Biotech). The filters were then incubated with 2 μg ml^{-1} of the antibodies raised against the human C/EBPβ in 5% non-fat dry milk and 0.05% Tween-20 in phosphate-buffered saline (PBS), pH 7.4 overnight at 4°C. As a control, we used the antibody against TFIID previously described. Proteins were developed by peroxidase-conjugated anti-rabbit secondary antibodies (ZYMED Laboratories) (1:1500) and immunodetected by a chemiluminescence system (ECL Plus^{TM}, Amersham Pharmacia Biotech).

Purification of the E. histolytica *proteins that bind to the C/EBP sequence*

E. histolytica nuclear proteins that bind to the C/EBP sites of the *EhPgp1* gene core promoter were partially purified under non-denaturing conditions using a DNA-binding protein purification kit (Boehringer Mannheim) and NE from trophozoites of both clones. Concatameric polynucleotides were prepared by self-ligation of the C/EBPIII probe (133 ng μl^{-1}) using one unit of T4 DNA ligase (Gibco-BRL). The oligomer was coupled to magnetic particles coated with streptavidin as described by the manufacturer. After dialysis and concentration with Centricon concentrators (Amicon), 50 μg of NE from trophozoites were mixed with the particles. After extensive washes, proteins bound to the particles were eluted with elution buffer containing 2 M KCl. All fractions were analysed by 10% SDS-PAGE (Laemmli, 1970). Gels were silver stained or transferred to nitrocellulose membranes for Western blot assays. Purified fractions were submitted to EMSA as described above.

Confocal and immunofluorescence microscopy

Trophozoites were grown overnight on sterile coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and blocked with 1% (w/v) BSA. The fixed trophozoites were incubated with a 1:500 dilution of the rabbit polyclonal antibodies against the human C/EBPβ and then, with a 1:250 dilution of a goat anti-rabbit polyclonal antibody labelled with fluorescein isothiocyanate (FITC) (Sigma). Finally, cells were counterstained with propidium iodide in PBS (1 μg ml^{-1}) for 5 min and examined through a Nikon microscope attached to a laser confocal scanning system MRC 1024 (Bio-Rad). As negative controls, we omitted the first antibody.

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