Physiology and molecular genetics of multidrug resistance in *Entamoeba histolytica*

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Abstract *Entamoeba histolytica* presents the evolutionarily conserved multidrug-resistance (MDR) phenotype, discovered in mammalian cells. MDR cells overexpress the membrane P-glycoprotein, which excludes unrelated drugs from the cytoplasm. *E. histolytica* mutants exhibit cross-resistance to unrelated drugs, which are pumped out from the cytoplasm. In drug-resistant trophozoites, the constitutively expressed *EhPgp1* gene appears to be up-regulated by a C/EBP-like factor and a multiprotein complex that were not found in drug-sensitive trophozoites. The drug-induced *EhPgp5* gene, on the other hand, appears to be up-regulated by AP-1 and HOX factors. Here we review the main physiological and molecular facts of the MDR phenotype in *E. histolytica*.

INTRODUCTION

The ability of mammalian cells and microorganisms to develop resistance to drugs has become a world health problem of increasing proportions in cancer and infectious diseases and has fueled the development of new drugs to fight cancer cells and microbial pathogens. The study of drug-resistance phenotypes has advanced our understanding of several cellular processes.

Survival of cancer cells and microbial pathogens in the presence of drugs can occur by several mechanisms:[1]

- Mutations in proteins that metabolize drugs.
- Membrane alterations that decrease drug accumulation in the cytoplasm.
- Extra-chromosomally encoded, drug inactivating enzymes.
- Failure of cells to undergo apoptosis in the presence of drugs.
- Changes in cell-cycle that modify cell responses to drugs.
- Changes in cytotoxic targets that abrogate the drug effect.
- Fast and efficient repair of damage induced by drugs, allowing the cell to escape.

Surface membrane proteins, overexpressed in drug-resistant cells, can transport a variety of chemically unrelated drugs. Elucidation of the function of the efflux proteins and their involvement in drug-resistance has been an important development in the field. These proteins are energy-dependent pumps that transport drugs out of the cell before they reach their target. Two major mechanisms have been described to explain cross-resistance in drug-selected cell lines:

1. Overexpression of a surface glycoprotein (P-glycoprotein)[2,3] producing the multidrug-resistance (MDR) phenomenon.
2. Expression of the multidrug-resistance associated protein (MRP),[4] which was identified as the glutathione conjugate transporter.[5,6]

The MDR phenotype is present in about 50% of cancers and is a frequent cause of failure chemotherapy in cancer treatment. It has also been described in *Plasmodium*[7–10] *Leishmania*,[11–15] *Trypanosoma cruzi*,[16] *Trichomonas vaginalis*,[17] *Giardia lamblia*[18] and *Entamoeba histolytica*.[19,20] *Mdr*-like genes have been cloned from all of these parasites (Table 1).[7,12,21–24] However, most of P-glycoproteins have been predicted from *mdr* homology and have not been functionally analyzed, except for *P. falciparum*, whose P-glycoprotein has been expressed in yeast as a recombinant protein.[25]

The P-glycoprotein is an ATP-dependent transporter that belongs to the ATP-binding cassette (ABC) family. It has two homologous halves, each containing six transmembrane regions, forming a putative membrane channel composed of six loops and two ATP-binding domains that couple ATP hydrolysis to transport. Some members of this protein class can transport diverse hydrophobic substances with aromatic rings (Fig. 1).[26]

The MDR phenotype represents an evolutionary conserved mechanism; *mdr* genes are most likely derived from duplication of bacterial genes. For example, the *Escherichia coli hlyB* gene encodes a protein involved in the transport of the haemolysin protein and is closely related to a half mammalian *mdr* gene.[27,28] Thus, it is not surprising that protozoan parasites express the MDR phenotype with physiological and molecular similarities to that of transformed mammalian cells.

MDR in parasites

Multidrug-resistant parasite lines exhibit a remarkable similarity with the mammalian MDR phenotype (Table 2):

Table 1 ***mdr-like* genes identified in protozoan parasites**

Organism	*pgp* gene(s)	Resistance to:
P. falciparum	*pfmdr1*	Mefloquine
	pfmdr2	Halofantrine
		Quinine
L. tarentolae	*ltpgpA*	Arsenite
L. mexicana	*lmpgpA*	Antimonite
		Pentostam
L. donovani	*ldmdr1*	Vinblastine
L. enretii	*lemdr1*	Puromycin
*E. histolytica**	*EhPgp1*	Emetine
	EhPgp2	Colchicine
	EhPgp5	Iodoquinol
	EhPgp6	Diloxanide
T. vaginalis	*Tvpgp1*	Metronidazole

*The specificity of the *EhPgp* genes for each drug has not been determined.

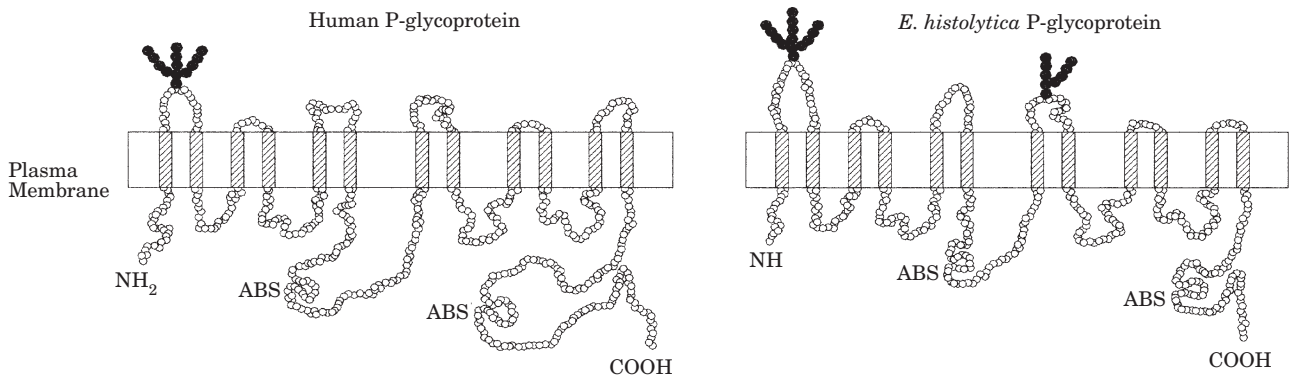

Fig. 1 **Comparison of the predicted models for human and *E. histolytica* P-glycoproteins derived from sequence analysis. Filled-in balls: putative glycosylation sites. ABS: ATP binding sites.**

Table 2 **MDR phenotype in mammals and protozoan parasites**

Phenotype	Mammals	Parasites				
		P	L	T	E	G
Resistance to more than one drug	Y	Y	Y	Y	Y	Y
Reversal of drug-resistance by verapamil	Y	Y	N	?	Y	?
Reduced drug accumulation in the cytoplasm	Y	Y	Y	?	Y	Y
Resistance based upon energy-dependent efflux of the drug	Y	Not tested in parasites				
mdr genes encode for a P-glycoprotein	Y	Y	Y	Y	Y	?
MDR correlates with a P-glycoprotein in the plasma membrane	Y	Not tested in parasites				
Expression of a 4.5 kb *mdr* mRNA	Y	Y	Y	Y	Y	?
Increased drug-resistance correlates with:						
Increased *mdr* gene expression	Y	Y	Y	Y	Y	?
mdr gene amplification	Y	Y	Y	N	Y	?
MDR is conferred after *mdr* gene transfection	Y	Y	Y°	?	Y°	?

°Resistance was conferred only at low levels. P, *Plasmodium falciparum*; L, *Leishamania donovani*; T, *Trichomonas vaginalis*, E, *Entamoeba histolytica*; G, *Giardia lamblia*.

- Many of the drug-resistant protozoa display an efflux mechanism, which prevents drug accumulation in their cytoplasm.[18,19,30–33]
- Parasites selected for their resistance to a single drug show resistance to other unrelated drugs.[10,19,29,34–37]
- Calcium channel blockers, such as verapamil,[19,38] reverse drug resistance.
- Over-transcription or amplification of parasite *mdr*-like genes correlates with increased drug resistance.[7,10,24,39,40]
- Transfection experiments, introducing *mdr* genes in sensitive parasites or in other type of cells, confer drug resistance to them.[15,33,41–43]

All these findings, coming from many laboratories, confirm the presence of *mdr* like genes in protozoa. In particular, transfection experiments have bolstered the role of *mdr* genes in drug resistance and have confirmed the MDR phenotype as an evolutionary conserved mechanism.

Amoebiasis and drugs used against *E. histolytica*

E. histolytica is the protozoan parasite responsible for human amoebiasis. It infects 500 million people, provokes 50 million cases of dysentery or liver abscesses and kills 100 000 humans each year around the world.[44] Amoebiasis is a protozoan disease second in mortality only to malaria.[44] It is controlled primarily by drug treatment of symptomatic individuals, using mainly metronidazole and emetine.

Metronidazole is the preferred drug in countries with a high incidence of amoebiasis, such as Mexico and India. It has been used for more than 30 years against anaerobic bacteria and protozoa and is also the first-line treatment of *G. lamblia* and *T. vaginalis* infections. In amoebiasis, metronidazole is given to adults in 1 g dose, twice per day, for 5–10 days. After administration, the drug could be found in high concentrations in liver, intestine, stomach and kidney. It provokes nausea, diarrhea, abdominal pain, anorexia, headache, fatigue and rash. In bacterial tests, metronidazole was shown to be mutagenic.[45]

Inside the cell, metronidazole and other nitroimidazoles reduce their nitro group into reactive free radicals and nitroso compounds. These interact with DNA, destroying the helical structure and generating DNA breaks.[46,47] Only anaerobic organisms can activate the drug efficiently under anaerobic

conditions, because oxygen removes the donor electron that acts on the nitro group. The electrons responsible for metronidazole activation in protozoa are mainly produced by pyruvate:ferredoxin:oxidoreductase (PFO) and are transferred to the drug by ferredoxin. The PFO enzyme has been cloned in *G. lamblia* (GenBank accession number L27221), *T. vaginalis* and *E. histolytica*.[48,49] Analysis of metronidazole-resistant *Trichomonas* isolates indicated that the drug was inefficiently converted to its activated form. A lower PFO activity was confirmed in these parasites.[50]

Emetine was for many years one of the major drugs used in the treatment of the principal forms of human amoebiasis: dysentery and liver abscesses. Emetine inhibits ribosome movement along mRNA by blocking the aminoacyl-tRNA transfer reaction in protein biosynthesis.[51,52] Emetine is more effective against trophozoites than cysts. The therapeutic dose is 1 mg/kg/day in adults for 10 days. Like metronidazole, emetine is found at high concentrations in blood during treatment and is excreted in urine. Patients taking emetine usually present abdominal pain, nausea and vomit.

The undesired effects of anti-amoebiasis drugs have led to increased efforts for developing new drugs and an efficient vaccine against *E. histolytica*. Additionally, differences in drug susceptibility have been reported for *E. histolytica* strains in vivo[53–55] and in vitro.[19,20] There are also case reports of failed drug treatments,[56,57] suggesting clinical drug resistance. However, drug susceptibility of clinical isolates to amoebicidal drugs has not been routinely determined because trophozoites are difficult to culture from stool samples. Due to the aggressiveness of this parasite, an outbreak caused by drug-resistant trophozoites would be a major problem. Furthermore, the development of drug-resistant amoebae in infected patients would represent an additional burden in health care cost and planning of public health policies in poor countries affected by *E. histolytica*. Due to the relevance of drug resistance in *E. histolytica*, it is important to study the molecular basis of this phenotype in drug-resistant trophozoites.

E. Histolytica EMETINE-RESISTANT MUTANTS

The development of drug-resistant mutants has been fundamental to the understanding of the physiological and molecular mechanisms responsible for the MDR phenotype in parasites. In 1985, our laboratory mutagenized by ethylmethane sulphonate a clonal population (clone A)[34] of strain HM1:IMSS (58) and selected trophozoites that were able to grow at lethal concentrations (12 μM) of emetine. Twenty emetine-resistant mutants were thus obtained that showed differences in their drug-resistance phenotype. Two different types of resistant trophozoites were identified: (1) Clones C9, resistant to 35 μM emetine but sensitive to colchicine; and (2) Clones C2, resistant to 90 μM emetine and cross-resistant to colchicine,[19,34] diloxanide and iodoquinol,[35] but susceptible to metronidazole. Although metronidazole-resistant *T. vaginalis*[17] and *G. lamblia*[29] isolates have been reported, we could not generate *E. histolytica* metronidazole-resistant mutants. Recently, Samarawickrema et al.[55] obtained a metronidazole-resistant *E. histolytica* population (HTH-56:MUTM strain), following continuous exposure to steadily increasing drug concentrations. The HTH-56:MUTM strain did not present decreased PFO activity, but had a marked increase in superoxide dismutase activity.

The characterization of clones C9 suggested that they have a possible alteration in the protein synthesis machinery. The cross-resistance displayed by clones C2 to colchicine and other chemicals led us to suspect that they had the MDR phenotype. Further experiments demonstrated that indeed clones C2 share a number of characteristics with multidrug-resistant cancer cells and parasites including *Plasmodium*,[7] *Giardia*[18] and *Leishmania* (Table 2).[14]

Physiology of multidrug-resistance in trophozoites of clone C2

The MDR phenotype in transformed mammalian cells is manifested by cross-resistance to several drugs, decreased drug accumulation in the cytoplasm and reversion of drug-resistance by calcium channel blockers (Table 2). The decrease or lack of drug accumulation in the cytoplasm of resistant cells is indirect evidence of alterations in the plasma membrane, frequently related to changes in permeability and transport. Drug uptake experiments, using radiolabeled drugs, demonstrated that trophozoites resistant to 200 μM emetine accumulated significantly less ^{3}H-emetine and ^{3}H-colchicine (drugs to which C2 trophozoites show cross-resistance), than the wild-type clone A, which is sensitive to 8 μM emetine (Fig. 2). In contrast, amoebae incubated with the radioactive drugs in the presence of verapamil (10 μM), a calcium channel blocker, efficiently accumulated the drugs in their cytoplasm. In fact, in the presence of verapamil, the level of radioactivity in drug-resistant cells was very close to that obtained for wild-type trophozoites.[19] *E. histolytica* strains and clones derived from a cloned population exhibit significant differences in their emetine-resistance level. For example, clone L6 (strain HM1:IMSS) is killed by 3 μM emetine, while clone A (strain HM1:IMSS) survives in this concentration (Fig. 2).

GENES INVOLVED IN MDR PHENOTYPE IN *E. Histolytica* (*EhPgP* GENES)

The physiological results obtained with trophozoites of clone C2 suggested that they express the MDR phenotype. However, it was important to demonstrate the presence of *mdr*-like genes in this parasite. As the phenotype is a conserved mechanism, the sequence of *mdr* genes from different species displays high homology, mainly in the regions corresponding to the ABC sites of the P-glycoprotein (Fig. 1). Oligonucleotides were constructed similar to the most conserved ABC regions in human and mouse genes, but with the codon usage of *E. histolytica*.[59] mRNA from the emetine-resistant trophozoites, together with the oligonucleotides, were used to identify cDNA fragments with the reverse transcriptase. The cDNA fragments were then amplified by the polymerase chain reaction (PCR) and four different DNA fragments of the expected size were obtained. The predicted amino acid sequence of the DNA products displayed 46–56% identity to the corresponding fragment of the human P-glycoprotein.[60] Interestingly, the same fragments were only 30–35% identical to the predicted *P. falciparum* P-glycoprotein.[61]

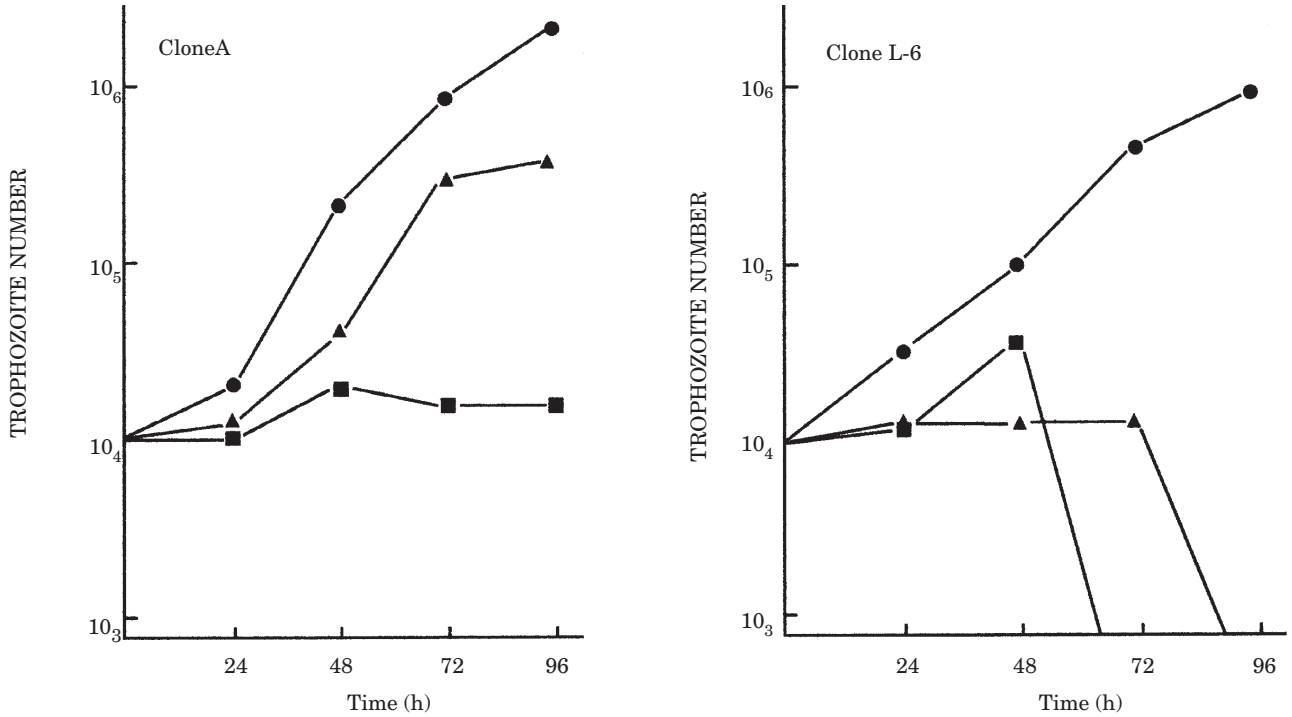

Fig. 2 **Sensitivity of different *E. histolytica* clones to emetine. Trophozoites (10^4) of clones A and L6 (strain HMI:IMSS) were incubated in TYI-S-33 medium with different emetine concentrations. (●) Without drug, (▲) 3 μM and (■) 8 μM emetine.**

Using the PCR-amplified DNA fragments, four different *mdr*-like genes (*EhPgp1*, *EhPgp2*, *EhPgp5* and *EhPgp6*) and two pseudogenes (*EhPgp3* and *EhPgp4*) were isolated from a genomic library.[23,24] The complete open reading frame of the *EhPgp* genes encoded for EhPGP-like proteins ranging between 1282 and 1301 amino acids. *EhPgp* genes sequence confirmed that *mdr* genes are highly conserved in organisms distant in the evolutionary chain, such as *E. histolytica* and *Homo sapiens* whose proteins showed 22–27% homology.[24] The hydropathy plots of the EhPGP proteins[23] were nearly superimposable onto each other and onto the hydropathy plot of the human MDR protein, revealing the archetypal P-glycoprotein membrane topology of two alike halves, each consisting of six putative transmembrane domains followed by a nucleotide binding motif (Fig. 1).

In transformed mammalian cells, the overexpression of P-glycoprotein and the MDR phenotype are due to amplification or augmented transcription of the *mdr* genes. Our first experiments using *E. histolytica* trophozoites of clone C2, grown in 90 μM emetine, did not show *EhPgp* gene amplification. Southern blot experiments, using DNA from clone A and clone C2 digested with different endonucleases and hybridized with an *EhPgp* fragment, displayed a high number of bands (Fig. 3), confirming that the *EhPgp* genes constitute a multigene family (Table 1). No differences in position, number or intensity of DNA bands were found in these experiments, suggesting no DNA re-arrangements for *EhPgp* genes in the drug-resistant mutant.[60]

EhPgp gene copy number and transcription

The EhPgp gene family in *E. histolytica* is the largest *mdr* gene family found in an organism (Table 1). Southern blot experiments indicated that there is a unique copy for *EhPgp1*, *EhPgp2* and *EhPgp6* genes. Two bands were visible with the *EhPgp5* probe, suggesting that there are at least two copies of this gene, or that the probe cross-reacted with an unidentified *EhPgp* gene.[24]

As no DNA amplification or re-arrangements were detected in emetine-resistant trophozoites, the other possible explanation for the MDR phenotype in clone C2 was over-transcription of the *EhPgp* genes. Hybridization of mRNAs with a probe specific for *EhPgp1* gene revealed a 4–4.5 kb transcript present in similar amount in clone C2 grown at different emetine concentrations (0–200 μM), but absent in the wild-type clone A.[24] Further, primer extension experiments demonstrated that the *EhPgp1* gene is transcribed at a basal amount in clone A.[61] The specific probe for *EhPgp2* gene did not detect any band in clones C2 and A, suggesting that this gene is not transcribed. *EhPgp5* transcript was found only in clone C2 grown at high drug concentration. The quantity of the transcript was higher when the amount of emetine in the culture medium of the trophozoites was increased.[24]

These results indicate that the MDR phenotype in trophozoites grown in up to 200 μM emetine is controlled at the transcriptional level by the overexpression of *EhPgp1* and *EhPgp5* genes.[24] Our more recent results indicate that clone

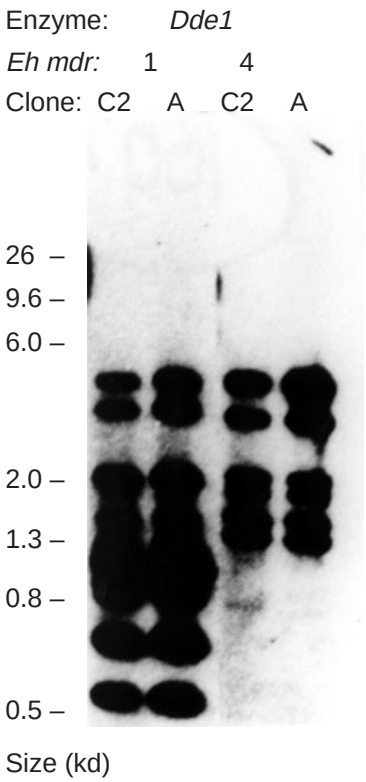

Fig. 3 **Southern blot of DNA from drug-resistant (C2) and drug-sensitive (A) trophozoites. Total DNA was digested with Dde1 restriction enzyme, transferred to nylon membranes and hybridized with two fragments[1,4] labeled with ^{32}P, corresponding to two different *mdr* genes.**

C2 trophozoites grown at higher drug concentration have amplified the *EhPgp* gene.[62] Interestingly, this amplification is exhibited only by some trophozoites and the gene copy number vary from trophozoite to trophozoite (our unpublished data). In other systems overproduction of P-glycoprotein is also mediated by *mdr* gene amplification.[1,39] However, amplification per se seems not to be sufficient to activate expression of a gene that is normally turned off or transcribed at very low level.[63] In mammalian mutant cell lines, *mdr* gene amplification frequently occurs at 100 or 1000-fold excess of drug concentration; that is, after cells have reached high *mdr* mRNA levels, which supports the fact that the regulation of *mdr* gene expression is primarily controlled at the transcriptional level.[61,64–66]

The differential expression of *EhPgp1* and *EhPgp5* genes suggests particular control mechanisms of the MDR phenotype in *E. histolytica*. The *EhPgp1* gene is constitutively expressed in drug-resistant mutants, whereas the *EhPgp5* gene is induced by the presence of the drug. MDR cell lines exhibit an altered pattern of drug resistance according to the different selection schemes.[66] In MDR rodent cell lines, the distinct MDR profiles have been explained, in part, by the presence of two *mdr* genes, each conferring different but overlapping MDR profiles.[67] Thus, we can expect that drug-resistant *E. histolytica* trophozoites, selected with a different scheme to the one used to select clones C2, could present a dissimilar molecular pattern in *EhPgp* genes expression and a different MDR profile.

EhPgp gene expression

The contribution of each *EhPgp* gene to the MDR phenotype in drug-resistant trophozoites is an important issue to elucidate. On the other hand, *EhPgp* genes provide an excellent model to study factors involved in constitutive and induced gene expression regulation in *E. histolytica*. Transfection systems in *E. histolytica* were developed in 1994.[68,69] Later, in 1996, the wild-type clone A was transfected with a plasmid containing the coding regions of bacterial Neomycin phosphotransferase gene (Neo), the *EhPgp1* gene and the 5′ and 3′ ends of the *actin* gene.[43] Transfected trophozoites resistant to G418 only grew in 40 μM emetine, supporting the hypothesis that *EhPgp1* gene participates in emetine resistance at low drug concentration.

The understanding of the mechanisms and factors involved in *EhPgp1* and *EhPgp5* genes expression in drug-resistant trophozoites lies mainly in three areas: (1) alterations in the gene coding regions; (2) mutation in the regulatory regions; and (3) differences in the transcription factors interacting with the regulatory regions.

As the *EhPgp* genes have not been isolated from clone A, it is not possible to know if mutations in their coding regions in drug-resistant trophozoites are responsible for the MDR phenotype. In addition, no significant differences between clones A and C2 were found in the 1000 bp region upstream the ATG start codon in *EhPgp1* and *EhPgp5* genes.[61,64] These results indicate that mutations in the regulatory regions do not participate in the drug-resistance mechanism of the clone C2 trophozoites. However, transfection experiments and studies of the nuclear factors interacting with the *EhPgp* gene promoters have explained the differences in transcription of mutant and wild-type genes.[61,64]

EhPgp1: a gene constitutively expressed

Like the human *MDR1* gene, the *EhPgp1* gene is constitutively expressed. *MDR1* mRNA has been detected in several normal tissues, suggesting a role for its encoded protein in toxin or steroid transport.[70] Several tumors, including leukemia and lymphoma, express high levels of the *MDR1* gene. Thus, tissue-specific factors appear to be important in the regulation of P-glycoprotein expression. *EhPgp1* promoters from drug-susceptible and drug-resistant trophozoites are 99.7% identical. However, there is a higher amount of the *EhPgp1* transcript in the drug-resistant trophozoites than in susceptible ones, suggesting that transcription factors are important for the overexpression of this gene. As other promoters of genes constitutively expressed,[71] the *EhPgp1* promoter does not have the TATA box motif found in other *E. histolytica* genes.[72] Instead, it has two putative Inr sequences that appear to have the same functions as the TATA box.[73,74] The *hgl5* gene, which encodes the *E. histolytica* N-acetylgalactosamine-inhibitable lectin, has a GAACTAA sequence, identical to the one displayed by *EhPgp1* promoter at position –18. This sequence is an Inr element necessary for gene transcription.[75] The human *MDR1* promoter also lacks a TATA box and has an Inr element.[71] By analogy, we suggest that there might be certain similarities in the regulatory

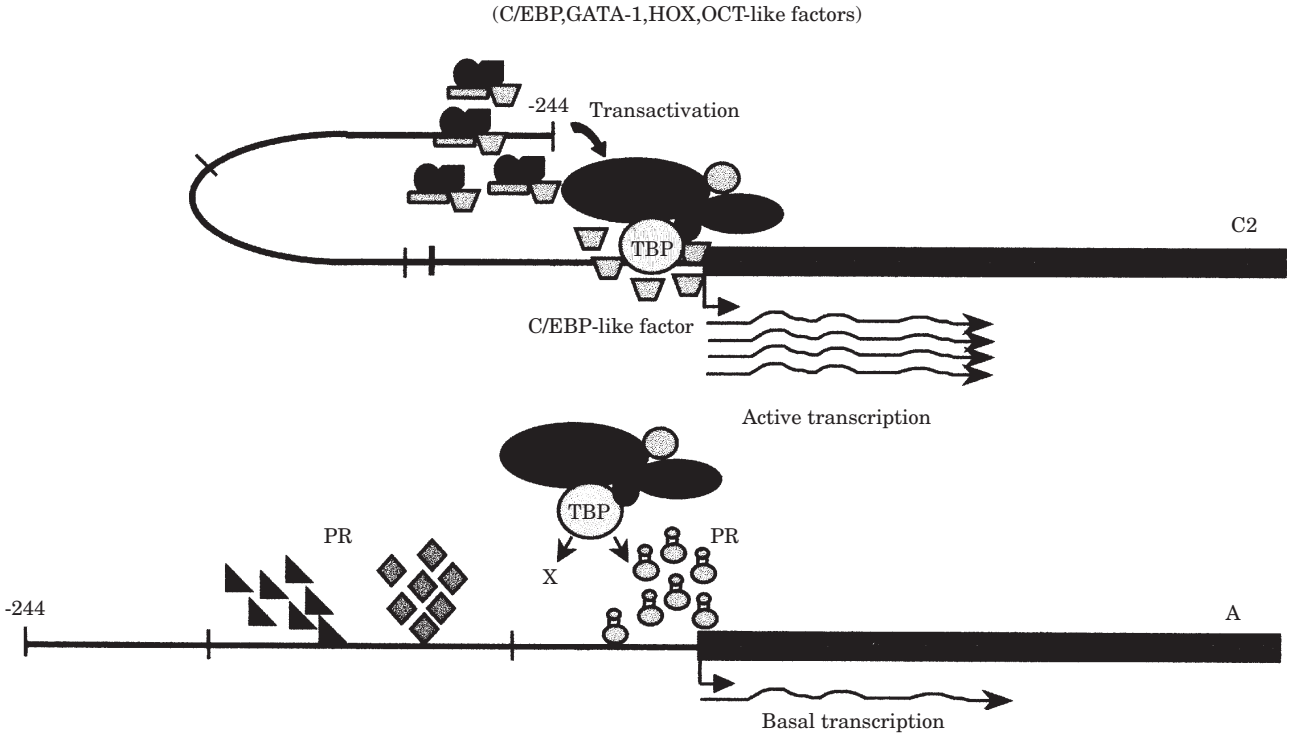

Fig. 4 **Working model proposing a regulatory mechanism for the constitutively expressed EhPgp1 gene. C2: (clone C2) A C/EBP-like factor (▽) bound close to the transcriptional initiation complex (–54 to –43), may act as a transcription activator. A multi-protein complex formed by C/EBP, GATA-1, HOX and OCT-like factors (▽) (–144 to –244) transactivate the EhPgp1 transcription. A (Clone A): the C/EBP and multi-protein complex are not present. DNA binding proteins (◣, ◆, ♂) may act as transcriptional repressors. Wavy arrows: *EhPgp1* transcript in both clones. Small arrow: Correct transcription initiation site. TBP, TATA binding protein; PR, putative repressor.**

mechanisms of the human *MDR1* gene and the *E. histolytica EhPgp1* gene.

The *EhPgp1* promoter core is located in the 250 bp region upstream ATG (Fig. 4). This fragment, cloned in front of the chloramphenicol acetyl transferase (CAT) gene, is able to drive the synthesis of the enzyme in *E. histolytica* drug-resistant trophozoites. However, poor CAT expression was detected in transfected drug-susceptible trophozoites.[61] Putative transcription factors present in clone C2 but absent in clone A trophozoites may be good candidates for the regulation of *EhPgp1* transcription. In the *EhPgp1* promoter, a C/EBP-like sequence located close to the second Inr site forms a complex with a C/EBP-like protein from nuclear extracts of drug-resistant trophozoites (clone C2). The C/EBP protein has been described as an activator in mammalian genes[76,77] and C/EBP consensus sequences have been found in the human *MDR3* and in the mouse *mdr1* promoters, probably acting as *cis*-elements regulating their transcriptional activity.[78] In the region of –144 to –244 bp upstream ATG of the *EhPgp1* gene, a protein complex is formed exclusively with nuclear extracts from clone C2 trophozoites (Fig. 4). This complex was competed by GATA-1, C/EBP, OCT and HOX consensus-binding sequences and may be participating in transcription regulation, since deletion of this region abrogates transcription (our unpublished data). On the other hand, repressors in nuclear extracts from clone A, absent, diminished or modified in clone C2, can not be discarded in studies of the regulation of *EhPgp1* gene expression.[61]

EhPgp5: an induced gene

While the *EhPgp1* gene is constitutively transcribed in drug-resistant trophozoites, *EhPgp5* gene expression is induced when drug concentration is increased in the culture medium. A 1000 bp sequence of the *EhPgp5* promoter from drug-resistant and susceptible trophozoites is 99.6% identical, suggesting that, as in the *EhPgp1* gene, nuclear factors may be regulating its expression. In contrast to the *EhPgp1* gene, the *EhPgp5* gene promoter has a TATA box-like motif at –31 bp of the transcription initiation site.[64] The core promoter of *EhPgp5* gene is also located in the first 250 bp upstream of the transcription initiation site, three bases upstream of ATG.[64] Functional CAT assays demonstrated that the promoter is active at a very low rate in the clone C2 grown without drug. Its activity is increased when trophozoites are cultured in the presence of emetine, and it is turned off in the drug-susceptible clone A.[64] A transcript without an open reading frame is detected in trophozoites grown in the absence of the drug, suggesting that an accurate selection of the transcription initiation site may be a regulating mechanism of the *EhPgp5* gene expression (Fig. 5).

As occurs with the *EhPgp5* gene, expression of the mouse *mdr1b* gene is induced by the presence of drug in

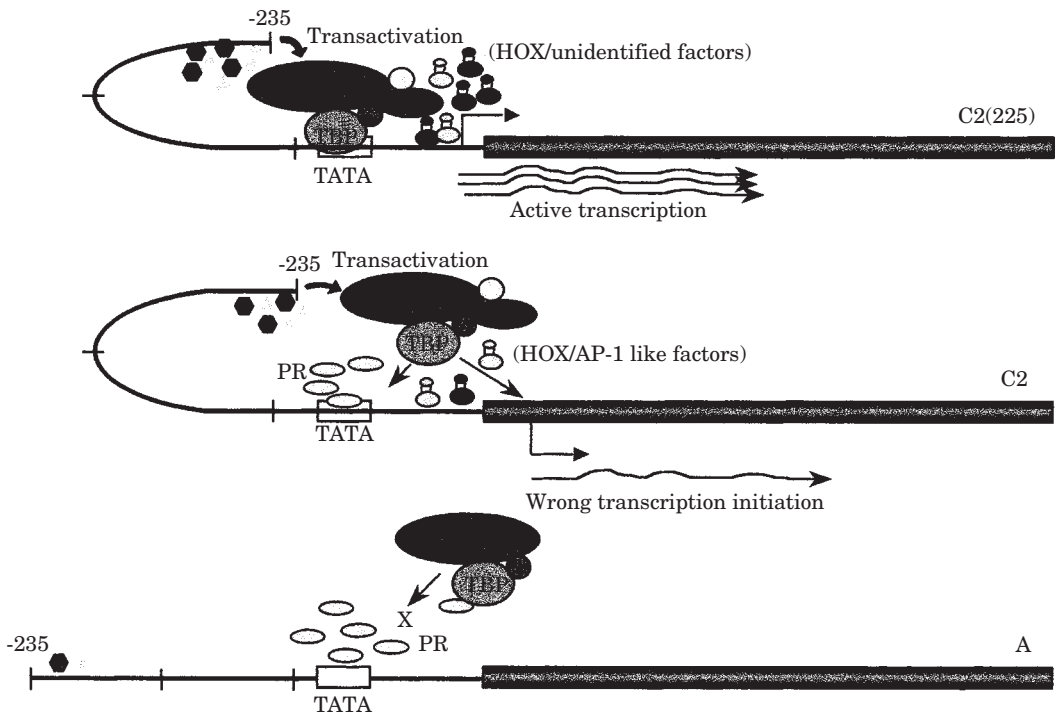

Fig. 5 **Working model proposing a regulatory mechanism for the induced expression of *EhPgp5* gene. C2(225): An HOX and an unidentified factor (♙, ♟) bound close to the transcriptional initiation complex may act as activators. Two complexes (●, ▲) in the region of –235 to –125 transactivate the *EhPgp5* transcription. Clone C2: AP-1/HOX-like factors (♙, ♟) act as activators. A putative repressor (⬭) (PR) was identified close to the TATA box sequence, causing the wrong selection of the transcription initiation site. Clone A: the HOX/AP-1 and HOX-unidentified complexes are absent. The PR (⬭) is in higher amount avoiding the positioning of the transcriptional initiation complex. Wavy arrows: *EhPgp5* transcript. Small arrow: transcription initiation sites. TBP, TATA binding protein; C2(225), clone C2 grown in the presence de 225 μM emetine; C2, clone C2 grown without drug.**

the medium.[79] The AP-1 factor plays an important role in the positive regulation of this gene and the hamster *pgp1* gene.[80] The AP-1 factor has been described as an activator in the mouse *mdr1b* gene promoter.[79] In the *EhPgp5* gene promoter (Fig. 5) there are consensus sequences for AP-1 and HOX-like factors that form complexes with nuclear extracts from drug-resistant trophozoites grown in the absence of the drug. The HOX-like factor and an unidentified complex augment with nuclear extracts from trophozoites grown at high drug concentrations. These complexes are not detected in drug-susceptible trophozoites (Fig. 5). Their differential detection suggest that they may have a role in the *EhPgp5* gene induction. HOX-like proteins, which recognize the ATATTAA motif, have been implicated in the developmental stage-specific activation of the γ-globin gene, promoting specific protein-protein interaction between factors bound to the promoter region.[81] Other unidentified nuclear factors expressed only in the drug-resistant trophozoites could also have a role in the induced expression of *EhPgp5* gene. Additionally, an unidentified DNA-protein complex found exclusively in the drug-susceptible trophozoites might be down-regulating the *EhPgp5* gene expression.[64] This unidentified factor may interfere with the TATA-binding protein provoking the loss of promoter activity in clone A and the wrong selection of initiation site in trophozoites of clone C2 grown without drug.[64] These results, in concordance with the DNA-protein interaction analysis, suggest that the expression of *EhPgp5* gene in clone C2 is regulated by transcriptional factors induced by the presence of emetine.[64]

A HYPOTHETICAL WORKING MODEL TO UNDERSTAND MDR PHENOTYPE IN *E. Histolytica*

How are the *EhPgp1* and *EhPgp5* genes co-regulated to produce the MDR phenotype in *E. histolytica*? Without doubt, more experimental work is necessary to fully understand this. We can hypothesize that the P-glycoprotein encoded by the *EhPgp1* gene (EhPGP1) could be the first pump responsible for the extrusion of drugs in clone C2. It confers basal drug resistance. On the other hand, the P-glycoprotein encoded by the *EhPgp5* gene (EhPGP5) could be functionally more efficient in conferring high-level drug resistance, or it may be more efficiently transcribed. This would explain why resistance to emetine increases during *EhPgp5* gene induction. In other systems it has been demonstrated that the switching of *mdr* genes is accompanied by other cellular alterations such as elevated glutathione S-transferase activity,[82,83] but this has not been explored in *E. histolytica*. It is also possible that the different proteins encoded by *EhPgp* genes may have different affinities for a variety of drugs. It

will be of interest to determine in future studies how all the EhPGP proteins cooperate to give the MDR phenotype.

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