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Biochimica et Biophysica Acta 1770 (2007) 1605 – 1611
Biochimica et Biophysica Acta BBA
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# *Trypanosoma cruzi* as a model system to study the expression of exogenous genes coding for polyamine biosynthetic enzymes. Induction of DFMO resistance in transgenic parasites

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Received 26 April 2007; received in revised form 8 August 2007; accepted 10 August 2007
Available online 6 September 2007

**Abstract**

*Trypanosoma cruzi*, the etiologic agent of Chagas' disease, is a polyamine auxotroph organism because its genome contains neither ornithine decarboxylase (ODC) nor arginine decarboxylase (ADC) genes, presumably lost during evolution. After transformation with a recombinant plasmid bearing the complete coding region of *Crithidia fasciculata ODC* gene, the transgenic parasites were able to synthesize putrescine and simultaneously became susceptible to α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC. We have studied the emergence of DFMO-resistant *T. cruzi* after one-step selection of ODC-transformed parasites cultivated in the presence of high levels of the drug (5 mM). Our results have indicated a duplication of the *ODC* gene copy number in the drug-resistant cell line. The ODC transcripts and the corresponding translation products showed very significant increases (about 7- and 25-fold, respectively) in DFMO-resistant parasites, while the ODC enzymatic activity was 5 times higher than in drug-sensitive *T. cruzi*. The unequal increases of ODC protein and enzymatic activity in DFMO-resistant protozoa strongly suggest that in addition to gene amplification and enhanced transcription and translation, the assembly of ODC polypeptide chains into dimeric active enzyme molecules might also contribute to regulate the development of DFMO resistance.


*Keywords:* *Trypanosoma cruzi*; Ornithine decarboxylase; Polyamine biosynthesis; DFMO resistance; Transgenic parasites

## 1. Introduction

Polyamine biosynthetic enzymes have been considered as probable good targets for antiparasitic chemotherapies [1,2]. Studies carried out in several laboratories have shown that wild-type *Trypanosoma cruzi* epimastigotes are unable to synthesize putrescine de novo [3–6]. These parasites do not contain ornithine decarboxylase (ODC) enzymatic activity [2–4], which catalyses the first step of polyamine biosynthetic pathway in mammalian cells as well as in most protozoan organisms [2,7]. We have previously reported that *T. cruzi* epimastigotes also lack arginine decarboxylase (ADC) activity [6,8], the enzyme involved in the other pathway known to synthesize putrescine in bacteria and plants [7]. All these results were confirmed at physiological and biochemical levels by cultivating different strains of *T. cruzi* epimastigotes in a semi-defined medium (SDM-79) almost free of polyamines [5,9]. Under these conditions, parasite proliferation was arrested after several passages, when polyamine endogenous pools of protozoa were markedly depleted. At this point growth could be resumed by supplementing the medium with putrescine, cadaverine or spermidine, but not with ornithine, lysine or arginine [9]. Although these basic amino acids are efficiently taken up, they are not polyamine precursors in wild-type *T. cruzi*, which therefore behaves as one of the few eukaryotic organisms auxotrophic for polyamines [3,4,10].

The absence of both, ODC and ADC enzymatic activities in *T. cruzi* epimastigotes might be due to the presence of ODC and ADC inhibitors in the internal medium of *T. cruzi* or to the repression of *ODC* and *ADC* gene expressions inside the parasites. We have investigated both possibilities after successful transformations of wild-type *T. cruzi* epimastigotes

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doi:10.1016/j.bbagen.2007.08.013

with recombinant plasmids bearing the complete coding region of heterologous *ODC* or *ADC* genes [5,8,11]. In both cases, we have observed good levels of expression of the foreign genes in the transgenic parasites, pointing to the absence of enzymatic inhibitors or repressors that could block ODC or ADC expression.

Previous experiments on Southern hybridization of wild-type *T. cruzi* DNA with specific probes homologous to conserved regions of *ODC* and *ADC* genes from many organisms, strongly suggested the absence of these sequences in the *T. cruzi* genome [5,8]. The same conclusion was supported by bioinformatic analysis using the available data from the *T. cruzi* genome project [8]. In addition, after careful phylogenetic analyses, Steglich and Schaeffer have recently concluded that a common ancestor of *T. cruzi* and several African trypanosomes has lost its *ODC* gene during evolution, and that *Trypanosoma brucei* and other related trypanosomatids (but not *T. cruzi*) were then able to recover a new copy of a homologous gene by horizontal transfer from a vertebrate organism [12].

In the present work we have used the ODC-transformed parasites as a tool to investigate for the first time the induction of transgenic *T. cruzi* resistance to DFMO and the involved mechanisms. In addition, we have performed a biochemical and molecular characterization of the DFMO-resistant protozoa. The same system of ODC-transgenic *T. cruzi* might also be useful to investigate the effects of reduced endogenous levels of polyamines on the physiology of trypanosomatid parasites.

## 2. Materials and methods

### 2.1. Materials

Brain–heart infusion, tryptose and yeast extracts were purchased from Difco Laboratories (Detroit, MI, USA). Minimal essential medium (SMEM), amino acids and vitamins were from Gibco/BRL (Gaithersburg, MD, USA). Bases, haemin, pyridoxal 5′-phosphate, polyamines, HEPES buffer and antibiotics were obtained from Sigma (St. Louis, MO, USA). Fetal calf serum was purchased from Natocor (Carlos Paz, Córdoba, Argentina). L[1-$^{14}$C]ornithine (58 Ci/mol) and [$^{32}$P] dCTP [α P] (3000 Ci/mmol) were from Amersham Life Sciences (Buckinghamshire, UK). DFMO was a generous gift from Merrell Dow Research Institute (Cincinnati, OH, USA).

### 2.2. Parasite cultures

*T. cruzi* epimastigotes, strain Tul 2 [13] was cultivated at 28 °C in the rich medium BHT [14] or in a semi-defined medium (SDM-79), which contains only traces of polyamines [5,15]. Using the latter medium, wild-type strains of *T. cruzi* are unable to grow continuously for long periods of time, and proliferation stops after several passages due to a marked depletion of the parasite polyamine internal pools [5]. All cultures were supplemented with haemin (20 mg/l), 10% heat-inactivated fetal calf serum and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin). Parasite growth was followed by cell counting and cultures were diluted weekly to 10–15 × $10^6$ cells/ml using fresh medium in the absence or presence of DFMO at the indicated concentrations.

### 2.3. Preparation of parasite extracts and ODC assay

Parasites were collected at the exponential phase of growth by centrifugation at 3000×*g* for 10 min, washed once with PBS and resuspended ($10^9$ cells/ml) in 50 mM HEPES sodium buffer solution, pH 7.0, containing 1 mM DTT, 0.5 mM EDTA and 0.2 mM pyridoxal 5′-phosphate. Cells were lysed by addition of Nonidet P-40 at a final concentration of 0.5% (v/v) and a subsequent incubation for 30 min at 0 °C. After a 10-s sonication to break down DNA, cell extracts were centrifuged for 20 min at 20,000×*g* and the supernatant liquids were collected and stored at −70 °C.

ODC activity was measured as previously described by the release of $^{14}CO_2$ from radioactive ornithine [16]. Protein concentration of enzyme preparations was determined according to Bradford [17] using BSA as standard.

### 2.4. Construction of the recombinant plasmid pODC7 and parasite transfection

A DNA segment about 2.2 kbp long containing the complete coding region of the *ODC* gene from *Crithidia fasciculata* was cloned as previously described [5,18] in the expression vector pRIBOTEX [19]. Plasmid pODC7 [5] bearing the ODC ORF inserted in the sense orientation was used either as closed circles or after linearization with the restriction enzyme *Nhe*I to transform wild-type *T. cruzi* epimastigotes according to a protocol already published [20,21]. After electroporation of 3 × $10^8$ parasites with 50–100 μg of pODC7 followed by dilution with BHT medium containing 10% fetal calf serum, parasites were incubated at 28 °C for 48 h before adding Geneticin (G418, 250 μg/ml) which allows the selection of transformed protozoa. Samples of *T. cruzi* cultures were collected before and after different times of electroporation and subsequent cultivation in the absence or presence of DFMO to measure ODC enzymatic activities and to prepare DNA and RNA for hybridization analyses, or total extracts for Western assays.

### 2.5. Sensitivity to DFMO of ODC-transformed T. cruzi cultures

In order to measure the sensitivity of different cultures to DFMO we have followed essentially the procedure previously described [22]. The transformed parasites were cultivated by repeated passages in SDM-79 medium in the absence (control) and presence of different concentrations of DFMO. The initial parasite density of cultures was 5 × $10^6$ cells/ml, and every 3 days, they were diluted to the initial cell concentration with fresh medium containing the appropriate levels of the inhibitor. After eight rounds of dilution and subsequent cultivation in the absence and presence of DFMO, cells were counted 72 h after the last dilution and the percentages of growth inhibition relative to the corresponding control were calculated.

### 2.6. Southern and Northern hybridization analyses

Total DNA from wild-type and ODC-transformed *T. cruzi* was prepared as previously described [23]. With this procedure, it is possible to recover genomic as well as episomal DNA. After digestion with restriction enzymes the resulting DNA fragments were separated by electrophoresis on 1% agarose gels and then transferred onto nylon membranes (Hybond $N^+$, Amersham).

Preparations of total RNA were carried out with TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) [24], fractionated by denaturing agarose gel electrophoresis and transferred onto nylon membranes.

DNA and RNA samples for hybridization analyses were obtained from parasites cultivated for 30 days in the absence of DFMO.

Hybridization assays were performed by standard procedures using a $^{32}$P-labeled DNA probe (810 bp) specific for *ODC* gene which was prepared by PCR amplification using DNA from *C. fasciculata* as template and the pair of primers 1C (5′-CCGGAATTCCCGCACTTTGCCGTGAAGTGCAAC-3′) and 7C (5′-CCGGAATTCGTAGAGACCGTCGTTCACGTAGTA-3′), corresponding to the nucleotide sequence of the 5′ and 3′ limits of an internal segment of the *C. fasciculata ODC* gene.

### 2.7. PCR analyses of transformed parasites DNA

In order to elucidate whether the transforming plasmid was integrated into the genome of transgenic parasites or remained as free episomes after transformation, samples containing 50–100 ng of total DNA from DFMO-sensitive and resistant parasites were amplified by PCR using two sets of primers: (1) the forward primer T7 (Promega, Madison, WI, USA), specific for the promoter region of the pRIBOTEX vector, and the reverse primer RIB, complementary to

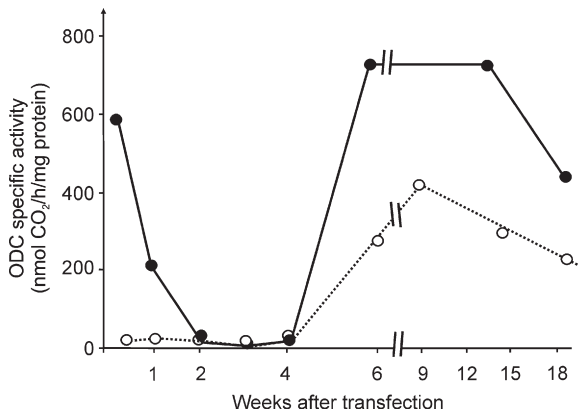


Fig. 1. Time course of ODC activity after electroporation. *T. cruzi* epimastigotes were transformed with closed circles (●) or linearized (○) pODC7 plasmid and the enzymatic activities were measured at the indicated times.

an internal sequence of the parasite ribosomal locus [25], and (2) with the pair of T7 and the reverse primer 2C (5′-CCGGAATTCGAGCTGCTTA-CACGGGTTCGCGAA-3′) complementary to an internal segment of *C. fasciculata ODC* gene. PCR amplification reactions and analyses of their products were performed as previously described [11].

### 2.8. Detection of ODC protein by Western assays

Parasite extracts from DFMO-sensitive and resistant ODC-transformed *T. cruzi* were prepared as described above. Samples corresponding to DFMO-resistant parasites were obtained 30 days after removal of the drug. Aliquots containing 20 to 50 μg of total protein were analysed by SDS-polyacrylamide gel electrophoresis (PAGE), subsequent blotting to a nitrocellulose membrane (Hybond-C extra, Amersham) and immuno-detection with a polyclonal antibody against *C. fasciculata* ODC, kindly provided by Dr. L. Persson (Department of Physiological Sciences, University of Lund, Sweden). Cruzipain, a house-keeping *T. cruzi* protein, was used as loading controls in Western assays. The antibody against cruzipain was a gift from Dr. J.J. Cazzulo (University of San Martin, Argentina). Proteins reacting with antibodies were detected using the Super Signal West Dura kit (Pierce Biotechnology, Inc., Rockford, IL, USA) followed by X-ray film exposure.

## 3. Results and discussion

### 3.1. Heterologous ODC gene expression in transgenic parasites

*T. cruzi* behaves as a null mutant of *ODC* gene and constitutes an appropriate system to study the expression of foreign genes coding for the same or other related enzymes. After transformation of wild-type *T. cruzi* epimastigotes with the recombinant plasmid pODC7 bearing the ORF of *C. fasciculata* ODC, the transgenic parasites were able to synthesize putrescine from ornithine and at the same time became susceptible to DFMO which inhibits ODC specifically [5].

*T. cruzi* epimastigotes were transformed with the recombinant plasmid pODC7 as closed circles or linearized plasmid forms, which contained the complete coding region of *C. fasciculata ODC* gene [5,18]. Geneticin (G418) was added to cultures after 48 h for the selection of plasmid-containing protozoa and ODC enzymatic activity of the transfected *T. cruzi* was measured at different times after electroporation. Parasites transformed with the closed recombinant plasmid showed high levels of ODC activity early after transfection that decreased markedly during the following weeks, while protozoa containing the open plasmid gave a rather low and almost constant ODC activity during the first period post-transfection. At 5 or 6 weeks after transformation, cultures in the continuous presence of the selection agent G418 showed that the enzyme activities in all cases increased up to high values which remained at elevated levels for several months. The results shown in Fig. 1, representative of two independent transfections, indicate that transformation with the recombinant plasmid as closed circle forms was able to elicit transient and stable expressions of the heterologous gene, whereas the linearized transforming plasmid gave mainly a stable expression. These differential effects of closed and opened plasmids might be explained by a rapid although not complete degradation of the latter by intracellular exonucleolytic enzymes early after transfection. We cannot discard the possibility that an undetectable fraction of pODC7 remained as closed circles even after exhaustive digestion with the restriction enzyme *Nhe*I, and that these plasmid forms were responsible for the stable *ODC* gene expression.

All subsequent results were obtained with parasites transformed with the closed circular form of pODC7 plasmid.

### 3.2. Induction of DFMO resistance in ODC-transformed parasites

Wild-type *T. cruzi* is auxotrophic for polyamines [4,5], while ODC-transformed parasites become autotrophic for these substances, since the transgenic protozoa can grow continuously in the semi-defined medium without the addition of exogenous polyamines [5]. These transformed *T. cruzi* behaved as sensitive to DFMO, which was able to block the proliferation of the transgenic parasites growing in the synthetic medium after inhibiting the activity of the newly expressed heterologous ODC (Fig. 2). However, after a period of growth inhibition of several weeks caused by the drug, the continuous presence of DFMO at a concentration of 5 mM elicited the spontaneous reinitiation of growth with the concomitant selection of DFMO-resistant parasites which grew normally even in the presence of high concentrations of the drug as shown in Figs. 2 and 3. We

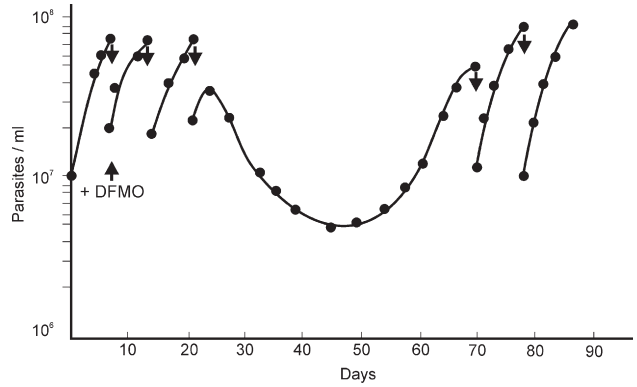


Fig. 2. Effect of DFMO on the growth of ODC-transformed parasites cultivated in SDM-79 medium. After 7 days of culture 5 mM DFMO was added. At the times indicated by downward arrows cultures were diluted with fresh medium containing the inhibitor.

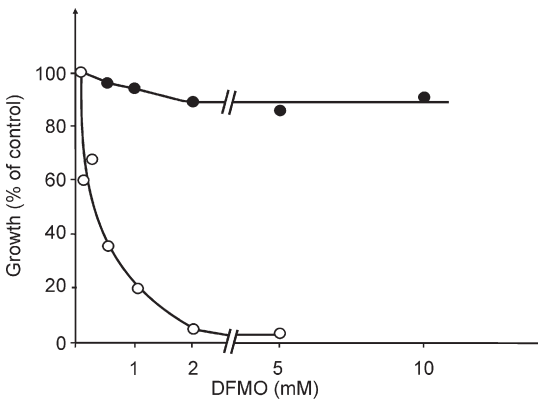


Fig. 3. Effect of DFMO on the growth rate of drug-sensitive (○) or resistant (●) transformed parasites. ODC-transfected *T. cruzi* were cultivated in the presence of increasing concentrations of the drug under the conditions described in Materials and methods. The results were plotted as percentages of growth in the absence of the inhibitor and were the average of duplicate experiments.

have used a one-step selection with high drug doses during the induction of DFMO resistance in the ODC-transformed parasites as previously reported for *Leishmania mexicana* [22]. This procedure differs markedly from the slow stepwise selection followed by other authors for different drugs and protozoa [26–28].

### 3.3. Sensitivity of transgenic T. cruzi to DFMO

ODC-transfected parasites previously cultivated for several months in the absence or presence of 5 mM DFMO were washed carefully and subsequently grown without the drug for 30 days. Different concentrations of DFMO were then added to various aliquots of these cultures and each condition was maintained for eight passages by repeated dilution to the same initial concentration of cells as described in Materials and methods. The number of parasites was counted 3 days after the last passage and the percentages of proliferation relative to the control cultures growing in the absence of inhibitor were calculated. Fig. 3 shows that the growth of ODC-transformed protozoa permanently cultivated in the absence of DFMO was markedly reduced (about 50%) at 0.1 mM DFMO, whereas transgenic parasites previously selected by high doses of the inhibitor did not alter their proliferation rate even in the presence of a concentration 100-fold higher.

### 3.4. ODC enzymatic activity in DFMO-sensitive and resistant transgenic parasites

The induction of resistance to DFMO in cultures of ODC-transformed *T. cruzi* incubated with high levels of the inhibitor for long periods of time was probably due to the increase of the enzyme-specific activity (Fig. 4). We have discarded the possibility that the emergence of DFMO resistance might be due to an impairment of the drug uptake by parasites because the *in vivo* inhibition of ODC activity by DFMO showed the same kinetics in DFMO-sensitive and -resistant ODC-transformed *T. cruzi* (a 90% of enzyme inhibition was measured in both cases after parasites incubation for 2 h in the presence of 5 mM DFMO). We have also ruled out a change of the target enzyme structure with a presumably concomitant decrease of the affinity for the inhibitor in the ODC of DFMO-resistant parasites. This conclusion is in agreement with the kinetic parameters measured for the enzymes of DFMO-sensitive and resistant protozoa. In both cases we have obtained approximately the same values of the apparent $K_m$ for ornithine (0.87 and 0.77 ± 0.10 mM) and $K_i$ for DFMO (90 and 100 ± 10 μM) with drug-sensitive and -resistant parasites, respectively. In contrast to these results, the ODC-specific activity was markedly higher in DFMO-resistant *T. cruzi* (Fig. 4B). In order to compare ODC activities in DFMO-sensitive and -resistant parasites, we have first cultivated the latter in the absence of the inhibitor for several weeks to allow the excretion of the intracellular free drug, therefore avoiding the inhibition of newly synthesized enzyme molecules. Fig. 4A indicates that ODC activity of resistant cells increased steadily during a period of about 30 days after the removal of DFMO from the cultures. Under these conditions, ODC-specific activity of drug-resistant parasites was 5-fold higher than in DFMO-sensitive cells (Fig. 4B), and this increase can account for the alteration of the parasite susceptibility to the drug [22]. It is interesting to mention that the enzyme of both kinds of protozoa could be completely inhibited by DFMO when this compound was added to the ODC assay (Fig. 4B).

### 3.5. ODC gene amplification in DFMO-resistant transformed T. cruzi

Although many different mechanisms of drug resistance have been described [29,30], gene amplification and transport alterations are the most frequent in parasitic protozoa. In order to elucidate the molecular mechanisms involved in the induction of DFMO resistance in transformed *T. cruzi*, total DNA was obtained from DFMO-sensitive and -resistant transgenic parasites and digested with *Bam*HI and *Sac*I restriction enzymes. The separated DNA fragments were subjected to hybridization

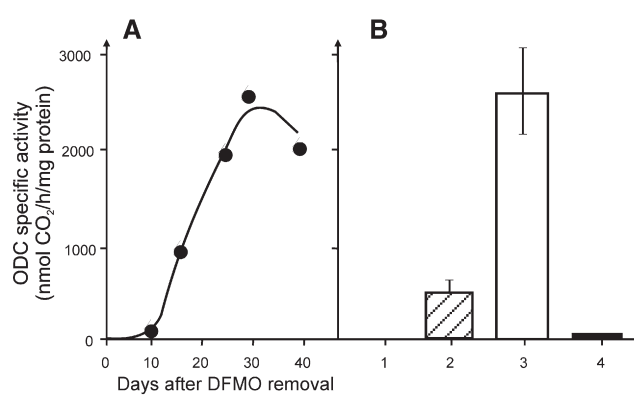


Fig. 4. ODC-specific activity in DFMO-sensitive and -resistant transgenic parasites. (A) DFMO was removed from cultures of drug-resistant transformed protozoa and ODC activity was measured at different times thereafter. (B) ODC-specific activity in extracts of wild-type (1), DFMO-sensitive (2) and resistant parasites 30 days after drug removal, in the absence (3) or presence (4) of DFMO added to the enzyme assay. The standard deviation for three experiments is indicated.

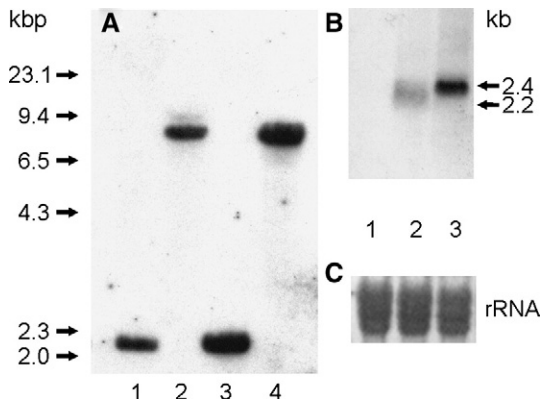


Fig. 5. Southern and Northern hybridization analyses of DNA and RNA from ODC-transformed *T. cruzi*. (A) 15 μg of DNA from DFMO-sensitive (lanes 1 and 2) or -resistant (lanes 3 and 4) parasites was digested with *Bam*H I (lanes 1 and 3) or *Sac*I (lanes 2 and 4) restriction enzymes and then subjected to electrophoresis on a 1% agarose gel. (B) 20 μg of total RNA obtained from wild-type *T. cruzi* (lane 1) and DFMO-sensitive (lane 2) or -resistant (lane 3) transformed parasites were subjected to electrophoresis on a 1% agarose/2.2 M formaldehyde gel. After transference to nylon membranes, Southern and Northern hybridization assays were carried out with the radioactive ODC-specific probe described in Materials and methods. (C) The methylene-blue stained rRNA bands used as loading controls are shown.

assays with the radioactive probe specific for ODC described in Materials and methods. We have obtained one hybridizing fragment in each case, of 2.2 and 8.2 kbp for digestions with *Bam*HI and *Sac*I, respectively (Fig. 5A). The labeled band signals corresponding to DFMO-resistant parasites were stronger than those derived from the drug-sensitive cell line, and densitometric scanning of autoradiograms indicated that the *ODC* gene copy number in DFMO-resistant transgenic *T. cruzi* was about 2-fold higher than in drug-sensitive parasites.

### 3.6. ODC messenger RNA in the transgenic parasites

Total RNA samples prepared from DFMO-sensitive and resistant ODC-transformed *T. cruzi* were subjected to Northern hybridization analysis with the same specific probe already mentioned. Broad ODC mRNA bands with a size of about 2.2–2.4 kb were detected, which might indicate two mRNA species (Fig. 5B). After normalization for loading rRNA controls (Fig. 5C), the relative intensities of bands indicated that ODC mRNA was around 6 to 8-fold more abundant in DFMO-resistant transgenic *T. cruzi.*

### 3.7. Genome organization of ODC-transformed parasites

To ascertain whether the transforming plasmid pODC7 was integrated into the parasite genome or remained as a free episome, we have carried out two different PCR amplification assays as detailed in Materials and methods. We could predict that if the recombinant plasmid remained free as an extrachromosomal element, the product of the amplification reaction with the pair of primers T7 and 2C should be a DNA segment of 1940 bp (Fig. 6A). On the other hand if total integration of pODC7 into the parasite genome had occurred the PCR assay with primers T7 and RIB should give a DNA segment of 890 bp

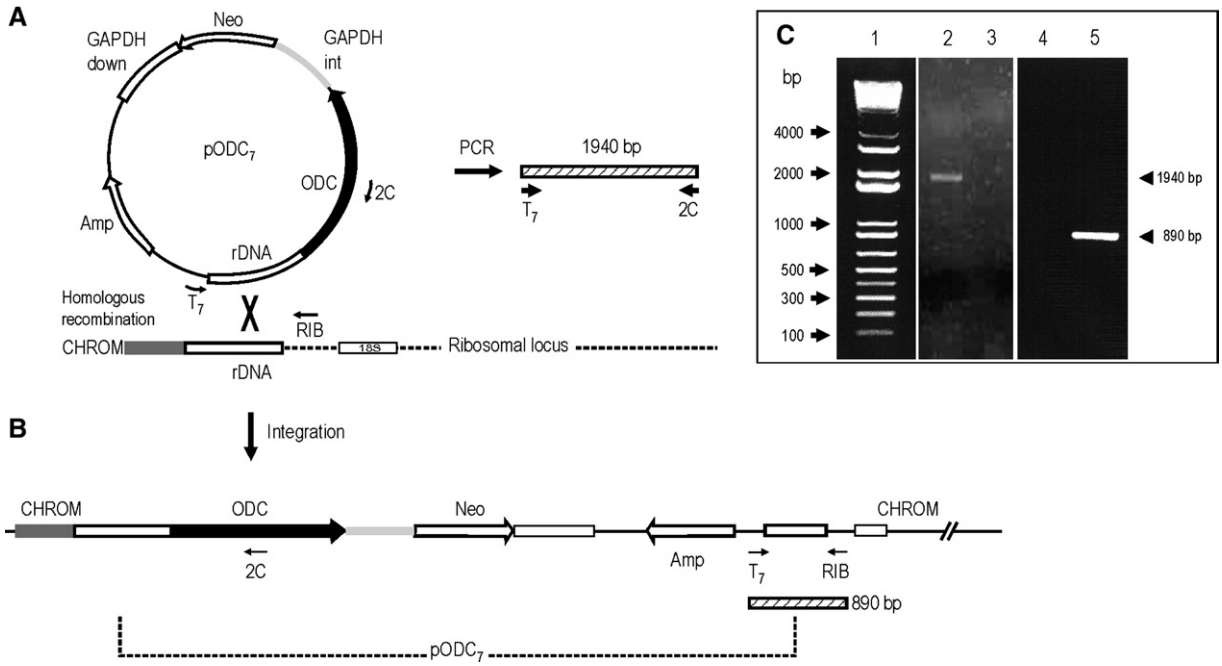


Fig. 6. Genome organization of ODC-transformed *T. cruzi* studied by PCR amplification. Schematic diagrams predicting the fate of the plasmid pODC7 in transgenic parasites as free extrachromosomal elements (A) or after integration of one plasmid copy into the parasite genome (B). The hatched horizontal segments represent the expected PCR amplification products for DNA samples corresponding to each type of genome organization. The primers annealing sites are indicated by arrows. (C) PCR amplification products obtained as described in Materials and methods with DNA from DFMO-sensitive transformed parasites collected after 3 h (lanes 2 and 3) or 6 weeks (lanes 4 and 5) after transformation, using the pair of primers T7 and 2C (lanes 2 and 4) or T7 and RIB (lanes 3 and 5). DNA ladder standard (lane 1). PCR products were analysed by electrophoresis on agarose gels containing ethidium bromide and observed under UV light.

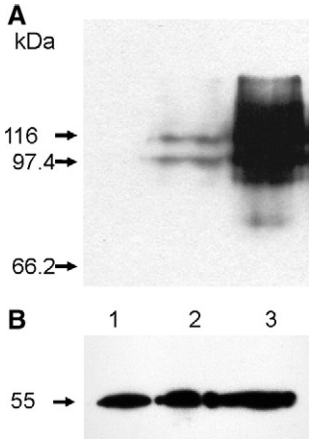


Fig. 7. Western assays of ODC protein in extracts of wild-type *T. cruzi* (lane 1) and DFMO-sensitive (lane 2) or -resistant (lane 3) transformed parasites (A). Cruzipain bands detected with a specific antibody were used as loading controls (B). Experimental details as described in Materials and methods.

(Fig. 6B). The PCR approach to study the genome organization in transformed parasites was validated by working with DNA from transgenic *T. cruzi* collected 3 h or 6 weeks after electroporation. In the former case the transforming plasmid remains as a free episome while in the latter it is integrated into the parasite genome. After agarose gel electrophoresis of PCR products obtained with both pairs of primers, we have only detected the 1940- or 890-bp products, with the sizes expected for the free episome or the integration of one plasmid copy into the parasite genome, respectively (Fig. 6C). DNA preparations from DFMO-sensitive and -resistant transgenic parasites gave the same results shown in Fig. 6C. We cannot as yet discard that more than one copy of pODC7 plasmid arranged in tandem might be integrated into the genome of DFMO-resistant transformed parasites.

Experiments of Southern hybridization and PCR amplification analyses during the 48 h following the parasite transfection showed an intense degradation of the free episome during this period, before the integration of the remaining pODC7 plasmid by homologous recombination, presumably at a rRNA locus of the parasite genome [19]. We have been able to confirm the integration of the transforming plasmid into the parasite genome by pulsed-field gel electrophoresis (PFGE) of chromosomes prepared from DFMO-sensitive and -resistant protozoa, followed by hybridization with the labeled ODC-specific probe. The radioactive bands obtained in both cases have the same mobility as a parasite chromosome bearing an rRNA locus (C.C., unpublished results).

### *3.8. ODC protein in extracts of DFMO-sensitive and -resistant transgenic T. cruzi*

The translation product corresponding to the expression of the foreign *ODC* gene in the transformed protozoa was detected by Western assays carried out on parasite extracts subjected to PAGE followed by a reaction with an ODC-specific antibody, as described in Materials and methods. Fig. 7 shows that the extracts from DFMO-resistant parasites contained an amount of ODC protein remarkably higher (25–30 times) than that present in drug-sensitive transformed protozoa. It is interesting to mention that protein bands corresponding to ODC showed a molecular size of 105–110 kDa, while the predicted value for the *C. fasciculata* ODC is 79 kDa [18]. We have recently found that the heavier forms of ODC protein seem to contain ubiquitin since anti-ubiquitin antibodies recognized the 105- to 110-kDa polypeptide bands as well as those corresponding to other proteins (N.S.G., unpublished work). The various protein bands seen in Fig. 7 might correspond to multiple forms of poly-ubiquitinated polypeptides. A protein similar to mammalian antizyme was not found in trypanosomatid parasites [31]; therefore, ODC degradation in these protozoa could be ubiquitin-dependent [32].

Our results seem to indicate that several mechanisms, namely gene amplification and a concomitant enhancement of transcription and translation might contribute to the emergence of DFMO resistance in ODC-transgenic *T. cruzi*. The discrepancy found in the ratios of ODC protein amounts and the ODC-specific enzymatic activities between DFMO-resistant and -sensitive parasites (Figs. 7 and 4B, respectively) strongly suggests a hypothetical additional regulation at the level of polypeptide chains assembly to produce enzymatically active dimeric molecules. However, we cannot rule out the contribution of other unknown factors.

## Acknowledgements

We thank Drs. Sara H. Goldemberg and Maximiliano Juri Ayub for their helpful discussions and Edith Trejo and Carlos Zadikian for their technical assistance. This work was partially supported by grants from the University of Buenos Aires, Agencia Nacional de Promoción Científica y Tecnológica and the National Research Council (CONICET, Argentina).

## References

[1] O. Heby, S.C. Roberts, B. Ullman, Polyamine biosynthetic enzymes as drug targets in parasitic protozoa, Biochem. Soc. Trans. 31 (2003) 415–419.
[2] S. Müller, G.H. Coombs, R.D. Walter, Targeting polyamines of parasitic protozoa in chemotherapy, Trends Parasitol. 17 (2001) 242–249.
[3] I.D. Algranati, C. Sánchez, S. González, Polyamines in *Trypanosoma cruzi* and *Leishmania mexicana*, in: S.H. Goldemberg, I.D. Algranati (Eds.), The Biology and Chemistry of Polyamines, Oxford Univ. Press, Oxford, 1990, pp. 137–146.
[4] M.R. Ariyanayagan, A.H. Fairlamb, Diamine auxotrophy may be a universal feature of *Trypanosoma cruzi* epimastigotes, Mol. Biochem. Parasitol. 84 (1997) 111–121.
[5] C. Carrillo, S. Cejas, N.S. González, I.D. Algranati, *Trypanosoma cruzi* epimastigotes lack ornithine decarboxylase but can express a foreign gene encoding this enzyme, FEBS Lett. 454 (1999) 192–196.
[6] C. Carrillo, S. Cejas, A. Huber, N.S. González, I.D. Algranati, Lack of arginine decarboxylase in *Trypanosoma cruzi* epimastigotes, J. Eukaryot. Microbiol. 50 (2003) 312–316.
[7] S.S. Cohen (Ed.), A Guide to the Polyamines, Oxford University Press Inc., New York, 1998.
[8] C. Carrillo, M.P. Serra, C.A. Pereira, A. Huber, N.S. González, I.D. Algranati, Heterologous expression of a plant arginine decarboxylase gene in *Trypanosoma cruzi*, Biochim. Biophys. Acta 1674 (2004) 223–230.
[9] C. Carrillo, S. Cejas, M. Cortés, C. Ceriani, A. Huber, N.S. González, I.D. Algranati, Sensitivity of trypanosomatid protozoa to DFMO and metabolic

turnover of ornithine decarboxylase, Biochem. Biophys. Res. Commun. 279 (2000) 663–668.

[10] T. Cook, D. Roos, M. Morada, G. Zhu, J.S. Keithly, J.E. Feagin, G. Wu, N. Yarlett, Divergent polyamine metabolism in the Apicomplexa, Microbiology 153 (2007) 1123–1130.

[11] M.P. Serra, C. Carrillo, N.S. González, I.D. Algranati, Modulation of oat arginine decarboxylase gene expression and genome organization in transgenic *Trypanosoma cruzi* epimastigotes, FEBS J. 273 (2006) 628–637.

[12] C. Steglich, S.W. Schaeffer, The ornithine decarboxylase gene of *Trypanosoma brucei*: evidence for horizontal gene transfer from a vertebrate source, Infect. Genet. Evolut. 6 (2006) 205–219.

[13] E.L. Segura, E. Subias, M. Esteva, P. Cabeza Meckert, A. Brozina, R.P. Laguens, Características de infectividad de tres poblaciones de cultivo de *Trypanosoma cruzi*, Medicina (Buenos Aires) 40 (1980) 97–102.

[14] J.J. Cazzulo, B.M. Franke de Cazzulo, C.J. Engel, J.J.B. Cannata, End products and enzyme levels of aerobic glucose fermentation in trypanosomatids, Mol. Biochem. Parasitol. 16 (1985) 329–343.

[15] R. Brun, M. Schonenberger, Cultivation and *in vivo* cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium, Acta Trop. 36 (1979) 289–292.

[16] C.P. Sánchez, N.S. González, I.D. Algranati, Stable ornithine decarboxylase in promastigotes of *Leishmania mexicana*, Biochem. Biophys. Res. Commun. 161 (1989) 754–761.

[17] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[18] F. Svensson, C. Ceriani, E. Lövkvist Wallström, I. Kockum, I.D. Algranati, O. Heby, L. Persson, Cloning of a trypanosomatid gene coding for an ornithine decarboxylase that is metabolically unstable even though it lacks the C-terminal degradation domain, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 397–402.

[19] S. Martínez-Calvillo, I. López, R. Hernández, pRIBOTEX expression vector: a pTEX derivative for a rapid selection of *Trypanosoma cruzi* transfectans, Gene 199 (1997) 71–76.

[20] S. Hariharan, J. Ajioka, J. Swindle, Stable transformation of *Trypanosoma cruzi*: inactivation of the PUB 12.5 polyubiquitin gene by targeted gene disruption, Mol. Biochem. Parasitol. 57 (1993) 15–30.

[21] N.Y. Lu, G.A. Buck, Expression of an exogenous gene in *Trypanosoma cruzi* epimastigotes, Mol. Biochem. Parasitol. 44 (1991) 109–114.

[22] C.P. Sánchez, J. Mucci, N.S. González, M.M. A.Ochoa, I.D. Zakin, α-Difluoromethylornithine-resistant cell lines obtained after one-step selection of *Leishmania mexicana* promastigote cultures, Biochem. J. 324 (1997) 847–853.

[23] E. Medina-Acosta, G.A.M. Cross, Rapid isolation of DNA from trypanosomatid protozoa using a simple "mini-prep" procedure, Mol. Biochem. Parasitol. 59 (1993) 327–330.

[24] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.

[25] H.A. Lorenzi, M.P. Vázquez, M.J. Levin, Integration of expression vectors into the ribosomal locus of *T. cruzi*, Gene 310 (2003) 91–99.

[26] J.C. Coderre, S.M. Beverley, R.T. Schimke, D.V. Santi, Overproduction of a bifunctional thimidylate synthetase-dihydrofolate reductase and DNA amplification in methotrexate-resistant *Leishmania tropica*, Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 2132–2136.

[27] T. Coons, S. Hanson, A.J. Bitonti, P.P. McCann, B. Ullman, Alpha-difluoromethylornithine resistance in *Leishmania donovani* is associated with increased ornithine decarboxylase activity, Mol. Biochem. Parasitol. 39 (1990) 79–90.

[28] A. Olmo, R. Arrebola, V. Bernier, D. González-Pacanowska, L.M. Ruiz-Pérez, Co-existence of circular and multiple linear amplicons in methotrexate-resistant *Leishmania*, Nucleic Acids Res. 23 (1995) 2856–2864.

[29] P. Borst, M. Ouellette, New mechanisms of drug resistance in parasitic protozoa, Annu. Rev. Microbiol. 49 (1995) 427–460.

[30] B. Papadopoulou, C. Kundig, A. Singh, M. Oullette, Drug resistance in *Leishmania*: similarities and differences to other organisms, Drug Resist. Updat. 1 (1998) 266–278.

[31] S.B. Hua, X. Li, P. Coffino, C.C. Wang, Rat antizyme inhibits the activity and does not promote the degradation of mouse ornithine decarboxylase in *Trypanosoma brucei*, J. Biol. Chem. 270 (1995) 10264–10271.

[32] L. Persson, A. Jeppsson, S. Nasizadeh, Turnover of trypanosomal ornithine decarboxylases, Biochem. Soc. Trans. 31 (2003) 411–414.