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Differential Gene Expression in Benznidazole-Resistant *Trypanosoma cruzi* Parasites

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We analyzed the differential gene expression among representative *Trypanosoma cruzi* stocks in relation to benznidazole exposures using a random differentially expressed sequences (RADES) technique. Studies were carried out with drug pressure both at the natural susceptibility level of the wild-type parasite (50% inhibitory concentration for the wild type) and at different resistance levels. The pattern of differential gene expression performed with resistant stocks was compared to the population structure of this parasite, established by random amplified polymorphic DNA analysis and multilocus enzyme electrophoresis. A RADES band polymorphism was observed, and over- or underexpression was linked to the resistance level of the stock. The analysis of RADES bands suggested that different products may be involved in benznidazole resistance mechanisms. No significant association was found between phylogenetic clustering and benznidazole susceptibility. Benznidazole resistance may involve several mechanisms, depending on the level of drug exposure.

Chagas' disease, or American trypanosomiasis, is caused by the flagellate protozoan *Trypanosoma cruzi*. Almost 18 million people are infected by this parasite in Latin American countries (57).

Drug resistance is one of the most important clinical problems that affect not only bacterial diseases produced by staphylococci, enterococci, pneumococci, or streptococci but also parasitic diseases and, more specifically, those produced by pathogenic protozoa such as *Plasmodium falciparum*, *Giardia lamblia*, *Trichomonas vaginalis*, *Leishmania* spp., and *Trypanosoma brucei*. Drug resistance has a strong impact on chemotherapy for Chagas' disease, increasing the number of treatment failures in patients and greatly limiting the treatment options.

The natural resistance of *T. cruzi* to chemotherapeutic drugs has already been reported (10, 11, 12, 38) among the different drug susceptibilities (2, 10, 12, 36, 41, 49, 53). Among the different techniques that have been used to study the differential expression of genes involved in cellular drug resistance, the differential display method is one of the most widely used (15, 54). Currently, it is possible to compare genes that are differentially expressed during the life cycle of parasites by studying mRNA polymorphism through RNA differential display (28) or representation of differential expression, which consists of the selection of specific genes through PCR amplification of hybrid selected sequences (18).

The mRNA populations can be studied either by subtractive enrichment (56), which requires large amounts of mRNA, or by differential screening of a cDNA library (22), which is less sensitive but which can identify moderately to highly expressed sequences. Both methods can be used to identify genes that play key roles in a broad spectrum of biological (21, 58) and pathological (23) processes.

For the study of developmentally regulated genes in African trypanosomes, the random amplified differentially expressed sequences (RADES) technique has been used, in which the mRNA is reverse transcribed into cDNA, which is then used as the template in PCR protocols (32). The advantage of this technical approach resides in the simultaneous use of various combinations of one decameric primer and one oligo(dT) primer, which allows the simultaneous analysis of many samples. This technique has also been used to study gene expression during concanavalin A-induced cell death in *Trypanosoma brucei rhodesiense* (55).

The goal of this study was to analyze by the RADES technique differential gene expression in a sample of *T. cruz*i stocks selected to be representative of the entire genetic variability of the parasite (3, 48), with regard to transient benznidazole exposure or benznidazole-induced resistance, and to explore the possible association between specific differential gene expression and *T. cruzi* phylogenetic subdivisions.

MATERIALS AND METHODS

Biological material. Seventeen stocks of *T. cruzi* were used. Previous analysis by multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAPD) analysis have shown that *T. cruzi* natural populations are subdivided into two main genetic clusters and five lesser genetic clusters (3, 30) as a result of predominant clonal evolution (48). The stocks were selected to be representative of the entire genetic variability of the parasite (Table 1). All stocks were cloned by micromanipulation, with verification under a microscope. Epimastigote culture was carried out at 27°C in liver infusion tryptose (LIT) medium supplemented with fetal calf serum (FCS; 10%). Cultured epimastigotes were harvested by centrifugation (2,800 × *g* for 20 min, 4°C) and then washed twice in phosphate-buffered saline solution. The final centrifugation was at 15,000 × *g* for

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TABLE 1. *Trypanosoma cruzi* stocks used in this work

Genetic cluster	Stock	Stock code	Host	Country
I (19/20[a])	Cutia cl1	1	*Dasylprocta aguti*	Brazil
I	P209 cl1	2	Human (chronic phase)	Bolivia
I	OPS cl11[b]	3	Human (acute phase)	Venezuela
I	Tehuantepec cl2[b,c]	19	Triatominae	Mexico
IIb (32[a])	Mas1 cl1	4	Human (chronic phase)	Brazil
IIb	IVV cl4[b]	5	Human (chronic phase)	Chile
IIb	CBB cl3[b]	6	Human (chronic phase)	Chile
IId (39[a])	Sc43 cl1	7	*Triatoma infestans*	Bolivia
IId	SO3 cl5	8	*Triatoma infestans*	Bolivia
IId	Mn cl2[b]	9	Human (chronic phase)	Chile
IIe (43[a])	Tula cl2[b]	10	Human	Chile
IIe	Tulahuen Fluc resistant[d]	11	Human	Chile
IIe	Tulahuen Fluc sensitive[d]	12	Human	Chile
IIa (27[a])	DogTheis	14	*Canis familiaris*	United States
IIc (36[a])	X109/2	16	*Canis familiaris*	Paraguay
IIc	X110/8	17	*Canis familiaris*	Paraguay
IIc	M6241 cl6[b]	18	Human	Brazil

[a] According to the numbering by Tibayrenc and Ayala (48).
[b] Benznidazole-resistant stocks induced by in vitro drug pressure.
[c] Stock kindly provided by P. Nirdé (37).
[d] Stocks kindly provided by F. S. Buckner (8). Fluc, fluconazole.

TABLE 2. Sequences of primers[a] used in RAPD and RADES analyses

Primer	Sequence	Primer	Sequence
A2	5′-TGCCGAGCTG-3′	N4	5′-GACCGACCCA-3′
A3	5′-AGTCAGCCAC-3′	N15	5′-CAGCGACTGT-3′
A4	5′-AATCGGGCTG-3′	N18	5′-GGTGAGGTCA-3′
A7	5′-GAAACGGGTG-3′	R1	5′-TGCGGGTCCT-3′
A9	5′-GGGTAACGCC-3′	R2	5′-CACAGCTGCC-3′
A10	5′-GTGATCGCAG-3′	R8	5′-CCCGTTGCCT-3′
B1	5′-GTTTCGCTCC-3′	R10	5′-CCATTCCCCA-3′
B5	5′-TGCGCCCTTC-3′	U13	5′-GGCTGGTTCC-3′
B11	5′-GTAGACCCGT-3′	U16	5′-CTGCGCTGGA-3′
B18	5′-CCACAGCAGT-3′	U19	5′-GTCAGTGCGG-3′
F13	5′-GGCTGCAGAA-3′	U20	5′-ACCTCGGCAC-3′

[a] The primers were from Operon Technologies.

20 min at 4°C; the pellets were then weighed and stored at −80°C until characterization.

Genetic characterization of the stocks. Genetic characterization was carried out by MLEE and RAPD analysis. A total of 19 different isoenzymatic systems corresponding to 22 polymorphic loci were scored. Isoenzymes were revealed by electrophoresis in cellulose acetate plates (Helena System) as previously described by Ben Abderrazak et al. (4) and Barnabé et al. (3). DNA extraction and RAPD analyses of *T. cruzi* were performed as described by Brisse et al. (7). For RAPD analysis, a total of 22 different primers that give reproducible bands and that correspond to the primers in the A, B, F, N, R, and U kits from Operon Technologies were used (Table 2).

Growth kinetics of epimastigotes. The growth kinetics of epimastigotes were determined by flow cytometry, as previously described by Vergnes et al. (51). For genetic analysis, all parasites were harvested during the same growth phase so that reliable comparisons could be made. Epimastigotes (10^6 cells ml^{-1}) in exponential growth phase were cultured in 24-well cell culture plates. The plates were incubated at 27°C for 15 days. Samples were harvested daily and stained with a propidium iodide solution in phosphate-buffered saline (1×). The mean cell number and viability were determined by flow cytometric analysis with a FACScan flow cytometer (Becton Dickinson) equipped with an argon-ion laser tuned to 488 nm. Growth curves were computed by using a three-parameter logistic equation, also known as a sigmoidal dose-response, with Prims 3.0 (Graphpad Software Inc., San Diego, CA).

IC_{50} determinations. The 50% inhibitory concentration (IC_{50}) corresponds to the drug concentration necessary to inhibit the parasite growth by 50%. Inhibitory concentrations were assessed by the combined use of two methods, namely, the MTT-PMS [3-(4,5-dimethylthiazol-2-yl)-2,5-deiphenyl-2H-tetrazolium bromide–phenazine methosulfate] micromethod and flow cytometry (53). The IC_{50} for the parasites exposed to the concentration of benznidazole corresponding to the natural IC_{50} was called the IC_{50wt}. This IC_{50wt} was also used for the pulsed exposure procedure, in which epimastigotes were exposed to benznidazole at the concentration that corresponds to their specific natural chemosensitivity level (IC_{50wt}) for 72 h at 27°C, prior to gene expression analyses.

Induction of benznidazole resistance in *T. cruzi* parasites. Two characteristic levels of induction can be distinguished: (i) the therapeutic resistance level, which corresponds to the concentration of benznidazole in plasma during a chemotherapy course in humans (50 μM), and (ii) the chemoresistance level, which corresponds to the concentration of benznidazole in which all induced benznidazole-resistant parasites that grow in culture medium containing drugs at levels above those for therapeutic resistance are chemoresistant. In vitro benznidazole resistance was induced in cloned *T. cruzi* epimastigotes by the continuous drug pressure protocol described by Nirdé et al. (37). Drug-resistant parasites were grown in L30TC culture medium (60% LIT medium and 30% TC-100 insect medium; Invitrogen), 10% inactivated FCS, and the appropriate benznidazole concentration in a 25-cm^2 tissue culture flask at 27°C (37). Six stocks were induced up to the therapeutic resistance level. One clone (stock 19, Tehuantepec cl2; Table 1) was induced with up to 200 μM of drug, and several intermediate levels were used (15, 50, 100, and 200 μM). Control wild-type stocks were grown under the same culture conditions (L30TC, 10% FCS) with the vehicle alone (dimethyl sulfoxide).

Drug resistance stability was tested to verify that resistant phenotypes were stable. All resistant stocks were grown for at least 3 months in L30TC and 10% FCS without drug, and then benznidazole was added to verify their viability and growth.

Differential gene expression by the RADES technique. The RADES technique (32) was used to study gene expression in benznidazole-exposed or benznidazole-resistant *T. cruzi* parasites. Parasites were therefore harvested during the exponential growth phase, and their mRNA was extracted. The RADES approach was used to study gene expression in stocks exposed to their specific IC_{50} levels of benznidazole (pulse exposure), therapeutic-resistant stocks, and chemoresistant stocks in comparison with that in wild-type stocks.

mRNA isolation and ss cDNA synthesis. mRNA from epimastigotes was isolated by use of the Dynabeads mRNA Direct kit, according to the manufacturer's instructions (Dynal Biotech). mRNA was quantified by spectrometry (at 260 nm) and was then retrotranscribed into single-strand (ss) cDNA by using an avian myeloblastosis virus reverse transcriptase (Promega) and an oligo(dT_{18}) primer. Reverse transcription was carried out in a PTC-100 Thermocycler (MJ Research) for 1 h at 42°C. Double-strand (ds) cDNA was generated by using a primer specific for the conserved 39 nucleotides (nt) at the 3′ end of the miniexon SL of all *Trypanosoma* mRNAs (18), which contain 23 nt of the miniexon sequence and the oligo(dT_{18}) primer. The optimal conditions for ss cDNA amplification were 16 μM SLc (5′-GATACAGTTTCTGTACTATATT G-3′), 16 μM oligo(dT_{18}), 166.66 μM deoxynucleoside triphosphates, 1 U *Taq* polymerase (Boehringer Mannhein), 10 μl 10× Tampon, and 100 ng ss cDNA in a 100-μl total volume. Thirty-five cycles consisting of 10 cycles at 61°C and 25 cycles at 45°C (denaturation for 30 s at 94°C, annealing for 1.5 min at 61 or 45°C, and elongation for 3 min at 72°C) were used; this was followed by a 5-min final extension at 72°C. After amplification, the ds cDNA was purified with QIAquick PCR purification kit (QIAGEN) and was quantified by spectrometry. The ds cDNA quality was analyzed by electrophoresis in 1% agarose gels in 0.5× TAE buffer (Tris acetate, 40 mM, pH 8.3; 1 mM EDTA).

ds cDNA amplification. The ds cDNA was amplified with single arbitrary 10-mer primers (32); 57 different primers corresponding to those in panels A ($n = 8$), B ($n = 7$), F ($n = 8$), N ($n = 10$), R ($n = 8$), and U ($n = 10$) kits from Operon Technologies Inc. were screened; but only 22 primers that produced reproducible profiles were selected (Table 2). The amplification reaction volumes were 60 μl and contained 100 μM each deoxynucleoside triphosphates, 10 μM primer, 0.9 U *Taq* polymerase (Boehringer Mannhein), 6 μl of 10× buffer, and 20 ng ds cDNA. Forty-five cycles (denaturation for 1 min at 94°C, annealing for 1 min at 36°C, and elongation for 2 min at 72°C) were followed by a final elongation step of 7 min at 72°C. The products obtained by the RADES technique were analyzed by electrophoresis in 1.6% agarose gels in 0.5× TAE buffer. All experiments were performed at least twice with two independent cDNA samples to test the reproducibility of the technique.

Gene cloning. Selected products obtained by the RADES technique were purified from agarose gels (QIAquick gel extraction kit; QIAGEN), ligated into the pGEM-T vector (pGEM-T Easy Vector System I; Promega), and transformed into high-efficiency competent *Escherichia coli* strain JM109 (Promega). Screening for blue and white colonies was used to identify recombinant plasmids, which were subsequently purified (QIAprep Spin Miniprep kit; QIAGEN).

Sequencing. Products cloned by the RADES technique were analyzed by automated sequencing by the dye terminator method (ABI PRISM 310 genetic analyzer; Applied Biosystems).

Gene identification. When it was necessary, the sequences were corrected with Chromas 2.23 software (Technelysium Pty. Ltd., 1998-2002). The sequences were compared to those in the GenBank and EMBL sequence databases by using the BLAST (basic local alignment search tool) program. For nucleotide sequences, BLASTN and BLASTX (1) searches of the sequences in the National Center for Biotechnology Information (NCBI) site (BLAST with eukaryotic genomes) were used. cDNA sequences were translated into amino acid sequences with the Six Frame Translation of Sequence program at the Baylor College of Medicine Human Genome Sequencing Center Search Launcher from the ExPaSy Molecular Biology Server (13). In order to determine the possible identities and/or homologies, predicted coding sequences were searched by BLASTP at ExPaSy (SIB BLAST Network service) against the putative protein banks (Swiss-Prot, TrEMBL, and TrEMBL_NEW) (6). Generally, hits with BLASTN scores of over 500 with E values of <1e−05 and hits with BLASTX and BLASTP scores of over 45 with E values of <1e−04 were considered significant, although some exceptions were made upon inspection of the alignments.

Data analyses. For MLEE, RAPD analysis, and the RADES technique (at the IC_{50wt}, i.e., pulsed exposure), Jaccard's genetic distances (17) between pairs of stocks were calculated by using the Genetic Tools Box software designed in our laboratory. The neighbor-joining method (43) and the unweighted pair-group method with arithmetic averages (45) were used to cluster the genotypes with the Neighbor software from the PHYLIP package (J. Felsenstein, PHYLIP [Phylogeny Inference Package]), version 3.57c, University of Washington, Seattle, 1995). Phylogenetic trees were visualized by using the TREEVIEW software (39). Correlations between IC_{50}s and genetic distances were tested by the Mantel test (25) with ADE-4 software (version 2001 [47]).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper are available in the GenBank, EMBL, and DDBJ databases under the accession numbers AJ748750 to AJ748761.

RESULTS

Growth kinetics of resistant stocks. The growth kinetics of the wild-type clone, which exhibited the highest growth rate with a doubling time of 29 h, are shown in Fig. 1. The growth rate was lower for all the drug-resistant epimastigotes than for the wild type. The doubling time was as high as 35 h in the presence of 200 μM benznidazole, which was the maximum concentration tested in this study. Except for the 15 μM chemosensitive level, the growth rate decreased when the drug resistance increased. It should be mentioned here that the viabilities of the resistant parasites did not change in comparison to those of their sensitive counterparts.

Gene expression of stocks exposed to benznidazole at the IC_{50} (pulsed exposure). Analysis of the RADES profiles obtained at the IC_{50wt} for each stock showed that most of the primers produced polymorphic patterns (Fig. 2a). However, a monomorphic pattern could be found, i.e., with primer F13, as illustrated in Fig. 2b. Of 22 primers tested, 14 exhibited differential gene expression. Seven primers (primers A2, A4, A9, B5, B18, F13, and N4) produced differential bands which were either enhanced or totally induced in the exposed stocks, as illustrated in Fig. 3a. The last seven primers (primers A3, A10, B1, N4, N18, R1, and U13) showed an absence or a decrease in the intensity of one of the specific bands, as shown in Fig. 3b for stock 11.

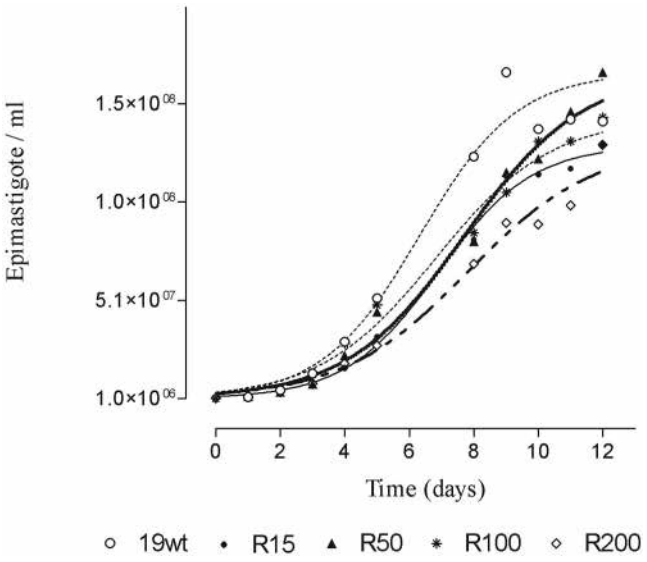

FIG. 1. Growth kinetics of wild-type and resistant stock 19 (Tehuantepec cl2) (Table 1) at different levels of benznidazole resistance (15, 50, 100, and 200 μM).

Within the same genetic cluster, pulsed exposure to benznidazole allowed the observation of differential band expression, which can be different from one stock to another (Fig. 4, lane 3+). For example, the pattern seen for stock 3, i.e., the presence of an additional band after exposure to the drug (Fig. 4, lane 3+), was not recorded for the other stocks of the same cluster (Fig. 4). At the pulse exposure level, the stocks exposed to benznidazole showed a differential banding pattern. However, differential bands could not be specifically associated with any *T. cruzi* genetic subdivision. In addition, when all expressed bands were analyzed, correlation studies showed that the entire gene expression at this specific level either in the presence ($r = -0.063$; $P = 0.593$) or in the absence ($r = -0.044$; $P = 0.684$) of drug was not related to the natural benznidazole susceptibilities of the stocks. In contrast, the overall gene expression revealed by the total RADES profiles showed a strong link with *T. cruzi* genetic clustering (52). This was tested by evaluating the correlation between RADES genetic distances on the one hand and the MLEE and RAPD distances on the other hand by a nonparametric Mantel test. For RAPD analysis, the results were $r = 0.822$ ($P < 10^{-4}$) and $r = 0.789$ ($P < 10^{-4}$) in the presence and the absence of benznidazole, respectively; and for MLEE the results were $r = 0.465$ ($P < 10^{-4}$) and $r = 0.677$ ($P < 10^{-4}$) in the presence and the absence of benznidazole, respectively.

Gene expression of resistant stocks at the therapeutic level (50 μM). Eight of 22 primers (primers A2, A3, A7, B1, B5, N4,

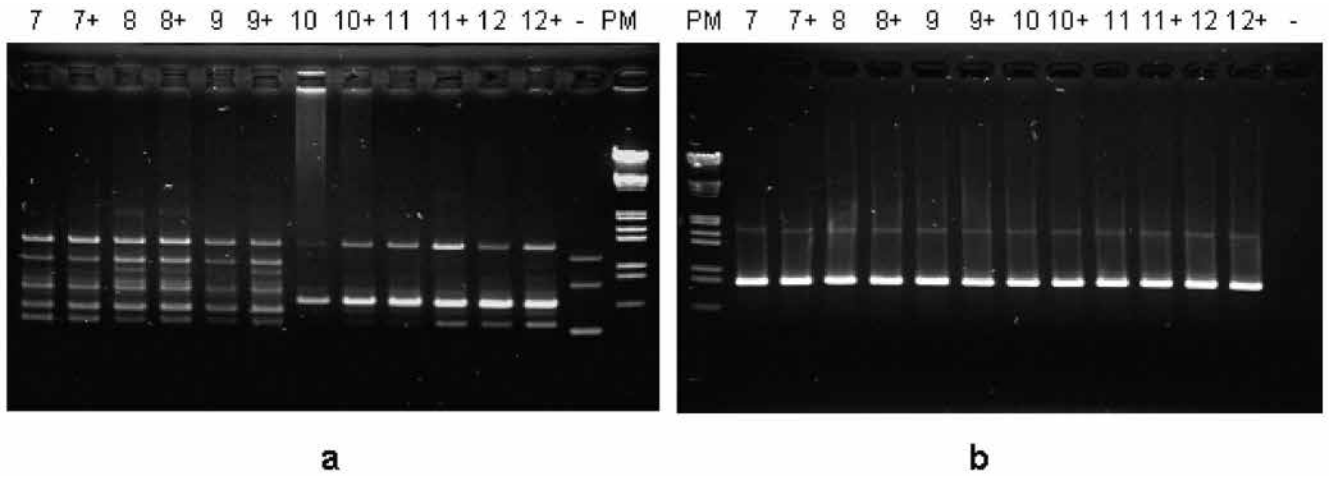

FIG. 2. RADES profiles: examples of polymorphic profiles obtained with primer A3 (a) and monomorphic profiles obtained with primer F13 (b) for six different stocks of *T. cruzi* exposed to benznidazole (lanes with plus signs) at their specific IC_{50} compared to those obtained for their sensitive counterparts (lanes without plus signs). Lanes PM, molecular weight markers (EcoRI- and HindIII-digested bacteriophage λ DNA). The numbers that designate the stocks are the same as those in Table 1.

N18, and R2) produced differential bands at the therapeutic level (50 μM benznidazole). In this analysis, 10 differential bands were found. Among these differential bands, three were expressed only in resistant parasites, four were expressed only in sensitive parasites, and the other three were found to be overexpressed in resistant parasites, as illustrated in Fig. 5. Figure 5 shows the RADES profiles obtained with primers R2 (Fig. 5a) and A7 (Fig. 5b) with benznidazole-resistant stocks and their sensitive counterparts (wild type). At this therapeutic level, differential bands were generated by the sensitive stock (Fig. 5a, see wt) or overexpressed band products were generated by the resistant stocks (Fig. 5b, see 3R and 6R). Interestingly, as shown in Fig. 5, these differential bands were not associated with one specific genetic cluster. In addition, after calculation of the sizes of the specific bands on agarose gels, this differential expression was not found to correspond to the differential bands found for the parasites after benznidazole pulse exposure. Furthermore, only four of six stocks showed differential gene expression between sensitive and resistant stocks, whereas two stocks did not show any differential gene expression.

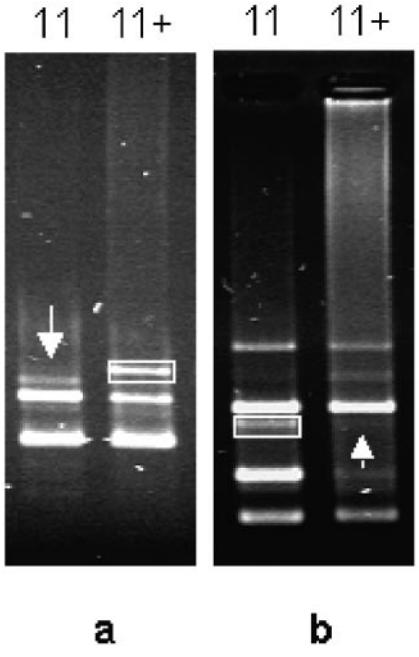

FIG. 3. Example of differential RADES profiles obtained with the primers B18 (a) and N4 (b) for stock 11 of *T. cruzi* exposed to benznidazole (lanes with plus signs) at its specific IC_{50} compared to those for the sensitive counterpart (lanes without plus signs). Arrows and rectangles indicate the differential bands. The numbers that designate the stock are the same as those in Table 1.

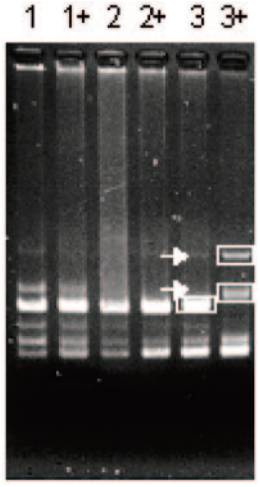

FIG. 4. RADES profiles obtained with primer N4 for stocks 1, 2, and 3 at their specific IC_{50}s (lanes with plus signs) compared to those for the sensitive counterparts (lanes without plus signs). Arrows and rectangles indicate the differential bands. The numbers that designate the stocks are the same as those in Table 1.

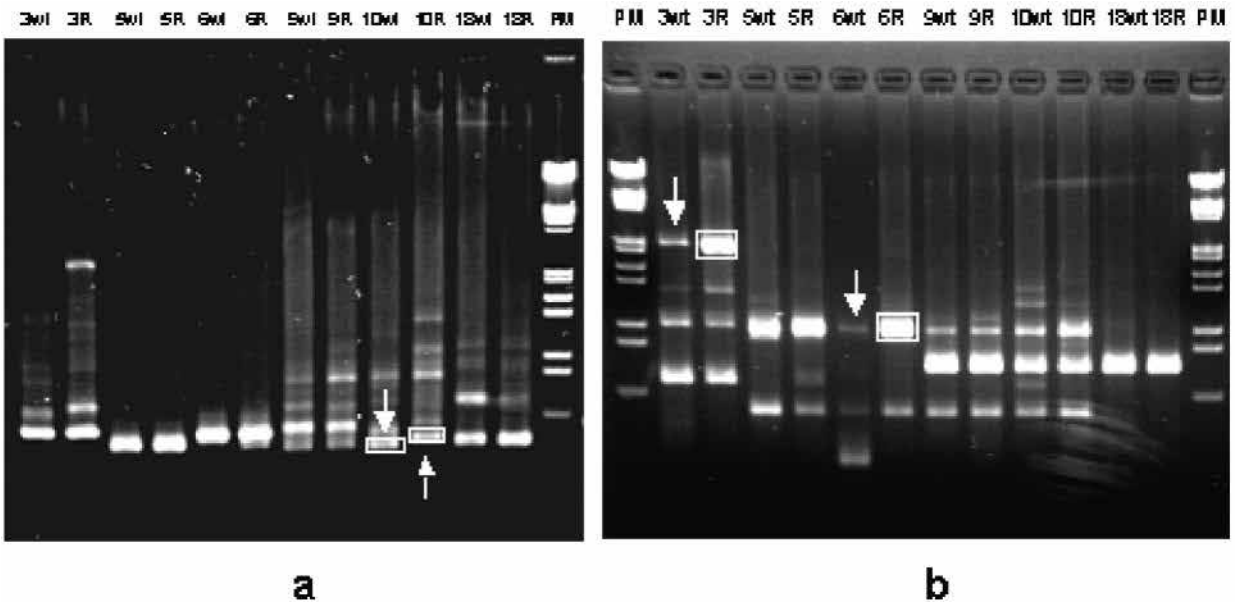

FIG. 5. RADES profiles obtained with primers R2 (a) and A7 (b) for six resistant stocks at the therapeutic level (lanes R) and the corresponding wild-type stocks (lanes wt). Lanes PM, molecular weight markers (EcoRI- and HindIII-digested bacteriophage λ DNA). Arrows and rectangles indicate the differential bands. The numbers that designate the stocks are the same as those in Table 1.

Modulation of gene expression in a benznidazole-resistant stock. We studied the impact of increasing levels of benznidazole resistance (i.e., 15 μM, 50 μM, 100 μM, and 200 μM) on the modulation of the differential bands obtained with different primers, as shown in Fig. 6. Typical results obtained with the primers that produced differential bands (i.e., primers A2, A7, A9, U13, and U16) are shown for primers A7 (Fig. 6a), A9 (Fig. 6b), and U16 (Fig. 6c). Seven differential bands were found, three of which were expressed only by the chemoresistant parasite for all resistance levels tested; an additional three bands were overexpressed (Fig. 6a to c). In contrast, one band was modulated by an increasing amount of benznidazole resistance (Fig. 6b), and one band was dependent upon the level of benznidazole resistance. Its intensity decreased as the drug resistance level increased (Fig. 6c).

Results for differentially cloned and sequenced bands. The selected differential bands expressed in benznidazole-resistant parasites, which were cloned and sequenced, are listed in Table 3.

For the bands that were overexpressed in resistant parasites, a sequence comparison was made by using the alignments between both the reverse and the forward sequences. Thus, we obtained identities of 98.1% with the forward sequences A2 and O1 (see the fragment code in Table 3) and identities of 98.9% with the reverse sequences A1 and O2 for the wild-type and resistant lines of stock 3 (OPS cl11) (Table 1) at a therapeutic level (50 μM).

The sequences A1 and A2 (Table 3), obtained with resistant lines from stock 3 (Table 1, OPS cl11), were then aligned with sequences C1 and C2 (Table 3), obtained from the resistant lines of stock 19 (Table 1, Tehuantepec cl2). Identities of 97.5% and 99.5% were obtained for the forward and for the reverse sequences, respectively.

Table 4 shows the differential RADES band sequences, which displayed significant alignment with sequences from the genome data banks by using BLASTN, BLASTX, or BLASTP.

BLASTX provided significant scores for sequences C1 and F1 (Table 3), which were found in two other flagellate protozoa, *Trypanosoma brucei* and *Leishmania major*, respectively; however, the complete identification of the proteins as well as their precise functions remain unknown.

The most significant match (obtained by the use of BLASTP; Table 4) for sequence D2 corresponds to a coiled-coil flagellar MBO2 protein from *Chlamydomonas reinhardtii* (a unicellular green alga), and for sequence F1 the most significant match corresponded to a hypothetical protein from *Leishmania major* (44). Interestingly, the latter sequence presents identities (score = 33.9; E value = 1) with a putative ABC transporter from *Sinorhizobium meliloti* (a soil and rhizosphere bacterium).

DISCUSSION

The induction of benznidazole resistance in *T. cruzi* epimastigotes was associated with a decrease in their growth rate, which confirmed previous data (37). In addition, the growth rate of epimastigotes decreased when the level of drug resistance increased from the therapeutic resistance level (50 μM) to higher resistance levels.

Information on the molecular mechanisms for benznidazole resistance in *T. cruzi* is scarce. Nevertheless, some have attempted to analyze the behavior of naturally chemosensitive parasites and/or parasites with induced resistance to further elucidate the possible mechanisms involved in these phenomena (8, 12, 33, 35, 37). Additionally, some studies have suggested that the natural resistance of *T. cruzi* to nitroheterocyclic derivatives may be an important factor in explaining the low rates of successful cure detected (12, 34). Furthermore,

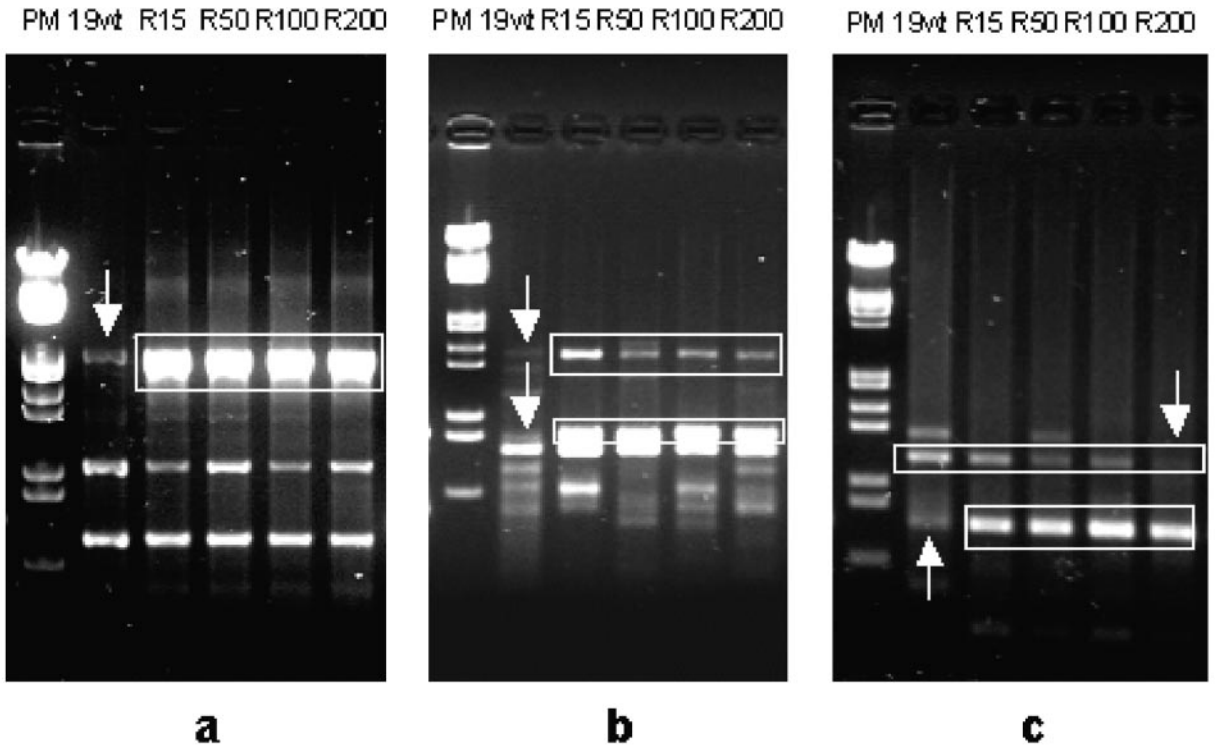

FIG. 6. RADES profiles obtained with primers A7 (a), A9 (b), and U16 (c) for wild-type (wt) and resistant (R) stock 19 at different chemoresistance levels (15, 50, 100, and 200 μM). Lanes PM, molecular weight markers (EcoRI- and HindIII-digested bacteriophage λ DNA). Arrows and rectangles indicate the differential bands. The numbers that designate the stock are the same as those in Table 1.

failure in the treatment of patients might be due to differential responses that depend on the biological properties of either the parasite or the vertebrate host, or both (29).

It was demonstrated that resistance to benznidazole or nifurtimox in *T. cruzi* parasites was increased when parasites were isolated from mice treated with the same drugs (26, 33). It has also been suggested that the biological properties of *T. cruzi* stocks could be linked to their overall genetic variability

TABLE 3. Selected, cloned, and sequenced differentially expressed RADES bands obtained from benznidazole-resistant stocks and corresponding wild-type stocks

Resistance	Band no.	Stock[a]	10-mer primer	Band size (nt)	Primer M13 used	Fragment code	Sequence size (nt)	GenBank accession no.
Therapeutic level	1	OPScl11 R50	A7	2,027	Reverse Forward	A1 A2	623 650	AJ748750 AJ748751
	1	OPS cl11 wt	A7	2,027	Forward Reverse	O1 O2	618 658	AJ748760 AJ748761
	4	Tula cl2 wt	R2	450	Reverse	I1	450	AJ748758
	3	Tula cl2 R50	R2	500	Reverse	J1	500	AJ748759
Chemoresistance level	1	Tehuantepec cl2 R200	A7	2,027	Reverse Forward	C1 C2	718 602	AJ748752 AJ748753
	1	Tehuantepec cl2 R100	A2	1,584	Reverse	D2	692	AJ748754
	2	Tehuantepec cl2 R200	U13	1,600	Reverse	E3	573	AJ748755
	1	Tehuantepec cl2 wt	U16	1,200	Reverse	F1	543	AJ748756
	2	Tehuantepec cl2 R200	U16	650	Reverse	G1	562	AJ748757

[a] R, benznidazole-induced resistant stock; wt, wild-type stock.

TABLE 4. Sequences producing alignments with sequences from differentially expressed bands in resistant and wild-type stocks

Band code[a]	Results of BLASTN search of NCBI database[b]	Score (E value[c])	Results of BLASTX search of NCBI database[d]	Score (E value[c])	Results of BLASTP search of ExPASy[e]	Score (E value[c])
C1	gnl|TIGR_5693|	1221	gnl|Sanger_5691|TRYP10.20020325; prot_TRYP10.0.00 0155_142	125	tr Q9GYC6	33
	1101817	(0.0)	*T. brucei* unfinished protein	(8e-30)	Possible calmodulin-binding protein L3856.01 of *Leishmania major*	(3.2)
C2	gnl|TIGR_5693|	758	gnl|Sanger_5691|TRYP10.20020325; prot_TRYP10.0.000155_142	37	tr Q8F1N1	32
	1083441	(0.0)	*T. brucei* unfinished protein	(0.003)	Conserved hypothetical protein LA3101 of *Leptospira interrogans*	(6.3)
D2	gnl|TIGR_5693|	1052	gb|AAA20179.1|	34	tr Q8S4W6	60
	1072671	(0.0)	Glycoprotein 96-92 *L. major*	(0.018)	Coiled-coil flagellar protein MBO2 of *Chlamydomonas reinhardtii*	(4e−08)
E3	gnl|TIGR_5693|	887	pir||PH1919	30	tr|AE017232|AAS03827|5092833872 C11E48 (MAP1510)	42
	1103328	(0.0)	FL-160-4 protein of *T. cruzi* (fragment)	(0.32)	Hypothetical protein, *Mycobacterium paratuberculosis*	(0.008)
F1	gnl|TIGR_5693|	952	embCAB77682.1 hypothetical protein L4803.04 of *L. major*	134	tr Q9NEA0 hypothetical 51.4-kDa protein L4803.04 *Leishmania major*	134
	1102691	(0.0)		(9e-33)		(8e−31)
G1	gnl|TIGR_5693|	871	gnl|Sanger_5691|TRYP10.20020325; prot_TRYP10.0.000041_125	30	tr Q8IJK4	32
	1102811	(0.0)	*T. brucei* unfinished protein	(0.24)	Hypothetical protein PF10_0191 *Plasmodium falciparum* (isolate 3D7)	(4.3)
I1	gnl|TIGR_5693|	504	gnl|Sanger_5691|TRYP10.20020325; prot_TRYP10.0.003381_3	30	tr Q9ZZR2	36
	1084883	(1e−142)	*T. brucei* unfinished protein	(0.21)	Succinate dehydrogenase iron-sulfur protein SDHB of *Cyanidioschyzonmerolae* (red alga)	(0.22)
J1	gnl|TIGR_5693|	527	gnl|Sanger_5691|TRYP10.20020325; prot_TRYP10.0.003823_17	27	tr Q9ZZR2	42
	1084883	(1e−149)	*T. brucei* unfinished protein	(1.7)	Succinate dehydrogenase iron-sulfur protein SDHB of *Cyanidioschyzon merolae* (red alga)	(0.004)

[a] Code corresponding to selected differential bands (fragment code, Table 3).
[b] BLASTN search of the NCBI site (BLAST with eukaryotic genomes) by use of the *T. cruzi* genome gene bank.
[c] E values of ≤1e−05 for BLASTN and E values of ≤1e−04 for BLASTX and BLASTP were considered positive.
[d] BLASTX search of the NCBI site (BLAST with eukaryotic genomes) by use of the *L. major, T. brucei*, and *T. cruzi* genome gene banks.
[e] BLASTP search of the ExPASy Molecular Biology Server from translated sequences.

(20, 41). However, our previous study showed that the level of natural chemosensitivity of this parasite is not associated with its genetic diversity (53). Here we have found variability in the differential gene expression of resistant parasites belonging to the same genetic cluster. It was not possible to demonstrate a link between drug resistance and genetic clustering by MLEE or RAPD analysis.

The RADES technique was initially proposed for use for the identification of developmental genes involved in life cycle stages (32) and differentially expressed genes in the induced cell death mechanisms in African trypanosomes (55). Here, we validated the RADES technique, which was used to identify the precise genes that are differentially expressed in epimastigotes of *T. cruzi* as a result of benznidazole exposures.

It is noteworthy that the differential bands expressed in a stock do not correspond to those expressed in the other stocks from the same genetic cluster. As for natural resistance, this result is not in agreement with the proposed hypothesis that chemoresistance might be linked to *T. cruzi* clonal variability (50). The random distribution of differential bands suggests that each stock acts independently of its own genetic cluster when it is submitted to a brief stress.

Indeed, it looks as though the parasite naturally had its own mechanism of drug susceptibility or resistance: for a temporary drug stress, it may be able to develop transient mechanisms; for a stabilized and higher level of chemoresistance than IC_{50wt}, it could be able to initiate new mechanisms for a long period of time.

Whereas differentially expressed bands showed no apparent link with the genetic subdivisions evidenced by MLEE and RAPD analysis, the overall RADES profiles of the parasites showed a strong association with these genetic subdivisions not only in the absence of benznidazole but also in presence of benznidazole (52). Our results therefore favor the hypothesis that the mechanisms involved in natural drug sensitivity (chemosensitivity) are different from those involved in induced chemoresistance. Several mechanisms that act together to produce the chemoresistance state could be involved in the resistance of benznidazole in *T. cruzi*.

Many differentially expressed sequences are apparently nonidentified genes that correspond to hypothetical proteins. A few sequences have low significance scores but belong to known protein families. We thus focused our study on a limited number of genes corresponding to this case.

Analysis of the D2 fragment (Table 3) has shown a 27% identity with a coiled-coil flagellar protein from *Chlamydomonas reinhardtii*. This MBO2 protein is involved in a Ca^{2+}-dependent waveform conversion (46). Possibly, the role of the MBO2p-like protein involves alteration of the waveform of the flagella in response to changes in the Ca^{2+} concentration. It is noticeable that many Ca^{2+}-binding proteins in trypanosomes have been localized in the flagellum, an organelle that seems to be crucial for calcium signaling (14, 24). Additionally, the flagellum emerges from an invagination called a flagellar pocket, in which intense endocytic and exocytic activity takes place (9). Interestingly, previous studies have shown that a Ca^{2+}-binding protein induces a low level of drug resistance in cancer cells (40). It is therefore reasonable to suppose that a Ca^{2+}-dependent protein could be involved in benznidazole resistance in *T. cruzi*.

A C1 fragment sequence (Table 3) showed significant identity with a calmodulin-binding protein from *L. major* (16). It is remarkable that two sequences (fragments C1 and D2) from different differentially expressed bands and from two different primers showed links with Ca^{2+}-binding proteins. Ca^{2+} is used as a major signaling molecule in a broad range of microorganisms, including parasitic protozoa that infect humans (31). In these parasites, the Ca^{2+} apparently plays an important role in cell physiology and it is critical for cellular invasion.

Plasma membrane Ca^{2+}-ATPases are activated by the Ca^{2+}-binding protein calmodulin in mammals, but biochemical evidence of a role of these enzymes in *T. cruzi* has been reported (5). Our results suggest that a possible Ca^{2+}-binding protein plays a role in metabolism pathways as a result of drug stimulation or drug resistance. A dependent cell death pathway has also been described in *T. brucei* (42). A role for Ca^{2+} signaling in differentiation has been postulated on the basis of changes in intracellular Ca^{2+} concentrations observed upon differentiation of *T. cruzi* (19). In this way, we have observed some parasite morphological changes as well as a decrease in flagellar movements in resistant parasites by comparison with wild types (unpublished data). Several different pathways are probably involved in benznidazole resistance in *T. cruzi* parasites.

An F1 fragment sequence (Table 3) showed 40% identity with a hypothetical 51.4-kDa protein from *L. major*. It is noteworthy that this sequence was shown to be similar to both a sperm-binding protein from *Sus scrofa* (wild pig) and a putative ABC transporter from *Sinorhizobium meliloti*. However, the levels of identity were not significant. More information is necessary to ascertain whether other ABC transporters (Pgp-170-like transporter [27]) are involved in benznidazole resistance.

Our results have provided preliminary but important information about differential gene expression in sensitive and resistant stocks of *T. cruzi*. Although no significant association was found between induced drug resistance and phylogenetic clustering, important differences in drug susceptibility were observed among stocks. This deserves further investigation and might be taken into account for future treatments.

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