TRYPANOSOMA CRUZI: EVALUATION OF A RAPD SYNAPOMORPHIC FRAGMENT AS A SPECIES-SPECIFIC DNA PROBE

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ABSTRACT: A methodological approach is proposed to select rapidly DNA sequences characterized by a well defined specificity and potentially interesting to be used as diagnostic probes or as taxonomic and phylogenetic markers. A fragment amplified from a diversified sample of Trypanosoma cruzi stocks by the RAPD (random amplified polymorphic DNA) method with the A8 primer, previously found to be monomorphic in all stocks, was separated in 2 fragments using polyacrylamide electrophoresis. RFLP (restriction fragment length polymorphism) analysis of the 750-bp fragment common to all stocks revealed some sequence heterogeneity within the T. cruzi species, whereas hybridization experiments showed a high homology between fragments amplified from different T. cruzi stocks. These results suggest that sequence analysis will allow the design of internal primers to be used as probes to target specific taxonomic levels (clone, family of related clones, or species) and for diagnosis.

Natural populations of Trypanosoma cruzi, the agent of Chagas’ disease, have a clonal structure as previously demonstrated by extensive population genetic studies (Tibayrenc et al., 1986). This model does not imply that genetic recombination is absent, but rather indicates that such genetic recombination is not frequent enough for altering significantly the uniparental propagation of the parasite and, consequently, its clonal evolution. Clones are stable in space and time, and their clonality permits their use as epidemiological markers. The accumulation of genetic differences among the clonal lineages has an impact on some biological and medical properties of clones or families of related clones (Laurent, 1994; Revollo, 1995). It is, therefore, useful to identify clones in epidemiological surveys or experimental studies with reliable and easy-to-use markers.

At present, T. cruzi stocks are characterized by 3 main methods: (1) multilocus enzyme electrophoresis (MLEE) on 22 loci (Tibayrenc et al., 1986); (2) amplification of hypervariable sequences in kinetoplast DNA minicircles (Sturm et al., 1989; Veas et al., 1991; Brounère et al., 1992); and (3) random amplified polymorphic DNA (RAPD) (Tibayrenc et al., 1993).

MLEE is the most widely used method, but it presents 2 drawbacks. First, it is time-consuming and, second, parasites must be amplified in culture after their isolation from chagasic patients, mammalian hosts, or vectors. This introduces a bias, because a selection of genotypes may occur during the culture step. Although amplification of hypervariable sequences from kinetoplast DNA minicircles allows characterization of stocks directly in biological samples, several steps are required during the procedure: hypervariable sequences must be amplified, then labeled before being used as probes to hybridize Southern blots of amplified DNA from reference stocks (Brounère et al., 1995). RAPD is a recently developed method that has been used to characterize many different organisms (Welsh and McClelland, 1990; Williams et al., 1990; Tibayrenc et al., 1993). It is a relatively straightforward procedure, but requires meticulous sample processing to avoid contamination with foreign DNA. RAPD analysis of T. cruzi has shown that some RAPD fragments are specific for a given level of phylogenetic divergence (a clone, a family of related clones, or the whole species) (Tibayrenc et al., 1993). In particular, it was observed that the A8 primer generated a 1-banded pattern monomorphic in all T. cruzi stocks and was different from the patterns observed for other species of parasitic protozoa. The present study was designed to explore the value of this A8 fragment as a taxonomic and epidemiologic tool.

MATERIALS AND METHODS

Parasite stocks

A total of 6 T. cruzi stocks was studied (Table I). They were selected to be representative of the extensive genetic variability of the parasite and correspond to clonal genotypes 17, 19, 27, 30, 32, and 39 described by Tibayrenc et al. (1986) (Fig. 1). Stocks corresponding to 14 other parasitic protozoa taxa (Table I) were included in the study in order to evaluate the species specificity of the A8 fragment.

Parasite culture

Trypanosoma cruzi epimastigotes were grown in LIT medium supplemented with 10% inactivated fetal bovine serum (FBS) at 28 C, as described Castellan et al. (1967). Trypanosoma cruzi marenkellei epimastigotes were grown in the same conditions as T. cruzi stocks whereas Trypanosoma rangeli epimastigotes were obtained in NNN biphasic medium at 28 C (Tibayrenc and Le Ray, 1984). Leishmania promastigotes cultures were kept at 28 C in RPMI-1640 medium supplemented with 10% inactivated FBS for Leishmania braziliensis and Leishmania guyanensis or 20% FBS for Leishmania infantum, Leishmania major, and Leishmania tropica. Procyclic forms from Trypanosoma brucei and Trypanosoma congolense were grown in Cunningham medium supplemented with 20% inactivated FBS at 28 C according to Cunningham (1977). Plasmodium falciparum stock was kept in continuous culture using the method described by Trager and Jensen (1976).

Preparation of cell pellets

For trypanosomatids, cultures were harvested at the late log phase and washed 3 times in phosphate-buffered saline (PBS): 1.75 mM KH2PO4, 10.1 mM NaH2PO4, 2.7 mM KCl, 137 mM NaCl, pH 7.4. After the last high-speed centrifugation (15,000 g), the supernatant was removed and parasites were frozen in liquid nitrogen to be stored and to facilitate cell lysis.

For Plasmodium falciparum, cultures were harvested at parasitemia levels of about 5–10%. Infected red blood cells were washed 3 times in PBS. Intracellular parasite forms were released by lysis of host erythrocytes with 0.15% saponin for 15 min on ice and recovered by centrifugation (15,000 g). Red cell walls and hemoglobin were discarded by several washings in large volumes of PBS until the supernatant was clear. Parasite pellets were frozen in liquid nitrogen.
### TABLE I. List of the parasite stocks* used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stock reference</th>
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<tbody>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>X10 c1 (17)</td>
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<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>P209 c1 (19)</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>CanIII c1 (27)</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>ESMERALDO (30)</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>Tzu18 c12 (32)</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>SC43 c1 (39)</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi marenkellei</em></td>
<td>B3</td>
</tr>
<tr>
<td><em>Trypanosoma rangeli</em></td>
<td>Basel</td>
</tr>
<tr>
<td><em>Trypanosoma brucei brucei</em></td>
<td>MTRG/UG/66/Eatro 1125</td>
</tr>
</tbody>
</table>

*Numbers in parentheses after *T. cruzi* stock names refer to the genotype numbering established by Tibayrenc et al. (1986) on the basis of 15 isoenzyme loci (see also Fig. 1).

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**DNA preparation**

Parasite pellets (100 mg) were resuspended in 300 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM ethylenediaminetetraacetic acid [EDTA], 0.5% sodium dodecyl sulfate [SDS]) and incubated for 1.5 hr at 37 C with RNase A (100 µg/ml) and for 2 hr at 50 C with proteinase K (100 µg/ml). After 2 phenol and 3 chloroform extractions, DNA was precipitated in 70% ethanol and redissolved in sterile MilliQ® water. DNA concentration was measured by spectrophotometry at 257 nm. Genomic DNA samples were stored at −20 C.

**DNA amplification (RAPD)**

Amplification reactions were performed in a volume of 100 µl according to Williams et al. (1990). Briefly, genomic DNA samples (10 ng) were used as template for polymerase chain reaction (PCR) in buffer containing 0.2 µM of the A8 primer (5'-GTGACGTAGG-3'; Operon Technologies Kit A), 4 X 100 µM dNTP, 1.5 mM MgCl₂, and 1.5 U Taq polymerase (Boehringer Mannheim GmbH, Mannheim, Germany). The amplification program was achieved on a PTC-100-60 thermocycler (MJ Research Inc., Watertown, Massachusetts, USA) and consisted of 45 cycles at 94 C for 1 min, 36 C for 1 min, and 72 C for 2 min and a final elongation step at 72 C for 7 min. Amplified DNA was kept at 4 C before use.

**DNA electrophoresis**

Amplification products were separated by electrophoresis in 1.6% agarose gels with TAE buffer (40 mM Tris-acetate, pH 7.4, 1 mM EDTA) at 5 V/cm or in 5% polyacrylamide gels with TBE buffer (45 mM Tris-borate, pH 7.5, 1 mM EDTA) at 5 V/cm. DNA fragments were visualized by ethidium bromide staining.

**Elution of DNA**

The 750-bp fragment from each stock was retrieved from a precut polyacrylamide gel slice by electroelution using the electroeluter 422® (Bio-Rad, Richmond, California) under conditions recommended by the supplier. Briefly the elution was done at constant current (15 µA/tube). After 1 phenol, 1 phenol-chloroform, and 1 chloroform extractions,
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DNA fragment was precipitated in 70% ethanol and redissolved in sterile MilliQ® water. DNA concentration was determined (as described).

**Restriction fragment length polymorphisms**

In order to obtain enough material for RFLP analysis, the 750-bp fragment was reamplified with the A8 primer under the same conditions as described above. Reamplified DNA was purified by chromatocentrifugation through a Quick Spin Column Sephadex® (Boehringer Mannheim) according to the manufacturer's instructions. Eluted DNA was precipitated in 70% ethanol and redissolved in sterile MilliQ® water. The DNA concentration was measured (as described). The 750-bp fragment (500 ng) was digested with each (1 U) of the following restriction enzymes: Acc I, Alu I, Bam HI, Eco RI, Hae III, Hha I, Hind III, Hpa II, Kpn I, Pst I, Rsa I, Sal I, Sau3A, Sca I, Sma I, and Xba I, under conditions recommended by the suppliers. Digested DNA (250 ng) was analyzed after electrophoresis on agarose gels, as described above.

**DNA probe labeling**

Amplified DNA products were purified by chromatocentrifugation (as described). Purified DNA was precipitated in 70% ethanol and redissolved in sterile MilliQ® water. The DNA concentration was measured (as described) and labeled with 32P by nick translation (Boehringer Mannheim).

**Southern blot and dot blot**

Amplified DNA was separated by electrophoresis in 1.6% agarose gels and transferred onto nylon membranes (Hybond N+®, Amersham, Buckinghamshire, U.K.) according to the alcalin method described by the manufacturer. Dot blot was performed as follows: genomic DNA (1 μg) was denatured by heating at 95°C for 10 min followed by rapid cooling in ice water for 10 min. After addition of 1 volume (100 μl) of cold 20X SSC (0.3 M tri-sodium citrate, 3 M NaCl, pH 7.0), samples were applied onto a nylon membrane, using a minifold vacuum filtration (“dot blot”) apparatus (BRL, Bethesda, Maryland). The membrane was then laid onto 3MM filter paper prewetted with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 min before transfer onto filter paper soaked in neutralizing solution (0.5 M Tris-HCl, pH 7.2, 1.5 M NaCl, 1 mM EDTA) for 1 min. The membrane was then air dried and DNA fixed by placing the membrane onto 3MM filter paper soaked in 0.4 M NaOH for 30 min. The membrane was finally rinsed in 5X SSC for 1 min under gentle agitation, air dried, and stored in the dark until used.

**RESULTS**

Tibayrenc et al. (1993) have previously reported that amplification with the A8 primer generated a 1-banded pattern that was identical for all T. cruzi stocks screened. Trypanosoma cruzi marenkellei, although exhibiting a more complex overall pattern, exhibited the same band as T. cruzi stocks. In this study, polyacrylamide gel electrophoresis of A8 primer-amplified DNA revealed 2 different profiles (Fig. 2): the P209 cl1, CAN-III, ESMERALDO (not shown), and SC43 cl1 T. cruzi stocks, as well as the T. cruzi marenkellei stock, exhibited 2 fragments of 750 bp and 760 bp, respectively, whereas the X10 cl1 and TU18 cl2 T. cruzi stocks showed the 750-bp fragment only.

In order to determine whether the 750-bp fragment, which is common to all T. cruzi stocks plus the T. cruzi marenkellei stock, is homogeneous, it was submitted to RFLP analysis. Among the 16 restriction enzymes tested, Hae III, Hha I, Hpa II, Pst I, and Sau3A gave 2 different restriction patterns that were specific for T. cruzi and T. cruzi marenkellei stocks. Polymorphism detected by Pst I is shown in Figure 3a. Rsa I revealed a polymorphism among the T. cruzi stocks: the 750-bp fragment from the X10 cl1 and P209 cl1 stocks was digested into 2 subfragments of 550 bp and 200 bp (Fig. 3b). For the other T. cruzi stocks, the 750-bp fragment remained undigested.

The homology between the different 750-bp fragments was assessed by hybridizing a southern blot of amplified products with the A8 fragment taken from the SC43 cl1 stock (genotype 39) as a probe. High stringency hybridization conditions revealed a difference of signal intensity between the T. cruzi stocks and the T. cruzi marenkellei stock, whereas the signal intensity was quite similar among all T. cruzi stocks (Fig. 4).

Dot blot hybridization of the A8 fragment from the T. cruzi SC43 cl1 stock, with genomic DNA from a panel of parasites belonging to various species (Table I) revealed no signal for T. brucei s.l., T. congolesens, Leishmania spp., and P. falciparum.
FIGURE 3. RFLP patterns of the 750-bp A8 fragment obtained from different Trypanosoma cruzi stocks and from T. v. marinkellei. The numbers correspond to the clonal genotypes (see Fig. 1 and Table I). UD is the undigested AS fragment amplified from the SC43 clone and used as control DNA.

a: Digestion with PstI distinguished T. cruzi m. from all T. cruzi stocks.

b: Digestion with RsaI revealed a polymorphism among T. cruzi stocks. This enzyme distinguished stocks with the genotypes 17 and 19 (Tibayrenc et al., 1986) from the other T. cruzi stocks. This corroborates the known picture of T. cruzi subspecies variability, which clearly shows that this taxon is subdivided into 2 main phylogenetic lineages (Tibayrenc, 1995).

The variability detected by RFLP confirms that the size identity of PCR fragments does not imply that the sequences involved are necessarily identical. However, this heterogeneity...
T. cruzi genomic DNA, it seems that the A8 fragment exists in only 1 copy or a limited number of copies in the parasite genome. Now it is generally believed that random amplified fragments correspond to highly or moderately repetitive sequences in the genome. At least for the A8 fragment of T. cruzi, our results do not corroborate this hypothesis.

Various DNA sequences have been proposed as diagnostic probes or taxonomic markers for T. cruzi, e.g., highly repeated sequences (195 bp) (Moser et al., 1989; Russomando et al., 1992; Brenière et al., 1993), kinetoplast minicircle DNA sequences (Sturm et al., 1989; Avila et al., 1991, 1993; Veas et al., 1991; Brenière et al., 1992; Britto et al., 1993; Wincker et al., 1994), intergenic spacer (IGS) or nontranscribed spacer (NTS) sequences (Dietrich et al., 1990; González et al., 1994), 18S rRNA genes (Hernández et al., 1990; Clark, 1992; Souto and Zingales, 1993; Clark and Pung, 1994), or 24S rRNA genes (Benavides et al., 1993). These markers were generally selected with a goal of species identification and did not address the problem of subspecific variability of T. cruzi.

In this study, we used information obtained from previous population genetic studies on T. cruzi to provide a convenient framework to rapidly select RAPD fragments exhibiting a given phylogenetic specificity (species, subspecies, natural clone).

Future work will involve looking for other potentially interesting RAPD fragments in the species T. cruzi and sequencing the 750-bp A8 fragment in several natural clones of the parasite. This will make it possible to select primers for the identification of T. cruzi or given subdivisions of the species without culture.
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LITERATURE CITED


