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Strains and clones of *Trypanosoma cruzi* can be characterized by pattern of restriction endonuclease products of kinetoplast DNA minicircles

(Chagas disease/rapid method for kinetoplast DNA isolation/schizodemes/zymodemes)

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ABSTRACT A simple method was developed for the characterization of different strains of *Trypanosoma cruzi*. *T. cruzi* stocks isolated from vectors or by hemoculture from patients with Chagas disease could be grouped in subpopulations having similar patterns of restriction endonuclease products of kinetoplast DNA minicircles. We designate such subpopulations by the term "schizodemes." Furthermore, it is shown that, from a given *T. cruzi* strain, clones with different biological properties can be isolated and identified by their restriction patterns.

The hemoflagellate *Trypanosoma cruzi* is the etiological agent of Chagas disease, a pleomorphic clinical entity that is an important cause of morbidity and mortality in Central and South America. In some patients the infection is devastating from the very beginning with death occurring after a short acute phase; meningoencephalitis and myocarditis are prominent findings. In other cases, however, the acute phase is oligosymptomatic or even silent and may evolve without detectable sequels. Between these two extremes, in most cases the course has a variable acute phase which subsides in a few weeks, to be followed years later by digestive or cardiac symptoms or both (1).

The exact causes of this clinical pleomorphism are not known, although evidence suggests that both host and parasite factors may be involved (1). In terms of the parasite, differences have been found among several strains of *T. cruzi* in regard to morphology, virulence, pathogenicity, and tissue tropism (1). Particularly well documented are the differences between the "polar" Y (2) and CL (3) strains. Isozyme analysis has also been used to distinguish strains of *T. cruzi*. Miles *et al.* (4–6) examined *T. cruzi* stocks isolated from humans and sylvatic animals in Brazil and found three distinct isozyme groups (or zymodemes). Romanha *et al.* (7, 8) studied *T. cruzi* cultures obtained from humans in the city of Bambuí (Minas Gerais, Brazil) and were able to group the stocks into four isozyme groups. Variations in kinetoplast DNA (kDNA) buoyant density between strains of *T. cruzi* and strains and species of *Leishmania* have been reported, and this classification method has been used by Chance (9) and Baker *et al.* (10).

Comparison of restriction endonuclease-generated fragments of kDNA has been proposed as another method for the intrinsic classification of trypanosomes (11, 12). We show here that stocks of *T. cruzi* isolated from patients with Chagas disease can be characterized by restriction digests of the kDNA networks.

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MATERIALS AND METHODS

Cells. With the exception of the strains Y and CL (13), all the *T. cruzi* stocks used in this study were obtained from patients who had positive serologic tests against Chagas disease and who were from the city of Bambuí (Minas Gerais, Brazil). Stock cultures were initiated with cells from 30 ml of blood, grown to stationary phase in liver infusion/tryptose medium (14) at 27°C, and stored frozen in liquid nitrogen. Clinical histories are available for each human isolate used in this study. For kDNA isolation, 30- to 60-ml cultures were initiated with frozen stabilates, and the cells were washed in Krebs–Ringer/Tris buffer and stored as pellets at −70°C until processed. Y and CL cells were grown in infusion/tryptose medium. Cloning of cells on infusion/tryptose agar was performed as described (15). With the exception of the CL clones in Fig. 5 the other cultures were not cloned. Classification of the stocks by isozyme patterns was done as described (7, 8), using eight soluble enzymes.

Preparation of kDNA. A modification of the method described for *L. tarentolae* was used (16). *T. cruzi* cells were suspended in 0.15 M NaCl/0.1 M EDTA, pH 8.0 (SE buffer), at a maximum density of 1.2×10^9 cells per ml. Pronase (10 mg/ml in SE buffer, predigested at 37°C for 30 min) was added to 0.5 mg/ml and sarkosinate (30% stock solution) was added to 3%, and the lysate was incubated at 60°C for 3 hr. Lysates were diluted with SE buffer and sheared through an 18-gauge needle at 25 psi or by hand with a syringe. Network DNA was pelleted in the SW 27 rotor (27,000 rpm, 1 hr), in the Sorvall HB-4 rotor (9000 rpm, 2 hr), or in an Eppendorf Microfuge ($13,000 \times g$, 1 hr) at 5°C. The pellets were resuspended in the original volume in 10 mM Tris·HCl/1 mM EDTA, pH 7.9 (TE buffer) and were recentrifuged as above. The pellets were then resuspended in TE buffer (400 μl for up to 1.2×10^{10} cells) and extracted twice with phenol/chloroform, 1:1 (vol/vol), saturated with 50 mM Tris·HCl/0.1 M NaCl, pH 7.4, and four times with ether, ethanol precipitated, dried, and resuspended in TE buffer at 100 μl/10^9 cells. All reagents were analytical grade.

This method of kDNA isolation is not optimal for preparation of intact *T. cruzi* maxicircle DNA.

Restriction Endonuclease Digestion and Gel Electrophoresis. Aliquots (5–10 μl) of the kDNA preparation were digested in the appropriate buffers. Restriction endonucleases were purchased from New England BioLabs (Beverly, MD) and Bethesda Research Laboratories (Rockville, MD) or were pre-

Abbreviation: kDNA, kinetoplast DNA.

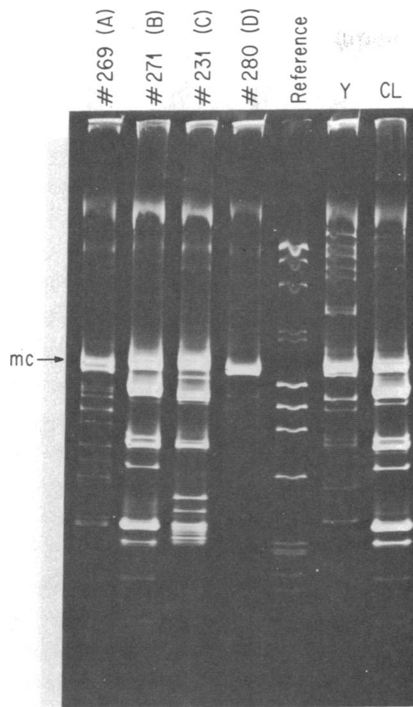

FIG. 1. Acrylamide gradient gel profiles of *Eco*RI digests of kDNA from the Y and CL strains and from four stocks that represent zymodeme groups A–D. The reference DNA is a mixture of *Hin*dIII-digested λ phage and *Hae* III-digested replicative form DNA from φX174. MC, Unit-length minicircle band.

pared according to Greene *et al.* (17). Electrophoresis was performed in 3.0% + 3.5–10% acrylamide linear gradient gels (18) or in 2% agarose gels. Gels were stained with ethidium bromide and photographed (19).

Labeling of kDNA and Hybridization. kDNA was labeled by nick translation (20) using [α-^{32}P]dCTP (Amersham). Transfer of DNA fragments from agarose gels to diazobenzylmethoxy-paper (21) and hybridization with the labeled probe was done according to standard procedures (22).

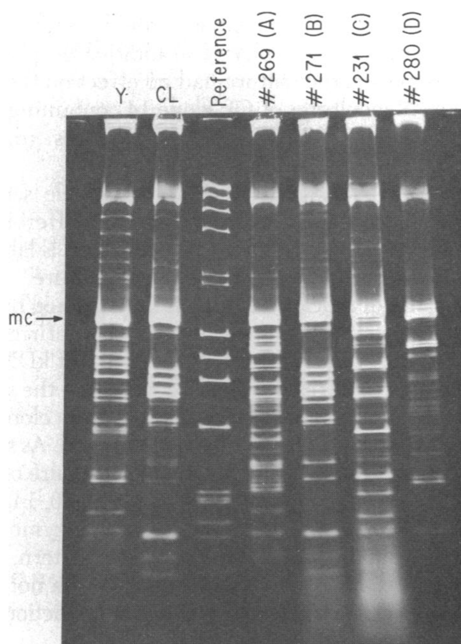

FIG. 2. Profiles of *Hin*fI digests of kDNA from the strains and stocks in Fig. 1.

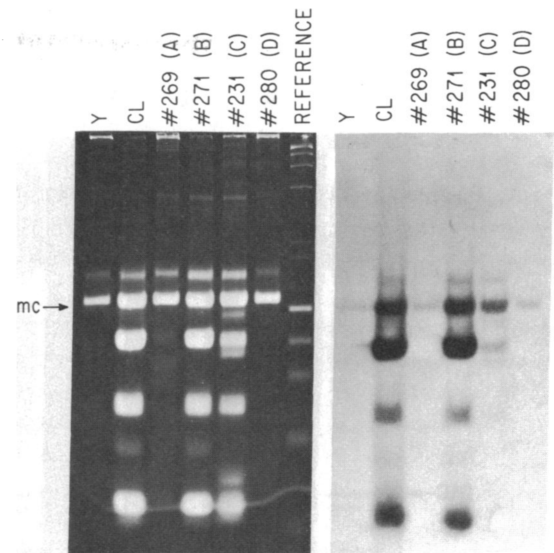

FIG. 3. Blot hybridization of *Eco*RI digests of the kDNA of the strains and stocks of Fig. 1 with nick-translated CL strain kDNA. The gel was 2% agarose. (*Left*) Ethidium bromide-stained gel. (*Right*) Autoradiogram.

RESULTS

Rapid kDNA Isolation Procedure. The protocol developed to prepare kDNA is simple and rapid. Minor nuclear DNA contamination is likely but this does not interfere with the visualization of the kDNA minicircle restriction profile. The yield of kDNA is high: cells from a standard 30-ml culture of *T. cruzi* in infusion/tryptose medium at a density of 45–70 × 10^6 cells per ml yielded enough kDNA for >25 electrophoretic runs.

Screening kDNA of Y and CL Strains with Restriction Enzymes. kDNA from the Y and CL "polar" strains was tested with *Bam*HI, *Bgl* I, *Hin*dIII, *Pst* I, *Sal* I, *Sma* I, *Xho* I, *Ava* I, *Bgl* II, *Bst*EII, *Hin*cII, *Hpa* I, *Xba* I, *Alu* I, *Ava* II, *Fnu*DII, *Hha* I, *Kpn* I, *Mbo* I, *Eco*RI, *Hae* III, *Hin*fI, *Msp* I, and *Taq* I. The last five enzymes gave the best patterns for comparative purposes, with a high percentage of fragments smaller than minicircle size. *Taq* I was unique in that it cleaved most of the minicircles more than once, giving rise to patterns in which a band with one-quarter of the molecular weight of the minicircle predominated.

Comparison of Restriction Patterns of Y and CL kDNA and of kDNA from Representative Stocks from Four Zymodeme Groups. Figs. 1 and 2 show the *Eco*RI and *Hin*fI digests of kDNA from the Y and CL strains and of kDNA from stocks 269, 271, 231, and 280, which represent randomly selected representatives of zymodeme groups A, B, C, and D. Quantitative as well as qualitative differences among the restriction profiles are apparent, except that the digests of strain CL and stock 271, were identical with all enzymes tested.

Evidence that similarities in restriction profiles represent similarities in DNA sequences was obtained by a blot hybridization experiment (23) with a nick-translated CL strain kDNA probe (Fig. 3). The CL probe hybridized with the 271 kDNA fragments to the same extent as it did with the homologous CL kDNA fragments; it hybridized with the 231 kDNA fragments to a lesser extent. The Y, 269, and 280 kDNAs showed little hybridization. The hybridization results agree well on a qualitative level with the differences in patterns shown in Figs. 1 and 2.

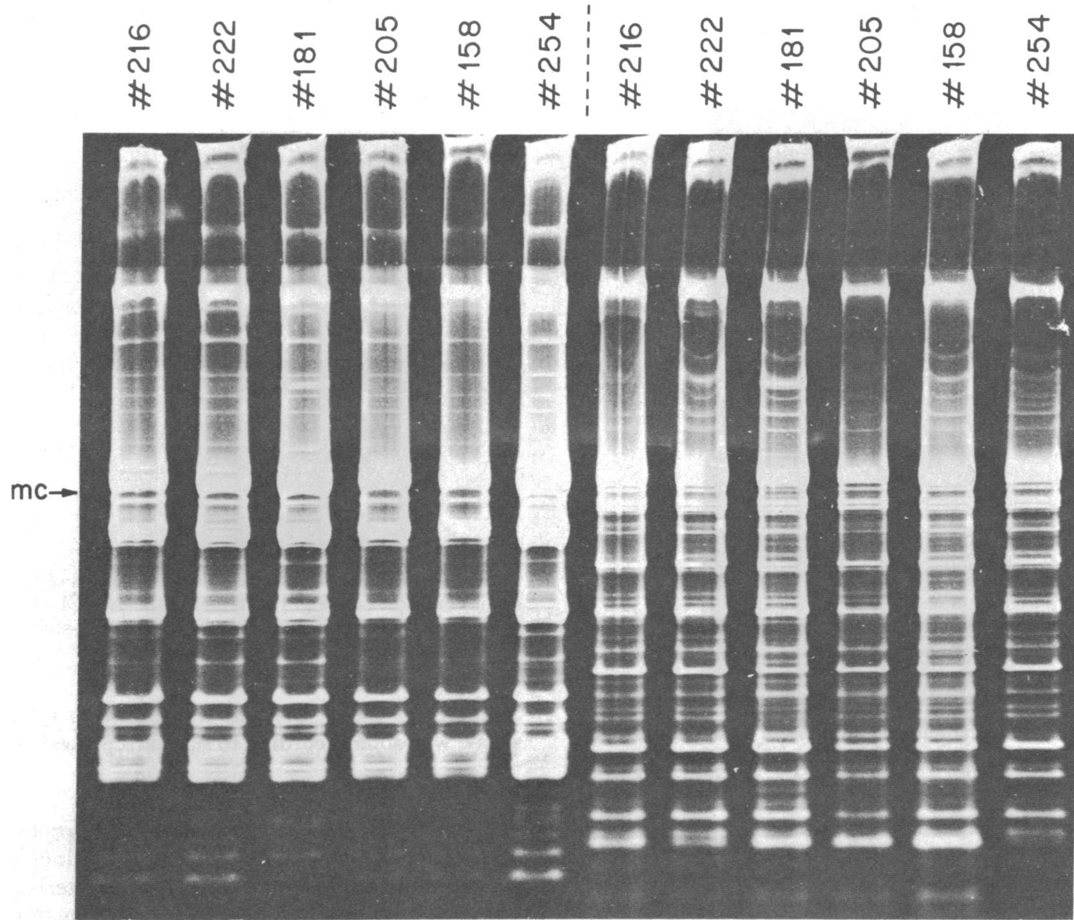

FIG. 4. Comparison of acrylamide gradient gel profiles of *Eco*RI (*Left*) and *Hin*fI (*Right*) digestions of kDNA from six human isolates of *T. cruzi* belonging to zymodeme group C.

An interesting feature of the restriction profiles shown in Figs. 1 and 2 is that in some cases different enzymes give rise to major bands with similar mobilities corresponding to multiples of one-fourth the minicircle unit length, as noted previously with different *T. cruzi* strains (12, 24–26). This has been interpreted in terms of the existence of repetitive sequences in *T. cruzi* kDNA minicircles (12, 24–26) and seems to be unique for *T. cruzi*.

Evidence for the Extent of Genetic Stability of *T. cruzi* kDNA Minicircle Sequences. We have shown that there can be striking differences between *T. cruzi* strains, but it is important to establish the approximate rate of sequence change under different laboratory conditions as well as the actual extent of variability in nature.

The analysis of six randomly selected *T. cruzi* stocks belonging to zymodeme group C is shown in Fig. 4. The six *Eco*RI and *Hin*fI restriction patterns are clearly similar when not identical [*Msp* I and *Bsp* RI digests were also identical (data not shown)]. They are also similar to the profile of stock 231 (Figs. 1 and 2). These results, together with the similarity of profiles of strain CL kDNA [isolated from an insect vector in south Brazil (3)] and stock 271 kDNA [isolated from a patient in southeast Brazil (Fig. 1)], suggest a rather good genetic stability of kDNA minicircle restriction patterns.

Another qualitative estimate of the rate of change of *T. cruzi* kDNA minicircle sequences was obtained by comparisons of kDNA from CL cultures kept at −70°C for 2 years with kDNA from the same cells after 2 years in continuous culture or after 2 years in mice. No differences in the restriction profiles were apparent after the 2-year period of serial culture, but a few minor changes were detected after 2 years serial passage in the mouse host (Fig. 5).

Fig. 5 also demonstrates that the CL kDNA minicircle restriction patterns did not change with growth conditions (logarithmic phase vs. stationary phase). In addition the proportion of trypomastigotes in the culture had no effect on the kDNA restriction profile: cultures of CL clone 14 containing 60% or 2.4% trypomastigotes gave identical profiles (data not shown).

We conclude that *T. cruzi* kDNA minicircle sequences change rapidly enough in nature to produce differences between strains but not so rapidly as to preclude a laboratory analysis after establishment of a stock hemoculture.

Heterogeneity of Stock CL Strain as Determined by kDNA Minicircle Restriction Patterns. A striking confirmation of the usefulness of strain classification by means of kDNA minicircle restriction patterns was the discovery that the standard laboratory CL strain is heterogeneous. Several clones were obtained from the stock CL culture and analyzed. As shown in Fig. 5 our CL laboratory strain is actually a mixture of at least two subpopulations, one represented by clones 10, 14, 18, and 20 and the other by clones 11, 12, and 16. By isozyme analysis the former showed the parental B zymodeme pattern, whereas the latter showed the C zymodeme pattern (data not shown). Three subclones of clone 10 showed identical restriction profiles as the parental clone (data not shown).

The presence of zymodeme group C cells in the stock CL culture was not detected previously by restriction analysis; this

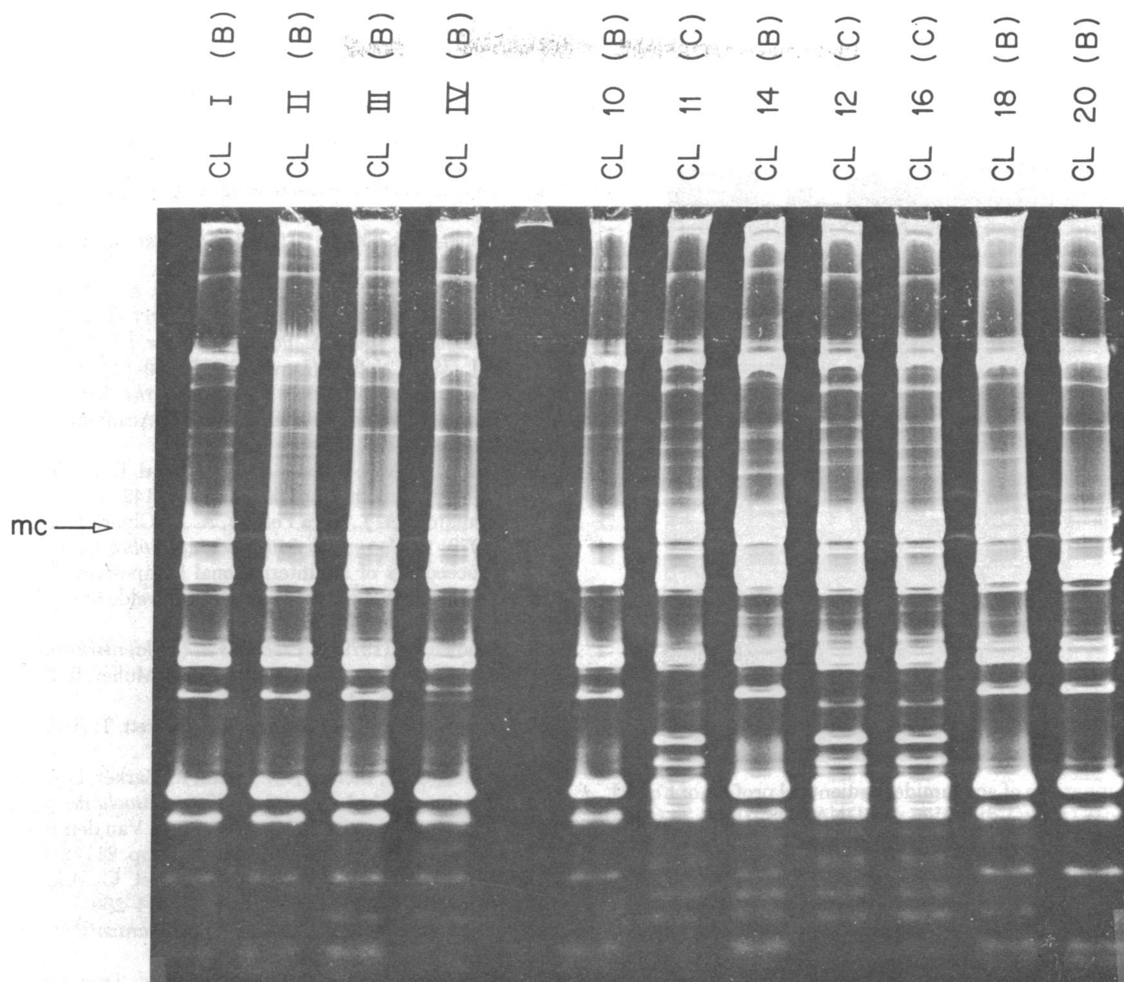

FIG. 5. Acrylamide gradient gel profiles of *Eco*RI digests of kDNA from several CL control cultures and clones. I, CL cells kept in serial culture for 2 years (1978–1980) and harvested in stationary phase; II, CL cells harvested in logarithmic phase; III, CL cells from a culture kept at −70°C for 2 years (1978–1980); IV, CL strain kept in mice for 2 years (1978–1980) and grown for 10 passages in culture before analysis; CL 10, 11, 14, 12, 16, 18, and 20, clones from parental CL strain. The zymodeme groups are given in parentheses.

implies that the group C cells represent a small but stable minority of the cells in the stock CL culture.

Correlation of Ability of Clones to Undergo Trypomastigote Differentiation with kDNA Restriction Patterns. In our study we noted a correlation between the ability of CL clones to undergo the epimastigote–trypomastigote transformation and the minicircle restriction profile. Clones 10, 14, 18, and 20, which display identical restriction patterns, produced 3%, 60%, 13%, and 19% trypomastigotes in stationary phase, respectively. On the other hand, clones 11, 12, and 16, which have a different restriction profile, produced 0%, 0.3% and 0% trypomastigotes (Fig. 5).

A similar situation was found for clones of one of the Y laboratory strains. Analysis of six strain Y clones showed that only one clone produced trypomastigotes in culture (47% trypomastigotes in stationary phase) and this exhibited a different minicircle restriction profile from the others (data not shown).

Proposal of a New Terminology To Classify Hemoflagellates into "Schizodemes." We propose to designate those populations displaying identical or similar kDNA minicircle restriction patterns by the term "schizodeme" (*schizo,* to split) (27, 28).

The question arises as to the correlation of zymodemes and schizodemes among natural populations of *T. cruzi*. The analysis of six zymodeme C stocks in Fig. 4 and the C stock (no. 231) in Figs. 1 and 2 indicate that zymodeme C seems to be composed of a single schizodeme. However, the analysis of three zymodeme A stocks shown in Fig. 6 shows that these stocks belong to three different schizodemes. A recent analysis of 35 zymodeme A stocks yielded 26 separate schizodeme groups with the enzymes *Eco*RI and *Msp* I; analysis of 14 zymodeme B stocks indicates that these belong to three separate schizodeme groups with the enzymes *Eco*RI and *Msp* I (unpublished data). In a few cases, samples belonging to different zymodemes could be grouped in the same schizodeme (data not shown). We conclude that schizodeme analysis will supplement and extend zymodeme analysis in the classification of different strains of *T. cruzi*.

DISCUSSION

We have presented evidence that strains of *T. cruzi* can be classified by means of kDNA minicircle restriction enzyme profiles in high-resolution acrylamide gradient gels. Leon *et al.* (26) reported extensive differences in kDNA restriction profiles between two isolates of the Y strain of *T. cruzi* and published profiles of Y strain kDNA; they concluded that the minicircle digestion pattern might not be a stable and reliable criterion for strain characterization. We believe that our control experiments have settled this question for the strains that we

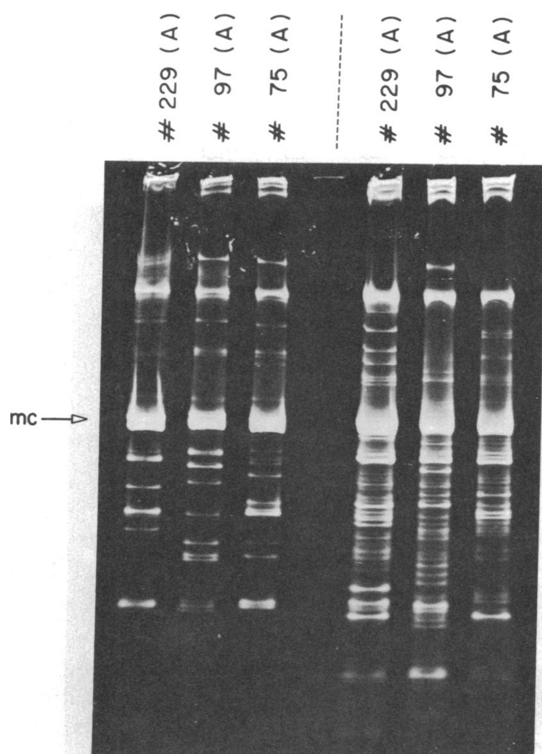

FIG. 6. Comparison of acrylamide gradient gel profiles of *Eco* RI (*Left*) and *Hin*fI (*Right*) digestion of kDNA from three *T. Cruzi* human isolates belonging to zymodeme group A.

have examined. However, we have also found differences between restriction digests of several presumptive Y strain cultures obtained from different laboratories (unpublished data) and we have found considerable schizodeme heterogeneity among Bambuí stocks which belong to the A zymodeme group. The possibility of laboratory strain mixup is always present, but the additional possibility of strain-dependent hypervariability of kDNA minicircle sequences should also be considered. The importance of preventing the occurrence of partial digestion should also be stressed.

The kDNA schizodeme concept should complement and extend isozyme analysis, kDNA buoyant density analysis, and immunological assays for the classification of parasitic hemoflagellates. Preliminary results indicate that this procedure is also useful for the classification of the pathogenic *Leishmania* (unpublished data). The method for schizodeme analysis is simple and rapid. With the availability of lyophilized restriction enzymes and commercially prepared acrylamide gradient gels, it could be adopted for use in clinical laboratories.

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