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Genomic Variation of *Trypanosoma cruzi*: Involvement of Multicopy Genes

WILMA WAGNER* AND MAGDALENE SO

Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037

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By using improved pulsed field gel conditions, the karyotypes of several strains of the protozoan parasite *Trypanosoma cruzi* were analyzed and compared with those of *Leishmania major* and two other members of the genus *Trypanosoma*. There was no difference in chromosome migration patterns between different life cycle stages of the *T. cruzi* strains analyzed. However, the sizes and numbers of chromosomal bands varied considerably among *T. cruzi* strains. This karyotype variation among *T. cruzi* strains was analyzed further at the chromosomal level by using multicopy genes as probes in Southern hybridizations. The chromosomal location of the genes encoding α- and β-tubulin, ubiquitin, rRNA, spliced leader RNA, and an 85-kilodalton protein remained stable during developmental conversion of the parasite. The sizes and numbers of chromosomes containing these sequences varied among the different strains analyzed, implying multiple rearrangements of these genes during evolution of the parasites. During continuous in vitro cultivation of *T. cruzi* Y, the chromosomal location of the spliced leader gene shifted spontaneously. The spliced leader gene encodes a 35-nucleotide RNA that is spliced in *trans* from a 105-nucleotide donor RNA onto all mRNAs in *T. cruzi*. The spliced leader sequences changed in their physical location in both the cloned and uncloned Y strains. Associated with the complex changes was an increase in the infectivity of the rearranged variant for tissue culture cells. Our results indicate that the spliced leader gene clusters in *T. cruzi* undergo high-frequency genomic rearrangements.

The kinetoplastid *Trypanosoma cruzi* is the etiologic agent of Chagas' disease, an infection which affects several million people in Mexico and Central and South America. The parasite undergoes three main morphologic changes as it cycles between an insect vector (epimastigote form) and its vertebrate host (extracellular blood stage trypomastigote and intracellular amastigote forms). A number of antigens are expressed throughout the life cycle of *T. cruzi*; as expected, some antigens are specific to each morphologic stage (15, 16, 28, 39). In addition, heterogeneity exists at various levels among different strains of the parasite (10, 41). Genetic polymorphisms have been observed among strains in terms of isoenzyme electrophoretic mobility (22), kinetoplast DNA restriction patterns (24), and DNA content (9). *T. cruzi* strains also vary in their degree of tissue tropism and infectivity for mice (29; H. Galliard, Prog. Prot. Ecerpta Med. Found. [Amsterdam], p. 143, 1965). Such variation may be responsible for the different clinical symptoms of Chagas' disease, and the genetic basis of this variability is unknown.

Characterization of *T. cruzi* at the cytogenetic level has been difficult because the chromosomes of the parasite do not condense during nuclear division. In three-dimensional reconstructions from electron micrographs of serial sections, 10 dense plaques were detected during nuclear division. The association of these plaques with the equatorial spindle suggested that they may have a kinetochorelike function and that the *T. cruzi* nucleus contains at least 10 chromosomes (35).

Previous studies on DNA complexity and of the chromosomal location of single-copy genes encoding housekeeping enzymes suggested that *T. cruzi* is diploid (6, 14, 19). However, meiosis has never been demonstrated, and the distribution of marker genes in *T. cruzi* populations suggests that meiotic recombination is restricted (40, 41). The development of pulsed-field gel electrophoresis has allowed the preliminary separation of *T. cruzi* chromosomes (2, 14) and the tentative localization of the genes for α- and β-tubulin, rRNA, and the spliced leader to compressed chromosomes in the megabase-pair (Mbp) size range (11). Pulsed-field studies of chromosomes of *T. cruzi* Dm28c suggested that the chromosomal migration patterns of the epimastigote and the metacyclic trypomastigote stages of the parasite were similar (2).

In this report we describe optimized conditions for the separation of chromosomes of several *T. cruzi* strains with the contour clamped homogeneous electric field (CHEF) system (7). With this technique we are able to separate chromosomes of up to 1.6 Mbp. Our studies indicate that the electrophoretic migration pattern of chromosomes of each *T. cruzi* strain is unique and extend earlier studies (2, 11, 14) indicating that, within any strain, the migration pattern does not vary during developmental conversion of the parasite. Using Southern hybridizations of CHEF-separated chromosomes, we localized several multicopy genes encoding rRNA (12), spliced leader (SL) RNA (8, 23, 36), ubiquitin (37), α- and β-tubulin (25), and an 85-kilodalton (kDa) trypomastigote-specific protein (30). The results of these studies indicate that the location of these genes is stable during developmental conversion of the parasite but suggest that multiple translocations of these sequences have occurred during parasite evolution. However, during continuous in vitro cultivation of both epimastigote and trypomastigote forms of the Y strain, we observed spontaneous complex rearrangements of the SL gene cluster. These rearrangements are associated with an apparent increase in infectivity of the trypomastigotes for tissue culture cells.

* Corresponding author.

MATERIALS AND METHODS

Parasite strains. The parasite strains *T. cruzi* Brazil W. F. and the clones 1.2, 3.2, and 4.1 (38), *T. cruzi* Tulahuen zymodeme 3 (21), and *Trypanosoma rangeli* d'Alessandro (42) were provided by G. Harth (Medical Research Foundation, Palo Alto, Calif.). *Leishmania major* WR567 (12), *T. cruzi* Silvio X10 clone 4 zymodeme 1 (32), *T. cruzi* Y zymodeme 2 (34), and the clone H6 (27) were obtained from S. Nickel (Johns Hopkins Medical School, Baltimore, Md.). *Trypanosoma dionisii* (31) was a gift from K. Petry (Fred Hutchinson Cancer Research Center, Seattle, Wash.).

Cell cultures. Epimastigotes of trypanosomatids and promastigotes of *L. major* were grown at 27°C in supplemented RPMI medium (32) and maintained for continuous growth by 1:10 dilutions after the cultures reached a density of 2×10^7 cells per ml. Epimastigotes of the *T. cruzi* strains were converted into metacyclic trypomastigotes by culturing epimastigotes at 27°C in Grace's insect tissue culture medium (GIBCO Laboratories, Grand Island, N.Y.) (15). Metacyclic trypomastigotes were separated from epimastigotes as described previously (15). BALB/3T3 clone A31 mouse fibroblast cells (received from American Type Culture Collection, Rockville, Md.) were infected with metacyclic trypomastigotes for 3 days at 37°C (33) in complemented Dulbecco modified Eagle medium (GIBCO). Depending on the strain, the infected fibroblasts were incubated for an additional 2 to 10 days at 33°C until the release of trypomastigotes. Tissue culture trypomastigotes were maintained by repetitive infection of mouse 3T3 fibroblasts.

Isolation of total DNA for pulsed-field electrophoresis. Parasites (5×10^8) were pelleted in a J-6 rotor for 15 min at 4°C at 2,400 rpm. The pellet was washed one time in 48 mM NaOH–36 mM NaCl–61 mM NaH_2PO_4, suspended in 1 ml of 50 mM EDTA (pH 8.0), and mixed with 1% low-melting-point agarose (FMC Corp., Rockland, Maine) to a final concentration of 0.6% agarose. After solidification of the agarose, 5 ml of 0.49 M EDTA–10 mM Tris hydrochloride (pH 8.0) and 0.2% *N*-lauroylsarcosine supplemented with 1 mg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) per ml was added. For cell lysis, the agarose blocks were incubated for 48 h at 56°C. Agarose blocks were stored in 0.5 M EDTA (pH 8.0) at 4°C for more than 2 years without detectable degradation of the chromosomes.

Pulsed-field separation of DNA. Agarose blocks containing approximately 10^7 parasites were loaded on gels consisting of 1% agarose (Bio-Rad Laboratories, Richmond, Calif.) in 0.5× TBE (0.1 M Tris base, 0.1 M boric acid, 2 mM EDTA). Electrophoretic separation was performed under CHEF conditions (7; with a 0.01-in.-diameter size and a modification in the electrical wiring recommended by A. Link, Washington University School of Medicine, St. Louis, Mo.) while 3.2 liters of 0.5× TBE buffer was circulated through a cooling system (Forma Scientific CH/P) to keep the temperature of the buffer at 13°C. Pulse length, separation time, and voltage were changed as indicated for each separation.

Estimation of sizes of chromosomes. The sizes of chromosomes were estimated by comparison with chromosomes from *Saccharomyces cerevisiae* 334 (Beckman), separated simultaneously with the chromosomes of parasites. The sizes of chromosomes that hybridized with the same gene probe were determined in all strains simultaneously within the same gel under the separation conditions described in the legend to Fig. 1A, except for the values of chromosomes from *T. cruzi* Y and its clone H6, which hybridized with the SL homologous gene probe in the size range between 970 and 1,100 kbp; these values were estimated under the separation conditions used for the experiment in Fig. 1C.

Gene probes used for hybridization. The 18S rRNA gene subcloned as a *Bgl*II-*Pst*I fragment from *T. cruzi* into pUC18 for detection of the rRNA cluster (17) was kindly provided by R. Hernandez (Department of Developmental Biology, Mexico City, Mexico). The α- and β-tubulin gene cluster subcloned as a *Hin*dIII fragment into pGEM2 from *Trypanosoma brucei* (25) was a gift from M. Muhich (California Institute of Technology, Pasadena). J. Swindle (University of Tennessee, Memphis) kindly provided the gene probe for ubiquitin as a *Pvu*II fragment subcloned into the *Sma*I site of pBS (37). The oligonucleotide probes we used were an oligonucleotide identical with nucleotides 23 to 47 of the coding region of an 85-kDa surface protein (30) isolated from *T. cruzi* (TGG GGG CGA GGG AGA CAG CGG) and oligonucleotides homologous to nucleotides −2 to +36 of both strands of the SL gene (CCA ATA TAG TAC AGA AAC TGT ATC AAT AAT AGC CTT AG and the complementary strand) in *T. cruzi* (8). Kinetoplast DNA was isolated by standard procedures (25), labeled by nick translation (20), and used for the detection of kinetoplast DNA in pulsed-field gels.

Nucleic acid hybridization. Nick translation of plasmids and kinase labeling of oligonucleotide fragments were performed by standard protocols (20). For Southern blots, DNA was transferred onto nylon membranes (ICN Pharmaceuticals, Inc., Irvine, Calif.) by depurination of the DNA for 15 min in 0.25 N HCl at room temperature, denaturation in 0.2 N NaOH–0.6 M NaCl three times for 10 min each, and neutralization of the agarose gels in 25 mM sodium phosphate buffer (pH 6.5) three times for 10 min each. Transfer of DNA was allowed for at least 18 h in neutralization buffer. The DNA was covalently bound to nylon membranes by drying the filters at 37°C. A buffer consisting of 1% bovine serum albumin, 1 mM EDTA, 0.5 M sodium phosphate buffer (pH 7.2), and 7% sodium dodecyl sulfate was used for prehybridization (for 2 h) and hybridization (for a minimum of 12 h). Each filter was washed for 15 min each in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate, 0.5× SSC–0.5% sodium dodecyl sulfate, and 0.1× SSC–0.1% sodium dodecyl sulfate. Prehybridization, hybridization, and the washes were performed at the same temperature as follows: at 65°C for the gene probes for the rRNA, ubiquitin, kinetoplast DNA, and the α- and β-tubulin; at 42°C for the SL gene homologous oligonucleotides; and at 50°C for the oligonucleotide homologous to the 85-kDa protein. Densitometric analysis of the hybridizing signals was performed with an LKB UltraScan XL laser densitometer.

DNA digestions. Before digestion of DNA that was embedded in agarose, the blocks were treated as described previously (4). The samples were incubated overnight with the restriction enzymes *Not*I, *Sal*I, and *Xho*I at 37°C and analyzed in 1% agarose gels under pulsed-field conditions.

Infection with variant trypomastigotes. We mixed the trypomastigote variant 1 (original strain from a frozen stock passaged one time through 3T3 mouse fibroblasts) and variant 2 (strain after 9 months of continuous cultivation) in ratios of 1:1, 2:1, and 4:1. 3T3 mouse fibroblast cells were infected with a total of 2 parasites per cell for 3 h at 37°C. Trypomastigotes that did not invade fibroblast cells during this time were removed by washing the cells at least three times with medium. After one cycle of infection, the released trypomastigotes were isolated as described earlier, and their

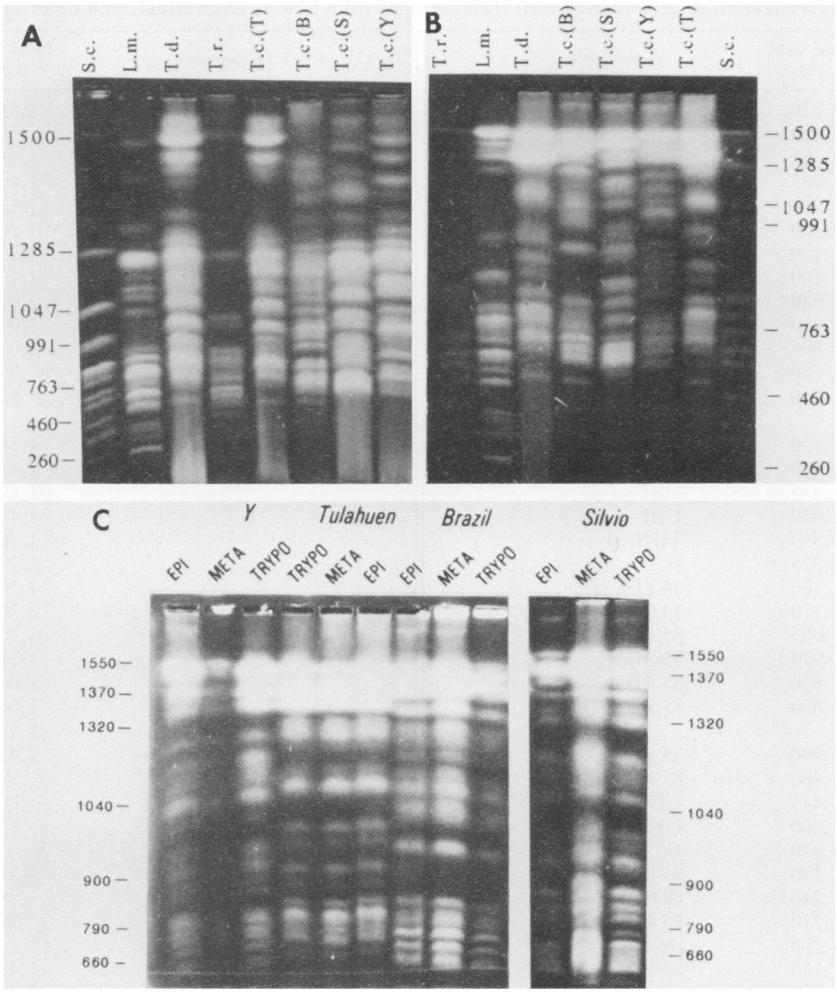

FIG. 1. Ethidium bromide-stained gels of CHEF-separated chromosomal DNA of kinetoplasids. (A and B) Chromosomes from promastigotes of *L. major* (L.m.) and epimastigotes of *T. dionisii* (T.d.), *T. rangeli* (T.r.), and the *T. cruzi* (T.c.) strains Tulahuen (T), Brazil (B), Silvio (S), and Y. *S. cerevisiae* (S.c.) chromosomes were used as molecular size markers (kilobase pairs). (A) Fractionation of 1.0- to 1.6-Mbp chromosomes. Prerunning conditions with a pulse length of 90 s were applied for 1 h at 50 V, 1 h at 100 V, and 1 h at 150 V. The chromosomes were separated for 21 h with a 3-min pulse, for 24 h with a 6-min pulse, and for 50 h at 50 V with a 20-min pulse. (B) Separation of 260- to 1,000-kbp chromosomes. The chromosomes were separated with a pulse length of 90 s for 1 h at 50 V, 2 h at 100 V, and 36 h at 150 V. (C) Comparison of the chromosomes from epimastigotes (EPI), metacyclic trypomastigotes (META), and tissue culture trypomastigotes (TRYPO) of *T. cruzi* strains under the separation conditions described for panel B, except that separation at 150 V was prolonged for a total of 42 h.

karyotypes were examined by probing chromosomal blots with the SL gene probe.

RESULTS

Chromosome organization. We used the CHEF system to separate chromosomes of four different *T. cruzi* strains belonging to three zymodeme groups. The chromosomal electrophoretic pattern of *T. cruzi* chromosomes was also compared with those of several other protozoan parasites: *T. dionisii*, a bat isolate that also belongs to the subgenus *Schizotrypanum*; *T. rangeli*, a nonpathogenic kinetoplastid; and the obligatory intracellular parasite *L. major*.

To achieve good resolution of the *T. cruzi* chromosomes, we needed to use three different sets of pulse conditions. Figure 1 shows gels run under these conditions, with *Saccharomyces cerevisiae* 334 chromosomes as markers. The chromosome migration patterns differed with the *T. cruzi* strain and involved chromosomes of all sizes (Fig. 1A and B), whereas the patterns were the same among the different developmental forms of each strain (Fig. 1C). We were able to resolve 20 to 24 ethidium-stained bands, depending on the *T. cruzi* strain. That the intensity of the bands was not proportional to the estimated sizes of the chromosomes suggests that some chromosomes are very similar in size or that several chromosomes are present in multiple copies. Southern hybridization of separated chromosomes with kinetoplast DNA as a probe revealed that the kinetoplast DNA did not enter the gel under our electrophoresis conditions (data not shown).

Chromosomal localization of multicopy genes. To examine the chromosome size variation further, we performed Southern hybridization on whole chromosomes separated by the pulse-field conditions shown in Fig. 1A (Table 1). Gene probes encoding the ubiquitin, α- and β-tubulin, rRNA, and

TABLE 1. Hybridization of gene probes with chromosomes from four *T. cruzi* strains and other kinetoplastids[a]

Organism	Band size (kb)	Gene probe(s)	Organism	Band size (kb)	Gene probe(s)
T. cruzi Silvio	1,520	UBI	*T. cruzi* Tulahuen	1,535	85 kDa
	1,480	UBI		1,500	85 kDa
	1,385	UBI		1,480	TUB
	1,285	85 kDa		1,460	UBI, 85 kDa
	1,280	rRNA		1,320	rRNA
	1,260	TUB, sl, 85 kDa		1,260	85 kDa
	1,120	85 kDa		1,250	rRNA
	1,100	85 kDa		1,190	sl
	980	85 kDa		1,090	85 kDa
	910	85 kDa		1,040	TUB
	875	85 kDa		1,025	85 kDa
	680	85 kDa		990	85 kDa
	640	85 kDa		820	85 kDa
T. cruzi Brazil	1,590	rRNA	*T. dionisii*	1,540	UBI, 85 kDa
	1,480	rRNA		1,480	TUB
	1,445	UBI		1,490	85 kDa
	1,420	UBI		1,470	85 kDa
	1,350	TUB, sl		1,460	UBI
	1,225	sl		1,290	85 kDa
	1,200	85 kDa		1,280	rRNA
	1,170	TUB		1,260	85 kDa
	1,005	85 kDa		1,165	85 kDa
	940	85 kDa		1,110	85 kDa
	820	85 kDa		1,065	sl
	650	85 kDa		1,040	TUB
				1,035	85 kDa
T. cruzi Y	1,490	85 kDa		1,020	sl
	1,460	85 kDa		995	85 kDa
	1,445	UBI		865	85 kDa
	1,400	UBI		835	85 kDa
	1,320	sl, TUB			
	1,290	85 kDa, TUB	*T. rangeli*	1,540	rRNA
	1,240	rRNA		1,280	rRNA
	1,210	85 kDa		590	TUB
	1,200	rRNA			
	1,130	85 kDa	*L. major*	1,480	UBI
	1,100	sl*		1,280	rRNA
	1,040	sl*		1,240	TUB, rRNA
	1,020	sl*		590	TUB
	1,010	sl*			
	970	sl*			
	700	85 kDa			

[a] UBI, Ubiquitin; TUB, α- and β-tubulin; 85 kDa, 85-kDa trypomastigate-specific surface protein of *T. cruzi*; sl, stable SL gene locations; sl*, variable locations of the SL gene; rRNA, 18S rRNA.

an 85-kDa surface protein of *T. cruzi* hybridized to the same chromosomes from different developmental stages of the *T. cruzi* strains analyzed (data not shown). However, among the different strains, the sizes and numbers of chromosomes hybridizing to these probes varied significantly. To examine the significance of these apparently heterogeneous loci, we compared the chromosomal locations of genes between different *T. cruzi* strains with those of other kinetoplastids when possible. Since we used predominantly *T. cruzi* genes as probes, those that are not mentioned for *L. major* and *T. rangeli* did not hybridize under high-stringency conditions (Table 1).

The rRNA gene probe hybridized to either one or two chromosomes in all the parasites analyzed, suggesting, in a most likely diploid organism, size variations of homologous chromosomes. In contrast, the probe containing the 5′ end of the coding region for the 85-kDa protein hybridized to multiple chromosomes in all the trypanosome strains and indicated that translocations of these sequences had occurred or that multiple related genes have evolved.

The ubiquitin probe hybridized to three chromosomal loci in the Silvio strain and one or two chromosomes in the other *T. cruzi* strains. Whereas two chromosomal loci could be explained by size variations of homologous chromosomes, the three chromosomal loci in the Silvio strain suggest that the ubiquitin gene had undergone translocation during the evolution of the parasite.

In three of the four *T. cruzi* strains analyzed, the probes encoding tubulin and the spliced leader hybridized to the same chromosomal band. The size of this band varied among the different *T. cruzi* strains and indicated that both genes are encoded on the same chromosome. In these strains additional chromosomal loci of the spliced leader and the tubulin genes could be detected. However, on these additional chromosomes both genes were physically unlinked. Furthermore, in the Tulahuen strain the sl probe did not hybridize to any of the chromosomal loci of the tubulin genes. These results suggest that translocations of these multicopied clustered genes must have occurred during evolution in *T. cruzi*.

Spontaneous variations in chromosomes encoding the

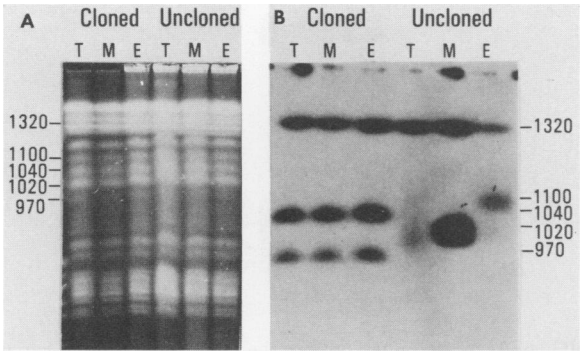

FIG. 2. Chromosomal localization of the SL gene cluster in the uncloned strain *T. cruzi* Y and the cloned derivative H6. (A) Ethidium bromide-stained gel of chromosomes isolated from *T. cruzi* Y and H6 and separated as described in the legend to Fig. 1C. T, Trypomastigotes; M, metacyclic trypomastigotes; E, epimastigotes. The chromosomes from epimastigotes of the uncloned Y strain were isolated after 5 months of continuous cultivation. (B) Chromosomes from the same gel shown in panel A were used for hybridization with the SL probe. The sizes of the SL-hybridizing chromosomes (kilobase pairs) were estimated by comparison with chromosomes from *S. cerevisiae* 334.

spliced leader gene cluster in *T. cruzi* Y. An oligonucleotide homologous to nucleotides −2 to +36 of the SL gene (8) was also used as probe in Southern hybridization of pulsed-field-separated chromosomes of the different developmental forms of several strains of *T. cruzi*. In *T. cruzi* the SL gene is encoded in multiple copies arrayed in tandem, with a 610-bp unit length of the repeat (8). As expected for a diploid organism, the SL probe hybridized to either one or two chromosomes of different sizes in the *T. cruzi* strains analyzed (Table 1). The chromosomal location of the SL gene probe immediately upon developmental conversion was stable in all strains tested. In *T. cruzi* Y, the SL probe initially hybridized to a 1,320-kbp chromosome and a 1,020-kbp chromosome. During constant cultivation of an originally uncloned isolate from strain Y (35), differences were observed over time in the hybridization patterns of the SL probe with chromosomes of epimastigotes. After 5 months of continuous cultivation, the probe hybridized to a 1,320-kbp chromosome and a 1,100-kbp chromosome (Fig. 2B).

Since fluctuations in a mixed population could be responsible for these results, we analyzed chromosomes from epimastigotes and tissue culture trypomastigotes of a cloned derivative of the Y strain (H6) in similar Southern blots. No difference in the sizes and numbers of chromosomes were observed between the original Y strain and its cloned H6 derivative shown in Fig. 2A. The SL probe hybridized to chromosomes of 1,320, 1,040, and 970 kbp in the original H6 clone (variant 1) (Fig. 2B); this hybridization pattern was identical in tissue culture trypomastigotes, metacyclic trypomastigotes, and epimastigotes of the H6 clone. Changes in the chromosomal SL loci were detected in H6 trypomastigotes after 7 months of cultivation. In the trypomastigote population the SL probe hybridized to chromosomes of 1,320, 1,040, 1,010, and 970 kbp (Fig. 3). The diffuse hybridization pattern of the SL probe to the 1,010- and 970-kbp chromosomes from trypomastigotes in the early stages of cultivation could be a reflection of telomere expansion and contraction or some other microheterogeneity in chromosome size. After 9 months of cultivation, in the new variant (variant 2) the SL probe hybridized only to chromosomes of

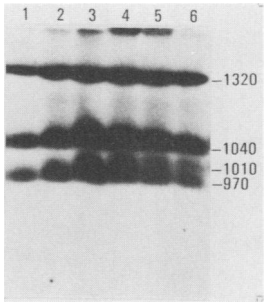

FIG. 3. Variation of the hybridizing pattern of the SL probe to chromosomes (kilobase pairs) of H6 tissue culture trypomastigotes cultured continuously. Chromosomes from parasites were isolated at various times during continuous cultivation. The chromosomes were separated as described in the legend to Fig. 1C and hybridized with the SL probe. Lanes: 1, original cell culture; 2 through 6, cell cultures after 4, 5, 6, 7, and 8 months of cultivation, respectively.

1,320, 1,040, and 1,010 kbp (Fig. 4B). In these variants, no changes in the size or number of chromosomes could be detected.

The chromosomal location of the SL gene cluster also changed during constant cultivation of H6 epimastigotes. Initially, the SL probe hybridized to chromosomes of 1,320, 1,040, and 970 kbp. After 12 months of cultivation, the SL probe hybridized to a chromosome of 1,320 kbp and two chromosomes of 970 kbp (Fig. 4A).

Nature of the size variation in chromosomes encoding the SL gene cluster. To examine the nature of the size variability of chromosomes encoding the SL gene cluster, Southern blots were performed on chromosomal DNA digested with various restriction enzymes. Whole chromosomes were extracted from epimastigotes and tissue culture trypomastigotes of the H6 clone, enclosed in agarose blocks, and digested with restriction enzymes *Sal*I, *Not*I, and *Xho*I, enzymes that we previously established do not cut within the tandem repeat of the SL gene cluster (data not shown). The DNA in the enzyme-treated blocks was then separated on a 1% agarose gel for Southern hybridization experiments with the SL probe (Fig. 5). In epimastigotes (Fig. 5B) and trypomastigotes (Fig. 5A) from the early stages of cultivation, the SL probe hybridized to five restriction fragments 340, 270, 130, 35, and 30 kbp in size. In epimastigotes from the early stages of cultivation (variant 1), the SL probe hybridized to a 340-kbp DNA fragment; in epimastigotes

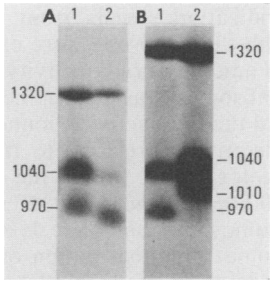

FIG. 4. Altered chromosomal location of SL genes during continuous cultivation of epimastigotes (A) and tissue culture trypomastigotes (B) of the H6 clone. Chromosomes extracted from the original cultures (variants 1, lanes 1) and cultures after 12 months of cultivation (variants 2, lanes 2) were separated as described in the legend to Fig. 1C and hybridized with the SL probe. The sizes of hybridizing chromosomes are given in kilobase pairs.

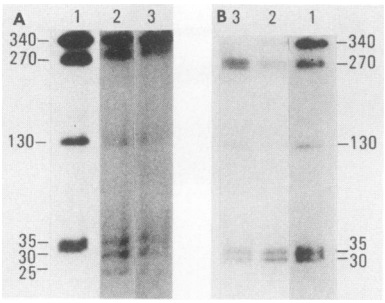

FIG. 5. Changes in the genomic organization of the SL gene clusters in H6 cultures of epimastigotes and trypomastigotes over time. Chromosomes isolated at various times during continuous cultivation from trypomastigotes (A) and epimastigotes (B) were digested with *Xho*I, *Sal*I, and *Not*I. The DNA fragments were separated by pulsed-field electrophoresis for 1 h at 50 V, 1 h at 100 V, and 19 h at 150 V with a pulse length of 10 s and hybridized with the SL probe. DNA was extracted from trypomastigotes (A) and epimastigotes (B). Lanes: 1, original cultures; 2 and 3, parasite cultures after 7 months (lanes 2) and 12 months (lanes 3) of cultivation. Molecular sizes in kilobase pairs were estimated by comparison with λ *Hin*dIII and chromosomes from *S. cerevisiae* 334.

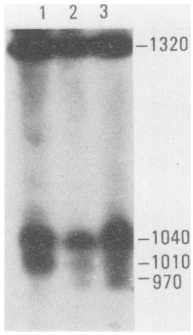

FIG. 6. Increased infectivity of the H6 trypomastigote variant 2 for mouse 3T3 fibroblasts. Fibroblasts were infected with mixtures of the original culture (variant 1) and variant 2 in ratios of 1:1 (lane 1), 2:1 (lane 2), and 4:1 (lane 3). The trypomastigotes released from these fibroblasts were analyzed by hybridization of the SL probe to their chromosomes separated under conditions described in Fig. 1.C. The sizes of hybridizing chromosomes are indicated in kilobase pairs.

from late stages of cultivation (variant 2) the probe hybridized to a fragment of 260 kbp (Fig. 5B). The SL-associated changes were more complex in the trypomastigotes. In early cultivation trypomastigotes (variant 1) the SL probe hybridized to a 270-kbp fragment; after 7 months of growth the SL probe hybridized to a 275-kbp fragment, and in late stage cultivation trypomastigotes (variant 2) the probe hybridized to a 290-kbp fragment (Fig. 5A). In addition a 25-kbp fragment hybridized with the SL probe after 7 months of cultivation.

No differences in the sizes and numbers of chromosomes from different isolates were detected. The changes in the sizes of restriction fragments containing the SL sequence in late-stage trypomastigotes total 42 kbp and could account for the 40-kbp increase in size of the chromosomes hybridizing with the SL probe. However a change in the hybridization intensity of the 1,010-kbp chromosomal band, containing approximately two chromosomes as estimated by the ethidium bromide stain intensity of this band, was not detectable. Furthermore, if amplification of the SL repeats were responsible for a 40-kbp size increase of the chromosome in late-stage trypomastigotes, the altered chromosome would contain 65 additional copies of the SL repeat with an accompaning dramatic increase in the SL hybridization signal. The hybridization signals from the chromosomal Southern blots with the SL probe were examined by densitometry. The total amount of radioactivity bound to all three hybridizing chromosomes was determined and assigned a value of 100%, and the radioactivity bound to each chromosome was then determined relative to the total. In early-stage trypomastigotes, 49 ± 2% of the radioactivity was bound to the 1,320-kbp chromosome, 35 ± 1% was bound to the 1,040-kbp chromosome, and 16 ± 1% was bound to the 970-kbp chromosome. The distribution of the radioactivity bound to chromosomes isolated from late-stage trypomastigotes after 12 months of continuous cultivation did not alter significantly; 42 ± 7% of the radioactivity bound to the stable 1,320-kbp chromosomal SL locus, 37 ± 2% bound to the 1,040-kbp chromosome, and 21 ± 3% bound to the 1,010-kbp chromosomal location of the SL gene. Since the ratio of labeled SL probe bound per chromosome did not alter significantly, we conclude that translocation of the SL repeat must have occurred during continuous cultivation of the H6 trypomastigotes.

A similar situation was observed with the SL hybridizing chromosomes from epimastigotes. In variant 2 of the epimastigotes, the size of an SL-hybridizing chromosome had decreased 70 kbp, whereas the restriction fragment hybridizing to the SL probe decreased 80 kbp. No differences in the chromosomal banding pattern in the two epimastigote variants was detectable, and the ratio of the hybridizing sl signals to chromosomes remained the same, suggesting translocation of the SL sequences.

Selective advantage of trypomastigote variant 2. To determine whether the change in location of the SL gene is reflected in any biological differences of the parasite, we determined the infectivity of the two H6 trypomastigote variants for tissue culture fibroblasts in vitro. Variant 1 (original strain from a frozen stock) and variant 2 (strain after 9 months of continuous cultivation) were mixed in ratios of 1:1, 2:1, and 4:1 and used to infect mouse 3T3 fibroblast cells. After one cycle of infection, Southern hybridization with the SL probe was performed on the chromosomes of released parasites (Fig. 6), and the hybridization signals from the chromosomes were examined by densitometry. In mixed infections with equal numbers of both variants, variant 2 appeared to have a selective advantage and overgrew variant 1. In an infection ratio of 2:1, variant 2 was still predominant. Only in an infection ratio of 4:1 were equal numbers of both variants released.

DISCUSSION

Characterization of *T. cruzi* nuclear DNA by pulsed-field electrophoresis has only recently been made possible. We have established conditions for the CHEF system for the separation of *T. cruzi* chromosomes. Compared with other recent reports (2, 11, 14), our improved conditions have allowed us to use this system to examine the number and size of chromosomes of up to 1.6 Mbp in length of several *T. cruzi* strains as well as the chromosome migration patterns of the different developmental forms of the parasite. Our data indicate that the size and number of chromosomes varied with each *T. cruzi* strain analyzed.

Variation among strains of *T. cruzi* (with respect to

infectivity and other biological parameters) has been reported previously (9, 10, 22, 29, 41). Whether these variations are a reflection of the karyotype differences is unknown.

In previous reports the nuclear DNA content in a single *T. cruzi* nucleus measured by microspectrofluorometry was estimated to be 2×10^8 bp (19), and the total DNA content of single cells in clonal populations of several *T. cruzi* strains determined by flow cytometry suggested that large parts of the parasite genome undergo frequent duplication or deletion (9). Furthermore, karyotypic variation has been reported among the epimastigotes of several clonal populations of the Miranda strain (11). We compared the karyotypes of various clones of the Brazil strain (data not shown) and did not detect such differences. Our results suggest that frequent duplication or loss of total chromosomes is not a common basis for the genomic size variability of the parasite.

In contrast to this interstrain variation in chromosome banding pattern, karyotype differences were not observed among the developmental stages of a single strain. Thus, the observed differences in chromosomal pattern among different strains do not involve rapid changes in chromosomal organization during developmental conversion. Similar results were recently reported by Aymerich et al. (2) for *T. cruzi* Dm28c. Taken together, these results suggest that the chromosomal banding pattern is a useful method for the characterization of *T. cruzi* strains.

Contrary to a previous report (11), we did not observe minisized chromosomes in the *T. cruzi* strains analyzed with our separation conditions. The smallest chromosomes we could separate in trypanosomes of the subgenus *Schizotrypanum* were 500 kbp, significantly larger than those of *T. rangeli* and *L. major* (420 and 270 kbp, respectively).

Gene probes encoding ubiquitin, α- and β-tubulin, rRNA, and an 85-kDa surface protein of *T. cruzi* (30) hybridized to the same chromosomes in different developmental stages of the same *T. cruzi* strains. However, the sizes and numbers of chromosomes hybridizing to these gene probes varied with the strains. Our hybridization results suggested that translocations of the genes encoding ubiquitin, α- and β-tubulin, and an 85-kDa surface protein occurred in the different *T. cruzi* strains during evolution.

In three of four *T. cruzi* strains analyzed, the chromosomal loci containing the spliced leader sequences remained constant. During continuous cultivation of epimastigotes and trypomastigotes of the Y strain (both cloned and uncloned variants), we detected differences in the hybridization pattern of the SL probe with the chromosomes. The changes in the location of the SL sequences did not involve developmental conversion of one stage to the other. However, it was associated with an apparent increase in infectivity of the rearranged variant for tissue culture cells.

The appearance of different-sized chromosomes hybridizing with the SL probe is most likely not due to growth-associated expansion and contraction of telomeres of the same chromosomes. This latter process involves approximately 20 kbp of trypanosome telomeres (3, 43), whereas the difference in size between the altered chromosomes hybridizing with the SL gene was 40 kbp in trypomastigotes and 70 kbp in epimastigotes.

Variation in the sizes of chromosomes caused by amplification of the SL gene has been observed in *L. major* (18). Our phenomenon cannot be explained by a simple amplification of SL-encoding DNA, since the ratio of labeled SL probe bound per chromosome remained the same.

In the SL rearranged variants, the chromosomal banding pattern in pulsed-field gels remained the same, indicating transposition of the SL gene cluster. The SL-associated changes were complex in trypomastigotes and suggested intra- and interchromosomal rearrangements. Retrotransposable insertion elements have been found in the SL cluster in African trypanosomes (1, 5, 26) and *Crithidia fasciculata* (13). Remarkably, although the target site of integration between these elements varied by only 2 bp, the retrotransposons showed no sequence homology. These observations suggest that the SL gene cluster is a main target for genomic rearrangements mediated by a variety of transposable elements (1, 5, 13). Our data indicate that the appearance of the SL sequences in chromosomes of different sizes is most likely due to a translocation of the DNA between chromosomes. Whether translocations involving other genes also occur in *T. cruzi* is unknown, since there are so few markers identified for this trypanosome.

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