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Genetics

Comparison of maxicircle DNAs of *Leishmania tarentolae* and *Trypanosoma brucei*

(kinetoplast DNA/evolution of maxicircle DNA/divergent region of maxicircle DNA)

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ABSTRACT The conserved portions of the maxicircle DNAs of *Leishmania tarentolae* and *Trypanosoma brucei* are organized in a basically colinear manner over a 15- to 17-kilobase region that is interrupted by two small less-homologous sequences. The most highly conserved regions are those encoding the *9S* and *12S* genes. An approximately 12-kilobase region directly upstream of the *12S* gene in the *L. tarentolae* maxicircle showed no sequence homology with the *T. brucei* maxicircle and also was not transcribed. An approximately 6-kilobase region in the *T. brucei* maxicircle in the same relative location also showed no sequence homology with the *L. tarentolae* maxicircle. We propose that evolution of maxicircle DNA occurs mainly within this "divergent region."

The mtDNA or kinetoplast DNA (kDNA) of the kinetoplastid protozoa (1, 2) consists of a catenated network of minicircles and maxicircles. The minicircles comprise approximately 95% of the mass of the network, are usually heterogeneous in sequence, and are probably not transcribed (3-5). The maxicircles are present at 20-50 copies per network, are homogeneous in sequence, and are extensively transcribed (6-9). Maxicircle DNA contains the genes for the putative mitochondrial miniribosomal RNAs and also contains sequences homologous to several structural genes from yeast (10) and *Zea mays* (11). In the African trypanosome *Trypanosoma brucei*, the maxicircle appears to be evolving in a complex fashion involving both base substitutions and insertions/deletions of up to 1.5 kilobases (kb) in a region of the molecule termed the "silent" or "variable" region (12).

Interspecific size variation of maxicircle DNA is much more pronounced, ranging from 3 to 17 kb (2). Conservation of the *9S* and *12S* gene sequences has been shown for several species (13-16), and conservation of other maxicircle sequences has also been reported for *T. brucei* and *Trypanosoma cruzi* (17) and for *T. brucei* and *Crithidia luciliae* (15).

To investigate the question of maxicircle evolution, we have examined in detail the extent of sequence homology between the 22-kb maxicircle of *T. brucei* 366D and the 30-kb maxicircle of *L. tarentolae*. We show that the organization of homologous regions is similar and that the two genomes differ mainly by insertions/deletions of nonhomologous sequences within one portion of the maxicircle that we have termed the "divergent region."

MATERIALS AND METHODS

Cells and Cell Culture. A clonal strain of *L. tarentolae* was grown as described (18). The 366D strain of *T. brucei* was grown as the procyclic form in modified Steiger's REI medium as described (16). Stationary phase cells were harvested for kDNA isolation.

Isolation of DNA. kDNA was isolated from *L. tarentolae* and *T. brucei* cells as described (16). The kDNA was purified by two ethidium bromide/CsCl gradients. *Eco*RI-linearized *L. tarentolae* maxicircle DNA was isolated from digested network DNA by the Hoechst 33258 dye/CsCl method described previously (19). The purified maxicircle DNA was digested with *Msp* I and the fragments were separated in agarose and isolated by electroelution into dialysis bags. *T. brucei* kDNA was digested with *Hin*dIII and the 9.3-kb maxicircle A fragment (see Fig. 2) was isolated by electroelution after separation in agarose. The 6.5-kb B and the 6.1-kb C maxicircle *Hin*dIII fragments were obtained from the pBR322 clones, pTbM-2 and pTbM-1, described previously (16). *Hin*dIII-excised inserts were recovered by electroelution following electrophoresis through agarose. The *Hha* I subfragments, a and b, of the *L. tarentolae* maxicircle *Msp* I fragment 3 were cloned into the *Eco*RI site of pBR322 after treatment with the Klenow fragment of *Escherichia coli* DNA polymerase I and *Eco*RI linker addition. Insert DNA was isolated from *Eco*RI-digested plasmid by agarose electrophoresis and electroelution. The 6.6-kb *Eco*RI/*Bam*HI fragment (see Fig. 6) of the *L. tarentolae* maxicircle was obtained from the pBR322 clone, pLt120 (6). Purified 120 insert DNA was digested with *Sau*3A and the fragments were isolated by electroelution after separation in agarose. Control hybridizations indicated no significant cross-contamination.

Restriction Endonuclease Digestion and Gel Electrophoresis. Restriction endonucleases were purchased from Bethesda Research Laboratories and New England BioLabs. Digestions were carried out under the conditions suggested by the suppliers. Electrophoresis was carried out in submerged horizontal agarose gels in a buffer of 0.09 M Tris borate/2.5 mM EDTA, pH 8.3, in the presence of ethidium bromide at 0.5 μg/ml. Gel illumination and photography were as described (20). *Hin*dIII-digested λ DNA and *Hae* III-digested ϕX replicative form DNA were used as molecular weight standards.

DNA Hybridizations. DNA fragments were transferred unidirectionally onto nitrocellulose (Schleicher & Schuell, BA85) as described (21). After blotting, the filters were washed in 0.30 M NaCl/0.030 M Na citrate, pH 7.2, and baked at reduced pressure at 80°C for 2 hr. Probe DNAs were labeled by nick-translation (22) with [α-^{32}P]dATP and [α-^{32}P]dCTP to specific activities of approximately 10^8 cpm/μg. Hybridizations were carried out with approximately 5×10^6 cpm of denatured probe per filter in either 0.75 M NaCl/0.075 M Na citrate, pH 7.2/0.2% $NaDodSO_4$/sonicated denatured salmon sperm DNA (0.5 mg/ml)/poly(rA) (0.5 mg/ml)/0.02% Ficoll/0.02% polyvinylpyrollidine/0.02% bovine serum albumin/50% formamide (HMA medium) (8) or 1 M NaCl/0.01 M Tris·HCl, pH 7.4/0.2% Ficoll/0.2% polyvinylpyrollidine/0.2% bovine serum albumin

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Abbreviations: kDNA, kinetoplast DNA; kb, kilobase(s).

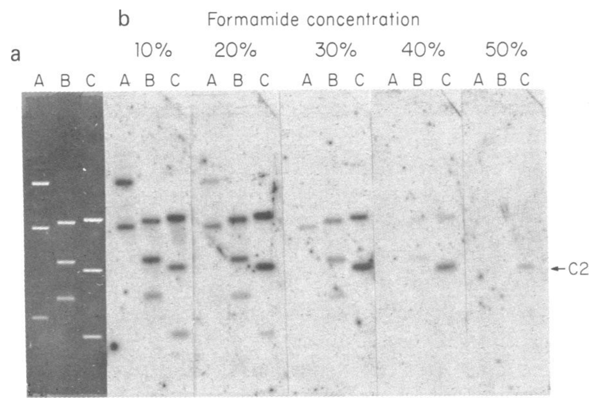

FIG. 1. Hybridization of ^{32}P-labeled *Eco*RI-linearized maxicircle DNA from *L. tarentolae* with *T. brucei* maxicircle DNA. (*a*) *Hin*dIII *T. brucei* maxicircle fragments were digested with various restriction endonucleases, electrophoresed through 0.8% agarose, and blotted onto nitrocellulose. (*b*) Hybridizations were carried out at 37°C in HHM medium at the indicated formamide concentrations. Lanes: A, *Eco*RI-digested fragment A; B, *Pst* I/*Bgl* II-digested fragment B; C, *Hae* III-digested fragment C.

(HHM medium) (23) at various formamide concentrations at 37°C for 36–40 hr. Washing was at hybridization-equivalent stringencies calculated as described (23). Filters from a given experiment were exposed for the same period of time to Kodak XAR-5 film at 23°C or to preflashed film at −70°C with a Quanta II intensifier screen.

RNA Hybridizations. Total RNA from a Renografin-purified kinetoplast fraction of early logarithmic phase *L. tarentolae* cells was isolated as described (14) and separated by electrophoresis in 1.5% agarose/2.2 M formaldehyde. The RNA was blotted onto nitrocellulose (Schleicher & Schuell, BA83) and hybridized with approximately 5×10^6 cpm of denatured ^{32}P-labeled probe DNA in HMA medium at 37°C as described (8). The filters were washed at a hybridization-equivalent stringency, exposed to Kodak XAR-5 film at 23°C for the same length of time, and then boiled briefly to remove the probe and rehybridized with ^{32}P-labeled pLt120 insert probe as an internal control for the absence of RNA degradation.

RESULTS

Hybridization of Total Maxicircle DNA from *L. tarentolae* with *T. brucei* Maxicircle Fragments. The results of hybridization of ^{32}P-labeled *L. tarentolae* total *Eco*RI-linearized maxicircle DNA to *T. brucei* maxicircle fragments (see Fig. 2 for map) at increasing stringencies are shown in Fig. 1. At the least stringent condition of 10% formamide, the *L. tarentolae* maxicircle probe hybridized to all *T. brucei* fragments except A3. *T. brucei* fragments A1 and C3 showed decreased hybridization at 20% formamide, whereas at 50% formamide the only hybridization observed was with the C2 fragment. The absence of detectable sequence homology between *T. brucei* fragment A3 and *L. tarentolae* maxicircle DNA was confirmed by back hybridization at low stringency of blots of digested *L. tarentolae* maxicircle DNA with ^{32}P-labeled gel-isolated *T. brucei* A3 fragment (data not shown).

These results indicate that all regions of the *T. brucei* maxicircle except A3 have some extent of sequence homology with the *L. tarentolae* maxicircle, with the most conserved region being *T. brucei* fragment C2, which is known to contain the *9S* and *12S* RNA genes (5).

Hybridization of Specific *L. tarentolae* Maxicircle Fragments with *T. brucei* Maxicircle Fragments. Gel-isolated *Msp* I fragments of the *Eco*RI-linearized *L. tarentolae* maxicircle (Fig. 2) were used to probe blots of *T. brucei* maxicircle fragments. Hybridizations were carried out at different stringencies to evaluate the relative stabilities of the heterologous hybrids. As shown in Fig. 3*a* and diagrammed in Fig. 2, the *L. tarentolae* fragment 1 probe hybridized with the three contiguous *T. brucei* fragments A1, A2, and B1 at 20% formamide but only (weakly) with fragment A2 at 40% formamide. The *L. tarentolae* fragment 5 probe hybridized with a single *T. brucei* fragment, B1, through 40% formamide. The *L. tarentolae* fragment 3 probe hybridized strongly at 20% formamide with fragments B1 and B2 as well as weakly with fragments B3 and C1;

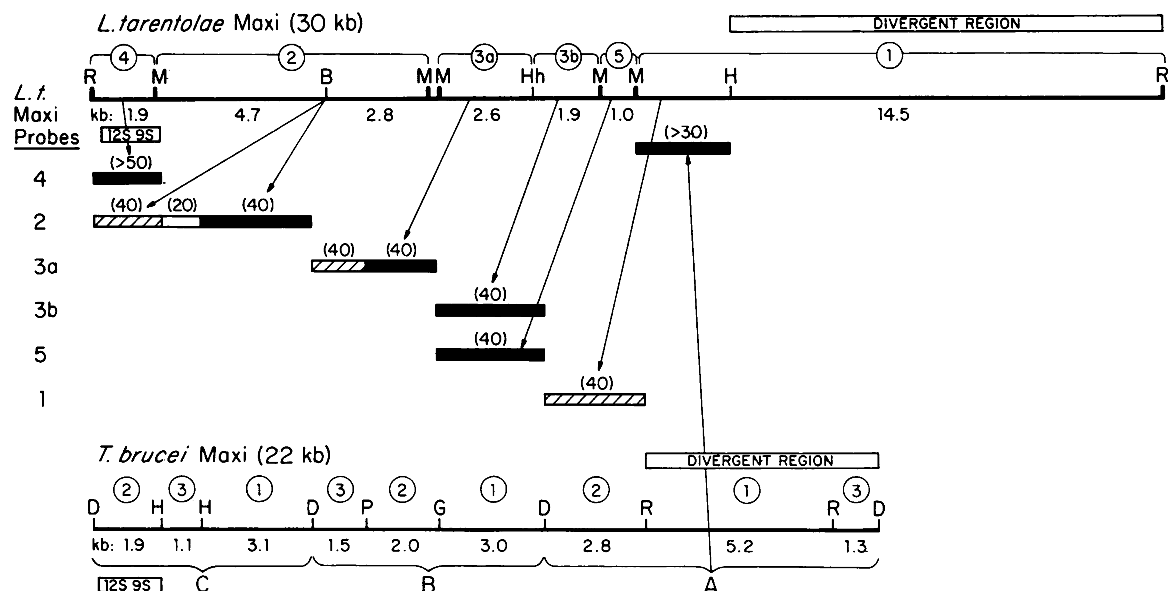

FIG. 2. Diagram of results of the hybridization experiments. Partial restriction maps of the *L. tarentolae* and *T. brucei* maxicircles are drawn to scale and are aligned at the *9S* and *12S* genes. The *L. tarentolae* fragments used as probes are indicated as are the *T. brucei* subfragments of the *Hin*dIII A, B, and C fragments. Strong hybridization is indicated by black boxes and weak hybridization by cross-hatched boxes; percent formamide is given in parentheses. Also indicated are the conserved, transcribed region of the *L. tarentolae* fragment 1 and the approximate boundaries of the divergent region of both maxicircles. B, *Bam*HI; D, *Hin*dIII; G, *Bgl* II; H, *Hae* III; Hh, *Hha* I; M, *Msp* I; P, *Pst* I; R, *Eco*RI.

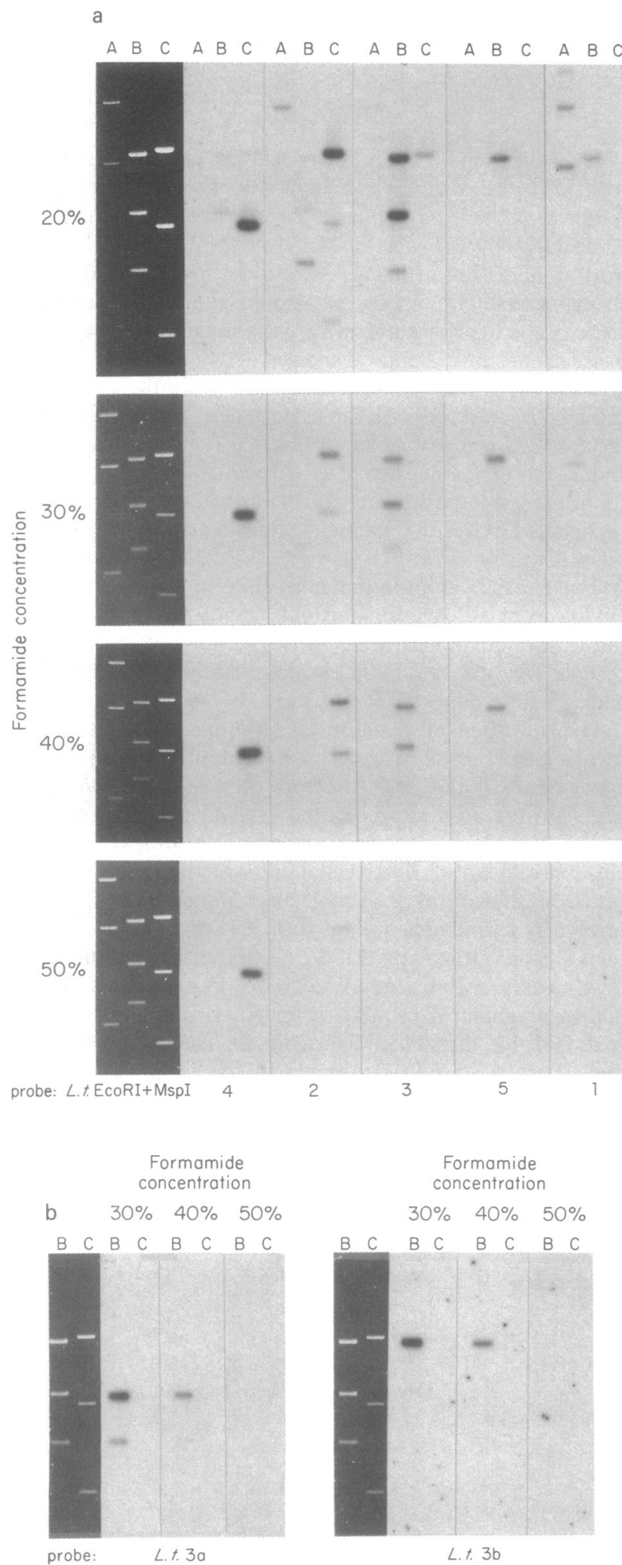

FIG. 3. Hybridization of specific maxicircle fragments from *L. tarentolae* to digests of the three *Hin*dIII *T. brucei* maxicircle fragments. (*a*) Hybridizations were carried out at 37°C in HHM medium at the indicated formamide concentrations with ^{32}P-labeled gel-isolated *Msp* I fragments of the *Eco*RI-linearized *L. tarentolae* (*L. t.*) maxicircle. For each group of filters, representing a single stringency, the autoradiograph was exposed to yield the same approximate intensity for the bound *L. t.* fragment 4 probe. (*b*) Hybridization of *Hha* I subfragments a and b of *L. t.* fragment 3 to *T. brucei* maxicircle fragments from the *Hin*dIII B and C regions. Hybridizations were carried out as in *a*. Lanes are designated as in Fig. 1.

only the B1 and B2 hybrids were stable at 40% formamide. The *L. tarentolae* fragment 2 probe hybridized with many of the *T. brucei* fragments at 20% formamide but, at 40% formamide, hybridization was observed only with C1 and, to a lesser extent, with C2. The *L. tarentolae* fragment 4 probe hybridized with the single *T. brucei* fragment C2 through 50% formamide. As shown in Fig. 2, the *L. tarentolae* fragment 4 and the *T. brucei* fragment C2 both encode the genes for the 9S and 12S RNAs, implying again that these are the most conserved genes in the maxicircle DNAs of these species.

A more detailed hybridization analysis of the *L. tarentolae* fragment 3 region and a summary of the results are shown in Figs. 2 and 3*b*. Two *Hha* I subclones of this fragment (3a and 3b in Fig. 2) were used as hybridization probes versus fragments from the *Hin*dIII B and C regions of the *T. brucei* maxicircle. The *L. tarentolae* fragment 3a probe hybridized with *T. brucei* fragment B2 and weakly with fragment B3. These hybrids persisted through 40% formamide and did not form in 50% formamide. The *L. tarentolae* 3b probe hybridized selectively to *T. brucei* fragment B1 through 40% formamide.

Localization of the Homologous Region Within the 14.5-kb *L. tarentolae* Fragment 1. The *T. brucei Hin*dIII A fragment was labeled and used as a probe to define more precisely the region of sequence homology with the 14.5-kb *L. tarentolae* fragment 1. As shown in Figs. 2 and 4, hybridization at 30% formamide was limited to that portion of *L. tarentolae* fragment 1 directly adjacent to fragment 5, with the region of homology extending no further than the *Hae* III site in fragment 1.

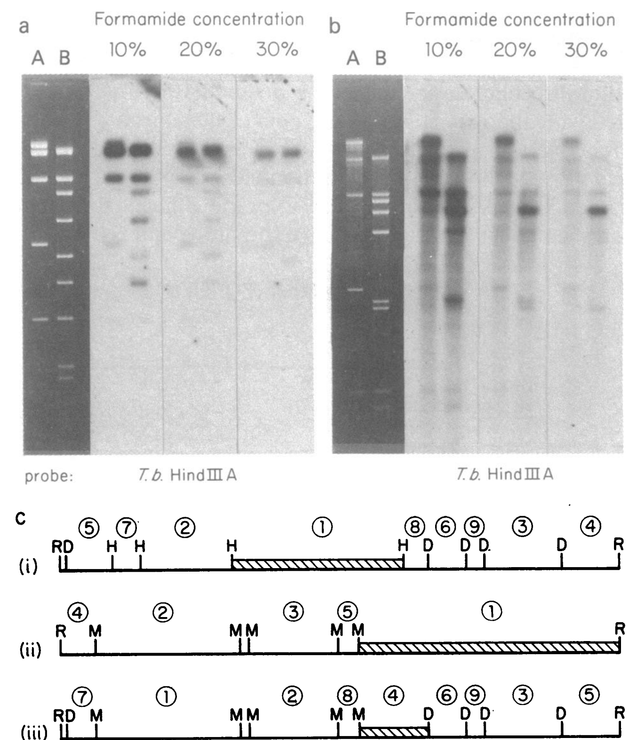

FIG. 4. Hybridization of the *T. brucei Hin*dIII A maxicircle fragment to *L. tarentolae* maxicircle DNA. *Eco*RI-linearized *L. tarentolae* maxicircle DNA was digested with *Hae* III or *Hae* III/*Hin*dIII (*a*, lanes A and B) or with *Msp* I or *Msp* I/*Hin*dIII (*b*, lanes A and B) and electrophoresed through 1.0% agarose. Hybridizations were carried out at 37°C in HHM medium at the indicated formamide concentrations. (*c*) The results of hybridization at 30% formamide are diagrammed with the main hybridizing bands shown as hatched marks. *i*, *Hae* III/*Hin*dIII (or *Hae* III alone); *ii*, *Msp* I; *iii*, *Msp* I/*Hin*dIII. D, *Hin*dIII; H, *Hae* III; M, *Msp* I; R, *Eco*RI.

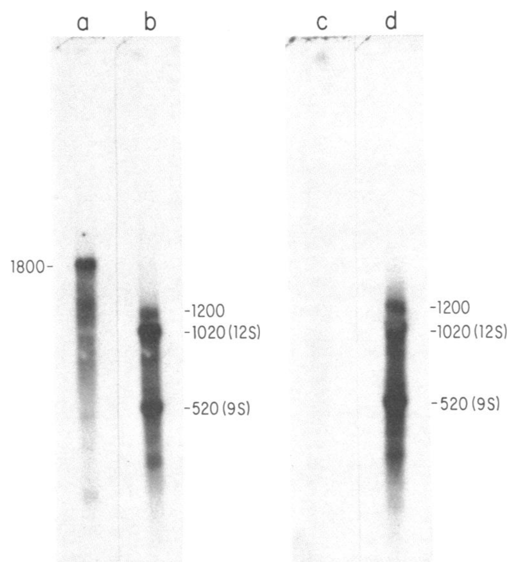

FIG. 5. Transcription of subfragments of the *L. tarentolae Msp* I maxicircle fragment 1. Total kinetoplast RNA was separated in formaldehyde/agarose, blotted onto nitrocellulose, and hybridized at 37°C in HMA medium with ^{32}P-labeled *Hae* III subfragments of the *L. tarentolae* fragment 1. Lanes a and c: the probes were the 2.4- and the 12.1-kb subfragments. Lanes b and d: the blots in lanes a and c boiled and rehybridized with ^{32}P-labeled pLt120 insert DNA.

Localization of the Transcribed Region of *L. tarentolae* Fragment 1. It was shown previously (8) that *L. tarentolae* fragment 1 hybridizes to a major 1,800-nucleotide RNA species and to additional minor species ranging in size from 1,400 to 250 nucleotides. To localize these transcripts within the 14.5-kb fragment 1, the 2.4- and 12.1-kb *Hae* III subfragments of fragment 1 were used as probes in a RNA blot analysis. As shown in Fig. 5, the 2.4-kb probe hybridized to the major 1,800-nucleotide transcript, as well as to several minor transcripts, whereas the 12.1-kb probe showed little hybridization. The lack of hybridization of the 12.1-kb probe was not due to RNA degradation, as a control rehybridization of the filters with a pLt120 insert probe showed the expected (8) RNA species (Fig. 5) homologous to the 120 region.

Analysis of the Region Containing the *9S* and *12S* RNA Genes. *Sau*3A subfragments of the cloned 6.6-kb *Eco*RI/*Bam*HI *L. tarentolae* fragment (pLt120) were labeled and used as probes versus blots of digested *T. brucei Hin*dIII B and C fragments (see map in Fig. 6). Hybridization was carried out under stringent conditions (HMA medium/50% formamide) with hybrids being evaluated at successively higher wash stringencies. As shown in Fig. 6, both the 12S-encoding *Sau* fragment 2 probe and the 9S-encoding *Sau* fragment 4 probe hybridized strongly to the *T. brucei* C2 fragment. The *12S* hybrid melted at 55°C whereas the *9S* hybrid melted at 60°C. In addition, weak hybridization was observed between the *L. tarentolae Sau* fragment 5 probe and the *T. brucei* C2 fragment (melting temperature, 60°C), between the *L. tarentolae Sau* fragments 7, 3, and 1 probes with the *T. brucei* C2 fragment (melting temperature, 55°C), and between the *L. tarentolae Sau* fragments 1 and 5 probes with the *T. brucei* C1 fragment (melting temperature, 55°C).

DISCUSSION

The conserved regions of the maxicircle DNAs of *L. tarentolae* and *T. brucei* are organized similarly in both genomes and extend over a region of 15–17 kb that is interrupted by two small less-homologous regions. The strongest homology was between

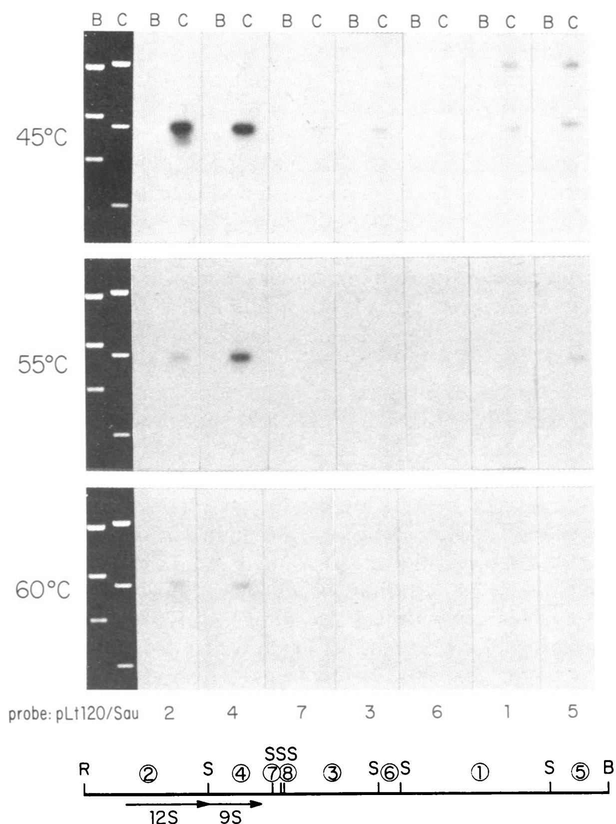

FIG. 6. Hybridization of *Sau*3A subfragments of the 120 region of the *L. tarentolae* maxicircle to *T. brucei* maxicircle fragments from the *Hin*dIII B and C regions. Lanes: B, *Pst* I/*Bgl* II-digested fragment B; C, *Hae* III-digested fragment C. Hybridizations were carried out at 37°C in HMA medium and the blots were washed in 15 mM NaCl/1.5 mM Na citrate, pH 7.2, at the indicated temperatures. A *Sau*3A map of the 120 region is shown with the approximate position and direction of transcription of the *9S* and *12S* genes (8). Note that the *Sau*6 fragment has been moved to between the *Sau*3 and *Sau*1 fragments in accordance with the recent results of a sequence analysis of the 120 region (unpublished data).

the genes coding for the 9S and 12S RNAs, and the weakest homology was in the region upstream from the *12S* gene, which on the basis of these results we have termed the divergent region.

Hybrids formed between the heterologous *12S* genes were less stable than hybrids between the *9S* genes (Fig. 6). This is most likely due to the relatively high A+T content of the *12S* gene (8, 25) rather than to sequence mismatch.

Tentative localizations of several structural genes in the maxicircle of *L. tarentolae* have been reported (10); all lie within the conserved regions of the genome. In the case of *T. brucei*, the *COXII* gene has been tentatively localized by hybridization with a *Zea mays* probe to a *Taq* I fragment situated within fragment C1 (11). This localization however does not agree with the fact that *L. tarentolae* fragment 3a, which was shown to hybridize to the yeast *COXII* gene (10) has sequence homology with *T. brucei* fragment B2. This discrepancy must be resolved by sequence analysis. The only other possible example of noncolinearity of gene sequence between the two genomes is the hybridization of the *L. tarentolae Sau*1 and *Sau*5 subfragments of the 120 region to *T. brucei* fragment C1 as well as C2 (Fig. 6).

Sequence divergence is most extensive within the *T. brucei Hin*dIII A fragment and the *L. tarentolae* fragment 1. Significant cross-homologies within these regions were limited to the

2.8-kb *T. brucei* A2 fragment and the 2.4-kb *Msp* I/*Hae* III subfragment of the *L. tarentolae* fragment 1. The *T. brucei* A1 fragment has previously been shown to exhibit a size variation of up to 1.5 kb in different strains of *T. brucei* (12).

Transcription of the *L. tarentolae* fragment 1 was also limited to the 2.4-kb *Hae* III subfragment (Fig. 5). In the case of *T. brucei*, Stuart and Gelvin (9) showed that the A3 fragment lacked any detectable transcriptional activity.

The correlation of lack of interspecies sequence homology with absence of transcription implies that the divergent region of the maxicircle may serve a function other than encoding structural genes. It is of some interest that the *T. brucei* A1 fragment and the 12S proximal portion of the *L. tarentolae* fragment 1 have proved refractory to cloning in either plasmid (6, 9) or phage vectors (24). It may also be relevant that a partial denaturation map of the *L. tarentolae* maxicircle (8) showed more than seven small denaturation loops in the portion of fragment 1 adjacent to the *12S* gene, implying a possible repetitive sequence. We have recently confirmed this by direct sequence analysis of two small cloned fragments from the divergent region of *L. tarentolae* (unpublished data). Borst *et al.* (26) have also obtained evidence by heteroduplex analysis in the electron microscope for the existence of imperfect tandem repeats within the divergent region of the *T. brucei* maxicircle. We propose that the major mode of sequence evolution of maxicircle DNA of the kinetoplastid protozoa involves insertions/deletions within the nontranscribed divergent region. The actual molecular mechanism of this phenomenon remains to be explained but clearly is associated with the presence of repetitive sequences.

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