Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 565–569, January 1987
Microbiology

Kinetoplast DNA minicircles: Regions of extensive sequence divergence

(*Leishmania*/hybridization/taxonomy/mitochondrial DNA/diagnosis)

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Communicated by George H. Hitchings, October 8, 1986

ABSTRACT **Previous work has shown that the kinetoplast minicircle DNA of *Leishmania* species exhibits species-specific sequence divergence and this observation has led to the development of a DNA probe-based diagnostic test for leishmaniasis. In the work reported here, we demonstrate that the minicircle is composed of three types of DNA sequences with differing specificities reflecting different rates of DNA sequence change. A library of cloned fragments of kinetoplast DNA (kDNA) from *Leishmania mexicana amazonensis* was prepared and the cloned subfragments were found to contain DNA sequences with different taxonomic specificities based on hybridization analysis with various species of *Leishmania*. Four groups of subfragments were found, those that hybridized with a large number of *Leishmania* sp. as well as sequences unique to the species, subspecies, or isolate. Analysis of nested deletions of a single, full-length minicircle demonstrates that these different taxonomic specificities are contained within a single minicircle. This implies that different regions of a single minicircle have DNA sequences that diverge at different rates. These sequences represent potentially valuable tools in diagnostic, epidemiologic, and ecological studies of leishmaniasis and provide the basis for a model of kDNA sequence evolution.**

Human leishmaniasis is a spectral disease caused by one of several *Leishmania* species and subspecies. The clinical manifestations of the disease depend at least in part on the particular infecting *Leishmania* species (1–4). Leishmaniasis is a zoonotic disease and different subspecies of *Leishmania* have different natural animal reservoirs and insect vectors (4). Treatment of the disease as well as studies of the complex epidemiology depend on rapid, accurate identification of the *Leishmania* sp. in biological samples. We have described a DNA probe-based method for the rapid diagnosis of leishmaniasis in biological samples (5) and have used this technique to test >200 patients with cutaneous leishmaniasis in Manaus, Brazil (6). This technique uses isolated kinetoplast DNA (kDNA), the unusual mitochondrial DNA, as the probe and is able to distinguish the two major New World agents of leishmaniasis, *Leishmania braziliensis* and *Leishmania mexicana*, but does not discriminate among the subspecies within these complexes. This is an important distinction because certain subspecies are associated with different clinical manifestations of the disease and it is important to distinguish these early in the infection for proper treatment. We describe here the refinement of these DNA probes through the use of recombinant DNA techniques to produce DNA probes specific for the species *L. mexicana*, and the subspecies *L. mexicana amazonensis*, and probes specific for the strain of parasite from which they were derived.

Leishmania are members of the protozoic order Kinetoplastida. The defining characteristic of this order is a highly unusual, concatenated mitochondrial DNA structure, the kDNA (10). It consists of two types of circular DNA molecules, the maxicircle [20,000–40,000 base pairs (bp)], which encodes mitochondrial proteins and is present in 10–20 copies, and the minicircle (<1000 bp), which has no known coding function and is present in 10,000 copies. The minicircles within any given organism do not have identical DNA sequences; instead, the minicircles fall into different sequence classes. The number of sequence classes is species dependent, ranging from one in *Trypanosoma equiperdum* to >300 in *Trypanosoma brucei*. Previous work indicates that there are an intermediate number of minicircle sequence classes in *Leishmania* sp., with one or two predominant minicircle sequences (7). One of the major questions to be addressed is the origin and maintenance of these minicircle sequence classes.

The kDNA minicircle appears to undergo rapid DNA sequence evolution. Previous work has shown species-related restriction site heterogeneity within the kDNA (11–17) and we (18, 19) and others (20, 21) have cloned species- or isolate-specific fragments from visceral and Old World cutaneous strains of *Leishmania*. This work demonstrated that within the kDNA network, fragments of DNA existed with different taxonomic specificities but did not distinguish between these sequences being present in different classes of minicircles or in a single minicircle. Here we demonstrate that DNA sequences of different taxonomic specificity are present in a single minicircle of *L. mexicana amazonensis*. We present two potential models for kDNA sequence divergence.

MATERIALS AND METHODS

Cultivation of Parasites. Promastigotes were cultured in Schneider's *Drosophila* medium (22) supplemented with 15% heat-inactivated fetal calf serum (Flow Laboratories).

Isolation of kDNA. Promastigotes (2×10^{10}) were harvested by centrifugation at $1000 \times g$ and resuspended in a buffer containing 0.1 M NaCl, 20 mM EDTA, and 10 mM Tris·HCl (pH 8). The suspension was made 1% in $NaDodSO_4$ and chromosomal DNA was sheared by passage through a 21-gauge needle. The sample was incubated with 10 mg of proteinase K per ml (Boehringer Mannheim) for 1 hr at 37°C. The kDNA networks were pelleted at $100,000 \times g$ for 60 min, extracted with phenol/chloroform, and resolved in a CsCl density gradient as described (5).

Restriction Digestion. Digestions were carried out under conditions suggested by the manufacturer (New England Biolabs) and resolved by electrophoresis in 1.8% agarose.

Plasmid Constructions. Random fragments of kDNA from *L. mexicana amazonensis* strain PH8 were generated by partial digestion of kDNA networks with *Rsa* I, *Fnu*DII, *Hae* III, and *Alu* I. Fragments were then cloned into pBR322 at the

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Abbreviation: kDNA, kinetoplast DNA.
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*Hin*dIII site after it had been end-filled (23). Recombinants were differentially screened by hybridization with nick-translated kDNA from PH8 and from the *L. mexicana mexicana* strain L11. Plasmids pMAS2, pMAS12, and pMAS9 were chosen for further study.

Full-length minicircles from PH8 were produced by digestion of the kDNA network with *Dra* I, which cuts most minicircles only once, and these were cloned into the *Dra* I site of pBR322. A set of nested deletions was derived from one minicircle clone, pMAT13. The plasmid was opened at the *Hin*dIII site in pBR322 and digested with BAL-31 exonuclease, end-filled, digested with *Ava* I, and treated with calf intestinal phosphatase to remove 5′ phosphates. The tetracycline-resistance gene was replaced by ligation of these deleted plasmids to the *Eco*RI/*Ava* I fragment of pBR322. The chimeric plasmids were used to transform *Escherichia coli*, strain HB101, and selected for resistance to tetracycline. Recombinants were screened by hybridization with nick-translated kDNA from PH8 and the deletions were sized by measuring the *Dra* I/*Cla* I fragment in 1.8% agarose gels and by restriction mapping on 7.5% polyacrylamide gels.

Hybridization. Methods for nick-translation, hybridization, and preparation of dot blots are as described (5), except that 500 μg of denatured herring sperm DNA per ml was used in all hybridizations.

RESULTS

kDNA Contains Sequences of Different Taxonomic Specificity. Previous work had demonstrated that kDNA isolated from *L. mexicana amazonensis* strains contains DNA sequences that distinguish the two major forms of cutaneous leishmaniasis in the New World. This kDNA hybridizes with all isolates of the *L. mexicana* complex but not with isolates of the *L. braziliensis* complex (ref. 6; Table 1). As can be seen in Fig. 1 *Left* and Table 1, this result is confirmed with kDNA isolated from PH8 (*L. mexicana amazonensis*), which hybridizes with itself (lane 1) and with isolated kDNA from *L. mexicana mexicana* (lane 2) and not with kDNA from *L. braziliensis* (lane 3). In addition, PH8 kDNA reacts with an isolate of *Leishmania major* (lane 4), the causative agent of Old World cutaneous leishmaniasis. In other experiments, PH8 kDNA shares some sequence homology with a subset of strains from the Old World that cause either cutaneous or visceral disease (see Table 1 and data not shown).

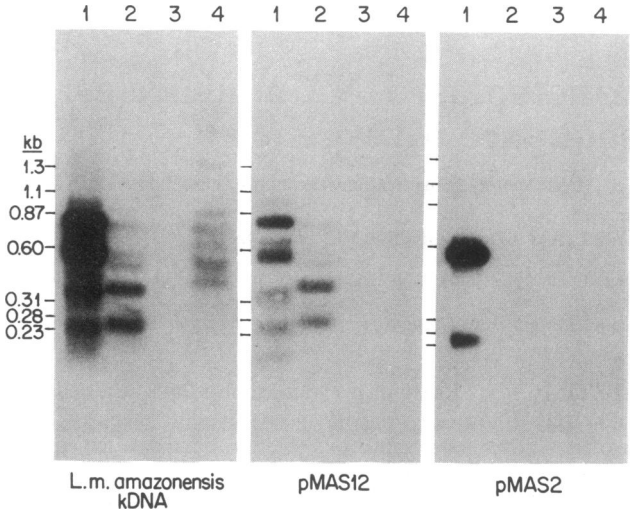

FIG. 1. Hybridization of cloned PH8 kDNA fragments to total kDNA from *Leishmania* reference strains. kDNA (500 ng per lane) was digested with *Hae* III and *Alu* I, resolved by electrophoresis in a 1.8% agarose gel, and transferred to nitrocellulose. Lane 1, *L. mexicana amazonensis* (PH8); lane 2, *L. mexicana* (L11); lane 3, *L. braziliensis guyanensis* (M4147); lane 4, *L. major* (WR309). Filters were hybridized to radiolabeled PH8 kDNA (*Left*), pMAS12 (*Center*), or pMAS2 (*Right*). Shown is an autoradiogram. kb, Kilobase pairs.

The purpose of these experiments was to develop DNA probes that had more restricted hybridization specificities. There were two separate goals, one to develop a DNA probe that could distinguish the *L. mexicana* complex from all others and one to develop DNA probes that could distinguish the subspecies of *L. mexicana* from one another. These are important distinctions because certain subspecies are associated with specific clinical manifestations of the disease and

Table 1. Hybridization of cloned PH8 kDNA fragments to *Leishmania* promastigotes

Strain	*Leishmania* species	Source	PH8	pMAS12	pMAS2	pMAS9
PH8	*m. amazonensis*	Brazil, Lutzomyia	++++	++++	++++	++++
M4588	*m. amazonensis*	Brazil, Proechymis	++++	+++	++	++
M4065	*m. amazonensis*	Brazil, man	++++	+++	+++	−
H21	*m. amazonensis*	Brazil, man	++++	+++	++++	++
M4435	*m. amazonensis*	Brazil, man	++++	+++	+++	−
IM84	*m. amazonensis*	Brazil, man	+++	+++	+	−
IM1043	*m. amazonensis*	Brazil, man	++++	+++	+++	−
WR303	*m. amazonensis*	Brazil, man	++++	+++	++	−
Josefa	*m. amazonensis*	Brazil, man	++++	+++	+++	−
Raimundo	*m. amazonensis*	Brazil, man	++++	+++	+++	++
MTD	*m. amazonensis*	Brazil, man	++++	+++	+++	++
M6331	*m. aristedisi*	Panama, rodent	+	++	−	−
L11	*m. mexicana*	Honduras, Nyctomys	++	++	−	−
Emilio	*m. mexicana*	Dominican Republic, man	++	++	−	−
Isabel	*m. mexicana*	Dominican Republic, man	++	++	−	−
WR309	*major*	Israel, man	+	−	−	−
LRCL-134	*aethiopica*	Ethiopia, man	+	−	−	−
WR352	*donovani*	India, man	+	−	−	−
LV-9	*donovani*	Ethiopia, man	++	−	−	−
M2904	*b. braziliensis*	Brazil, man	−	−	−	−
WR120	*b. panamensis*	Panama, man	−	−	−	−
M4147	*b. guyanensis*	Brazil, man	−	−	−	−

Leishmania promastigotes were spotted onto nitrocellulose filters and hybridized to nick-translated probes, PH8 kDNA, pMAS12, pMAS2, and pMAS9. Hybridization intensities were rated as previously (19). *m.*, *mexicana*; *b.*, *braziliensis*.

early identification of the subspecies is important for treatment and for disease transmission studies. As can be seen in Table 1, when PH8 kDNA is used as a hybridization probe, it reacts most strongly with other isolates of *L. mexicana amazonensis* and weakly with other *L. mexicana* subspecies or with isolates from Old World cutaneous or visceral leishmaniasis.

Based on the differential hybridization intensities of total kDNA, we hypothesized that only a subset of the PH8 kDNA sequences was found in the other *Leishmania* strains. To test this, a recombinant DNA library was prepared from restriction enzyme-digested PH8 kDNA. This library was screened in duplicate with radiolabeled PH8 kDNA and L11 (*L. mexicana mexicana*) kDNA to identify unique subfragments of the PH8 kDNA. As can be seen in Fig. 1 *Center* and *Right* and Table 1, PH8 kDNA contains at least three subfragments with different taxonomic specificities, species specific (pMAS12), subspecies specific (pMAS2), and group specific (pMAS9).

The clone pMAS12 contains a sequence that hybridizes with all isolates of *L. mexicana* but does not share any homology with the isolates of Old World *Leishmania* species. In Fig. 1 *Center*, it is clear the pMAS12 hybridizes with the same major restriction fragments in lanes 1 and 2 as does total PH8 kDNA. We interpret this result to indicate that pMAS 12 contains a sequence common to many minicircle classes and does not represent a minor variant class specific only to the *L. mexicana* complex.

The clone pMAS2 contains a sequence that is specific to *L. mexicana amazonensis* and does not hybridize with any other species or subspecies. Fig. 1 *Right* demonstrates that this sequence is homologous to only a subset of the major restriction fragments recognized either by total PH8 kDNA or pMAS12. The Southern blot indicates that the pMAS2 sequence is derived from a major minicircle class.

Finally, a subfragment of PH8 kDNA (clone pMAS9) recognizes only a subset of the isolates of *L. mexicana amazonensis*. This type of hybridization specificity will probably not be very useful in diagnosis but may be very important in future transmission and epidemiological studies. Analogous fragments have been found in *L. major* and *Leishmania donovani* (18–21)—namely, fragments that recognize a single isolate or small group of isolates. This result indicates that at least some portion of the minicircle population is undergoing rapid sequence divergence such that even very closely related organisms contain divergent minicircle sequences.

DNA Sequence Divergence Occurs Within a Single Minicircle. The results from the random restriction fragment cloning experiments demonstrated that the minicircle population from a single organism contains kDNA subfragments with different taxonomic specificities. This result suggested that within the minicircle population, there were different rates of DNA sequence divergence. The purpose of these experiments was to investigate the mechanism of this divergence. We first asked whether this divergence was found within a single minicircle or was represented in different minicircle classes. To answer this question, a full-length minicircle library was generated from PH8 kDNA after digestion with the restriction enzyme *Dra* I, which has a single restriction site in most minicircles of PH8 kDNA. A single cloned minicircle (pMAT13) was then analyzed. A nested set of deletions was generated from one end of the linearized cloned minicircle using BAL-31 exonuclease. Maps of the deletions are shown in Fig. 2. These deleted minicircles were then used to probe a panel of *Leishmania* sp.

As can be seen in Fig. 3, different taxonomic specificities are contained within the single cloned minicircle DNA. In Fig. 3 *Left*, the single cloned minicircle has a similar hybridization pattern to that of total PH8 kDNA (see Fig. 1 and Table 1). A *L. mexicana* species-specific hybridization probe is obtained if ≈230 bp are deleted from one end of the linearized minicircle DNA. Thus, the cross-reaction with strains of Old World cutaneous and visceral leishmaniasis can be localized to a maximum of 230 bp from the *Dra* I site in the minicircle. This may be analogous to the conserved region of minicircle DNA described in other kinetoplastida organisms (7, 24) and may be important to minicircle function.

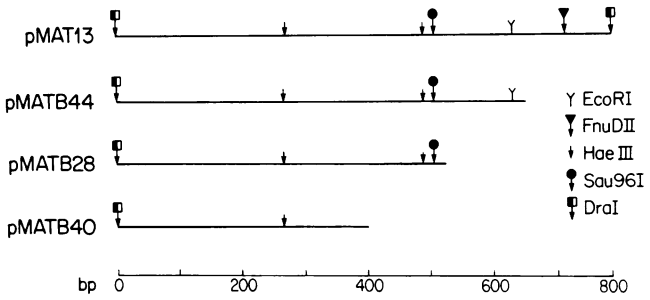

FIG. 2. Nested deletions of a unit length minicircle. The deletions were generated and sized by measuring the *Aha* III/*Cla* I fragment on a 1.8% agarose gel or by restriction analysis on 7.5% polyacrylamide gels.

The most striking result is presented in Fig. 3 *Right*, in which an isolate-specific probe is generated when ≈400 bp of the major PH8 minicircle are deleted. The result implies that half of the DNA sequences within a single minicircle have diverged such that this fragment no longer hybridizes with a closely related organism (Josefa) of the same subspecies, *L. mexicana amazonensis*. Thus, it appears that there is a gradient of DNA sequence divergence within a single minicircle DNA molecule. There is a highly conserved region near the *Dra* I site that is homologous with presumably similar regions in several different species. At ≈230 bp to the left (in the linearized minicircle) of the *Dra* I site, there is a segment of DNA that is common to *L. mexicana* and is thus a species-specific sequence. Finally, there is a very specific sequence that comprises about one-half of the cloned minicircle and exhibits the greatest amount of DNA sequence divergence.

DISCUSSION

We have demonstrated that species-, subspecies-, and isolate-specific sequences can be isolated from *L. mexicana amazonensis*, strain PH8 kDNA using recombinant DNA techniques. Analogous random cloning experiments have

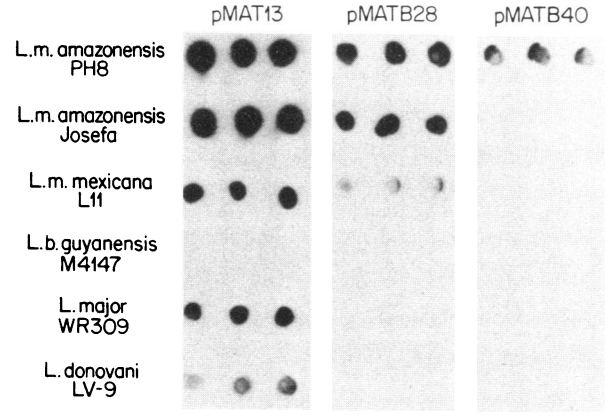

FIG. 3. Hybridization of deleted minicircle clones to *Leishmania* strains. Filters were hybridized to nick-translated probes, pMAT13 (*Left*), pMATB28 (*Center*), and pMATB40 (*Right*). Shown is an autoradiogram.

shown similar results in *L. major* and *L. donovani* (18–21)—namely, the presence of subfragments of the total minicircle DNA that show different taxonomic specificities. We have further demonstrated in the work presented here that most of these unique specificities are found within a single, cloned minicircle DNA and arranged in a defined order along the length of that minicircle DNA.

Species- and subspecies-specific hybridization probes have obvious usefulness in the diagnosis and management of clinical leishmaniasis. Clinical manifestations of leishmaniasis are dependent at least in part on the species or subspecies of infecting *Leishmania* organism and thus specific diagnosis is necessary for proper clinical treatment. These probes should be able to provide this diagnosis directly from primary lesion material without the need for isolation and culturing of the organism. Probes specific for a small number of isolates, on the other hand, may be particularly useful in studying the epidemiology and ecology of leishmaniasis within a single narrowly defined focus of the disease. The general approach of isolating kDNA fragments of varying hybridization specificities may also prove useful in the diagnosis of diseases caused by other kinetoplastic protozoa (e.g., African or American trypanosomiasis).

We have examined the organization of the variable and conserved sequences within the minicircles. The $\approx 10^4$ minicircles within a single *Leishmania* organism are heterogeneous and fall into a number of different sequence classes. Within a given sequence class, the minicircle sequences are identical (24, 25). Sequence analysis of different classes of minicircles in *Leishmania tarentole* (7) and *T. brucei* (26) has revealed that minicircles of different classes share ≈ 150 bp of homology but are widely divergent throughout the rest of their length. Our results show (see Fig. 1) that the species-specific clone pMAS12 is present in many minicircle classes, whereas the subspecies-specific clone pMAS2 is found only in a subset of minicircle classes.

We also found that sequences with different taxonomic specificities are present in a defined order along the length of a single clone minicircle, pMAT13. This clone contains a region of <230 bp that is conserved across species boundaries and that may be analogous to conserved regions found in other kinetoplastida minicircles (24, 26, 27). There thus appears to be a gradient of sequence variation extending leftward, with sequences conserved in the *L. mexicana* complex present in the next 170 bp and sequences that have the highest divergence in the leftmost 400 bp (Fig. 4*A*).

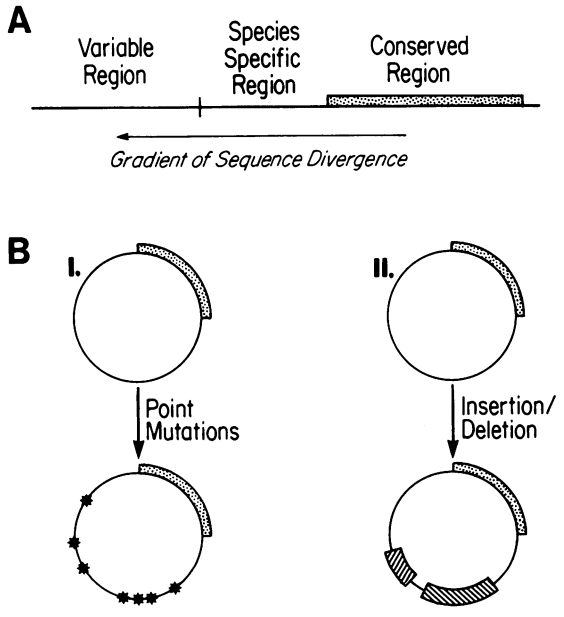

FIG. 4. Models of sequence variation in kDNA minicircles. The stippled box represents the conserved region. (*A*) Gradient of sequence variation along the linearized minicircle. (*B*) I: rapid accumulation of point mutations (*) in the variable region. II: introduction of new sequences (hatched boxes) into minicircles.

The maintenance of homogeneous sequence classes in the face of rapid sequence variation presumably requires a mechanism whereby mutations in a single minicircle are transmitted to other minicircles. A similar phenomenon occurs in other mitochondrial systems and a similar mechanism may exist here (8, 9). In addition, some mechanism must exist for the generation of rapid sequence divergence in one portion of the minicircle model with the maintenance of relatively conserved regions within the same molecule. We propose two general models (Fig. 4*B*) for this sequence divergence, one, a high rate of point mutations along the entire length of the minicircle followed by selection for the apparent important sequences in the conserved region of the minicircle. The second model involves a high frequency of recombination or DNA sequence rearrangement in the variable region. These models make very clear predictions concerning the DNA sequence of the variable region of two closely related minicircles that differ only in the variable region. In the first model, the two sequences will be related to one another in sequence but with a large number of single base-pair changes. In the second model, the sequences will be unrelated to one another, having been derived by recombination with other minicircle, maxicircle, or nuclear sequences. Ideally these experiments would be done with cloned isogeneic *Leishmania* organisms that differ only in the minicircle variable region. No such organisms are available and experiments to generate such *in vitro* variation have thus far been unsuccessful. The initial test of the model can be done by using two closely related isolates of *L. mexicana amazonensis* containing minicircles that by hybridization analysis of cloned minicircles are very homologous to one another in the relatively conserved 400 bp of the minicircle but differ in the variable region.

We thank R. Lainson, J. Shaw, U. Lopes, D. McMahon-Pratt, J. Arias, and the Walter Reed Army Institute of Research for materials. We thank U. Lopes, C. French, and T. Unnasch for helpful discussions and J. David for enthusiastic support. This work received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, the John D. and Catherine T. MacArthur Foundation, National Institutes of Health Grants 5P01 AI16305 and AI 21365-011A, and a grant from the Rockefeller Foundation Great Neglected Disease Program. D.F.W. is a Burroughs-Wellcome Scholar in Molecular Parasitology. W.R. was supported by National Institutes of Health Predoctoral Training Grant 5T32GMO07226-10.

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