Kinetoplast DNA in Trypanosomid Flagellates

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I. Introduction

This review will emphasize recent developments in the application of recombinant DNA technology to problems of the structure, replication, and transcription of the unusual mitochondrial DNA in the kinetoplastid protozoa known as kinetoplast DNA. Kinetoplast DNA will be discussed in terms of its two molecular components, minicircles and maxicircles, and species-dependent variations will be emphasized.

II. Minicircle DNA: Structure and Complexity

The minicircle component of the kinetoplast DNA is the most unusual aspect of this mitochondrial genetic system. Circular DNA molecules are not uncommon in nature nor in other mitochondrial genetic systems, but nowhere in nature does one find thousands of small circles catenated together into a single giant network of DNA such as found in the kinetoplastid protozoa. The function of minicircle DNA is still a mystery, but much has been learned recently about the structure and replication of this DNA in several kinetoplastid species: *Trypanosoma brucei, Trypanosoma equiperdum, Leishmania tarentolae, Crithidia fasciculata,* and *Crithidia luciliae*. In general minicircle DNA from all these species has the following general characteristics: (1) circles exist catenated with each other, although there is a small percentage of unattached circles that may have a functional significance; (2) within any one species the circles are of fairly uniform size although the size varies from species to species; (3) there is a species-dependent variable amount of sequence heterogeneity among the minicircles from any one clonal population; (4) the sequence heterogeneity is expressed on the level of the individual minicircle as a variable region and a constant region and sequence changes among the minicircle population in a kinetoplastid species occur rapidly in nature; and (5) there is no apparent sequence homology between the minicircles of a given species and the maxicircle DNA. In addition to these general common properties of minicircle DNA, there are many differences between the various kinetoplastid species, and these differences may prove

to have phylogenetic significance. Therefore, I will discuss results from each species in succession.

A. *Trypanosoma brucei*

From the initial optical reassociation studies of Steinert *et al.* (1976) it was realized that the kDNA of *T. brucei* was perhaps the most complex in terms of sequence heterogeneity and the most rapidly changing in terms of evolution. The DNA reassociated with a single copy complexity of 300 times the size of the minicircle, and no hybridization between different *T. brucei* strains was observed. The high complexity of kDNA from *T. brucei* was confirmed by the S_1 C_0t analysis of Donelson *et al.* (1979) in which [^{3}H]kDNA renatured with a single copy $C_0t_{1/2}$ 316 times the $C_0t_{1/2}$ when a cloned 1.1-kb minicircle (pkT51) was the excess driver (Fig. 1). However, there was a minor component (15%) of the kDNA which renatured at a rate suggestive of repetitive sequences. Renaturation of a cloned minicircle, pkT51, in the presence of excess DNA from two other cloned minicircles, pkT3 and pkT204, showed that approximately 25% of the sequence of the pkT51 insert is homologous to sequences in both the pkT3 and pkT204 inserts (Fig. 1). Stuart (1979) confirmed that there are approximately 300 different minicircle sequence classes in *T. brucei* 164 kDNA and showed that the minicircle classes differ in relative abundance (Stuart and Gelvin, 1982). For example, a *Hin*dIII cut minicircle cloned into the *Hin*dIII site of pBR322 renatured at a rate implying the existence of 500 copies per network, whereas *Bam*HI cleaved uncloned minicircles renatured at a rate implying the existence of 60 copies per network (Fig. 2).

However, assuming a *T. brucei* network mass of 4.2×10^9 Da that is composed of 15% maxicircle DNA of 22-kb molecules and 85% minicircle DNA of 1.1-kb molecules, one can calculate that there are approximately 45 maxicircles and 5500 minicircles (Stuart and Gelvin, 1980). Furthermore, assuming that there are about 300 different minicircle sequence classes, this would imply about 18 copies of each sequence class if the classes are of equal size. The finding (Stuart and Gelvin, 1980) that one cloned minicircle is present in 500 copies implies that there is a wide divergence in frequency of the different sequence classes in *T. brucei*.

The sequence heterogeneity of *T. brucei* minicircle DNA shown by C_0t analysis is evidenced also by restriction enzyme analysis on gels (Fig. 3). Digestion of *T. brucei* kDNA with most restriction enzymes yields low release of unit-length linearized minicircles with no smaller bands (Borst *et al.*, 1980a, 1981a; Brunel *et al.*, 1980a; Fairlamb *et al.*, 1978; Simpson and Simpson, 1980). Most of the DNA remains catenated in the network

that does not enter the gel. This low release of mainly once-cut minicircles by most restriction enzymes is due both to the presence of many minicircle sequence classes and to the high percentage of AT in the minicircle DNA of *T. brucei*. However, digestion with *Taq*I, *Alu*I, or *Mbo*I does release most of the minicircles from the network and produce fragments of less than unit length, implying that sites for these enzymes are present more than once in conserved regions of the minicircles. The apparently single bands of unit-length minicircle DNA released by digestion with enzymes such as *Eco*RI, *Pst*I, *Hpa*II, *Hae*III, *Hin*dIII, and *Hha*I were

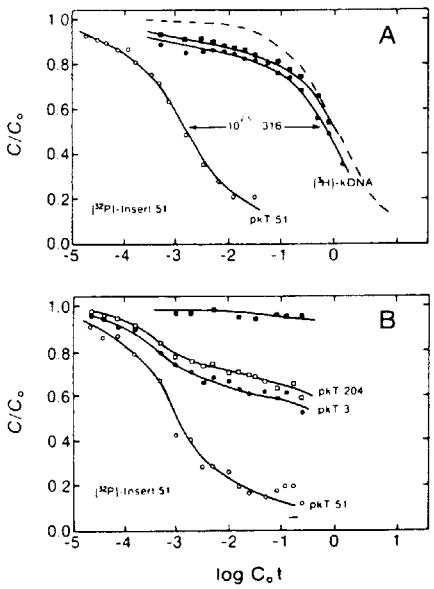

FIG. 1. Renaturation kinetics of the *Trypanosoma brucei* kDNA insert of pkT51 which had been labeled *in vitro* with ^{32}P. (A) The renaturation of ^{32}P-labeled pkT51 insert DNA in the presence of an excess of pkT51 DNA (open circles) or an excess of total network kDNA (solid circles). The network kDNA was labeled with ^{3}H *in vitro* prior to shearing so that its renaturation could also be monitored (solid squares). The broken line is a model curve generated by the standard renaturation equation when it is assumed that a single-copy DNA sequence has a log $C_0t_{1/2}$ = 0.15. (B) The renaturation of ^{32}P-labeled pkT51 insert DNA (open circles), pkT3 DNA (solid circles), pkT204 DNA (open squares), or pBR322 DNA (solid squares). The initial concentration, C_0, of the excess unlabeled driver DNAs refers to the concentration of only the inserted kDNA sequence when a pkT recombinant plasmid is the driver DNA. In all of the experiments C_0 was chosen so that the final time point was taken 11 hours after the start of the reaction. About 4000 ^{32}P cpm were sampled at each time point. The pBR322 sequence does not drive the renaturation of the kDNA insert 51 within the time span of the renaturation experiments (solid squares). Reprinted from Donelson *et al.* (1979) with permission.

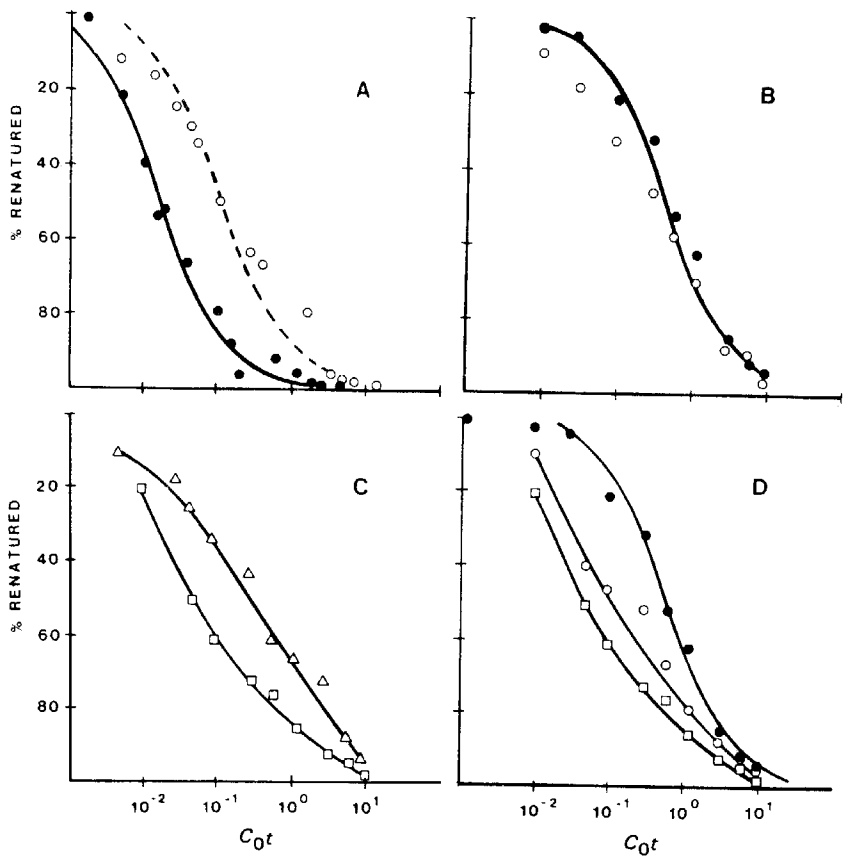

FIG. 2. Renaturation kinetics of nick-translated kDNA sequences of *T. brucei*. (A) kDNA and homologous driver (open circles) compared to lambda DNA standard (solid circle). Both lines are theoretical single component curves. (B) Maxicircle (open circles) and cloned maxicircle fragment (solid circles) driven by kDNA. (C) Cloned *Hin*dIII minicircle (open squares) and *Bam*HI minicircle (open triangles) sequences driven by kDNA. (D) Cloned *Hin*dIII minicircle (open squares), kDNA (open circles), and cloned maxicircle segment (solid circles) driven by kDNA. The curve through the cloned maxicircle segment points is a theoretical single component curve. Reprinted from Stuart and Gelvin (1980) with permission.

shown to be composed of 5–10 closely spaced bands by acrylamide gel electrophoresis (Simpson and Simpson, 1980) (Fig. 4), implying either a substantial minor length heterogeneity or the existence of gel mobility anomalies, as shown to exist in *L. tarentolae* minicircle DNA (Challberg and Englund, 1980; Simpson, 1979).

Analysis of cloned minicircle DNA has greatly assisted our understand-

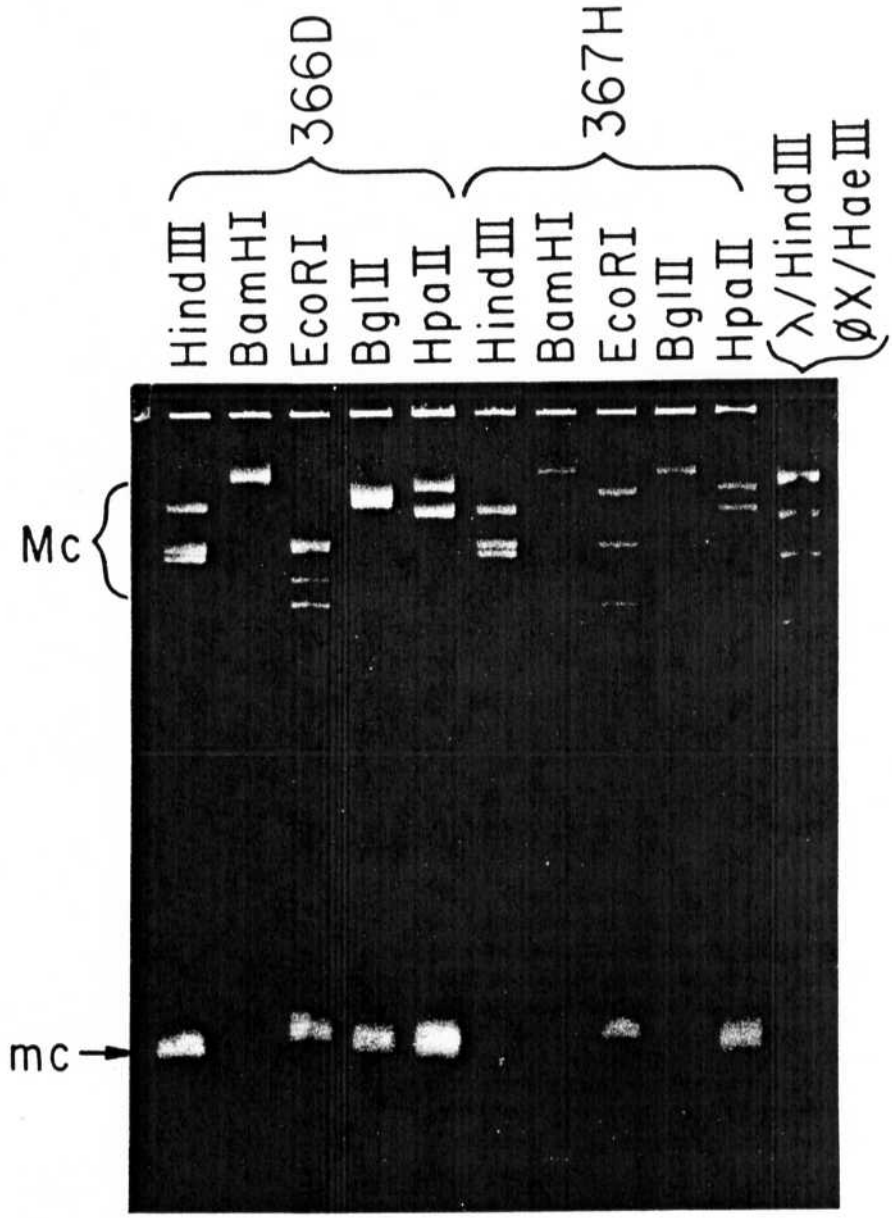

FIG. 3. Comparison of digestions of kDNA from two clonal strains (366D and 367H) of *T. brucei* procyclics in 0.8% agarose. The minicircle (mc) and maxicircle (Mc) regions are indicated. The molecular weight references are λ/*Hin*dIII and ϕXRF/*Hae*III fragments. Reprinted from Simpson and Simpson (1980) with permission.

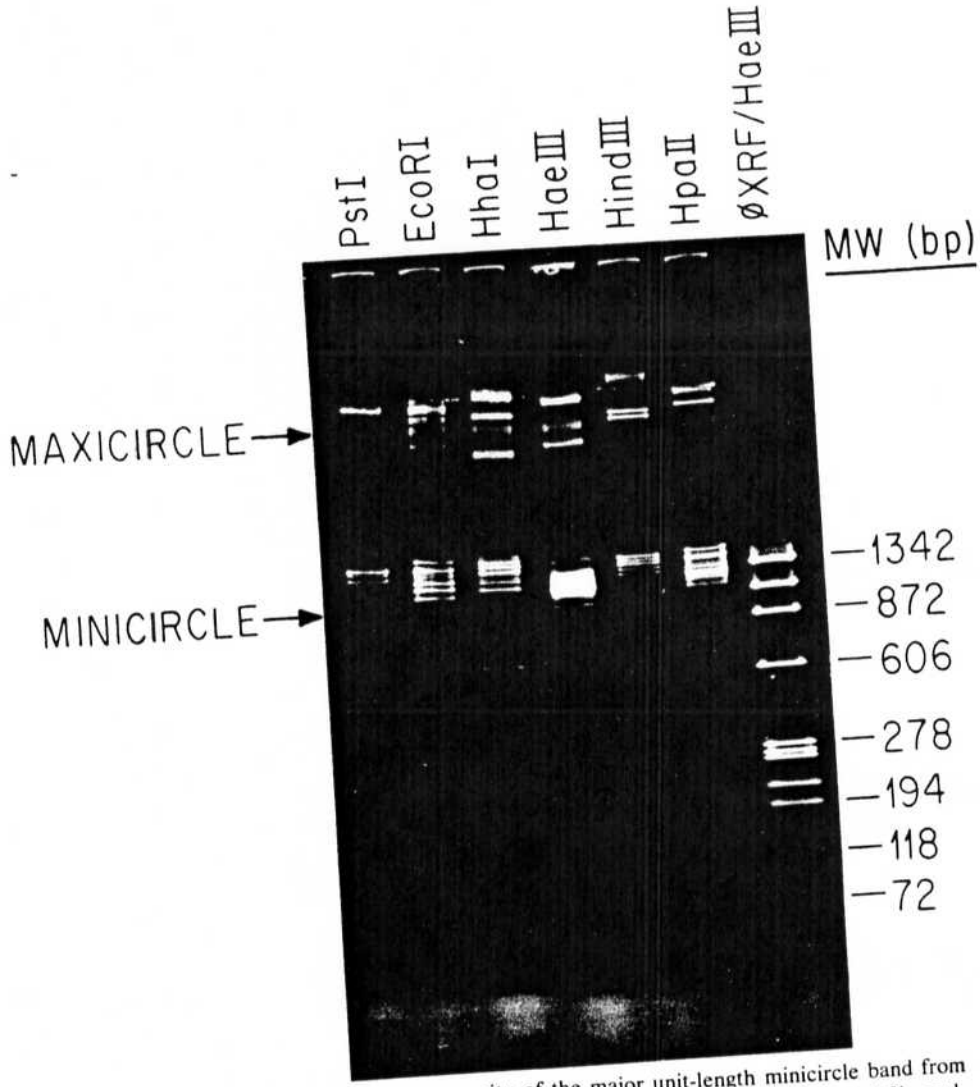

FIG. 4. Demonstration of heterogeneity of the major unit-length minicircle band from 366D procyclic *T. brucei* kDNA in an acrylamide gradient gel. Conditions: 3.5–10% acrylamide with a 3.0% stacking region. The molecular weight references are ϕXRF/*Hae*III fragments. Reprinted from Simpson and Simpson (1980) with permission.

ing of the observed sequence heterogeneity. Donelson *et al.* (1979) cloned minicircles from *T. brucei* clone 18E2 either by C-tailing minicircles released by digestion with *Hae*III, or with a mixture of *Hpa*II, *Hha*I, and *Alu*I and cloning into the *Pst*I site of pBR322, or by direct ligation of *Hin*dIII- or *Pst*I-released minicircles into the appropriate sites in pBR322. One minicircle, pkT3, was labeled *in vitro* and used as a probe in a Southern blot hybridization of kDNA digested with several enzymes. Hybridization was observed in all minicircle bands released by digestion with six enzymes. Similar results were obtained by colony hybridization, using three minicircle clones as probes. In general the probes hybridized strongly to themselves and weakly to all the other minicircle clones. Brunel *et al.* (1980a,b) cloned *Hin*dIII minicircles from *T. brucei* 427–60 in pBR322 and also *Eco*RI minicircles in λgtWES/λB (together with a maxicircle fragment, or with λB) and tested the cloned inserts for digestion with nine enzymes. The only sites found were single sites for *Eco*RI, *Hha*I, *Hae*III, and two to three sites for *Taq*I; all four of the clones studied contained different patterns of restriction sites. However, when the λ clones were spotted onto filters and probed with five of the pBR322 clones, some hybridization was observed with all clones, implying common sequences.

Simpson and Simpson (1980) cloned *Bam*HI-released minicircles from *T. brucei* 366D into pBR322 and tested 25 clones by colony hybridization using one, pTb7, as a probe. Four of the 25 clones showed strong hybridization, but all showed some hybridization, implying the existence of more than one semihomologous minicircle sequence class with a single *Bam*HI site.

Some heteroduplex studies of *T. brucei* minicircles have been reported, but the small size of these molecules precludes obtaining much information from this method (Brunel *et al.*, 1980a; Donelson *et al.*, 1979).

A direct confirmation and extension of the hybridization results was reported by Chen and Donelson (1980) in a sequence analysis of two cloned minicircles from *T. brucei* (Fig. 5). pk51 was derived from a digest of kDNA with a mixture of *Hha*I, *Hpa*II, and *Alu*I, tailed with G and inserted into the *Pst*I site of pBR322. pk201 was released from kDNA by digestion with *Pst*I and ligated directly into the *Pst*I site of pBR322. Both molecules contained approximately 72% AT, as expected from the low buoyant density of the kDNA (ρ = 1.690 g/ml). The most striking aspect was a nearly perfect homology of 122 bp. There were also 12 other common regions containing 50% AT. pk201 contained a decanucleotide sequence repeated 5 times in tandem. The only region of dyad symmetry was an 11-bp sequence in pk51. There was a high frequency of termination codons, TAA, TGA, and TAG, in all reading frames in both mole-

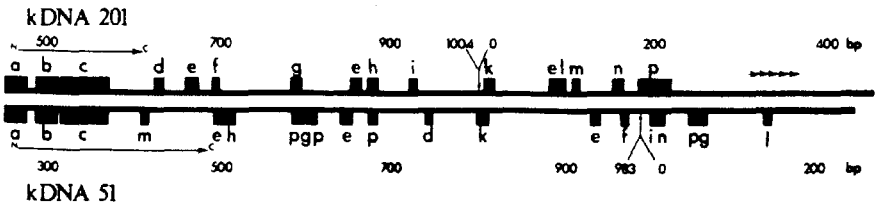

FIG. 5. Diagram showing the relative locations of perfect homologies between *T. brucei* cloned minicircle kDNAs 201 and 51 that are equal to or greater than 10 bp. The two circular kDNA sequences are represented by two lines aligned so that each begins with the first position of the 122-bp near-perfect homology. The other letters indicate the other smaller corresponding regions of homology. The numbers indicate nucleotide positions. The horizontal arrows with N at the left end and C at the right show the largest regions in the two kDNAs that could potentially code for a polypeptide. The five tandem arrows above the lines on the right indicate five repeats of a decanucleotide sequence in kDNA 201. Reprinted from Chen and Donelson (1980) with permission.

cules; the longest open reading frames were 52 amino acids in pk201 and 71 amino acids in pk51. Interestingly, these small open reading frames encompassed the 122-bp common region. The sequence data nicely confirmed the evidence from the C_0t analysis that the different minicircle classes had approximately 25% of their sequences in common, since summation of all the perfect homologies of 10 bp or greater gave a value of 27%.

B. *Trypanosoma gambiense*

Riou and Barrois (1981) found that the minicircles from the kDNA networks of *T. gambiense* were similar to those from *T. brucei* in terms of cleavage by restriction enzymes. The only enzymes that cleaved the majority of the network minicircles and produced fragments of less than unit length were *Taq*I, *Hin*fI, and *Xba*I. The patterns with these enzymes indicated extensive minicircle sequence heterogeneity. They also found that the minicircles hybridized somewhat with minicircle DNA from the Pasteur Institute strain of *T. equiperdum*.

C. *Trypanosoma equiperdum*

Unlike most other kinetoplastid species, the kDNA minicircles from *T. equiperdum* are homogeneous in base sequence. This was first shown by Riou and Barrois (1979) for a strain of *T. equiperdum* from the Pasteur Institute and by Frasch *et al.* (1980) for a strain from the ATCC (30019). However, these two strains differ signficantly in the maxicircle compo-

nents, indicating a possible molecular pleomorphism among strains of this species.

A unique circular restriction enzyme map for the uncloned minicircle from the Pasteur *T. equiperdum* strain was derived by Riou and Barrois (1979) and the complete nucleotide sequence was reported by Barrois *et al.* (1982). The molecule contained 73% AT, had a 6-fold tandem repeat of a 12-bp sequence, and had three small dyad symmetries of 9–11 bp distributed equidistantly from each other. In terms of possible codogenic function, the longest open reading frames were 18 and 22 amino acids (Fig. 6). A sequence of 130 bp was strikingly homologous to the 122-bp common sequence to *T. brucei* minicircles pkT51 and pkT201; it differed from pkT201 by 12 bp and from pkT51 by 15 bp. The remainder of the *T. equiperdum* molecule had no significant homology with the *T. brucei* sequences. This cross-species partial sequence conservation is intriguing in terms of the functional significance of the minicircle. Both Chen and Donelson (1980) and Barrois *et al.* (1982) speculated that the conserved

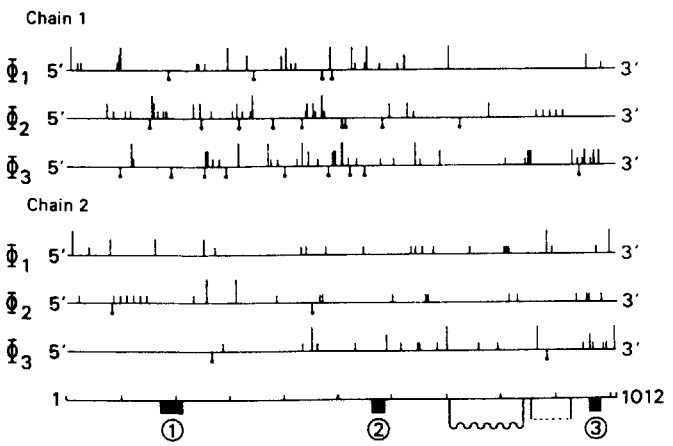

FIG. 6. Diagram showing the distribution of nonsense (vertical bars) and initiation (inverted bars with solid circles) codons in the six reading frames of *T. equiperdum* minicircle DNA. Three reading frames called ϕ_1, ϕ_2, and ϕ_3 were initiated from the 5′ end of each DNA strand. On chain 1, ϕ_1 is initiated by its first triplet AAT, ϕ_2 by ATC, and ϕ_3 by TCA. On chain 2, ϕ_1 is initiated by its first triplet ATT, ϕ_2 by TTC, and ϕ_3 by TCT. The length of the kDNA minicircle does not correspond to a multiple of three nucleotides. Therefore, going through *Hin*fI site, phase ϕ_1 is changed into phase ϕ_2. Vertical bars have different lengths, depending on the stop codon (small-sized vertical bar, TAA; medium-sized vertical bar, TAG; large-sized vertical bar, TGA). (Bottom) Schematic representation of the minicircle, with the locations of the three dyad symmetries (circled numbers 1, 2, and 3), the DNA region homologous to *T. brucei* minicircles (wavy line), and the six repeats of 12 bp each (dashed line). Reprinted from Barrois *et al.* (1982) with permission.

region may contain the origin of replication. *T. equiperdum* is apparently a recent derivative of *T. brucei* that has lost the ability to undergo cyclical transmission through the insect host [I^- (Opperdoes *et al.*, 1976)], and hence it is not entirely surprising that the *T. brucei* minicircle common region is conserved. It is of great interest, however, that minicircle sequence heterogeneity does not occur in *T. equiperdum*, and this may be related to defects in maxicircle DNA of *T. equiperdum*, that will be discussed below (Frasch *et al.*, 1980).

D. *Trypanosoma evansi*

This is another example of a species that contains kDNA minicircles of a single sequence class. In addition, the networks appear to lack maxicircle DNA (Fairlamb *et al.*, 1978). Frasch *et al.* (1980) reported that the *Mbo*I agarose gel restriction profiles of kDNA from *T. evansi* strains were very similar to that from *T. equiperdum* ATCC 30019 and suggested that there is a basic minicircle sequence characteristic of the *T. brucei* type trypanosomes, that is, obscured in *T. brucei* itself by the extensive sequence heterogeneity.

E. *Trypanosoma mega*

T. mega kDNA minicircles show extensive sequence heterogeneity by both restriction enzyme analysis and reassociation kinetics. Steinert and Van Assel (1980) have calculated that the network may contain approximately 70 different semihomologous sequence classes.

F. *Trypanosoma lewisi*

Two minicircles from *T. lewisi* bloodstream forms were cloned in M13 vectors and completely sequenced (Ponzi *et al.*, 1984). The molecules contain 1018 bp and show a conserved region of 95 bp that is repeated twice in the molecule. The conserved regions are oriented approximately symmetrically within the minicircle. A 15-mer sequence (5′-GGGGTTG-GTGTAATA-3′) within the conserved region was found to be also present in the published *T. brucei* and *T. equiperdum* minicircle sequences. The longest polypeptides that could be encoded by the conserved regions of the two *T. lewisi* minicircles are 27 and 26 amino acids, and these amino acid sequences show no relation to the amino acid sequences derived from the *T. brucei* and *T. equiperdum* minicircle sequences (Ponzi *et al.*, 1984).

G. *Trypanosoma cruzi*

The first evidence on kDNA minicircle sequence heterogeneity came from studies on *T. cruzi* (Riou and Yot, 1975, 1977). The minicircle of *T. cruzi* kDNA is intermediate in size between *T. brucei* and *Crithidia* and exhibits a sequence heterogeneity that is extensive but less than that of *T. brucei* or *T. mega* minicircle DNA. The *T. cruzi* minicircle has the unique property of an intramolecular repetitive sequence (conserved region) that is repeated four times with some variation. The initial evidence for this was the fact that several restriction enzymes give major bands with similar mobilities corresponding to multiples of one quarter of the unit minicircle length (Leon *et al.*, 1980; Mattei *et al.*, 1977; Morel *et al.*, 1980; Riou and Gutteridge, 1978; Riou and Yot, 1975, 1977). The existence of a quarterly repetitive conserved sequence has been confirmed by direct sequence analysis of minicircle DNA cloned into the *Eco*RI site of pBR325 and M13mp7 (Van Heuverswyn *et al.*, 1982).

Saucier *et al.* (1981) reported that network kDNA from log phase *T. cruzi* culture forms has a low-density shoulder in CsCl when the kDNA was isolated without use of a proteolytic digestion step (Fig. 7). The kDNA from stationary phase cells lacked this low-density shoulder. They attributed this density shift to a bound protein since the shift was eliminated by pronase digestion of the DNA preparation and they suggested that the bound protein was involved in kDNA replication or in the organization of the kDNA nucleoid body *in situ*. Since 95% of the kDNA is minicircle DNA, it is likely that the bound protein is linked to minicircle DNA. It should be of great interest to extend these findings to other kDNA systems, to localize the position of the bound protein, and to determine the functional significance of this phenomenon.

H. *Crithidia* Species

kDNA from *C. fasciculata*, *C. luciliae*, and *C. acanthocephali* has been studied in detail. *Crithidia* spp. contain the largest kDNA minicircles (2.3 kb) in the kinetoplastid protozoa (Renger and Wolstenholme, 1972; Simpson *et al.*, 1974). The sequence heterogeneity of the minicircle DNA, however, is less than that found in *T. brucei*. Reassociation kinetic analysis has yielded values for the complexity of network DNA from *Crithidia* of 1–2.5 times the unit minicircle size (Fouts *et al.*, 1975; Hoeijmakers *et al.*, 1982b; Kleisen and Borst, 1975b), with no evidence for the presence of two or more components with different complexities (Kleisen and Borst, 1975b). In addition, the decrease in melting temperature of a self-annealed sonicated kDNA preparation from *C. luciliae* (Kleisen *et al.*,

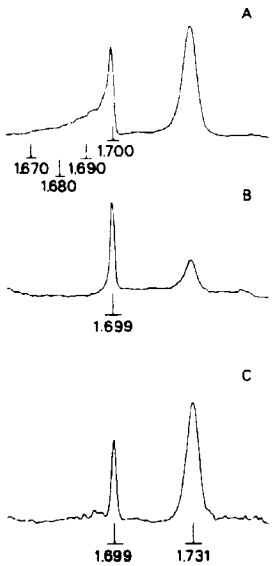

FIG. 7. Equilibrium density centrifugation in CsCl gradients of kDNA from *T. cruzi* and *Micrococcus luteus* DNA (ρ = 1.731 g/cm^3). (A) Untreated sample extracted from trypanosomes grown for 5 days; (B) same sample dialyzed against SSC to remove CsCl and incubated with pronase (1 mg/ml for 2 hours at 37°C); (C) untreated sample extracted from trypanosomes grown for 9 days. Reprinted from Saucier *et al.* (1981) with permission.

1976a) was only 1°C, implying that only 2% of the base pairs were involved in the sequence heterogeneity.

A qualitative estimate of more than 13 semihomologous minicircle sequence classes has been made from analysis of restriction profiles in gels (Kleisen *et al.*, 1976a). An apparent minor length heterogeneity of unit-length linearized minicircles has also been reported (Kleisen *et al.*, 1976a), although it has not been shown that this length heterogeneity is real and is not due to the gel mobility anomalies first observed with *Leishmania* minicircle DNA.

Hoeijmakers *et al.* (1982b) reported the results of an informative electron microscopic heteroduplex analysis of denatured and annealed minicircles of *C. luciliae* that were released from the networks by *Hin*dIII digestion. In approximately 40% of the reannealed minicircles they found heteroduplex structures characteristic of sequence rearrangements. The majority of these rearrangements were interpreted as inversions and the remainder as translocations, insertions, and deletions. The rearrangements were not at random locations but were localized to four minicircle segments (Fig. 8). In addition, there was a region (65–95% of the molecule

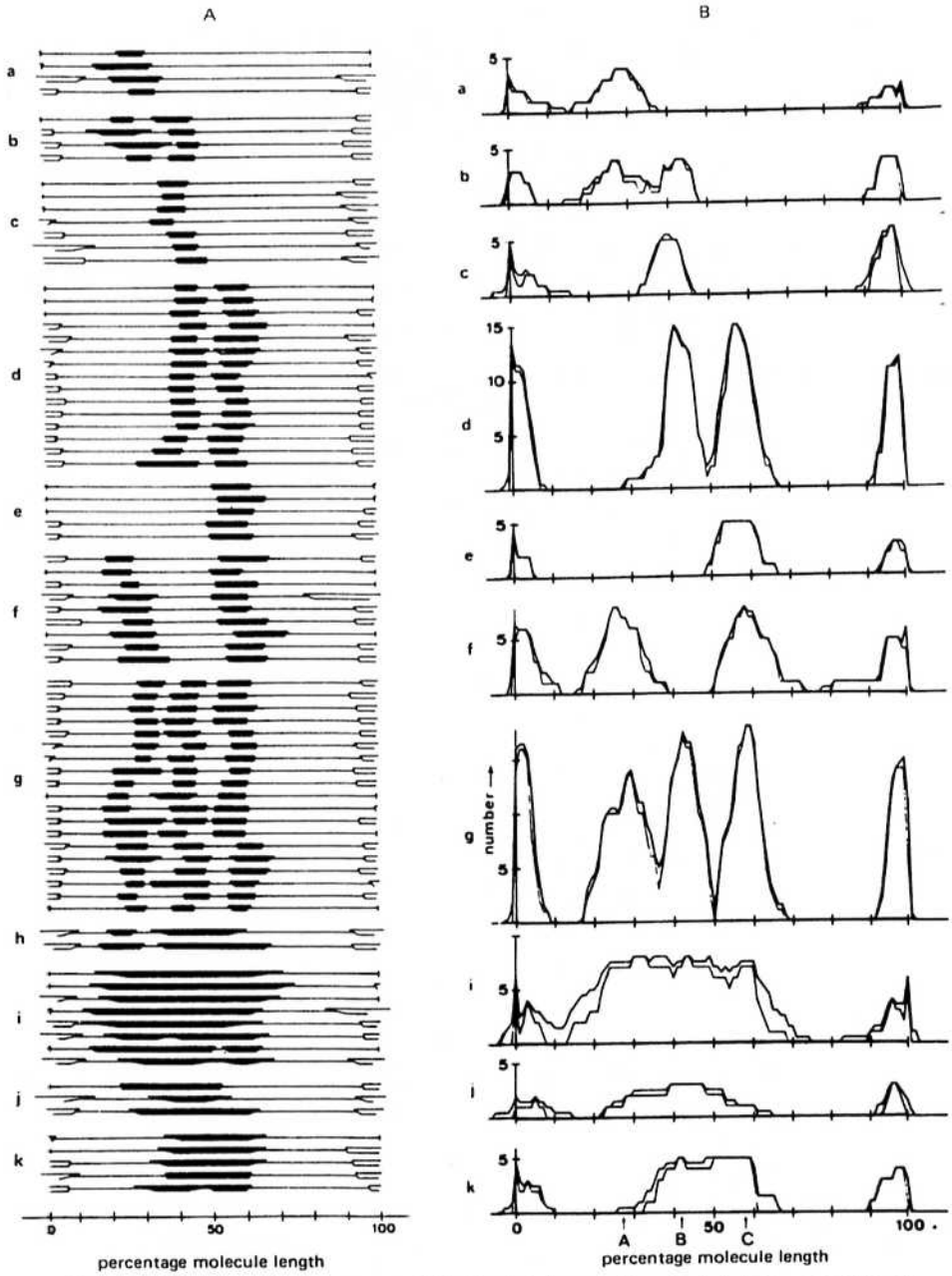

FIG. 8. (A) Heteroduplex maps of 81 *C. luciliae* renatured minicircle molecules of classes $I^{a,b,c}$ and III. Ninety molecules of the heteroduplex categories $I^{a,b,c}$ and, in part, III (only molecules with apparent deletions replaced by inserts of different size) were traced and divided into groups on the basis of overlap of hetero- and homoduplex segments. Eighty-one molecules (90%) could be classified in the groups shown (a–k). Thin single line,

in the map in Fig. 8) that showed no rearrangements and could correspond to the "constant region" such as found in *T. brucei* minicircles. They concluded that "specific segmental rearrangements form the main basis of the minicircle sequence heterogeneity in *Crithidia*" (Hoeijmakers and Borst, 1982). In order to correlate these results with the previous report of complete renaturation of *Crithidia* kDNA and only a 2% base pair mismatch of the self-annealed DNA (Kleisen *et al.*, 1976a), it must be assumed that the minicircle fragments used in the renaturation analysis were smaller than the rearranged segments found in the electron microscope study. The rearrangement model for the generation of minicircle diversity implies the existence of extensive recombination between minicircles in the network. Some evidence for this was previously obtained by a density shift experiment using *C. acanthocephali* kDNA (Manning and Wolstenholme, 1976). In addition "fused dimer" type minicircle structures have been observed in electron micrographs of minicircle DNA from several kinetoplastid species (Simpson and da Silva, 1971), which are similar to structures involved in genetic recombination.

The nature of the minicircle sequence heterogeneity in *Crithidia* (Hoeijmakers and Borst, 1982) was tested by redigestion of specific bands with a second enzyme. By this method it was shown that the bands in the primary digests are largely homogeneous, implying again that the sequence heterogeneity is caused by rearrangements rather than by accumulation of point mutations.

I. *Leishmania tarentolae*

The minicircles in the *Leishmania* are the smallest reported to date. *L. tarentolae* minicircles are about 870 bp in size, show extensive sequence heterogeneity by restriction enzyme analysis, and renature with a $C_0t_{1/2}$

homologous duplex region; parallel thin lines, single strands of a heteroduplex terminal segment; black box, internal heterologous region. The longest strand of a heteroduplex region is always shown above the base line, the smallest below. All molecules are normalized to a uniform length by putting the mean size of the upper and lower strands of each molecule (respectively the longest and shortest combination of strands) to a value of 100%. For absolute measurements the size of a *C. luciliae* minicircle was used (0.76 μm). The correction factor for dsDNA was determined from size measurements of circular ds minicircles (0.76 μm ± 0.03 SD) present in the same spreading. For ss regions the correction factor was based on the average size of ss circles and linears present in the spread preparation (0.72 μm ± 0.07 SD). (B) Histogram of heteroduplex regions of the groups of (A). Number of molecules versus percentage minicircle size. The designation of the histograms corresponds with that of the groups of (A). The two molecules constituting group h were not included here. The arrows labeled A, B, and C represent the three sites where the small heteroduplex areas are preferentially localized. Reprinted from Hoeijmakers *et al.* (1982b) with permission.

corresponding to a complexity between one and two times the minicircle length (Wesley and Simpson, 1973b). Reannealed open minicircles showed no decrease in the melting temperature and contained no regions of strand separation in the electron microscope (Wesley and Simpson, 1973a). Open circles did exhibit a multiphasic melting curve, however, that implied intramolecular sequence heterogeneity (Wesley and Simpson, 1973a). Intermolecular sequence heterogeneity is evidenced by restriction enzyme analysis (Challberg and Englund, 1980; Simpson, 1979) and also by cloning and sequencing of several sequence classes. The sequence heterogeneity of minicircle kDNA from *L. tarentolae* can best be visualized by high-resolution acrylamide gradient gel electrophoresis (Simpson, 1979) as shown in Fig. 9. The rich complexity of the banding pattern is almost obliterated by electrophoresis in agarose, but the major bands are visible (Figs. 10 and 11). Challberg and Englund (1980) concluded from analysis of minicircle fragments in agarose gels that there were three major sequence classes (Table I). Class I minicircles were linearized by *Hpa*II and represented 70% of the total DNA; class II minicircles were linearized by *Hind*II and represented 15% of the DNA; and class III minicircles were linearized by *Hpa*II, could be separated from linearized class I by agarose gel electrophoresis, and represented 7% of the DNA. A remaining 4% of the DNA consisted of minor classes and

TABLE I
COMPONENTS OF *L. tarentolae* KINETOPLAST DNA NETWORKS[a]

Component	Approximate percentage of total kDNA	*Hpa*II fragments	Enzyme used to obtain full-length fragment
Class I minicircles	70	α	*Hpa*II
Class II minicircles	15		*Hind*II
Class III minicircles	7	β	*Hpa*II
Other minicircles	4	—	—
Maxicircles	4	—	—

[a] To determine the percentage of kDNA in each component, a *Hpa*II digest of [^{3}H]kDNA was electrophoresed on a 1–4% agarose tube gel. The gel was stained with ethidium bromide to locate the bands and sliced, and the slices were counted. The percentages in each component were calculated from the total radioactivity recovered (about 75% of the original sample). Approximately 4% was found in three high-molecular-weight fragments presumed to be derived from maxicircles. Another 4% was spread throughout the gel and is assumed to correspond either to low-molecular-weight maxicircle fragments or to fragments from minor classes of minicircles. No corrections were made for possible variations in base composition of the different components. Reprinted from Challberg and Englund (1980) with permission.

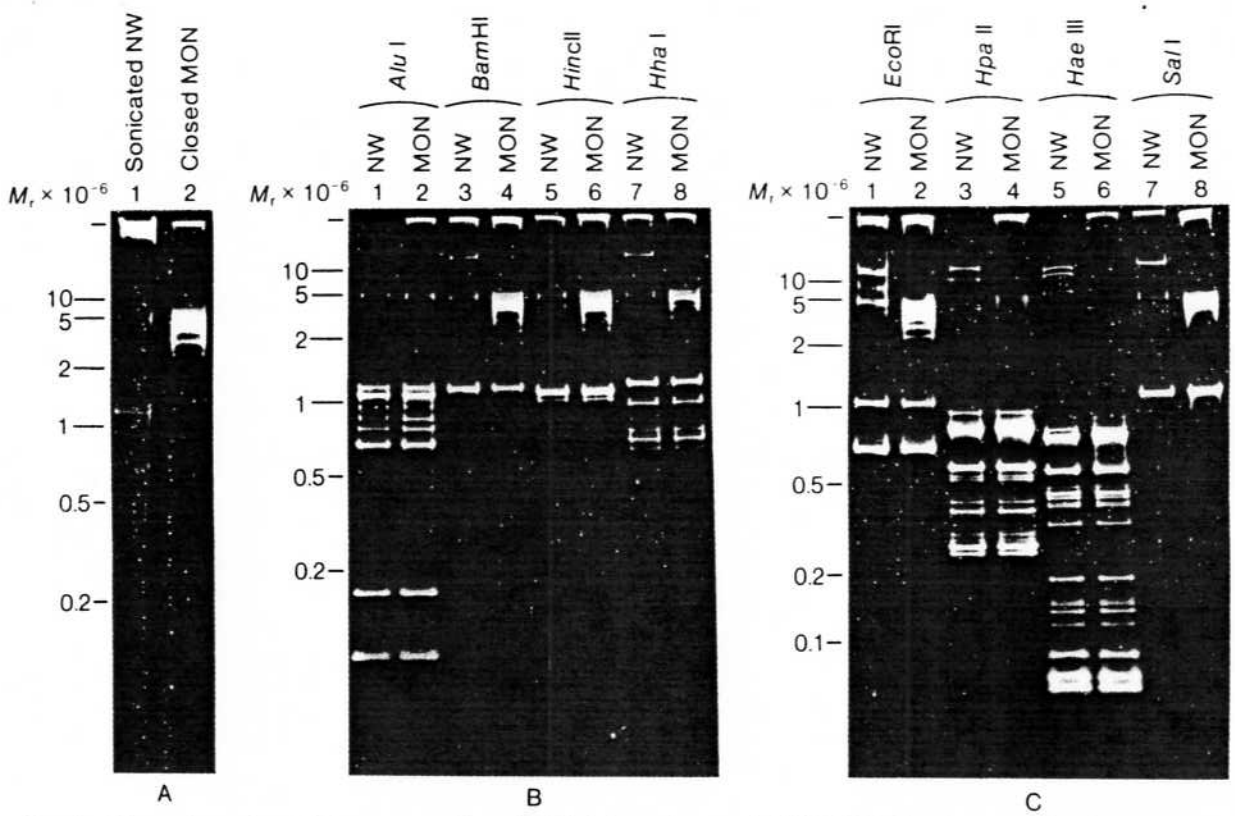

FIG. 9. Comparison of restriction enzyme-digested minicircles and network (NW) DNA from *L. tarentolae* by acrylamide gradient electrophoresis. (B and C) Closed monomeric minicircles (MON) and closed networks were digested with several restriction enzymes and electrophoresed on 3.5–10% acrylamide gradient gels with 3% stacking gels. (A) Mildly sonicated network DNA and undigested closed monomeric minicircles were electrophoresed. Several reference DNAs (not shown) were electrophoresed in each gel to obtain the molecular-weight scales. Reprinted from Simpson (1979) with permission.

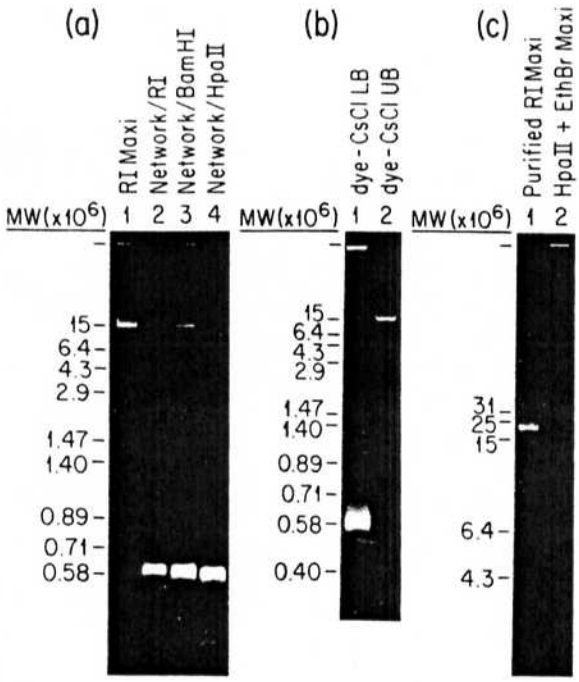

FIG. 10. Agarose gel electrophoresis of digested kDNA. (a) A 0.8% agarose gel of purified *L. tarentolae* *Eco*RI-digested maxicircle DNA and total network kDNA digested with *Eco*RI, *Bam*HI, and *Hpa*II. This *Eco*RI maxicircle preparation was purified by a single cycle of Hoechst 33258/CsCl separation and has some contaminating minicircle linear molecules migrating as if their molecular weight is approximately 0.6×10^6. The reference DNAs (not shown) were λ/*Hin*dIII fragments and φX174 RF/*Hae*III fragments. (b) A 0.8% agarose gel of the lower band (LB) and upper band (UB) from the Hoechst 33258/CsCl gradient of *Eco*RI-digested kDNA shown in Fig. 18; same reference fragments as in (a). (c) A 0.5% agarose gel of purified *Eco*RI maxicircle DNA and once-cleaved, permuted maxicircle linear molecules produced by digestion of kDNA with *Hpa*II in the presence of ethidium bromide (EthBr). The reference DNAs (not shown) were λ DNA, T7 DNA, and λ/*Hin*dIII fragments. Reprinted from Simpson (1979) with permission.

another 4% of maxicircle DNA. Evidence for the homogeneity of the sequence classes came from the construction of unique restriction enzyme maps for class I and class II uncloned gel-isolated linearized fragments for the enzymes *Hae*III, *Mbo*I, and *Hpa*II (Fig. 12). In addition, both class I and class II fragments reassociated with a $C_0t_{1/2}$ equivalent to a complexity of about 850 bp, and the reassociated molecules melted off hydroxyapetite at the same temperature as the native molecules. Further-

more, when class I linears were used as the driver and class II linears as the tracer, there was an acceleration of the rate of annealing of class II molecules, indicating sequence homology between class I and class II molecules. The heterologous hybrid showed a decrease in the melting temperature, indicating a 10–15% base pair mismatch. Recent Maxam–Gilbert sequence analysis of class I and class II linears isolated from a gel

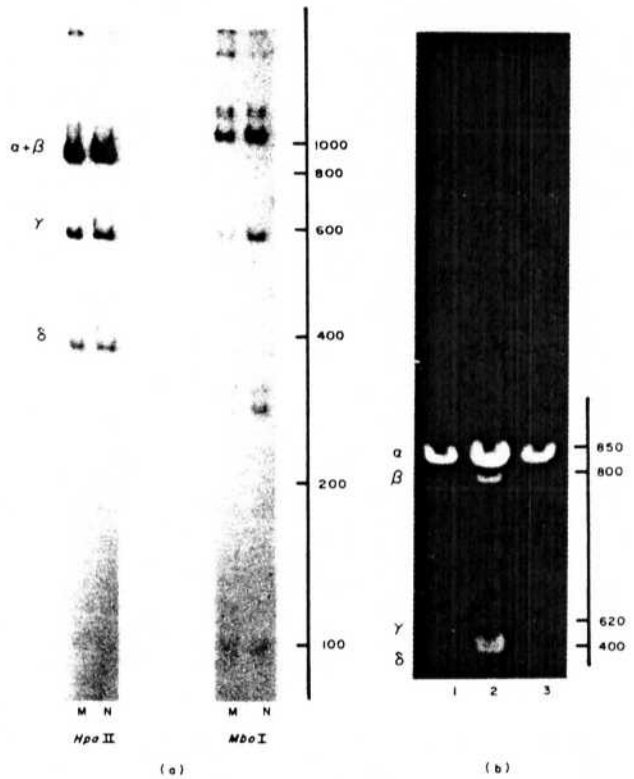

FIG. 11. Restriction enzyme cleavage products of *L. tarentolae* kDNA. (a) Fragments produced from digestion of purified minicircles (M) or intact networks (N) were 5′ terminally labeled with ^{32}P and electrophoresed on an 8% polyacrylamide gel which was then autoradiographed. (b) Fragments were electrophoresed on a 1.4% agarose slab gel which was then stained and photographed. Lane 1 is an *Hpa*II digest of the *Hin*dII-resistant component of networks. Lane 2 is an *Hpa*II *Hin*dII double digest of intact networks. Appropriate controls showed that all digests are complete. The base-pair scales were established using restriction fragments of SV40 DNA, but many kDNA frgments do not electrophorese as expected from their size. Control experiments showed that *Hpa*IIα and *Hpa*IIβ migrate together on the 8% polyacrylamide gel. Numbers of base pairs are indicated on the right-hand sides of the gels. Reprinted from Challberg and Englund (1980) with permission.

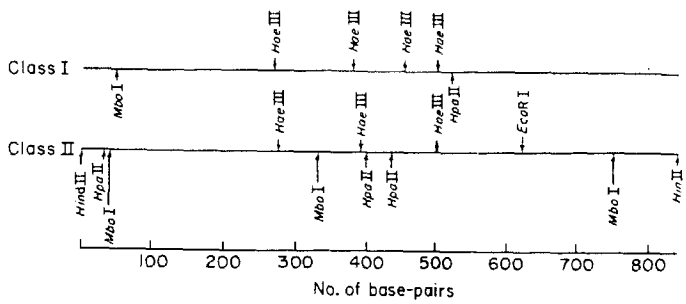

FIG. 12. Restriction enzyme cleavage site maps for *L. tarentolae* class I and class II minicircles. The maps are aligned arbitrarily to maximize similarities. Class I minicircles do not contain *Hin*dII or *Eco*RI sites. Reprinted from Challberg and Englund (1980) with permission.

has confirmed the homogeneity of class II but has shown class I to be heterogeneous (P. T. Englund, personal communication). The reason that this heterogeneity was not evidenced in the earlier analysis of Challberg and Englund (1980) is probably due to the limited number of enzymes used to construct the restriction map. Heterogeneity is clearly apparent in high-resolution acrylamide gels of digested kDNA from *L. tarentolae*, but it is nevertheless obvious that the sequence diversity in *Leishmania* is less than that in *T. brucei* and probably less than that in *Crithidia*.

The minicircle sequence diversity in *L. tarentolae* was also demonstrated by cloning different sequence classes in *Escherichia coli* and direct sequence analysis (Simpson *et al.*, 1979, 1980; Kidane *et al.*, 1984). Total kDNA was digested with *Bam*HI and cloned into pBR322. One unit-length minicircle clone, pLt12, was selected and used as a probe in colony hybridization to detect nonhomologous minicircle clones. Two *Hin*dIII minicircle clones in pBR322 were selected by this method, pLt26 and pLt154. An *Eco*RI minicircle, pKSR1, and a *Pst*I minicircle, pLtP1, were also cloned into pBR322. All minicircle inserts were subcloned into M13mp7, M13mp8, or M13mp9, in both orientations. The cloned minicircles were shown to be semihomologous by colony and blot hybridization. Restriction maps were constructed and sequence analysis performed. pKSR1 and pLt19 were shown to be the identical minicircle cloned at the *Eco*RI or the *Bam*HI site. It was shown by sequence analysis (Kidane *et al.*, 1984) that pLt19-KSR1, pLt26, and pLt154 contained a conserved sequence of 160–270 bp (Fig. 13). Barker *et al.* (1982) have also cloned and sequenced a minicircle from *L. tarentolae*, which has almost exactly the same sequence as the pLt19 clone derived in our laboratory.

A 14-mer sequence (5′-AGGGGTTGGTGTAA-3′) within the conserved region of the pKSR1, pLt26, and pLt154 minicircles was noted by

Kidane *et al.* (1984) to be conserved in the minicircle sequences reported from *T. brucei, T. equiperdum,* and *T. cruzi.* As noted above, this sequence is present also in the two cloned minicircles from *T. lewisi* and may represent a universal conserved minicircle sequence in all trypanosomatids. Evidence from Ntambi and Englund indicates that this "universal" minicircle sequence may represent an origin of replication. They found that newly replicated *T. equiperdum* minicircles which have recatenated to the network contain a single gap about 10 nt in size which overlaps the "universal" sequence.

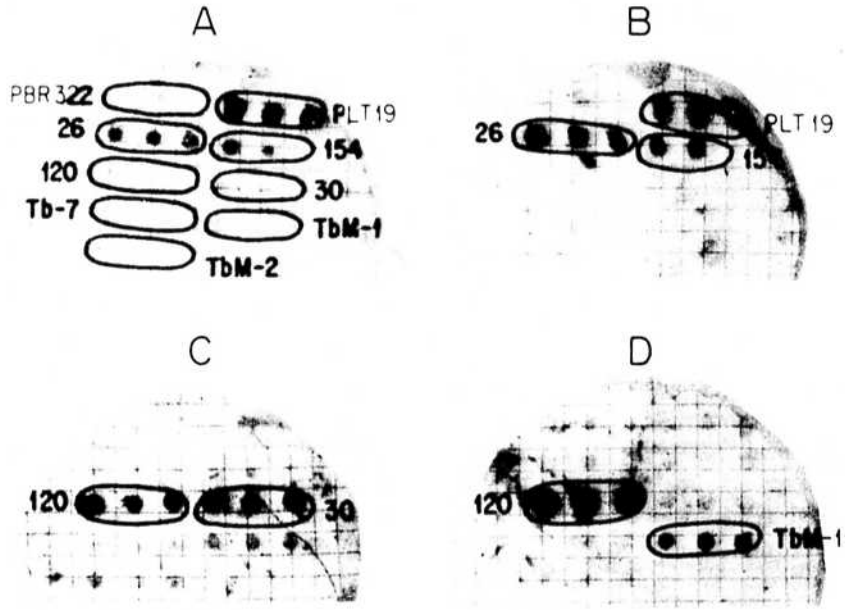

FIG. 13. Colony hybridization of recombinant clones containing *L. tarentolae* kDNA fragments and 366D procyclic *T. brucei* kDNA fragments. The clones were spotted in triplicate on each of four filters as indicated. The ^{32}P-labeled probes were (A) total kDNA from *L. tarentolae;* (B) total closed monomeric minicircle DNA from *L. tarentolae;* (C) purified *Bam*HI-linearized maxicircle DNA from *L. tarentolae;* (D) purified insert DNA from pLt120 plasmid, which contains a 6.6-kb fragment of *L. tarentolae* maxicircle DNA containing the 9 and 12 S RNA genes. pLt19, 26, and 154 are *L. tarentolae* kDNA unit-length minicircle clones; pLt120 and 30 are *L. tarentolae Eco*RI/*Bam*HI and *Hin*dIII maxicircle clones. TbM-1 is the *Hin*dIII C fragment maxicircle clone from *T. brucei;* TbM-2 is the *Hin*dIII B fragment maxicircle clone from *T. brucei.* Tb-7 is a unit-length minicircle clone from *T. brucei.* The original filters were inserted under the autoradiographs for photography. The relative positions of the colonies are as indicated in A by circling the three colonies of each type. In B, C, and D only those colonies showing strong positives are circled. Reprinted from Simpson and Simpson (1980) with permission.

Kidane *et al.* (1984) showed by a combination of quantitative gel electrophoresis and examination of T ladders of minicircles from *L. tarentolae* kinetoplast DNA randomly cloned into M13 at several sites that approximately 50% of the total minicircle DNA could be assigned into five sequence classes. The pKSR1-Lt19 sequence class was shown to represent 26% of the total minicircle DNA and a portion of the sequence was identical to the revised sequence (Marini *et al.*, 1983) of the cloned 414-bp *Mbo*I fragment of Marini *et al.* (1982), implying that the pKSR1 minicircle probably was identical to the class II minicircle of Challberg and Englund (1980).

Arnot and Barker (1981) have analyzed kDNA from *Leishmania tropica major, Leishmania aethiopica,* and *Leishmania SP48* by agarose gel electrophoresis and by blot hybridization and have concluded that the minicircles from *L. tropica major* (700 bp) are smaller than those from *L. tarentolae* and that rapid minicircle sequence evolution is occurring in these Old World leishmanias.

An interesting anomaly in electrophoretic behavior of minicircle DNA from *L. tarentolae* was noted in the initial studies (Challberg and Englund, 1980; Simpson, 1979). The observation was that the mobility of full-length uncloned linearized minicircle DNA and certain fragments was different in acrylamide than in agarose gels, leading to large discrepancies in apparent molecular weights. Similar behavior was observed with cloned minicircles and minicircle fragments. Simpson *et al.* (1980) and Kidane *et al.* (1984) showed that the larger *Sma*I fragment of the cloned minicircle, pLt19, had an apparent size in 5% acrylamide 1.5 times the apparent size in 1.5% agarose. However, cleavage of this fragment at the *Eco*RI site produced two fragments which ran properly in acrylamide. This phenomenon was studied in detail by Marini *et al.* (1982), Kidane *et al.* (1984), Wu and Crothers (1984), and Hagerman (1984). The *Mbo*I fragment A from class II minicircles of *L. tarentolae* was cloned in pBR322 as pPE103 and found by sequence analysis to contain 414 bp (Marini *et al.*, 1982, 1983). This fragment had an apparent size in 1% agarose of 450 bp and an apparent size in 12% acrylamide of 1380 bp. It eluted from Sephacryl 500 with an apparent size of 375 bp and behaved like a fragment of less than 309 bp in electric dichroism experiments. Marini *et al.* (1983) concluded that the molecule had an unusually compact configuration and proposed that it contains a region of smoothly bent B-DNA which may be caused by sequence periodicities. The sequence of this fragment was shown to contain a significant 10-nt periodicity of pdApdA, which could cause a bending of the molecule. They suggested that the curvature of the minicircle DNA is functionally significant in terms of binding a protein to the bent region, or of facilitating condensation of the network into the kDNA nucleoid body.

This argument was strengthened by the experiments of Wu and Crothers (1984), who constructed circularly permuted fragments of the same *Mbo*I minicircle fragment by cloning a double tandem repeat into a pBR322 plasmid and digesting with different restriction enzymes which only cut once. The relative gel mobilities of the permuted linearized plasmids were compared in acrylamide and agarose. By this method the "bend" was mapped to a site 148 nt from the *Mbo*I site, which corresponds to approximately 20 nt from the *Eco*RI site in pKSR1. This localization agrees well with that obtained by Kidane *et al.* (1984) by restriction enzyme digestion of circular pKSR1 minicircle DNA and comparative agarose–acrylamide gel electrophoresis. Wu and Crothers (1984) point out a $CA_{5-6}T$ tract repeated at a 10-nt interval four times at that locus in the sequence, and they attribute the bending of the molecule to this sequence periodicity. Hagerman (1984), however, has proposed an alternative physical explanation for the observed sequence dependent bend in the pKSR1 minicircle, which is based on the "purine clash" model of Calladine (1982). Further work is needed to distinguish between these theories.

Similar anomalies in electrophoretic behavior were noted for *T. brucei* minicircle DNA by Chen and Donelson (1980) and for *T. equiperdum, Herpetomonas muscarum,* and *C. fasciculata* by Ntambi *et al.* (1984) and may be a general property of kDNA minicircles.

J. *Phytomonas davidi*

Phytomonas davidi kDNA minicircles, which are 1.1 kb in size, renature with a $C_0t_{1/2}$ corresponding to a complexity of approximately 1.3 times the size of the minicircle and the reannealed circles melt 0.7–1.8°C below the native melting temperature, indicating no more than 2–4% base pair mismatch (Cheng and Simpson, 1978). Physically purified closed monomeric minicircles exhibit at least 13 bands of superhelical molecules in acrylamide–agarose gels in a distinctly non-Gaussian banding pattern which could be explained by the existence of a minor minicircle length heterogeneity or mobility abnormalities, as seen in *L. tarentolae*.

III. Evolution of Minicircle Sequence Heterogeneity

Minicircle sequence evolution has been studied both by examination of different strains or isolates from nature and by comparison of the same strain kept under nonselective laboratory conditions. Steinert *et al.* (1976) showed that two isolates of *T. brucei* did not cross hybridize. Borst *et al.* (1980a, 1981a) showed that the *Taq*I agarose restriction profiles of kDNA

Reference
#269 (A
#271 (B
#231 (C
#280 (D

digests from nine strains of *T. brucei* were clearly different, but that four clones of *T. brucei* strain 427 yielded identical restriction profiles.

A comparison of the minicircle restriction profiles of kDNA from *C. fasciculata* and *C. luciliae* in high-resolution acrylamide gradient gels showed hardly any bands in common (Hoeijmakers and Borst, 1982). Cross hybridization of blots of *C. luciliae* kDNA digests with a labeled specific gel isolated fragment of *C. fasciculata* minicircle DNA showed weak hybridization of a specific subset of bands. Use of total *C. fasciculata* kDNA as a probe gave weak hybridization to most *C. luciliae* bands. Since the maxicircle DNA from these species were shown to be greater than 90% homologous, Hoeijmakers and Borst (1982) suggested that *C. luciliae* and *C. fasciculata* represent different strains of the same species which have undergone extensive minicircle sequence evolution.

The actual rate of change of minicircle sequences in both *C. fasciculata* and *C. luciliae* was monitored qualitatively by examination of kDNA from cells grown in the laboratory over a 2-year period (Hoeijmakers and Borst, 1982) at intervals of 9–12 months. kDNA from the uncloned *C. fasciculata* culture showed many small changes in restriction bands and the cloned *C. luciliae* showed a few changes. The changes were not due to methylation differences or to the presence of gaps or nicks in the molecules and were not caused by different maxicircle DNA concentrations in the networks.

Camargo *et al.* (1981) analyzed kDNA restriction profiles and other properties of 13 species of monoxenic trypanosomatids. They found interspecific but not intraspecific differences and concluded that this method can be used for species identification of insect trypanosomatids. However, the absence of restriction profile differences between three laboratory strains of *C. fasciculata* does not necessarily imply that this species shows no natural minicircle sequence evolution, since the actual history of these three laboratory strains is uncertain. Examination of *Crithidia* strains freshly isolated from nature is necessary to answer this important question.

Different strains or stocks of *T. cruzi* isolated from human patients and animals in Brazil were shown to exhibit distinctly different restriction profiles in high-resolution acrylamide gradient gels (Morel *et al.*, 1980) (Fig. 14). Control experiments showed that strain CL cultures showed no

FIG. 14. Acrylamide gradient gel profiles of *Hin*fI digests of kDNA from the Y and CL strains of *T. cruzi* and from four stocks of *T. cruzi* (from patients) that represent zymodeme groups (A–D). The reference DNA is a mixture of λ/*Hin*dIII and ϕXRF/*Hae*III. The unit-length minicircle band is indicated by mc. Reprinted from Morel *et al.* (1980) with permission.

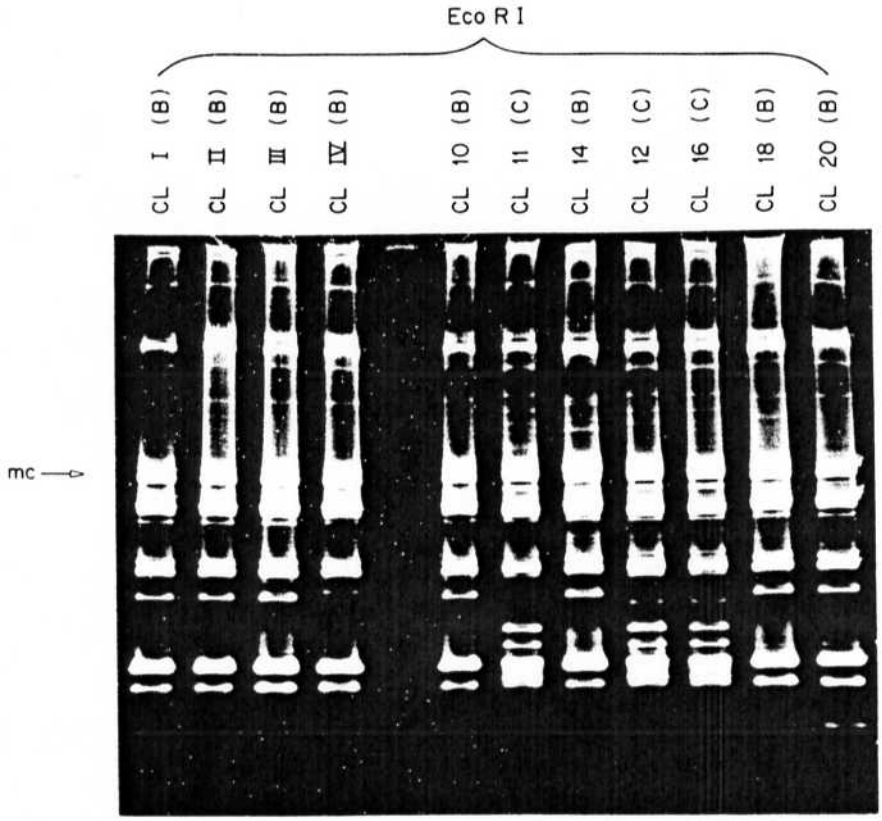

FIG. 15. Acrylamide gradient gel profiles of *Eco*RI digests of *T. cruzi* kDNA from several CL control cultures and clones. I, CL cells kept in serial culture for 2 years (1978–1980) and harvested in stationary phase; II, CL cells harvested in logarithmic phase; III, CL cells from a culture kept at −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. Reprinted from Morel *et al.* (1980) with permission.

large-scale changes in restriction profiles with several enzymes after 2 years of continuous laboratory passage either in culture or in mice (Fig. 15). There were also no differences in restriction profiles of kDNA from cells in early log phase or in stationary phase, or from cells which had a large proportion of trypomastigotes or epimastigotes. In addition, six randomly isolated human-derived stocks of *T. cruzi* from the same area that belonged to the zymodeme group C showed identical restriction profiles. Morel *et al.* (1980) concluded that *T. cruzi* minicircle sequences change rapidly enough in nature to produce differences between strains, but not so rapidly as to preclude establishment of a stable hemoculture. Morel *et*

al. (1980) proposed to designate subpopulations of *T. cruzi* having similar minicircle DNA restriction profiles by the term "schizodemes."

Frasch *et al.* (1981) have used the kDNA restriction profile method to compare stocks of *T. cruzi* and the presumed closely related *Trypanosoma rangeli* in agarose gels. They found that *Bsp*RI and *Msp*I gave patterns that allowed the differentiation of these species and concluded that these enzymes cut within the variable region of the minicircles. On the other hand, *Taq*I cut within the repetitive constant region, yielding profiles that were identical for the two species. Sanchez *et al.* (1984) extended this analysis to seven additional *T. cruzi* isolates and introduced the use of cloned minicircle and maxicircle fragments as hybridization probes. No correlation between different schizodeme patterns and infectivity for mice was apparent.

The rate of change of minicircle sequences in *L. tarentolae* was studied by examination of kDNA restriction profiles from cells frozen at intervals of 4 and 2 years (Simpson *et al.*, 1980). Profiles for the enzymes *Hha*I, *Hae*III, and *Msp*I were examined in acrylamide gradient gels (Fig. 16). The only changes observed were approximately two *Hha*I bands out of 19 during the period 1973–1977.

Arnot and Barker (1981) have attempted, with some success, to distinguish different species of Old World *Leishmania* and an unknown human isolate by blot hybridization of digested kDNA with labeled *L. aethiopica* kDNA. Lopes *et al.* (1984) have likewise attempted, with some success, to distinguish different species and strains of New World *Leishmania* by comparison of restricted profiles in acrylamide gels.

Wirth and Pratt (1982) found no detectable hybridization between the kDNA of two subspecies of *Leishmania brasiliensis* and three subspecies of *Leishmania mexicana,* nor between *L. mexicana* and *L. tropica*. They also showed that whole organisms could be spotted directly onto nitrocellulose filters and hybridized with nick-translated kDNA probes. This led to the development of a technique termed "touch blots," in which fragments of lesions from infected animals are touched to the filters and then the filters are hybridized with the labeled kDNA probe. Sufficient amastigotes are transferred to the filter by this method to provide a strong hybridization signal (Wirth and Pratt, 1982). This technique may prove to have clinical importance in the diagnosis of dermal leishmaniasis.

Handman *et al.* (1983) employed schizodeme analysis in acrylamide gels to characterize four infective and noninfective clones of *L. tropica* derived from a stock that is infective for mice. They found that the clones formed two schizodemes that were closely related in terms of cross hybridization of minicircle sequences, implying that the noninfective clones were derived from the original infective presumptive parental clone.

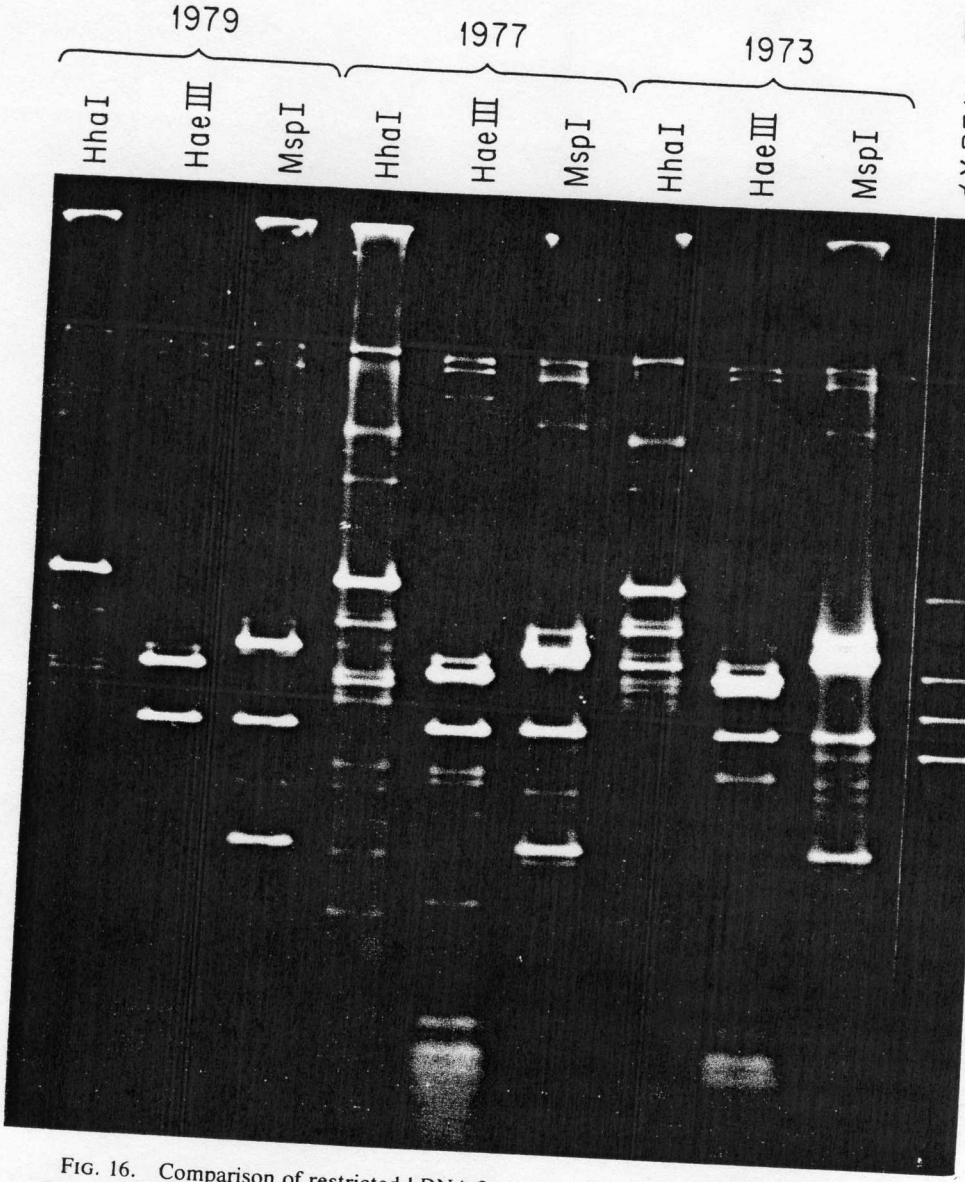

FIG. 16. Comparison of restricted kDNA from *L. tarentolae* cells kept in serial culture from 1973–1979 versus kDNA from stabilates frozen in 1973 and 1977. The gels are 3.5–10% acrylamide gradient gels with a 3% stack. The gel was stained with ethidium bromide. Reprinted from Simpson *et al.* (1982b) with permission.

As stated previously, the kDNA minicircles in two strains of *T. equiperdum* show an absence of sequence heterogeneity and at least one of these minicircles has a 130-bp region almost identical to the common region in *T. brucei* minicircle clones pkT51 and 201 (Barrois *et al.*, 1982). The suggestion has been made that the absence of minicircle sequence heterogeneity is somehow related to the observed deletion in the maxicircle DNA seen in strain ATCC 30019 (Frasch *et al.*, 1980). However, the Pasteur strain of *T. equiperdum* seems to contain an intact maxicircle at least at the level of overall size and restriction map (Riou and Saucier, 1979).

T. evansi represents another I^- trypanosome that is unable to synthesize mitochondrial enzymes and differentiate into the procyclic form, and the kDNA of this species was shown by restriction analysis to contain catenated minicircles of an apparent single-sequence class and no maxicircle DNA at all (Fairlamb *et al.*, 1978). *T. equinum* is also unable to differentiate into the procyclic form in nature and contains an altered form of kDNA. As visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining and electron microscopy, the mitochondrial DNA appears as scattered as clumps of material instead of the well-organized kinetoplast nucleoid body (Cuthbertson, 1981). In addition, a cellular DNA is present at the same buoyant density as kDNA, but it consists of a heterogeneous collection of circular molecules from 0.1 to 9.7 μm in size. Similar molecular forms have been observed in several strains of dyskinetoplastic *T. equipardum* (Frasch *et al.*, 1980; Hajduk and Cosgrove, 1979; Riou and Pautrizel, 1977), and it was shown that there was no sequence homology of this DNA with kDNA from *T. brucei* or normal *T. equiperdum*. However, in another dyskinetoplastic strain of *T. equiperdum*, a complete absence of kDNA was reported (Riou *et al.*, 1980). Furthermore, there is some evidence that the DNA in dyskinetoplastic *T. equiperdum* that bands in CsCl at the low kDNA buoyant density is a nuclear satellite DNA present in all brucei-type trypanosomes that is normally obscured by the kDNA band. Borst *et al.* (1980b) found that there is a small fraction of *T. brucei* nuclear DNA that is resistent to cleavage by most enzymes, but is digested by *Alu*I or *Hin*dII into 180-bp repeats and which bands at the same density in CsCl as kDNA. They also showed that the low-density DNA in one strain of dyskinetoplastic *T. equiperdum* that banded at the density of kDNA was similarly digested by *Alu*I into 180-bp fragments and multimers. It was suggested (Borst *et al.*, 1980b) that the presence of this nuclear satellite DNA could explain the observations of apparently abnormal kDNA in dyskinetoplastic trypanosomes. However, the presence of DAPI-staining clumps in the mitochondria of certain dyskinetoplastic trypanosomes remains to be explained. In general, there

is a clear correlation of the presence of a single minicircle sequence class with defects or absence of functional maxicircle DNA, but the meaning of this correlation is not clear. Frasch *et al.* (1980) suggested that minicircle sequence heterogeneity is related to recombination between maxicircle DNA and minicircle DNA, but there is as yet no evidence for this type of interaction. It is clear that in normal strains extensive minicircle sequence changes occur under natural conditions to produce strain differences. Some changes can be seen in periods of several years under laboratory conditions, but cultures are stable enough for purposes of strain differentiation by comparison of minicircle restriction profiles. The actual mechanism of minicircle sequence change is uncertain, although there is some evidence for segmental rearrangements being important, at least in *Crithidia*. Studies are required on the relative stabilities of the constant region and the variable region, on the exact nature of sequence changes between strains and species, and on the possible role of maxicircle–minicircle interactions in minicircle sequence changes.

IV. Transcription of Minicircle DNA

There is one report of a 240-nt transcript of the minicircle DNA of *C. acanthocephali* (Fouts and Wolstenholme, 1979), but this has not yet been confirmed by Northern blot or S_1 nuclease analysis. Attempts to demonstrate a minicircle transcript in *T. brucei* and *C. luciliae* by hybridization of *in vitro* labeled total cell RNA to kDNA blots have been negative (Hoeijmakers and Borst, 1978; Hoeijmakers *et al.*, 1981). In all cases, only maxicircle fragments showed hybridization. In addition, hybridization of kinetoplast RNA blots with *in vitro* labeled minicircle clones has also proved negative both for *L. tarentolae* (L. Simpson, G. Kidane, and A. Simpson, unpublished results) and *T. brucei* (Stuart and Gelvin, 1980). In the Northern blots there often is a band with the mobility of minicircle DNA that hybridizes with the minicircle probes, but it was shown that this band represents contaminating minicircle DNA in the RNA preparations.

The evidence against minicircle DNA having a codogenic function includes the apparent lack of stable transcripts, the rapid rate of sequence changes in nature, and the presence of many termination codons in the *T. brucei* and *T. equiperdum* sequences reported to date. However, the small open reading frames that cover the conserved regions of the two *T. brucei* clones (Chen and Donelson, 1980) and the three *L. tarentolae* clones (Kidane *et al.*, 1984) leave open the possibility that minicircle DNA is transcribed but that transcription is limited to certain points in the

life cycle, or that the transcripts are unstable. Further work is needed to settle this important question.

There is, however, a recent report by Shlomai and Zadok (1984) that suggests that minicircles of *C. fasciculata* contain long open reading frames which are expressed in the cell. kDNA minicircle fragments obtained by *Fnu*DII and *Alu*I restriction enzyme digestion were inserted into the *Sma*I site of the pORF expression vectors. These vectors contain the 5′ end of the *E. coli ompF* gene, which provides a strong promotor, translation initiation site, and a signal sequence for export. In addition, they contain the out-of-frame *E. coli lacZ* gene lacking the 5′ end. Several kDNA minicircle fragments were able to realign the *ompF* and *lacZ* genes in frame and induce the expression of β-galactosidase activity in the *E. coli* cells. The size of the kDNA inserts was from 550 to 720 nt and all showed cross hybridization. Expression of a tribrid fusion protein in the bacterial cells was established by observation of a new protein species of 135,000 Da in SDS-polyacrylamide gels. The new fusion protein was extracted from gels, and antibodies were raised in rabbits. The sera were absorbed with bacterial extracts carring pORF vectors lacking the kDNA inserts but expressing β-galactosidase. The presence of a reactive protein within the *Crithidia* cells was determined by indirect immunofluorescence. The site of reactivity was the flagellar pocket. Western blots of *Crithidia* total cell proteins revealed three polypeptides which reacted with the antisera. Two of these polypeptides contained sugar residues, as seen by labeling with glucosamine. These results imply that certain *Crithidia* kDNA fragments are transcribed and translated and that the protein product may be associated with the kDNA itself, which is situated next to the flagellar pocket. However, this must be confirmed by a sequence analysis of the kDNA fragments cloned, and it must be established that these are derived from minicircles and not from maxicircles which are known to be transcribed and translated.

V. Replication of Minicircle Kinetoplast DNA

The hypothesis of Englund (1978) that closed minicircles are removed from the network at random by a specific topoisomerase, replicated as free circular molecules, and then recatenated as nicked circles at the periphery of the network, perhaps at the two sites described in *Crithidia* by autoradiography experiments (Simpson and Simpson, 1976; Simpson *et al.*, 1974), has been adequately reviewed (Englund, 1981; Englund *et al.*, 1982). The hypothesis predicts the existence of specific topoiso-

merases which can decatenate closed circles and recatenate nicked circles. Marini *et al.* (1980) and Kayser *et al.* (1982) showed that both T_4 phage topoisomerase and DNA-gyrase can decatenate protein network DNA from *C. fasciculata* and *T. cruzi* into monomeric minicircles and maxicircles. Riou *et al.* (1982) have partially purified two types of topoisomerase activities from a nuclear fraction of *T. cruzi*, the first of which relaxes supercoiled circles and the second of which induced catenation of circles in the presence of ATP.

Shlomai and Zadok (1983) reported a DNA topoisomerase activity in *C. fasciculata* which catalyzes a reversible interlocking of duplex circles into huge catenane forms resembling naturally occurring networks. There was a specific requirement for covalently closed circles as substrates for decatenation, but no preference for closed circles for catenane formation. These properties suggested to Shlomai and Zadok (1983) a potential role for this topoisomerase in the replication of kinetoplast DNA minicircles. However, the intracellular localization of this or any other topoisomerase from hemoflagellates has not yet been established.

Recent work from Englund's laboratory (Kitchin *et al.*, 1984, 1985) has shown more details of the minicircle replication pattern in the case of *C. fasciculata*. After θ-type replication of released minicircles, the two progeny molecules have different structures. One contains a nascent strand with a single nick or gap, while the other contains a nascent strand composed of small fragments which are separated by gaps. The former are nicked several times and the latter are partially repaired prior to recatenation to the network. The two progeny are attached to the network at different rates. Once attached, convalent closure ensues. In a related study done with *T. equiperdum* minicircles, Ntambi and Englund (1985) found that newly replicated reattached minicircles possess a gap of about 10 nt which overlaps the "universal" 14-mer sequence and that appears to be bounded by a ribonucleotide, implying that this site represents an origin of replication of the minicircle.

VI. Maxicircle DNA: Isolation, Cloning, and Restriction Mapping

The maxicircle component of the kinetoplast DNA appears to represent the homolog of the informational mitochondrial DNA found in other eukaryotic cells. The maxicircle DNA is present as 20–50 apparently identical circular molecules catenated to the network. The molecular size varies from 20 kb in *T. brucei* to almost 39 kb in *T. cruzi*. This DNA has

been the object of investigation in several laboratories and the results will be discussed on a species basis.

A. *Trypanosoma brucei*

The kDNA network in *T. brucei* is comparatively small (4.5 × 3 μm) when spread by the Kleinschmidt method for electron microscopy, and maxicircle molecules can often be visualized as long edge loops extending out from the network. Fairlamb *et al.* (1978) showed that digestion of the network with *Pst*I or nuclease S_1 would selectively remove the edge loop molecules and not affect the network structure, implying that minicircle catenation represents the major structural stabilizing force of the network.

The size of the maxicircle was calculated both by summation of fragments released from network DNA by digestion with several enzymes and by measurement in the electron microscopy of the *Pst*I linearized maxicircle to be 20–22 kb. Stuart (1979) showed that *Bam*HI digestion would release linearized maxicircle molecules and a small percentage of linearized minicircles from the network kDNA of *T. brucei* 164 and he used this method to enrich for maxicircle DNA by isopycnic banding in CsCl–EthBr. Stuart (1979), Simpson and Simpson (1980), and Borst and Fase-Fowler (1979) showed that linearized maxicircle DNA from several *T. brucei* strains banded in CsCl at a lower density than the kDNA network or the minicircle DNA (1.682 versus 1.690 g/ml), implying that the maxicircle DNA is lower in %GC than the minicircle DNA. This difference in %GC has been used to preparatively isolate linearized maxicircle DNA by addition of the AT-intercalating dye, Hoechst 33258, to the CsCl density gradient (Simpson and Simpson, 1980). The release of a low-density band in CsCl gradients has also been used to calculate the percentage of maxicircle DNA in total kDNA from *T. brucei*. Values reported range from 10 (Borst and Fase-Fowler, 1979) to 20 (Stuart, 1979) to 39% (Simpson and Simpson, 1980). Despite the range in these values, the relative percentage of maxicircle DNA is clearly greater in *T. brucei* kDNA than in kDNA from other species, in agreement with the greater ease of edge loop visualization in network preparation.

Unique circular restriction maps have been constructed for maxicircle DNA from several *T. brucei* strains: strain 427-60 (Borst and Fase-Fowler, 1979), strain 164 (Stuart, 1979), and strain 366D (Simpson and Simpson, 1980) (Fig. 17). The most striking aspect of these maps was the relative absence of restriction sites from a 5- to 6-kb region of the 20- to 22-kb molecule, termed the "silent region" or "variable region" by Borst

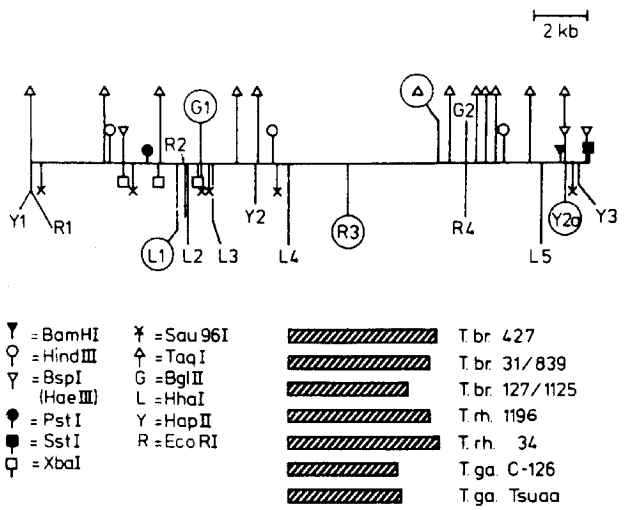

FIG. 17. The linearized physical map of the maxicircle from *T. brucei* kDNA. The length is 20.5 kb. Polymorphic sites are circled; circled open triangle is site Q6. The hatched bars represent the size of the variable region. The *Taq*I map is incomplete and there are several additional fragments in the span R4–R1. T. br., *T. brucei brucei;* T. rh., *T. brucei rhodesiense;* T. ga., *T. brucei gambiense*. Reprinted from Borst *et al.* (1980a) with permission.

and Fase-Fowler (1979) or the "divergent region" by Muhich *et al.* (1983). Direct gel comparisons of kDNA digests from nine stocks have revealed that there are two basic types of sequence changes (Borst *et al.*, 1980a, 1981a):

1. Restriction site polymorphisms, apparently randomly localized.
2. Deletions or insertions of up to 1.5 kb within the divergent region.

Site polymorphisms were also observed between strains 366D and 367H (Fig. 3) versus strain 164 (Simpson, 1979; Stuart, 1979). Four human infective stocks of *T. brucei* (*rodesiense* and *gambiense*) were also examined by Borst *et al.* (1981a). They found that the human infective stocks could not be distinguished from the *T. brucei brucei* stocks on the basis of maxicircle DNA site changes or deletions and they concluded that the human infective forms are host-range variants of the same species by this criterion. In terms of the rates of nucleotide substitution in maxicircle DNA from all *T. brucei* stocks examined, the variation (0.01–0.03 substitutions per base pair) was similar to that observed with mitochondrial DNA within a single species of mice or gophers. However, the nature of the deletions and insertions within the divergent region is not yet understood and this may affect these calculations.

Reassociation complexity analysis of maxicircle DNA from *T. brucei* has confirmed the sequence homogeneity of these molecules as compared to the minicircle DNA and the kinetic complexity compares well to the calculated size of the molecule (Stuart and Gelvin, 1980).

Much of the maxicircle DNA of *T. brucei* has been cloned in bacterial plasmids and phages. Brunel *et al.* (1980a) cloned the two smaller *Eco*RI fragments (*Eco*2 and *Eco*3) from strain 427-60 in λgt WES/λB, but were unable to clone the largest *Eco*RI (*Eco*1) fragment, although this is the optimal size for this cloning vector. Simpson and Simpson (1980) cloned the two smaller *Hin*dIII fragments from strain 366D in pBR322. These fragments cover essentially the same region of the maxicircle as the cloned *Eco*RI fragments of Brunel *et al.* (1980a). Stuart and Gelvin (1982) cloned 80% of the maxicircle from strain 164 as small fragments in pBR322 and pBR325; the only fragment that Stuart and Gelvin (1982) were unable to clone was the R4-R1 (Fig. 17) fragment from the divergent region.

Riou and Barrois (1981) examined the kDNA from a strain of *T. gambiense* and showed that maxicircle DNA of density 1.684 g/ml was liberated from network DNA by digestion with *Pst*I and that this represented 17% of the network DNA. A restriction map was constructed for seven

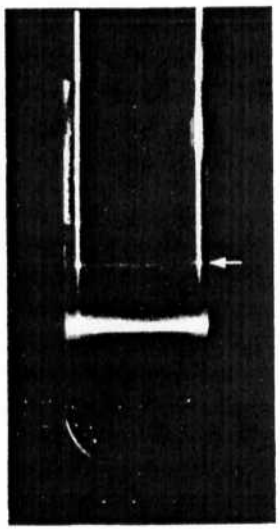

FIG. 18. Hoechst 33258 dye/CsCl equilibrium gradient of *Eco*RI-digested *L. tarentolae* network kDNA. Ti60 rotor, 48 hour, 40,000 rpm, 20°C; long-wave-length ultraviolet illumination. Arrow indicates low-density band. Reprinted from Simpson (1979) with permission.

enzymes and reassociation kinetic analysis gave a complexity that agreed well with the size of the molecule.

B. *Trypanosoma evansi*

Isolated kDNA networks from this species, which is normally I^- in the terminology of Opperdoes *et al.* (1976) and does not undergo development in an insect vector, appear to completely lack maxicircle DNA edge loops in the electron microscope (Fairlamb *et al.*, 1978).

C. *Trypanosoma equiperdum*

This is another I^- species which does not undergo development in an insect vector and which has unusual kDNA molecular species. As discussed previously, the minicircle component of the kDNA is homogeneous in terms of sequence. The maxicircle component of the kDNA has been studied from two strains of *T. equiperdum*. The Pasteur strain was shown by Riou and Saucier (1979) to contain maxicircle DNA that is 23 kb in size and to be homogeneous by reassociation kinetic analysis, with a density in CsCl of 1.685 g/ml. A restriction enzyme map of this molecule was similar but not identical to those constructed for *T. brucei* maxicircle DNA. A comparison by C_0t analysis of the extent of sequence homology between the maxicircle DNA from a *T. gambiense* strain and this *T. equiperdum* strain (Riou and Barrois, 1981) indicated approximately 10% base sequence homology. On the other hand, maxicircle DNA isolated from the ATCC strain 30019 of *T. equiperdum* (Frasch *et al.*, 1980) was found to have a size of only 12.6 kb and to have significant sequence homology only with the large *T. brucei* *Eco*RI fragment (*Eco*1) and a portion of the smallest *Eco*RI fragment (*Eco*3). The cross-species homology was determined by hybridization of Southern blots of *T. equiperdum* maxicircle DNA with the two cloned *T. brucei* *Eco*RI maxicircle fragments, *Eco*2 and *Eco*3, and with total poly(A)$^+$ RNA from *T. brucei*. The conservation of the *Eco*1 *T. brucei* fragment was deduced only from the conservation of the *Eco*RI, *Hpa*II, and *Hin*dIII sites and the overall size; however, new restriction sites are present in the *T. equiperdum* sequence. The differences between the findings of Riou and Saucier (1979) and Frasch *et al.* (1980) possibly indicate an inherent pleomorphism of *T. equiperdum* with regard to the maxicircle DNA. However, it is clear that in general there is a strong correlation between the I^- state and defects in the maxicircle DNA.

There is one report by Hadjuk and Vickerman (1981) of the absence of any detectable alteration in the maxicircle DNA of a *T. brucei* strain that

was rendered I^- by repeated passage in mice. They were not able to detect any gross changes in restriction sites or fragment sizes, as compared to the I^+ parental strain. This observation does not invalidate the hypothesis since the supposed maxicircle damage may be in the form of point mutations or the damage may be to a nuclear gene.

D. *Trypanosoma mega*

Maxicircle DNA has been identified in *T. mega* (Borst *et al.*, 1977) by the release of restriction fragments from digested kDNA and by the rare appearance of free 24-kb circles in kDNA preparations visualized in the electron microscope. Linearized 26-kb maxicircle molecules could also be released from the network DNA by nuclease S_1 digestion. Large circles of intermediate length between minicircular and maxicircular size were also seen rarely, the identity of which is unknown.

E. *Trypanosoma cruzi*

Maxicircle DNA in *T. cruzi* networks (Leon *et al.*, 1980) was shown to have a size of approximately 39 kb by summation of fragments released by digestion with several enzymes. Direct visualization of intact circular maxicircle DNA in the electron microscope was reported by Kayser *et al.* (1982), who used topoisomerase II from *Micrococcus luteus* to decatenate *T. cruzi* kDNA networks into monomeric minicircles and maxicircles. The size of the released maxicircle molecules was 38 kb, which agreed well with the size determined from gel analysis. *T. cruzi* kDNA networks appear to be relatively fragile to handling in the laboratory, unlike those from *T. brucei* or *Crithidia,* but similar to those from *Phytomonas* and *Leishmania*.

F. *Leishmania tarentolae*

The first indication of the existence of maxicircle DNA in the kDNA of *L. tarentolae* was from the reassociation analysis of Wesley and Simpson (1973b), who showed by C_0t fractionation that total kDNA contained a component that had a complexity more than 10 times that of the minicircle DNA and represented approximately 5% of the total kDNA. Gel analysis of kDNA digests showed the maxicircle size to be approximately 30 kb and to represent 5.4% of the total kDNA network. Maxicircle DNA has been isolated from *L. tarentolae* network DNA by digestion with a single cleaving restriction enzyme, such as *Eco*RI or *Bam*HI, and separated from network DNA and released minicircles by centrifugation in CsCl in

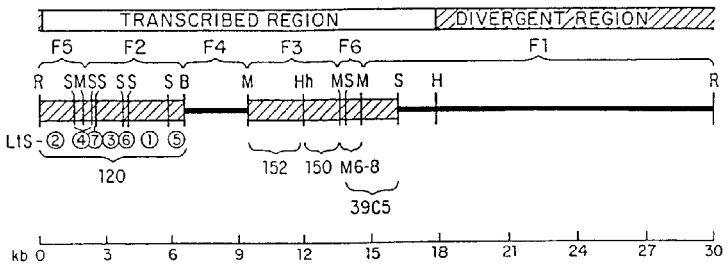

FIG. 19. Partial restriction map of the maxicircle DNA of *L. tarentolae*. The cloned and sequenced fragments within the transcribed region are indicated by cross-hatched boxes and the plasmid numbers 120, 152, 150, M6-8, and 39C5. Restriction site symbols: B, *Bam*HI; H, *Hae*III; Hh, *Hha*I; M, *Msp*I; R, *Eco*RI; S, *Sau*3A. The *Msp*I/*Bam*HI subfragments of the *Eco*RI-linearized maxicircle are designated as F1–F7. Reprinted from de la Cruz *et al.* (1984) with permission.

the presence of Hoechst 33258 (Fig. 18). This method has been proposed as a general method for maxicircle DNA isolation due to the universal density difference found between maxicircle and minicircle DNA (Simpson, 1979). The density difference in *L. tarentolae* is larger than that in *T. brucei* since the minicircle DNA in *L. tarentolae* has a higher %GC than the minicircle DNA in *T. brucei*.

A unique circular restriction enzyme map was constructed for maxicircle DNA liberated from the network by either *Eco*RI or *Bam*HI digestion (Masuda *et al.*, 1979) (Fig. 19). Intramolecular heterogeneity of AT composition was demonstrated first by CsCl buoyant analysis of several *Hpa*II maxicircle fragments (Simpson, 1979) and more precisely by partial denaturation mapping using the electron microscope (Simpson *et al.*, 1982a). The most striking features of the partial denaturation map are the large AT-rich regions located 10–20 kb from the single *Eco*RI site and the six to seven short AT-rich regions located within the divergent region (Fig. 20).

The *in situ* closed circular nature of the maxicircle DNA of *L. tarentolae* was demonstrated by the ability of ethidium bromide to restrict the digestion by *Hpa*II of the intact molecule to only one of the several *Hpa*II sites (Simpson, 1979).

Several maxicircle fragments have been cloned in bacterial plasmids, but a large region has proved so far relatively refractory to cloning. A 6.6-kb *Eco*RI/*Bam*HI fragment (pLt120) was cloned in pBR322, and two adjacent 2.6-kb and 1.9-kb *Hha*I subfragments of the *Msp*I/*Bam*HI fragment 3 were cloned in pBR322 using *Eco*RI linkers (pLt150 and pLt152) (de la Cruz *et al.*, 1984). In addition, the 1.8-kb *Hin*dIII fragment 4 was also cloned in pBR322 (pLt30) (Masuda *et al.*, 1979) and the 0.9-kb *Hin*dIII

fragment 5 was cloned in M13mp8 (pDAH54) (D. Hughes and L. Simpson, unpublished results). The pLt120, pLt150 pLt152, pLt30, and PDAH54 inserts were subcloned in M13 and sequenced (de la Cruz *et al.*, 1984; Muhich *et al.*, 1985). The *Msp/Bam* fragments F4, F6, and a portion of F1 have also been cloned and sequenced (L. Simpson, A. M. Simpson, V. de la Cruz, and N. Neckelmann, unpublished results).

G. *Crithidia* Species

Maxicircle DNA was first visualized as rare circular molecules 33 kb in size in *C. luciliae* kDNA preparations spread for electron microscopy (Steinert and Van Assell, 1975). Unequivocal demonstration of maxicircle

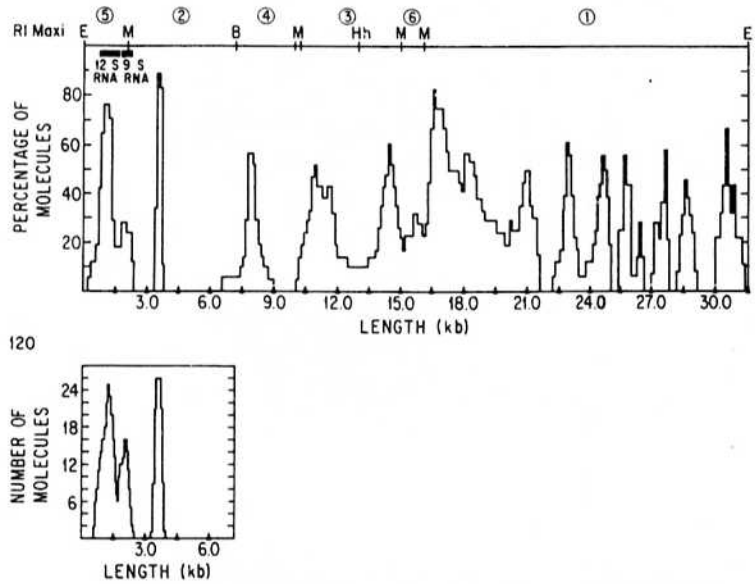

FIG. 20. Partial denaturation maps of the *L. tarentolae* RI Maxi molecule and the pLt120 insert. The histogram of the RI Maxi is aligned with that of the 120. Both maps were determined by electron microscopy. The 120 map is presented in terms of the numbers of full-length 120 insert molecules showing denaturation at each length increment. However, in the case of the RI Maxi, very few instances of full-length partially denatured molecules were found. Usually molecules were fragmented, frequently in high AT regions. Twenty-eight molecules ranging in size from 17 to 34 kb were measured, length-normalized by the internal standards, and aligned so as to maximize correlation of the pattern of denaturation loops. The percentage of molecules denatured at increments along the length is plotted, since the absolute number of molecules at each length varied due to this fragmentation. The localizations of the *Msp*I and *Bam*HI sites are indicated as are the localizations of the 9 and 12 S RNA genes. Reprinted from Simpson *et al.* (1980) with permission.

DNA as a minor component of kDNA networks was first accomplished by Kleisen *et al.* (1976b) using gel restriction analysis of kDNA from *C. luciliae*. Liberation of once-cleaved maxicircle DNA by nuclease S_1 digestion confirmed the closed circular nature of the molecule *in situ*. The size of 32 kb obtained by restriction analysis agreed well with that obtained by electron microscopy.

From the proportion of minor high-molecular-weight bands in a digest of total kDNA, Kleisen *et al.* (1976b) calculated that 3–5% of the kDNA was maxicircle DNA. An accurate measurement of the relative number of maxicircles and minicircles in a network from *C. fasciculata* was performed by Marini *et al.* (1980) who counted the numbers of such molecules after complete decatenation by T_4 topoisomerase. They found 25 maxicircles and 5785 minicircles in a field of decatenated form I network DNA. Since it can be calculated from the value for DNA per network and the size of the minicircle that there are approximately 5000 *Crithidia* minicircles per network, this follows that there are about 22 maxicircles per network (about 6% of the kDNA). Marini *et al.* (1980) did not observe any intermediate-size circles in their preparation. Weislogel *et al.* (1977) showed that selective removal of maxicircle DNA from the network of *C. luciliae* by digestion with *Pst*I, which cuts the maxicircle DNA once and cleaves only 6% of the minicircle DNA, had little effect on the structural appearance of the network in the electron microscope, showing, as in the case of *T. brucei*, that the network is held together mainly by minicircle catenation.

A restriction map of the maxicircle DNA from *C. luciliae* (Hoeijmakers *et al.*, 1982a) (Fig. 21) has been reported. An interesting observation in this work was that a 6-kb restriction fragment had different electrophoretic mobility in the presence and absence of ethidium bromide (Fig. 21).

A comparison of restriction sites in the maxicircle DNA from *C. luciliae* and *C. fasciculata* showed a sequence homology of more than 96% (Hoeijmakers *et al.*, 1982a), which is similar to the diversity found between maxicircle DNA from different strains of *T. brucei* (Borst *et al.*, 1981a). From this evidence Hoeijmakers *et al.* (1982a) proposed that *C. luciliae* and *C. fasciculata* represent different varieties of the same species rather than different species. This is an interesting approach to kinetoplastid taxonomy, but the conclusion ignores the extensive biological, morphological, and enzymatic evidence that these are separate species.

Maslov *et al.* (1982) have constructed a restriction map of the 24.5-kb maxicircle DNA of *Crithidia oncopelti* and have cloned a 12-kb *Bam*HI fragment in a bacterial plasmid (pCo52). Maslov *et al.* (1984) have constructed detailed restriction maps of maxicircle DNA from *C. luciliae*,

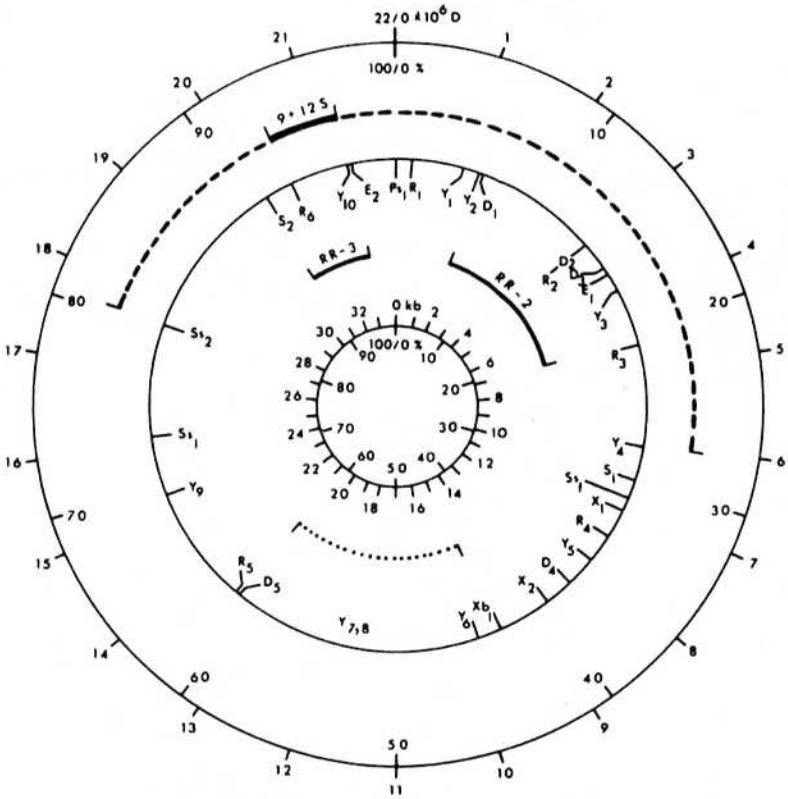

FIG. 21. The physical map of the maxicircle of *C. luciliae*. The exact order of the *Hap*II fragments Y_6Y_7, Y_7Y_8, and Y_8Y_9 is not determined. Regions that show hybridization with total cellular RNA in Southern blots are indicated on the outside of the circular map. The solid line represents strong hybridization due to the 9 and 12 S RNAs; the dashed line indicates regions showing weak hybridization. The regions hybridizing with the cloned *T. brucei* maxicircle fragments RR-2 and RR-3 (λTbR-2 and TbR-3) are indicated on the inside. The maxicircle segment that is associated with the difference in fragment electrophoretic mobility in the presence or absence of ethidium bromide is indicated by a dotted line on the inside. The map of *C. fasciculata* maxicircle is identical to that of *C. luciliae* except for an additional *Sst*I site (Sst_f) and some of the *Hap*II sites. A complete *Hap*II map for the *C. fasciculata* maxicircle has not been constructed, but most if not all of the differences are located between map units 30 and 70. D, *Hin*dIII; E, *Hae*III; Ps, *Pst*I; R, *Eco*RI; S, *Sal*I; Ss, *Sst*I; X, *Xho*I; Xb, *Xba*I; Y, *Hap*II (*Msp*I). Reprinted from Hoeijmakers *et al.* (1982a) with permission.

Leptomanas pessoai, and *Leishmania gymnodactyli* and have compared these molecules by cross hybridization (see Section X,C).

H. *Herpetomonas samuelpessoa*

S. Gomes, H. Van Heuversvyn, R. Mueller, L. Simpson, A. Simpson, and C. Morel (unpublished results) have cloned approximately 50% of the *Pst*I-linearized maxicircle of *H. samuelpessoa* as *Eco*RI fragments in pBR322. One fragment was shown to contain the 9 and 12 S RNA genes and these were subcloned into M13. Gomes and Morel (1982) reported that the cloned *Eco*RI maxicircle fragments of *H. samuelpessoa* exhibit similar anomalous mobility properties in gel electrophoresis as minicircle DNA. All of these fragments gave larger apparent sizes in acrylamide than in agarose, ranging from 25 to 400% increases.

VII. Replication of Maxicircle DNA

A. Replication by Rolling-Circle Model

Riou and Barrois (1981) reported linear DNA molecules connected to maxicircle edge loops in 6% of the kDNA networks isolated from a strain of *T. gambiense*. They also observed three double-branched edge loop molecules. These structures could represent either broken Cairn's forms or rolling-circle intermediates in the replication of the maxicircle. Similar results were reported by Hajduk *et al.* (1984) with *C. fasciculata* kDNA. They observed rolling-circle-type edge loops in replicating networks and also showed that a population of free maxicircle linears are preferentially labeled by [^{3}H]thymidine in a 20-minute pulse. They suggested that maxicircles replicate by a rolling-circle intermediate, which produces free linear molecules that then recircularize and become catenated with network molecules. The putative origin of replication of the leading strand was localized by the pulse-labeling results to a site within the divergent or nontranscribed region.

B. Autonomous Replication Sequences in Maxicircle DNA

Fragments exhibiting autonomous replicating sequence (ars) activity in yeast have been isolated from the maxicircle DNA of *T. brucei* (Davison and Thi, 1982), *C. fasciculata* (Koduri and Ray, 1984), and *L. tarentolae* (Hughes *et al.*, 1984). The *C. fasciculata* fragment has been subcloned to a 189-nt fragment (Cf 189ars) that still retains strong ars activity (Kim and

Ray, 1984). The Cf 189ars fragment was found to be strikingly similar in nucleotide sequence (78% similarity) to a portion of one of the four fragments from the *L. tarentolae* maxicircle that showed strong ars activity (Hughes *et al.*, 1984). The relative localization of this sequence in the *L. tarentolae* maxicircle with respect to the 9 and 12 S ribosomal RNA genes was similar to that in the *C. fasciculata* maxicircle, implying an overall conservation of gene order in these species. Hughes *et al.* (1984) suggested that this sequence represented the origin of replication of the lagging strand of the maxicircle.

VIII. Transcription of Maxicircle DNA

A. *Trypanosoma brucei*

Transcription of the maxicircle DNA of *T. brucei* has been studied by two methods: hybridization of *in vitro* labeled total cell RNA or labeled cDNA to DNA blots and hybridization of labeled cloned maxicircle DNA fragments to RNA blots. In addition, three cDNA clones were shown to hybridize to DNA blots and in two cases the transcripts were identified. Hoeijmakers *et al.* (1981) showed that *in vitro* labeled total cellular RNA (strain 427) hybridized mainly to a 2.0-kb maxicircle fragment from sites D3 to E2. Stuart and Gelvin (1982) showed that labeled cDNA synthesized from total cell RNA templates hybridized mainly to the H1-H2 segment, but in addition to all segments of the maxicircle (strain 164) except the 1.2-kb H1-R1 segment. A cDNA probe made from RNA from bloodstream *T. brucei* gave identical results except for a weaker signal.

Northern blot experiments were performed by both Hoeijmakers *et al.* (1981) and Stuart and Gelvin (1982). Hoeijmakers *et al.* (1981) hybridized the two cloned *Eco*RI fragments, *Eco*2 and *Eco*3, which cover approximately 50% of the molecule, to RNA blots and were able to visualize approximately eight RNA species. The *Eco*3 clone hybridized to two major and four minor RNA species, whereas the *Eco*2 clone hybridized to two minor RNAs. The two major RNAs in the *Eco*3 fragment correspond to the 9 and 12 S RNAs first isolated from a purified kinetoplast fraction of *L. tarentolae* (Simpson and Simpson, 1978) and represent the mitochondrial ribosomal RNAs. The minor transcripts were enriched by poly(A) selection and the transcript sizes varied from 1.1 to 0.36 kb. Stuart and Gelvin (1982) confirmed and extended these results, and, by using as probes six smaller cloned maxicircle fragments representing approximately 80% of the molecule and one gel-isolated maxicircle fragment representing the remaining 20%, were able to visualize approximately 18

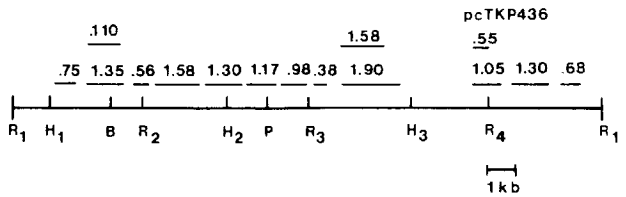

FIG. 22. Diagram summarizing *T. brucei* 164 maxicircle transcript sizes and the approximate location of their coding sequences on the maxicircle. pcTKP 436 is a cDNA clone containing a 550-bp insert. It hybridized to a 1.05-kb transcript and the H3-R4 maxicircle segment but not to the R4-R1 segment. Reprinted from Stuart and Gelvin (1980) with permission.

total transcripts, some of which are probably identical (Fig. 22). The transcript sizes ranged from 1.9 to 0.45 kb, implying that the RNA preparation examined by Hoeijmakers *et al.* (1981) was somewhat degraded. In no case was any evidence obtained for transfer RNAs, although it is possible that hybridization conditions employed were too stringent.

Three total cell RNA cDNA clones were found by Hoeijmakers *et al.* (1981) to hybridize to maxicircle fragments. Clones Tck-1 and Tck-2 proved to be clones of the same transcript, the 570-bp R-2b transcript from the *Eco*2 region. Another clone, Tck-3, hybridized to a maxicircle region 5′ to the 12 S gene, but no transcript could be seen on Northern blots. Stuart and Gelvin (1982) identified one cDNA clone, pcTKP 436, that represented part of the 1.05-kb transcript which overlaps the R4 site. None of the remaining seven transcripts of Hoeijmakers *et al.* (1981) were localized within the *Eco*2 and *Eco*3 segments, but Stuart and Gelvin (1982) were able to localize the transcripts more precisely since they used smaller DNA probes and several of the transcripts appeared to overlap two adjacent DNA probes (Fig. 22).

No major differences between maxicircle transcripts from bloodstream forms and procyclic forms were observed by Hoeijmakers *et al.* (1981), but conclusions to this effect are precluded by the low resolution of the gels and the apparent degradation of the RNA preparations used. It is clear, however, that the poly(A)$^+$ maxicircle transcripts are significantly lower in abundance in bloodstream forms than in procyclic forms. Stuart and Gelvin (1982) were able to detect a 0.98-kb transcript that seemed to be procyclic-specific. Simpson and Simpson (1980) showed that 9 and 12 S RNAs could be isolated from kinetoplast fractions of both stages of the life cycle of *T. brucei* strain 366D, and they mapped the location of these genes to the identical segment shown by Hoeijmakers *et al.* (1981) and Stuart and Gelvin (1982) to give the major hybridization with total cell RNA.

The relative orientation of the 9 and 12 S RNA genes in *T. brucei* maxicircle DNA was determined by Hoeijmakers *et al.* (1981). This orientation implied a transcription from the 12 S gene to the 9 S gene, a situation unlike all other adjacent ribosomal transcription units known, in which the direction of transcription is from small rRNA to large rRNA.

Expression of the cloned 6-kb *Eco*2 maxicircle fragment of *T. brucei* in *E. coli* minicells was demonstrated by Brunel *et al.* (1980b). Two polypeptides, 10.3 and 13.5 Da, were synthesized under control of a promotor localized within the maxicircle fragment. However, it is possible that these products are prematurely terminated due to the different pattern of codon usage in the mitochondrial genetic system versus that in *E. coli* (Benne *et al.*, 1983; de la Cruz *et al.*, 1984).

B. *Leishmania tarentolae*

Transcription of maxicircle DNA was first demonstrated by hybridization of 9 and 12 S RNAs isolated from a kinetoplast fraction to a discrete region of the maxicircle DNA of *L. tarentolae* (Simpson and Simpson, 1980). These RNAs represented the major steady-state transcripts found in purified kinetoplast fractions and were also the major species labeled *in vivo* in a 1-hour pulse of [^{3}H]uridine. The 9 and 12 S RNAs lacked poly(A) tails, and *in vivo* transcription of these species was inhibited by acriflavin and rifampin. RNA species with identical mobilities in denaturing gels were observed in kinetoplast fractions of *P. davidi* (Cheng and Simpson, 1978) and *T. brucei* (Simpson and Simpson, 1980). Despite the small size of these transcripts, it is fairly certain that these represent the mitochondrial ribosomal RNAs. The evidence for this statement is that they represent the major maxicircle transcripts, that these genes represent the most conserved region of the maxicircle molecule in all species so far examined, and that secondary structures can be constructed which conform well to the *E. coli* rRNA models (de la Cruz *et al.*, 1985a).

At least 10 polyadenylated *L. tarentolae* maxicircle transcripts have been identifed by Northern blots (Simpson *et al.*, 1982a) (Figs. 23 and 24). Transcript sizes ranged from 1.8 to 0.25 kb. The DNA probes were M13 *Sau*3A subclones of the pLt120 region, and gel isolated *Msp*I/*Bam*HI restriction fragments of the purified RI Maxi DNA. Most of the maxicircle was transcriptionally active, although there was an absence of transcripts directly upstream of the 12 S gene and fewer transcripts from the 14.5-kb *Msp*/*Bam* fragment 1. Muhich *et al.* (1983) have localized the transcription within the *Msp*I/*Bam*HI fragment 1 to the 2.4-kb *Msp*I/*Hae*III subfragment adjacent to fragment 6, with the remaining 12.1 kb of fragment 1 being inactive. Transcription of the pLt120 region and most of the *Msp*/

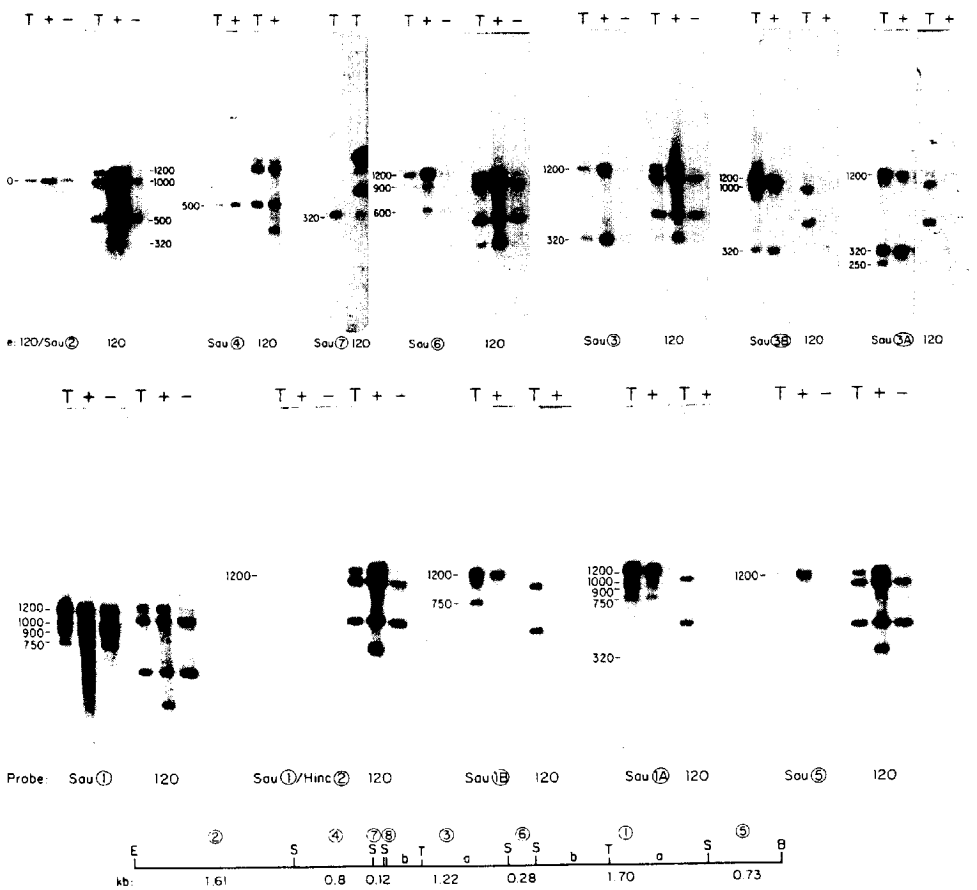

FIG. 23. Detailed Northern blot analysis of the 120 region of the maxicircle of *L. tarentolae*. Total kRNA (T), poly(A)$^+$-kRNA (+), and, in some cases, poly(A)$^-$-kRNA (−) were run in 1.5% agarose formaldehyde gels and blotted onto S∂S BA83 filters which were probed with the indicated DNA fragments. All the probes but Sau (7) were gel-isolated fragments of the pLt120 cloned insert DNA. The Sau (7) probe was the RF from an M13mp7 clone of 120/*Sau*3A fragment 7. Identical results were obtained using as probes RFs of all *Sau*3A M13 clones of the 120 region. The filters were boiled to remove the probes and rehybridized with 120 insert probe and reexposed. Note that several RNA preparations were used for different blots and the extent of poly(A)$^+$ selection varied as can be seen by the 120 control hybridizations. The blots are presented in the same sequence as the fragments are localized in the molecule. Reprinted from Simpson *et al.* (1982a) with permission.

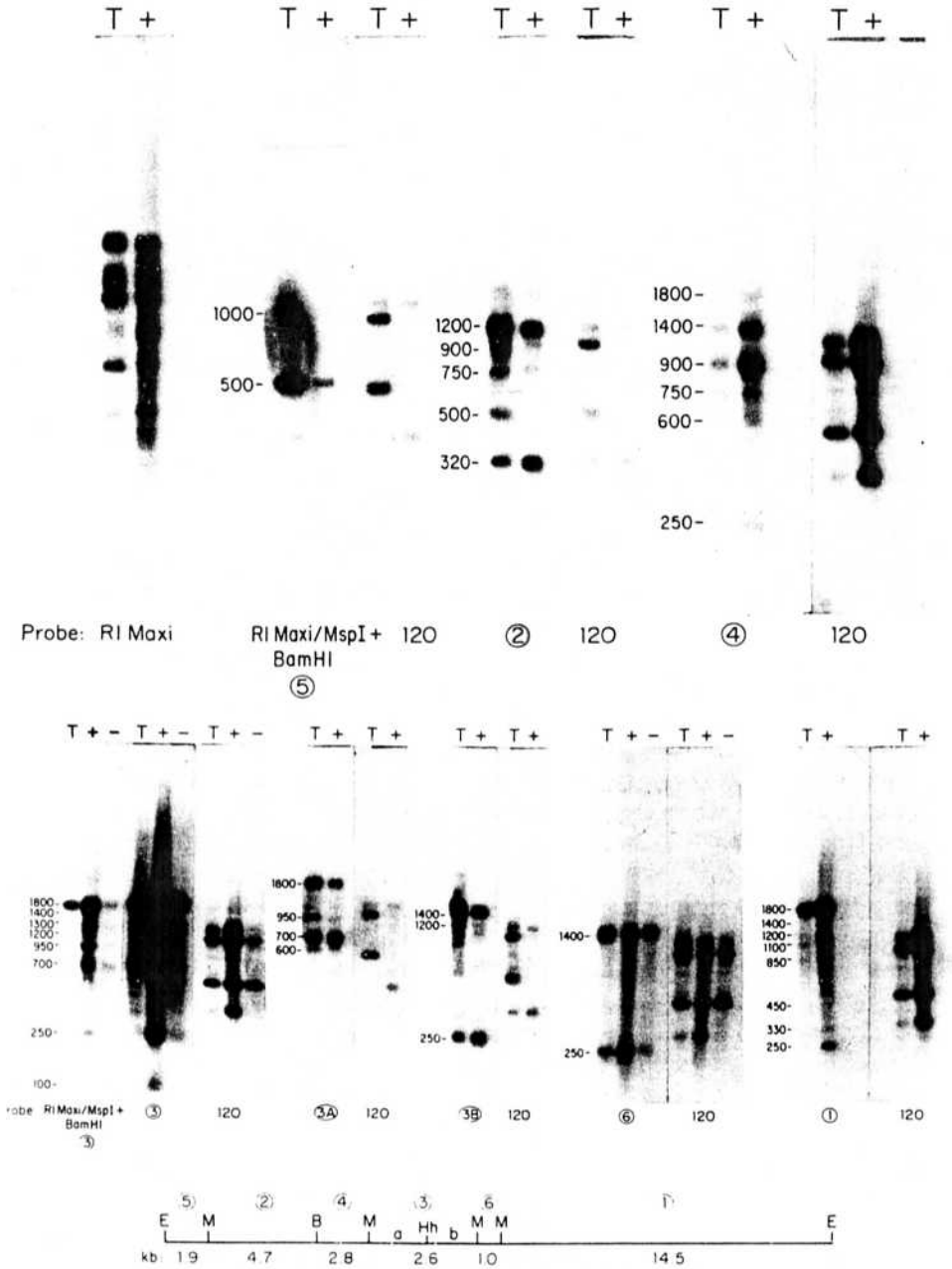

FIG. 24. Detailed Northern blot analysis of the RI Maxi molecule of *L. tarentolae*. Total kRNA (T), poly(A)$^+$-kRNA (+), and, in two cases, poly(A)$^-$-kRNA (−) were run in 1.5% agarose–formaldehyde gels and blotted onto S∂S BA83 filters which were probed with the indicated *Msp*I/*Bam*HI gel-isolated fragments of the RI Maxi. The blots are presented in the same sequence as the fragments are localized in the molecule. The blot of probe F3 was reexposed so as to visualize the low-molecular-weight RNA species. Rehybridizations of the blots were performed with the 120 probe as in Fig. 23. Reprinted from Simpson *et al.* (1982a) with permission.

Bam fragment 3 region was shown to be from one strand and in the direction 12 to 9 S, as in the case of *T. brucei*. However, transcription of the 1.8-kb RNA (the mRNA for the *COI* gene) in the *Msp/Bam* fragment 3 was from the opposite strand (de la Cruz *et al.*, 1984).

Identification of specific stable RNAs as the transcripts of identified maxicircle structural genes was accomplished by Northern blot hybridizations using synthetic oligonucleotides, and the 5′ ends of the RNAs were determined by primer runoff experiments (Simpson *et al.*, 1985). The 5′ ends of the RNAs are located 20–64 nt from the putative translation initiation codons. The extent of RNA processing, if any, is not known.

IX. Kinetoplast Ribosomes

A. Isolation of Ribosomes

Isolation of mitochondrial ribosomes from kinetoplastid protozoa has proved to be a difficult and as yet unsolved problem, in spite of earlier reports to the contrary. Hanas *et al.* (1975) reported that mitochondrial ribosomes in *T. brucei* sedimented at 72 S, as compared to the cytoplasmic 84 S ribosomes, and that mitochondrial protein synthesis was blocked selectively by chloramphenicol. Laub-Kuperszteijn and Thirion (1974) reported the mitoribosome of *C. luciliae* sedimented at 60 S, and that 50% of total cellular protein synthesis was chloramphenicol-sensitive. Kleisen and Borst (1975a) were unable to confirm this report, and Spithill *et al.* (1981) showed that chloramphenicol had nonspecific effects on cellular respiration and produced secondary inhibition of both cytoplasmic and putative mitochondrial protein synthesis. The existence of 68 S kinetoplast ribosomes and 23 and 16 S kinetoplast ribosomal RNAs from *C. oncopelti* and *C. fasciculata* was reported in a series of papers from the laboratory of G. N. Zaitseva (Zaitseva *et al.*, 1979a,b). However, there is no confirmatory evidence that these RNAs are transcribed from maxicircle DNA, and, on the other hand, there is evidence for the ribosomal nature of the 9 and 12 S RNAs. Several laboratories have attempted to isolate mitoribosomes from *Crithidia*, *T. brucei*, and *L. tarentolae* without success. Further work must be performed to resolve this question.

B. DNA Sequences of 9 and 12 S RNA Genes

The DNA sequences of the 9 and 12 S RNA genes from *T. brucei* and *L. tarentolae* have been reported (Eperon *et al.*, 1983; de la Cruz *et al.*, 1985a,b). Little sequence homology to known ribosomal genes was ob-

served, although tentative secondary structures could be formed for both species, which agreed well with the *E. coli* 16 and 23 S rRNA models (see below). Comparison of the *T. brucei* and the *L. tarentolae* sequences shows a nucleotide similarity of 84% for the 9 S RNA and 81% for the 12 S RNA. With regard to the unmatched bases, the ratio of transversions to transitions for the 9 S RNAs is 1.66 and for the 12 S RNAs is 2.81; assuming no large base ratio effect, this possibly implies that these two species are separated in evolution by a greater distance than previously presumed, since Cann *et al.* (1984) showed that in the case of primate mitochondrial genes, transitions predominate in recently diverged species, whereas transversions predominate in distantly diverged species.

C. Secondary Structures of 9 and 12 S RNAs

Eperon *et al.* (1983) proposed a tentative partial secondary structure for domain VI of the 12 S RNA from *T. brucei*, in terms of the *E. coli* model of Noller *et al.* (1981). De la Cruz *et al.* (1985a,b) have established the precise 5′ and 3′ ends of the 9 and 12 S RNAs of both *L. tarentolae* and *T. brucei* and proposed a complete secondary structure for the 9 S (Fig. 25) and a partial structure for the 12 S RNAs which agreed well with the *E. coli* models. The striking aspect of these structures is that many domains are severely reduced or even completely missing, but the overall secondary structure is well conserved in spite of little actual absolute sequence similarity. It is presumed that an analysis of the specific conserved regions in these minimal ribosomal RNAs may lead to a better understanding of the detailed function of ribosomal RNA in general.

X. Genomic Organization of Maxicircle DNA

A. Maxicircle Structural Genes

The first evidence for the existence of nonribosomal structural genes in the maxicircle was obtained by low stringency heterologous hybridization of *L. tarentolae* maxicircle fragments with labeled yeast petite mitochondrial DNA probes known to contain part of all of the genes for cytochrome oxidase subunits I (COI), II (COII), III (COIII), F_0–F_1 ATPases 6 (ATP6) and 9 (ATP9), and cytochrome *b* (CY*b*) (Simpson *et al.*, 1982b). Regions homologous to COI, COII, and CY*b* were localized fairly accurately. The ATP6 probe hybridized to a broad region of the maxicircle and no hybridization was observed with the ATP9 probe. The COIII hybrid melted at a low temperature, implying extensive mismatch. Candidates

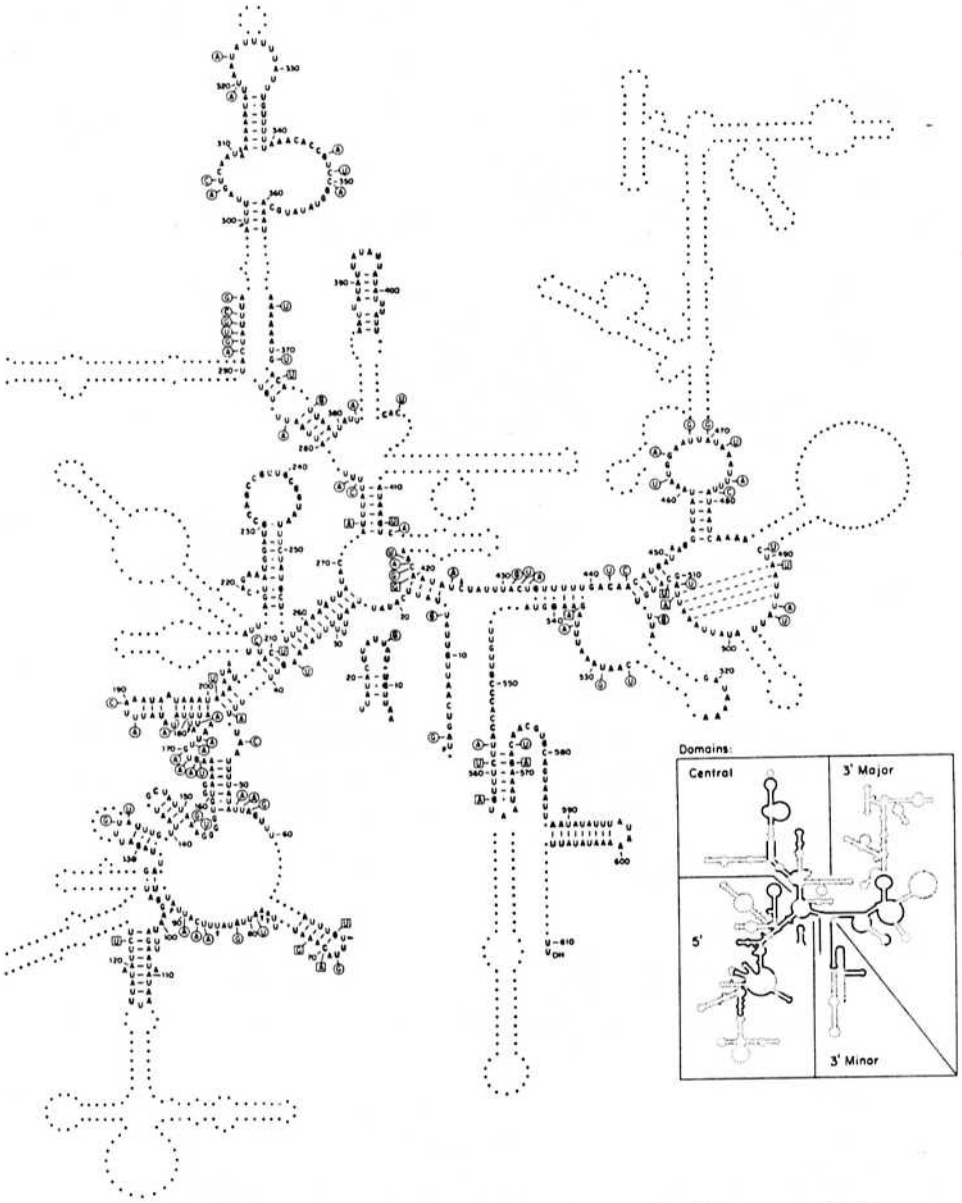

FIG. 25. Secondary structure of the 9 S rRNA from *L. tarentolae*. The pattern of helices was initially determined by a comparison of the *T. brucei* (Eperon *et al.*, 1983) and *L. tarentolae* sequences using the reversed homology matrix of Pustell and Kafatos (1982) and was confirmed by a comparison with the generalized secondary structure pattern for the *E. coli* 16 S small subunit rRNA (Noller *et al.*, 1982; Maly and Brimacombe, 1983; Woese *et al.*,

for transcripts of these presumptive structural genes were suggested (Simpson *et al.*, 1982a). Johnson *et al.* (1982) used maize and yeast petite COII probes to localize the presumptive structural gene for COII on the maxicircle of *T. brucei*. This localization, however, did not agree with the cross-species hybridization of specific *L. tarentolae* maxicircle fragments performed by Muhich *et al.* (1983), nor with a subsequent sequence analysis by Benne *et al.* (1983). The cross-species hybridization indicated the presence of the *CYb* gene rather than the *COII* gene and this was substantiated by the sequence results of Benne *et al.* (1983) and Johnson *et al.* (1984). The reason for this discrepency is unknown.

The first nonribosomal maxicircle structural gene sequence to be obtained was that for the *CYb* gene of *T. brucei* (Benne *et al.*, 1983; Johnson *et al.*, 1984). Benne *et al.* (1983) and Hensgens *et al.* (1984) have reported the sequence of the entire transcribed region of the *T. brucei* maxicircle. The *COI*, *COII*, and *CYb* genes were identified, in addition to 10 unidentified reading frames and three genes homologous to unidentified reading frames 1, 4, and 5 from the human mitochondrial genome (HURF1, HURF4, and HURF5). The genomic organization, shown in Fig. 26, is similar to that of the *L. tarentolae* maxicircle, as expected from the cross-species hybridization results of Muhich *et al.* (1983).

In the case of *L. tarentolae*, the DNA sequence of approximately 80% of the transcribed region of the *L. tarentolae* maxicircle is known (de la Cruz *et al.*, 1984; L. Simpson, A. M. Simpson, V. de la Cruz, and N. Neckelmann, unpublished results). Seven structural genes were identified in this sequence; the genomic localizations of the *COI*, *COII*, *COIII*, and *CYb* genes agreed with those predicted by the heterologous hybridizations. In addition to the genes for COI, COII, COIII, and CYb, three open reading frames were identified as homologous to unidentified reading frames 1, 4, and 5 from the human mitochondrial genome (HURF1, HURF4, and HURF5). Several unidentified open reading frames were also observed. The *L. tarentolae* genes were localized by comparison of the translated amino acid sequences with those of known mitochondrial genes. The *ATP6*, *ATP8*, and *ATP9* genes have not been identified within

1983). Nucleotides conserved between the 9 S sequences and *E. coli* are shown in bold face. Nucleotide positions that are not represented by the 9 S RNA on the *E. coli* model are indicated by small circles. The numbers refer to the *L. tarentolae* sequence, the 5′ nt being position one. Mismatches in the *T. brucei* sequence are indicated adjacent to the *L. tarentolae* sequence. Nucleotides within circles are noncompensatory changes with regard to base pairing, and nucleotides within boxes are compensatory changes. Deletions in the *T. brucei* sequence are indicated by solid triangles and insertions are indicated by lines pointing between the appropriate *L. tarentolae* nucleotides. The locations of the four domains are indicated in the inset. Reprinted from de la Cruz *et al.* (1985a) with permission.

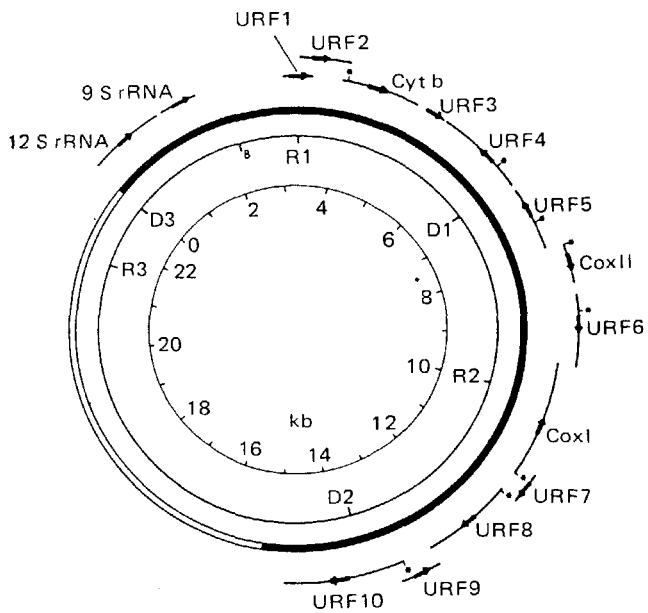

FIG. 26. Partial gene map of *T. brucei* maxicircle DNA. The arrangement of genes is derived from various studies. Arrows indicate direction of transcription of each gene. The black bar indicates the sequenced area (B, *Bam*HI). Reprinted from Hensgens *et al.* (1984) with permission.

the maxicircle sequences reported to date. The maxicircle genes showed different levels of sequence conservation as compared to mammalian and fungal sequences, with *COI* being the most conserved and *COIII* the least conserved. The genomic organization of the published, identified, and sequenced *L. tarentolae* maxicircle genes is shown in Fig. 27. This organization is unique and unlike that in either mammalian, insect, or fungal systems.

From a comparison of the translated amino acid sequences of the maxicircle genes with the sequences of known proteins from other organisms, Benne *et al.* (1983), Hensgens *et al.* (1984), and de la Cruz *et al.* (1984) concluded that UGA = tryptophan is the only apparent deviation from the universal genetic code.

A striking feature of the identified maxicircle structural genes is a pronounced strand asymmetry; the T : A ratio of the coding strand is approximately 2.0 for the six identified genes and also for several open reading frames (de la Cruz *et al.*, 1984).

Transcripts of the structural genes in both *L. tarentolae* and *T. brucei* have been identified and the 5′ ends mapped (Benne *et al.*, 1983; Simpson

et al., 1982a, 1985). Unlike the human mitochondrial situation where the 5′ end of the transcript is flush with the initiation codon, in *L. tarentolae* there are substantial 5′-untranslated sequences in all the maxicircle transcripts. There are no indications of intervening sequences in any of the identified structural genes.

There is, however, a −1 frameshift in the C-terminal portion of the COII reading frame, which is present at the same location in the maxicircle DNA sequences from both *T. brucei* and *L. tarentolae* (Hensgens *et al.*, 1984; de la Cruz *et al.*, 1984). This conserved frameshift is not understood. Possible explanations include the presence of a pseudogene, or a specific insertion of U's at the RNA level (R. Benne, personal communication).

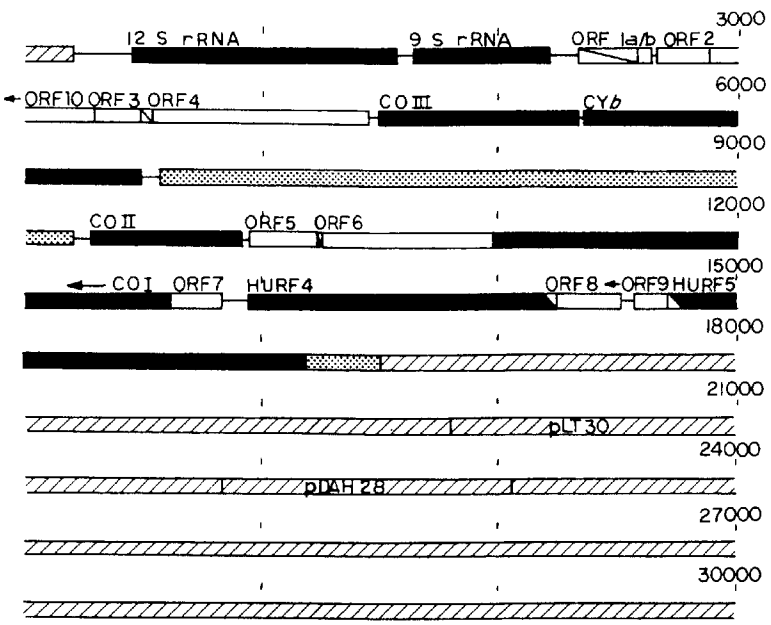

FIG. 27. Geonomic organization of the maxicircle DNA of *L. tarentolae*. The non-transcribed divergent region is indicated by cross-hatching and the identified genes are indicated by dark shading. Unidentified open reading frames (ORFs) are blank. The portions of the transcribed region that have not yet been sequenced are indicated by stipling. All identified genes are transcribed left to right except for COI, which is transcribed in the reverse direction (arrow). The identified genes are the large and small ribosomal RNAs (12 and 9 S RNAs), subunits I, II, and III of the cytochrome oxidase complex (COI, COII, and COIII), cytochrome *b* (CY*b*), and sequences homologous to human mitochondrial unidentified reading frames 4 and 5 (HURF4 and HURF5). Open reading frames which are putative genes are indicated as ORF1–10. Reprinted from de la Cruz *et al.* (1984) with permission.

B. Evolution of Maxicircle DNA: Structural and Ribosomal Genes

Conservation of the 9 and 12 S ribosomal genes has been demonstrated for several species including *L. tarentolae, T. brucei, C. fasciculata,* and *C. luciliae*. Cross-species hybridization of maxicircle sequences outside the 9 and 12 S regions has also been demonstrated, but the homologies are less striking. Hoeijmakers *et al.* (1982a) used the *T. brucei Eco*2 and *Eco*3 maxicircle probes and found adjacent regions in the *C. luciliae* maxicircle showing hybridization with the *T. brucei* probes. Muhich *et al.* (1983) examined in detail sequence homologies between maxicircle DNA of *T. brucei* and *L. tarentolae,* which differ by almost 10 kb in size. They found that the genomic organizations of the transcribed regions were similar, but that the nontranscribed regions were completely nonhomologous. Comparison of the *CYb* gene sequences from the *L. tarentolae* and *T. brucei* maxicircles shows a nucleotide similarity of 83% and an amino acid identity of 84%. Of the nonidentical codons, 26% are third base substitutions, 96% of which are silent. In regard to nucleotide changes, the transversion/transition ratio is 1.0, again possibly implying a distant divergence time for these species.

One striking difference between the *L. tarentolae* and the *T. brucei* maxicircle genomes is the absence of the *COIII* gene in *T. brucei*. De la Cruz *et al.* (1984) were unable to detect this gene in *T. brucei* maxicircle DNA by hybridization, using a *L. tarentolae* maxicircle gene probe, and the gene has not yet been identified in the *T. brucei* maxicircle sequences (Benne *et al.*, 1983; Hensgens *et al.*, 1984). It may be relevant that the *L. tarentolae COIII* gene is the most diverged of the identified maxicircle genes, as compared to the human and yeast homologous genes.

C. Evolution of Maxicircle DNA: Divergent Region

The first indication of the existence of a region in the maxicircle that served a noncoding function was the finding that maxicircle DNA from different strains of *T. brucei* differed by as much as 1 kb, and that this size variation was localized in one region of the maxicircle that was termed the "variable" region (Borst *et al.*, 1980a, 1981a). This region appeared to be "nonclonable" in bacterial plasmids or phage vectors and was found to show little if any transcription (Brunel *et al.*, 1980; Stuart and Gelvin, 1980; Simpson and Simpson, 1980).

Muhich *et al.* (1983) compared the genomic organization of the maxicircle DNA of *T. brucei* and *L. tarentolae* and found that there was one large region of nonhomology. This region was within the *Msp/Bam* fragment 1,

which corresponds in relative location to the *T. brucei* variable region. They concluded that evolutionary changes in maxicircle DNA occur mainly in one region, which they termed the "divergent region," and probably involve insertions and deletions. A similar conclusion was reached by Maslov *et al.* (1984) who cross hybridized maxicircle fragments from *C. oncopelti, C. luciliae, Lp. pessoai,* and *L. gymnodactyli,* using labeled maxicircle fragments from *C. oncopelti* as probes. Maslov *et al.* (1984) constructed a general model of the structural organization of maxicircles in which the molecule is composed of a 17-kb conservative region common to all species and a divergent region represented by the remainder of the molecule. They conclude that the major size differences between the maxicircle molecules from different species are due to length variability of the divergent regions.

Two contiguous fragments totaling 2.76 kb from the divergent region of *L. tarentolae* have been cloned and sequenced (M. Muhich, N. Neckelmann, and L. Simpson, unpublished results). Extensive tandem repeats were seen, both short (AAATT, AATAATAT) and long (ATATTAAAACAAGTTATTCCC), some of which are present throughout the divergent region as shown by blot hybridization. The presence of these repetitive sequences is consistent with the lack of transcription of this region and the observed rapid rate of sequence change in nature. Similar conclusions were reached by an electron microscopic analysis of heteroduplexes formed with maxicircle DNA of *T. brucei* (Borst *et al.*, 1982). The molecular mechanism for the rapid rate of sequence change of the divergent region is unknown, but any mechanism must take into account the existence of multiple tandem repeats.

XI. Unusual Kinetoplast DNAs

The minicircle/maxicircle organization pattern of kinetoplast DNA appears to be correlated to the existence of the highly organized kinetoplast DNA nucleoid bodies observed in thin sections of intact cells by electron microscopy. Several kinetoplastid protozoa, however, lack this characteristic nucleoid body structure in the mitochondrion and instead possess a structure reminiscent of the bacterial nucleus consisting of one or more masses of "isotropically arranged bundles of isotropic fibrils" (Vickerman, 1977). This condition has been termed "pankinetoplastic" by Vickerman (1977) and has been observed in the free-living kinetoplastid, *Bodo,* as well as in the parasitic kinetoplastids, *Cryptobia vaginalis* and *Herpetomonas ingenoplastum.* The mitochondrial DNA of *H. ingenoplastum* has been isolated and characterized (Borst *et al.*, 1981b). It consists of

two components, a minor class of 36-kb circles, which show sequence homology with the 9 and 12 S genes of *T. brucei*, and a major class of 17- and 23-kb circles, which exhibit sequence heterogeneity and have no sequences in common with *Herpetomonas muscarum* maxicircle or minicircle DNA. It was hypothesized that the 36-kb circles represent defective maxicircles and the 17- and 23-kb circles represent an evolutionary precursor of the small minicircles found in other kinetoplastids.

XII. Conclusions

The minicircle represents the unique aspect of the mitochondrial DNA of the kinetoplastid protozoa, but the significance of this DNA is not yet understood, although much is now known about its structure and replication. In spite of the lack of understanding of the function of the minicircle DNA, the extensive sequence divergence observed to occur in nature has been employed to distinguish different strains and species of these parasites. The maxicircle DNA, on the other hand, represents the informational molecule in the mitochondrion of the kinetoplastid protozoa and has been shown to contain sequences homologous to several known mitochondrial structural genes. The basic genomic organization of the maxicircle DNA has been shown to be conserved in representative species from several kinetoplastid genera, with evolutionary changes probably occurring mainly by insertions/deletions and rearrangements within one region of the molecule, which we have termed the "divergent region."

The genomic organization of the maxicircle DNA has some similarities with the organization of the human mitochondrial genome and the yeast mitochondrial genome, but it is unique in several respects, implying that the evolution of mitochondrial genomes has progressed along several pathways in different organisms but that the basic informational content has remained fairly constant. The high divergence of the identified maxicircle genes from those of mammalian, fungal, and insect systems implies a relatively ancient branching of the kinetoplastid protozoa from other eukaryotic lines, an interpretation consistent with the phylogenetic analysis from cytochrome *c* data by Schwartz and Dayhoff (1978).

There has been a large amount of progress in our understanding of the kinetoplast genome in the last few years, but much remains to be investigated. I would make the following suggestions as possible fruitful lines for future research:

1. Evolution of minicircle sequence diversity. Comparison of rates of change of the constant and variable regions. Mechanisms for the generation of sequence changes (e.g., recombination, mutation).

2. Examination of the possibility of recombination between minicircles and maxicircles, especially within the divergent region of the maxicircle.

3. Investigation of the role, if any, of the minicircle sequence-dependent "bending" in terms of packaging of DNA in the nucleoid body or interaction with specific proteins.

4. Investigation of the protein apparently bound to minicircle DNA in terms of sequence localization and function.

5. Isolation and characterization of the mitochondrial topoisomerases responsible for decatenation of closed minicircle and catenation of nicked minicircles.

6. Investigation of the *Crithidia* minicircle open reading frames apparently responsible for the production of three polypeptides localized to the vicinity of the flagellar pocket.

7. Investigation of the replication of maxicircle DNA.

8. Comparative study of the detailed maxicircle genomic organization in representative species of several genera.

9. Examination of the evolution of specific mitochondrial genes, such as the ribosomal genes, by comparison of sequences from representative species of several genera.

10. Examination of the possibility of regulation of maxicircle transcription in different stages of the life cycle, particularly in the African trypanosomes.

11. Examination of the possibility of processing of maxicircle transcripts.

12. Examination of the possibility of interactions between the kinetoplast and nuclear genomes during the mitochondrial biogenesis that occurs in the life cycle of *T. brucei*.

13. Molecular events occurring during the development of dyskinetoplastic cells after treatment with dyes.

14. Characterization of the mitochondrial DNA in "pankinetoplastic" cells, including free-living species.

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