CHARACTERIZATION OF THE MOLECULAR COMPONENTS IN KINETOPLAST-MITOCHONDRIAL DNA OF *TRYPANOSOMA EQUIPERDUM*

Comparative Study of the Dyskinetoplastic and Wild Strains

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ABSTRACT

The structure of the kinetoplast DNA of *Trypanosoma equiperdum* has been studied and compared to the structure of the circular mitochondrial DNA extracted from a dyskinetoplastic strain of *T. equiperdum*. In *T. equiperdum* wild type, the kinetoplast DNA constitutes ~6% of the total cellular DNA and is composed of ~3,000 supercoiled minicircles of 6.4×10^5 daltons and ~50 circular supercoiled molecules of 15.4×10^6 daltons topologically interlocked. The buoyant density in CsCl of the minicircles is 1.691 g/cm^3. The large circles have a buoyant density of 1.684 g/cm^3, are homogeneous in size and are selectively cleaved by several restriction endonucleases which do not cleave the minicircles. The cleavage sites of six different restriction endonucleases have been mapped on the large circle. The minicircles are cleaved by two other restriction endonucleases, and their cleavage sites have been mapped. The mitochondrial DNA extracted from the dyskinetoplastic strain of *T. equiperdum* represents 7% of the total DNA of the cell and is composed of supercoiled circles, heterogeneous in size, and topologically associated in catenated oligomers. Its buoyant density in CsCl is 1.688 g/cm^3. These molecules are not cleaved by any of the eight restriction endonucleases tested. The reassociation kinetics of in vitro labeled kDNA minicircles and large circles has been studied. The results indicate that the minicircles as well as the large circles are homogeneous in sequence and that the circular DNA of the dyskinetoplastic strain has no sequence in common with the kDNA of the wild strain.

KEY WORDS *Trypanosoma equiperdum* · dyskinetoplasty · kinetoplast DNA · core kinetoplast DNA · restriction endonuclease mapping · reassociation kinetics

The kinetoplast is the specialized portion of the mitochondrial apparatus of trypanosomes which contains DNA in high concentration as revealed by light microscopy after staining. In all trypanosomes species studied so far, the kinetoplast DNA (kDNA) has been isolated as a complex network composed of numerous interlocked minicircles and long molecules without apparent free ends (26). In some species these long DNA molecules

form loops at the edge of the network (9, 18). These loops, of molecular weight varying with the species of trypanosomes, have been named maxi-circles (10). The genetic function of kDNA is yet unknown and there is at present little direct evidence for its in vivo transcription. However, Simpson and Simpson (27) have recently isolated from *Leishmania tarentolae* two RNA species of kinetoplast-mitochondrial origin. These RNAs were found to hybridize selectively to the maxicircle sequences of the kDNA.

In several species of trypanosomes, especially in the subgenus Trypanozoon, spontaneously dyskinetoplastic mutants have been isolated (30) which might be helpful to clarify the problem of the biological role of kDNA. In these mutants, which have the same infectivity as the wild type, no kDNA can be observed after cytological staining, but dispersed clumps of intramitochondrial DNA can be seen with DAPI (4,6-diamidino-2-phenylindole) staining (8). Furthermore, it has not been possible to isolate minicircles from a dyskinetoplastic strain of *T. equiperdum*. However, circular supercoiled DNA heterogeneous in size and of about the same buoyant density as the wild type kDNA has been purified from this dyskinetoplastic strain (20). It has been assumed that this DNA is of mitochondrial origin.

We have characterized by several techniques the components of kDNA networks of *T. equiperdum* wild type comparative to the circular DNA extracted from the dyskinetoplastic strain. Our results presented in this paper show that the long DNA molecules of the wild type network are supercoiled circles topologically linked to the network. These circles constitute ~30% of the total kDNA and have a low buoyant density in CsCl. They have a homogeneous molecular weight of 15.4 megadaltons. The thirteen sites of cleavage by six different restriction enzymes have been mapped. The minicircles are not cleaved by these six enzymes, but two other restriction enzymes were found which cleave them into, respectively, one and two fragments. By contrast, the circular DNA extracted from the dyskinetoplastic strain is not cleaved to a significant extent by any of the eight restriction enzymes tested. Its buoyant density is slightly smaller than that of the minicircles and higher than that of the large circles. Reassociation kinetics studies have shown that the mitochondrial DNA of the dyskinetoplastic strain has no sequence in common with the kDNA minicircle or large circle of the wild strain. Furthermore,

these studies indicate that the kDNA minicircles and large circles form two classes of molecules homogeneous in sequence.

MATERIALS AND METHODS

Strain and Obtention of Trypanosomes

The kinetoplastic strain of *T. equiperdum* was obtained from Institut Pasteur in 1961. The dyskinetoplastic strain was provided by Dr. E. Tobie who isolated this mutant from blood of rats infected with wild type trypanosomes (29). Trypanosomes were maintained in Wistar rats by syringe passage every 3–4 d or stored at −190°C in rat blood and glycerol. Trypanosomes were inoculated into Wistar rats. Maximal parasitaemia was reached in 3–4 d and blood was withdrawn. Trypanosomes were separated from red and white cells by passage through a DEAE cellulose column as described by Lanham and Godfrey (12) and washed three times with 0.15 M NaCl, 0.015 M Na$_3$ citrate.

Preparation and Fractionation of kDNA and mtDNA

Total DNA was extracted from the two strains, and kDNA and mtDNA were fractionated in propidium diiodide (PDI) CsCl gradients in conditions previously described (21). PDI was extracted with isopropanol (14).

In Vitro Synthesis of ^{32}P-labeled kDNA

Nick translation of kDNA was carried out as described by Mackey et al. (13) in the presence of α^{32}PdCTP. The reaction mixture contained in 20 μl of final volume, 250 ng of kDNA, ~30 μCi of α^{32}PdCTP (New England Nuclear Corp., Boston, Mass., sp. act. 2–3000 Ci/mmol) and 9 units of *Escherichia coli* DNA polymerase I (Boehringer Mannheim, Biochemicals, Indianapolis, Ind.). The incorporation was followed by measuring the acid-precipitable radioactivity, and the specific activity of the synthesized DNA was determined at the end of the reaction. The labeled DNA was purified by gel filtration and centrifuged in an alkaline sucrose gradient as described (13). The size of DNA fragments was estimated (28) relative to sheared ^{3}H-labeled *Trypanosoma cruzi* nuclear DNA of known size ($S_{20,w}$ = 5.9). The three peak fractions after dialysis were used for the renaturation kinetics studies.

Restriction Endonuclease Cleavage of kDNA and Gel Electrophoresis

Eco R1, *Hind* III, *Hae* III, *Hpa* II, *Pst* I, *Hinf* I and *Bam* H1 were purchased from New England Biolabs (Beverly, Mass.). *Taq* I prepared according to the method of Sato et al. (23) was a generous gift of Dr. J. Feunteun. The reactions were carried out in the follow-

ing buffers: 6.6 mM Tris HCl, pH 7.5, 6.6 mM MgCl$_2$, 50 mM NaCl, and 6.6 mM 2-mercaptoethanol for Hae III, Hinf I, Pst I, and Taq I; 10 mM Tris HCl, pH 7.4, 7 mM MgCl$_2$, 10 mM NaCl, and 14 mM 2-mercaptoethanol for Hpa II; 100 mM Tris HCl, pH 7.2, 5 mM MgCl$_2$, 50 mM NaCl, and 2 mM 2-mercaptoethanol for Eco R1; 20 mM Tris HCl, pH 7.4, 7 mM MgCl$_2$, and 60 mM NaCl for Hind III; 20 mM Tris HCl, pH 7.5, 7 mM MgCl$_2$, and 2 mM 2-mercaptoethanol for Bam H1. Incubation was usually carried out for 2 h at 37°C except for Taq I which was used at 60°C as described by Sato et al. (23). Double-digest experiments were carried out sequentially. The solution was extracted with CHCl$_3$-isoamyl alcohol after the first incubation. The DNA was precipitated with ethanol, dissolved in the appropriate buffer, and incubated with the second restriction enzyme. λ DNA and SV 40 DNA were used as substrates to check the activities of these restriction endonucleases. The DNA fragments were separated by electrophoresis in vertical slab gels. 2% agarose gels were used to measure the molecular weight of the fragments up to 1.6 kilobase pairs (kb) and 0.5 % agarose, 1.8 % polyacrylamide gels for larger fragments. After migration, they were located by EthBr (10 μg/ml) staining and photographed under UV illumination. The fragments of λ DNA generated by cleavage with Hind III (33) and of T. cruzi kDNA generated by cleavage with Hae III (21) were used as size standards. The molecular weights of the four fragments of T. cruzi kDNA were determined by electron microscopy relative to Φ X174 RF DNA (5,375 base pairs according to Sanger et al. [22]). Φ X174 DNA was a generous gift of Dr. O. Croissant.

Reassociation Kinetics

^{32}P-labeled DNA was heated for 20 min at 100°C in 30 mM Na phosphate pH 6.8 and reassociation was carried out at 68°C in 1.25 M NaCl, 80 mM Na phosphate pH 6.8 and 0.08% SDS. 100-μl fractions were collected at various times and diluted with 0.9 ml of 0.14 M Na phosphate pH 6.8, 0.4% SDS. The fraction of single- and double-stranded DNA was determined by hydroxylapatite chromatography as described by Sharp et al. (25).

Electron Microscopy of DNA

kDNA networks and mtDNA were spread using the microdiffusion technique previously described (4) or the Kleinschmidt technique modified as described (17) and examined under a Philips EM 300 electron microscope. Ultrathin sections of trypanosomes were prepared as described by Delain et al. (5).

Analytical Ultracentrifugation

A Spinco model E analytical ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) was used. Sedimentation coefficients were measured with an ANJ rotor at 6,000 rpm by band sedimentation

analysis at 20°C using 3 M CsCl, 1 mM Na$_3$ EDTA (pH 8.0) as bulk solvent. Each cell was filled with 15 μl of the DNA solution previously dialysed against 2 M NaCl, 1 mM Na$_3$ EDTA. The corrected values of sedimentation coefficients were calculated according to Bruner and Vinograd (3). Equilibrium density centrifugation in CsCl gradient was performed at 44,000 rpm at 25°C using Micrococcus lysodeikticus DNA as density marker. Scans were obtained using the Beckman photoelectric scanning system.

RESULTS

Ultrastructure of Wild Type and Dyskinetoplastic Strain of T. Equiperdum

Fig. 1 shows electron micrographs of ultrathin sections of the kinetoplast region of T. equiperdum. In Fig. 1 A, the kinetoplast of the wild strain with its kDNA can be easily seen, surrounded by the mitochondrial membrane. The kDNA appears as a concave sheet of fibrillar material, dense to electrons, with the fibrils oriented in a direction parallel to the small axis of the kinetoplast. The mitochondrial apparatus, poorly developed as a general characteristic of the subgenus Trypanozoon (26), forms a vesicle extending from the kinetoplast. In the dyskinetoplastic trypanosomes, mitochondrial vesicles similar to those found in the wild type are also observed at the base of the flagellum (Fig. 1 B). However, the organized mass of DNA fibrils is absent; instead, we observe a diffuse mass of electron-dense material in the mitochondrial vesicle. This could be the mitochondrial DNA in a molecular organization different from that of the kDNA network of kinetoplastic organisms, as already described (20).

Characterization of the Circular DNA Molecules of the Dyskinetoplastic Strain

We have previously analysed the circular DNA extracted from a dyskinetoplastic strain of T. equiperdum (20). This DNA presumably of mitochondrial origin (mtDNA) is organized in catenated oligomers composed of circular molecules of different lengths ranging from 0.5 to 40 μm. An electron micrograph of this DNA is shown in Fig. 2. The total DNA extracted from this strain has been analysed by equilibrium density centrifugation. As shown in Fig. 3, the nuclear DNA is heterogeneous in density. The main component has a density of ρ = 1.706 g/cm^3 and the minor component a density ~5-6 mg/cm^3 smaller. The mtDNA has a density of ρ = 1.688 g/cm^3 and
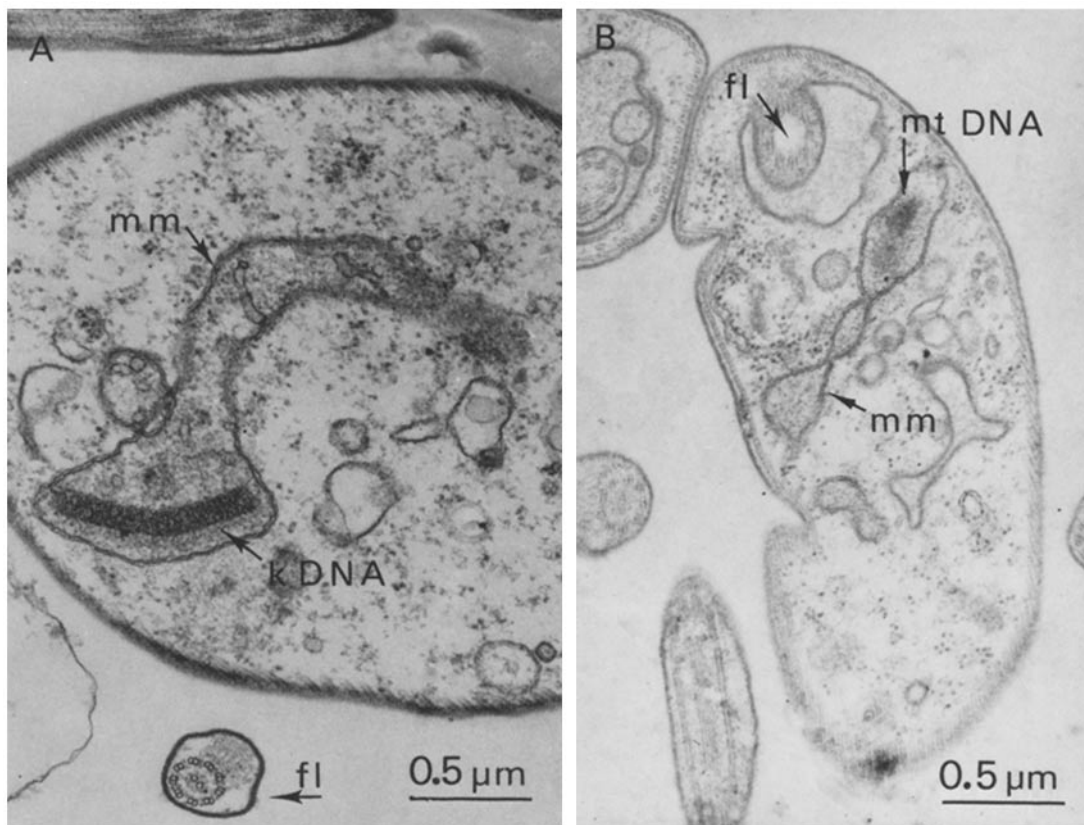
Figure 1 Electron micrograph of ultrathin sections in the kinetoplast region of *T. equiperdum*. (*A*) kinetoplastic strain; (*B*) dyskinetoplastic strain; *fl*, flagellum; *mm*, mitochondrial membrane; mtDNA, mitochondrial DNA; kDNA, kinetoplast DNA. Bar, 0.5 μm. × 32,000.

accounts for ~7% of the total DNA as estimated by measuring the area under the peaks of the photoelectric scanner tracing.

When this mtDNA is analysed by electrophoresis on agarose gels, a significant fraction of the DNA remains on the top of the gel. This is probably due to its catenated structure. However, several bands are observed. The mtDNA was incubated with the restriction endonucleases *Bam* H1, *Pst* 1, *Hae* III, *Hpa* II, *Hind* III, *Eco* R1, and *Hinf* I and analysed by agarose gel electrophoresis as shown in Fig. 4. The band pattern is identical with the seven enzymes tested and does not differ from that of the untreated mtDNA. This result indicates that the mtDNA has not been cleaved to a significant extent by any of these enzymes. The cleavage pattern obtained by digestion with *Taq* I differs from that obtained with untreated mtDNA. The disappearance of the fastest band is probably due to the nicking of closed circles during incuba-

tion carried out at 60°C with this enzyme. This mtDNA is hydrolysed by pancreatic DNase as shown in Fig. 3*C*.

Purification and Characterization of the kDNA Networks

When the total DNA extracted from *T. equiperdum* wild type is analysed by equilibrium centrifugation in a CsCl gradient (Fig. 5*A*) at least two components are observed: kDNA with a density ρ = 1.690 g/cm^3 and nuclear DNA banding at ρ = 1.707 g/cm^3 with a shoulder corresponding to DNA of lower density. The kDNA which accounts for ~6.0 ± 1.0% of the total DNA was purified by equilibrium density centrifugation in CsCl gradient containing PDI. The upper band contains mostly nuclear DNA and occasionally a small amount of form II kDNA networks. The lower band containing the kDNA networks form
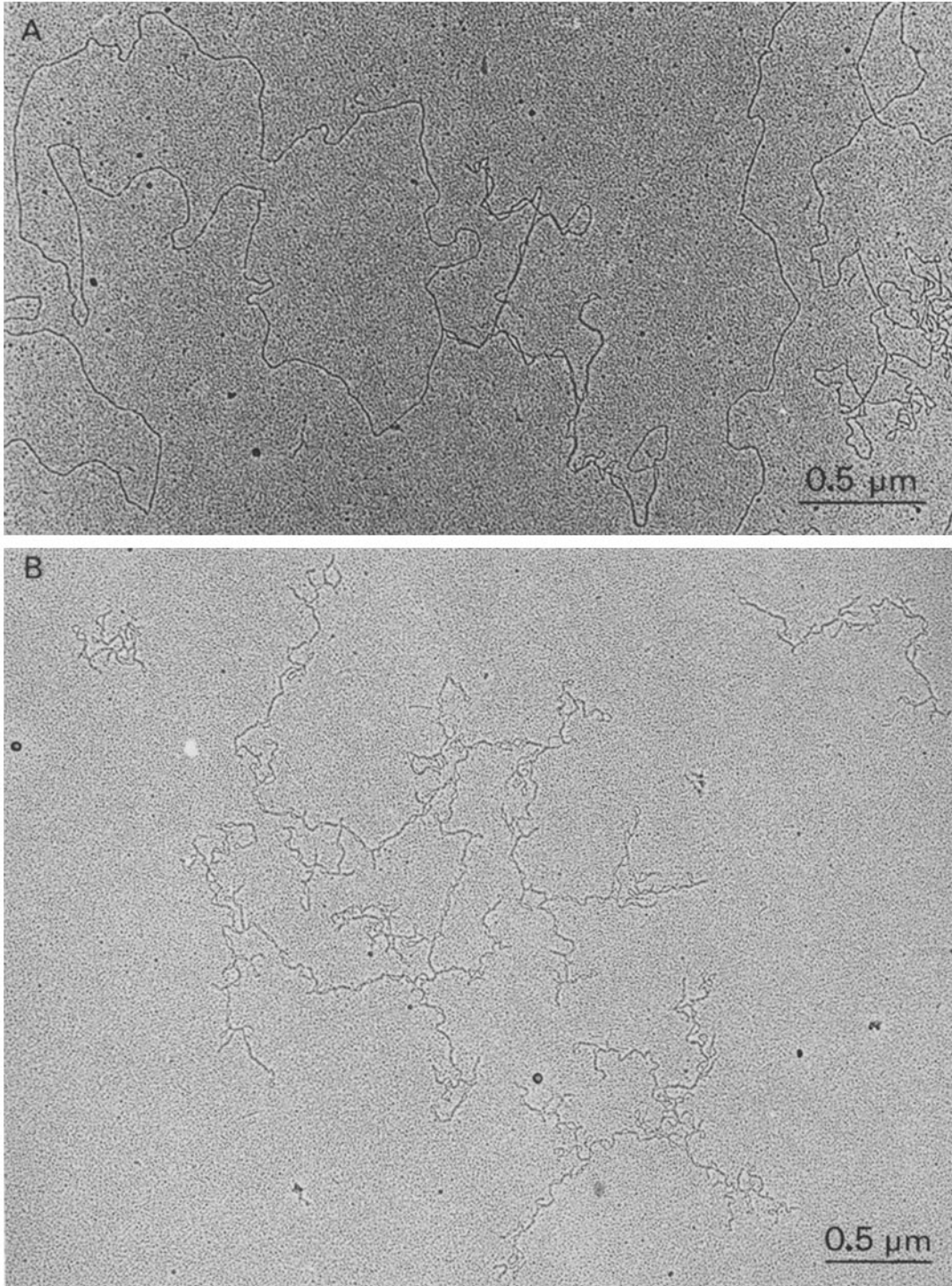
FIGURE 2   Electron micrograph of mtDNA extracted from the dyskinetoplastic strain of *T. equiperdum* and spread by the microdiffusion technique. (*A*) dimeric catenane of one supercoiled molecule and one nicked circular molecule. Bar, 0.5 μm. × 39,000. (*B*) supercoiled mtDNA. Bar, 0.5 μm. × 33,000.
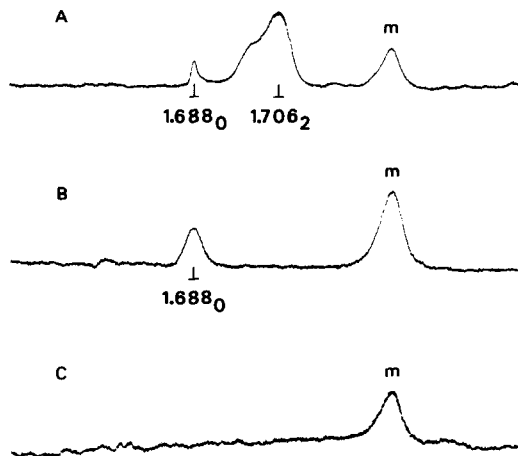
FIGURE 3 Equilibrium density centrifugation in CsCl gradient of dyskinetoplastic *T. equiperdum* DNA. (*A*) total DNA; (*B*) mtDNA form I; (*C*) mtDNA form I treated with pancreatic DNase (250 U/ml of pancreatic DNase Sigma, for 30 min at 37°C in 5 mM $MgCl_2$, 15 mM NaCl, pH 7.4). *Micrococcus lysodeikticus* DNA (ρ = 1.731 g/cm^3) was used as density marker.
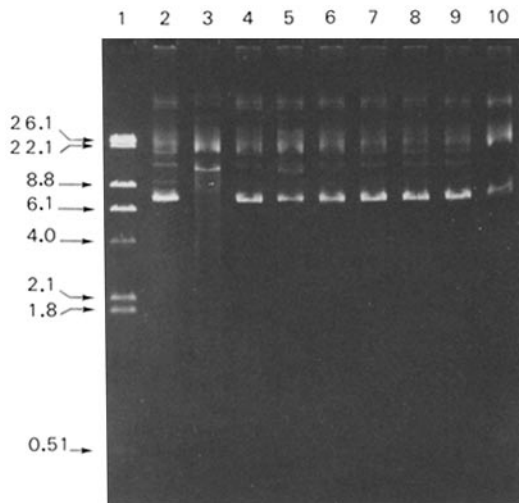

FIGURE 4 Electrophoresis in 0.5% agarose-1.8% polyacrylamide gel of the mtDNA from the dyskineto-plastic strain of *T. equiperdum*. The molecular weight scale is in kilobases (kb). (1) λ DNA fragments obtained by *Hind* III cleavage. The 26.1 kb fragment results of the aggregation of the 22.1 and 4.0 kb fragments by their sticky ends; (2) unreacted mtDNA; (3) *Taq* I; (4) *Hinf* I; (5) *Pst* I; (6) *Bam* H1; (7) *Hind* III; (8) *Eco* R1; (9) *Hae* III; (10) *Hpa* II.

I was collected and, after spreading, the kDNA networks were examined by electron microscopy. The micrograph presented in Fig. 6 shows one

kDNA network with the typical structure already described for *T. equiperdum* (18, 20) and other species of trypanosomes (15, 32). Two distinct components are apparent in the network: numerous interlocked minicircles of 0.31 μm (6.4 × 10^5 daltons) and long DNA segments without apparent free ends. Some of these long DNA molecules are tangled and stretched inside the network. Other long DNA molecules anchored to the network form loops of different lengths protruding from the periphery of the network. About 5-12 such loops can be observed per kDNA network. In the electron micrograph shown in Fig. 6, the minicircles as well as the DNA loops appear to be essentially devoid of supercoils. In contrast, when the kDNA networks are spread on a hypophase containing EthBr, the DNA loops and minicircles
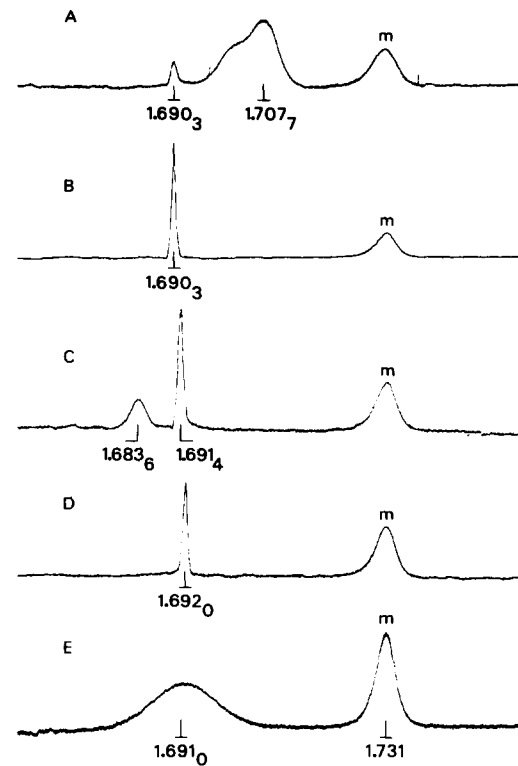

FIGURE 5 Equilibrium density centrifugation in CsCl gradient of wild type *T. equiperdum* DNA. (*A*) total DNA; (*B*) intact kDNA form I; (*C*) kDNA form I cleaved by endonuclease *Bam* H1; (*D*) Core-kDNA form I obtained after *Bam* H1 cleavage and CsCl-EthBr centrifugation as shown in Fig. 9; (*E*) 0.97 kb linear fragments obtained by cleavage with *Hinf* I of core kDNA minicircles. *Micrococcus lysodeikticus* DNA (ρ = 1.731 g/cm^3) was used as density marker.
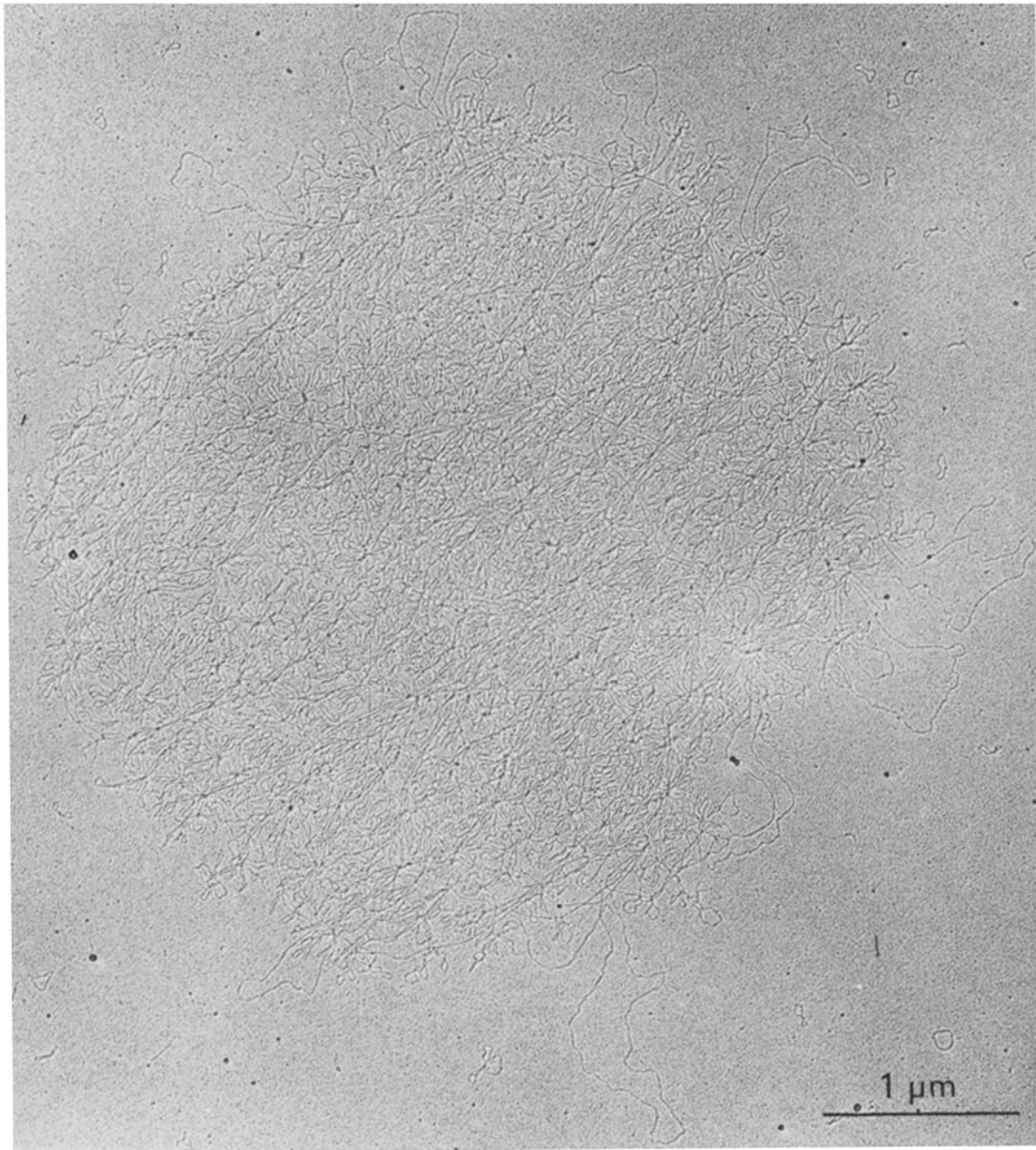
FIGURE 6 Electron micrograph of intact kDNA network form I from wild type. *T. equiperdum* spread by the microdiffusion technique. Bar, 1 μm. × 29,000.

are supercoiled (Fig. 7). The buoyant density in CsCl of the purified kDNA network (Fig. 5B) is equal to 1.690 g/cm³ (average of six determinations). The networks have a sedimentation coefficient of $S_{20,w}$ = 1,530 as measured by band sedimentation in 3 M CsCl.

Cleavage by Restriction Endonucleases of the Intact kDNA Networks

When the intact kDNA network is analysed by gel electrophoresis, most of the DNA does not enter the gel (Fig. 8). However, two fast bands

are usually observed corresponding, respectively, to free nicked minicircles and linear molecules of the same size as the minicircles in small amount. In some cases, a third band faster than the two others, composed of free supercoiled minicircles, is also present. The incubation of kDNA networks with *Bam* H1, *Pst* I, *Hae* III, *Hpa* II, *Hind* III, and *Eco* R1 generates one or several DNA fragments of relatively high molecular weight. We

have occasionally observed that the amount of free minicircles and linear molecules is slightly higher than in unreacted kDNA and varies with the different enzymes. However, a significant fraction of the kDNA remains on the top of the gel. It is unlikely that the digestion of the kDNA was incomplete since control DNAs incubated in the same conditions give the usual band patterns. As shown below, the material that does not enter
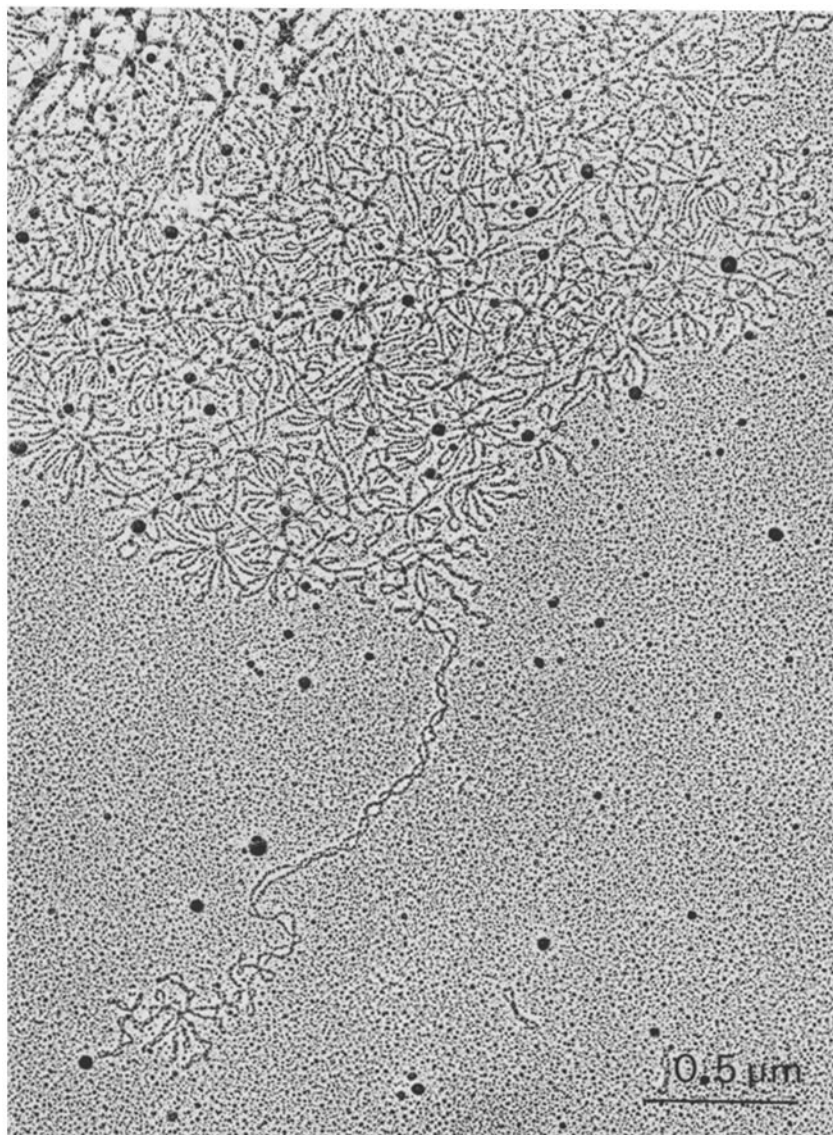

FIGURE 7   Electron micrograph of a part of intact kDNA network spread according to the modified Kleinschmidt technique on a hypophase containing 5 μg/ml of EthBr in distilled water. Bar, 0.5 μm. × 41,000.
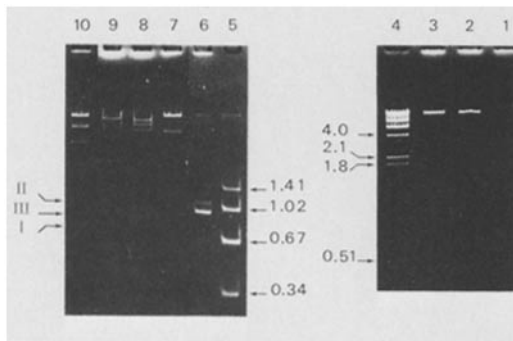
FIGURE 8   2% agarose gel electrophoresis of the fragments generated by cleavage of kDNA network by restriction endonucleases, (1) unreacted kDNA; (2) *Bam* H1; (3) *Pst* I; (7) *Hae* III; (8) *Hind* III; (9) *Eco* R1; (10) *Hpa* II. (4) λ DNA fragments obtained by *Hind* III cleavage and (5) *T. cruzi* kDNA fragments obtained by *Hae* III cleavage were used as size standards (molecular weights are given in kb). The arrows indicate the position of supercoiled minicircles (I), nicked minicircles (II) and linear fragments of the size of the minicircle (III) present in small amount. The forms II and III (0.97 kb) are clearly visible in channel (6) obtained with a kDNA sample partially degraded upon storage.

the gel is kDNA networks containing essentially interlocked, covalently closed minicircles, which we will refer to as core kDNA. The unique fragment generated by *Bam* H1 has a molecular weight of 15.4 × 10^6 daltons as measured by electron microscopy using PM2 DNA as a standard of 6.30 × 10^6 daltons (11). This measurement is confirmed by the molecular weight determination of the fragments generated by the various restriction endonucleases as shown in Table I. These results indicate that the loops are circles of uniform length.

The cleavage of kDNA form I by endonuclease *Bam* H1 generates two components of different buoyant density as observed by centrifugation at equilibrium in a CsCl gradient (Fig. 5C). The linear fragment liberated by *Bam* H1 has a buoyant density of 1.683_5 g/cm^3 (average of three determinations) corresponding presumably to a low GC content. From the area under the peak of the photoelectric scanner tracing (Fig. 5C), the linear fragments account for 33% of the intact networks.

Characterization of the Core kDNA Networks

The kDNA networks form I were digested with

restriction endonuclease *Bam* H1 and the DNA was fractionated by equilibrium density centrifugation in CsCl-EthBr gradient. As shown in Fig. 9, two bands were obtained. The lower band was collected and the DNA was analysed by electron microscopy, analytical ultracentrifugation, and gel electrophoresis. The DNA of the lower band appears in the electron microscope as networks of interlocked minicircles in which the long DNA molecules have been stripped off (Fig. 10). The minicircles are covalently closed as indicated by the position of the band in the CsCl-EthBr gradient. This kDNA network fraction resistant to *Bam* H1 cleavage which has been observed to be unable to migrate in gel electrophoresis is the core kDNA. The buoyant density of the core kDNA is found equal to 1.691_6 g/cm^3 (average of five determinations) and its sedimentation coefficient is $S_{20,w} = 1,310$ corresponding to ~86% of the value of the intact network.

When the intact kDNA network is digested with *Hinf* I and *Taq* I (Fig. 11), small fragments are obtained and no core network remains at the top of the gel in contrast with the other endonucleases tested. When the core network is incubated with *Taq* I, two bands are observed after gel electrophoresis corresponding to fragments of 0.80 kb and 0.17 kb. The sum of these fragments is equal to the size of the kDNA minicircles. This indicates that there are two sites for *Taq* I in the minicircles. When the intact network is digested with *Taq* I, several larger fragments are observed in addition to the fragments generated by the cleavage of the minicircles. Similarly, digestion of the intact network with *Hinf* I generates several large fragments and one linear fragment of the size of the minicircle. To determine whether the core network is completely cleaved by *Hinf* I, ^{32}P-labeled core kDNA was digested with this enzyme and analysed by gel electrophoresis (Fig. 12). In the undigested sample, most of the radioactivity is found at the top of the gel; however, a significant fraction of the radioactivity migrates in the form of linear fragments of the size of the minicircle which are generated during the labeling procedure. After digestion, >99% of the core kDNA is cleaved in linear fragments of the size of the minicircles. The linear fragments generated during the labeling procedure are cleaved by *Hinf* I into smaller fragments migrating faster than the main peak (Fig. 12B). These results indicate that all the minicircles possess the cleavage sites recognized by *Taq* I and *Hinf* I and do not exhibit the heterogeneity observed in *T. cruzi* (17, 21) and in

TABLE I

Molecular Weights Expressed in kb of the Fragments Generated by Restriction Endonuclease Cleavage of kDNA Large Circles

	Restriction endonuclease									
Fragment	Bam H1	Pst_I	Hae_III	Hpa_II	Hind_III	Eco R1	Bam H1 + Pst_I	Bam H1 + Hpa_II	Pst_I + Hpa_II	Bam H1 + Hind_III
[1]	23.3	23.2	18.1	14.2	9.5	12.3	17.4	13.4	14.2	9.5
[2]			5.4	6.0	7.7	6.5	5.4	6.0	4.7	6.0
[3]			23.5	3.2	6.0	4.5	22.8	3.2	3.2	3.9
[4]				23.4	23.2	23.3		0.72	1.28	3.6
[5]								23.32	23.38	23.0

	Pst_I + Hind_III	Hind_III + Hpa_II	Bam H1 + Eco R1	Pst_I + Eco R1	Bam H1 + Hae_III	Pst_I + Hae_III	Eco R1 + Hae_III	Hind_III + Hae_III	Hpa_II + Hae_III
[1]	9.5	9.5	12.3	12.3	18.1	18.1	12.3	9.5	13.1
[2]	7.7	4.4	6.5	4.8	5.4	5.4	4.8	4.3	4.8
[3]	4.4	3.2	3.6	4.5	23.5	23.5	3.5	3.8	3.2
[4]	1.6	2.9	0.98	1.66			1.66	3.6	1.19
[5]	23.2	2.9	23.38	23.26			1.06	1.66	0.75
		22.9					23.32	22.83	23.04

Molecular weights determined by gel electrophoresis. The molecular weights of the fragments generated by cleavage with a single endonuclease were confirmed by electron microscopy.

other species of trypanosomes (2). The core kDNA was digested sequentially with *Taq* I and *Hinf* I. Three fragments of 0.57, 0.22, and 0.19 kb were obtained (data not shown) and the mini-circle map was drawn using this result (Fig. 15). The buoyant density in CsCl of the 0.97 kb linear fragment obtained by cleavage with *Hinf* I of the core kDNA minicircles is equal to 1.691 g/cm^3 (Fig. 5E). The width of the band is related to the low mol wt of the DNA. Furthermore, the shape of this band indicates the absence of residual networks of core kDNA.

Restriction Cleavage Map of Large kDNA Circles

The effect of various restriction endonucleases on the kDNA networks was studied and the size of the fragments generated by cleavage of the large circles was determined by gel electrophoresis (Fig. 13, Fig. 14, and Table 1). Since each enzyme has a limited number of cleavage sites per DNA molecule, it is relatively straightforward to draw a physical map of this molecule (Fig. 15). The unique cleavage site of *Bam* H1 is taken as origin and the unique cleavage site of *Pst* I is used as secondary reference. The molecule is cleaved into three fragments by *Hpa* II: double-digest experi-
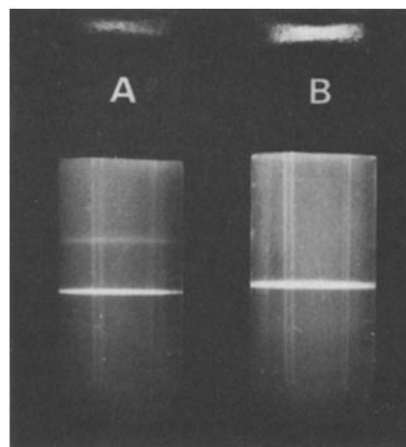

FIGURE 9   Photograph of CsCl-EthBr gradient centrifugation of intact kDNA network form I (B) and cleaved by *Bam* H1 (A).

ments show that fragment *Hpa* II [1] is cleaved by *Bam* H1 and that fragment *Hpa* II [2] is cleaved by *Pst* I. From these results, the three *Hpa* II cleavage sites are unambiguously mapped relative to *Bam* H1 and *Pst* I sites. Similarly, fragment *Hind* III [2] is cleaved by *Bam* H1 and fragment *Hind* III [3] is cleaved by *Pst* I. The position of the three *Hind* III cleavage sites can be assigned
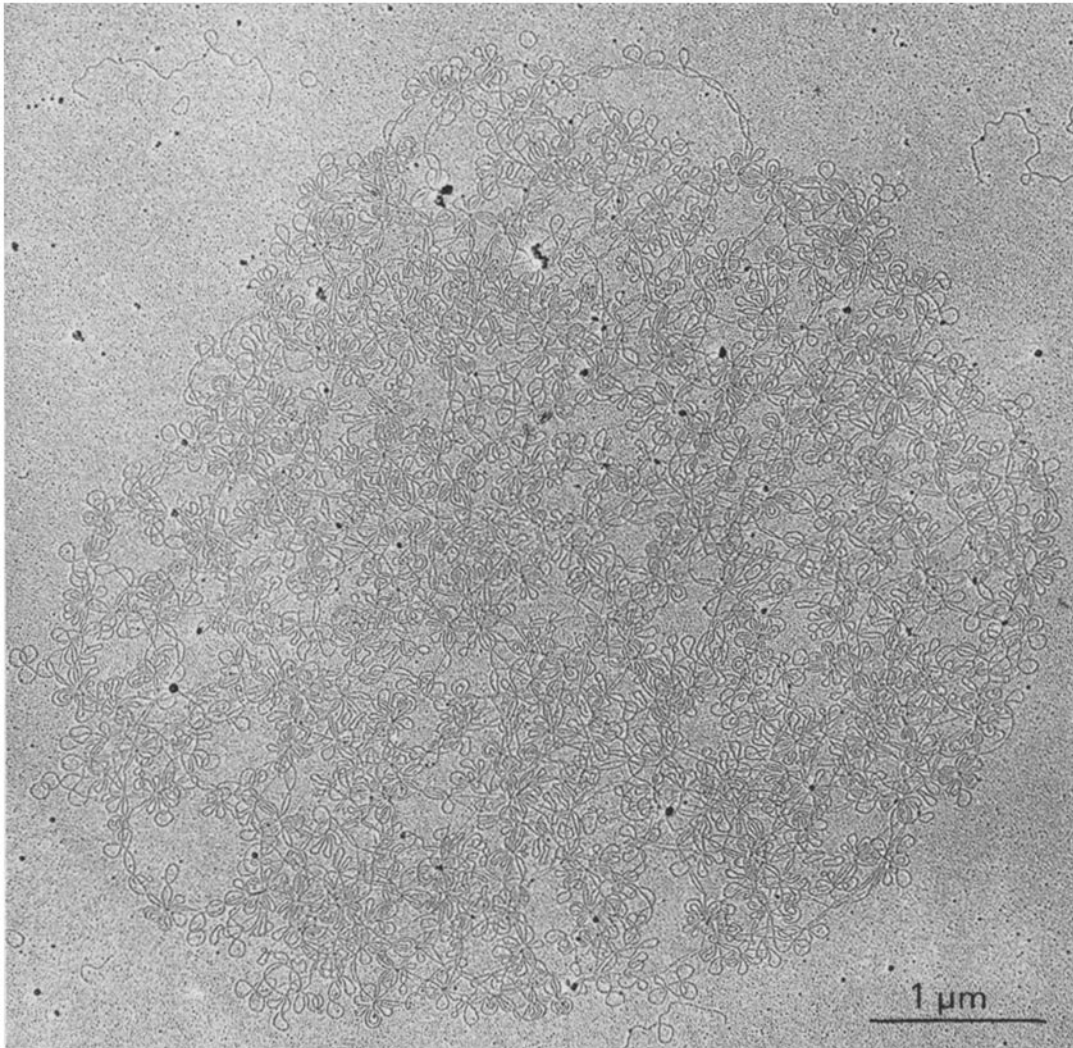
FIGURE 10 Electron micrograph of the core kDNA network form I resistant to endonuclease *Bam* H1 spread by the Kleinschmidt technique. Bar, 1 μm. × 25,000.

from these data. As shown in Fig. 15, one *Hpa* II site and one *Hind* III site are very close and undistinguishable with our technique. The relative position of the six *Hpa* II and *Hind* III sites is confirmed by the result of the double-digestion with these two restriction enzymes. Fragment *Eco* R1 [3] is cleaved by *Bam* H1, and fragment *Eco* R1 [2] is cleaved by *Pst* I, and the 3 *Eco* R1 cleavage sites are located relative to *Bam* H1 and *Pst* I sites using these data. The data of Table I indicate that the two *Hae* III cleavage sites are very close to, respectively, the *Bam* H1 site and the *Pst* I site. However, comparison of the cleav-

age pattern of fragment *Eco* R1 [3] by *Bam* H1 and the cleavage pattern of the same fragment by *Hae* III suggests that the *Hae* III site is situated between *Bam* H1 and *Hpa* II sites at ~50–100 base pairs from the *Bam* H1 site. For the second *Hae* III site, comparison of the length of fragment *Pst* I/*Hind* III [4] and the length of fragment *Hind* III/*Hae* III [5] indicates that the *Pst* I site is closer to the *Hind* III site and that the *Hae* III site is closer to the *Hpa* II site. This is confirmed by the comparison of the length of fragment *Pst* I/*Hpa* II [4] and the length of fragment *Hae* III/*Hpa* II [4]. The distance between the *Pst* I and *Hae* III sites
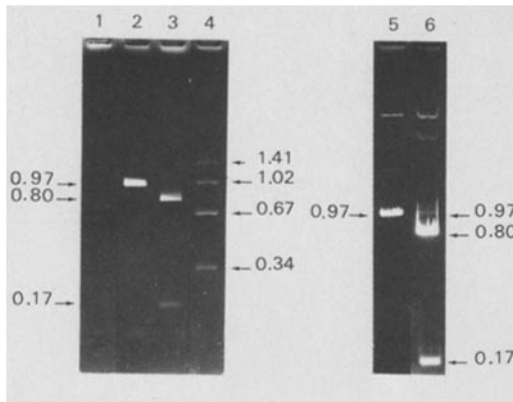
FIGURE 11 2% agarose gel electrophoresis of the fragments generated by cleavage of core kDNA and intact kDNA network by restriction endonucleases. (1) control core kDNA; (2) core kDNA + *Hinf* I; (3) core kDNA + *Taq* I; (5) intact kDNA + *Hinf* I; (6) intact kDNA + *Taq* I; (4) *Hae* III fragments of *T. cruzi* kDNA (molecular weights are indicated in kb).

can be roughly estimated from these data as 50–100 base pairs.

Reassociation Kinetics Studies

kDNA minicircles were labeled in vitro with ^{32}P and the reassociation kinetics of the labeled fragments was studied. The reannealing of labeled DNA alone and in the presence of a 1-, 5-, and 10-fold molar excess of unlabeled kDNA minicircles follows a simple second-order kinetics (25) as shown in Fig. 16. This result indicates the presence of a single class of nucleotide sequence in the minicircle population. The time required for half renaturation of the DNA (t $^1/_2$) is equal respectively to 15.1 h, 7.2 h, 2.73 h, and 1.57 h in the four experiments of Fig. 16. The average value of Cot $^1/_2$ calculated from these measurements is 3.0 × 10^{-4} M × s (the DNA concentration Co, being expressed in moles of nucleotides per liter and the time, t, in seconds). We estimate that the accuracy of our measurements permits the detection of 0.3 copy of kDNA minicircle sequence. When reassociation is carried out in the presence of a 45-fold molar excess of unlabeled mtDNA (assuming an average mol wt of 14 × 10^6 daltons (20) for this DNA) the rate of the reaction is not significantly different (Fig. 16B). We conclude that the mtDNA contains in the average <7 × 10^{-3} copy of the minicircle sequence per molecule. Similar experiments were carried out using in vitro ^{32}P-labeled kDNA large circle fragments. The rean-

nealing reaction follows a simple second-order kinetic as shown in Fig. 17. The time of half renaturation is equal to 11.9 h, 4.1 h, and 2.0 h, respectively, in the absence of unlabeled DNA and in the presence of a 2- and 5-fold molar excess of unlabeled kDNA large circle fragment. The average value of Cot $^1/_2$ deduced from these measurements is 5.9 × 10^{-3} M × s. The same Cot $^1/_2$ value within experimental error is observed in the presence of an 11-fold molar excess of unlabeled mtDNA (Fig. 17). This result indicates that the mtDNA contains in the average <0.03 copy of the kDNA large circle sequence per molecule.

DISCUSSION

The kinetoplast DNA usually observed by light microscopy after cytological staining, by direct examination of ultrathin sections of trypanosomes in the electron microscope and after purification by various physicochemical techniques is absent from the naturally occurring dyskinetoplastic *T. equiperdum*. Moreover, in this strain, clumps of intramitochondrial DNA can be revealed with fluorescent dyes (8) or detected by electron mi-
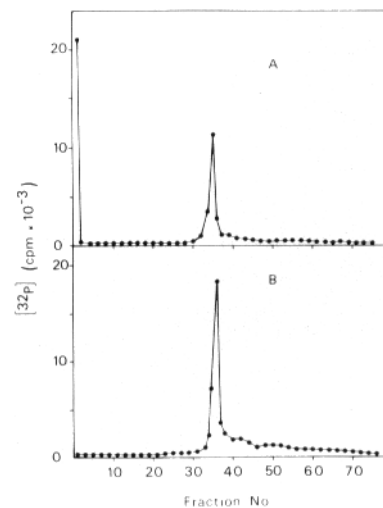

FIGURE 12 2% agarose gel electrophoresis of ^{32}P-labeled core kDNA intact (*A*) and after cleavage with *Hinf* I (*B*). Purified core kDNA was labeled in vitro with ^{32}P as described in Materials and Methods to a specific activity of 3.9 × 10^7 cpm/μg. After 2 h of digestion with *Hinf* I, the DNA was analysed by gel electrophoresis. The gel was cut into 1.7-mm slices and the radioactivity of each fraction was measured. Unlabeled fragments of core kDNA generated by *Hinf* I cleavage were used as markers. The linear fragments of 0.97 kb comigrated with fractions 35–36.
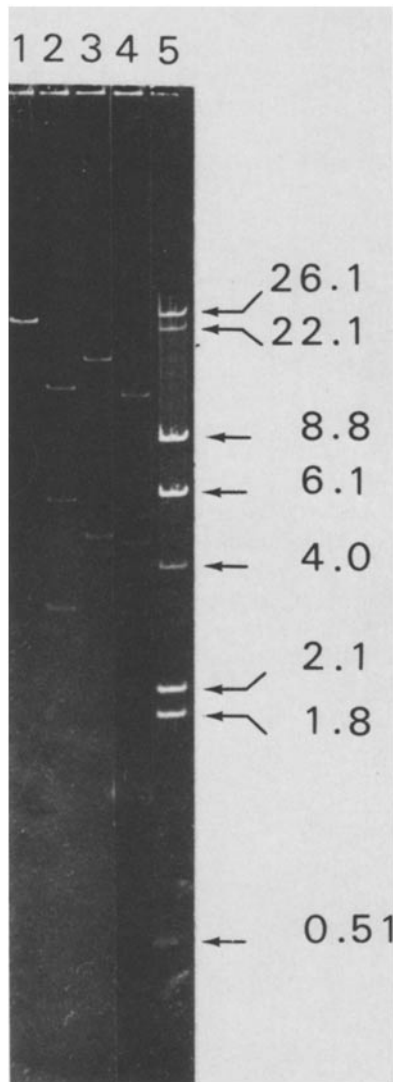
of circular molecules heterogeneous in length. The present study confirms this observation. Furthermore, our results indicate that the mtDNA has a buoyant density slightly smaller than that of the
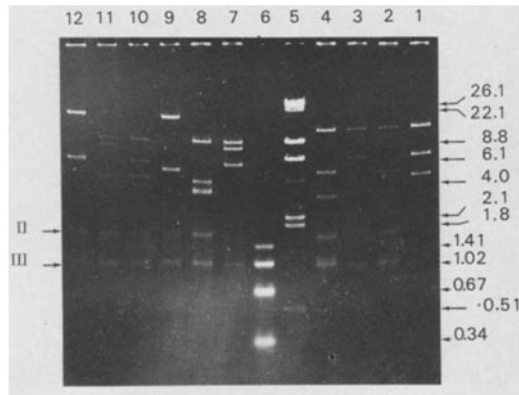

FIGURE 14 Molecular weight determination by agarose-polyacrylamide gel electrophoresis of the fragments generated by cleavage of kDNA network by (1) *Eco* R1; (2) *Eco* R1 + *Pst* I; (3) *Eco* R1 + *Bam* H1; (4) *Eco* R1 + *Hae* III; (7) *Hind* III; (8) *Hind* III + *Hae* III; (9) *Hae* III; (10) *Hind* III + *Bam* H1; (11) *Hind* III + *Pst* I; (12) *Bam* H1 + *Pst* I; (5) *Hind* III fragments of λ DNA; (6) *Hae* III fragments of *T. cruzi* kDNA (molecular weights are indicated in kb). II and III indicate, respectively, the position of nicked minicircles and linear fragments of the size of the minicircles (0.97 kb).

FIGURE 13 Molecular weight determination by agarose-polyacrylamide gel electrophoresis of the fragments generated by cleavage of kDNA network by (1) *Bam* H1; (2) *Hpa* II; (3) *Hae* III; (4) *Hpa* II + *Hae* III; (5) λ DNA fragments obtained by *Hind* III cleavage (molecular weights are indicated in kb).

croscopy of ultrathin sections. We have obtained evidence (Riou, G. Manuscript in preparation) for the presence of DNA in these clumps by the method of selective destaining of DNA as described by Bernhard (1) and Delain et al. (5). We have shown in a previous report (20) that the mtDNA extracted from the dyskinetoplastic *T. equiperdum* is in the form of catenated oligomers
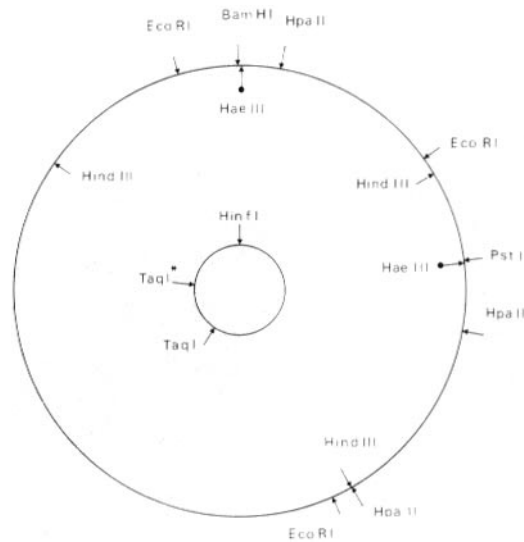

FIGURE 15 Restriction cleavage maps of kDNA minicircle and large circle of *T. equiperdum* wild type. The two maps are not drawn to the same scale. (*) This site is cleaved by incubation at 60°C but not at 37°C.
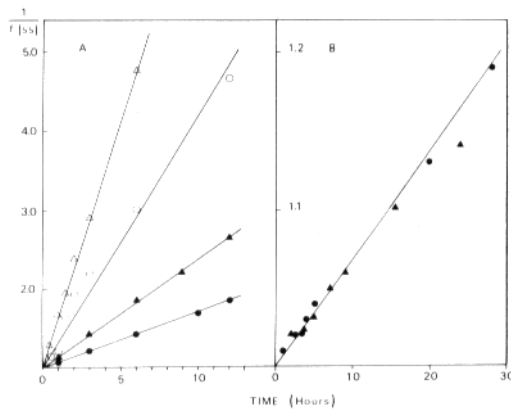
FIGURE 16 Reassociation kinetics of in vitro labeled kDNA minicircle fragments. kDNA minicircle fragments obtained by cleavage of core kDNA with *Hinf* I and purified by low-speed centrifugation were labeled with ^{32}P as described in Materials and Methods. The DNA had a specific activity of 4.7×10^7 cpm/μg, migrated as a single band of 0.97 kb in agarose gel electrophoresis and gave, at alkaline pH, single-strand fragments of ~400 nucleotides. The reaction mixture contained 1.75 ng of labeled DNA per ml (panel A) alone (−●−) or with 1.75 ng/ml (−▲−), 8.75 ng/ml (−○−), 17.5 ng/ml (−△−) of unlabeled kDNA minicircle fragments added. In panel B, the reaction mixture contained 0.2 ng of labeled DNA per ml alone (−●−) or with 0.2 μg/ml of unlabeled mtDNA fragments of the dyskinetoplastic strain added (−▲−). Unlabeled fragments prepared by sonication had an average size of ~400–500 nucleotides as measured by analytical ultracentrifugation at alkaline pH. The reciprocal of the fraction of the single-strand DNA ($1/f_{ss}$) is plotted as a function of time.

kinetoplast minicircles of the wild strain and is resistant to cleavage by eight different restriction endonucleases.

The kDNA of *T. equiperdum* contains two components topologically linked differing by their buoyant density, molecular weight, and susceptibility to restriction endonucleases. Upon incubation with various endonucleases, one component is selectively cleaved and liberated from the network, whereas the 6.4×10^5-dalton minicircles which have been extensively described (16, 18) remain intact and topologically associated in a core kDNA network. When the kDNA networks are examined in the electron microscope, the endonuclease-sensitive component appears as long loops protruding at the periphery of the network and long segments stretched in the network. These loops and long segments are liberated

from the network by a unique double-strand scission upon digestion with restriction endonucleases. We conclude that they are circles topologically interlocked in the network. Moreover, our results indicate that these kDNA large circles are covalently closed.

As the kDNA large circles have a buoyant density 7 mg/cm³ smaller than that of the intact network, it is possible to estimate their relative amount in the network by analytical ultracentrifugation in a CsCl gradient after selective cleavage by *Bam* H1. The measurement of the area under the peaks indicates that the kDNA large circles account for 33% of the intact kDNA network. The expected buoyant density for a network composed of 67% of minicircles ($\rho = 1.691$ g/cm³) and 33% of large circles ($\rho = 1.684$ g/cm³) is $\rho = 1.689$ g/cm³. The buoyant density of the intact kDNA network actually measured is 1-2 mg/cm³ higher. Similarly, the buoyant density of the 0.97 kb linear fragments obtained by cleavage of the minicircle is slightly smaller than that of the core kDNA. These differences can be explained by
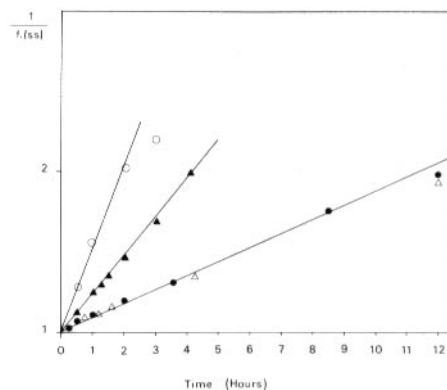

FIGURE 17 Reassociation kinetics of kDNA large circles. The linear form of kDNA large circles obtained by cleavage with *Bam* H1 was isolated by equilibrium density centrifugation in CsCl-EthBr gradient and purified by low-speed centrifugation. The DNA labeled with ^{32}P to a specific activity of 2.0×10^6 cpm/μg had a size of ~500 nucleotides as measured by alkaline sucrose gradient centrifugation. The reaction mixture contained 45 ng of labeled DNA per ml. Reassociation was studied in the absence of unlabeled DNA (−●−), in the presence of 0.135 μg/ml (−▲−), 0.270 μg/ml (−○−), of unlabeled kDNA large circle fragments and in the presence of 0.45 μg/ml (−△−) of unlabeled mtDNA fragments of the dyskinetoplastic strain. The reciprocal of the fraction of single-strand DNA ($1/f_{ss}$) is plotted as a function of time.

assuming that the kDNA in the intact network and, to a smaller extent, in the core network has a higher density than a DNA of the same chemical composition but in a less compact conformation. We note that a comparable phenomenon has been observed with circular DNA of high negative superhelical density which also has presumably a very compact structure (31). From the measurement of the buoyant density, a composition of 32% GC base pairs can be estimated for the minicircles and 24% for the large circles using the data of Schildkraut et al. (24), provided no abnormal bases are present. Simpson and Simpson (27) have isolated from the kinetoplast of *L. tarentolae* two RNA species containing ~20% G+C and have shown that these RNAs are transcription products of the kDNA large circles. This result indicates that the *L. tarentolae* maxicircles have sequences composed of AT base pairs in high proportion.

The total kDNA content of *T. equiperdum* has been previously found equal to 7.7×10^{-14} g/cell (19). Assuming that the kDNA represents 6% of the total DNA and taking the relative amount of large kDNA circles as equal to 30%, we find that one kinetoplast is a network of ~3,000 minicircles of 6.4×10^5 daltons topologically interlocked with ~50 large circles of 15.4×10^6 daltons. These results have been obtained with kDNA networks under form I. kDNA form II has been isolated from trypanosomes in exponential growth and has been shown to contain about twice the amount of DNA of the form I (Bénard, J., G. Riou, and J. M. Saucier. Manuscript in preparation). The data of Englund (6) indicate that in the case of *Crithidia fasciculata* it is the replicative form of kDNA.

Recently, Fairlamb et al. (7) reported the absence of DNA loops in the *T. equiperdum* kinetoplast observed by electron microscopy and analysed by gel electrophoresis after incubation with restriction endonuclease *Pst* I. A different origin of the *T. equiperdum* strain used by these authors might explain the discrepancy between their results and our own observation. However, in *T. brucei*, another trypanosome species belonging to the subgenus Trypanozoon, these authors have described maxicircles of 12-13 $\times$ 10⁶ daltons as a component of the kDNA networks. It is interesting to note that these maxicircles are cleaved by *Pst* I in one fragment and by *Eco* R1 in three fragments. It is possible that the two smaller fragments generated by *Eco* R1 cleavage of the *T. brucei* maxicircles have the same molecular weight

as those generated by *Eco* R1 cleavage of *T. equiperdum* kDNA. This might be an indication that the large circles of these two species are derived from one another by deletion or insertion of a DNA fragment of ~3 $\times$ 10⁶ daltons. A restriction enzyme cleavage map will probably be very useful to study the phylogenetic relationship between the different species of Trypanosomatidae.

Reassociation reactions of the kDNA minicircle and large circle follow a simple second-order kinetics indicating that these DNAs are homogeneous in sequence. The kinetic complexity of the kDNA large circle calculated from the measured Cot ½ value (34) is found equal to 20 times the kinetic complexity of the kDNA minicircle in agreement with the molecular weight ratio of these DNAs. The reassociation reaction of *T. cruzi* kDNA minicircles follows complex kinetics indicating sequence heterogeneity of these molecules (21). Sequence heterogeneity is also apparent in gel electrophoresis patterns of the fragments obtained after restriction endonuclease cleavage of the minicircles of *T. cruzi* (21) and other species of trypanosomes (reviewed in reference 2). In contrast, cleavage of *T. equiperdum* core kDNA minicircle with *Hinf* I and *Taq* I is complete as shown by the gel electrophoresis pattern of ³²P-labeled kDNA (Fig. 12B) and by equilibrium density centrifugation (Fig. 5E). These observations confirm our conclusion regarding the existence of a single class of minicircular molecules.

We have mapped in the kDNA minicircle three sites of cleavage by two restriction endonucleases and in the kDNA large circles thirteen sites recognized by six different restriction enzymes which do not cleave the minicircles. The limited number of cleavage sites per DNA molecule has made this mapping relatively easy. However, there is some inaccuracy in the determination of the size of the fragments which have an electrophoretic mobility close to that of the minicircle and the linear fragments of the size of the minicircle. For this reason, there is some uncertainty in the respective location of two cleavage sites when their distance is smaller than 0.2 kb. The map in Fig. 15 shows the most probable positions of the different cleavage sites: it is interesting to note that a region corresponding to ~40% of the large circles is not cleaved by any of the six enzymes tested. This may be because this region is particularly poor in GC base pairs.

The results of the present study indicate that

the mtDNA of the dyskinetoplastic *T. equiperdum* is very different from the kDNA of the wild strain. Reassociation kinetics studies strongly suggest that the mtDNA has no common sequences with kDNA minicircles or large circles. Since we have not detected any kDNA in the dyskinetoplastic *T. equiperdum* or mtDNA in the wild strain, a surprising consequence of this observation is that this genetic material is not necessary for the viability of parasites.

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