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 x 10^5 daltons and ~50 circular supercoiled molecules of 15.4 x 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 · dyskinetoplastic · 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 dyski-
etoplastic 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-phen-
ylindole) staining (8). Furthermore, it has not been possible to isolate minicircles from a dyski-
etoplastic 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 dyskine-
toplasmic strain of T. equiperdum. These circles constitute about 30\% of the total kDNA.

We have characterized by several techniques the components of kDNA networks of T. equiper-
dum 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 about 30\% of the total kDNA and have a low buoyant density in CsCl.

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 
\(-190^\circ\text{C}\) in rat blood and glycerol. Trypanosomes were inoculated into Wistar rats. Maximal parasitaemia was reached in 3-4 d and blood was withdrawn. Trypano-

Preparation and Fractionation of kDNA and mtDNA

Total DNA was extracted from the two strains, and kDNA and mtDNA were fractionated in propidium diodide (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 $^{32}$PdCTP. The reaction mixture contained in 200 µl of final volume, 250 ng of kDNA, 30 µCi of $^{32}$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 ($S_{0,20} = 5.9$). The three peak fractions after dialysis were used for the renaturation kinetics studies.

Restriction Endonuclease Cleavage of kDNA

Eco RI, Hind III, Hae III, Hpa II, Pst I, Hind 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-
Reassociation Kinetics

Analytical Ultracentrifugation

Analytical Ultracentrifugation

Electron Microscopy of DNA

Characterization of the Circular DNA
Molecules of the Dyskinetoplastic Strain
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 I, Hae III, Hpa II, Hind III, Eco R1, and HinfI 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 incubation carried out at 60°C with this enzyme. This mtDNA is hydrolysed by pancreatic DNase as shown in Fig. 3C.

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. 5A) at least two components are observed: kDNA with a density $\rho = 1.690 \text{ g/cm}^3$ and nuclear DNA banding at $\rho = 1.707 \text{ g/cm}^3$ 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 catenate 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 MgCl₂, 15 mM NaCl, pH 7.4). Micrococcus lysodeikticus DNA (ρ = 1.731 g/cm³) was used as density marker.

FIGURE 4 Electrophoresis in 0.5% agarose-1.8% polyacrylamide gel of the mtDNA from the dyskinetoplastic 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) Hind 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 × 10⁶ 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/cm³) was used as density marker.
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

![Electron micrograph of intact kDNA network form I from wild type T. equiperdum spread by the microdiffusion technique. Bar, 1 μm. × 29,000.](image_url)
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; (4) Hind III; (5) Eco RI; (6) Hpa II; (7) Hae III; (8) Hind III; (9) Eco RI; (10) Hpa II. (4) λ DNA fragments obtained by Hind III cleavage and (5) T. cruzi kDNA fragments obtained by HaeIII 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 $15.4 \times 10^6$ daltons as measured by electron microscopy using PM2 DNA as a standard of $6.30 \times 10^6$ daltons (11). This measurement is confirmed by the molecular weight determination of the fragments generated by the various restriction endonucleases as shown in Table 1. 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 g/cm³ (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 g/cm³ (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...
Molecular Weights Expressed in kb of the Fragments Generated by Restriction Endonuclease Cleavage of kDNA Large Circles

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 minicircle 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/cm³ (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 I). 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.
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 cleavage 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...
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 + HinfI; (3) core kDNA + Taq I; (5) intact kDNA + HinfI; (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 $t_{1/2}$ calculated from these measurements is $3.0 \times 10^{-3}$ M x 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 $14 \times 10^6$ 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 $<7 \times 10^{-3}$ copy of the minicircle sequence per molecule. Similar experiments were carried out using in vitro $^{32}$P-labeled kDNA large circle fragments. The reannealing 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 $t_{1/2}$ deduced from these measurements is $5.9 \times 10^{-3}$ M x s. The same Cot $t_{1/2}$ 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-
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 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 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).

**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 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 Hinfl and purified by low-speed centrifugation were labeled with ³²P as described in Materials and Methods. The DNA had a specific activity of 4.7 × 10⁶ 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/fₐ) is plotted as a function of time.

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 ³²P to a specific activity of 2.0 × 10⁶ 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 (– – –), 0.45 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/fₐ) 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 × 10¹⁰-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 (ρ = 1.691 g/cm³) and 33% of large circles (ρ = 1.684 g/cm³) is ρ = 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...
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 \times 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 \times 10^6 daltons topologically interlocked with ~50 large circles of 1.54 \times 10^9 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 Crithididae 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 10^9 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 10^9 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 1/2 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 detected no 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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REFERENCES
1. Bernhard, W. 1969. A new procedure for electron microscopical cytology. J. Ultrastruct. Res. 27:250-265.
2. Borst, P., and A. H. Fairlamb. 1976. DNA of parasites, with special reference to kinetoplast DNA. In Biochemistry of Parasites and Host-Parasite Relationships. H. Van den Bosch, editor. Elsevier North-Holland Biomedical Press, Amsterdam. 169-191.
3. Bruner, R., and J. Vinograd. 1965. The evaluation of standard sedimentation coefficients of sodium RNA and sodium DNA from sedimentation velocity data in concentrated NaCl and CaCl2 solutions. Biochem. Biophys. Acta. 108:18-29.
4. Delain, E., C. Brack, A. Lacome, and G. Rou. 1972. Organization of the DNA in the kinetoplast of Trypanosoma cruzi. In Comparative Biochemistry of Parasites. H. Van den Bosch, editor. Elsevier North-Holland Biomedical Press, Amsterdam. 169-191.
5. Delain, E., C. Brack, G. Rou, and B. Festy. 1971. Ultrastructural alterations of Trypanosoma cruzi kinetoplast induced by the interaction of a trypanocidal drug (hydroxyaminthidine) with the kinetoplast DNA. J. Ultrastruct. Res. 37:200-218.
6. Englund, P. T. 1978. The replication of kinetoplast DNA networks in Crithidia fasciculata. Cell. 14:157-168.
7. Fairlamb, A. H., P. O. Weisselug, J. H. H. Holmnaers, and P. Borst. 1978. Isolation and characterization of kDNA from bloodstream form of Trypanosoma cruzi. J. Cell Biol. 76:293-309.
8. Haidar, S. L. 1971. Influence of DNA complexity on the progression of the trypanosomatids. Prog. Mol. Subcell. Biol. 6:153-160.
9. Lang, D., T. N. Taylor, D. C. Dorvan, and D. M. Gray. 1976. Dehydrated circular DNA: electron microscopy of ethanol condensed molecules. J. Mol. Biol. 106:57-107.
10. Lannam, S. M., and D. G. Gottfried. 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE cellulose. Exp. Parasitol. 28:521-534.
11. Mackey, J. K., K. H. Brackmann, M. R. Green, and M. Green. 1977. Preparation and characterization of highly radioactive in vitro labeled adenovirus DNA and DNA restriction fragments. Biochemistry. 16:4478-4483.
12. Ono, D. A., and H. B. Gray. 1973. Sedimentation and intrinsic viscosity behavior of PM2 bacteriophage DNA in alkaline solution. Biopolymer. 12:1387-1419.
13. Pautznel, H. C., and D. R. Wolstenholme. 1970. Kinetoplast deoxyribonucleic acid of the hemoflagellate Trypanosoma lewisi. J. Cell Biol. 47:790-802.
14. Pautznel, H. C., and D. R. Wolstenholme. 1971. Kinetoplast and other satellite DNAs of kinetoplastic and dyskinetoplastic strains of Trypanosoma. J. Cell Biol. 50:333-340.
15. Rou, G., and W. E. Guttmann. 1978. Comparative study of kDNA in culture, blood and intracellular stages of Trypanosoma cruzi. Biochem. Biophys. Res. Commun. 84:790-795.
16. Rou, G., A. Lacome, C. Brack, E. Delain, and R. Pautznel. 1977. Importance de la méthode d'extraction dans l'isolement de l'ADN de kinetoplaste de trypanosome. C.R. Acad. Sci. Paris. Sér. D. 275:2150-2153.
17. Rou, G., and R. Pautznel. 1969. Nuclear and kinetoplast DNA from Trypanosoma. J. Protozool. 16:509-513.
18. Rou, G., and R. Pautznel. 1977. Isolation and characterization of circular DNA molecules heterogeneous in size from a dyskinetoplastic strain of Trypanosoma equiperdum. Biochem. Biophys. Res. Commun. 78:1084-1091.
19. Rou, G., and P. Yot. 1977. Heterogeneity of the kinetoplast DNA molecules of Trypanosoma cruzi. Biochemistry. 16:2350-2356.
20. Sanger, F., G. M. Air, B. G. Barrell, N. L. Brown, A. R. Coulson, J. C. Fields, C. A. Hutchinson III, P. M. Slocomb, and M. Smith. 1977. Nucleotide sequence of bacteriophage-4 X174 DNA. Nature (Lond.) 265:687-695.
21. Sato, S., C. A. Hutchinson III, and J. L. Haber. 1977. A thermostable sequence specific endonuclease from Thermus aquatissim. Proc. Natl. Acad. Sci. U. S. A. 74:542-546.
22. Scherwartz, C. L., J. MacKer, and P. Doty. 1962. Determination of the base composition of deoxyribonuclide acid and its buoyant density in CaCl2. J. Mol. Biol. 4:430-443.
23. Sharp, F. A., U. Petterson, and J. Salmbrook. 1974. Viral DNA in transformed cells. I. A study of the sequences of adenovirus 2 DNA in a line of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. 86:704-716.
24. Simpson, L. 1972. The kinetoplast of the hemoflagellates. Int. Rev. Cytol. 32:139-207.
25. Simpson, L., and A. M. Simpson. 1978. Kinetoplast RNA of Leishmania species. Cell. 14:169-178.
26. Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. 11:373-390.
27. Tomi, E. J. 1971. Loss of the kinetoplast in a strain of Trypanosoma cruzi. Exp. Mol. Med. 7:231-254.
28. Vickerman, K., and T. M. Preston. 1976. Comparative cell biology of the kinetoplastid flagellates. In Biology of the Kinetoplastidae. W. H. R. Lumsden, and D. A. Evans, eds. Academic Press, Inc., N. Y. vol. I. 15-130.
29. Wang, J. C. 1974. The degree of unwinding of the DNA helix by ethidium. I. Titration of twisted PM2 DNA molecules in alkaline cesium chloride density gradients. J. Mol. Biol. 87:751-811.
30. Weisselug, P. O., J. H. H. Holmnaers, A. H. Fairlamb, C. M. Kleihorst, and P. Borst. 1977. Characterization of kDNA networks from the insect trypanosome Crithidia luciliae. Biochem. Biophys. Acta. 478:167-179.
31. Weisnero, P. K., R. H. Redeker, D. Carroll, D. D. Brown, A. Dutsch, T. Higashinakagawa, and J. B. David. 1974. Amplified ribosomal DNA from Xylophaga lavis has heterogeneous spacer lengths. Proc. Natl. Acad. Sci. U. S. A. 71:2823-2827.
32. Weisner, J. G., and N. Davidson. 1968. Kinetics of renaturation of DNA. J. Mol. Biol. 31:349-370.