A Nicked Form of Kinetoplast DNA in Leishmania tarentolae*

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The mitochondrial DNA of the protozoan Leishmania tarentolae, known as kinetoplast DNA, contains thousands of minicircles linked in a two-dimensional network. When kinetoplast DNA from exponentially growing cells is centrifuged to equilibrium in a CsCl/ethidium bromide gradient, it is resolved into two discrete components, Form I and Form II. Nearly all of the minicircles in Form I networks are covalently closed and all of those in Form II networks are open. These forms are indistinguishable from each other when examined by electron microscopy and they appear identical when analyzed by gel electrophoresis after digestion with the restriction enzymes Hae III or Hpa II. However, Form II networks sediment roughly 50% faster than Form I networks on a neutral sucrose gradient, indicating that Form II networks are larger in size or more compact in conformation, or both. Analysis of denatured Form II DNA by sedimentation or electron microscopy indicates that nearly all of its minicircles have one or more interruptions in both strands. Since the majority of the Form II minicircles can be closed by DNA ligase, most of these interruptions must be nicks. Experiments with S, nuclease indicate that some small gaps may also exist in Form II minicircles. 5'-Terminal nucleotide analysis of Form II minicircles does not suggest that the interruptions are at specific locations in the minicircles. The significance of the two forms of kinetoplast DNA has not yet been determined, but it is possible that Form II is an intermediate in replication of this DNA.

Kinetoplast DNA is an extremely complex structure which is found in the mitochondria of trypanosomes and related protozoa (1). Its major structural components are minicircles, which in the case of Leishmania tarentolae are only about 1000 base pairs in size (2). Nearly all of the minicircles are linked together in massive networks, and each network contains roughly 15,000 minicircles (3). There appears to be only one network/mitochondrion, and only one mitochondrion/cell (3).

Although it was originally assumed that all minicircles within a network are identical, recent investigations involving restriction enzyme analysis and reassociation kinetics have revealed that networks contain numerous types of minicircles which have nonidentical but probably related nucleotide sequences (4-11). Analysis of kDNA with restriction enzymes and also by electron microscopy has recently revealed a second type of structural component in networks (5, 12, 13). This component, known as the maxicircle, accounts for less than 5% of the total kDNA and has a molecular weight of about $22 \times 10^6$ in kDNA of Crithidia luciliae and about $13 \times 10^6$ in kDNA of Trypanosoma brucei (8). Although nothing is known about the genetic function of maxicircles and minicircles, Borst and co-workers have speculated that maxicircles carry the genes which are characteristic of mitochondrial DNA in other eukaryotic species (8).

Despite these recent advances in our understanding of the sequence complexity of kDNA, there is still very little known about the mechanism by which the networks grow in size during the period of DNA synthesis, and about the way in which the kDNA segregates to form two networks within the daughter cells during cell division. These processes cannot be understood until the replicative intermediates of kDNA are identified and their structures are determined. As an initial approach to these questions, we have investigated the structure of a form of kDNA which usually accounts for about one-quarter of the kDNA in dividing cells, but which is absent in stationary phase cells (14). These networks, known as Form II, contain minicircles which are nicked or gapped, whereas the major species of kDNA, known as Form I, contains minicircles which are covalently closed. An additional difference between these two types of kDNA is that Form II networks sediment faster than Form I networks. We describe here our comparison of Form I and Form II kDNAs. Some of our conclusions have already been published in preliminary form (10).

EXPERIMENTAL PROCEDURES

Materials

Leishmania tarentolae was obtained from American Type Culture Collection (ATCC 30143), and was grown in Difco brain heart infusion medium supplemented with hemin (20 μg/ml) (15). Stock cultures were grown in cotton-stopped Erlenmeyer flasks at 27°C in a

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The abbreviation used is: kDNA, kinetoplast DNA.
water bath shaker, and larger cultures (5 or 10 liters) for isolation of kDNA were grown in a New Brunswick Microferm Fermentor (27); rotor speed, 200 rpm; air, 10 liters/min. For small stock cultures, the medium was autoclaved for 20 min. For large scale preparations using the fermentor, the medium was filter-sterilized (Millipore type GS) and it contained 20 units/ml of penicillin, 100 mcg/ml of streptomycin (both from Microbiological Associates), and 0.1 ml/liter of Dow Antifoam C. [PH]Thymidine (20 mcCi/mmol; New England Nuclear) was added at a concentration of 2 mcCi/ml together with the inoculum (about 5 x 10^5 cells/5 liters of medium). Cells were harvested by centrifugation when they had grown to a cell concentration between 5 x 10^5/ml and 2 x 10^6/ml. They were growing exponentially even at the latter density.

kDNA was isolated by differential centrifugation of a cell lysate essentially as described by Simpson and Berliner (18), and nearly all of the residual nuclear DNA was removed by preparative centrifugation in a CsCl gradient. For this gradient each sample, containing up to 200 mcg of DNA in a solution (refractive index 1.4002) prepared from 3.0 g of CsCl and 4.0 ml of 25 mM Tris/HC1, pH 8.0, was centrifuged at 44,000 rpm in a Spinco 50 rotor for 24 hours at about 4°C. Fractions were collected from the bottom of the tube and assayed by spotting 2-μl samples on Whatman No. 3MM filter disks. These were washed sequentially in 5% trichloroacetic acid, ethanol, and ether, and after drying were counted with a toluene fluid in a scintillation counter. Recoveries of DNA from CsCl gradients were in the range of 60 to 90%. After pooling the appropriate fractions, the kDNA was centrifuged to equilibrium in a CsCl/ethidium bromide gradient for the purpose of separating Form I and Form II kDNAs. DNA in the presence of this dye was kept in subdued light. Each sample, containing about 150 mcg of kDNA in a solution (refractive index 1.3883) prepared from 5.0 g of CsCl, 1.0 mg of ethidium bromide, and 5.0 ml of 20 mM Tris/HC1, pH 8, was centrifuged and assayed as described for the preparative CsCl gradients. Recoveries of kDNA were also in the range of 60 to 90%. After pooling the Form I and Form II DNA fractions, the ethidium bromide was removed by extraction with water-saturated l-butanol and the DNA was dialyzed against 50 mM NaCl, 10 mM Tris/HC1 (pH 8), and 1 mM EDTA.

The specific radioactivity of kDNA ranged from 4000 to 10,000 cpm/μg, and in any preparation the specific radioactivities of Form I and Form II kDNAs were about the same.

Methods

Enzymatic Modifications of kDNA—3'-Terminal labeling of kDNA was carried out with T4 DNA polymerase and [α-32P]dATP (15). The reaction mixture (100 μl) contained kDNA (1.6 μg of Form I or 0.7 μg of Form II), 70 mM Tris/HC1 (pH 7.8), 7 mM MgCl2, 10 mM 2-mercaptoethanol, 0.12 mM [α-32P]dATP (5000 cpm/μmol; synthesized as described previously (18)), and 20 units of T4 DNA polymerase (Fraction VI (23)). After 60 min at 11°C, the reactions were terminated by chilling, addition of 10 μl of 0.5 M EDTA, and phenol extraction. The solutions were dialyzed first against 1.0 M NaCl, 10 mM Tris/HC1 (pH 7.8), 1 mM EDTA to remove unincorporated [α-32P]dATP, and then against 50 mM NaCl, 10 mM Tris/HC1 (pH 7.8), 1 mM EDTA.

5'-Terminal labeling of Hae III digests of kDNA was carried out with T4 polynucleotide kinase and [γ-32P]ATP (24). The kDNA was first digested with Hae III at 37°C for 2 h in a reaction mixture (48 μl) containing 0.17 μg of Form I or 0.27 μg of Form II kDNA, 6 mM Tris/HC1 (pH 7.4), 6 mM NaCl, 6 mM MgCl2, 6 mM 2-mercaptoethanol, and 1 unit of Hae III (Bethesda Research Laboratories). These conditions were sufficient for complete digestion. The reactions were stopped by the addition of 10 μl of 0.1 M EDTA. To remove phosphomononucleotides from the DNA fragments, 2 x 10^7 unit of Escherichia coli alkaline phosphatase ( Worthington BAPC, assayed as described in the Worthington catalog and rechromatographed on DEAE-cellulose (20)) was then added and the incubations were continued at 37°C for 1 h. The solutions were deproteinized by extracting three times with an equal volume of neutralized phenol, and then they were dialyzed against 10 mM Tris/HC1 (pH 8.0), 1 mM EDTA. The fragments were rephosphorylated with [γ-32P]ATP and polynucleotide kinase (21). The reaction mixtures (100 μl) contained the dephosphorylated fragments (0.17 μg of Form I or 0.27 μg of Form II), 1 μM [γ-32P]ATP (1000 to 2000 Ci/mmol), 50 mM Tris/HC1 (pH 9.4), 10 mM MgC12, 5 mM dithiothreitol, 5% glycerol, 0.1 mM spermidine, and 2.1 units of T4 polynucleotide kinase using a method similar to that described above except that the DNA was denatured by boiling prior to the alkaline phosphatase treatment. The [32P]DNA was degraded to 5'-mononucleotides using pancreatic DNase and snake venom phosphodiesterase in sequential reactions (24), and the nucleotides were separated by paper chromatography (16).

Electron Microscopy—DNA was prepared for microscopy by the formamide technique of Davis et al. (25). When using this method in the presence of ethidium bromide, the dye concentration in the spreading solution was 100 μg/ml and in the hypophase was 1 μg/ml. Micrographs were taken on a JEM 100B electron microscope, and molecules were measured on projected micrograph images using a digital length calculator (Numonics Corp.). Calibration was with a grating replica from E. F. Fullam (2160 lines/mm).

RESULTS

When purified kDNA from exponentially growing Leishmania tarentolae is centrifuged to equilibrium in a CsCl/ethidium bromide gradient, two discrete components are observed (10, 14). We have designated these components Form I and Form II (Fig. 1). The relative amounts of the two species are somewhat variable, although usually the amount of Form II is much greater than that of Form I. Nevertheless, by using a preparative CsCl gradient, purified kDNA was isolated essentially free of contaminating material (16, 17).

FIG. 1. Preparative CsCl/ethidium bromide gradient of [3H]-

The pH of all buffers was measured at room temperature and at a concentration of 50 mM.
II is roughly one-half of the amount of Form I. The DNA which bands between Forms I and II is usually in the range of 15 to 20% of the total. Form I DNA contains minicircles which are nearly all covalently closed, and Form II DNA contains open minicircles with either nicks or gaps (10, 14). The purpose of the experiments described in the following paragraphs is to compare the properties of these two forms of kDNA.

Electron Microscopy of Form I and Form II DNA – When examined by electron microscopy, both forms appear as networks which are typical of kinetoplast DNA (Fig. 2). Both are composed primarily of 0.3-μm minicircles which are catenated, fused in figure-8 structures, joined in rosettes, or linked in other complex structures. Both also contain some "linear" DNA which may be part of maxicircles (5, 12, 13). Form II
preparations contain occasional free linear molecules of random length which are not joined to the networks, but those are probably contaminating nuclear DNA. After examining many micrographs of several different preparations of kDNA, we are unable to detect any characteristic differences between Forms I and II. Unfortunately, the sensitivity of Leishmania networks to breakage makes it difficult to examine the size of intact networks by this technique. The only way that the two forms can be distinguished by electron microscopy is by spreading the DNA in the presence of ethidium bromide (Fig. 2). In this case nearly all (>80%) of the minicircles in Form I, which are covalently closed, become tightly twisted. In contrast, virtually all of the minicircles in Form II, which contain single strand breaks, remain untwisted.

**Restriction Enzyme Analysis of Forms I and II**—Because the minicircles in kDNA networks are heterogeneous in nucleotide sequence (4–11), gel electrophoresis of restriction enzyme digests reveals complex patterns of fragments. We compared digests of Forms I and II kDNA for the purpose of determining whether the same pattern of fragments is obtained from both forms. As shown in Fig. 3, the two Hae III digests are similar if not identical, and the same result was obtained with Hpa II digests. Most of the fragments shown in the Hae III digests (Fig. 3) derive from minicircles. However, electrophoresis of Hpa II digests on 1.4% agarose gels reveals three fragments with a combined molecular weight of about 20 × 10^6 and which presumably derive from maxicircles. These fragments are also identical in both forms of kDNA. These results support the possibility that the same nucleotide sequences are present in the two forms of kDNA and that the different classes of circles are present in the same ratio.

**Sedimentation of Form I and Form II kDNA in Neutral Sucrose Gradients**—One of the most striking properties of kDNA is its enormous sedimentation coefficient (3, 27). After zone sedimentation of Form I kDNA in a neutral sucrose gradient, most of the DNA is found in a single peak which sediments about 2.5 times faster than T7 phage. (The S_{20,w} of the phage is 487 S (28).) This peak probably consists of intact networks, and the slower sedimenting shoulder probably contains fragments of networks (Fig. 4A). Similar results with Form I DNA have been reported by Simpson and Berliner (5). Zone sedimentation of Form II kDNA reveals that it sediments in a broad peak almost 4 times faster than T7 phage and about 1.5 times faster than Form I DNA (Fig. 4B).

**Sedimentation of Form I and Form II kDNA in Alkaline Sucrose Gradients**—Zone centrifugation of Form II kDNA in an alkaline sucrose gradient reveals that virtually all of the DNA sediments as short fragments which are equal to or less than about 1000 nucleotides in size (Fig. 5B). When Form I DNA is centrifuged under the same conditions, most of the DNA is found in the pellet, and only a small fraction (5 to 20%) in several preparations) sediments together with fragments of about 1000 nucleotides (Fig. 5A). The experiments in Fig. 5 were performed with kDNA which was both uniformly labeled with 3H and 3'-terminally labeled with 32P, and the distribution of these isotopes after centrifugation reveals several facts about the structure of the DNAs. With Form I DNA, no 32P is found in the major component which sediments to the bottom of the tube. This absence of 32P was expected if the rapidly sedimenting DNA consists of networks of covalently closed circles which are not susceptible to terminal labeling. All of the 32P in Form I DNA is found in the peak sedimenting in the position of a marker of about 1000 nucleotides and, since the ratio of 32P/3H is constant across this peak, nearly all the fragments released from Form I DNA must be about this size.

With Form II DNA, both isotopes are found only in fragments which sediment together with or slower than the 1000 nucleotide marker. The breadth of the 32P peak and the increasing 32P/3H ratio in the trailing shoulder indicate that the fragments derived from Form II DNA are heterogeneous in size with a maximum of about 1000 nucleotides.

The experiment in Fig. 5A confirms Simpson and Berliner's observation (3) that Form I networks are stable in alkali but, since some linear fragments of about 1000 nucleotides are released, a small fraction of the minicircles within these networks must contain either one single strand break or one such break in each strand. In contrast, nearly every minicircle in Form II DNA contains single strand breaks and, after denaturation in alkali, each of the segments sediments independently. The appearance of the small fragments in the gradients in Fig. 5, A and B, is not due to the presence of alkali-labile bonds in the kDNA minicircles because similar results were found with kDNA which was denatured by boiling and then centrifuged on a neutral sucrose gradient. Results identical to those in Fig. 5 were also obtained with kDNA which had been 5'-terminally labeled with polynucleotide kinase by a procedure similar to that described under "Methods."

**Single Strand Interruptions in Form II kDNA**—In agreement with the findings from the alkaline sucrose gradients, electron microscopy of Form II kDNA after heat denaturation reveals that nearly all of the DNA was converted to short single strands. Analysis of 1151 molecules on four micrographs showed that most of these strands were linear, heterogeneous in size, and shorter than a kDNA minicircle (1091 molecules; see histogram in Fig. 6 for a representative distribution of lengths). A very small fraction of the DNA was minicircular (34 molecules), another small fraction included longer linear molecules of various sizes which may have originated in kDNA networks or which may have been nuclear DNA contaminants (24 molecules), and virtually none were oligomeric minicircles (2 molecules). Since only a small fraction of circular structures survive heat denaturation, it can be concluded that most minicircles within Form II networks have interruptions in both strands. Because of the heterogeneity in size of the short linear strands formed by alkali (Fig. 5B) or by heat denaturation (Fig. 6), most of the minicircular strands probably have multiple interruptions.

**Effect of Ligase on Form II kDNA**—To test whether the interruptions in the minicircle strands of Form II kDNA are nicks or gaps, we treated this DNA with Escherichia coli DNA ligase. This enzyme seals nicks in a duplex DNA molecule, provided the apposing strands contain a 3'-hydroxyl and a 5'-phosphate (29). We assayed the extent of ligation of Form II kDNA by CsCl/ethidium bromide centrifugation and by electron microscopy of molecules spread in the presence of ethidium bromide. Neither assay detects individual acts of ligation, but reveals only the ligation of all interruptions within a given minicircle. The centrifugation assay (Fig. 7) shows that ligased Form II kDNA bands in a broad peak at a position near that of Form I DNA. This result indicates that many of the minicircles in all of the networks were covalently closed by ligase and therefore every interruption in those minicircles is a nick with appropriate termini. When the same preparation of ligased Form II kDNA was examined by electron microscopy in the presence of ethidium bromide, we found that about 50% of the minicircles had been covalently closed by the ligase treatment. The resistant minicircles presumably contain...
Fig. 3 (left). Comparison of Hae III digests of Form I and Form II kDNAs by gel electrophoresis. Digests were prepared and the fragments were 5'-terminally labeled as described under "Methods." The samples contained fragments (roughly 20,000 cpml) in 15 µl of 5 mM Tris/HCl (pH 8.0), 10% glycerol, 0.5 mM EDTA, 0.02% bromphenol blue. The electrophoresis system was described by Danna and Nathans (26) except that the gel was 8% polyacrylamide. After electrophoresis the gel was dried and autoradiographed for 40 h using Kodak XS-1 film. The arrow indicates the position of the bromphenol blue dye marker (26 cm from the origin). Fragment size was estimated by comparison with a Hae III digest of SV40 DNA (manuscript in preparation).

Fig. 4 (center). Sedimentation of [3H]kDNA in neutral sucrose gradients. The samples (0.1 ml) contained 0.4 µg of Form I or Form II [3H]kDNA and 0.36 A260 unit of a T7 phage marker. They were layered on 5-ml gradients which contained 10 mM Tris/HCl (pH 8.0), 1.0 mM EDTA, 1.0 M NaCl, and a linear gradient of 5 to 20% sucrose. A 0.2-ml cushion at the bottom of the tubes contained 80% sucrose in the same buffer. The samples were centrifuged at 15,000 rpm for 25 min at about 5°C in a Spinco SW 50.1 rotor. Fractions were collected from the bottoms of the tubes. After locating the T7 phage by A260 measurements on fractions diluted with 0.2 ml of water, the fractions were spotted on Whatman No. 3MM filters, washed, and counted as described under "Methods." Recovery of kDNA from these gradients was about 60%. The Savg of T7 phage is 457 S (28).

Fig. 5 (right). Sedimentation of kDNA in alkaline sucrose gradients. Strands were uniformly labeled with [3H] and 3'-terminally labeled with [32P] as described under "Methods." The samples, in 0.1 ml of 0.1 M NaOH, contained about 0.7 µg of Form I DNA (Panel A) or about 0.2 µg of Form II DNA (Panel B). The gradients (5.0 ml) contained 0.2 M NaOH, 0.8 M NaCl, 1.0 mM EDTA, and a linear gradient of 5 to 20% sucrose; a 0.25-ml cushion contained 80% sucrose in gradient buffer. The samples were centrifuged at 50,000 rpm for 7 h at about 5°C in a Spinco SW 50.1 rotor. Fractions were collected and analyzed for acid-insoluble radioactivity as described in the legend to Fig. 4 and under "Methods." In a control experiment, a Hpa II digest of Form I kDNA was used as a sedimentation marker. The major fragment in this digest is about 1000 base pairs (101, and its position relative to the alkali-denatured kDNA is shown by the arrow. Recovery of Form I kDNA was about 50% for [3P] and 25% for [3H] (additional [3H] is in the pellet), and that for Form II kDNA was about 70% for both isotopes. In another experiment with Form II [3H]kDNA, in which centrifugation was at 25,000 rpm for 1 h and in which rigid precautions were taken to minimize losses of DNA, the total recovery of DNA was 101% and 85% was in the peak corresponding to fragments of 1000 nucleotides or less.

5'-Terminal Nucleotide Analysis of Form II kDNA—In a preliminary approach to the question of whether the interruptions in the Form II minicircles are at specific locations, we identified the 5'-terminal nucleotides on Form II kDNA which had been 3'-terminally labeled with [32P]phosphate. We found that 14% of these nucleotides are dAMP, 31% are dCMP, 43% are dGMP, and 11% are dTMP. Because of the heterogeneity in minicircles, it is possible that different 5' termini are pres-
The ligase treatment was for 4 h as described under "Methods." The DNA (Panel B), and 0.4 pg of ligase-treated Form II DNA (Panel C). Samples were 0.3 pg of Form I DNA (Panel A), 0.4 pg of Form II extent of ligation had reached a limit because another sample, when by equilibrium centrifugation in CsCl/ethidium bromide gradients.

a terminal nucleotide analysis on Hpa II fragments of 5' ent in different species of minicircles. We therefore conducted terminally labeled Form II kDNA. We found a distribution of radioactive nucleotides similar to that shown above when a mixture of Hpa II Fragments A and B were analyzed, and also when a mixture of Fragments C and D were analyzed (see Fig. 10 for a definition of these fragments). Therefore, terminal nucleotide analysis does not provide any evidence that the interruptions in Form II minicircles are at specific locations, but neither does it prove that they are completely random.

Effect of S, Nuclease on Forms I and II kDNA – S, nuclease degrades non-base-paired DNA, and we used this enzyme to test whether Form II kDNA might have single-stranded regions in the form of gaps. Although S, attacks double-stranded DNA at the site of nicks, the rate is less than that at single-stranded regions (31, 32). The effect of this enzyme on kDNA was first measured using sedimentation in neutral sucrose gradients (Fig. 8). S, clearly has an effect on Form I networks as shown by the conversion of the radioactivity to a more slowly sedimenting form (Fig. 8A). However, it has a much more striking effect on Form II networks in that about 65% of the radioactivity is converted to a form which sediments at a rate equal to or less than that of the T7 phage marker (Fig. 8B).

S, digests of kDNA were also analyzed by gel electrophoresis (Fig. 9). Undigested kDNA, in the form of networks, is unable to enter gels and remains trapped on the upper surface. When an S, digest of Form I DNA was electrophoresed on an agarose gel, nearly all of the DNA remained on top but a single minor component entered the gel (Fig. 9A). From the mobility of this fragment on an 0.8% agarose gel, in comparison with known standards, we estimate its molecular weight to be about 23 x 106. This fragment may derive from minicircles, as Kleisen and co-workers have previously observed that S, causes the release of this component from Crithidia luciueae networks (12). With Form II DNA, we electrophoresed digests prepared with several enzyme levels, and similar patterns were ob-
A Form I
A Nicked Form of Kinetoplast DNA
B Form II

FIG. 9. Effect of S1 nuclease on kDNA as measured by gel electrophoresis. A, Form I kDNA was incubated in a solution (110 μl) containing 1.8 μg of kDNA, 0.8 μg of sonicated and denatured λ-[32P]DNA, and 2.5 units of S1 nuclease. A control solution contained neither enzyme nor λ-[32P]DNA. After 30 min at 37°C, the solutions were treated with 3 μl of 0.5 M EDTA and 2 μl of 2 M Tris base. About 90% of the [32P]DNA had been converted to an acid-soluble form during the first 15 min of reaction. A sample of each solution (30 μl) was diluted with 60 μl of water and 10 μl of a solution containing 20% glycerol, 1% sodium dodecyl sulfate, and 0.03% bromphenol blue. The samples were then placed on 1.4% agarose tube gels (12 x 0.6 cm). The gel buffer contained 40 mM Tris/HCl (pH 8.0), 5 mM sodium acetate, 1 mM EDTA, and 0.4 μg/ml of ethidium bromide. Electrophoresis was at 75 V at room temperature and was stopped when the dye had migrated about 10 cm to the position marked D. The gels were then illuminated with an ultraviolet light and photographed with a Polaroid camera. Gel 1 is the control sample and Gel 2 is the sample containing S1. The arrow indicates the position of the faint band in Gel 2. B, Form II kDNA was incubated in solutions (25 μl) containing 0.3 μg of kDNA and variable amounts of S1 nuclease. Samples 1 to 7 contained 0, 0.05, 0.13, 0.50, 1.25, 5, and 15 units, respectively. After 15 min at 37°C, the samples were treated with 5 μl of 50 mM EDTA, 50 μl of water, and 10 μl of a solution containing 20% glycerol, 1% sodium dodecyl sulfate, and 0.03% bromphenol blue. They were electrophoresed as described above except that the dye had migrated about 8 cm to the position marked D. The band at about 1 cm in all of the gels is primarily nuclear DNA which contaminated this Form II preparation.

TABLE I
Composition of S1 digest of kDNA (Form II)

| Fragment Type | Number of unit-lengths per graph* | Approximate percentage of total DNA |
|---------------|----------------------------------|-----------------------------------|
| Linears       | 176 ± 23                         | 28                                |
| Circles       | 35 ± 9                           | 6                                 |
| Dimers        | 10 ± 5                           | 2                                 |
| Trimmers      | 10 ± 4                           | 2                                 |
| Tetramers     | 3 ± 2                            | <1                                |
| Pentamers     | 5 ± 3                            | <1                                |
| Oligomers (>pentamers) | 391 ± 255 | 62                                |
| Acid-Soluble* |                                  | <0.5                              |

* Unit length refers to molecules approximately 1000 base pairs (see histogram in Fig. 10 for a size distribution of linear molecules and monomeric circles). These values are averages ± S.D. from counting six random photographs made from one grid. The counts of the larger oligomers (some with hundreds of minicircular units) are approximate because of the difficulty in counting the closely packed circles.

† Long linear molecules are not included in this tabulation (a total of 29 long molecules were found in nine photographs of this grid). They ranged in size from about 0.4 to about 7 μm, and the average was 2 μm. A histogram indicated that there was no regularity in the size distribution of this DNA. These molecules may derive from kDNA associations (e.g., from maxicircles) or they may be contaminating nuclear DNA.

‡ Acid-soluble DNA was measured in a separate experiment in which 1.5 μg of DNA was incubated with 10 units of S1 for 30 min. Acid-soluble DNA is defined as that which migrates to the front of a polyethyleneimine cellulose thin layer plate when eluted with 2 M HCl.

Fig. 10. Histogram of fragments formed by S1 nuclease digestion of kDNA (Form II). Linear molecules longer than 0.3 μm were rare and are not included in the histogram (see footnote b in Table I). The difference in average size between the circular molecules (Panel A) and the linear molecules (Panel B) may not be significant due to the possibility of a systematic error in identifying the exact position of the ends of the short linear molecules.

proportional to the level of enzyme used (Fig. 9B). For example the bands in Gel 4 seem only slightly less intense than those in Gel 7, even though the amount of enzyme used in these two digests differed by a factor of 30. Therefore, the...
pattern seen in Gel 4 must represent a nearly limit digest. The nonlinearity between extent of degradation and enzyme concentration indicates that some sites on the Form II network are cleaved readily at low enzyme concentrations, but that other sites are cleaved much more slowly. One possible interpretation of this result is that the very sensitive sites are gaps, and the less sensitive sites are nicks.

Table I indicates the types of fragments which exist in an S, digest of Form II kDNA as determined by electron microscopy. Roughly 62% of the DNA in the digest remains as large oligomers and small networks (some with several hundred minicircle units), and about 28% is in the form of linear molecules. These linear molecules are rather homogeneous in length and are roughly the same size as minicircles (see histogram in Fig. 10). The rest of the digest consists of minicircles and small oligomers. All of the minicircles in this S, digest, whether free or part of an oligomer or small network, must contain single strand breaks, as none of them appear twisted when examined by electron microscopy in the presence of ethidium bromide. Virtually no acid-soluble material is released from Form II kDNA by S, nuclease (Table I).

The histidine kinase, S, which is involved in the regulation of the kinetoplast DNA replication in Trypanosoma cruzi, is known to be sensitive to the presence of ethidium bromide. The restriction enzymes Hae III and Hpa II (Fig. 3). However, as determined by equilibrium centrifugation and electron microscopy in the presence of ethidium bromide (Figs. 1 and 2), by sedimentation in an alkaline sucrose gradient (Fig. 5), and by electron microscopy after heat denaturation (Fig. 6), virtually every minicircle in Form II kDNA contains one or more interruptions in both of its strands. In contrast, almost all of the minicircles in Form I kDNA are covalently closed. A second difference between Form I and Form II kDNAs is the fact that Form II sediments at neutral pH about 1.5 times faster than Form I kDNA. This large difference in sedimentation velocity could be due either to a more compact conformation or to a larger size of Form II networks.

Most of the interruptions in Form II minicircles are nicks which are susceptible to ligase (Figure 7). However, the resistance of some minicircles to this enzyme, as well as the susceptibility of some to cleavage by low concentrations of S, nuclease, suggests that some minicircles may contain gaps as well. If gaps do exist, they must be very small, because S, digestion did not release significant amounts of acid-soluble nucleotides. It is likely that there is no more than one gap, or several tightly clustered gaps, in most minicircles because the linear fragments produced by S, digestion are nearly all about the same size as a minicircle (Fig. 10). The terminal nucleotide analysis of Form II 5'-32P-labeled kDNA did not suggest that the interruptions are at unique sites, but because of the presence of multiple types of minicircles and of multiple gaps within many of the minicircles, there remains a possibility that some of the gaps are at specific locations.

Several facts make it unlikely that the nicks or gaps in Form II DNA are caused by nuclease activity during isolation of the DNA. First, lysis of nonradioactive cells by the standard procedure (3) in the presence of Form I 3HkDNA caused no change in the banding pattern of this DNA in a CsCl/ethidium bromide gradient. Second, direct assay of nicking activity, in extracts of Leishmania prepared by sonication, revealed insignificant levels of such an enzyme even under conditions which could be expected to favor its activity. Finally, the fact that Form II kDNA sediments faster than Form I kDNA (Fig. 4) would not be a likely consequence of nicking during the isolation procedure. Simpson and Berliner have already shown that nicking of Form I kDNA with pancreatic DNAse causes a gradual shift of all networks to a lower density position in a CsCl/ethidium bromide gradient, but this treatment does not affect the velocity of sedimentation of the networks through a neutral sucrose gradient (3). Furthermore, closure of the nicks in minicircles in Form II networks with E. coli ligase does not significantly alter its sedimentation rate in neutral sucrose gradients. These facts, together with the argument that a nuclease which introduced random nicks during the isolation procedure would not be expected to attack all of the circles within some networks and to leave other networks virtually unscathed, indicate that Form II is not an artifact of isolation, but instead is a natural component of Leishmania mitochondria which may be an intermediate in some process such as replication or recombination. Riou and Delain have also reported a nicked form of kDNA in Trypanosoma cruzi (33).

Very few facts are known about the mechanism of replication of kDNA, but some of the available information is summarized in the following paragraph. The Leishmania tarentolae cell cycle takes about 10 to 12 h, and kDNA synthesis occurs during a period of 3 to 4 h prior to cell division (15). Density shift experiments have indicated that each minicircle is replicated by a semiconservative mechanism once during each cell cycle and that recombination between minicircles may occur as well (14, 34). These observations are consistent with the finding that the surface area of networks isolated from synchronized cultures of Crithidia fasciculata or Leishmania tarentolae, as estimated by light microscopy, approximately doubles in size during the period of DNA synthesis (14). Pulse labeling of Crithidia fasciculata or Leishmania tarentolae kDNA, followed by light microscope autoradiography, has indicated that DNA synthesis occurs at two sites on the periphery of the network which are situated 180° apart (35). Longer pulses result first in labeling of the entire periphery and then in uniform labeling of the entire network (14). Analysis of pulse-labeled kDNA in a CsCl/ethidium bromide gradient reveals that it bands in the position of Form II kDNA or in an intermediate position between Forms I and II, but that during a chase of several hours it is converted into a species which bands together with covalently closed minicircles. No radioactivity is found in individual covalently closed minicircles isolated from sonicated pulse-labeled networks until after a chase of 5 to 4 h (35), which indicates that there is a delay of several hours between the synthesis of a minicircle and its covalent closure.

We can speculate that prior to replication all minicircles in a network are covalently closed, but that after each minicircle is nicked...

3 Nicking activity was assayed in extracts of exponentially growing cells which were prepared by sonication at 0°C (3.3 x 10^8 cells in 0.6 ml of 50 mM Tris/HCl [pH 8.1], 5 mM 2-mercaptoethanol). Assay mixtures (100 μl) contained 0.5 μg of Form I 3HkDNA, extract containing 117 μg of protein, 50 mM Tris/HCl (pH 8.1), 5 mM 2-mercaptoethanol, and 10 mM MgCl2. The protein/kDNA ratio used in this assay is more than half that which exists in vivo. After 30 min at 37°C, the solution was deproteinized by Sarkosyl/propanol treatment (3) followed by phenol extraction. One sample was centrifuged to equilibrium on a CsCl/ethidium bromide gradient, and the DNA banded in a position which was not distinguishable from untreated Form I kDNA. A second sample was tested for acid solubility of the 3HkDNA, and none (<1% of the total) was

A Nicked Form of Kinetoplast DNA 6215

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replicated its daughters remain nicked or gapped. It is possible that the nicks serve in vivo to distinguish replicated minicircles from those not yet replicated, and therefore prevent them from undergoing more than a single round of replication during each generation. As replication continues, the number of minicircles in the network increases until finally the product of replication contains double the normal number of minicircles, all of which are linked in a network. The final step in the replication process might include both the covalent closure of all minicircles and the cleavage of the network into daughter networks which will be segregated into the two daughter cells. No information is available on the mechanism of these processes or on their time of occurrence in the cell cycle.

It seems possible that Form I kDNA is from cells which have not yet begun the replication of their kDNA, and Form II kDNA is from cells which have completed or nearly completed the replication process. However, the Form II minicircles have not yet undergone covalent closure and the Form II networks have not yet been cleaved into daughter networks. Several properties of Form II networks are consistent with a role in the replication process. First, its larger sedimentation coefficient, relative to that of Form I, would be expected of a network which had increased in size. Second, the presence of single strand breaks in all Form II minicircles is consistent with Simpson and Simpson's finding that pulse-labeled thymidine is not found in covalently closed minicircles until after a chase of 3 to 4 h (35). Third, Form II kDNA is absent in stationary phase cells, which are not replicating their DNA (14). The fact that Form II kDNA usually accounts for roughly 25 to 30% of the total kDNA would imply that there was a relatively long delay between the completion of kDNA synthesis and the covalent joining of the minicircles, or else that the Form II kDNA was preferentially recovered during the isolation procedure.

There are many questions which remain about the structure and replication of kDNA. One question concerns the origin and function of the nicks in Form II kDNA. It is possible that the nicks are a consequence of a delay in joining of discontinuously synthesized Okazaki fragments, or it is possible that they result from a delay in completion of a recombination event. A second question concerns the structure of partly replicated networks. If we assume that in Form II kDNA most or all of the minicircles have replicated, then partly replicated networks might have some minicircles which are covalently closed and some which are nicked. It is likely that these networks would band in a CsCl/ethidium bromide gradient between Forms I and II. A third question concerns the origin of the linkages between the thousands of minicircles in a network, and the distribution of the maxicircles and various classes of minicircles throughout the network. Both catenane and figure-8 structures appear to be present and it is likely that these linkages between circles arise either from a replicational or a recombinational process. A final question concerns the mechanism by which the replicated network is cleaved into daughter networks. Some of these questions are now under investigation in our laboratory.

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A nicked form of kinetoplast DNA in *Leishmania tarentolae*.

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