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Isolation of maxicircle component of kinetoplast DNA from hemoflagellate protozoa

(Hoechst 33258 dye/CsCl equilibrium centrifugation/*Leishmania tarentolae*)

LARRY SIMPSON

Department of Biology and Molecular Biology Institute, University of California, Los Angeles, California 90024

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ABSTRACT **Maxicircle DNA has been isolated from the kinetoplast DNA of *Leishmania tarentolae* culture forms by buoyant separation in CsCl in the presence of the A+T-binding dye Hoechst 33258, after liberation from the kinetoplast DNA network by cleavage with the single-hit restriction endonuclease *Eco*RI. The purified linearized maxicircle DNA has a density in CsCl of 1.681 g/cm^3 (79% A+T) and a molecular weight of approximately 18–20 × 10^6. The maxicircle molecule exhibited intramolecular base composition heterogeneity ranging from 85% A+T to 74% A+T.**

The kinetoplast DNA (kDNA) of the hemoflagellate protozoa is an unusual mitochondrial DNA that consists of several thousand duplex minicircles and a smaller number of larger maxicircles, all catenated into a network structure that has a mass of approximately 10^{10} daltons and a sedimentation coefficient of 2000–5000 S (1–8). Minicircles have been isolated from sonicated (9–11), sheared (12), or restriction enzyme-digested networks (13); the minicircles have been found to possess a variable amount of sequence heterogeneity and a small length heterogeneity (13–16). The sequence heterogeneity in the case of *Crithidia* (13) and *Phytomonas* (17) involves no more than 2–4% of the base pairs. Maxicircles have been observed as rare free circular molecules (18) and as loops radiating out from the networks (19, 20) and can be isolated as high molecular weight fragments by gel electrophoresis of restriction enzyme digests of total kDNA (19, 21, 22). In the case of *Crithidia* (19, 22) and *Phytomonas* (17), the maxicircle DNA represents approximately 5% of the network DNA. In *Trypanosoma brucei* the maxicircle DNA represents a significantly higher percentage of the network DNA (20).

In this report I describe an effective method for isolating maxicircle fragments in high yield by buoyant separation in CsCl in the presence of the bisbenzimidazole dye Hoechst 33258, using the fact that this DNA is higher in percent A+T than the minicircle component of the kDNA.

MATERIALS AND METHODS

Isolation of kDNA. A modification of our standard isolation method (7) was developed to obtain large quantities of kDNA from *Leishmania tarentolae* culture forms: The sheared cell lysate from 12–15 liters of a fermentor culture (300 × 10^6 cells per ml) was centrifuged in a Sorvall GSA rotor for 5 hr at 13,000 rpm. The kDNA-enriched pellets were dissolved in 10 mM Tris·HCl (pH 7.9)/1 mM EDTA (Tris/EDTA), and then CsCl and ethidium bromide (EtdBr) were added (refractive index $n_D^{25°}$ = 1.3876, 300 μg of EtdBr per ml) and the tubes were centrifuged in the Beckman Ti 60 rotor for 40 hr at 40,000 rpm at 20°C. The lower DNA bands were collected by side puncture, the dye was removed by isopropanol extraction, and the DNA was precipitated with 3 vol of 70% (vol/vol) ethanol. The yield from 12 liters of culture was 4–6 mg of kDNA.

Cells. *L. tarentolae* clonal strain C-1 was grown in Difco brain heart infusion medium as described (23).

Gel Electrophoresis. The 3.5–10% gradient acrylamide gels that were used in these experiments were run as described (17). Horizontal agarose gels were run in an apparatus modified from that of McDonnel *et al.* (24). EtdBr staining and photography were performed as described (25). Agarose and acrylamide were purchased from Bio-Rad. The electrophoresis buffer for agarose and acrylamide gels was 90 mM Tris borate/2.5 mM EDTA (pH 8.3). Agarose and acrylamide gels were run at a constant voltage at 25°C without recirculation of buffer. Molecular weights were calculated by use of the least-squares curve-fitting program of Parker *et al.* (26), using the reference molecular weight values of Murray and Murray (27) and Sanger *et al.* (28). The phage λ, phage λ cleaved by *Hin*dIII, and phage T7 reference DNAs were heated to 70°C for 5 min prior to running to prevent aggregation.

Elution of DNA from Agarose. EtdBr-stained bands were cut into small pieces, which were extracted in 0.5 M NH_4 acetate/10 mM Mg acetate/0.1% sodium dodecyl sulfate/0.1 mM EDTA (29) at 37°C for 24 hr. The suspension was then centrifuged at 70,000 × *g* for 1 hr to collapse the agarose. The DNA in the supernate was extracted with phenol and precipitated with ethanol.

Isolation of Maxicircle DNA. The standard preparation involved the digestion of 3 mg of closed network kDNA in 5 ml of enzyme buffer with 400 units of *Eco*RI or *Bam*HI (New England BioLabs) at 37°C for 1 hr. Then 30 ml of Hoechst 33258 dye (American Hoechst, Somerville, NJ) stock solution (0.1 mg/ml) was added (approximately 1 μg of dye per μg of DNA) and the solution was left at 25°C for 10 min, after which 37 g of CsCl was added and the $n_D^{25°}$ was adjusted to 1.3950. The solution was then centrifuged in a Ti 60 rotor in two tubes at 20°C for 40 hr at 40,000 rpm. The upper bands were removed by side syringe puncture and the $n_D^{25°}$ was readjusted to 1.3935. A second centrifugation was performed in two tubes in a Beckman 50 rotor for 40 hr at 20°C. The upper bands were removed and extracted three times with isopropanol. After each extraction it was necessary to bring up the volume with water to avoid precipitation of CsCl. The DNA was then recovered either by direct precipitation with 3 vol of 70% ethanol or by dialysis versus Tris/EDTA and precipitation with 0.2 M NaCl and 2 vol of ethanol. The DNA pellet was dried and resuspended in Tris/EDTA for storage at −20°C.

Enzyme Digestion. All restriction enzymes were purchased from New England BioLabs. Digestions were carried out in 200-μl vol for 1 hr at 37°C (17). In order to visualize the once-cleaved maxicircle DNA in total network digestions, undigested

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Abbreviations: kDNA, kinetoplast DNA; EtdBr, ethidium bromide.

catenanes, which would remain at the origin and cause smearing, were removed prior to electrophoresis by centrifugation at 70,000 × *g* for 30 min. The DNA pellet was visualized and avoided by the addition of 10 μg of EtdBr to the digest prior to the centrifugation. The DNA in the supernate was recovered by ethanol precipitation. Maxicircle permuted linear molecules were released from closed network DNA of *L. tarentolae* by digestion with *Hpa* II in the presence of EtdBr. The optimal conditions were as follows: 100 μg of kDNA, 200 μg of EtdBr, 35 units of *Hpa* II, 200-μl vol, 1 hr at 37°C. The solution was then centrifuged as above to remove undigested catenanes, extracted with phenol, and precipitated with ethanol.

Isolation of Minicircles. Closed monomeric minicircles were isolated from sonicated closed networks of *L. tarentolae* by the alkaline sucrose technique of Wesley and Simpson (9).

Analytical CsCl Gradients. The gradients were prepared and analyzed as described (7).

RESULTS

Heterogeneity of the kDNA. Acrylamide gradient gel profiles of kDNA networks and monomeric minicircles digested with several restriction enzymes are shown in Fig. 1. The localization of purified closed monomeric minicircles in this gel system is shown in Fig. 1*A*, slot 2, and the localization of nicked monomeric minicircles produced by sonication of network DNA is shown in Fig. 1*A*, slot 1. Limit digestions of network and minicircle DNA with the tetranucleotide-recognizing enzymes—*Alu* I, *Hha* I, *Hpa* II, and *Hae* III—and with the hexanucleotide-recognizing enzymes—*Bam*HI, *Hin*cII, *Eco*RI, and *Sal* I—are shown in Fig. 1 *B* and *C*. The minicircle sequence heterogeneity, which has been described previously in several hemoflagellate species (13–15, 20, 30), is clearly apparent. In addition, several high molecular weight bands derived from the maxicircle component are seen in all network digests, especially in the *Hae* III and *Hpa* II digests in Fig. 1*C*, slots 3 and 5, which show almost complete digestion of the kDNA and therefore a lack of obscuring closed or open monomeric minicircles, which migrate in this system with some of the maxicircle bands.

The maxicircle fragment bands are seen more clearly in the agarose gel in Fig. 2*A*, which demonstrates that the maxicircle has single sites for *Eco*RI and *Bam*HI and at least three sites for *Hpa* II. The relative percentage of maxicircle DNA in total network DNA was measured by using kDNA labeled with ^{32}P *in vivo:* the three maxicircle-derived bands of *Hpa* II and *Hae* III digests represent, respectively, 5.8% and 5.4% of the total kDNA.

It is of some interest that the apparent molecular weight of the unit-length minicircle linear molecules is consistantly greater in acrylamide (Fig. 1) than in agarose (Fig. 2). The agarose value agrees well with that determined by electron microscopy (6). The reason for this discrepancy is not known.

The Maxicircle Contains More A+T Than the Minicircle. We have shown (10) that the CsCl buoyant density of purified minicircles from *L. tarentolae* is 1.705 g/cm^3, whereas the buoyant density of network DNA is 1.703 g/cm^3 (6). Because the maxicircle component of the kDNA represents 5.4–5.8%

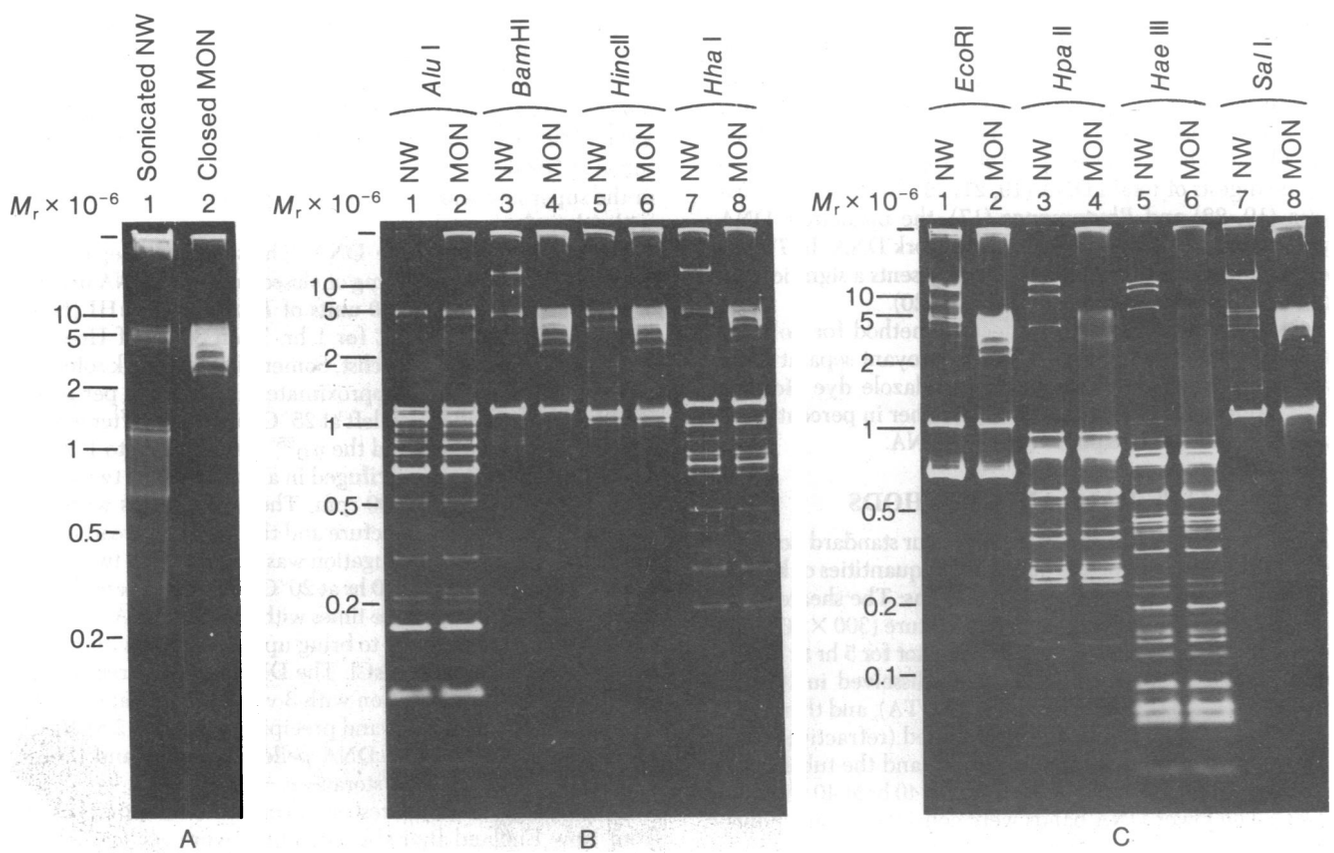

FIG. 1. Comparison of restriction enzyme-digested minicircles and network (NW) DNA by acrylamide gradient gel electrophoresis. (*B* and *C*) Closed monomeric minicircles (MON) and closed networks were digested with several restriction enzymes and electrophoresed on 3.5–10% acrylamide gradient gels with 3% stacking gels. (*A*) Mildly sonicated network DNA and undigested closed monomeric minicircles were electrophoresed. Several reference DNAs (not shown) were electrophoresed in each gel to obtain the molecular weight scales.

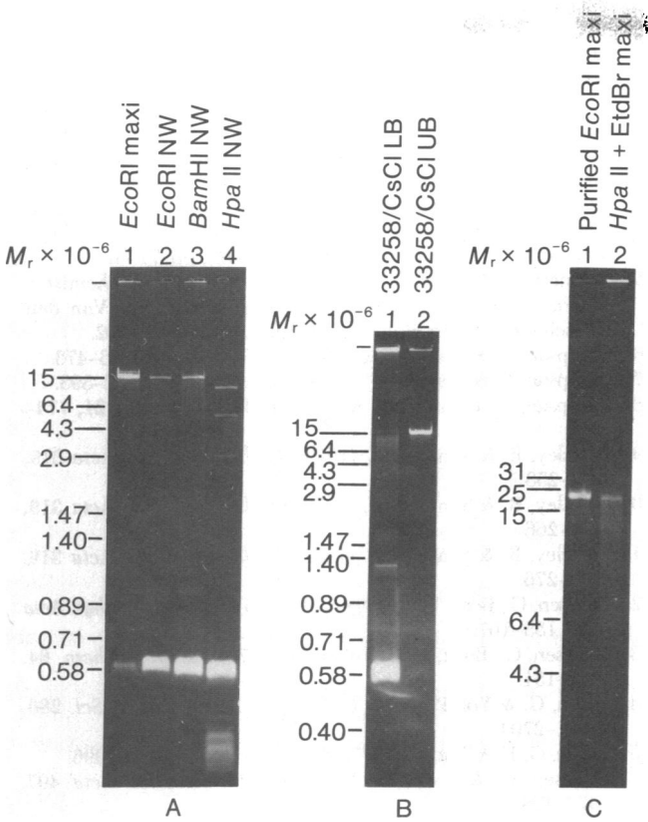

FIG. 2. Agarose gel electrophoresis. (*A*) A 0.8% agarose gel of purified *Eco*RI-digested maxicircle DNA and total network (NW) kDNA digested with *Eco*RI, *Bam*HI, and *Hpa* II. This *Eco*RI maxicircle preparation was purified by a single cycle of Hoechst 33258/CsCl separation and has some contaminating minicircle linear molecules migrating as if their molecular weight is approximately 0.6×10^6. The reference DNAs (not shown) were λ *Hin*dIII fragments and phage φX174 replicative form *Hae* III fragments. (*B*) A 0.8% agarose gel of the upper band (UB) and lower band (LB) from the Hoechst 33258/CsCl gradient of *Eco*RI-digested kDNA shown in Fig. 4; same reference fragments as in *A*. (*C*) A 0.5% agarose gel of purified *Eco*RI maxicircle DNA and once-cleaved, permuted maxicircle linear molecules produced by digestion of kDNA with *Hpa* II in the presence of EtdBr. The reference DNAs (not shown) were λ DNA, T7 DNA, and λ *Hin*dIII fragments.

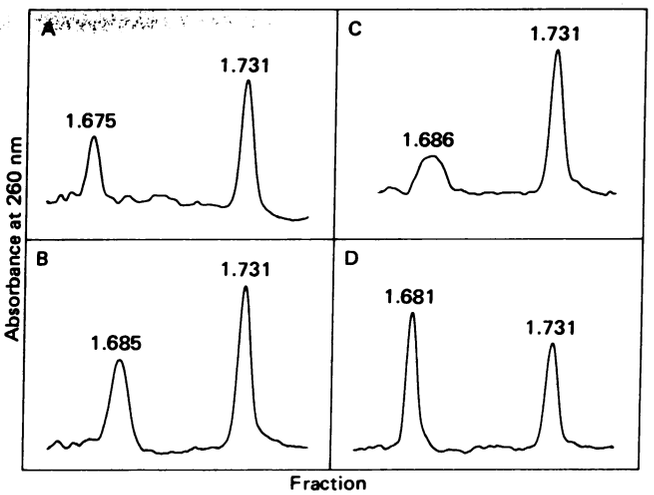

FIG. 3. Buoyant density of three maxicircle-derived fragments from *Hpa* II-digested network DNA. Densitometry tracings of analytical CsCl equilibrium gradients. Buoyant densities, in g/cm^3, are given above the peaks. The 1.731 g/cm^3 reference is *Micrococcus lysodeikticus* DNA. (*A*) *Hpa* II fragment A (largest fragment). (*B*) *Hpa* II fragment B. (*C*) *Hpa* II fragment C. (*D*) Purified *Eco*RI maxi DNA.

of the network, it follows that the maxicircle DNA possesses a low buoyant density and therefore a high percentage of A+T. This was verified by direct buoyant analysis of the three *Hpa* II maxicircle bands eluted from agarose (Fig. 3). The largest *Hpa* II fragment (fragment A) has a density of 1.675 g/cm^3 (85% A+T). The smaller two fragments (B and C) have densities of 1.685 g/cm^3 (75% A+T) and 1.686 g/cm^3 (74% A+T).

Isolation of Maxicircle DNA. We used the property of relative high A+T content to separate once-cleaved maxicircle DNA from the bulk of the network and minicircle DNA. Closed network DNA was digested with *Eco*RI or *Bam*HI and the digest was centrifuged in CsCl in the presence of the A+T-binding dye Hoechst 33258. As shown in Fig. 4*A*, a low-density band (arrow) was liberated. This band was recovered and shown to consist of a single DNA component having an approximate molecular weight of $18–20 \times 10^6$ (Fig. 2*A*, slot 1; Fig. 2*B*, slot 2; Fig. 2*C*, slot 1). The lower Hoechst 33258/CsCl band, which is a superposition of a sharp high molecular weight band and a broad low molecular weight band, consisted of undigested minicircles, minicircle fragments, and oligomeric catenanes that did not penetrate the gel (Fig. 2*B*, slot 1). Two centrifugations were usually required to completely purify the linear maxicircle DNA. The purified *Eco*RI-cleaved maxicircle DNA yielded a single band in buoyant CsCl at a density of 1.681 g/cm^3, which implies an A+T content of 79% (Fig. 3*D*). All results in this report refer to *Eco*RI-digested kDNA, but identical results were obtained with *Bam*HI-digested kDNA.

A *Hpa* II digest of kDNA was also subjected to Hoechst 33258/CsCl centrifugation. Two low-density minor bands were seen, the upper of which was found by gel electrophoresis to represent the large fragment A and the lower of which was found to represent the two smaller fragments B and C (data not shown). This separation is consistent with the different A+T contents of the three *Hpa* II fragments shown above.

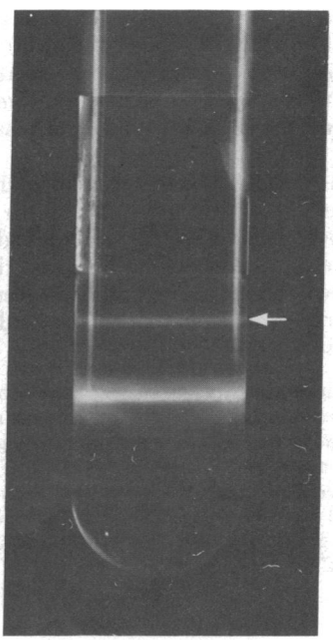

FIG. 4. Hoechst 33258 dye/CsCl equilibrium gradient of digested network kDNA. The kDNA was digested with *Eco*RI prior to centrifugation. Ti 60 rotor, 48 hr, 40,000 rpm, 20°C; long-wavelength UV illumination. Arrow indicates low-density band.

The purified *Eco*RI-cleaved maxicircle DNA migrated in 0.5% agarose with the single band released from total network DNA by digestion with *Eco*RI or *Bam*HI (Fig. 2*A*) and also with the single band released from network DNA by digestion with *Hpa* II in the presence of EtdBr (Fig. 2*C*). This latter band probably corresponds to the set of permuted once-cleaved maxicircle linear molecules, because Parker *et al.* (26) have shown that, under the right conditions, EtdBr limits the activity of a restriction enzyme to any one of the possible sites on a closed circular molecule.

This isolation method has also been applied to *Eco*RI-digested kDNA from *Crithidia fasciculata*, *Leptomonas* sp., and *Phytomonas davidi* and to *Bam*HI-digested kDNA from *Trypanosoma brucei*. In each case a minor upper band was visible in the Hoechst 33258/CsCl gradient (data not shown).

DISCUSSION

I have described a method for the isolation of maxicircle DNA based on the observation that the maxicircle component of the kDNA from at least five species has a higher content of A+T than the minicircle component. This property was employed for a buoyant separation of once-cleaved maxicircles or maxicircle fragments in CsCl in the presence of the A+T-binding fluorescent dye Hoechst 33258 (31–34). It is clear that the absolute separation of the maxicircle DNA from the network and minicircle DNA in the Hoechst 33258/CsCl gradient is a function of the difference in percent A+T between these two kDNA species. In the case of *L. tarentolae* kDNA, the difference is 24% A+T, leading to a large separation in the Hoechst 33258/CsCl gradient, whereas in the case of *T. brucei* kDNA the difference is only 8% A+T (unpublished results), leading to a smaller separation in the Hoechst 33258/CsCl gradient. All other hemoflagellate species examined (*Crithidia*, *Leptomonas*, *Phytomonas*) yielded separations comparable to the *L. tarentolae* separation.

This isolation method has the advantage over isolation in agarose gels of large-scale quantitative recovery and freedom from gel extractable impurities. Preparative isolation of the maxicircle DNA from the kDNA network is important in view of the probability that this component represents the analogue of the mitochondrial DNA in other systems (35). We have recently used this isolation method to develop a restriction enzyme map of the maxicircle DNA of *L. tarentolae* and to clone fragments of maxicircle DNA in the bacterial plasmid pBR322 (36).

The liberation of once-cleaved molecules by digestion with *Hpa* II in the presence of EtdBr implies that the maxicircle is a covalently closed circular molecule *in situ*. And the fact that a single cleavage of the maxicircle liberated the linearized molecule from the network implies that the molecule is retained within the network by catenation alone, as concluded previously by other workers (20–22).

Some of the preliminary restriction enzyme analysis of *L. tarentolae* kDNA was done in collaboration with Brad Hyman, and the results on *Crithidia*, *Leptomonas*, *Phytomonas*, and *Trypanosoma* were obtained in collaboration with P. Ting, B. Stevens, D. Freaney, and A. M. Simpson. I thank June Baumer for assistance with the analytical ultracentrifuge and A. M. Simpson for advice and discussion. This work was supported by grants from the National Institutes of Health (AI 09102 and AI 13027).

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