HETEROGENEITY IN SENSITIVITY TO CLEAVAGE BY THE RESTRICTION ENDONUCLEASES ECORI AND HINDIII OF CIRCULAR KINETOPLAST DNA MOLECULES OF CRITHIDIA ACANTHOCEPHALI

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ABSTRACT

Kinetoplast DNA (kDNA) of the protozoan *Crithidia acanthocephali* consists mainly of an association of ~27,000 covalently closed, 0.8-μm (1.58 × 10^6 daltons) circular molecules apparently held together in a particular structural configuration by topological interlocking. The sensitivities of circular kDNA molecules to the restriction endonucleases *Eco*RI and *Hin*dIII have been studied using agarose gel electrophoresis and electron microscopy. Digestion with *Eco*RI or *Hin*dIII of collections of single circular molecules obtained from sonicated kDNA associations resulted in a single cleavage of 9.3 and 12% of the molecules, respectively. Digestion of intact kDNA associations with *Eco*RI or *Hin*dIII resulted in cleavage of 9.2 and 10.4%, respectively, of the component circular molecules, but not in detectable disruption of the characteristic structure of the associations. Analysis of the products of sequential digestion of kDNA with the two enzymes indicated that ~8% of the circular molecules each contain a single site sensitive to *Eco*RI and a single site sensitive to *Hin*dIII; 1.5-3% contain only an *Eco*RI-sensitive site; 3-4% contain only a *Hin*dIII-sensitive site; and the remainder (~86%) are insensitive to either enzyme. Further, data obtained from sequential digestion experiments and from studies of the partial denaturation products of the circular molecules digested with *Eco*RI or *Hin*dIII indicated that when they occur the *Eco*RI site and the *Hin*dIII site are each at a unique position in all molecules, 10-13% of the circular contour length apart. Similar digestion products were found for kDNAs from different cloned organisms, suggesting that the four different kinds of circular molecules, in regard to *Eco*RI and *Hin*dIII sensitivity, are found in similar proportions in the kDNA association of different organisms.

KEY WORDS kinetoplast DNA · *Crithidia acanthocephali* · restriction enzymes · electron microscope mapping · nucleotide heterogeneity

Members of an order of protozoa, the Kinetoplastidia (9), are distinguished by the presence of a structurally complex DNA known as kinetoplast

DNA (kDNA) which is situated within an enlarged region of a mitochondrion. In the case of *Crithidia acanthocephali*, this DNA comprises on the average 27,000 covalently closed circular molecules, each 0.8 μm in contour length (mol wt 1.6 × 10⁶), which are held together apparently by topological interlocking in a definite ordered manner to form a structure termed an association (22, 32). Some long, possibly linear molecules are found associated with some associations. Associations of circles with some species specific variation in contour length (0.3-0.8 μm) of the component circular molecules, in the arrangement of the individual circular molecules, and in the degree to which linear DNA is associated with the associations, have been described for kDNA from a variety of species of *Trypanosoma* (17, 20-23) and *Crithidia* (22), and from *Leishmania tarentolae* (25).

Data obtained from a number of physicochemical analyses of *C. acanthocephali* kDNA were consistent with the view that all of the circular molecules are similar in base composition (7). Further, from a study of the kinetics of renaturation of *C. acanthocephali* kDNA (7) it was shown that the nucleotide sequence complexity of 70% of the DNA was equivalent to 1.6 times the molecular weight of a single circular molecule. This was interpreted as indicating that at least 70% of *C. acanthocephali* kDNA could comprise only one kind of circular molecule in regard to nucleotide sequences. However, a component accounting for ~15% of kDNA renatured at a distinctly slower rate than the majority, leaving open the possibility that molecules with a nucleotide sequence complexity ~12 times that of a circular molecule exist in kDNA associations. Similar conclusions concerning the sequence complexity of the kDNAs of *L. tarentolae* and *C. luciliae* have been reached by Wesley and Simpson (28, 29) and by Kleisen et al. (14, 15), respectively.

Recently, evidence has been presented from the results of a number of studies using bacterial restriction enzymes which indicates that nucleotide differences do occur between circular molecules of kDNA of *C. luciliae* (13-15) and species of *Trypanosoma* (24). Further, evidence has also been presented (1, 5, 14, 15, 27) which clearly indicates a distinct high molecular weight circular component to be present in the kDNAs of *Crithidia* and *Trypanosoma*.

In the present paper, we describe the results of

studies on the sensitivity of *C. acanthocephali* kDNA to the bacterial restriction enzymes *Eco*RI and *Hind*III. Our findings indicate that the circular molecules of this DNA are also heterogeneous in regard to the nucleotide sequences recognized by each of these enzymes.

MATERIALS AND METHODS

Culturing of Organisms

All experiments involved cells of the same strain of *Crithidia acanthocephali* as that used by Renger and Wolstenholme (22) and originally obtained from Dr. Helene Guttman at the University of Illinois, Chicago Circle. Maintenance of stocks and culturing of cells used for DNA extraction were carried out as described previously (7, 22).

Kinetoplast DNA Isolation

Details of the following techniques employed were as described previously (7): isolation of whole cell DNA; separation and isolation of purified kDNA associations; preparation of fractions consisting of covalently closed single circular molecules and of fractions consisting of a mixture of open single circular molecules and linear molecules of single circle length (unit-length linear molecules); preparative neutral cesium chloride and ethidium bromide-cesium chloride equilibrium density gradient centrifugation; analytical neutral cesium chloride equilibrium density gradient centrifugation.

Restriction Endonuclease Digestions

*Eco*RI restriction endonuclease was prepared as described by Greene et al. (8), and *Hind*III restriction endonuclease was prepared as described by Mantei et al. (18). Preparations of *Eco*RI and *Hind*III were also obtained from Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.

Between 25 and 125 units of *Eco*RI or *Hind*III were used to digest 0.25-0.88 μg of DNA in the previously described reaction buffers (8, 18) at 37°C for times ranging from 30 to 240 min. Some samples were sequentially digested with *Hind*III and *Eco*RI. All experiments were controlled by carrying out parallel incubations in which the enzyme was omitted. All reactions were terminated by adding 1/50 volume of 1 M EDTA, except when the digest was to be subjected to analysis by agarose gel electrophoresis, in which case 0.25 volume of a solution containing 5% sodium dodecyl sulfate, 25% glycerol, and 0.025% bromophenol blue was added.

That each enzyme preparation used was in fact cleaving all of the sensitive sites of the kDNA samples was ascertained as follows. Covalently closed, single circular kDNA molecules were mixed with approximately equimolar amounts of circular mitochondrial DNA (mtDNA) molecules isolated from the embryos of *Dro-*

sophila melanogaster (donated by Christiane M.-R. Fauron), and the mixture was digested with either *Eco*RI or *Hin*dIII for 30 min at 37°C. The products were examined and analyzed by electron microscopy. In both *Eco*RI and *Hin*dIII digests, circular molecules of the size expected for *D. melanogaster* mtDNA were absent. The only circular molecules that were present had the contour length expected for unit-length molecules of *C. acanthocephali* kDNA. In both digests, five classes of fragments were found. The molecular weights of four of these corresponded to values expected for the four fragments produced by complete *Eco*RI or *Hin*dIII cleavage of *D. melanogaster* mtDNA molecules (references 30, 16; Fauron and Wolstenholme, unpublished observations). The molecular weight of the fifth class of linear fragments corresponded to that expected for singly cleaved *C. acanthocephali* kDNA circular molecules. Also, the ratio of the number of linear fragments of the latter class to single circular kDNA molecules in the *Eco*RI and *Hin*dIII digests was 1:9.6 and 1:7.4, respectively.

Agarose Gel Electrophoresis

Electrophoresis of enzyme digests and control DNAs was carried out (8) using 1.2% agarose (Seakem MCI Biomedical, Division of Marine Coloids, Inc., Rockland, Maine) slab gels, and the products were visualized and photographed as previously described (8, 30).

Analysis of Restriction Enzyme-Digested, ^{32}P-labeled kDNA Associations

Native kDNA associations were isolated from cells grown to a titer of ~2 × 10^{7} cells/ml in medium containing 12.5 mCi/l of [32]P (New England Nuclear, Boston, Mass.) as described by Fouts et al. (7). Samples containing 2 μg of kDNA associations (specific activity, 1,600 cpm/μg) were digested with 25, 50 or 125 units of either *Eco*RI or *Hin*dIII for 60 min at 37°C and then electrophoresed on 1.2% agarose slab gels. The three regions of each gel which contained an ethidium-bromide-stained band representing associations, single circular molecules and unit-length linear molecules were cut out with a single-edged razor blade and placed on glass fiber filters in scintillation vials and dried at 60°C for 24 h. Radioactivity in each band was determined using 10 ml of 2,5-bis-2(5-tert-butyl-benzoxazoly)thiophene (scintillation grade, Packard Instrument Company, Inc., Downers Grove, Ill.) (8 g/l toluene), as a fluor in a Packard Model 3320 liquid scintillation system. As controls in each experiment, regions of gels free of fluorescence were cut out, dried, and the radioactivity was determined.

Partial Thermal Denaturation

Partial denaturation (10, 11) of open circular molecules and linear molecules which had been separated from covalently closed circular molecules by ethidium bromide-cesium chloride equilibrium centrifugation of restriction enzyme digests was accomplished by heating the DNAs in 0.05 M sodium phosphate (pH 7.8) and 10% formaldehyde at 52°C for 10 min exactly as described previously (31).

The results of control experiments such as have been described previously (30) indicated that single-stranded kDNA resulting from heating in the presence of formaldehyde had undergone shrinkage in length by a factor of 1.4 relative to the same nucleotide sequence in the native double-stranded form. Appropriate corrections were therefore made where relevant.

Electron Microscopy

Details of electron microscopy were as given previously (7), except that grids were photographed at original magnifications of 10,000 or 12,000. Determinations of the frequencies of circular and linear molecules in the various preparations were made directly from negatives on a viewing box (Ladd Research Industries, Inc., Burlington, Vt.). Length measurements of molecules were made on positive prints at a final magnification of ~125,000 using a graphics calculator (Numonics Corp., North Wales, Pa.).

RESULTS

Enzyme-Sensitive Sites in Isolated Single Circular Molecules and in Native Associations

Fractions shown by electron microscopy to comprise covalently closed, single circular kDNA molecules of *Crithidia acanthocephali* were digested separately with *Eco*RI and *Hin*dIII, and the products were analyzed by agarose gel electrophoresis and electron microscopy. The results are presented in Fig. 1 and Table I. Multiple bands were observed for the control sample in the agarose gel (Fig. 1A). Since examination of this sample in the electron microscope revealed only circular molecules, 89% of which were of a size consistent with a homogeneous molecular weight of 1.58 × 10^{6}, it seems most likely that the multiple bands represent covalently closed circular molecules with different winding numbers (3, 4, 12, 19). Digestion with either *Eco*RI or *Hin*dIII resulted in the appearance of a distinct new band on agarose gels at a position expected for linear molecules of approximately 1.6 × 10^{6} daltons, suggesting that each enzyme cleaves a fraction of the circular molecules at a single site. A second faint band was visible in gels of some *Eco*RI digests just below the more distinct new band, suggesting that a minor portion of the circular molecules contained
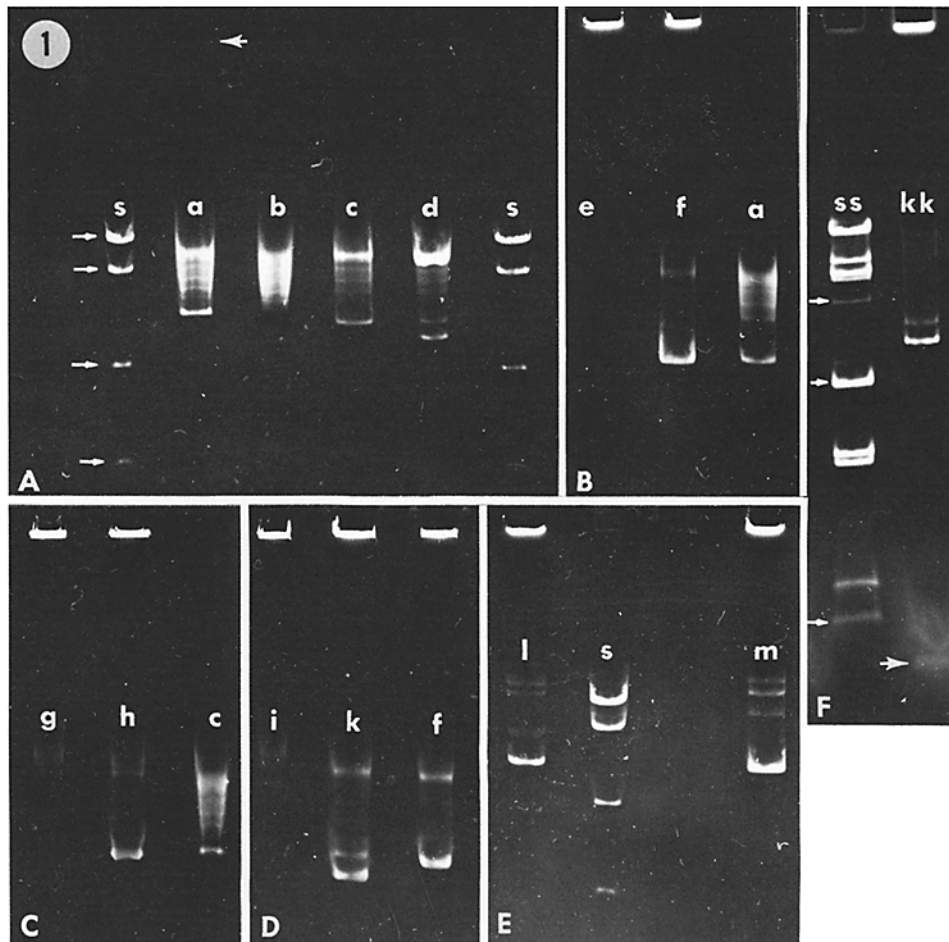
FIGURE 1 Fluorescent photographs showing the distribution of ethidium bromide-stained DNA bands in 1.2% agarose slab gels after electrophoresis. The origin is indicated by the arrowhead near the top of micrograph A and by the positions of the fluorescent bands (representing undigested kDNA associations) near the top of micrographs B-F. All enzyme incubations and controls were carried out at 37°C for 30 min, except where otherwise noted. The slots marked s contain EcoRI digests of *Drosophila melanogaster* mtDNA molecules which were included in gels A-E to provide molecular weight standards. The four bands (arrows) from top to bottom represent fragments of molecular weights of 7.3×10^6, 3.4×10^6, 1.14×10^6, and 0.57×10^6, respectively (30). (A) Slots a-d contain covalently closed single circular molecules of *C. acanthocephali* kDNA incubated in the absence of any enzyme (b); with EcoRI (a); with $Hind$III (c); and sequentially with $Hind$III (60 min) and EcoRI (60 min) (d). (B-D) Native kDNA associations incubated in the absence of enzyme (slots e, g, and i); with EcoRI (f); with $Hind$III (h); and sequentially with $Hind$III (60 min) and EcoRI (60 min) (k); slots a and d contain covalently closed single circular molecules digested with EcoRI or $Hind$III respectively. (E) Native kDNA associations digested with EcoRI (l) or $Hind$III (m). In each of these cases extra bands are visible, some of which by comparison with the standard, represent DNA molecules of higher molecular weight than single circular molecules. (F) Slot kk contains kDNA associations digested sequentially with $Hind$III (60 min) and EcoRI (60 min). A band corresponding to a linear fragment with a molecular weight of $\sim 0.18 \times 10^6$ dalton is visible in this case (large arrow). Slot ss contains a collection of standard DNA fragments (details given in reference 30). The fragments relevant to this experiment are shown by the three small arrowheads and from top to bottom correspond to linear fragments of 2.0×10^6 (EcoRI fragment of lambda bacteriophage DNA), 1.1×10^6, and 0.27×10^6 daltons (both $Hind$III fragments of SV40 DNA).

TABLE I

Data Concerning Cleavage by EcoRI and HindIII of Covalently Closed Single Circular Molecules, Open Single Circular Molecules, and Native Associations of kDNA of C. acanthocephali

Kinetoplast DNA preparation	Restriction enzyme	Percentage molecules cleaved
Covalently closed circular molecules*	EcoRI¶	9.5‡‡,§§
	HindIII¶	11.8‡‡,§§
Open circular molecules‡	EcoRI¶	11.2‡‡,‖‖
	HindIII¶	12.4‡‡,‖‖
Associations§	EcoRI**	9.2¶¶
	HindIII**	10.4¶¶
EcoRI-treated covalently closed circular molecules‖	EcoRI**	0‡‡
	HindIII**	3.2‡‡
HindIII-treated covalently closed circular molecules‖	EcoRI**	0‡‡
	HindIII**	3.0‡‡

* The preparation of covalently closed circular molecules used in this experiment actually comprised 89.2% single circular molecules (having an approximate molecular weight of 1.58×10^6), 8.8% catenated circular dimers, 0.5% catenated trimers and higher oligomers, and 1.5% unit length linear molecules.

‡ The preparation of open circular molecules used in this experiment actually comprised 81.7% single circular molecules, 7.7% catenated circular dimers, 0.5% catenated trimers and higher oligomers, and 10.1% unit length linear molecules.

§ ^{32}P-labeled, 1,600 cpm/μg.

‖ Obtained as the lower bands of cesium chloride-ethidium bromide gradients of covalently closed single circular molecules of kDNA previously digested with either EcoRI or HindIII.

¶ Digested for 30 min at 37°C.

** Digested for 60 min at 37°C.

‡‡ Calculated from the number of unit length linear molecules observed in the electron microscope preparations among an average of 2,279 molecules (unit length molecule equivalents) in each of the top two samples, and an average of 1,152 molecules in the remaining noted samples, after correction in each case for the proportion of unit length linear molecules (0.5-2.4% in covalently closed single circular preparations, and 9.9-10.6% in open circle preparations) present among an average of 1,087 molecules in undigested samples. Unit length linear molecules in the various digests had a range of molecular weights (± SD) from 1.57 ±0.05 (n = 100) to 1.58 ± 0.01 (n = 100) as determined by length comparisons with circular molecules found on the same grid square.

TABLE I—Continued

§§ The values observed when the digestion times, for EcoRI and for HindIII were increased to 120 min were 9.2 and 12.1%, respectively.

‖‖ Corrections have not been made for enzyme cleavages of unit length linear molecules known to be present in the preparation of open circular molecules before enzyme digestions.

¶¶ Calculated from the mean proportion of ^{32}P radioactivity in the ethidium bromide-stained band representing unit length linear molecules in six gels (EcoRI) and eight gels (HindIII), and in each case corrected for background radioactivity in the same gel and for radioactivity in the position expected for unit length linear molecules in gels of undigested associations. Background radioactivity was defined as the mean number of counts (60 cpm/band) in a portion of the gel equal in size to each of the portions containing an ethidium bromide-stained band, and taken from a region just below the lowest ethidium bromide-stained band.

a second EcoRI site. Analysis of electron microscope preparations (Table I) of samples of covalently closed circular molecules incubated in the absence or presence of a restriction enzyme confirmed the major conclusions drawn from the agarose gel analyses that each enzyme cleaves a portion of the molecules at a single site. EcoRI or HindIII digestion resulted in cleavage on the average of 9.5 and 11.8% respectively, of the circular molecules.

To test the possibility that circular molecules cleaved in this preparation were only those containing one or more phosphodiester bond breaks, enzyme digestions of fractions comprising collections of open circular and unit-length linear molecules were performed. In agarose gels (data not shown), DNA incubated in the absence of restriction enzymes separated into two bands, one at the position expected for unit-length linear molecules and a second representing the majority of DNA in the digestion and, therefore, representing the open circular molecules (Table I, footnote ‡) at the position found for the slowest-migrating component of the covalently closed circular molecules (Fig. 1A). A similar band pattern was observed for this DNA after digestion with EcoRI and HindIII. The results of electron microscope analyses of the digest (Table I) were similar to the results of the corresponding analyses for covalently closed circular molecules. Only 11.2% of open circular molecules were cleaved with EcoRI, and 12.4% were cleaved with HindIII.

Intact kDNA associations of *C. acanthocephali*

were digested either with EcoRI or with HindIII and the products were subjected to analysis by agarose gel electrophoresis and by electron microscopy. Most of the DNA in the samples of kDNA associations incubated in the absence of enzymes failed to penetrate the gels (Fig. 1 B-F), as might be expected due to the high molecular weight ($\sim$41 $\times$ 10^9 daltons [22]) of the associations. For these samples, however, a single faint diffuse band was apparent at a position just above that expected for single circular kDNA molecules. For samples incubated in the presence of either EcoRI or HindIII, similar results were obtained except that in each case a distinct band was visible at the position expected for single circular molecules, and a well-defined band was visible at the position expected for a linear fragment (molecular weight $\sim$1.6 $\times$ 10^6) resulting from a single enzyme cleavage.

In some gels of EcoRI digests and of HindIII digests of kDNA associations, faint bands were observed (Fig. 1 E) above the band representing the single circular molecules. Further experiments were not performed to determine whether these latter bands represented catenated oligomers comprising different numbers of 1.58 $\times$ 10^6 dalton circular molecules, or high molecular weight linear or circular molecules.

In electron microscope preparations of kDNA which had been incubated in the absence of enzyme, associations of circular molecules were found together with free circular molecules of $\sim$1.6 $\times$ 10^6 daltons. In electron microscope preparations of kDNA samples digested with either EcoRI or HindIII, associations having the same general appearance, in regard to individual rosette structure and arrangement of rosettes, as those seen in the control sample were found, together with single circular molecules. However, in contrast to what was observed in the control preparations, in each preparation of enzyme-digested kDNA an abundance of linear fragments with molecular weights (1.58 $\pm$ 0.02 $\times$ 10^6, n = 30 for EcoRI digestions, and 1.58 $\pm$ 0.03 $\times$ 10^6, n = 30 for HindIII digests) similar to that of the single-circular molecules was found.

These data suggest that, as was found for isolated single-circular molecules, of the circular molecules in the associations some are cleaved at a single site by EcoRI and some are cleaved at a single site by HindIII. The data further indicate that the enzyme cleavages do not result in disruption of the general structure of the associations.

To determine the fraction of kDNA associations released as unit-length linear molecules by either EcoRI or HindIII, ^{32}P-labeled associations were digested with either enzyme, and the distribution of radioactivity was determined amongst the products separated by electrophoresis. The results (Table I) indicated that EcoRI and HindIII liberate 9.8 and 11.3%, respectively, of the DNA of associations as unit-length linear molecules. As these values are similar to the respective frequencies of unit-length linear molecules produced by EcoRI and HindIII digestion of isolated single-circular molecules (Table I), the latter are indicated to be a random sample of the circular molecules of associations. Results from this experiment also indicated that no detectable single circular molecules were liberated from associations by EcoRI digestion, and only 1.4% single circular molecules were liberated from associations by HindIII digestion.

The Distribution and Relative Locations of EcoRI and HindIII Cleavage Sites on Circular Molecules

A sample containing $\sim$50 μg of covalently closed, single circular molecules was digested with EcoRI for 60 min at 37°C, and the product was centrifuged to equilibrium in a cesium chloride-ethidium bromide density gradient. The lower band, comprising covalently closed single circular molecules which had survived EcoRI digestion, and the upper band, comprising unit-length linear molecules (resulting primarily from EcoRI digestion of covalently closed circular molecules) and open, single circular molecules (presumably resulting from light-induced nicking of ethidium bromide-bound covalently closed circular molecules [2]), were separated. The covalently closed circular molecules were then further digested with either EcoRI or HindIII, and the products were examined and analyzed by electron microscopy. A similar experiment was performed beginning with covalently closed single circular molecules digested with HindIII. The results of the two experiments are summarized in Table I. Covalently closed single circular molecules which survived previous incubation with EcoRI or with HindIII were unaffected by a second incubation with the same enzyme. However, 3.2% of the molecules which survived EcoRI digestion were cleaved by HindIII, and 3.0% of the molecules which had survived HindIII digestion were cleaved by

EcoRI. These results confirm the existence of at least three kinds of circular kDNA molecule in regard to sensitivity to cleavage by EcoRI and HindIII: molecules which are resistant to both enzymes; molecules which are resistant to EcoRI but are cleaved at a single site by HindIII; and molecules which are resistant to HindIII but are cleaved at a single site by EcoRI. The existence of kDNA molecules which contain both an EcoRI site and a HindIII site is also indicated. If the EcoRI and HindIII sites are randomly distributed relative to each other among circular kDNA molecules, then the expected frequency of the molecules containing both sites would be about 1% (9.3 × 12%). However, if this were the case, then ~11% of the circular molecules surviving EcoRI digestion should contain a HindIII site and ~8% of the circular molecules surviving HindIII digestion should contain an EcoRI site. The observed values were in fact much lower, 3.2 and 3.0%, respectively.

To test directly for the existence of circular molecules which contain both EcoRI and HindIII-sensitive sites and to estimate the distance between these sites on the circular molecules, we digested intact associations and single circular molecules sequentially with HindIII and EcoRI and analyzed the products by agarose gel electrophoresis and electron microscopy. For both associations and single circular molecules, the banding patterns in agarose gels (Fig. 1A, D, and F) were similar to those found after digestion with a single enzyme, but in each case an extra band was visible. The electrophoretic mobility of this band indicated the component DNA molecules to have a molecular weight of ~1.4 × 10^6. A second band representing DNA fragments with a molecular weight of ~0.18 × 10^6 which would be expected if the 1.4 × 10^6 dalton band resulted from circular molecules cleaved once by each enzyme was detected in some gels (Fig. 1F). These data imply that the two enzyme sites, where they occur, are at unique relative locations on the circular molecule.

Electron microscope examination of the sequentially digested products of covalently closed single circular molecules revealed circular molecules and linear molecules. The latter were in three size classes, the means of which were equivalent to the length of a single circular molecule (99.9%), and to 87.8 and 13% of the length of a circular molecule (Fig. 2). The latter two length classes comprised approximately equal numbers
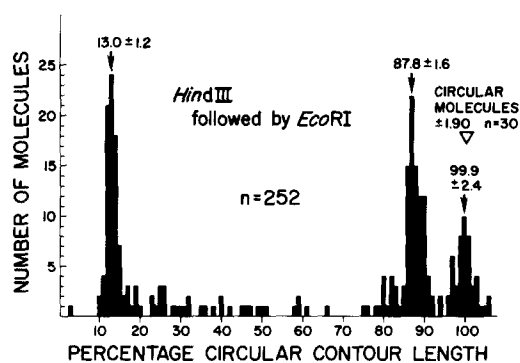

FIGURE 2 Frequency distribution of the lengths of linear fragments resulting from sequential digestion of covalently closed single circular molecules of C. acanthocephali kDNA with HindIII and EcoRI. The lengths are expressed as percentages of uncleaved circular kDNA molecules (open triangle; defined mean length of 100.0 (± 1.90%) taken at random from the same areas of the grid squares as the linear fragments. The mean and standard deviation of each of the three size groups of linear fragments is given.

of fragments (86:78), and the sum of their lengths was 100.8% of a circular molecule, suggesting that they are products of circular molecules which have been cleaved once by each enzyme at separate, unique sites, 13% of the contour length apart. The ratio of linear molecules of the 99.9, 87.8, and 13% size classes was 1:1.7:1.7. This is close to the ratio expected (1:1.6:1.6) for the three classes of fragments if 8% of the circular molecules contained sites cleaved by both enzymes, 1% contained only an EcoRI site and 4% contained only a HindIII site. A ratio of 19:1:1 would be expected if only 1% of the circular molecules (representing random distributions of EcoRI and HindIII sites between circular molecules) contained both an EcoRI and a HindIII site.

The Positions of the EcoRI and HindIII-Sensitive Sites Relative to Two A+T-Rich Regions in the kDNA Circular Molecule

We have previously found (J. E. Manning, and D. R. Wolstenholme. Unpublished observations) that open circular molecules of C. acanthocephali kDNA can be partially denatured at specific temperatures in 0.05 M sodium phosphate and 10% formaldehyde (10, 11), and that there is a common pattern of partial denaturation among molecules. The regions which denature at lower tem-

peratures are presumably rich in adenine and thymine (A+T) (6). We have used denaturation mapping to determine the relative positions of the EcoRI- and HindIII-sensitive sites and the A+T-rich regions.

Samples of two fractions each containing linear molecules and open single circular molecules, and obtained as the upper bands of the cesium chloride-ethidium bromide density gradients of EcoRI digested or HindIII digested covalently closed single circular molecules mentioned above, were used in these experiments. DNA from each sample was heated separately at 52°C in 0.05 M sodium phosphate and 10% formaldehyde for 10 min and prepared for electron microscopy by the protein monolayer technique. In each preparation, the first 30 circular molecules and the first 30 linear molecules to be observed on a grid square were photographed (Fig. 3). Denaturation maps of these molecules were constructed and are compared in Figs. 4 and 5. Two regions of denaturation were apparent in each circular molecule in both preparations (Fig. 3 A-E). These regions were of rather constant size (Figs. 4 and 5) and, for the two preparations, averaged 47.2 and 14.8% of the contour length of the circular molecule. The two double-stranded regions separating the two denatured regions of the circular molecules were also of constant size and averaged 16.8 and 21.4% of the contour length. These observations are consistent with the circular kDNA molecules being homogeneous in regard to the gross arrangement of their base sequences. Similar patterns of denaturation were found for linear molecules in both the EcoRI and the HindIII preparations (Fig. 3 F-K and Fig. 3 L-P, respectively). Each linear molecule in both EcoRI and HindIII preparations contained two regions of denaturation similar in size to those found in circular molecules (Figs. 3-5). Alignment of linear molecules from the EcoRI preparation by their two ends resulted in alignment of the two regions of denaturation (Fig. 4) separated by a double-stranded region of 17% of the molecule length. Regions of double-stranded DNA were also found at both ends of the molecule and averaged 10.3 and 9.6% of the molecule length. These data confirm that each linear molecule resulted from EcoRI cleavage at a unique position on the circular molecule which, in the denaturation map, lies in the larger double-stranded region, 9.6% of the contour length from the junction of this region with the smaller denatured region.

Alignment of linear molecules from the HindIII preparation by their ends also resulted in alignment of the two regions of denaturation (Fig. 5) which were again separated by a double-stranded region approximately equal in length (18.5%) to the smaller double-stranded region of circular molecules. In this case, however, a region of double-stranded DNA was found at only one end of each molecule. This region was equivalent in length (21.7%) to the larger double-stranded region of circular molecules. The denatured region seen as two single strands with separated ends (Fig. 3 L-P) at the end of each molecule was the smaller of the two denatured regions. These data indicate that the HindIII site, when it occurs on a circular molecule, does so at a position on the denaturation map inside the smaller denatured region but close to the junction of this region with the larger double-stranded region. Comparison of the data from the denaturation maps of linear fragments from the EcoRI and HindIII digests indicates that the two enzyme-sensitive sites are ~10% of the kDNA circular molecule's contour length apart.

Analysis of Clones

The kDNA used in all of the previous experiments was isolated from cultures initiated by inoculation of a large number of organisms from a stock culture. It was therefore not ruled out that the four different kinds of circular molecules detected in regard to enzyme sensitivity originated from different organisms, rather than from the kDNA association of each organism. To determine which interpretation was correct, organisms were serially diluted to 0.6 organisms per tube. Of 30 tubes inoculated, eight produced a culture (clone) of Crithidia. The clones were then grown in 500-ml volumes to a titer of 2 × 10⁷ cells per ml. Native kDNA associations were isolated from the individual cultures, digested with EcoRI, HindIII or sequentially with HindIII and EcoRI as described above, and the digestion products were analyzed by agarose gel electrophoresis. For each of the eight clones, for each kind of digest the patterns of DNA bands were identical to each other and to the respective digest pattern obtained for kDNA associations isolated from multiple organism-initiated cultures.

DISCUSSION

The results presented clearly indicate that the component circular molecules of the kDNA asso-
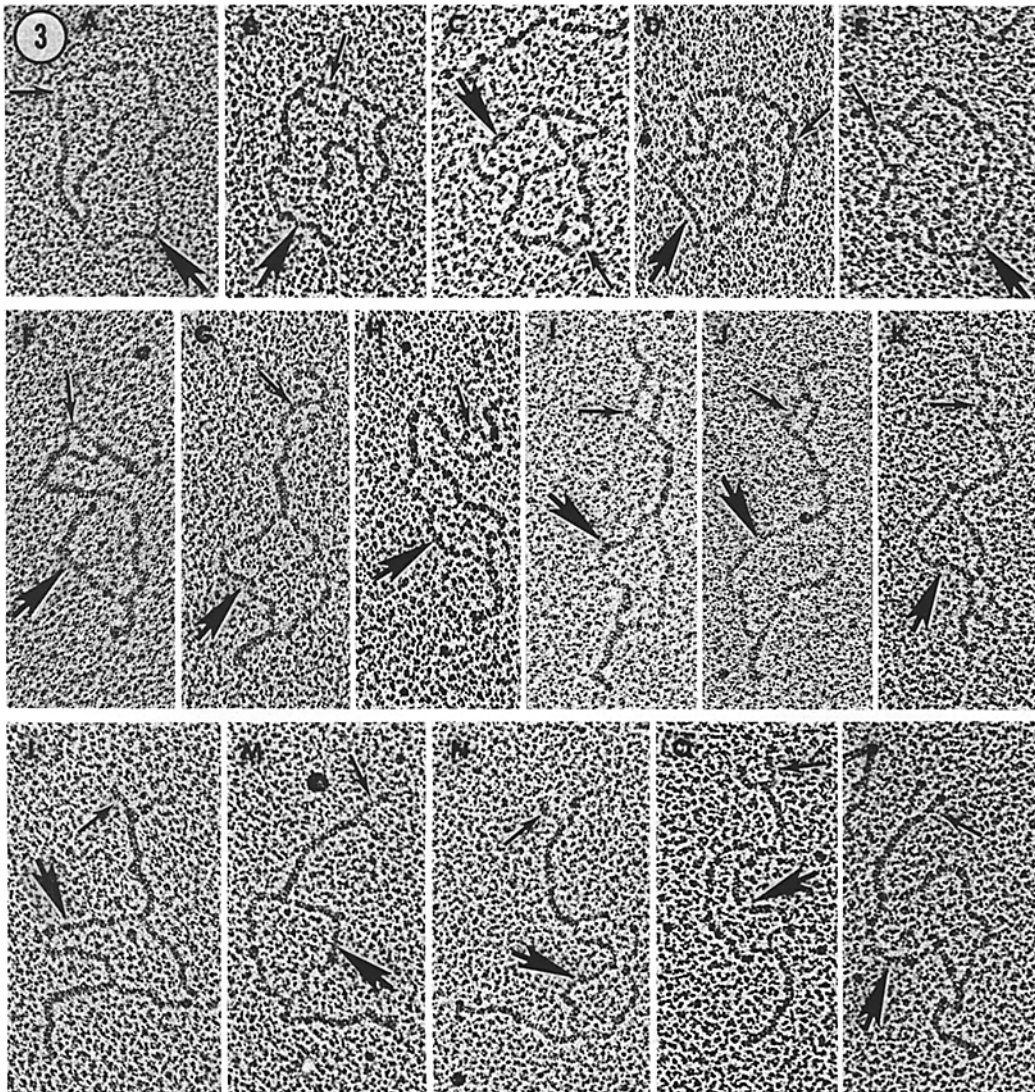
FIGURE 3   Electron micrographs of single molecules of kDNA of *C. acanthocephali* heated for 10 min at 52°C in 0.05 M sodium phosphate and 10% formaldehyde. Fig. 3 *A-E* and *F-K* are circular and linear molecules, respectively, from a preparation of the upper band of a cesium chloride-ethidium bromide equilibrium gradient of a sample of covalently closed single circular molecules which had been digested with *Eco*RI for 30 min at 37°C. The larger and smaller arrows indicate, respectively, the larger and smaller regions of denaturation of each molecule. Fig. 3 *L-P* are linear molecules from a preparation of the upper band of the cesium chloride-ethidium bromide equilibrium gradient of a sample of covalently closed single circular molecules which have been digested with *Hin*dIII for 30 min at 37°C. Two regions of denaturation are again visible in each molecule (arrows) but in each case the smaller of these regions is terminal in position (small arrows). All micrographs × 47,500.

ciations of individual *Crithidia acanthocephali* cells comprise four classes in regard to sensitivity to cleavage by the restriction enzymes *Eco*RI and *Hin*dIII. ~8% contain a single site sensitive to *Eco*RI and a single separate site sensitive to *Hin*dIII. Between 1.5 and 3% contain a single *Eco*RI-sensitive site but are insensitive to *Hin*dIII and 3-4% contain a single *Hin*dIII-sensitive site but are insensitive to *Eco*RI. The remaining circular molecules (~86%) are insensitive to either

EcoRI   52°C

CIRCULAR MOLECULES


```
|——— 46.1 ———|— 16.3 —|— 16.2 —|— 21.4 —|
       ±3.5        ±2.7     ±2.2     ±3.4
```

```
20      40      60      80      100
```

LINEAR MOLECULES


```
|10.3|——— 47.2 ———|—— 17.2 ——|—15.7—|—9.6—|
 ±1.5      ±2.4        ±3.0    ±1.6   ±1.3
```

```
20      40      60      80      100
```
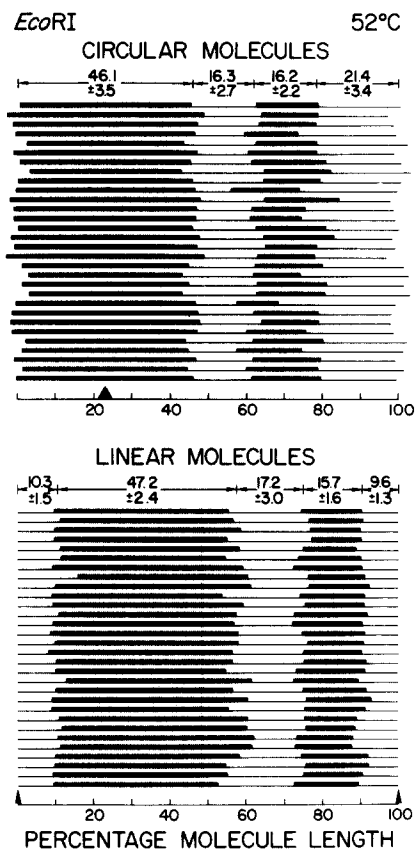
PERCENTAGE MOLECULE LENGTH

FIGURE 4 Denaturation maps of 30 open circular molecules (above) and 30 linear molecules (below) taken from a single preparation of the upper band of a cesium chloride-ethidium bromide equilibrium gradient of a sample of covalently closed single circular molecules of kDNA of *C. acanthocephali* which have been digested with *Eco*RI for 30 min at 37°C. Partial denaturation was accomplished by heating the open circular and linear molecules at 52°C for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. For the purpose of comparison, each circular molecule was converted to a linear rod. The lengths of the denatured (thick lines) and undenatured (thin lines) regions of each molecule were standardized by converting them to a percentage of the mean lengths of circular molecules. For denatured regions, this followed correction for the relative shrinkage of single-stranded DNA under the conditions employed. For the circular molecule, the midpoint of the longer denatured region was taken as a common point by which the molecules were aligned (point of solid triangle on the abscissa). The linear molecules were aligned by their ends (fine arrows on the abscissa). For both circular and linear molecules, direction was defined by placing the smaller region of denaturation to the right. The numbers above each set of molecules are the mean length ± standard deviation of each of the denatured and undenatured regions.

HindIII   52°C

CIRCULAR MOLECULES


```
|——— 48.2 ———|— 17.2 —|—13.3—|— 21.3 —|
      ±2.7       ±2.2    ±1.5    ±1.6
```

```
20      40      60      80      100
```

LINEAR MOLECULES


```
|— 21.7 —|——— 47.8 ———|— 18.5 —|—12.0—|
   ±1.3        ±2.0       ±1.7   ±1.0
```

```
20      40      60      80      100
```
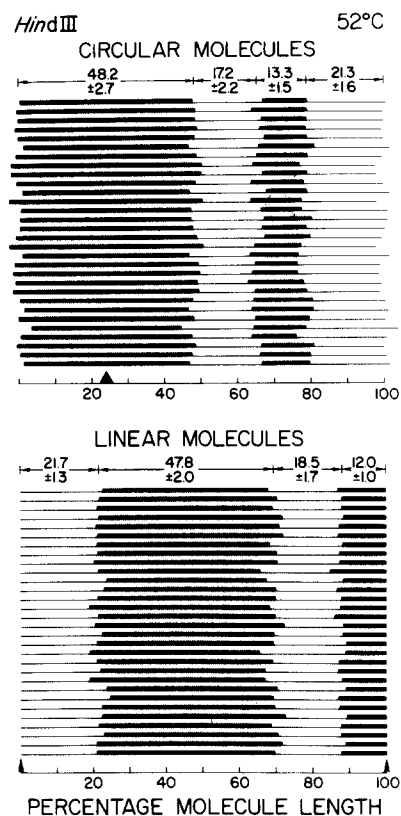
PERCENTAGE MOLECULE LENGTH

FIGURE 5 Denaturation maps of 30 open circular molecules (above) and 30 linear molecules (below) taken from a single preparation of the upper band of the cesium chloride-ethidium bromide equilibrium gradient of a sample of covalently closed single circular molecules of kDNA of *C. acanthocephali* which had been digested with *Hind*III for 30 min at 37°C. Other details are as given in the legend to Fig. 4.

enzyme. The occurrence in gels of some *Eco*RI digests of covalently closed circular molecules of a faint band below the band representing unit-length linear molecules leaves open the possibility that a small proportion of circular molecules contain two *Eco*RI sites.

It appears from the distribution of lengths of fragments resulting from sequential digestion of circular molecules with *Hind*III and *Eco*RI that, when a *Hind*III and an *Eco*RI site occur together on the same molecule, they are always located 13% of the circular contour length apart.

As it is expected that the molecules mapped in regard to partial denaturation include those containing both enzyme sites (~85% of the *Eco*RI-cleaved molecules and 65% of the *Hind*III-cleaved molecules) and those containing only a

single enzyme site, these data are consistent with all EcoRI sites being at the same position relative to the A+T-rich regions and with all HindIII sites being at a second unique position on different molecules. The distance between the HindIII site and the EcoRI site is indicated from these data to be ~10% of the circular contour length. Considering the possible errors which might be expected in estimating lengths of denatured regions in the two separate denaturation mapping experiments, and the possibility that small segments of denatured DNA at the ends of linear molecules produced by HindIII digestion may have gone undetected, the value of 10% is in reasonable agreement with the value of 13% indicated from the double-digest data (Fig. 2) as the distance apart on the circular molecule of the two enzyme sites. Fig. 6 summarizes the conclusions drawn above.

The finding of four classes of circular molecule using only two restriction enzymes, each of which recognizes a hexanucleotide sequence, suggests that nucleotide differences between circular molecules of C. acanthocephali kDNA may be extensive. However, we previously found that the nucleotide sequence complexity of at least 70% of C. acanthocephali kDNA was ~2.5 × 10⁶ daltons, only 1.6 times the molecular weight of a single
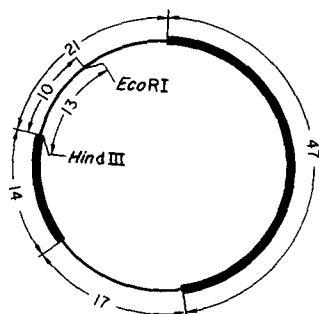

FIGURE 6 A map of the C. acanthocephali circular kDNA molecule showing the relative positions of the EcoRI-sensitive site, the HindIII-sensitive site, and the two regions of the molecule which denature (thick line) at 52°C in 0.05 M sodium phosphate and 10% formaldehyde, expressed as percentages of the contour length of the circular molecules. The values concerning denatured and double-stranded regions are mean values calculated from the data in Figs. 4 and 5. The value of 10% of the distance between the EcoRI and HindIII sites is also calculated from the data in Figs. 4 and 5. The value of 13% for the distance between EcoRI and HindIII sites is the mean size of the smallest fragment resulting from sequential digestion of circular molecules with the two enzymes (Fig. 2).

circular molecule. Assuming that the different sequence classes of circular molecule revealed by the present study are representative of the component with a sequence complexity of 2.5 × 10⁶ daltons, it seems most likely that individual circular molecules have much of their nucleotide sequence in common. Nucleotide differences between circular molecules may be present in nontranscribed or nontranslated regions of the molecule, leaving open the possibility that identical polypeptides (or non-messenger RNAs) are in fact coded for by all of the circular molecules of the kDNA of this species. While we have recently found evidence for a stable RNA (Fouts and Wolstenholme. Unpublished observations) coded by 10% of the H-strand of the C. acanthocephali kDNA circular molecule, we do not have information concerning the sequence complexity of this RNA.

It is noted in this regard that, while the simplest explanation of the observed heterogeneity in sensitivity to restriction enzymes is that circular molecules differ in nucleotide sequences, it is not ruled out that base modifications are involved in at least some cases.

Recently, evidence has been provided (13, 14, 27) from experiments involving agarose gel electrophoresis and ultracentrifugation that the circular molecules (molecular weight = 1.5 × 10⁶) of kDNA of Crithidia luciliae are heterogeneous in regard to their sensitivity to a number of restriction enzymes. HpaII digestion resulted in the production of 37 fragments with a combined molecular weight (24 × 10⁶) 16 times that of a circular molecule, and AluI digestion resulted in production of 21 fragments with a combined molecular weight (18 × 10⁶) 12 times that of a circular molecule. A combination of HindII and HindIII (HindII + III) cleaved 50% of the circular molecules, resulting in nine fragments with a combined molecular weight of 8 × 10⁶. EcoRI cleaved 20% of the circular 1.5 × 10⁶ dalton kDNA molecules of C. luciliae (28) compared to the 10% of the 1.6 × 10⁶ dalton kDNA circular molecules of C. acanthocephali cut by this enzyme in our experiments. However, in contrast to our findings that the product of EcoRI cleavage of C. acanthocephali circular kDNA molecules migrates mainly or exclusively as a single band, in the photograph presented by Kleisen and Borst (13), the EcoRI digest products of C. luciliae circular kDNA molecules appear in the agarose gel as two bands of approximately equal intensity

lying close together and close to the position expected for unit-length linear molecules. It was concluded (14) from the results of these studies on *C. luciliae* that at least 13 different circle sequence classes are present in unequal amounts in the kDNA of this organism. Further, from a consideration of the kinetics of renaturation of *C. luciliae* kDNA, it was argued that the heterogeneity involved less than 2% of the nucleotides of the 1.5×10^6 dalton circular molecules. This is consistent with the conclusions drawn above concerning the circular molecules of *C. acanthocephali*.

Riou and Yot (24) found that digestion of the 0.8×10^6 dalton circular kDNA molecules of *Trypanosoma cruzi* with *Eco*RI resulted in five fragments with a combined molecular weight greater than that of an individual uncleaved circular molecule. *Hpa*II and *Hind*II + III each cleaved a portion of the circular molecules of *T. cruzi* at a single site.

Kleisen et al. (13) electrophoresed open single circular and unit-length linear molecules of *C. luciliae* kDNA and observed three bands representing the circular molecules, and four bands representing the linear molecules. They interpreted these data as evidence for size heterogeneity of up to 4% for the component circular molecules of this kDNA. In contrast, we have not obtained evidence for heterogeneity of lengths of circular molecules of *C. acanthocephali* kDNA. The multiple bands observed in the present experiments for covalently closed circular molecules of *C. acanthocephali* kDNA are clearly best interpreted as resulting from differences in winding numbers (3, 4, 12, 19).

Structural disruption of the kDNA associations of *C. acanthocephali* either in regard to the characteristic individual rosette structures (22, 32) or to the arrangement of rosettes to form the association did not result from either *Eco*RI or *Hind*III digestion. This finding is consistent with the interpretation that the circular molecules which were cleaved by either enzyme were evenly distributed throughout the associations rather than being preferentially located in specific rosettes. Kleisen et al. (15) and Weislogel et al. (27) noted that in contrast to our observations, after digestion of *C. luciliae* kDNA associations with *Hind*II + III, or with *Eco*RI, although large associations of DNA remained, the rosette structure was no longer apparent. However, whereas *Eco*RI or *Hind*III resulted in cleavage of only 9.5 and 12.5% respectively (and liberation of 0 and 1.4% single circular molecules, respectively) of the circular molecules of *C. acanthocephali* kDNA, 50 and 20% of the circular molecules of *C. luciliae* kDNA were cleaved by *Hind*II + III and *Eco*RI respectively.

Our experiments did not determine whether the faint extra bands observed in some *Eco*RI and *Hind*III digests of kDNA associations, but not in digests of isolated single circular molecules, represent oligomers of 1.58×10^6 daltons circular molecules or linear molecules of lengths in excess of 1.58×10^6. Similar bands were found by Kleisen et al. (14, 15) and Weislogel et al. (27) after digestion of *C. luciliae* kDNA with restriction enzymes. By a series of extensive studies they provided convincing evidence that in the case of *C. luciliae* these extra bands are in fact the restriction enzyme digest products of a circular DNA component with a sequence complexity of about 26×10^6 daltons which accounts for 2-3% of the *C. luciliae* kDNA. Rare circular molecules of approximately this molecular weight (22×10^6) have also been found in isolated kDNA of *C. luciliae* by Steinert and van Assel (26). Similar studies to those carried out on *C. luciliae* have provided evidence for a distinct circular component of 16.1×10^6 and 12.2×10^6 daltons in the kDNAs of *T. mega* (1) and *T. brucei* (5).

Long (possibly linear) molecules have often been observed at the periphery of intact kDNA associations of a number of organisms including *C. luciliae* and *C. acanthocephali* (20, 21). In the latter organism, a component representing ~15% of kDNA was found to renature at a rate expected for a unique (double-stranded) nucleotide sequence of 18.0×10^6 daltons (7). However, the relationship of this component to the long linear molecules observed in electron micrographs of kDNA associations remains obscure as peripheral long linear molecules show partial denaturation patterns similar to kDNA circular molecules, and their presence appears to be limited to kDNA associations in which DNA synthesis is taking place (J. E. Manning and D. R. Wolstenholme. Unpublished observations).

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REFERENCES

1. Borst, P., F. Fase-Fowler, M. Steinert, and S. van Assel. 1977. Maxi-circles in the kinetoplast DNA of *Trypanosoma mega. Exp. Cell Res.* **110**:167–173.
2. Clayton, D. A., R. W. Davis, and J. Vinograd. 1970. Homology and structural relationships between the dimeric and monomeric circular forms of mitochondrial DNA from human leukemic leukocytes. *J. Mol. Biol.* **47**:137–157.
3. Depew, R. E., and J. C. Wang. 1975. Conformational fluctuations of DNA helix, *Proc. Natl. Acad. Sci. U. S. A.* **72**:4275–4279.
4. Dugaiczyk, A., H. W. Boyer, and H. M. Goodman. 1975. Ligation of *Eco*RI endonuclease generated DNA fragments into linear and circular structures. *J. Mol. Biol.* **96**:171–184.
5. Fairlamb, A. H., P. O. Weislogel, J. H. J. Hoeijmakers, and P. Borst. 1978. Isolation and characterization of kinetoplast DNA from bloodstream form of *Trypanosoma brucei. J. Cell Biol.* **76**:293–309.
6. Follett, E. A. C., and L. V. Crawford. 1968. Electron microscope study of the denaturation of polyoma virus DNA. *J. Mol. Biol.* **34**:565–573.
7. Fouts, D. L., J. E. Manning, and D. R. Wolstenholme. 1975. Physicochemical properties of kinetoplast DNA from *Crithidia acanthocephali, Crithidia luciliae* and *Trypanosoma lewisi. J. Cell Biol.* **67**:378–399.
8. Greene, P. J., M. C. Betlach, H. M. Goodman, and H. W. Boyer. 1974. The *Eco*RI restriction endonuclease. *In* DNA Replication. Methods in Molecular Biology. R. B. Wickner, editor. Marcel Dekker Inc., New York. **7**:87–105.
9. Honigberg, B. M., W. Balamuth, E. C. Bovee, J. O. Corliss, M. Gojdics, R. P. Hall, R. R. Kudo, N. D. Levine, A. R. Loeblich, J. Weiser, and D. H. Wenrich. 1964. A revised classification of the phylum protozoa. *J. Protozool.* **11**:17–20.
10. Inman, R. B. 1966. A denaturation map of the λ phage DNA molecule determined by electron microscopy. *J. Mol. Biol.* **18**:464–476.
11. Inman, R. B. 1967. Denaturation maps of the left and right sides of the lambda DNA molecules determined by electron microscopy. *J. Mol. Biol.* **28**:103–115.
12. Keller, W. 1975. Determination of the number of superhelical turns in simian virus 40 DNA by gel electrophoresis. *Proc. Natl. Acad. Sci. U. S. A.* **72**:4876–4880.
13. Kleisen, C. M., and P. Borst. 1975. Sequence heterogeneity of the minicircles of kinetoplast DNA of *Crithidia luciliae* and evidence for the presence of a component more complex than minicircle DNA in the kinetoplast network. *Biochim. Biophys. Acta.* **407**:473–478.
14. Kleisen, C. M., P. Borst, and P. J. Weijers. 1976a. The structure of kinetoplast DNA 1. The mini-circles of *Crithidia luciliae* are heterogeneous in base sequence. *Eur. J. Biochem.* **64**:141–151.
15. Kleisen, C. M., P. O. Weislogel, K. Fonck, and P. Borst. 1976b. The structure of kinetoplast DNA. 2. Characterization of a novel component of high complexity present in kinetoplast DNA networks of *Crithidia luciliae. Eur. J. Biochem.* **64**:153–160.
16. Klukas, C. K., and I. B. Dawid. 1976. Characterization and mapping of mitochondrial ribosomal RNA and mitochondrial DNA in *Drosophila melanogaster. Cell* **9**:615–625.
17. Laurent, M., and M. Steinert. 1970. Electron microscopy of kinetoplast DNA from *Trypanosoma mega. Proc. Natl. Acad. Sci. U. S. A.* **66**:419–424.
18. Mantei, H., H. W. Boyer, and H. M. Goodman. 1975. Mapping Simian Virus 40 mutants by construction of partial heterozygotes. *J. Virol.* **16**:754–757.
19. Pulleyblank, D. E., M. Shure, D. Tang, J. Vinograd, and H.-P. Vosberg. 1975. Action of nicking-closing enzyme in supercoiled and non-supercoiled closed circular DNA: Formation of a Boltzmann distribution of topological isomers. *Proc. Natl. Acad. Sci. U. S. A.* **72**:4280–4284.
20. Renger, H. C., and D. R. Wolstenholme. 1970. Kinetoplast deoxyribonucleic acid of the haemoflagelate *Trypanosoma lewisi. J. Cell Biol.* **47**:689–702.
21. Renger, H. C., and D. R. Wolstenholme. 1971. Kinetoplast and other satellite DNAs of kinetoplastic and dyskinetoplastic strains of *Trypanosoma. J. Cell Biol.* **50**:533–540.
22. Renger, H. C., and D. R. Wolstenholme. 1972. The form and structure of kinetoplast DNA of *Crithidia. J. Cell Biol.* **54**:346–364.
23. Riou, G., and E. Delain. 1968. Electron microscopy of the circular kinetoplastic DNA from *Trypanosoma cruzi:* occurrence of catenated forms. *Proc. Natl. Acad. Sci. U. S. A.* **62**:210–217.
24. Riou, G., and P. Yot. 1975. Etude de l'ADN. kinetoplastique de *Trypanosoma cruzi* a l'aide d'endonucleases de restriction. *C. R. hebd. Seances Acad. Sci. Ser. D. Sci. Nat.* **280**:2701–2704.
25. Simpson, L., and A. da Silva. 1971. Isolation and characterization of kinetoplast DNA from *Leishmania tarentolae. J. Mol. Biol.* **56**:443–473.
26. Steinert, M., and S. van Assel. 1976. Large circular mitochondrial DNA in *Crithidia luciliae. Exp. Cell Res.* **96**:406–409.
27. Weislogel, P. O., J. H. J. Hoeijmakers, A. H. Fairlamb, C. M. Kleisen, and P. Borst. 1977. Characterization of kinetoplast DNA networks from the insect Trypanosome *Crithidia luciliae. Biochim. Biophys. Acta.* **478**:167–179.
28. Wesley, R. D., and L. Simpson. 1973. Studies on kinetoplast DNA. II. Biophysical properties of minicircular DNA from *Leishmania tarentolae. Biochim. Biophys. Acta.* **319**:254–266.
29. Wesley, R. D., and L. Simpson. 1973. Studies on kinetoplast DNA. III. Kinetic complexity of kinetoplast and nuclear DNA from *Leishmania tarentolae. Biochim. Biophys. Acta.* **319**:267–280.
30. Wolstenholme, D. R. and C. M.-R. Fauron. 1976. A partial map of the circular mitochondrial genome of *Drosophila melanogaster:* location of *Eco*RI sensitive sites and the adenine-thymine rich region. *J. Cell Biol.* **71**:434–448.
31. Wolstenholme, D. R., R. H. Kirschner, and N. J. Gross. 1972. Heat denaturation studies of rat liver mitochondrial DNA: a denaturation map and changes in molecular configurations. *J. Cell Biol.* **53**:393–406.
32. Wolstenholme, D. R., H. C. Renger, J. E. Manning, and D. L. Fouts. 1974. Kinetoplast DNA of *Crithidia. J. Protozool.* **21**:622–631.