STUDIES ON KINETOPLAST DNA

III. KINETIC COMPLEXITY OF KINETOPLAST AND NUCLEAR DNA FROM LEISHMANIA TARENTOLAE

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SUMMARY

Quantitative renaturation kinetic studies of purified kinetoplast minicircular DNA from Leishmania tarentolae indicated that there is probably one but certainly no more than two classes of identically sized minicircles in terms of DNA base sequences. Total kinetoplast network DNA was found to consist mainly of repeated minicircle base sequences, but also to possess a component of a higher complexity (7.0 · 10^6 daltons) which comprised 5.4 % of the total network DNA. The most likely molecular candidates for this more complex species are the long DNA molecules seen in the kinetoplast networks. Nuclear DNA possessed three species of annealing components: presumed singlecopy sequences, moderately repetitive sequences, and a rapidly annealing component representing approx. 25 % of the nuclear DNA with a C_0t value which was even less than that of minicircles.

INTRODUCTION

The kinetoplast DNA of the hemoflagellate mitochondrion exists as a massive, sheet-like network of interlocked minicircles and long molecules. Minicircles, which vary in size from 0.29–0.80 μm in different species, represent the predominant molecular species of the kinetoplast DNA network (see review by Simpson¹). In the case of Leishmania tarentolae there are approx. 10^4 minicircles per network. In addition approx. 6–9 % of the total kinetoplast DNA of L. tarentolae consists of monomeric minicircles and small catenanes that are either free or loosely bound to the network. We have previously described several methods to isolate monomeric minicircles from both the class of “free” molecules and from the network itself (Wesley, R. D. and Simpson, L., preceeding article), and have investigated several physical properties of these unusual molecules (Wesley R. D., and Simpson, L., pre-

Abbreviation: SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0.
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ceeding article). Purified minicircles from either class formed a homogeneous population of circular duplex molecules with a contour length of $0.29 \pm 0.02\,\mu m$. We have previously described evidence from thermal chromatography and from CsCl equilibrium rebanding experiments that the multiphasic melting curve of open minicircles could be accounted for by intra- rather than intermolecular base sequence heterogeneity. The method of choice to provide a definite answer to this question is complexity analysis by quantitative renaturation kinetics. An equality of the kinetic complexity and the measured minicircle molecular weight would imply a lack of intermolecular heterogeneity in base sequences. The results of such a study are reported in this paper. We have also examined the kinetic complexity of total network DNA and nuclear DNA.

MATERIALS AND METHODS

Cultivation and labeling of cells

*L. tarentolae* (clonal strain Lt-C-1) was grown in defined Medium C (ref. 2) and labeled for 2.5 days by the addition of $[^3H]$thymidine (3.5 $\mu$Ci/ml, 3.0 $\mu$g/ml, Schwarz-Mann). Log phase cells were harvested, washed and stored frozen at $-20^\circ C$ as described previously (Wesley, R. D. and Simpson, L., first article, p. 237).

Isolation of DNA

Kinetoplast DNA. Cells ($1.2 \cdot 10^9$ cells/ml) in 0.2 M NaCl – 0.025 M EDTA, pH 8.2, were lysed with 3 % sodium dodecyl sulfate and pronase (1 mg/ml, self-digested at 37 °C for 90 min) at 60 °C for 3 h. To reduce the viscosity prior to differential centrifugation, the lysate was forced through a No. 20 gauge needle at 24 lb/inch², and then diluted with an equal volume of the lysis solution (Simpson, L., and Berliner, J., unpublished). The kinetoplast DNA networks were pelleted by centrifugation at 20 000 rev./min (20 °C) for 90 min in an SW 27 rotor and washed once in 0.15 M NaCl – 0.015 M sodium citrate, pH 7.0 (SSC) – 0.02 % sodium azide. The recovery represented 10 % of the total cell DNA. To guarantee purity, the kinetoplast DNA was centrifuged to equilibrium in CsCl in a Spinco No. 50 fixed-angle rotor at 33 000 rev./min (20 °C) for 68 h. Four-drop fractions, collected from the bottom, were diluted with 0.8 ml SSC and the $A_{260\,\text{nm}}$ read in a Gilford spectrophotometer. The single kinetoplast DNA band was shown to be free of nuclear DNA contamination by overloaded analytical CsCl equilibrium gradients.

Kinetoplast DNA minicircles remaining in the supernatant after the removal of networks were recovered by the method of Wesley and Simpson (first article, p. 237). Centrifugation in high-salt 4–18 % (w/w) alkaline sucrose gradients at 60 000 rev./min for 4 h (5 °C) in the SW 65 rotor was carried out to separate the final minicircle preparation into covalently closed molecules and minicircle single strands. In preparing all alkaline gradients the sucrose was dissolved in a solution consisting of 1 M NaCl, 0.01 M Tris–HCl, 0.001 M EDTA, 0.3 M NaOH (pH 13.0).

Closed minicircles were also prepared from sonicated kinetoplast DNA networks via a two-step process: (1) ethidium bromide–CsCl equilibrium centrifugation and (2) band sedimentation of the covalently closed molecules on a linear 5–20 % neutral sucrose gradient in SSC. Centrifugation conditions are described elsewhere (Wesley, R. D. and Simpson, L., first article, p. 237).
**Nuclear DNA.** Kinetoplast DNA networks were first removed from the cell lysate by differential centrifugation as described above, except that 3% sodium sarcosinate (Geigy Industrial Chemicals) was used to lyse the cells instead of sodium dodecyl sulfate. \(^3\)H-labeled nuclear DNA in the supernatant solution was incubated for 30 min at 50 °C with 20 μg/ml ribonuclease I and 20 units/ml ribonuclease T1 (both stock ribonuclease solutions were pretreated in 0.1 M sodium acetate buffer, pH 5.1, for 10 min at 90 °C). The DNA was then purified in the following manner: Sephadex G-100 chromatography (bed volume 900 ml) was used to remove the sarkosyl, degraded RNA and other low molecular weight material. The DNA in the excluded volume was concentrated by lyophilization, dialyzed against 0.1 × SSC and deproteinized by chloroform–isoamyl alcohol extractions. The nuclear DNA was then centrifuged to equilibrium in a CsCl density gradient at 36 000 rev./min (20 °C) for 60 h in a No. 50 rotor. Each tube contained approx. 300 μg DNA. At the end of the run fractions were collected from the bottom, diluted and the \(A_{260\ nm}\) read. The nuclear peaks were pooled, dialyzed against SSC and stored frozen at \(-20^\circ\ C\).

**ΦX174 DNA.** *Escherichia coli* HF 4704 was infected with ΦXam3 (a lysis-defective amber mutant of ΦX174) as described by Francke and Ray\(^3\). Chloramphenicol was added to the 200-ml culture 6 min after infection and \[^3\]H thymidine (a total of 2 mCi) was added in four equal portions at 1, 20, 35 and 50 min after infection. The culture was vigorously aerated for a total of 90 min. The intact RF-containing cells were then collected by centrifugation, washed twice in 0.05 M tetra-sodium borate – 0.006 M EDTA at 4 °C and resuspended by vortexing in 16 ml of the borate–EDTA solution.

Infected cells were lysed with lysozyme (100 μg/ml) and 2 cycles of freeze-thawing, and the viral DNA was solubilized by incubating for 2 h at 60 °C with pronase (200 μg/ml, self-digested) and 1% sodium dodecyl sulfate. To minimize shearing, the lysates were poured directly onto 34 ml high-salt sucrose gradients (5–20%, sucrose in 1 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 8.0) and centrifugation was carried out at 24 000 rev./min for 16 h at 5 °C in an SW 27 rotor. 1-ml fractions were collected from the top of the tube using an Isco Model D fractionator. To localize the RF I and RF II peaks, 10-μl aliquots were spotted onto Whatman 3MM filter discs. After drying, the DNA in the filters was precipitated with cold 5% trichloroacetic acid and the filters were then dehydrated in ethanol and ether, dried and counted in toluene-based Omnifluor (New England Nuclear) in a Beckman LS-230 scintillation counter. The peaks were separately pooled and incubated with ribonuclease I (50 μg/ml) at 40 °C for 15 min, and then dialyzed extensively against SSC.

To further separate the RF I and RF II species, preparative ethidium bromide–CsCl centrifugation was carried out in a No. 50 rotor at 40 000 rev./min at 20 °C for 48 h. Each tube had an initial density of 1.58 g/ml, and contained 308 μg/ml in a final volume of 6.5 ml. After the run, 5-drop fractions were collected from the bottom of the tube and the DNA peaks were identified by counting small aliquots on filters as described above.

Since the end-products of ribonuclease I digestion may have contaminated these preparations, the RF I molecules were re-incubated with ribonuclease I and T1 (10 μg/ml, 20 units/ml, 50 °C, 15 min) and passed through a Sephadex G-100 column (bed volume 110 ml). This final step increased the specific activity of the RF I preparation 3-fold when the DNA concentration was calculated by \(A_{260\ nm}\).
Sonication conditions

The small probe of Bronsonic III sonicator at maximum power was used in all experiments. It was necessary to clean the probe by sonication in water until no further ultraviolet absorbing material was released. Ar gas (Matheson) was first bubbled through the DNA samples (4–5 ml) for 15 min and then sonication was carried out at 4 °C in 15-s bursts. After every 2 min of sonication the solution was again saturated with Ar. Using these conditions, 3 min of sonication reduced L. tarentolae nuclear DNA and φX DNA to about minicircle-sized fragments.

X-irradiation of minicircles

14C-labeled minicircles, X-irradiated to produce single strand breaks, were used as a marker in the determination of DNA fragment sizes. A silicad-treated watch-glass containing 0.2 ml of DNA in 0.001 M histidine, 0.01 M sodium phosphate buffer (pH 7.8) was irradiated with 16,000 röntgens using a 0.25-mm aluminum filter at a dose rate of 2000 röntgens per min. With these conditions approximately 37% of the minicircles remained covalently closed giving rise to the maximum number of minicircles with one single-strand break.

Determination of fragment size

A 3H-labeled sample of unknown fragment size was mixed with X-irradiated 14C-labeled minicircles and then run on a 4–18% (w/w) alkaline sucrose gradient. Centrifugation was carried out in an SW 65 rotor 65,000 rev./min for 10 h at 4 °C. Fractions were collected onto Whatman 3MM filter discs from the bottom of the tube. The filters were processed and counted as described above. A series of three such determinations is shown in Fig. 2.

The linear single strand of 14C-labeled minicircles (slower sedimenting peak) was taken as the standard of known molecular weight (2.8 × 10^6 daltons) and the size of the DNA sample was estimated by the equation: \( D_1/D_2 = (M_1/M_2)^{0.4} \) where \( D \) is the distance sedimented and \( M \) is the molecular weight.

Renaturation studies

Renaturation of DNA was studied by the hydroxylapatite method of Britten and Kohne. A series of small columns, maintained at 60 °C in a water bath, and containing about 0.5 ml packed hydroxylapatite were first equilibrated with 0.12 M sodium phosphate buffer (pH 6.8). The DNA sample was denatured by boiling for 5 min and then was incubated at 60 °C in a closed tube. If incubations were to continue for more than 12 h, the sample was overlaid with mineral oil. At predetermined intervals, aliquots (>1000 cpm) were withdrawn and mixed with the upper layers of hydroxylapatite in the columns. In some instances positive air pressure was used to force the sample into the column. The single-stranded DNA was eluted by washing five times with 1 ml of 0.12 M phosphate buffer followed by removal of the renatured DNA by washing five times with 1 ml of 0.4 M phosphate buffer (pH 6.8). This procedure removed all the radioactivity from the columns.

To determine the percentage of DNA reassociated at each \( C_0f \) value, 100 µg of bovine serum albumin was added as carrier and the samples were precipitated with an equal volume of cold 10% trichloroacetic acid. After 30 min at 4 °C, the precipitated DNA was collected on Millipore filters, dried and counted.
RESULTS

Complexity of kinetoplast DNA minicircles

Because of the small size of the minicircle (838 ± 45 base pairs), the hydroxylapatite method of Britten and Kohne\textsuperscript{5} was chosen to measure the rate of reassociation of \textsuperscript{3}H-labeled DNA since low DNA concentrations could be used. \textit{\Phi}X RF DNA was used as a standard of known complexity.

To obtain DNA fragments of uniform size, sonicated minicircles and sonicated \textit{\Phi}X RF molecules were co-sedimented in separate tubes in neutral sucrose gradients as shown in Fig. 1. The positions of the two peaks in the minicircle sample

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Fig. 1. Band velocity sedimentation in 5-ml neutral 5-20% sucrose gradients of sonicated minicircles (a) and sonicated \textit{\Phi}X174 RF I molecules (b) to obtain uniform-size DNA fragments. Centrifugation was for 315 min at 60 000 rev./min (4 °C). Prior to renaturation studies, closed minicircles which had not been broken by sonication were removed by a second sedimentation in an alkaline sucrose gradient.

Fig. 2. Determination of the fragment size of sonicated DNA by band velocity sedimentation in alkaline sucrose gradients. Centrifugation was for 10 h at 65 000 rev./min (4 °C) in the SW 65 rotor. The nicked linear strand (838 ± 45 nucleotides, Simpson and da Silva\textsuperscript{4}) from X-irradiated \textsuperscript{14}C-labeled closed minicircles was used as a reference. The profiles were corrected for cross-over. (a) Sonicated \textit{\Phi}X RF DNA (Fractions 10–13 from Fig. 1). (b) Sonicated minicircles with one double-strand break (Fractions 10–13 from Fig. 1). (c) Minicircles sonicated to half monomer-size fragments (Fractions 6–9 from Fig. 1).
(Fig. 1a). representing half minicircle and minicircle sizes respectively, were used to select double stranded \(\Phi X\) fragments of corresponding lengths (Fig. 1b). Alkaline sucrose band velocity sedimentation of the selected double-stranded fragment classes demonstrated that the sonication had produced mainly double-strand breaks (Fig. 2).

The renaturation kinetics of sonicated minicircles having one double-strand break, open minicircles having one single-strand break, and sonicated \(\Phi X\) RF DNA of minicircular size are presented in Fig. 3. The smooth lines represent the best single-

![Graph](image)

**Fig. 3.** The kinetics of reassociation of denatured open minicircles \((C_{a}f_{1} = 9.05 \cdot 10^{-4}, \bigcirc-\bigcirc\), sonicated minicircles \((C_{a}f_{1} = 1.36 \cdot 10^{-3}, \triangle-\triangle\) and sonicated \(\Phi X\) RF DNA \((C_{a}f_{1} = 6.25 \cdot 10^{-2}\) , \(\bullet-\bullet\)). Renaturation conditions are described in Materials and Methods. These \(C_{a}f_{1}\) curves represent the best single-component fit of a second-order reaction to the data points as determined by a computer program. The initial DNA concentrations for the reassociation of sonicated minicircles \((0.025-0.100 \mu g/ml)\) and sonicated \(\Phi X\) RF DNA \((0.158-0.816 \mu g/ml)\) were varied over a 5-fold range indicating that the rate constants were not concentration dependent and that the reactions obeyed second-order kinetics. The \(C_{a}f_{1}\) value for sonicated \(\Phi X\) RF DNA \((760\) nucleotide size fragments compared to 840 nucleotides for sonicated minicircles) was not corrected for the small difference in fragment size.

component fits of second-order equations to the data as determined by a least squares computer program written by Dr Roy Britten. The ratio of the \(C_{a}f_{1}\) values of sonicated minicircles with one double-strand break and sonicated \(\Phi X\) RF of the same size was 4.6, with the actual values being \(1.36 \cdot 10^{-3}\) and \(6.25 \cdot 10^{-3}\), respectively. An identical comparison of half minicircle, sized fragments \((450\) nucleotides) obtained by more extensive sonication and by sizing with neutral sucrose band velocity sedimentation, gave essentially the same ratio between the \(C_{a}f_{1}\) values of minicircles and \(\Phi X\). Since the ratio of the contour lengths of \(\Phi X\) and minicircles is 6.19 (ref. 4), and the ratio of the complexities is 4.6, we conclude that the number of minicircle classes is between one and two, and is most likely one, considering the accuracy of the hydroxylapatite technique.
COMPLEXITY OF LEISHMANIA DNA

There was no difference between the $C_{oT}$ values obtained with "free" minicircles or with minicircles derived from networks. This can be seen in Fig. 3, where the sonicated minicircle curve contains the combined data from four separate preparations, two of "free" minicircles and two of network minicircles.

Open minicircles with one single-strand break reannealed more rapidly than sonicated minicircles with one double-strand break (Fig. 3), indicating that the molecular configuration of DNA affects the renaturation kinetics. Khoury and Martin have shown this to be true in the case of SV 40 DNA.

To determine the extent of base mismatching during renaturation, minicircles with one double-strand break were heat-denatured, annealed in 0.12 M phosphate buffer to a $C_{oT}$ value of 0.5, and then remelted. The melting curve was indistinguishable in terms of hyperchromicity and $T_m$ values from that of an open minicircle control. This result indicates a high degree of accurate base pairing.

Complexity of kinetoplast DNA networks

Kinetoplast DNA networks were sonicated to a mixture of minicircle-sized fragments and half minicircle-sized fragments, as determined by neutral sucrose band velocity sedimentation, and the remaining unbroken closed minicircles and closed catenated minicircles representing 22.4% of the total network DNA were removed by alkaline band velocity sedimentation. The remaining network DNA fragments were denatured by boiling and the reassociation kinetics followed by hydroxylapatite chromatography. The DNA renatured with a smooth $C_{oT}$ curve (Fig. 4) having a $C_{oT}$ value of 1.26 · 10^-3, which was essentially identical to that obtained with purified minicircles. However, the upper plateau of the network $C_{oT}$ curve indicated that more DNA had reassociated at a $C_{oT}$ value of 3 · 10^-5 than was the case for

![Graph](image)

Fig. 4. The renaturation kinetics of sonicated kinetoplast DNA networks (○○) and sonicated nuclear DNA (●●). Alkaline sucrose gradient sedimentation was used to remove 22.4% of the network DNA in the form of covalently closed molecules prior to renaturation. The remaining network DNA consisted of an approx. 2:1 ratio of minicircle size and half minicircle size fragments while the fragment size of nuclear DNA was approx. 840 base pairs (minicircle size). The curves were determined by computer analysis yielding a $C_{oT}$ value of 1.26 · 10^-3 for network DNA and $C_{oT}$ values of 0.435 and 17.5 for the two components of nuclear DNA.
minicircles. This suggests that approx. 6% or less of the DNA in L. tarentolae kinetooplast networks is present as a very simple DNA sequence or as a DNA conformation that renatures rapidly. However, no evidence was obtained for the presence of a large amount of higher complexity component, as would be expected if the non-minicircular network DNA contained different base sequences.

To clearly establish the presence or absence of a higher complexity component, the sonicated network DNA was renatured to a C₀t value of 0.015 and the 86% of the DNA that renatured was discarded. The kinetics of renaturation of the last 14% of the DNA (representing 11% of the original network DNA) are shown in Fig. 5. The renaturation required more than three C₀t decades, implying a heterogeneity in base sequences⁴. Computer analysis of the C₀t curve in Fig. 5 indicated the presence of two homogeneous components, one having a C₀tₗ value (pure) of 1.29 \times 10⁻³, which is equivalent to that of minicircles, and the second having a complexity 12.7 times greater than that of minicircular DNA and equivalent to a unique DNA sequence of 7.0 \times 10⁶ daltons. This higher complexity component represented 5.4% of the total original network DNA.

![Fig. 5. Renaturation kinetics of the slow renaturing portion of kinetooplast DNA networks. This sample was from the same sonicated network preparation as in Fig. 4, but was further fractionated after removal of the closed molecules by hydroxylapatite after reannealing to a C₀t value of 0.015. The remaining single-stranded DNA represented 11% of the original network DNA. A two-component computer fit of the data is shown and the percentage and observed C₀tₗ value for each component is indicated.](image)

The higher complexity kinetooplast DNA component was not an artifact of either small fragment size or contamination with nuclear DNA. To account for such a large difference in complexity on the basis of fragment size requires the unlikely possibility that a discrete group of fragments 5–6 nucleotides long were generated by sonication, assuming that the renaturation rate is inversely proportional to the square root of the single-strand molecular weight⁷. It is clear in addition that the kinetooplast DNA component renatured more rapidly than nuclear DNA in Fig. 4.
COMPLEXITY OF LEISHMANIA DNA

Complexity of nuclear DNA

The renaturation of nuclear DNA fragmented to minicircular size is shown in Fig. 4. As is the case in all eukaryotic cells, L. tarentolae nuclear DNA contains three classes of DNA sequences highly repetitive, moderately repetitive, and presumed single-copy sequences. The renaturation curve in Fig. 4 is best fitted by two components, comprising 13 % and 59 % of the nuclear DNA and representing the moderately repetitive and the possible single-copy sequences, respectively. In addition, approx. 25 % of the nuclear DNA has a C_{ot} value which is even less than that of minicircles, and it was demonstrated by alkaline sucrose band velocity sedimentation that this was not due to the presence of covalently closed molecules. This low C_{ot} nuclear DNA is possibly identical to the rapidly annealing nuclear DNA identified by CsCl equilibrium centrifugation by Simpson and da Silva.

DISCUSSION

The quantitative renaturation kinetics studies reported in this paper have indicated that there is probably only one but certainly no more than two classes of minicircles in terms of base sequences. Other evidence reported previously (Wesley, R. D. and Simpson, L., preceding article) suggested that there is only one class of minicircles. Furthermore, the evidence from the preliminary fingerprint analysis of minicircles (Appendix) was consistent with a total complexity of around 800 nucleotide pairs, as deduced from a comparison of the number of spots on the fingerprint with the number of spots obtained by Fellner et al. in fingerprints of 16-S rRNA.

Therefore the estimated 10^4 minicircles per mitochondrial genome reflect an extensive gene amplification unprecedented in other mitochondrial genomes. The physiological and genetic significance of this amplification remains obscure. It has been proposed that such a gene amplification has evolved in this group of protozoa as a result of selective pressures brought about by a biphasic parasitic life cycle which involves, in some species, a cyclical loss and development of mitochondrial membranes and enzymes. Thus the minicircle may represent an important mitochondrial gene whose product is required in large amounts at one point in the life cycle where a sudden biosynthesis of new mitochondrial material occurs.

Kinetoplast network DNA was found to consist mainly of the repeated minicircle base sequence, but also to possess a component of a higher complexity (7.0 · 10^6 daltons) which comprised 5.4 % of the kinetoplast genome. The most likely molecular candidates for this more complex species are the long molecules observed by Simpson and da Silva in deoxyribonuclease II treated kinetoplast DNA associations and by Simpson, L. and Berliner, J., (unpublished) in covalently closed kinetoplast DNA networks. There is a large discrepancy between the percentage of long DNA estimated by Simpson and da Silva to be 33 ± 10 % of the kinetoplast DNA, and the percentage of the higher complexity component as measured in this paper (5.4 %). This is due either to an overestimate of the percentage of long DNA, or to a heterogeneity in the long kinetoplast DNA molecules, with a sizeable portion of these molecules representing tandem repeats of minicircles.

In regard to the nuclear DNA of L. tarentolae, presumed single-copy DNA representing 59 % of the genome had a complexity (pure) approximately three times greater than that of E. coli DNA. Most striking was the fact that 25 % of the nuclear
DNA had an apparent complexity even lower than that of minicircles. However the similarity in sequence complexity does not reflect sequence complementarity since Simpson and da Silva\(^4\) were unable to detect the formation of any density hybrid between coannealed nuclear and kinetoplast DNA from \textit{L. tarentolae}. Previous findings of extensive homology between nuclear DNA and kinetoplast DNA in \textit{Leishmania enriettii} have been criticized on several grounds\(^1,10\).

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APPENDIX TO STUDIES ON KINETOPLAST DNA

APPENDIX

USE OF NUCLEIC ACID FINGERPRINTS TO ESTIMATE THE COMPLEXITY OF MINICIRCLE DNA

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In order to obtain independent evidence concerning the sequence complexity of the minicircle DNA from Leishmania tarentolae we have used the nucleotide fingerprinting techniques developed by Sanger and his collaborators (see refs 1 and 2). Nicked minicircle DNA was used as the template for in vitro synthesis of 32P-labeled DNA. This DNA was ribo-substituted by supplying rGTP in place of dGTP in the reaction mixture so that treatment with alkali would cleave the product specifically at G residues. The fragments obtained are separated by first electrophoresis on a cellulose acetate strip at pH 3.5. The material is then transferred to DEAE ion exchange paper and electrophoresis in the second dimension is carried out in 7% formic acid. Radioautography is used to determine the distribution of the radioactive fragments.

In Fig. 1a we show a fingerprint obtained in this way from minicircle DNA. In this experiment the fragments have been treated with bacterial alkaline phosphatase to increase their mobility on the ion exchange paper so that the larger fragments are better resolved. Since the radioactive label was introduced into the DNA as [2,32P]-dCTP, only fragments which contain internal C residues will be seen on the autoradiograph. This procedure separates fragments according to size and base composition and in some cases even resolves different sequence isomers.

How can we determine the complexity of minicircle DNA from such a fingerprint? First consider a comparison of similar fingerprints from 23-S ribosomal RNA and from a tRNA molecule. It is obvious that many more spots would be observed in the fingerprint of 23-S rRNA since it is by far the larger molecule. But the complexity of the molecule is not directly proportional to the number of spots since many spots will contain two or more fragments from different parts of the molecule. In practice we may minimize such difficulties by considering only those regions of the fingerprint pattern which contain fragments which are large enough and sufficiently well resolved that one may assume that most spots contain only one fragment from a single position within the nucleic acid molecule. In such regions the number of spots should be directly proportional to the complexity of the molecule analysed.

That this can be done with a great deal of success for molecules of sequence complexity comparable to that of minicircle DNA is shown by a detailed consideration of published fingerprints of 16-S ribosomal RNA. In this case Fellner et al. have determined the sequences and the molar yields of all fragments present in each spot of the fingerprint. Fig. 1b shows one such fingerprint obtained by Fellner et al. (in order to be able to make direct comparisons with Fig. 1a the figure has been re-
drawn to omit those spots which would not be radioactive if label were introduced on C alone.

The open circles show those radioactive spots which were found to contain only a single labeled fragment. Filled-in circles show those spots found to contain two or more radioactive fragments from different parts of the 16-S rRNA molecule. In the lower half of the fingerprint shown in Fig. 1b, it is readily apparent that too few of the spots are unique for a count of spots to give a very good estimate of the complexity of the molecule. The upper half of this fingerprint is much more interesting. The largest fragments run in this region and most of the spots observed are unique (42 spots are unique, 5 other spots contain more than one fragment). If 16-S ribosomal

![Diagram](image-url)

Fig. 1. (a) Fingerprints of fragments produced when ribo-G-substituted minicircle DNA (labeled with $[\alpha-\text{P}^3]dCTP$) is cleaved at G residues. Ribo-substituted minicircle DNA was synthesized and cleaved using methods previously described$^5$. The nonradioactive minicircle template was activated for synthesis by random nicking at a concentration of 85 $\mu$g/ml with $5 \times 10^{-7}$ $\mu$g/ml pancreatic deoxyribonuclease I (Worthington DPFF) in 0.05 M Tris-HCl, pH 7.5, 0.005 M MgCl$_2$, 400 $\mu$g/ml bovine serum albumin, for 15 min at 30 °C and then heated to 77 °C for 5 min to inactivate deoxyribonuclease$^5$. (b) Fingerprints of 16-S rRNA redrawn with permission from Fig. 1 of Fellner et al.$^4$ to show the radioactivity pattern expected if the $^{32}$P label had been introduced only adjacent to C
residues (as shown in the case in Fig. 1a). Filled-in circles show these spots found to contain two or more radioactive fragments from different parts of the 16-S rRNA molecule. The spots are numbered according to Fellner et al.4 and the sequences of each fragment may be found in Table III of that paper. The fragments with one or no U residues run in overlapping graticules in the lower half of the pattern. Many of the larger fragments contain two or more U residues and run in the upper half of the pattern.

RNA is representative, then the number of spots in the upper half of this type of fingerprint should be almost directly proportional to the complexity of the molecule analysed (assuming that the molecules analysed have similar base compositions and that their fragment size distributions are not atypical).

Comparison of the number of radioactive spots in the upper portions of Fig. 1a and 1b leads us to conclude that *L. tarentolae* minicircles and *Escherichia coli* 16-S rRNA are of very similar complexity. There are 47 distinct spots in the upper region of Fig. 1b (rRNA, size about 1500 nucleotides) and about 41–47 spots in the corresponding regions of Fig. 1a (minicircles, size about 800 base pairs = 1600 nucleotides).
The variability in the number of spots given for Fig. 1a arises from the difficulty in deciding whether certain spots should be counted as two overlapping spots or only one.

This is the result expected if all minicircle DNA molecules have the same sequence, but this sequence is not itself internally repetitious. The data seem to unambiguously rule out the possibility that there are two or more major kinds of minicircles, each with different sequences which are not internally repetitious. The data do not exclude the possibility that there are two or more major kinds of minicircles with different sequences so long as these sequences have the right amount of internal repetitiousness to yield the same overall sequence complexity.

In carrying out such experiments it is very important to insure that equimolar yields of the radioactive product will be obtained from all parts of the DNA sequence analysed or, if this is not possible, to at least choose an experimental design so that one can predict what fraction of the DNA template which will be represented in the final fingerprint and make appropriate corrections. In this case, since the template was a circular duplex of about 800 base pairs we could insure that all parts of both strands should be copied roughly equally (1600 nucleotide sequence complexity) by randomly nicking the circular molecules with a light deoxyribonuclease I digestion before the start of DNA synthesis. We take advantage of the fact that although DNA polymerase I has an absolute requirement for a primer with a 3'-OH end, it does not appear to have any preferences for starting at a particular sequence. Similar experiments could be carried out using RNA polymerase to make an RNA copy of the DNA template. However, such results might be extremely difficult to evaluate since RNA polymerase has strong preferences for preferentially synthesizing from one strand or the other and for starting and stopping synthesis at particular sequences.

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