Biochimica et Biophysica Acta, 319 (1973) 267–280

BBA 97773

STUDIES ON KINETOPLAST DNA

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

RONALD D. WESLEY* and LARRY SIMPSON

Molecular Biology Institute and the Department of Biology, University of California, Los Angeles, Calif. 90024 (U.S.A.)

(Received May 3rd, 1973)

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 ($7.0 \cdot 10^6$ 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 Simpson[1]). 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.

* Present address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colo. 80302 (U.S.A.).

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 [^{3}H]thymidine (3.5 μCi/ml, 3.0 μg/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 viscocity prior to differential centrifugation, the lysate was forced through a No. 20 gauge needle at 24 lb/inch^2, 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 $A_{260\,nm}$ 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 T_1 (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 °C.

ΦX174 DNA. Escherichia coli HF 4704 was infected with ΦXam3 (a lysis-defective amber mutant of ΦX174) as desbribed 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 tetrasodium 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-λ 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 T_1 (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 conditons, 3 min of sonication reduced *L. tarentolae* nuclear DNA and ΦX DNA to about minicircle-sized fragments.

X-irradiation of minicircles

^{14}C-labeled minicircles, X-irradiated to produce single strand breaks, were used as a marker in the determination of DNA fragment sizes. A siliclad-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 ^{3}H-labeled sample of unknown fragment size was mixed with X-irradiated ^{14}C-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 ^{14}C-labeled minicircles (slower sedimenting peak) was taken as the standard of known molecular weight ($2.8 \cdot 10^5$ daltons[4]) 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[5]. 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 overlayered 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_0t 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[5] was chosen to measure the rate of reassociation of ^{3}H-labeled DNA since low DNA concentrations could be used. ΦX RF DNA was used as a standard of known complexity.

To obtain DNA fragments of uniform size, sonicated minicircles and sonicated ΦX 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

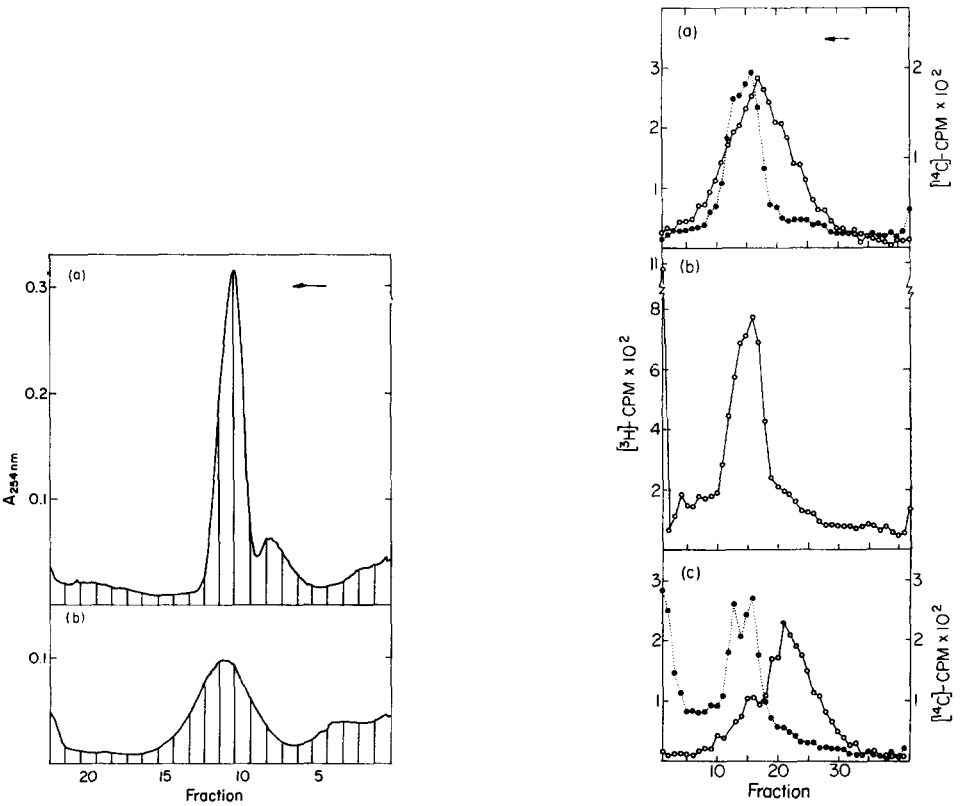

Fig. 1. Band velocity sedimentation in 5-ml neutral 5–20 % sucrose gradients of sonicated minicircles (a) and sonicated ΦX174 RF I molecules (b) to obtain uniform-size DNA fragments. Centrifugation was for 315 min at 60 000 rev./min (4 °C). Prior to renaturation studies, closed minicircles which had 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[4]) from X-irradiated ^{14}C-labeled closed minicircles was used as a reference. The profiles were corrected for cross-over. (a) Sonicated ΦX 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 ΦX 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 ΦX RF DNA of minicircular size are presented in Fig. 3. The smooth lines represent the best single-

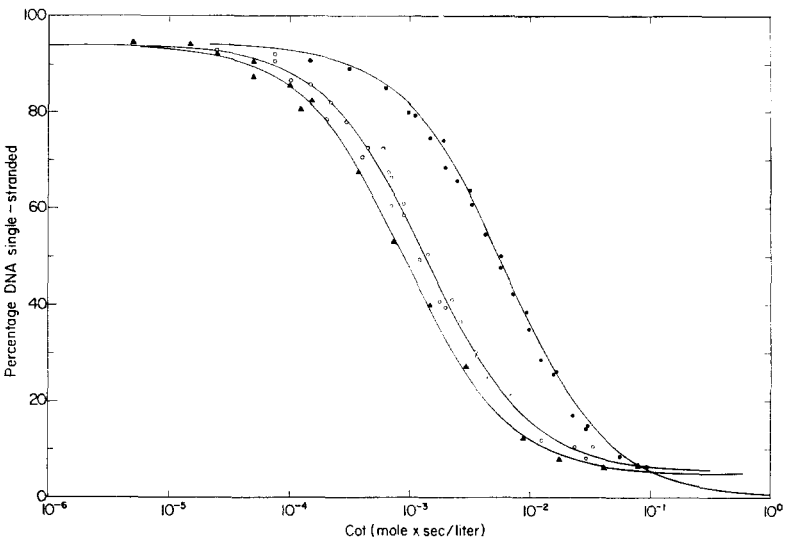

Fig. 3. The kinetics of reassociation of denatured open minicircles ($C_0t_{\frac{1}{2}} = 9.05 \cdot 10^{-4}$, ▲–▲), sonicated minicircles ($C_0t_{\frac{1}{2}} = 1.36 \cdot 10^{-3}$, ○–○) and sonicated ΦX RF DNA ($C_0t_{\frac{1}{2}} = 6.25 \cdot 10^{-3}$, ●–●). Renaturation conditions are described in Materials and Methods. These C_0t 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 μg/ml) and sonicated ΦX RF DNA (0.158–0.816 μg/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 $C_0t_{\frac{1}{2}}$ value for sonicated ΦX 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 $C_0t_{\frac{1}{2}}$ values of sonicated minicircles with one double-strand break and sonicated ΦX 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 $C_0t_{\frac{1}{2}}$ values of minicircles and ΦX. Since the ratio of the contour lengths of ΦX 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.

There was no difference between the $C_0t_{\frac{1}{2}}$ 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. Khowry and Martin[6] 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_0t 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 $C_0t_{\frac{1}{2}}$ curve (Fig. 4) having a $C^0t_{\frac{1}{2}}$ value of $1.26 \cdot 10^{-3}$, which was essentially identical to that obtained with purified minicircles. However, the upper plateau of the network C_0t curve indicated that more DNA had reassociated at a C_0t value of $3 \cdot 10^{-5}$ than was the case for

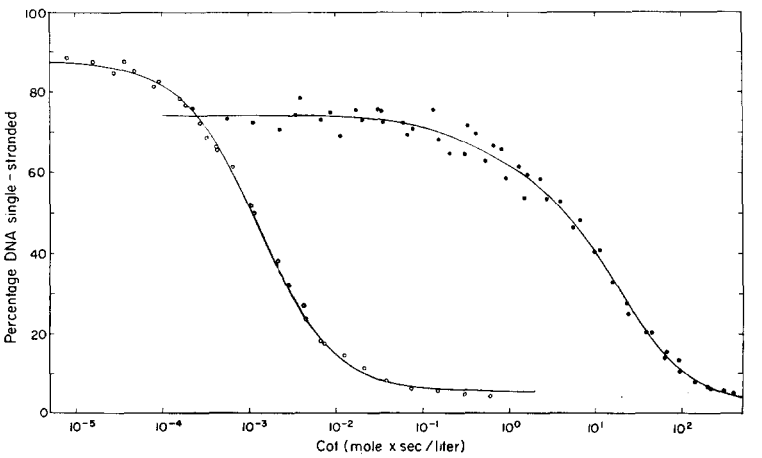

Fig. 4. The renaturation kinetics of sonicated kinetoplast DNA networks (○–○) and sonicated nuclear DNA (●–●). Alkaline sucrose gradient sedimentation was used to remove 22.4 % of the network DNA in the form of covalently closed molecules prior to renaturation. The remaining network DNA consisted of 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 $C_0t_{\frac{1}{2}}$ value of $1.26 \cdot 10^{-3}$ for network DNA and $C_0t_{\frac{1}{2}}$ 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* kinetoplast 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 C_0t 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 C_0t decades, implying a heterogeneity in base sequences[5]. Computor analysis of the C_0t curve in Fig. 5 indicated the presence of two homogeneous components, one having a $C_0t_{\frac{1}{2}}$ value (pure) of $1.29 \cdot 10^{-3}$, 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 $7.0 \cdot 10^6$ daltons. This higher complexity component represented 5.4 % of the total original network DNA.

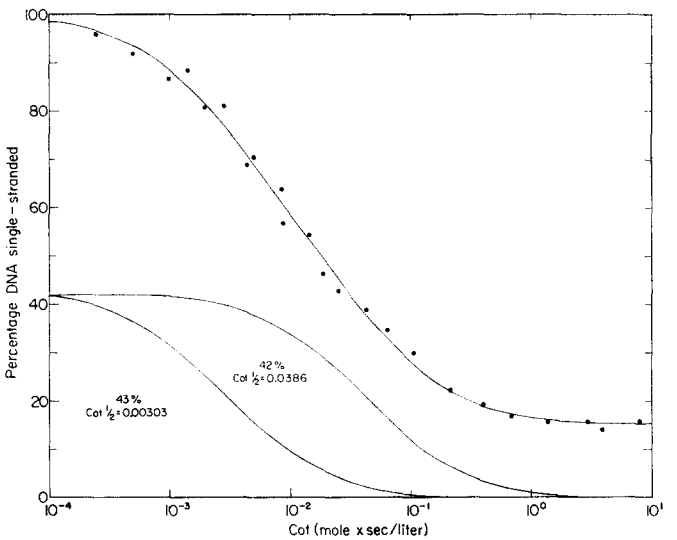

Fig. 5. Renaturation kinetics of the slow renaturing portion of kinetoplast DNA networks. This sample was from the same sonicated network preparation as in Fig. 4, but was further fractionated after removal of the closed molecules by hydroxylapatite after reannealing to a C_0t value of 0.015. The remaining 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 $C_0t_{\frac{1}{2}}$ value for each component is indicated.

The higher complexity kinetoplast 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 weight[7]. It is clear in addition that the kinetoplast DNA component renatured more rapidly than nuclear DNA in Fig. 4.

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_0t 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_0t nuclear DNA is possibly identical to the rapidly annealing nuclear DNA identified by CsCl equilibrium centrifugation by Simpson and da Silva[4].

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., preceeding 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.*[8] in fingerprints of 16-S rRNA.

Therefore the estimated 10^4 minicircles per mitochondrial genome[4] reflect an extensive gene amplification unprecedented in other mitochondrial genomes. The physiological and genetic significance of this amplification remains obscure. It has been proposed[1] 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 ($7.0 \cdot 10^6$ 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[4] 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 percenage of long DNA estimated by Simpson and da Silva[4] 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 *L. tarentolae*. Previous findings of extensive homology between nuclear DNA and kinetoplast DNA in *Leishmania enriettii* have been criticized on several grounds[1,10].

ACKNOWLEDGMENTS

We thank Dr Roy Britten for instructing us in the use of his computer program for fitting second-order curves to C_0t plots and for generous discussion and ideas. This research was supported by Grant No. AI-90102 from the National Institutes of Health, and by the University of California Biomedical Science Support funds. R. D. W. was supported by Training Grants No. GM-1531 and AI-00070 from the National Institutes of Health.

REFERENCES

1 Simpson, L. (1972) *Int. Rev. Cytol.* 32, 139–207
2 Trager, W. (1957) *J. Protozool.* 4, 269–276
3 Francke, B. and Ray, D. S. (1971) *Virology* 44, 168–187
4 Simpson, L. and da Silva, A. (1971) *J. Mol. Biol.* 56, 443–473
5 Britten, R. J. and Kohne, D. E. (1968) *Science* 161, 529–540
6 Khowry, G. and Martin, M. A. (1972) *Nat. New Biol.* 238, 4–6
7 Wetmur, J. G. and Davidson, N. (1968) *J. Mol. Biol.* 31, 349–370
8 Fellner, P., Ehresmann, C. and Ebel, J. P. (1970) *Nature* 225, 26–29
9 DuBuy, H. G. and Riley, F. L. (1967) *Proc. Natl. Acad. Sci. U.S.* 57, 790–797
10 Borst, P. and Kroon, A. M. (1969) *Int. Rev. Cytol.* 26, 107–119