STUDIES ON KINETOPLAST DNA
II. BIOPHYSICAL PROPERTIES OF MINICIRCULAR DNA FROM LEISHMANIA TARENTOLAE

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SUMMARY

Purified kinetoplast DNA minicircles from Leishmania tarentolae promastigotes have been studied in terms of several physical properties: contour length and molecular weight, superhelix density of covalently closed molecules, buoyant density in CsCl, sedimentation coefficients, and thermal melting characteristics. The minicircles studied represented a class of free or loosely bound kinetoplast DNA molecules, but these were found to differ in no significant way from the minicircles isolated from sonicated kinetoplast DNA networks. Naturally occurring open minicircles were found to possess a single nick per molecule. Evidence was obtained for intramolecular heterogeneity in base composition as the cause of an observed multiphasic melting curve of open minicircles.

INTRODUCTION

The kinetoplast DNA of Leishmania tarentolae and other hemoflagellates has been isolated in the form of sheet-like networks of interconnected minicircles and larger molecules by simple centrifugation techniques (Simpson, L. and Berliner, J., unpublished work and ref. 1). Most of the kinetoplast DNA of the cell is bound up in a single network. In the case of L. tarentolae, there are approx. $10^4$ minicircles of a molecular weight of $0.55 \times 10^6$ per network (Simpson, L. and Berliner, J., unpublished work). We have previously described a technique to isolate closed minicircles from networks by sonication and sucrose gradient sedimentation (Wesley, R. D. and Simpson, L., preceding article; ref. 2), and have investigated several physical properties of such minicircles. We have also described a class of free or loosely bound minicircles which represent approx. 6–9% of the total kinetoplast DNA and can be isolated in

Abbreviation: SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0.

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PHYSICAL PROPERTIES OF KINETOPLAST DNA MINICIRCLES

several ways (Wesley, R. D. and Simpson, L., preceding article). Several physical properties of “free” minicircles have been studied and are reported in this paper. “Free” minicircles are identical in all significant respects to those isolated from sonicated networks.

MATERIALS AND METHODS

**DNA isolation**

*Minicircles. L. tarentolae* (clonal strain Lt-C-1) cells were grown in Difco brain heart infusion medium or Medium C (ref. 3). Minicircular DNA was isolated by alkaline isolation Methods I or II (Wesley, R. D. and Simpson, L., preceding article). The purified minicircle preparations represented 6–9% of the total kinetoplast DNA.

*Networks.* To label the DNA, cells were grown for 3 days in Medium C in the presence of [3H]thymidine (1.2 μCi/ml, 1.8 μg/ml, Schwarz-Mann). 3H-labeled networks were isolated according to the method of Simpson and Berliner (unpublished). The recovery of kinetoplast DNA networks was approx. 50% of the kinetoplast DNA of the cell.

ΦX174 II. ΦX RF I molecules were isolated by the method of Rush and Warner and were converted into ΦX RF II by mild deoxyribonuclease I digestion as described elsewhere.

**Preparative centrifugation**

Preparative CsCl equilibrium centrifugation was carried out in a Spinco No. 50 fixed-angle rotor for 60 h at 33,000 rev./min (20 °C) in a Spinco Model 12-65B ultracentrifuge. Each tube contained 6.5 ml of DNA–CsCl (Harshaw) solution at an initial density of 1.713 g/ml. Four-drop fractions, collected from the bottom, were diluted with 0.8 ml 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0 (SSC) and the A260 nm read in a Gilford spectrophotometer.

Ethidium bromide–CsCl equilibrium centrifugation was carried out according to Radloff et al. The following conditions were employed: 3 ml final volume, 20 μg DNA, CsCl at an initial density between 1.55 and 1.58 g/ml, 330 μg/ml ethidium bromide, centrifugation for 48 h at 43,000 rev./min in a Spinco SW 50L rotor, 20 °C. Tubes were illuminated with a mineral-light lamp (Ultraviolet Products, Inc.) and photographs were taken with Kodak Tri-X film, using a Wratten 2A ultraviolet filter.

**Analytical centrifugation**

Buoyant density experiments were performed in a Spinco Model E ultracentrifuge at 44,770 rev./min at 25 °C for 20 h. Tracings of the ultraviolet films were made with a Joyce–Loebl recording microdensitometer and buoyant densities were calculated using Micrococcus lysodeikticus reference DNA, 1.731 g/ml (based on an assigned value of 1.710 g/ml for Escherichia coli DNA as a primary standard).

Band velocity sedimentations were performed in 1 M NaCl or 0.9 M NaCl–0.1 M NaOH (pH 12.6) at 44,770 rev./min (20 °C) using a 12-mm single sector, carbon-filled Epon. Type I band-forming centriple. s20,w values were calculated using the correction factors of Studier.
Sucrose gradient sedimentation

Linear 4–18 % (w/w) alkaline sucrose gradients were used to separate covalently closed molecules from nicked minicircles and to resolve circular and linear minicircle single strands. Sucrose (Schwarz-Mann, Ultra Pure) was dissolved in a solution consisting of 1 M NaCl, 0.01 M Tris–HCl, 0.001 M EDTA, and 0.3 M NaOH (pH 13.0).

To separate closed monomeric minicircles from nicked molecules, centrifugation was carried out in a Spinco SW 65 rotor at 60 000 rev./min (4 °C) for 4 h. Tubes were fractionated from the top with an Isco Model D Density Gradient Fractionator equipped with a flow cell of 0.5 cm optical path. To calculate the amount of DNA in each peak, the single-stranded peak was assumed to have a 36 % greater hyperchromicity.

To resolve circular and linear minicircle single strands centrifugation was carried out in the SW 65 rotor at 65 000 rev./min (4 °C) for 10 h. Six-drop fractions were collected from the bottom onto Whatman 3MM filter discs. The DNA on the filters was precipitated with cold 5 % trichloroacetic acid for 10 min, dehydrated in ethanol and ether, dried and counted in toluene-based scintillation fluid (Omnifluor, New England Nuclear) in a Beckman LS-230 scintillation counter.

Electron microscopy

Samples were prepared by a modified Kleinschmidt protein monolayer technique and examined in a Hitachi electron microscope as described elsewhere.

Melting curves

Thermal denaturation was performed in 1-ml stoppered cuvettes in a Gilford spectrophotometer equipped with an automatic blank compensator. The rate of temperature increase was 0.4 °C/min, as monitored by a Gilford thermistor. Corrections were made for thermal expansion.

Thermal chromatography

Hydroxylapatite, prepared by the method of Miyazawa and Thomas, was packed to a final volume of about 0.8 ml in a 0.5 cm × 30 cm water jacketed column (Glenco Scientific, Inc.). The temperature was controlled by a Haake water pump. Samples of radioactive DNA in 0.12 M sodium phosphate buffer (pH 6.8) were loaded onto the column at 60 °C and the column was washed twice with 1.5 ml of the buffer. Greater than 99.8 % of the DNA was retained on the column. Fractions at five degree intervals were collected between 60 and 80 °C, and fractions at one degree intervals were collected between 80 and 98 °C. The column was allowed to equilibrate for 7 min at each temperature interval and then washed twice with 1.5 ml of 0.12 M phosphate buffer. After reaching 98 °C, all of the DNA was removed from the column with 0.4 M phosphate buffer (pH 6.8).

Prior to precipitating the samples with 10 % trichloroacetic acid at 4 °C, 0.1 mg bovine serum albumin was added as carrier. After 30 min in the cold, the precipitate was collected on Millipore HA filters, dried and counted.
RESULTS

The preparation of “free” minicircles used contained a mixture of closed and open molecules in the ratio 60:40. This was the usual preparation obtained by alkaline lysis Methods I and II (Wesley, R. D. and Simpson, L., preceding article). For certain experiments, purified open and closed minicircles were prepared by an additional ethidium bromide–CsCl equilibrium centrifugation step.

Minicircle contour length and molecular weight

A frequency distribution of contour lengths of purified “free” minicircles is shown in Fig. 1. Electron micrographs revealed no regions of single-strandedness in any of the molecules, which had passed through a denaturation–reannealing step in the isolation procedure.

![Fig. 1. Length histogram of purified “free” minicircles. Tracings were made by projecting negatives of electron micrographs with a photographic enlarger and were measured with a map measurer. The lengths are shown in map centimeters, which are proportional to the true contour lengths.](image)

Measurement of minicircles from either the leading or trailing edge of the sucrose gradient monomer peak gave identical weight-average contour lengths, indicating a lack of any heterogeneity in minicircle size.

The molecular weight of minicircles was determined by cospreading with ΦX RF II molecules as shown in Fig. 2. The calculated average molecular weight of a minicircle was $0.55 \pm 0.04 \times 10^6$ giving a corresponding contour length of $0.29 \pm 0.02 \mu m$, assuming $1.92 \times 10^6$ daltons per $\mu m$ (Table 1). This standard deviation is expected for a homogeneous population of DNA molecules of this size$^{11}$. The calculated molecular weight is identical, within experimental error, to that previously determined by Simpson and da Silva$^2$ for *L. tarentolae* network minicircles which had not undergone alkaline denaturation.

Superhelix density of closed minicircles

Open and closed minicircles formed two broad bands at equilibrium in ethidium bromide–CsCl gradients in the SW 50 rotor. The large separation between these bands ($3.56 \pm 0.09$ mm; $n = 4$) suggested that minicircles have a low superhelical
Fig. 2. Open minicircles and ΦX RF II molecules co-spread on a water hypophase. The ratio of contour lengths is 6.19. The calculated molecular weight for minicircular DNA is $0.55 \times 10^6$, using $3.4 \times 10^6$ as the molecular weight of ΦX RF II (ref. 34). Reverse contrast.

**TABLE 1**

MEASUREMENT OF OPEN MINICIRCLES CO-SPREAD WITH ΦX174 RF II MOLECULES

<table>
<thead>
<tr>
<th>Molecular species</th>
<th>Measured length</th>
<th>($L_{o}^{\Phi X}$)</th>
<th>Mol. wt ($\times 10^6$)</th>
<th>($L_{o}^{mc}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open minicircles</td>
<td>4.7 ± 0.3 cm (167)</td>
<td>6.19</td>
<td>0.55 ± 0.04</td>
<td>0.29 ± 0.02 μm</td>
</tr>
<tr>
<td>ΦX174 RF II</td>
<td>29.1 ± 0.7 cm (79)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Measurements of map distances. The standard deviation and the number of determinations are included.

** Weight-average contour length: assuming $1.92 \times 10^6$ daltons per μm (Rhoades, et al.).

density. The open and closed forms of SV 40* and ΦX RF molecules centrifuged under identical conditions showed band separations of $2.80 \pm 0.10$ mm ($n = 3$) and $2.56$ mm, respectively.

The method of Gray et al. was used to calculate the superhelix density. Purified open and closed “free” minicircles and SV 40 I and II molecules were co-centrifuged in separate ethidium bromide–CsCl equilibrium gradients in an SW 50L

* The SV 40 was a gift from Dr W. Upholt.
rotor under standard conditions (Fig. 3). The calculated superhelix density of minicircles in concentrated salt solutions was $0.011 \pm 0.004$ (n = 3) turns per 10 base pairs. Assuming 838 ± 45 base pairs per molecule, we calculated the number of superhelical turns per molecule to be $1.0 \pm 0.3$, a value which is consistent with the appearance of minicircles in electron micrographs when spread on 0.25 M ammonium acetate (Fig. 4). Fig. 4 also shows the appearance of closed minicircles spread on water and on water containing ethidium bromide at 200 µg/ml. The “dead branch” appearance of the molecules when spread on the ethidium bromide hypophase is due to the super twisting caused by intercalation of dye molecules, and has been reported previously for closed chick liver mitochondrial DNA molecules by Ruttenberg et al.\textsuperscript{14}.

![DNA banding patterns in ethidium bromide-CsCl density gradients](image)

Fig. 3. DNA banding patterns in ethidium bromide–CsCl density gradients. (a) Purified minicircles. (b) SV 40 I and II. Photographs of fluorescent bands were taken with a SLR Topcon camera using Kodak Tri-X film. The tubes were centrifuged in the SW 50 rotor under standard conditions (see Materials and Methods.)

**Number of single strand breaks per open “free” minicircle**

Naturally occurring open minicircles were obtained from the standard preparation of “free” minicircles by ethidium bromide–CsCl equilibrium centrifugation and were sedimented through an alkaline sucrose gradient in order to estimate the number of single-stranded breaks. As shown in Fig. 5, the two peaks, corresponding to the circular and linear strands, were approximately equal in area, indicating that the molecules possess on the average a single nick. The fact that these molecules underwent a denaturation–renaturation step in the isolation procedure and did not form concatenates\textsuperscript{15} is again suggestive that there is only a single nick per molecule; on the other hand, minicircles which were randomly broken by sonication prior to denaturation and renaturation formed a sharp peak in buoyant CsCl (Fig. 6a), indicative of the formation of concatenates. However, this is not conclusive evidence as the formation of concatenates is most likely a concentration-dependent process and this was not tested in the case of the naturally occurring open minicircles.
Fig. 4. Electron micrographs of covalently closed minicircles spread on a hypophase of (a) redistilled water; (b) 0.25 M ammonium acetate; (c) water containing 200 μg/ml ethidium bromide.

**Buoyant properties**

Minicircles formed a single broad, symmetrical band when centrifuged to equilibrium in neutral CsCl (Fig. 6b). The buoyant density was $1.705 \pm 0.001$ g/ml,
Fig. 5. The resolution of circular and linear minicircle single strands by band velocity sedimentation on a 5-ml alkaline 4-18 % (w/w) linear sucrose gradient. Centrifugation was carried out at 65000 rev./min (4 °C) for 10 h in the SW 65 rotor. The faster sedimenting peak presumably represents circular single-stranded molecules. The leading shoulder of this peak possibly is due to contamination by singly nicked minicircle catenated dimers. Only a portion of the 62 fractions of this gradient is shown. The remainder of the gradient contained no radioactivity.

Fig. 6. Densitometer tracings of ultraviolet films obtained after analytical CsCl density centrifugation for 20 h at 44 770 rev./min, 25 °C. (a) Sonicated minicircles with one double-strand break which were heat-denatured and renatured for 2.8 h at 16 μg/ml, in 0.12 M sodium phosphate buffer (pH 6.8) at 60 °C. (b) Minicircles purified by alkaline cell lysis Method I (Wesley, R. D. and Simpson, L. preceding article).

corresponding to a G + C content of 45.6 % (ref. 16) assuming an absence of unusual bases.

Single strands of open minicircles were not resolved by either isopycnic centrifugation in alkaline CsCl, or the method of Hradecna and Szybalski17 utilizing poly-(I-G).

Sedimentation behavior

Table II presents the sedimentation coefficients derived by analytical-band

<table>
<thead>
<tr>
<th>Molecular species</th>
<th>s_{20}w (1 M NaCl)</th>
<th>s observed (0.9 M NaCl-0.1 M NaOH, pH 12.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open minicircles</td>
<td>10.0 ± 0.0 (2)</td>
<td>7.0 ± 0.1 (6)</td>
</tr>
<tr>
<td>Closed minicircles</td>
<td>10.2 ± 0.2 (6)</td>
<td>18.2 ± 0.4 (3)</td>
</tr>
<tr>
<td>Closed dimers</td>
<td>14.4 ± 0.4 (4)</td>
<td>26.1 ± 0.3 (3)</td>
</tr>
<tr>
<td>Closed trimers</td>
<td>17.9 ± 0.6 (3)</td>
<td>33.5 ± 0.1 (2)</td>
</tr>
</tbody>
</table>
velocity sedimentation of minicircles and catenated minicircles in neutral and alkaline NaCl solutions. The data are in agreement with the previously assigned molecular configurations for the various sedimenting components of sonicated kinetoplast DNA associations.

Open and closed monomeric minicircles, mixed in equal amounts, were not resolved by band sedimentation in neutral 1 M NaCl or in neutral 3 M CsCl; they sedimented as a single band with an $s_{20,w}$ of 10.0 for two preparations in 1 M NaCl. This lack of resolution is probably a function of both the small size and the low superhelix density of these molecules. The average $s_{20,w}$ value for closed minicircles was 10.2 ± 0.2 (n = 6).

The ratio of the $s_{20,w}$ values for closed monomeric minicircles at pH 7.0 and at pH 12.6 was 1.8. This ratio is consistent with results obtained with closed circular molecules of larger size. The ratio of the $s_{20,w}$ values of closed minicircles and closed catenated dimers was 1.4, which is in agreement with that reported by Wang and by Brown and Vinograd.

**Melting characteristics**

Purified monomeric minicircles containing open and closed molecules in the ratio 60:40 showed a multiphasic melting curve with a total average hyperchromicity of 18.9 ± 4.6 %, (n = 3), and three melting regions with $T_m$ values of 82.3 ± 0.6 °C, 85.4 ± 0.6 °C, 87.7 ± 0.6 °C. These $T_m$ values were slightly higher than those previously reported for kinetoplast DNA associations.

Purified open and closed monomeric minicircle preparations showed similar multiphasic melting curves but with markedly different hyperchromicities (Fig. 7).

![Fig. 7.](image)

(a) Melting curves of open (-○-) and closed (-●-) minicircles in SSC. (b) The differential melting curve of open minicircles. The ordinate represents the rate of melting, where

$$Y = \frac{A_{T1} - A_{T2}}{A_{100} - A_{25}}$$

$A_{T1}$, $A_{T2}$, $A_{100}$, and $A_{25}$ are the $A_{260}$ readings (corrected for thermal expansion) at temperatures $T_1$, $T_2$, 100 and 25 °C, respectively.
The open minicircles had a total hyperchromicity of $36.0 \pm 1.8\%$ ($n = 5$), whereas closed minicircles only showed a hyperchromicity of $5.2 \pm 1.0\%$ ($n = 3$). Alkaline sucrose gradient analysis of the melted closed minicircle preparations proved that the observed absorbance increase was due to the introduction of strand breaks during the melting process.

Melting of closed minicircles was possible in $7.2\ M\ NaClO_4$. In this solvent closed minicircles exhibited a broad melting curve with a hyperchromicity of $28\%$ and a $T_m$ of $71\ ^\circ C$, which corresponds to a $T_m$ in SSC of $106\ ^\circ C^{21}$. The $T_m$ of open minicircles in $7.2\ M$ perchlorate was $44\ ^\circ C$ and the hyperchromicity was $35\%$. The characteristic multiphasic melting curve observed in SSC was not present for either open or closed minicircles in $7.2\ M$ perchlorate.

There was very little mispairing in the open minicircles that had undergone thermal denaturation in SSC and were renatured at $60\ ^\circ C$ in the same solvent. A second melting of the same sample showed no lowering of the present hyperchromicity or the $T_m$ and renatured open minicircles showed no regions of strand separation in electron micrographs and had weight-average contour lengths identical to those of minicircles that had never undergone alkaline treatment (Table III).

**TABLE III**

**COMPARISON OF WEIGHT-AVERAGE CONTOUR LENGTHS PRIOR TO AND AFTER THERMAL DENATURATION**

Molecules were projected and traced using a photographic enlarger. The standard deviation and the number of measurements are included. Contour lengths are given in terms of measured map distances.

<table>
<thead>
<tr>
<th></th>
<th>Open minicircles</th>
<th>Closed minicircles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before denaturation</td>
<td>$5.50 \pm 0.25\ cm\ (50)$</td>
<td>$5.40 \pm 0.24\ cm\ (52)$</td>
</tr>
<tr>
<td>After denaturation</td>
<td>$5.51 \pm 0.21\ cm\ (54)$</td>
<td>$5.44 \pm 0.19\ cm\ (54)$</td>
</tr>
</tbody>
</table>

**Intramolecular base composition heterogeneity**

The multiphasic nature of the melting curve of open minicircles could be a result of either intramolecular or intermolecular heterogeneity in base composition. Several lines of evidence indicate the presence of only one type of minicircle possessing intermolecular heterogeneity.

If several classes of minicircles with different base compositions existed, then a separation by thermal chromatography on hydroxyapatite should be possible$^{10}$. In the experiment shown in Fig. 8a, $^3$H-labeled open minicircles and sonicated $^{14}$C-labeled nuclear DNA (average single-stranded fragment size of $10^3$ nucleotides) were applied to a hydroxyapatite column in $0.12\ M$ phosphate buffer at $60\ ^\circ C$ and eluted at one degree intervals between $80$ and $98\ ^\circ C$. The single peak obtained is indicative of intramolecular heterogeneity in base composition. The thermal chromatogram of sonicated kinetoplast DNA networks shown in Fig. 8b also had one peak, but showed some indication of an early melting shoulder.

Further evidence for a single type of minicircle was obtained by selecting the heavy and light edges of preparative CsCl buoyant equilibrium bands of open and
closed minicircles. The buoyant densities of closed minicircles from both edges showed no significant differences. Open molecules from both edges were examined by thermal melting analysis, and again no differences were apparent.

DISCUSSION

Minicircular DNA molecules of hemoflagellates range in size from 0.29–0.80 μm, the size being species-specific\(^{22}\). The 0.29 μm molecules from *L. tarentolae* are the smallest of the minicircular DNA molecules and have a size and complexity (Wesley, R. D. and Simpson, L., following article) large enough to code for only one protein with a molecular weight of 35,000 if transcribed and translated. We have studied several physical properties of a population of free or loosely bound minicircles isolated from *L. tarentolae*. These minicircles were identical in all significant respects to those isolated from sonicated networks.

Open minicircles representing approximately 40% of the isolated molecules possessed a single nick per molecule. By analogy with replication models proposed for viral circular DNA\(^{23}\) and mammalian mitochondrial DNA\(^{24}\), the introduction of a specific nick might represent a step in the replication of minicircles.

The low hyperchromicity (18.9%\(^{2}\)) of purified “free” minicircles preparations on thermal denaturation was due to the presence of covalently closed molecules. Renger and Wolstenholme\(^{25}\) have also shown that covalently closed kinetoplast DNA from *T. lewisi* had a high resistance to thermal denaturation; and both Renger and Wolstenholme\(^{25}\) and Simpson and da Silva\(^{26}\) have shown that this resistance is lost following sonication. DuBuy *et al.*\(^{26}\) have also reported a low thermal hyperchromicity for the kinetoplast DNA from *L. enriettii* which was probably due to the presence of covalently closed molecules.

The thermal melting curve of open minicircles was multiphasic, and this pattern
was not due to the presence of closed molecules, as has been suggested by Leffler et al.27 to account for the multiphasic melting curve of rat liver mitochondrial DNA. Bernardi et al.28 have suggested that an intramolecular heterogeneity in base composition is the cause of the multiphasic melting curves found for the mitochondrial DNAs from yeast petite mutants. A similar explanation may account for the multiphasic nature of the melting curve of L. tarentolae open minicircles, since several lines of experiments — thermal chromatography, analysis of CsCl equilibrium gradients band edges, and complexity analysis by renaturation kinetics (Wesley, R. D. and Simpson. L., following article) — all indicated the presence of only one type of minicircle. The evidence is consistent with the interpretation that minicircles have an intramolecular heterogeneity in base composition. This interpretation is supported by the results of Brack et al.29 with Trypanosoma cruzi kinetoplast DNA minicircles, which are 0.49 μm in contour length; they found that four specific regions of denaturation could be visualized in partially denatured molecules in electron micrographs.

It is of some interest that the buoyant density of purified minicircles (1.705 ± 0.001 g/ml; n = 11) was greater than that previously reported by Simpson and da Silva30 for isolated kinetoplast DNA associations (1.703 ± 0.0003 g/ml; n = 12). In addition, the thermal chromatogram of sonicated kinetoplast DNA networks indicated the presence of an early melting shoulder, which has been found to have a kinetic complexity greater than that of minicircular DNA (unpublished). These results suggest that the non-minicircular DNA component within the large networks has an average base composition slightly higher in A+T than that of purified minicircles.

Covalently closed minicircles were found to have an extremely low superhelix density which is the lowest yet observed for any mitochondrial DNA from any organism. The superhelix density is approximately three times lower than that reported for viral DNAs21,30,31, chick liver mitochondrial DNA14, and is even lower than the value for mouse mitochondrial DNA32, and the suggested values for HeLa cell and sea urchin egg mitochondrial DNAs12.

Covalently closed minicircles are readily isolated in large quantities for L. tarentolae by several methods. They present a homogeneous population of small duplex DNA molecules which are exceptionally suitable for such studies as DNA base sequencing, visualization of specific bases by electron microscopy, and the effect of molecular size on various properties of covalently closed molecules.

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