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MAXI-CIRCLES AND MINI-CIRCLES IN KINETOPLAST DNA FROM *TRYPANOSOMA CRUZI*

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Summary

Maxi-circles are a minor component of kinetoplast DNAs from all trypanosomatids studied, but they have not previously been found in *Trypanosoma cruzi*. We have spread intact kinetoplast DNA from the epimastigotes of strain Y in protein monolayers and analysed the mini-circle networks by electron microscopy. Long loops up to 10 μm were present, extending from the network rim; these are considered typical of maxi-circles. The presence of maxi-circles was proven by digestion of kinetoplast DNA with restriction endonucleases and S_1 nuclease. This released a minor DNA component, detectable by agarose gel electrophoresis, which hybridized to maxi-circle DNA from *Trypanosoma brucei*. The molecular weight of the linearized maxi-circle of *Trypanosoma cruzi* is $26 \cdot 10^6$, as judged from its electrophoretic mobility in 0.6% agarose.

Our restriction enzyme analysis of the mini-circles of *Trypanosoma cruzi* has confirmed their sequence heterogeneity and internally-repeated structure. We have found that more than 90% of the mini-circles are cut into 1/4 length molecules by endonuclease TaqI. Denaturation and renaturation of mini-circles, cut once with endonuclease MboI, mainly yields linear and circular molecules with single-stranded eyes and tails in electron micrographs. This shows that 1/4 repeats contain sub-segments in which sequence divergence is extensive.

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Our EcoRI and HapII digests differ in fragment size distribution from those previously reported. This suggests that this distribution may not be a stable characteristic of the Y strain.

Introduction

The pioneering work of Riou, Brack and their co-workers [1–5] with *Trypanosoma cruzi* first showed that the kinetoplast DNA of trypanosomes consists of a large network of catenated circles, called 'mini-circles' because of their small size (0.45 μm). These mini-circles show micro-heterogeneity in sequence [6–8], like the mini-circles from other kinetoplastid flagellates (see Ref. 9 for review). Electron microscopy [10] and restriction endonuclease digestion [11,12] of kinetoplast DNA networks from *Crithidia* later led to the identification of a second, minor component in the network, called the 'maxi-circle'. Maxi-circles vary in size from 6 μm in *T. brucei* to 11 μm in *Crithidia*, they lack sequence heterogeneity or internal sequence repetition and they probably are the trypanosome equivalent of mitochondrial DNA in other organisms [9].

Although long linear DNA was repeatedly observed in electron micrographs of kinetoplast DNA from *T. cruzi* [1,7], a distinct maxi-circle has not been found. In view of the postulated essential function of this kinetoplast DNA component [9], it should also be present in *T. cruzi*. We have, therefore, set out to find it, using methods previously employed to detect the maxi-circle in other trypanosome genera (see Refs. 9 and 13). A brief summary of our results was presented at recent symposia [13,14].

Methods

Growth and isolation of trypanosomes. *T. cruzi* Y strain [15] kept by weekly transfers in Warren's liquid medium [16], was grown in Roux bottles containing 100 ml medium. Epimastigotes were harvested as described elsewhere [17].

Isolation of kinetoplast DNA. The procedure of Fairlamb et al. [18] was followed with minor changes (see Ref. 19). 1 g of cells, wet wt., was suspended in 12 ml 100 mM NaCl, 250 mM sodium EDTA, 10 mM Tris-HCl, pH 8.0, and lysed with Sarkoxyl (1%) and incubated with pronase (2 mg/ml) overnight at 20°C, with gentle stirring. The lysate was then extracted twice with an equal volume of CHCl_3 /isoamylalcohol (24 : 1, v : v). The aqueous phase was carefully removed and the kinetoplast DNA was pelleted by centrifugation (Sorvall HB-4 rotor, 2 h at 11 000 rev./min and 5°C). The pellet was dissolved in 10 ml 100 mM NaCl, 100 mM sodium EDTA, 10 mM Tris-HCl, pH 8.0, and incubated successively at 37°C with 100 μg ribonuclease per ml (1 h) and 0.2 mg pronase per ml, 0.1% Sarkosyl (2 h). Further purification of the kinetoplast DNA was carried out by NaI gradient centrifugation as described [18]. When necessary, the kinetoplast DNA was concentrated by centrifugation in the Sorvall HB-4 rotor using Eppendorf tubes (11 000 rev./min, 45 min, 5°C). The invisible pellet was resuspended in a small volume of 10 mM Tris-HCl (pH 8.0). In some

experiments lyophilized cells were used with essentially similar results.

Digestion of DNA with restriction endonucleases and gel electrophoresis of kinetoplast DNA. The endonuclease digestion conditions and flat gel agarose electrophoresis procedures described in Ref. 19 were used. For visualization of maxi-circle fragments we usually (except for Fig. 1) removed undigested network remnants by centrifugation for 30 min at 11 000 rev./min and 5°C in Eppendorf tubes in the Sorvall HB-4 rotor. For analysis of mini-circle fragments, network remnants were not removed and linear 2.5%–8.0% acrylamide gradient gels were used [20].

Electron microscopy. For the spreading of the networks of kinetoplast DNA a modified version [21] of the Lang and Mitani technique [22] was used as described previously [18]. The length of the edge loops was measured on the electron micrographs with the aid of a Hewlett-Packard Digitizer, model 910-7A. All values were corrected using the 0.45- μ m mini-circles [7], present on the same grid, as internal length control. DNA was prepared by a formamide modification of the basic protein film technique [23]. 0.01 μ g kinetoplast DNA from *T. cruzi* was denatured 5 min at 65°C and renatured 15 min at room temperature in the presence of 60% formamide, 300 mM NaCl, 1 mM EDTA and 10 mM Tris-HCl (pH 8) (the final volume was 50 μ l). The renatured DNA was spread on an aqueous hypophase with the addition of 0.05% cytochrome *c* (final concentration) in 10 mM Tris-HCl (pH 8). Samples were picked, dehydrated, stained and examined as described previously [24].

*Origin of cloned *T. brucei* kinetoplast DNA fragments.* The EcoRI maxi-circle fragments of *Trypanosoma brucei* EATRO 427 kinetoplast DNA were cloned in lambda-gtWES · lambda-B by published procedures [25]. EcoRI restriction analysis of cloned DNA showed the presence of DNA fragments having the same electrophoretic mobility as the Eco-2 or Eco-3 maxi-circle fragments of *T. brucei* kinetoplast DNA. (For nomenclature of the fragments see Ref. 19). The inserted fragments hybridized with labelled total *T. brucei* kinetoplast DNA, but not with cloned mini-circle DNA of *T. brucei*. Mini-circles cut with HindIII were cloned into the HindIII site of plasmid pBR322 [26]. A full description of cloning procedures and clones will be presented in a later paper.

Hybridization experiments. After separation by electrophoresis in an agarose gel, the DNA fragments were denatured in situ and blotted onto a nitrocellulose filter by the method of Southern [27]. The filters were pre-incubated for 2 h at 58–60°C in the presence of a solution containing 50 μ g salmon-sperm DNA per ml and 0.3 M NaCl, 0.03 M sodium citrate (pH 7.0), 0.1% sodium dodecyl sulphate, 0.2% Ficoll, 0.2% polyvinylpyrrolidone and 0.2% bovine serum albumin [28]. The filters were then hybridized for 16 h in the same mixture and at the same temperature with $1 \cdot 10^6$ cpm of 32 P-labelled DNA probes, labelled by nick-translation [29]. The probes used were the lambda-gtWES · lambda-B ligated to either fragment Eco-2 or Eco-3 of the maxi-circle of *T. brucei* (or pBR322 DNA containing a cloned mini-circle of *T. brucei*; see preceding section). Nick-translated and denatured EcoRI fragments of phage ϕ 29 were also added to hybridize to the corresponding marker fragments, also transferred from gel to blot.

After hybridization, the filters were washed three times for 15 min at 58–

60°C in 0.3 M NaCl, 0.03 M sodium citrate (pH 7.0) containing 0.1% sodium dodecyl sulphate and three times at room temperature in the same solution without dodecyl sulphate.

Materials

The source of the restriction endonucleases and other materials is specified in Ref. 19.

Results

Detection of maxi-circle fragments by agarose gel electrophoresis

As a first approach to detect a maxi-circle in kinetoplast DNA networks from *T. cruzi*, the kinetoplast DNA was digested with restriction endonucleases and the fragments were separated by gel electrophoresis. With kinetoplast DNA from other trypanosomatid genera this yields distinctive minor bands of high molecular weight in addition to mini-circle oligomers, mini-circles and mini-circle fragments [9]. In initial experiments, no such 'extra' minor bands were observed in digests of *T. cruzi* kinetoplast DNA, confirming the negative experi-

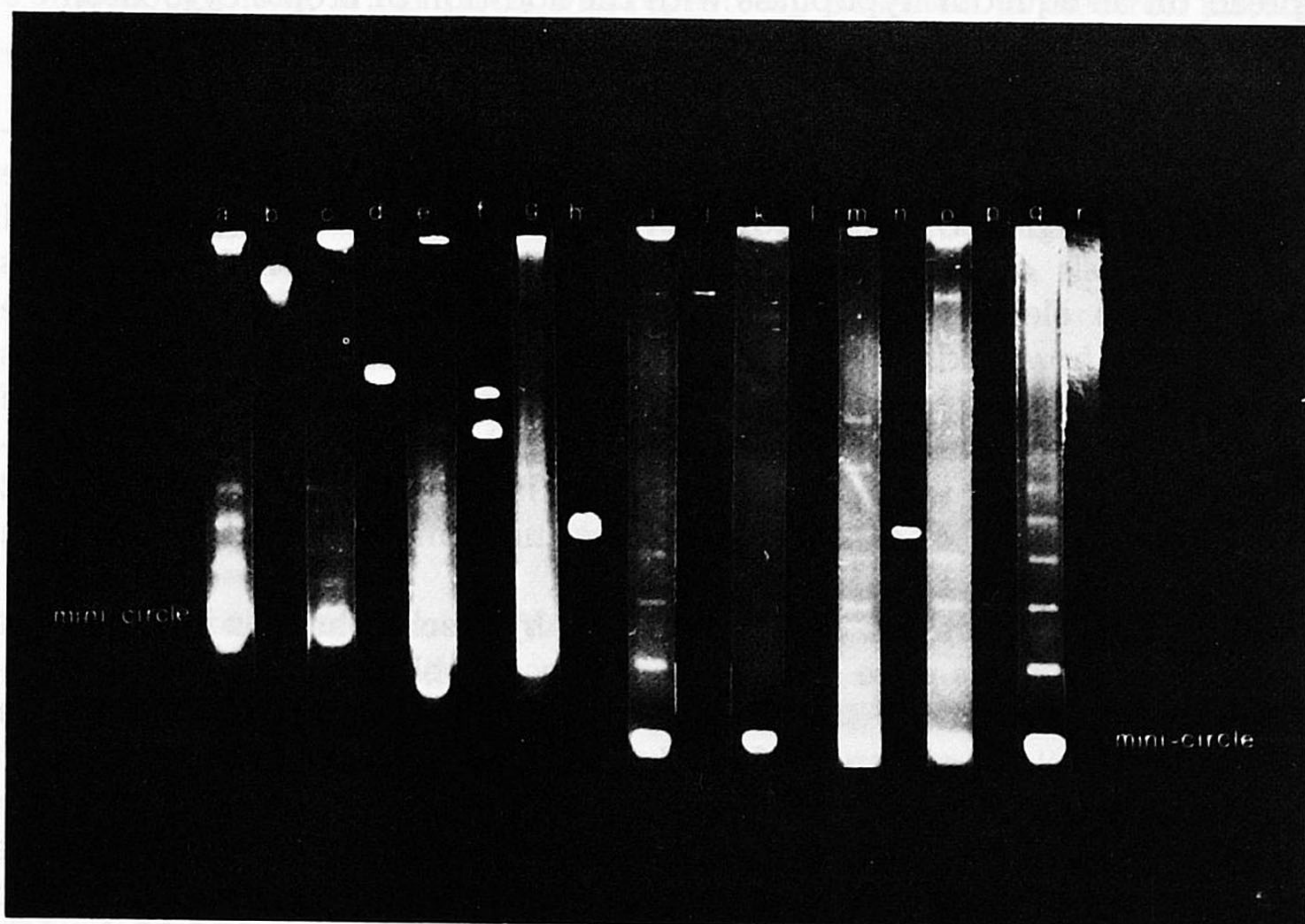


Fig. 1. Detection of maxi-circle fragments of kinetoplast DNA by gel electrophoresis and hybridization to maxi-circle fragments from *T. brucei*. *T. cruzi* kinetoplast DNA was digested with various endonucleases and the digests were separated by electrophoresis through a 0.5% agarose flat gel, containing ethidium bromide (see Methods). Lanes a, c, e, g, i, k, m, o and q show photographs of the fluorescence of the digests with endonuclease KpnI, BglI, EcoRI, HapII, Sall, BglI, EcoRI, HapII and S₁, respectively. The DNA in the gel was blotted onto nitrocellulose filters by the Southern procedure [27] and the blots were hybridized with DNA, labelled by nick-translation in vitro. The blots of lanes a-g were hybridized with the Eco-2 fragment of *T. brucei* maxi-circles and its cloning vector lambda-gtWES · lambda-B, the blots in lanes i-q with the Eco-3 fragment and its cloning vector. Lane b shows the autoradiogram of the hybridized blot of lane a, lane d the hybridized blot of lane c, etc.

TABLE I

MOLECULAR WEIGHTS OF MAXI-CIRCLE FRAGMENTS RELEASED FROM KINETOPLAST DNA WITH VARIOUS ENDONUCLEASES

The molecular weights were calculated from the electrophoretic mobility of each fragment in 0.6% agarose relative to the set of marker DNAs specified in Methods. The table presents average values \pm S.D. The number of experiments is given in brackets.

Fragment No.	Molecular weights ($\times 10^{-6}$) of fragments released by:					
	KpnI (3)	BglI (6)	HapII (3)	HindIII (2)	Sall (3)	EcoRI (4)
1	26 \pm 4	16 \pm 3	19 \pm 3	21	25 \pm 3	7.3 \pm 2
2		7 \pm 0.7	4 \pm 0.1	4.7		4.2 \pm 0.1
3			3.3	(1.7) *		2.7 \pm 0.2
4			(1.8) *	(1.2) *		
5			(1.3) *			
Total	26	23	27	26	25	14

* Fragments only detected by hybridization with *T. brucei* maxi-circle DNA (cf. Fig. 1).

ments in other laboratories. Electron micrographs of these kinetoplast DNA preparations contained large mini-circle associations, but no characteristic intact networks as observed with *Crithidia* or *T. brucei* kinetoplast DNA.

In subsequent experiments shear was avoided in the isolation of kinetoplast DNA. The results with these preparations are summarized in Fig. 1 and Table I. Fig. 1 shows the presence of faint 'extra' bands in agarose gels of kinetoplast DNA digested with restriction endonucleases or S_1 nuclease. The molecular weight of these 'extra' bands is characteristic for the restriction enzyme used and in each digest the added molecular weights of these fragments is about $26 \cdot 10^6$ (Table I) with the exception of the EcoRI digest.

To obtain more direct evidence that the 'extra' bands are derived from the maxi-circle of *T. cruzi* kinetoplast DNA, we have used our observation that maxi-circle sequences are conserved in evolution, whereas mini-circle sequences are not [9]. One would, therefore, expect to find cross-hybridization of the 'extra' bands in Fig. 1 with the maxi-circle but not with mini-circles of *T. brucei*. To test this, the gels in Fig. 1 were blotted onto nitrocellulose sheets and the immobilized DNA was hybridized to cloned fragments of the maxi-circle of *T. brucei*. Endonuclease EcoRI cuts the maxi-circle of *T. brucei* into three fragments; Eco-1 = 10.1 kilo base pairs, Eco-2 = 5.8 kilo base pairs and Eco-3 = 4.1 kilo base pairs [19]. We have cloned Eco-2 and Eco-3 in bacteriophage lambda and these fragments were labelled in vitro and used for the hybridization experiments included in Fig. 1. In each digest of *T. cruzi* kinetoplast DNA at least one 'extra' band shows hybridization and there are clear differences between the hybridization found with Eco-2 and Eco-3. There is a faint non-specific hybridization with mini-circle bands in some slots. No hybridization was observed with two cloned mini-circles of *T. brucei* (not shown).

When maxi-circle fragments are analysed on gels, over-estimation of molecular weights may result from trapping by the undegraded network remnants that remain in the slot. Therefore, the molecular weights of the KpnI and BglI maxi-circle fragments were also analysed after removal of network remnants

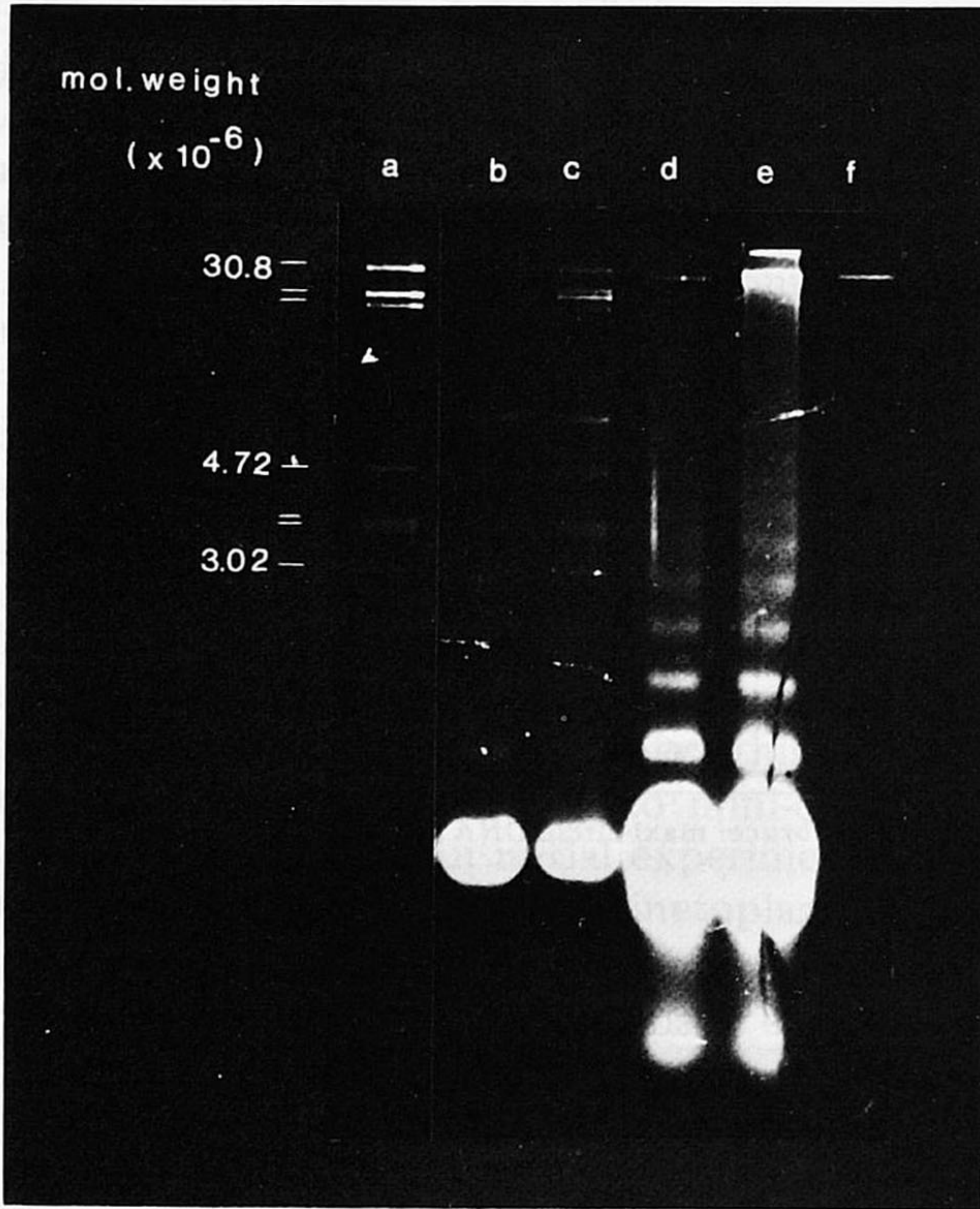


Fig. 2. Analysis of the molecular weight of maxi-circle fragments from *T. cruzi* by co-migration with added reference DNAs. The ethidium-stained 0.6% agarose gel contains: lane a, phage lambda DNA + an EcoRI digest of phage lambda DNA; lane b, kDNA \times BglI; lane c, kDNA \times BglI + marker DNA set of lane a; lane d, kDNA \times KpnI; lane e, kDNA \times KpnI + phage lambda DNA; lane f, phage lambda DNA. kDNA, kinetoplast DNA

and with internal reference DNAs, as shown in Fig. 2. Although the apparent molecular weight of the larger BglI maxi-circle band is decreased by $1 \cdot 10^6$ in the presence of the added phage lambda DNA, the molecular weights of the maxi-circle calculated from this experiment ($27 \cdot 10^6$ for the BglI digest, $28 \cdot 10^6$ for the KpnI digest) do not differ from those in Table I.

Electron microscopy of kinetoplast DNA networks

An example of a kinetoplast DNA network, spread by the protein monolayer technique, is presented in Fig. 3. In addition to catenated mini-circles, occasional edge loops much longer than mini-circles are visible, as shown. We have measured 11 of these loops; 80% were between 6 and 8 μm , the longest one was 10 μm . In addition, one free circle was found with a contour length of 6.7 μm .

Analysis of mini-circles

Riou and Yot [6,7] have previously reported limited sequence heterogeneity in the mini-circles of *T. cruzi*. In addition, they observed preferential production of 3/4-, 1/2- and 1/4-size molecules after digestion with EcoRI, HaeIII or HapII, suggesting the presence of an internally repeated structure. These results

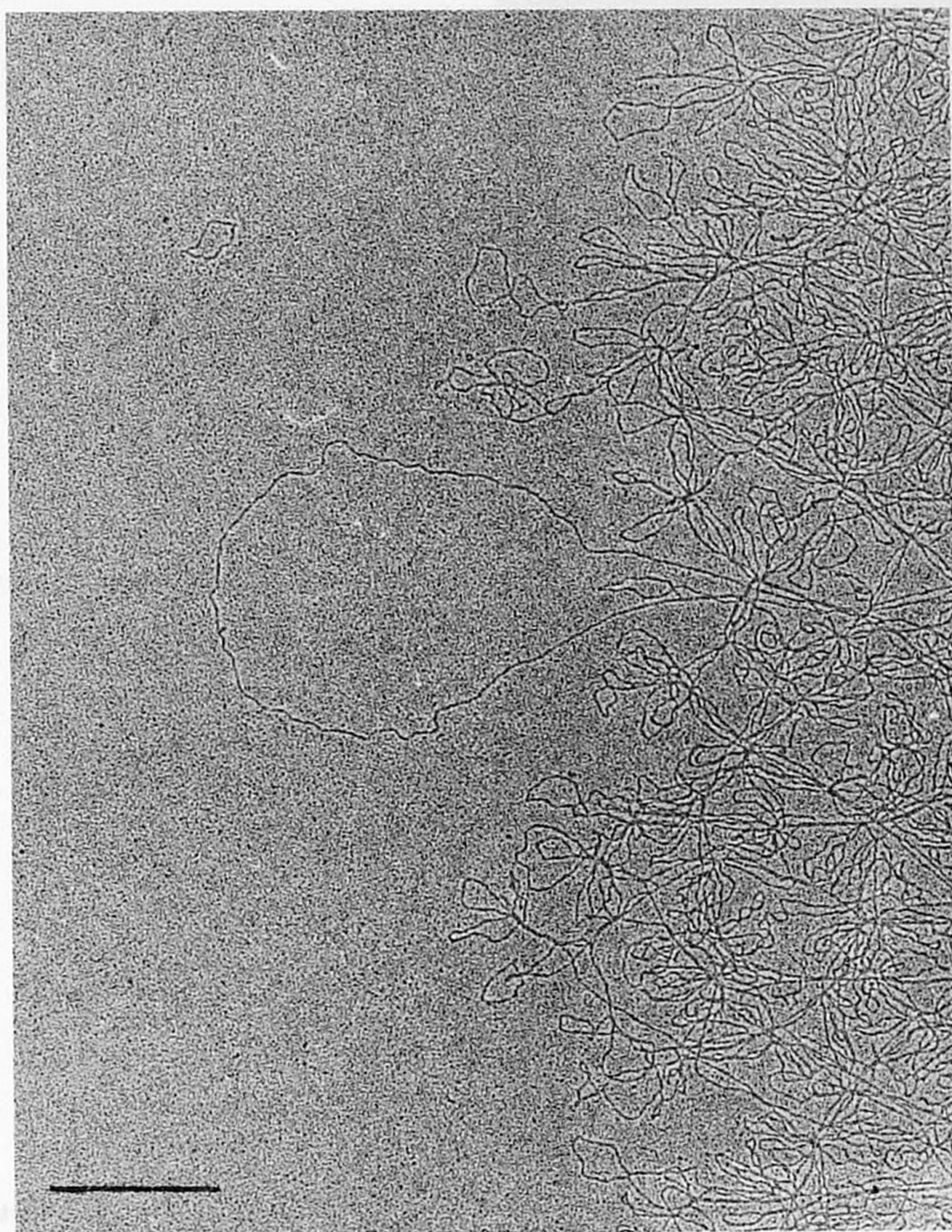


Fig. 3. Electron micrograph of the edge of a network, spread by a modified protein-monolayer technique as described in Methods. The bar is 0.5 μm .

are confirmed and extended by the data presented in Fig. 4 and Table II. The digests in lanes b–e of Fig. 4 do not change when the enzyme concentration is changed (not shown). The non-stoichiometric bands are, therefore, not partial digest products but caused by sequence heterogeneity. The internal repeat structure of the mini-circles is most clearly demonstrated by our finding that endonuclease *TaqI* cuts nearly all mini-circles into quarter molecules (lane b). The partial *TaqI* digest in lane e proves that the repeat size is indeed exactly one quarter of the circle contour length. Finally, the high resolution provided by the acrylamide gels used, shows that many bands are accompanied by a satellite band with a 5–8% lower molecular weight. We attribute this to the presence of a sub-population of smaller mini-circles with a deletion in the repeat.

If sequence heterogeneity in the major fraction of the mini-circles were limited to point mutations and other small alterations, one would expect that once-cut mini-circles would reform perfect hybrids after denaturation and renaturation. This was tested with mini-circles cut with endonuclease *MboI*. Electron micrographs of the renatured mini-circles showed that less than 5% of the molecules had formed fully-matched linears or circles of mini-circle size.

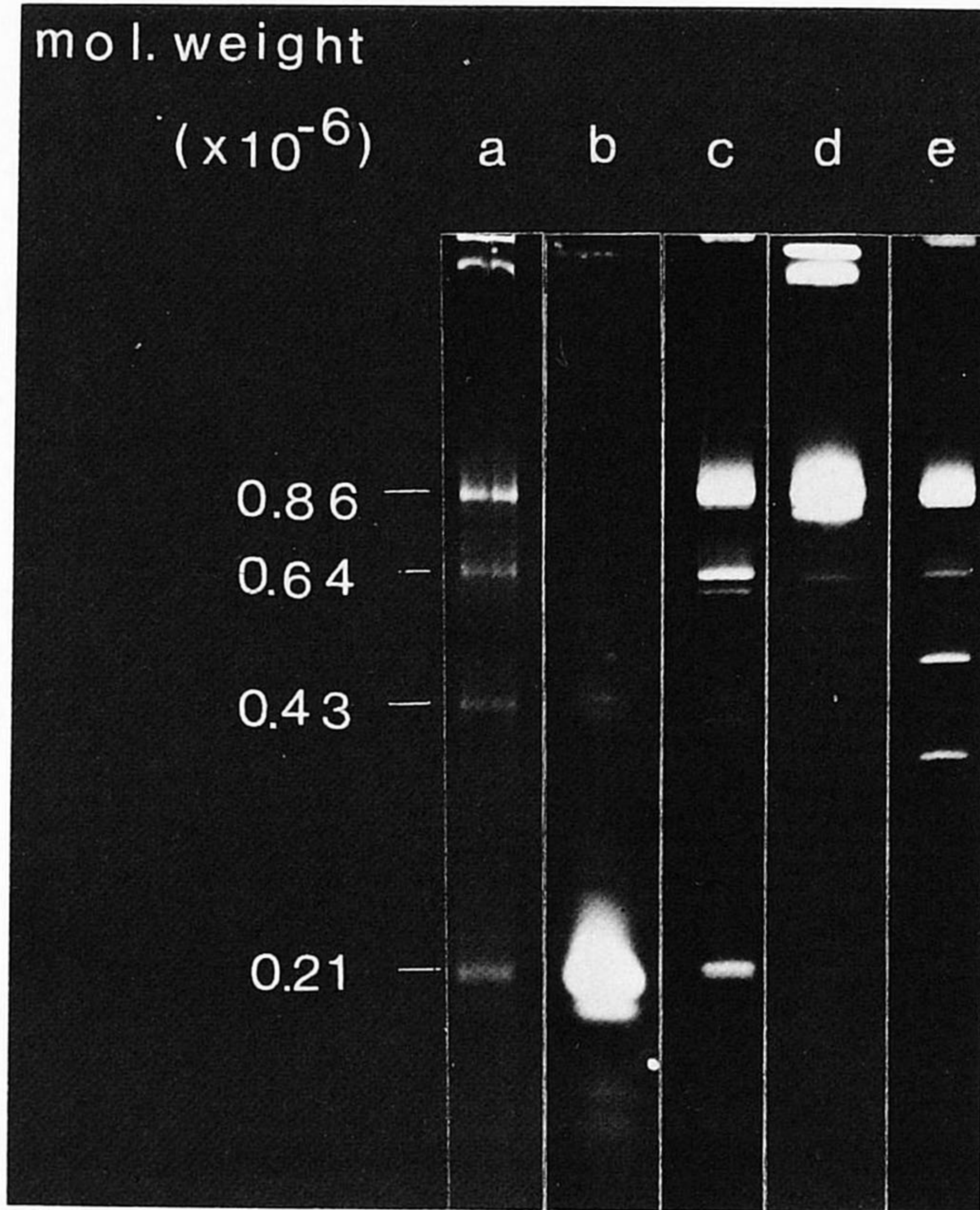


Fig. 4. Gel electrophoretic separation of mini-circle fragments, resulting from digestion of kinetoplast DNA networks with various restriction endonucleases. Fragments were separated by electrophoresis through a linear acrylamide gradient gel (2.5–8.0%). Lane a shows a partial TaqI digest; the other lanes complete digests with TaqI (b), HaeIII (c), AluI (d) and EcoRI (e).

TABLE II

MOLECULAR WEIGHTS OF MINI-CIRCLE DNA FRAGMENTS OBTAINED BY DIGESTING *T. CRUZI* KINETOPLAST DNA WITH VARIOUS RESTRICTION ENDONUCLEASES

Major fragments are underlined. The molecular weights were calculated either from the mobility of each fragment relative to a set of marker DNAs (see Methods) or to the partial TaqI digest of mini-circles (see Fig. 4).

Molecular weights ($\times 10^{-6}$) of fragments released by:

TaqI (partial digest)	TaqI	HaeIII	AluI	HapII	EcoRI
<u>-0.86</u>		<u>0.86</u>	<u>0.86</u> <u>0.80</u>	<u>0.86</u>	<u>0.86</u>
<u>-0.64</u>		<u>0.63</u> 0.58	0.62	<u>0.70</u> 0.63	0.72 0.64 <u>0.62</u> 0.59
<u>-0.43</u> 0.40	0.43 0.40	0.42 0.40			<u>0.48</u> 0.42 0.40
<u>-0.21</u> 0.20	<u>0.21</u> <u>0.20</u> 0.17 0.16	<u>0.21</u> 0.16	0.21	0.21 0.16	<u>0.37</u> 0.21

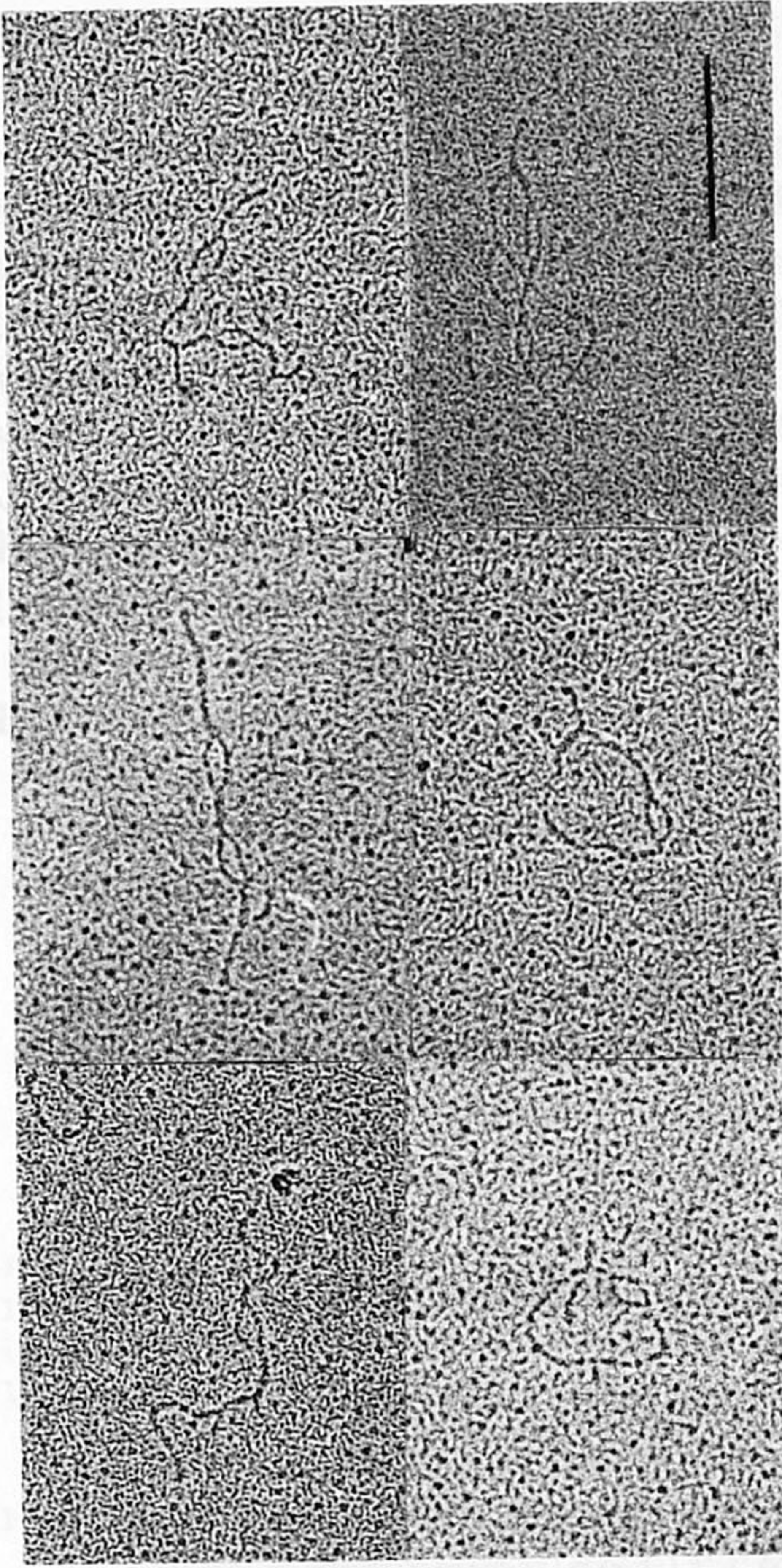


Fig. 5. Electron micrographs of renatured mini-circles. The kinetoplast DNA was digested with endonuclease MboI and after deproteinization the DNA was denatured, renatured and spread for electron microscopy as specified in Methods. The bar is $0.2 \mu\text{m}$.

More than 90% of all molecules contained single-stranded eyes or tails. A gallery of the major types present is assembled in Fig. 5. The size of the single-stranded eyes varied in 12 molecules between 0.06 and $0.08 \mu\text{m}$ with an average value of $0.073 \mu\text{m}$. This corresponds to 17% of the contour length of the mini-circle, assuming that the length of single- and double-stranded DNA is the same under our spreading conditions.

These results show that the $1/4$ repeats contain sub-segments in which sequence divergence is extensive.

Discussion

Our results establish the presence of a maxi-circle component in *T. cruzi* kinetoplast DNA. From Fig. 2 and Table I this maxi-circle appears to be $26 \cdot 10^6$ daltons, i.e. the largest yet found (see Ref. 13). The presence of edge loops in the networks up to $10 \mu\text{m}$ is in good agreement with this size estimate. Two discrepancies should be noted, however. First, we have found in electron

micrographs of spread kinetoplast DNA networks a single free circle and this had a contour length of 7 μm rather than the 13 μm expected. Free large circles ranging in size from 1 μm to 6.5 μm were previously observed in kinetoplast DNA preparations of *T. cruzi* by Riou and Yot [7] and they interpreted these to be circular oligomers of mini-circles. Second, the maxi-circle fragments in EcoRI digests add up to $14 \cdot 10^6$ daltons rather than $26 \cdot 10^6$ without clear evidence for double bands. Neither of these findings argue strongly against the $26 \cdot 10^6$ dalton size and it is highly unlikely that the unusually low mol% G + C (found for all maxi-circles analysed thusfar [13,30]) would lead to anomalously slow migration of long maxi-circle fragment in agarose. The ready cross-hybridization of the maxi-circle fragments from *T. brucei* and *T. cruzi* indicates that the *T. cruzi* maxi-circle has a low mol% G + C too, but this remains to be verified.

In our experience, the kinetoplast DNA networks of *T. cruzi* are more fragile than those from *Crithidia* species or *T. brucei*. Since network damage leads to preferential loss of the large maxi-circles, this may explain the low and variable amount of maxi-circle sequences found in purified networks and the failure of others to detect maxi-circle fragments in restriction digests of *T. cruzi* kinetoplast DNA. The Southern blotting procedure in conjunction with cloned kinetoplast DNA fragments should make it possible to detect minute amounts of kinetoplast DNA fragments even in total cell DNA. This should allow the comparison of restriction endonuclease recognition sites on the maxi-circle from different *T. cruzi* strains without requiring large amounts of relatively intact purified kinetoplast DNA networks.

It is of interest that the fragment distribution that we obtain by digesting mini-circles of the Y strain of *T. cruzi* with restriction enzymes, differs from that reported by others for the Y strain or other strains. Both Riou and Yot [7], Mattei et al. [8] and Riou and Gutteridge [31] only observed multiples of 1/4 molecules in all digests, whereas we find also other major bands, notably in the EcoRI and HapII digests. Moreover, the relative amount of the various HaeIII fragments in our experiments is rather different from those reported [7,8]. We have recently observed, however, the same mini-circle digestion pattern as in Refs. 7 and 8 with another isolate of our Y strain (unpublished data). Disregarding the unlikely possibility of strain mix-up, these results suggest that mini-circle digestion patterns may not be a stable and reliable criterion for strain characterization. This is in line with previous observations that the sequence evolution of mini-circles is rapid and that a change in restriction fragment patterns can even be observed with time in *Crithidia* cultures maintained in the laboratory [9,13].

Our results provide a base line for a more detailed analysis of the changes in kinetoplast DNA during the developmental cycle of *T. cruzi* and a further exploration of the usefulness of kinetoplast DNA sequence characteristics for the taxonomic classification of *T. cruzi* strains. Work along these lines is in progress.

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