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CHARACTERIZATION OF KINETOPLAST DNA NETWORKS FROM THE INSECT TRYPANOSOME *CRITHIDIA LUCILIAE*

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Summary

1. We have used the restriction endonucleases EcoRI and PstI to further characterize the structural components of intact kinetoplast DNA networks from stationary phase *Crithidia luciliae*.

2. Endonuclease PstI cuts less than 7% of the mini-circles (the major component in the network) and appears to give a single cut in the maxi-circles, allowing the isolation of this minor component in linearized form. The molecular weight of these linearized maxi-circles, determined by electron microscopy with phage PM2 DNA as internal standard, is $22 \cdot 10^6$.

3. Electron micrographs of intact networks show DNA considerably longer than the mini-circle contour length (0.8 μm) either in the network or attached to the edge. This long DNA never exceeds the size of maxi-circles (10.2 μm) and it is completely removed by treatment with either endonuclease PstI or endonuclease EcoRI (which cuts the maxi-circles and about 20% of the mini-circles). We conclude that the long DNA in these networks represent maxi-circles and that long circular oligomers of mini-circles are (virtually) absent.

4. Complete removal of maxi-circles with endonucleases PstI or EcoRI has little effect on the overall structure of the network. This shows that the mini-circles are not held together on maxi-circle strings.

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Introduction

The kinetoplast DNA (kDNA) of representatives of the sub-order *Trypanosomatina* is a most unusual form of mitochondrial DNA (mtDNA) (reviewed in refs. 1—7). It is found as a large clump of DNA in a specialized portion of the single mitochondrion of the unicellular flagellates, always in close apposition with the base of the flagellum. In *Crithidia*, an insect trypanosome that can easily be cultured on simple media, the total mass of the kDNA clump is about $2 \cdot 10^{10}$ [8]. Nevertheless, this DNA can be isolated as an intact network of catenated circular molecules, the compactness of the network making it relatively resistant to shear [8]. The major structural element in this network is a duplex DNA mini-circle with an average contour length of 0.8 μm [5].

The analysis of networks from *Crithidia luciliae* with restriction endonucleases led to two new findings: First, we established that the mini-circles are micro-heterogeneous both in size and in sequence [9,10]. Second, we discovered a novel structural component in the network, a maxi-circle with a unique non-mini-circle sequence and a molecular weight of about $20 \cdot 10^6$ [9, 11]. These maxi-circles are a constant and integral part of intact networks and represent 3—5% of the mass of the network in *Crithidia* [11]. Occasional free maxi-circles, not attached to networks, have been observed by Steinert and Van Assel [12]. More recently, maxi-circles have also been found in kDNA networks from *Trypanosoma brucei* [6,7] and *Trypanosoma mega* [13] and they are probably the "true" mtDNA of the *Kinetoplastida*.

In this paper we present the results of further experiments on kDNA networks from *C. luciliae*, mainly aimed at answering the following questions: (1) What is the exact molecular weight of the maxi-circle? (2) What is the effect of the removal of the maxi-circles and of specific mini-circle classes on network structure? (3) Are there other long DNA components in the network in addition to maxi-circles?

Some of the results of this work have been summarized in recent reviews [6,7].

Methods

Growth conditions and isolation of DNAs. kDNA was isolated from *C. luciliae* grown to stationary phase as described [14]. Viral DNAs were isolated by published procedures detailed previously [10].

Degradation of DNA with endonucleases. Endonucleases EcoRI, HapII and HindII + III were obtained and assayed as described [10,11]. Endonuclease HindIII was isolated from a HindII + III preparation by DEAE-cellulose column chromatography as suggested by Old et al. [15]. It was assayed as described for endonucleases HindII + III above, but with 5 mM instead of 10 mM Tris · HCl (pH 7.4) and 7 mM instead of 8 mM MgCl$_2$. Endonuclease PstI was isolated by the procedure described by Roberts et al. [16] for endonuclease HaeII with omission of the ammonium sulphate precipitation and assayed for 30 min at 37°C in 10 mM Tris · HCl (pH 7.5), 10 mM MgCl$_2$, 100 mM NaCl, 1 mM dithiothreitol. Following the incubation the solution was extracted once with an equal volume of buffered phenol; the phenol was removed by ether extraction, the ether with N$_2$ gas.

S_1 nuclease from *Aspergillus oryzae* was isolated according to the procedure of Vogt [17], omitting the Sephadex G-100 filtration and the sulphoethyl-Sephadex chromatography. The enzyme was assayed for 30 min at $45°C$ in 0.125 M sodium acetate (pH 4.7), containing 0.1 mM $ZnSO_4$, 0.4 M NaCl and 0.04% sodium dodecyl sulphate. After the incubation protein was removed with phenol as described above.

Equilibrium centrifugation of DNA in CsCl containing propidium diiodide. The separation of nuclease digests of kDNA into linear and open circular DNA (upper band) and closed circular DNA (lower band) was accomplished by making up the entire digest to 10 mM Tris · HCl (pH 7.5), 350 μg/ml propidium diiodide, adjusting the N_D^{25} to 1.3861 with solid CsCl and spinning to equilibrium (approx. 60 h) at 35 000 rev./min in a Spinco SW50.1 rotor. Following centrifugation, the bands were removed by gentle suction through a blunted 20 gauge hypodermic needle and the dye removed by dialysis as described [11] or by extraction with isopropanol (three times) as described by Hudson et al. [18].

Gel electrophoresis of DNA. The 0.5% agarose gels were electrophoresed and photographed as described [10]. Gels were usually run without ethidium bromide and stained afterwards for 20 min in electrophoresis buffer containing 0.5 μg ethidium per ml. In some experiments 0.1 μg ethidium per ml was included in the gel and 0.5 μg ethidium per ml in the tank and additional staining was omitted.

Before the nuclease digests of kDNA were layered on gels they were usually centrifuged for 25 min at 30 000 rev./min and $4°C$ in a Beckman-Spinco SW50.1 rotor, to remove large mini-circle associations that stay on top of the gel and tend to trap smaller DNA molecules. In some experiments centrifugation for 30 min at 12 000 rev./min and $4°C$ in a Sorvall HB-4 rotor was used.

The molecular weights of DNA fragments were determined from their mobility in gels relative to the set of reference DNAs used previously. Recently, Ito et al. [19] have carefully redetermined the molecular weights of the five fragments derived from phage ϕ29 DNA by digestion with endonuclease EcoRI. We have now used their values ($6.11 \cdot 10^6$, $3.89 \cdot 10^6$, $1.08 \cdot 10^6$, $0.54 \cdot 10^6$, $0.36 \cdot 10^6$) rather than the values determined by us before [10].

Electron microscopy. For the spreading of DNA a modified version [20] of the micro-diffusion technique of Lang and Mitani [21] was used. The spreading solution contained 3 μg cytochrome *c* and 1—2 μg kDNA per ml, 0.15 M ammonium acetate. Diffusion was for 40 min in an atmosphere saturated with formaldehyde vapour. Phage PM2 DNA, if included as length marker, was added to the spreading solution at a concentration of approx. 0.05 μg/ml. The DNA-protein film was picked up on carbon-coated 200-mesh grids, rotary shadowed at low angle with Pt : Pd (80 : 20) alloy and photographed with a Philips EM300 microscope at an operating voltage of 60 kV.

The relative lengths of DNA molecules were determined by projecting negatives of photographed molecules onto paper, tracing the resulting image and measuring the tracings with a Hewlett-Packard Digitizer, model 9107A. The relative lengths of DNA molecules were determined by using only the enlargement of the electron microscope (calibrated with a carbon replica of a diffraction grating containing 2160 lines per mm (E.F. Fullan)) and of projec-

tion. Absolute lengths were determined by comparison with co-spread phage PM2 DNA molecules with a contour length of 3.02 μm and a molecular weight of $5.91 \cdot 10^6$ [22].

Results

The molecular weight of the maxi-circle

We have determined the molecular weight of the maxi-circle in *C. luciliae* kDNA more precisely by electron microscopy, using the linear molecules liberated from intact kDNA networks by digestion with restriction endonuclease PstI. Unlike the restriction endonucleases HindII + III, EcoRI, HapII, AluI and HsuI, which cut the maxi-circle into three or more separate segments [11], the enzyme PstI appears to cut only at one position, allowing the linearized maxi-circle to be freed from the network as a single molecule. The evidence for this is 3-fold:

(1) As shown in slot c of Fig. 1, only one high-molecular-weight band is seen when a PstI digest of kDNA is electrophoresed on agarose gels. The only other band present in the gel is that of the mini-circles.

(2) Although the slow PstI band appears to migrate slightly ahead of the slow band generated by digestion of kDNA with S_1 nuclease (slots b and c of Fig. 1), the two bands co-migrate when present in the same slot (not shown). We have previously shown that the slow S_1 band contains the complete maxi-circle sequence [11].

(3) The maxi-circle was cut from the network with endonuclease PstI and partially purified in a CsCl-propidium diiodide gradient, which separates an upper band with linear DNA and open circles from a lower band containing closed circular DNA. This Pst-cut maxi-circle was redigested with other restriction enzymes and the results were compared with digests by the same set of enzymes of total kDNA. Whereas pre-cutting with endonuclease PstI had no detectable effect on the maxi-circle digests produced by endonuclease EcoRI (Fig. 1, slots d and e), it reduced the estimated mass of the largest HindIII fragment from $9.5 \cdot 10^6$ to $8.4 \cdot 10^6$ daltons (Fig. 1, slots f and g). The missing fragment is presumably too small to be visible as a separate band in the gel. In a similar fashion endonuclease PstI was found to cut only the smallest of the eight maxi-circle bands obtained with endonuclease HapII (cf. ref. 12). These results are compatible with the conclusion that there is only a single recognition site for endonuclease PstI per maxi-circle and that this site is close to an EcoRI site.

The size distribution of maxi-circles linearized with endonuclease PstI and measured by electron microscopy is shown in Fig. 2. The distribution is Gaussian and shows no evidence for size heterogeneity or degradation, in contrast to preparations of linearized maxi-circles obtained with S_1 endonuclease [11]. The mean contour length ($\pm$ S.D.) of 10.2 ± 0.5 μm corresponds to a corrected molecular weight of $22 \cdot 10^6$, using the circular DNA of phage PM2, co-spread with the kDNA, as internal standard for mass/length ratio.

This value is in good agreement with the molecular weight of the maxi-circle determined by electrophoresis in agarose gels [11].
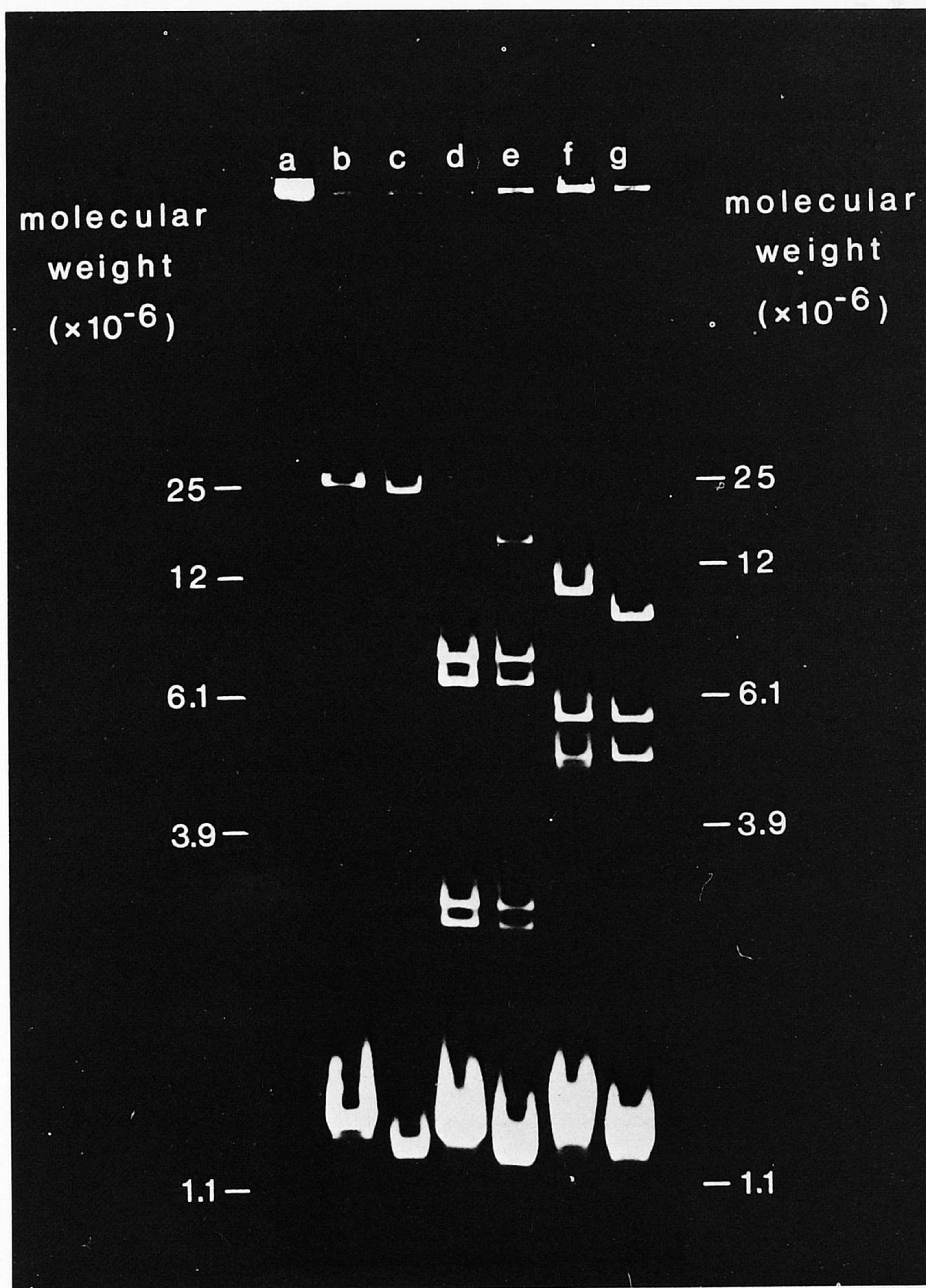
Fig. 1. 0.5% agarose gels of kDNA digested with various nucleases. The gel compares digests of total kDNA (slots b, d, f) with digests of the linearized maxi-circle liberated from the network with endonuclease PstI and purified by CsCl-propidium diiodide equilibrium centrifugation (slots c, e, g). a, 5 µg undigested kDNA; b, 4 µg kDNA digested with S_1 nuclease; c, 0.1 µg PstI upper band; d, 6 µg kDNA digested with endonuclease EcoRI; e, 0.4 µg PstI upper band, digested with endonuclease EcoRI; the faint band with an apparent molecular weight of $14 \cdot 10^6$ is a partial digestion product; f, 5 µg kDNA digested with endonuclease HindIII; g, 0.4 µg PstI upper band, digested with endonuclease HindIII.
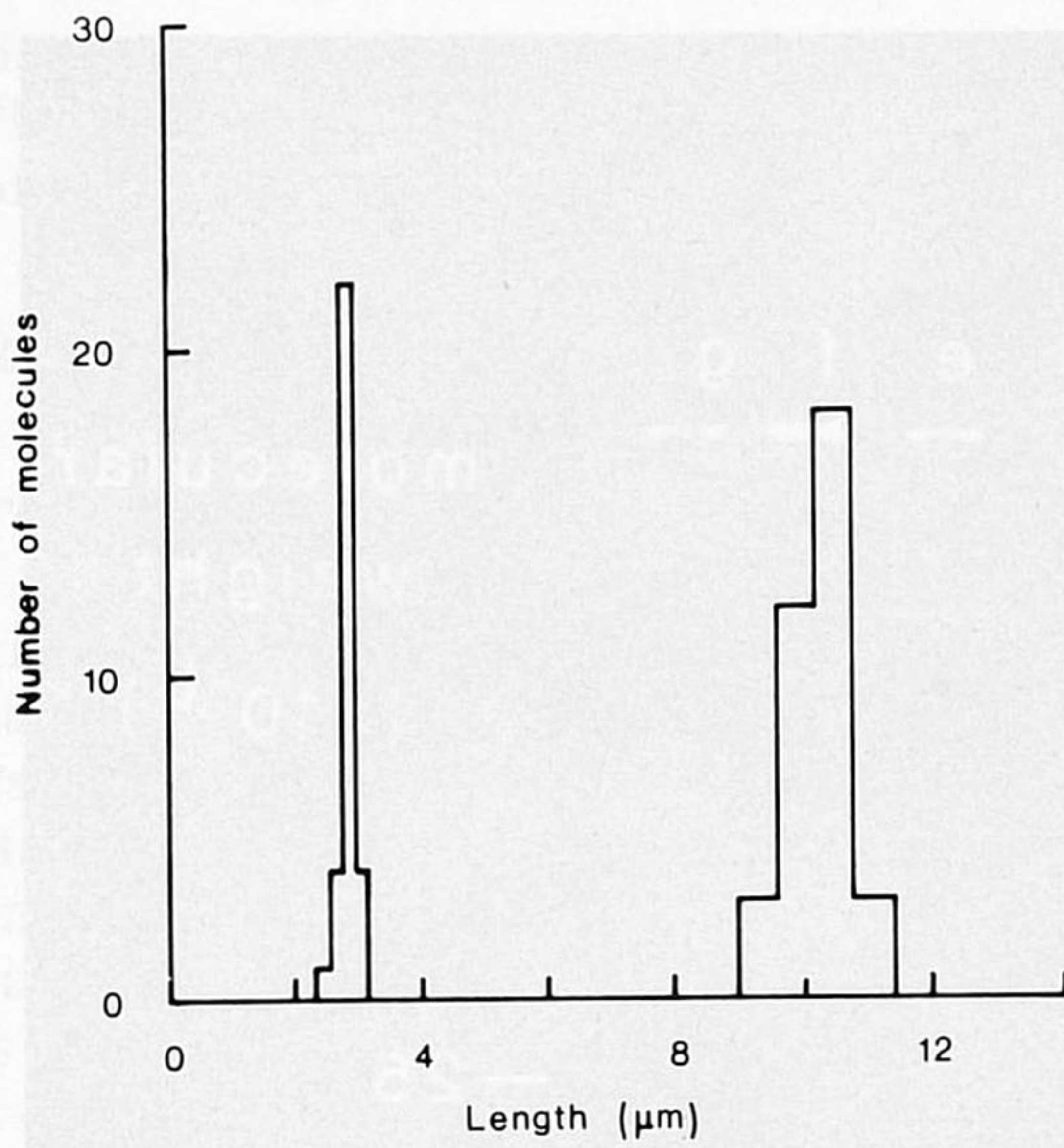
Fig. 2. Size distribution of the long linear molecules (linearized maxi-circles) liberated from kDNA networks by endonuclease PstI. 100 µg kDNA networks were digested with endonuclease PstI and the digest was centrifuged to equilibrium in CsCl containing propidium diiodide. The DNA in the upper band (linear DNA and open circles) was extracted with isopropanol, dialysed against 10 mM Tris · HCl (pH 7.5) and an aliquot was mixed with phage PM2 DNA and examined by electron microscopy as described in Methods. The histogram only includes molecules judged longer than 2 µm. The length of 31 circular phage PM2 DNA and 31 long linear molecules was measured. The peak at 3 µm represents the phage PM2 DNA.

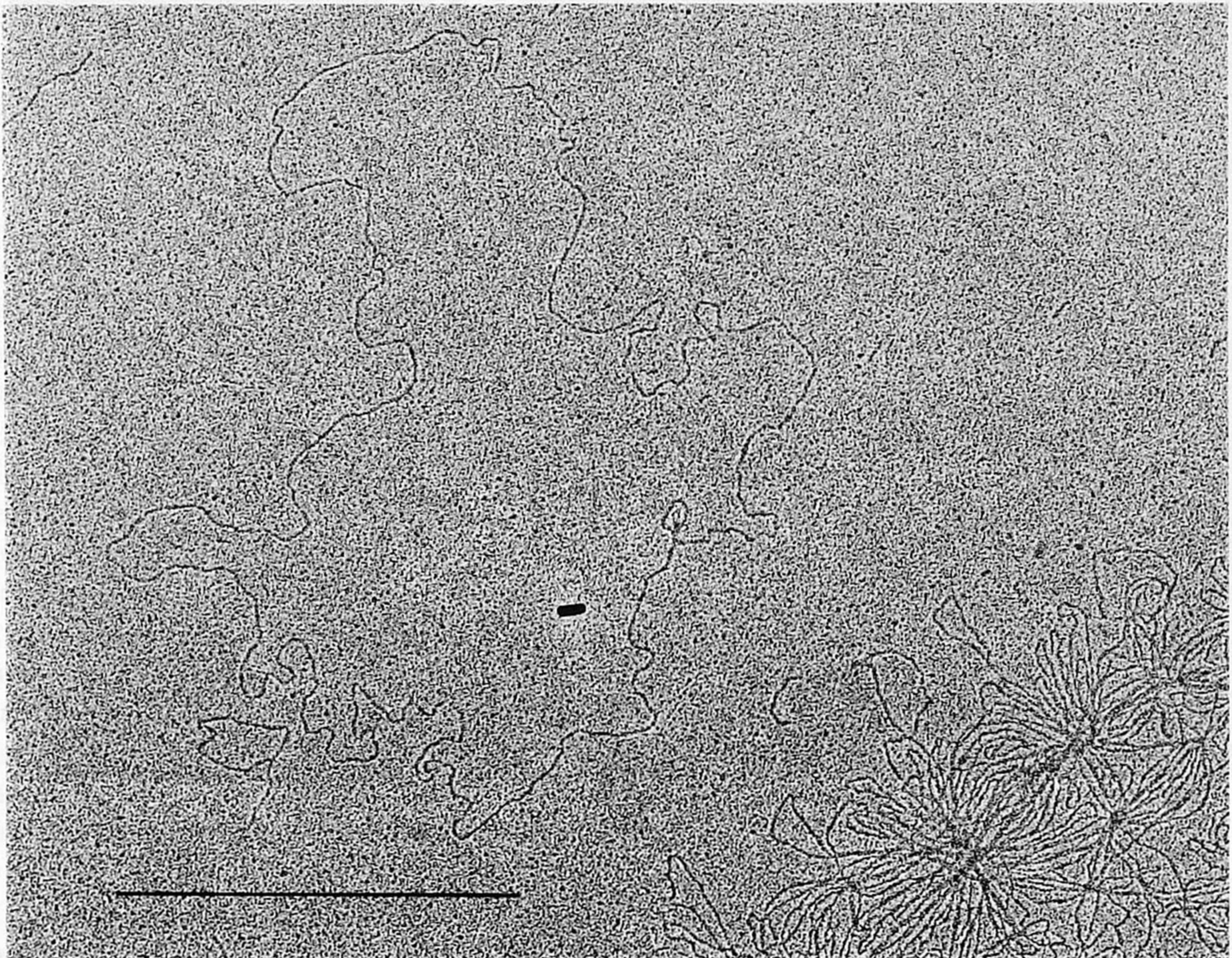Fig. 3. Electron micrograph of a free maxi-circle. The contour length is 11.3 µm (normalized using the 0.76-µm mini-circles present on the same grid). The bar is 1 µm.

Electron microscopy of free maxi-circles

We have previously presented evidence [11] that the complex DNA component, called maxi-circle, is indeed circular in intact networks. As the evidence for this was only indirect, we have looked if we could find the putative 10-μm circles in partly damaged networks. Indeed, three were found, one of which is shown in Fig. 3. To find these three about 50 grids of three different kDNA preparations were scanned, showing that such intact circles are only rarely liberated under our conditions.

The nature of the long DNA in networks

Electron microscopy of highly purified kDNA networks shows three types of long DNA, in addition to the characteristic interlocked mini-circle pattern which makes up the bulk of the network structure:

(a) Long internal strands without clear ends (Fig. 4a). Such strands cannot be identified with certainty in the compact intact networks, but they become visible in the loosened regions of networks damaged by ageing or by incubation with S_1 endonuclease.

(b) Long edge loops, extending from the edges of intact networks (Fig. 4b). These loops are rare in freshly isolated intact networks, they tend to increase at first with ageing, but they have disappeared in extensively aged preparations. Hence, the number of long edge loops in variable (Table I), though never more than 3—5 per network. The size of the loops is highly variable (Fig. 5), but no loops larger than 9 μm were ever seen. This is compatible with these edge loops be-

TABLE I

COMPLETE REMOVAL OF LONG DNA FROM kDNA NETWORKS (*C. LUCILIAE*) BY RESTRICTION ENDONUCLEASES

Networks were spread for electron microscopy as described in Methods. Networks were spread directly or after a standard exhaustive restriction endonuclease treatment with the enzymes indicated; a control (buffer) was incubated in the same way but without enzyme. Well-spread networks were randomly chosen and their edge scanned for loops or linear molecules clearly exceeding mini-circle length. Fifteen networks were scanned per experiment and n = number of experiments. The value for the maximal number of mini-circles cut after exhaustive digestion with endonucleases HindII + III was determined previously with ^{32}P-labelled isolated mini-circles. The fraction of mini-circles cut by the two other enzymes was determined semi-quantitatively as follows: the digested (unlabelled) kDNA was spun to equilibrium in a CsCl gradient containing propidium diiodide as described in Methods. The centrifuge tube was photographed and suitable negatives were scanned to determine the fraction of the total DNA in the upper band. The upper band was run through a 0.5% or 1% agarose gel to separate mini-circle fragments from maxi-circle fragments, the gel was photographed and the suitable negatives scanned to determine the relative proportion of DNA in the mini-circle band. The procedure measures the maximal fraction of mini-circles specifically cut by the enzyme, because mini-circles accidentally liberated from the network during incubation or handling are included in the fraction cut by the endonuclease.

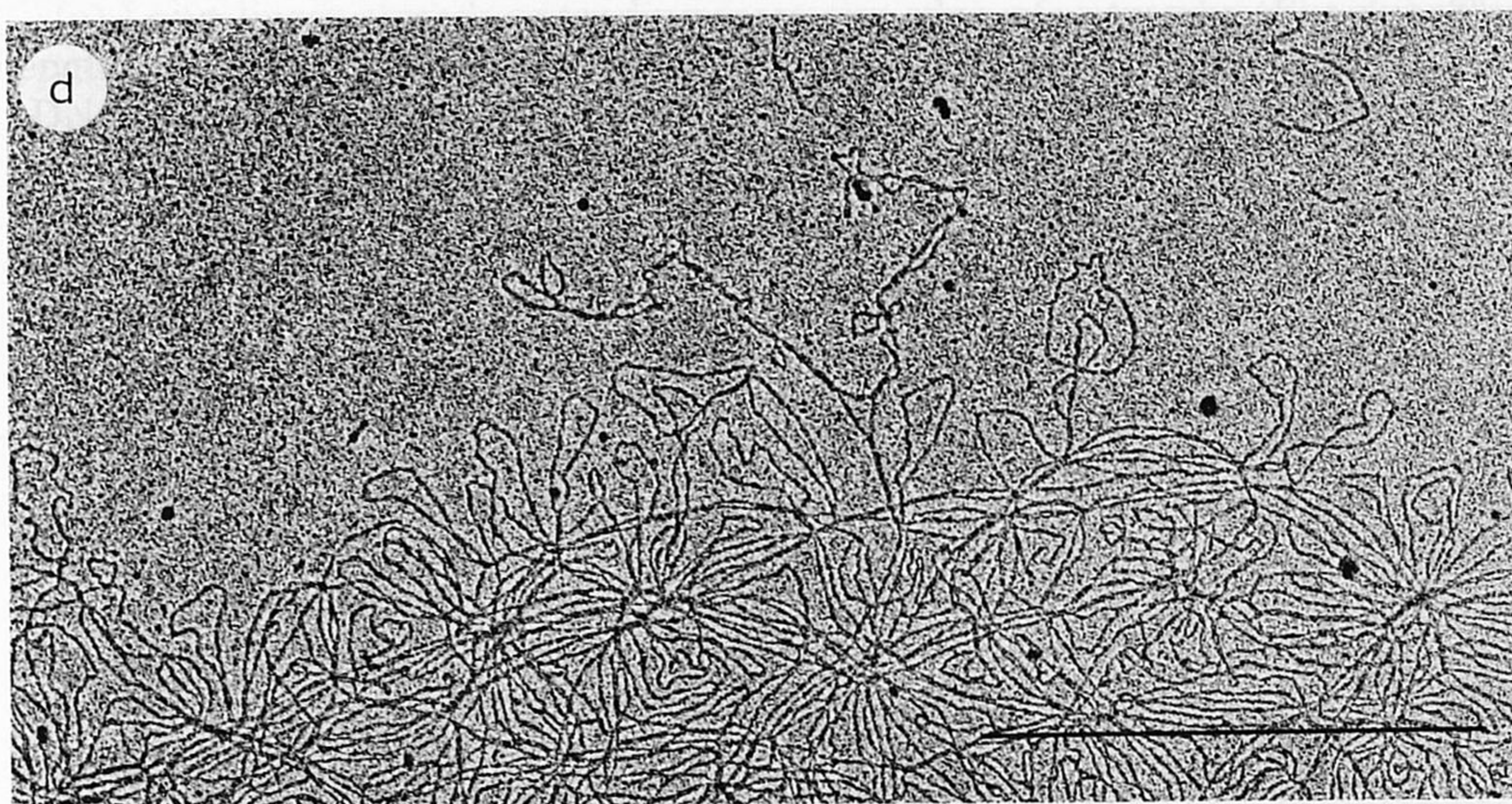| Treatment of kDNA	Average number of long molecules per network		Fraction of mini-circles cut by nuclease (%)
	Edge loops	Edge linears	
None (n = 3)	1.0—4.9	0—1.0	
Buffer (n = 1)	1.3	0.3	
HindII + III (n = 2)	0.0	0.0	<40
EcoRI (n = 2)	0.0	0.0	<20
PstI (n = 3)	0.0	0.0	< 7
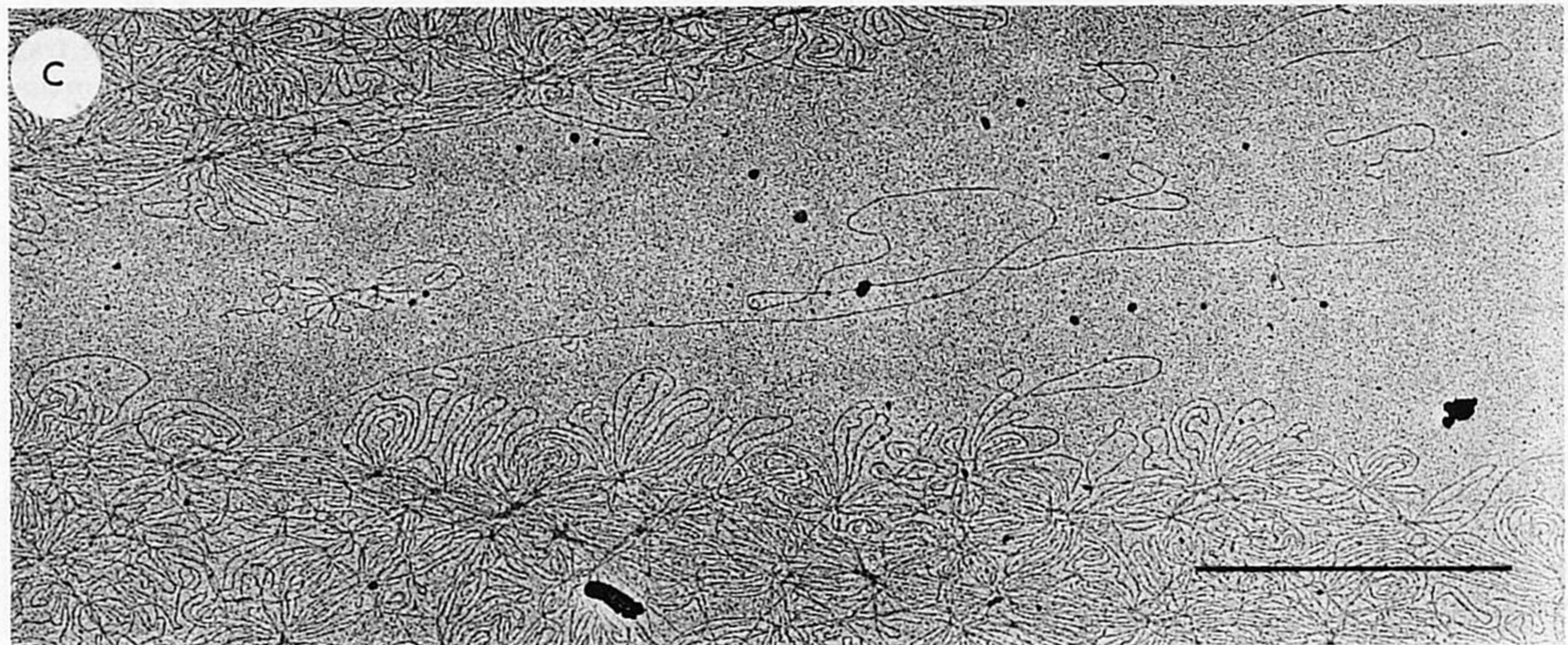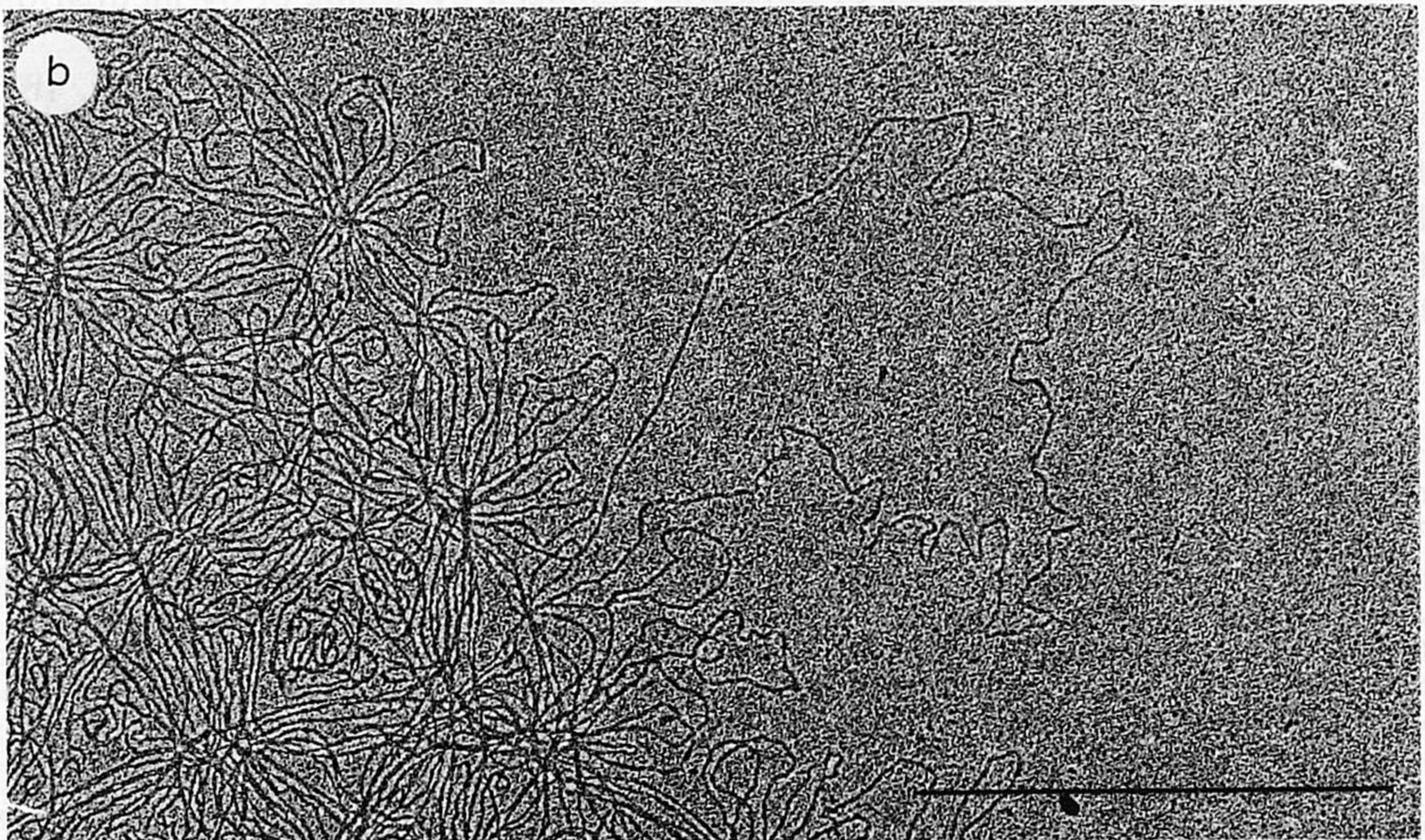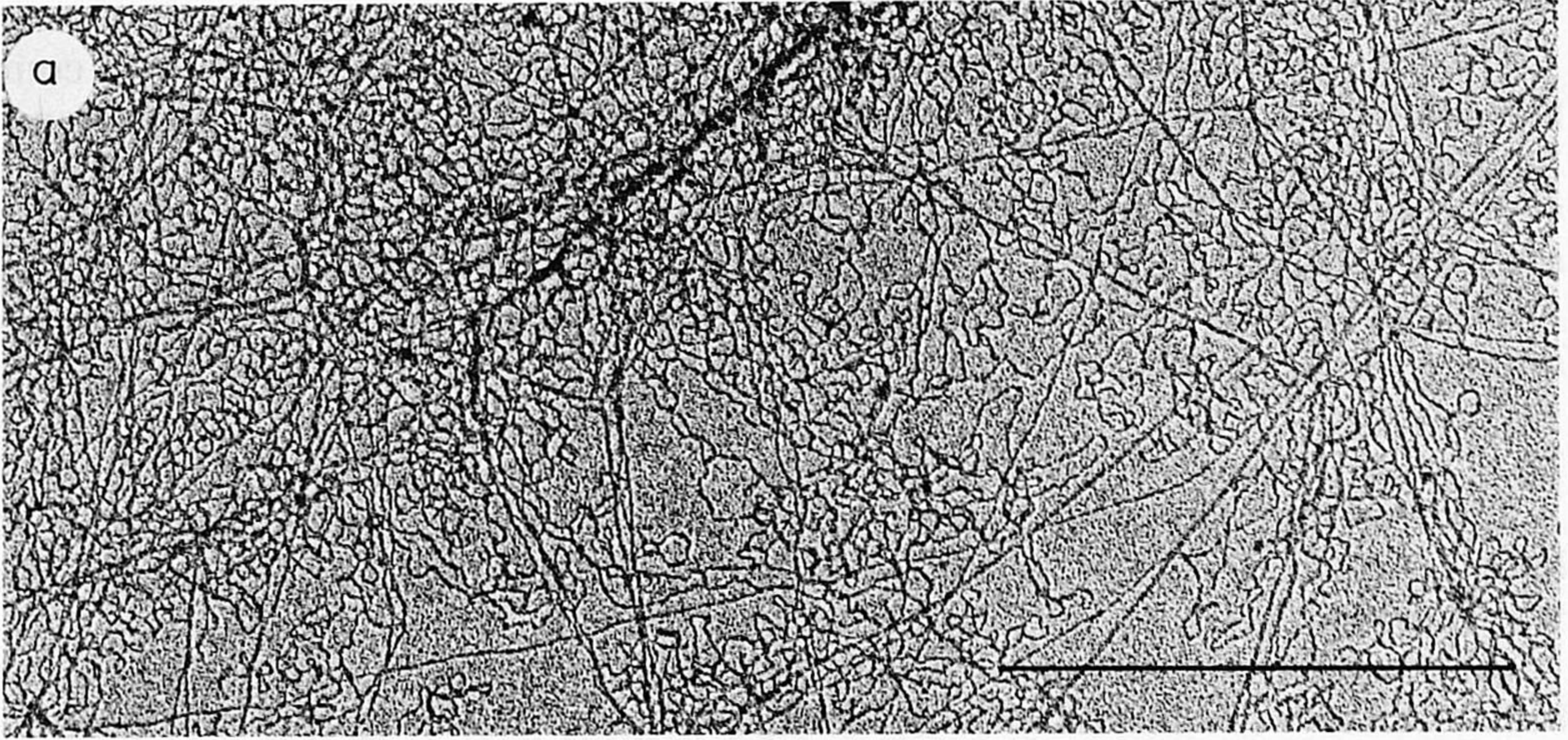
Fig. 4. Electron micrographs showing long DNA molecules in kDNA networks. a, internal long strand; b, long edge loop; c, long edge linear; d, twisted long edge loop. The bar is 1 µm.

ing part of 10-µm maxi-circles from the network. Because of the extreme density of DNA strands in the spread network it was impossible to follow the path of the loops into the network. Rarely the long loops had the characteristic twisted appearance of closed circular duplex DNA (Fig. 4d), indicating that they are part of a circular molecule.
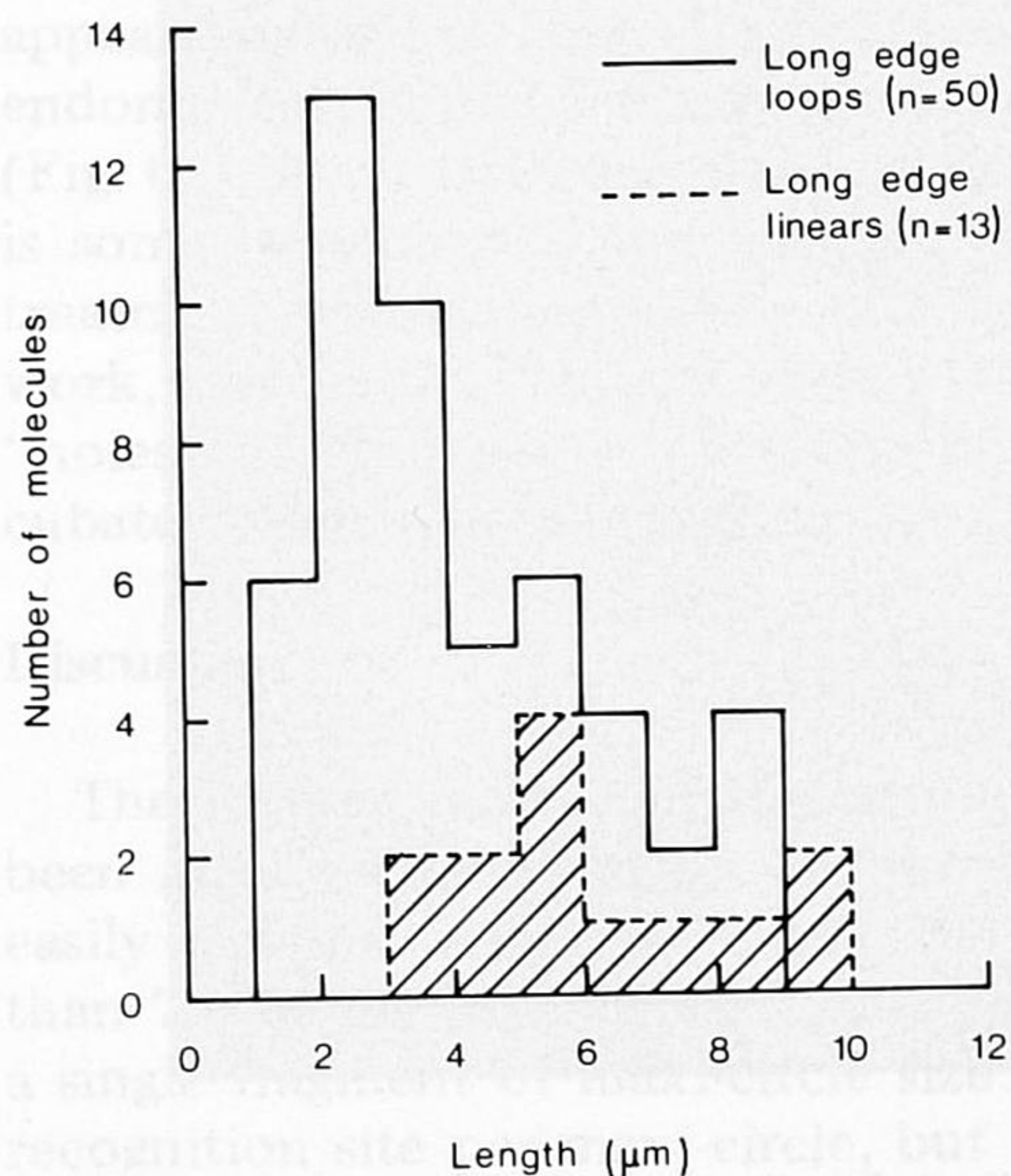

Fig. 5. Size distribution of long edge loops and linears attached to the rim of spread kDNA networks. Only molecules longer than mini-circle length are included.
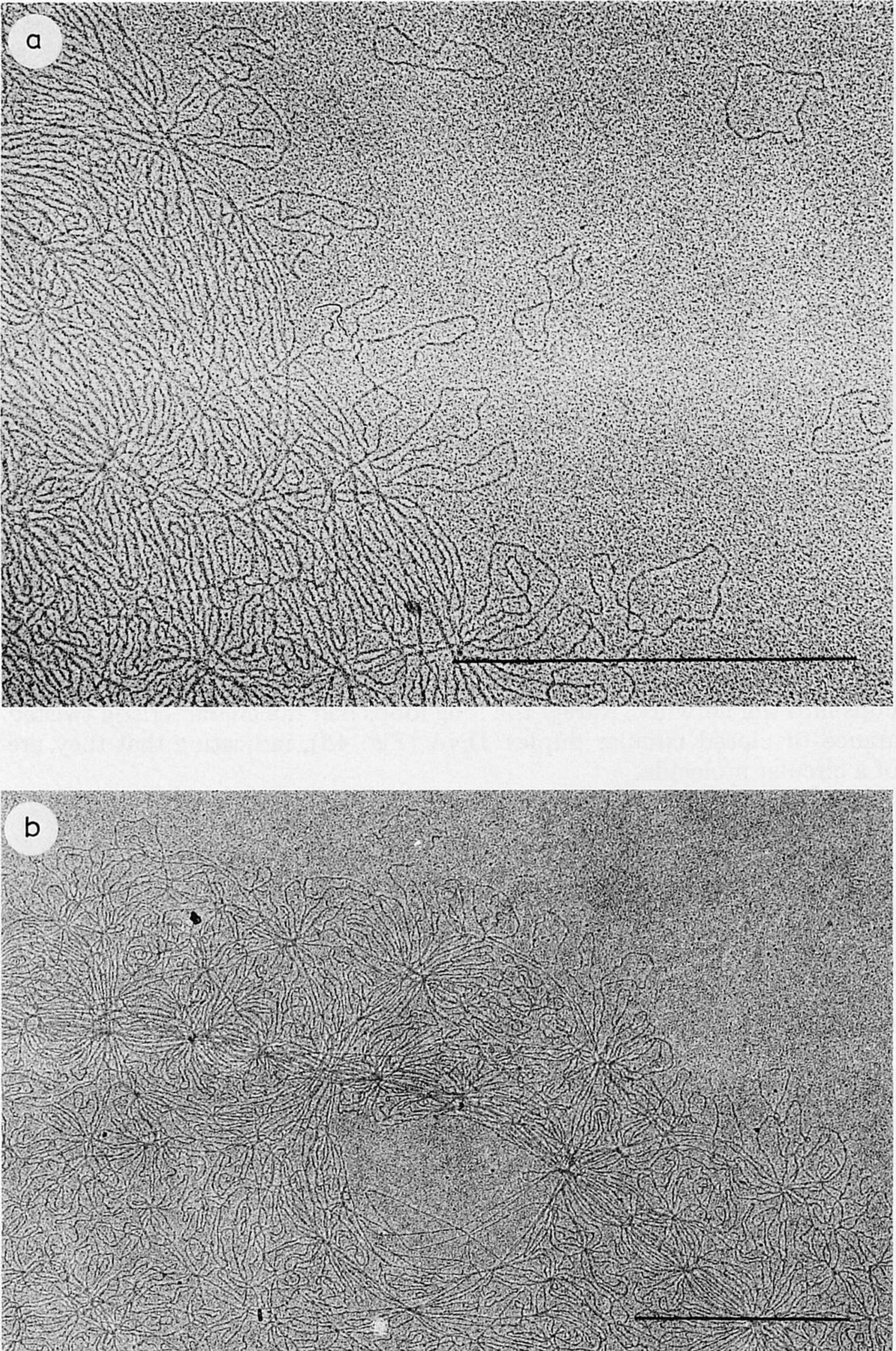
Fig. 6. Electron micrographs of kDNA networks after limit digestion with restriction endonucleases. a, network treated with endonuclease EcoRI; b, network treated with endonuclease PstI. The bar is 1 μm.

(c) Long linear strands extending from the edge of networks (Fig. 4c). The length of these molecules never exceeded 10 μm. Since they were found in kDNA preparations that contained virtually no free linear DNA and in which the networks were widely separated in the protein monolayer, we interpret these long edge linears as broken edge loops rather than extraneous linear DNA stuck to the network.

The long DNA in kDNA networks could represent maxi-circles, mini-circle oligomers or an as yet unidentified third DNA component in the network. To decide between these alternatives we examined networks after treatment with restriction endonucleases that cut the maxi-circles but only part of the mini-circle classes. This resulted in a complete removal of all long edge loops (Table I), long edge linears or long internal strands (not shown) from the network. With endonuclease PstI the cut long DNA was recovered as a homogeneous population of linear molecules of maxi-circle length (see (Fig. 2). Appropriate controls, included in Table I, show that this was not merely due to the incubation of networks at 37°C and the subsequent handling. These results give no evidence for the presence of long DNA in the network other than the maxi-circles.

After ageing of intact networks a band of high molecular weight appeared, that co-migrated in gels with the maxi-circle band generated by endonuclease PstI. The results of electron microscopy or EcoI digestion of this "slow input band" (not shown) are compatible with the conclusion that it consists of maxi-circles released from the network by a single, duplex break.

Effect of selective removal of mini-circle classes on network structure

The percentage of mini-circles cut from networks by the restriction endonucleases HindII + III, EcoRI and PstI are approx. 40, 20 and <7%, respectively (see Table I). Following digestion with endonucleases HindII + III networks are severely damaged, with a large amount of "loosening up" and disappearance of the characteristic "rosette" structure [10]. After treatment with endonuclease EcoRI the alterations in network structure are much less dramatic (Fig. 6a). Long DNA has gone as well as most of the "rosette" structure; there is some loosening of the network but the basic structure is unaltered. After treatment with endonuclease PstI hardly any alterations are found in the network, besides the removal of long DNA. The only peculiarity is the presence of "holes" or "thin spots" (Fig. 6b), but since these were also once found in unincubated control networks their significance is not clear.

Discussion

The further analysis of the structure of intact networks from *C. luciliae* has been greatly simplified by the use of endonuclease PstI. This enzyme can be easily isolated in large amounts free of other interfering nucleases, it cuts less than 7% of the mini-circles and it liberates the maxi-circle from the network in a single fragment of maxi-circle size. All data are compatible with a single PstI recognition site per maxi-circle, but we cannot rule out the presence of two or more sites clustered within 2% of the contour length, because the resulting small fragment(s) would have gone undetected with the methods used.

Electron microscopy of linearized maxi-circles liberated by endonuclease PstI yields a corrected molecular weight of $22 \cdot 10^6$ in good agreement with the molecular weight estimated from electrophoretic mobility of various endonuclease digests of the maxi-circle [11]. This correspondence indicates that major gene repetitions are absent in the maxi-circle and that its potential genetic information content (genetic complexity) is equivalent to its size. The fact that the maxi-circles linearized by endonuclease S_1, which does not cut circles at a specific nucleotide sequence, are of unit size, shows that circular dimers or higher oligomers of maxi-circles are rare or absent in our networks.

Free maxi-circles, not attached to networks, were very rare in our kDNA preparations and this is not unexpected. The isolation procedure strongly selects for rapidly sedimenting structures and would eliminate free circles. Liberation of intact maxi-circles during storage of purified kDNA is unlikely for the following reason: Our results strongly indicate that maxi-circles are attached to the network by catenation, because a single duplex cut is sufficient to liberate them. Liberation of intact circles would require scission of one or more mini-circles without scission of maxi-circles and, in view of the 15-fold difference in size, non-specific degradation of maxi-circles may be expected to occur more readily during ageing of kDNA than degradation of mini-circles. Our results do not give information on the existence of free maxi-circles in the intact trypanosome. This can now be verified, however, by renaturation analysis using purified, labelled maxi-circle sequences as probe.

Electron microscopy of kDNA networks after treatment with endonucleases PstI or EcoRI has provided new information on three points:

(1) Complete removal of maxi-circle sequences does not affect the overall structure of the network. This proves that the maxi-circles are not required to hold the mini-circles together.

(2) Removal of certain sub-sets of mini-circles from the networks with restriction endonucleases leads to a loosening of the network with no drastic change in network structure. This indicates that the sub-sets removed are fairly evenly spread through the network and that most mini-circles are catenated to more than one other mini-circle in agreement with previous suggestions.

The fact that the removal of about 20% of the mini-circles with endonuclease EcoRI leads to a disappearance of most of the mini-circle "rosette" structures at the edge of the networks, makes it unlikely that these "rosettes" are basic subunits of the network. This is more fully discussed elsewhere [7].

(3) Complete removal of maxi-circles and a minority of mini-circles removes all long DNA from the network. We conclude from this that the long DNA found in stationary phase networks (see refs. 1,23—29) is either maxi-circle DNA or contaminating nuclear DNA and that longer circular mini-circle oligomers are (virtually) absent. This conclusion is predominantly based on the experiments with endonuclease PstI which cuts less than 7% of the mini-circles. This enzyme removes all long DNA from the network and the linearized DNA set free contains only one class of homogeneous long linear DNA (linearized maxi-circles). If a substantial part of the long DNA in the network would consist of mini-circle oligomers one would expect such oligomers to yield linear DNA heterogeneous in length. The conclusion that (part of) the long edge loops of kDNA networks are due to maxi-circles has been reached indepen-

dently by Steinert et al. [28] and Brack et al. [29].

Although we think that the controversial issue (cf. refs. 1, 23—27) of the presence of long, tandemly repeated mini-circle oligomers has been settled by our experiments for stationary phase cells of *C. luciliae*, the possibility remains that such oligomers could arise as short-lived intermediates in replication or segregation of kDNA. The methodology developed in this and previous papers from our laboratory makes it now possible to tackle this intriguing problem.

Acknowledgements

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