The EMBO Journal vol.12 no.2 pp.403-411, 1993

The absence of supercoiling in kinetoplast DNA minicircles

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Communicated by P.Borst

Crithidia fasciculata **kinetoplast DNA is a mitochondrial DNA composed of 5000 minicircles and ~25 maxicircles, all catenated into a giant network. By comparing the linking number of minicircles released from the network by limited sonication with that of control minicircles, we demonstrate that not only does the elaborate catenation of the network not cause supercoiling, but that there is no minicircle supercoiling at all. The absence of catenation-induced supercoiling is explained by our finding [using electron microscopy (EM) and gel electrophoresis] that network minicircles are joined by only one interlock; single interlocking can be accommodated without helix distortion. EM revealed that propidium diiodide supertwists all the network minicircles and thereby condenses the network into a much smaller size while maintaining its planarity. At high dye concentration the network is condensed to a size comparable to that found *in vivo*. Nevertheless, network minicircles bind less propidium than free minicircles, indicating that catenation into a network restricts the supercoiling of individual rings. These studies show that the mitochondrion of trypanosomatids may be a unique niche in nature where a covalently-closed circular DNA is not supercoiled. This absence of supercoiling may be a major factor in promoting the formation of the network.**

Key words: *Crithidia fasciculata*/kinetoplast/minicircle/mitochondria/supercoiling

Introduction

Kinetoplast DNA (kDNA), the mitochondrial DNA in trypanosomes and related protozoan parasites, is unusual in both its structure and genetic function. kDNA is a giant network composed of several thousand minicircles and a few dozen maxicircles, which are all topologically interlocked. Each cell contains only one network within its single mitochondrion (for reviews see Ray, 1987; Simpson, 1987; Ryan *et al.*, 1988).

In the kDNA network of the trypanosomatid *Crithidia fasciculata*, maxicircles are 37 kb in length and the minicircles are 2.5 kb. Similar to the mitochondrial DNAs of other eukaryotes, maxicircles encode ribosomal RNAs and proteins involved in electron transport and oxidative phosphorylation (Simpson, 1987). An amazing property of trypanosomatids is the extensive editing of the maxicircle transcripts, a process in which uridine residues are added or deleted at many specific sites to produce translationally-competent reading frames (Benne *et al.*, 1986; Feagin *et al.*, 1988; Simpson and Shaw, 1989; Feagin, 1990; Simpson, 1990). Both maxicircles and minicircles can encode guide RNAs that determine the specificity of RNA editing (Blum *et al.*, 1990; Pollard *et al.*, 1990; Sturm and Simpson, 1990). Therefore, the maxicircles and minicircles cooperate in forming a functional transcript. In contrast to minicircles from most other trypanosomatids, those from *C.fasciculata* are nearly homogeneous in sequence, as judged by restriction mapping and direct sequence analysis (Sugisaki and Ray, 1987).

Electron microscopy of an isolated deproteinized *C.fasciculata* kDNA network shows a giant two-dimensional array of DNA molecules ~10 μm×15 μm. Although by EM the circles appear to be interlocked, they are too crowded to reveal any underlying organization. The complete decatenation of the network by topoisomerase II to individual maxicircles and minicircles proves that the circles are linked topologically (Marini *et al.*, 1980).

It is not known why kDNA is, thus far, the only DNA in nature that is organized into a network (for speculations see Borst, 1991). To understand the biological significance of the network or how it is replicated, it is essential to know its structure. As a first step in our investigation, we have examined the supercoiling of network minicircles. Negative supercoiling in other organisms is known to compact DNA in an orderly fashion and to promote DNA replication, transcription and recombination (Kanaar and Cozzarelli, 1992). We first investigated whether the elaborate catenation of minicircles in a network produces supercoiling. Although previous studies had indicated that minicircles released from networks by sonication are relaxed (Wesley and Simpson, 1973; Simpson and Simpson, 1974; Kleisen *et al.*, 1975), it is possible that the catenation-induced supercoiling was canceled out by unconstrained supercoiling of the opposite sign. We found that there was no catenation-induced supercoiling, a finding that can be explained by the fact that there are only single interlocks between neighboring minicircles. Single interlocks can provide strain-free connections. We also found that minicircles can be supercoiled to a limited extent by the addition of an intercalating agent causing a striking orderly compaction of the network.

Results

Does minicircle catenation induce supercoiling?

In a study of a family of model catenated dimers produced by recombination *in vitro*, Wasserman *et al.* (1988) demonstrated that catenation via more than one interlock causes supercoiling of each DNA circle. Subsequently,

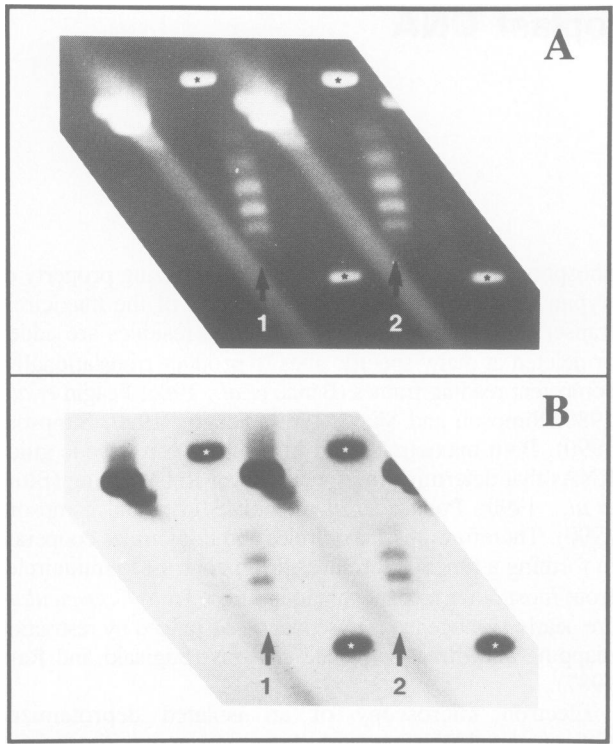


Fig. 1. Fractionation of minicircle linking number topoisomers by two-dimensional gel electrophoresis. Lane 1, analysis of supercoiling induced by network catenation. Unlabelled networks and tracer monomeric ^{3}H-labelled minicircles were mixed and treated with topoisomerase I to relax unconstrained supercoils. The SDS-quenched reaction was then briefly sonicated to release monomeric minicircles from the networks and Lk topoisomers were fractionated through two dimensions of agarose gel electrophoresis. Lane 2, analysis of total supercoiling of network minicircles. Unlabelled networks and tracer relaxed monomeric [^{3}H]minicircles were mixed, sonicated (without topoisomerase I treatment) and fractionated as described for lane 1. **A**. Photograph of ultraviolet fluorescence of ethidium bromide-stained DNA, showing minicircles derived from networks. **B**. Fluorograph of ^{3}H-labelled DNA, showing tracer monomeric minicircles. Control experiments showed that the ^{3}H-labelled tracer does not contribute significantly to the DNA signal detected by ethidium bromide staining. The asterisk represents ^{32}P end-labelled ΦX174 fragments generated by *Hpa*II digestion used for alignment of films and registration of ladders.

Monte Carlo simulations of the conformation of dimeric catenates showed that this supercoiling is an expression of the writhe resulting from the net winding of one DNA ring around the other, whereas the twist of the DNA is essentially unaltered (A.Vologodskii and N.R.Cozzarelli, unpublished observations). The kDNA network introduces the additional constraint that all minicircles lie essentially in a plane, so that catenation to multiple neighbors might itself produce supercoiling.

To determine whether the intricate catenation of minicircles in a *C.fasciculata* kDNA network introduces supercoiling, we used an assay similar to that of Wasserman *et al.* (1988). We mixed trace amounts of control monomeric ^{3}H-labelled minicircles with isolated kDNA networks present in a large molar excess. We treated this mixture with topoisomerase I to remove unconstrained supercoiling from both the network and control minicircles and to fix the linking number (Lk) of all circles under identical conditions. The control ^{3}H-labelled minicircles therefore define the distribution of relaxed topoisomers under these conditions. After liberating monomeric minicircles from the networks

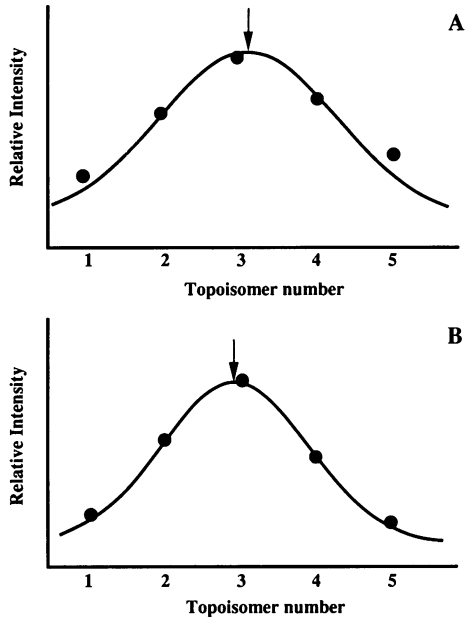


Fig. 2. Gaussian distribution of minicircle topoisomers from experiment measuring catenation-induced supercoiling. Topoisomers were quantified by densitometry of gels from an experiment similar to that in Figure 1A and B (lane 1). Digitized data were fitted to Gaussian curve using a least-squares analysis. **A**. Network minicircle topoisomers (mean of distribution, 3.12). **B**. Tracer monomeric ^{3}H-labelled minicircles (mean of distribution, 2.91). The difference between the means is statistically insignificant.

by brief sonication, we then compared their Lk with that of the control ^{3}H-labelled minicircles. The difference in these two values of Lk (ΔLk) is a quantitative measure of catenation-induced writhe. We used two-dimensional agarose gel electrophoresis to resolve Lk topoisomers and to separate them from the linear DNA fragments produced during the sonication (Figure 1).

The minicircle topoisomers released from networks by sonication and detected by ethidium staining are shown in Figure 1A (lane 1). The tracer ^{3}H-labelled minicircles, from the same experiment, appear in the fluorograph in Figure 1B (lane 1). We aligned these two ladders using fragments of ΦX174 RF [^{32}P]DNA (indicated by stars) that are detectable both by ethidium staining and fluorography. After measuring the relative amounts of topoisomers in each ladder by densitometry, we fitted these values to Gaussian curves using a least-squares method (Figure 2). In two experiments, the average ΔLk between network minicircles and the relaxed control minicircles was +0.06 (Table I). Therefore, there is no significant distortion of the minicircle DNA attributable to catenation into a network.

This conclusion depended on two control experiments. First, we showed that there was sufficient topoisomerase I to fully relax all the minicircles. At the end of the reaction we added negatively supercoiled pBR322 DNA to a portion of the mixture and incubated this for an additional 45 min. The pBR322 DNA was fully relaxed even with 3-fold less enzyme than used in the minicircle reactions (data not shown). Secondly, to prove that the topoisomerase I was completely inactivated by SDS prior to the release of minicircles from the network by sonication, we added a portion of the quenched reaction mixture to supercoiled pBR322 DNA. There was no further relaxation of this substrate (data not shown).

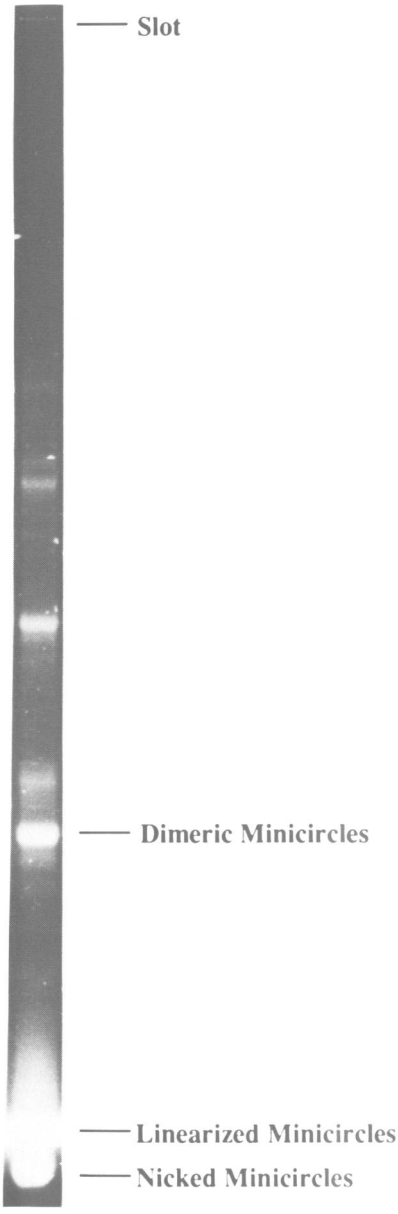


Fig. 3. Agarose gel electrophoresis of minicircle products produced by partial *Sst*II digestion of kDNA networks. DNA was detected by ultraviolet fluorescence after ethidium bromide staining. Linearized and nicked monomeric minicircles were identified by comparison with standards. Dimeric minicircles were identified by electron microscopy. Bands migrating more slowly than dimeric minicircles include oligomeric minicircles and maxicircle fragments.

The number of topological interlocks between network minicircles

The absence of catenation-induced supercoiling in *C.fasciculata* kDNA minicircles raised the possibility that there is only a single interlock between neighboring kDNA minicircles. A single interlocking of dimeric catenanes is accommodated without supercoiling (Wasserman *et al.*, 1988). Therefore by electron microscopy we measured the number of interlocks between pairs of network minicircles. Since minicircles are too crowded in intact networks for individual linkages to be readily evaluated by EM, we simplified this analysis by partially digesting kDNA networks with *Sst*II, an enzyme that singly cleaves nearly all of the minicircles. After subsequent nicking with DNase I to remove the confounding effect of supercoiling on electrophoretic mobility, we fractionated the digestion products by high resolution agarose gel electrophoresis (Figure 3). We identified the structure of the DNA in each band (including linearized minicircles and minicircle monomers, dimers and oligomers) by comparison of its electrophoretic mobility with that of markers or by visualization by EM. In the current study, we focused our attention on the catenated dimer band. Analysis of the more complex components in the digest will be presented elsewhere. To determine the number of interlocks we coated the DNA eluted from the gel with RecA protein before viewing by EM. This technique allows rigorous evaluation of strand overlay at crossover points (Krasnow *et al.*, 1983). As shown in the examples in Figure 4, all dimers examined were joined by a single interlock. EM also established that minicircles in higher oligomers were also joined by single interlocks (not shown). The electrophoresis in Figure 3 revealed a single band at the position expected for singly interlocked dimers; very little DNA was detected with the faster electrophoretic mobility expected for multiply linked dimers. We therefore conclude that the minicircles in a kDNA network are linked to their neighbors by only a single interlock.

Measurement of the total supercoiling of network minicircles

Thus far we have shown that catenation into a network does not introduce net supercoiling into the minicircles. Despite its complexity, the network does not cause any distortion of the conformation of the minicircles. We have not determined whether the network minicircles have an intrinsic supercoiling that was relaxed by the type 1 topoisomerase treatment. The minicircles could have been supercoiled before or after network formation. We addressed this question by comparing the Lk of network minicircles with that of control relaxed free minicircles. The experimental approach was identical to that used above, except that we did not treat with topoisomerase I before sonicating the mixture of networks and control monomeric ^{3}H-labelled minicircles that had been previously relaxed *in vitro* with topoisomerase II.

Figure 1A (lane 2) shows topoisomers of the network-derived minicircles (visualized by ethidium fluorescence), and Figure 1B (lane 2) shows those of the tracer monomeric ^{3}H-labelled minicircles (visualized by fluorography). In two experiments, the average difference in Lk was +0.5 (Table I). This value corresponds to a superhelical density (ΔLk divided by Lk of relaxed DNA) of +0.002. In comparison, the typical superhelix density of DNA in eukaryotic cells is −0.06 (Bauer, 1978). Even this low value for minicircles may result from the fact that the Lk of the network minicircles was fixed *in vivo* and that of the control minicircles was fixed *in vitro* under different ionic conditions. This difference, rather than network minicircle supercoiling, could easily account for the observed low value of the superhelical density (Bauer, 1978). These results confirm previous findings by dye-buoyant density centrifugation that minicircles released from networks by sonication are relaxed (Wesley and Simpson, 1973; Simpson and Simpson, 1974; Kleisen *et al.*, 1975).

Supercoiling of kDNA networks by intercalating dyes

Despite the absence of minicircle supercoiling in kDNA networks, it has been known for many years that these

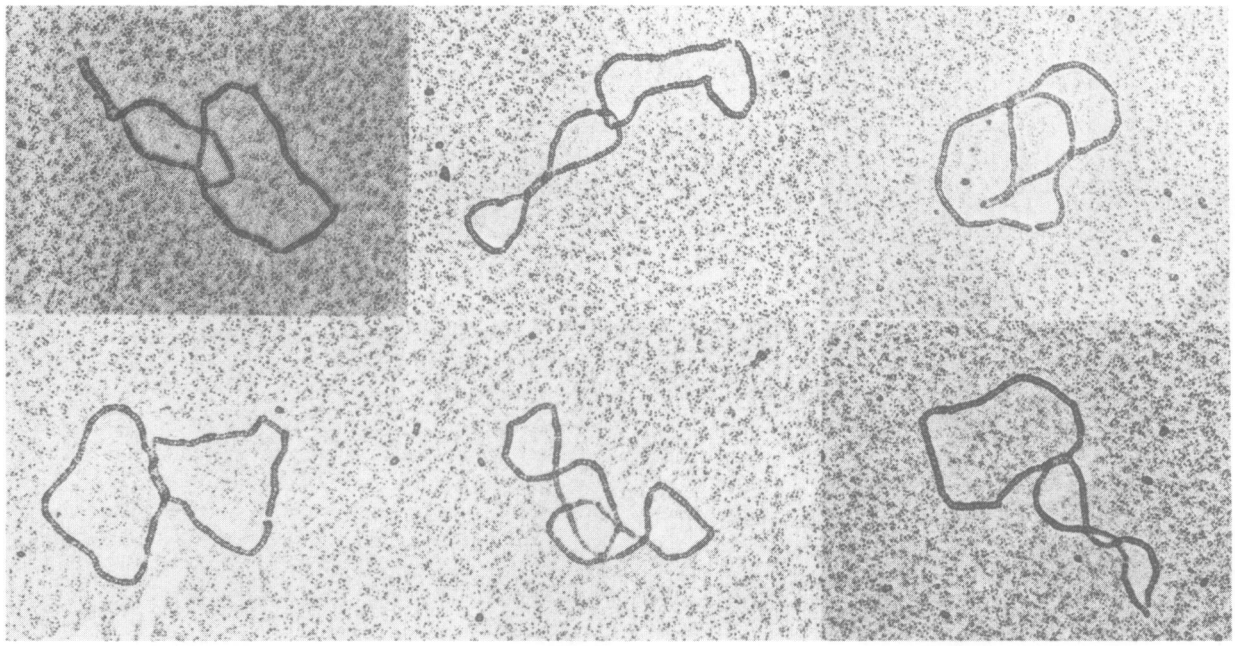


Fig. 4. Analysis of dimer band by electron microscopy. DNA purified from the dimer band from a gel like that in Figure 3 was evaluated by electron microscopy after RecA coating using the ethidium bromide technique (Thresher and Griffith, 1990). Primary magnifications on the microscope were ~20–30 000 ×. Each minicircle is 2.5 kb.

molecules can be positively supercoiled by the addition of intercalating dyes (Simpson and Da Silva, 1971; Delain *et al.*, 1972). These dyes reduce twist upon binding and therefore increase the writhe of covalently closed circular DNA. In this study, we examined the consequence of addition of propidium diiodide as a function of dye concentration. The electron micrographs in Figure 5 confirm that minicircles become highly supercoiled and that interlocking into a network does not prevent supercoiling of individual rings. As the concentration of dye increases, the network is condensed into a much smaller structure (Figure 5A, C, E, G and I; these same networks are shown at higher magnification in Figure 5B, D, F, H and J). The supercoiling reduces the distance between neighboring minicircles and causes an orderly compaction that maintains the planarity of the network except at the highest dye concentration tested, 500 μg/ml (Figure 5K). At that concentration, the network collapsed into a denser structure in which individual DNA strands could not be distinguished. To estimate the thickness of this structure, we visualized it after single-angle shadowing with palladium–platinum (Figure 5L); measurements of its size and shadow length indicate that the structure is a disk ~0.64 ± 0.07 μm in diameter and 0.27 ± 0.05 μm in thickness (23 measurements).

Maxicircles are rarely visible in *C.fasciculata* networks spread in the absence of dye, but they frequently protrude from the network edge when minicircles are compacted by the dye (especially see panels G and I). Some maxicircles are supertwisted in the presence of dye, indicating that they are covalently closed.

Restricted binding of intercalating dyes by kDNA networks

Given that kDNA networks can bind sufficient propidium diiodide to introduce substantial positive supercoiling, we next determined whether dye uptake by network minicircles is restricted by the structure of the network. This point has been investigated previously, but the results were controversial. In the case of *C.luciliae* networks, there was restricted dye uptake (Kleisen *et al.*, 1975), although the effect was not observed in networks from *Leishmania tarentolae* (Simpson and Berliner, 1974). We subjected a mixture of ^{32}P-labelled *C.fasciculata* kDNA networks and control ^{3}H-labelled monomeric minicircles to CsCl equilibrium gradient centrifugation in the presence of propidium diiodide. In the ^{32}P-labelled networks, 89% of the minicircles were covalently closed as judged by gel electrophoresis following decatenation by topoisomerase II. The ^{3}H-labelled monomeric minicircles were produced by decatenation of ^{3}H-labelled networks and consequently were fully relaxed under the decatenation conditions. As shown in Figure 6, the networks banded roughly two fractions below the monomeric minicircles, a buoyant density difference of ~0.02 g/cm^3. Dye uptake reduces buoyant density and therefore there must be less dye bound to minicircles in the network. Since there is only a very small difference in Lk between network minicircles and free minicircles relaxed *in vitro* by topoisomerase treatment (Table I), catenation of the minicircles must restrict the binding of propidium diiodide within the kDNA network. Treatment of the networks with *Pst*I, an enzyme that cleaves

Table I. Minicircle supercoiling

Experiment	ΔLk
Network-induced supercoiling	+ 0.06 ± 0.15
Total supercoiling	+0.50 ± 0.08

Linking differences (ΔLk) were determined as described in the text from data similar to that in Figure 2. ΔLk is the Lk of network-derived minicircles minus that of monomeric ^{3}H-labelled minicircles. Values shown for ΔLk are the mean of two experiments ± the differences from the mean.

the maxicircles in the network but only a very small fraction of the minicircles, had no detectable effect on the buoyant density of the networks (not shown). The restricted dye uptake is a result of higher order constraint imposed by the network structure, because no restriction could be present for singly interlocked dimeric catenates.

Discussion

Negatively supercoiled DNA is found throughout nature. The supercoiling arises either from wrapping of DNA around proteins as in nucleosomes (a physical constraint) or a non-zero Lk difference (a topological constraint). Often, both constraints apply because the winding of DNA around a protein introduces a compensatory supercoil elsewhere, which when relaxed by a topoisomerase causes a change in Lk. Indeed, nucleosome formation and topoisomerase relaxation are believed to be the primary cause of the linking number deficit of eukaryotic nuclear DNA (Germond *et al.*, 1975). In eukaryotes, the winding of DNA around nucleosomes compacts the genome into a more manageable size. In prokaryotes, supercoiling is induced by gyrase action on DNA. The resulting tension from superhelicity drives processes such as replication, transcription and recombination (Kanaar and Cozzarelli, 1992).

It now appears that the matrix of the trypanosomatid mitochondrion is a unique niche in which circular DNA is not supercoiled. Not only is there no supercoiling of network minicircles induced by catenation, but there is no minicircle supercoiling at all. Previously we had found that covalently-closed free minicircles, released from the network for the purpose of replication, are also fully relaxed (Kitchin *et al.*, 1985). It is possible, but not proven, that maxicircles are also not supercoiled. Our examination of hundreds of electron micrographs of *C.fasciculata* networks, in which maxicircles are occasionally visible as loops protruding from the network edge, has not revealed examples of maxicircle supercoiling. It is clear that some of the maxicircles are covalently closed since supercoiled maxicircles are visible in the presence of propidium (Figure 5). However, electron micrographs of networks from other species have, on occasion, revealed examples of maxicircles that appear to be supercoiled (Cheng and Simpson, 1978; Hoeijmakers and Weijers, 1980). It would be surprising if network maxicircles were supercoiled and minicircles were relaxed. However, selective maxicircle supercoiling could result from winding of maxicircles around a protein core in conjunction with action of a topoisomerase.

In this study we first investigated whether catenation of network minicircles itself causes supercoiling. Previous studies on dimeric catenanes revealed that the magnitude of catenation-induced supercoiling is proportional to the number of interlocks between the circles minus one: there was no supercoiling by a single interlock (Wasserman *et al.*, 1988). Using an assay similar to that of Wasserman *et al.* (1988) we found that there was no supercoiling induced by catenation into networks (Figures 1 and 2, Table I). This experiment could not rule out equal and opposite effects from the multiple neighbors of each minicircle. However, by EM of minicircle dimers that had been released from networks by partial restriction enzyme digestion, we showed that the strain-free connections were the result of single interlocks (Figure 4). Furthermore, we demonstrated that the minicircle dimers have the electrophoretic mobility expected for singly interlocked catenanes (Sundin and Varshavsky, 1980) (Figure 3).

The Lk experiments discussed thus far did not rule out any supercoiling in the network minicircles which could be relaxed by the topoisomerase I treatment. We therefore measured the total supercoiling of network minicircles by comparing the linking numbers of minicircles released from networks by sonication with those of free minicircles relaxed *in vitro* (Figure 1, Table I). This experiment revealed little supercoiling of network minicircles (a supercoiling density of +0.002). Even this low positive supercoiling may be an artifact of the method of measurement, as the linking number of the network minicircles was established inside the cell and that of the control monomeric minicircles was established *in vitro*. Our conclusion that *C.fasciculata* minicircles released from networks by sonication are nearly fully relaxed confirms previous findings, using dye-buoyant density centrifugation, for kDNA networks (Wesley and Simpson, 1973; Simpson and Simpson, 1974; Kleisen *et al.*, 1975).

Although network-bound minicircles are not supercoiled *in vivo*, they can be supercoiled as shown by EM of networks spread in the presence of an intercalating dye. As demonstrated by the electron micrographs in Figure 5, the networks are cup-shaped in the absence of dye (Figure 5A), but when the propidium diiodide concentration increases to 10 μg/ml (Figure 5E), they appear planar. Further increases in dye concentration maintain the planarity but promote an orderly shrinking of the network. At a propidium concentration of 500 μg/ml (Figure 5K), the network condenses into a structure in which individual DNA strands cannot be distinguished. This disk-shaped structure is ~0.64 μm in diameter and, as determined from the length of a single-angle shadow, ~0.27 μm in thickness (Figure 5L). This shape and size are comparable to that of the kDNA network *in vivo*, which is a disk ~1 μm in diameter and 0.3 μm in thickness (Kusel *et al.*, 1967; Anderson and Hill, 1969; Ferguson *et al.*, 1992). Therefore, the giant network has a natural tendency to condense into a disk-like structure similar to that present inside the cell. Nevertheless, the Lk measurements presented above rule out the possibility that supercoiling could account for condensation of the network *in vivo*. Instead, proteins in the mitochondrial matrix probably promote an orderly compaction of the network without supercoiling of individual rings.

Despite the efficient supercoiling of network minicircles by propidium diiodide, they bound significantly less dye than did relaxed monomeric minicircles as determined from buoyant density measurements in a CsCl dye gradient. As determined from the refractive index of the CsCl solution, the buoyant density of intact kDNA networks was ~1.62 g/cm^3, whereas that of control relaxed monomeric minicircles released by topoisomerase II was 1.60 g/cm^3 (Figure 6). The release of maxicircles from the network by *Pst*I digestion had no detectable effect on the buoyant density of the networks (not shown); therefore, the reduction in dye uptake was not a consequence of constraints introduced by catenation of the maxicircles into the network. The possible explanation that network minicircles are positively supercoiled was ruled out by direct measurement (Figure 1, Table I). Therefore, we conclude that the catenation of each minicircle to multiple neighbors reduces the number of positive supertwists that can be introduced into a network

minicircle, and therefore the amount of bound dye. As the network becomes positively supercoiled with increasing dye it condenses in size (Figure 5); the unfavorable energy of condensation opposes dye binding. Previous studies on *C. luciliae* kDNA also revealed that the networks banded in dye–CsCl gradients at a density greater than that of monomeric minicircles (Kleisen *et al.*, 1975); this effect was also thought to result from steric hindrance due to network

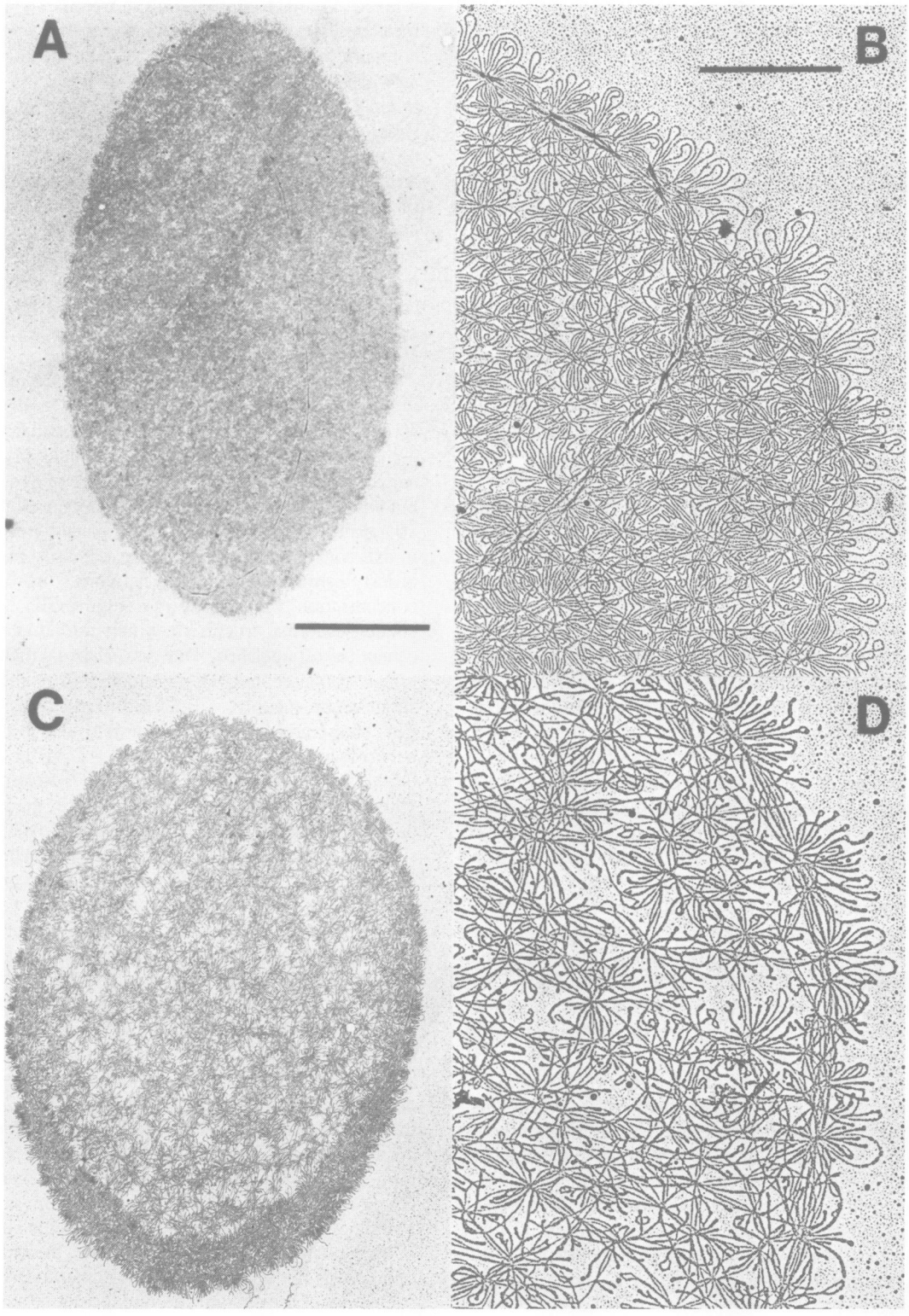
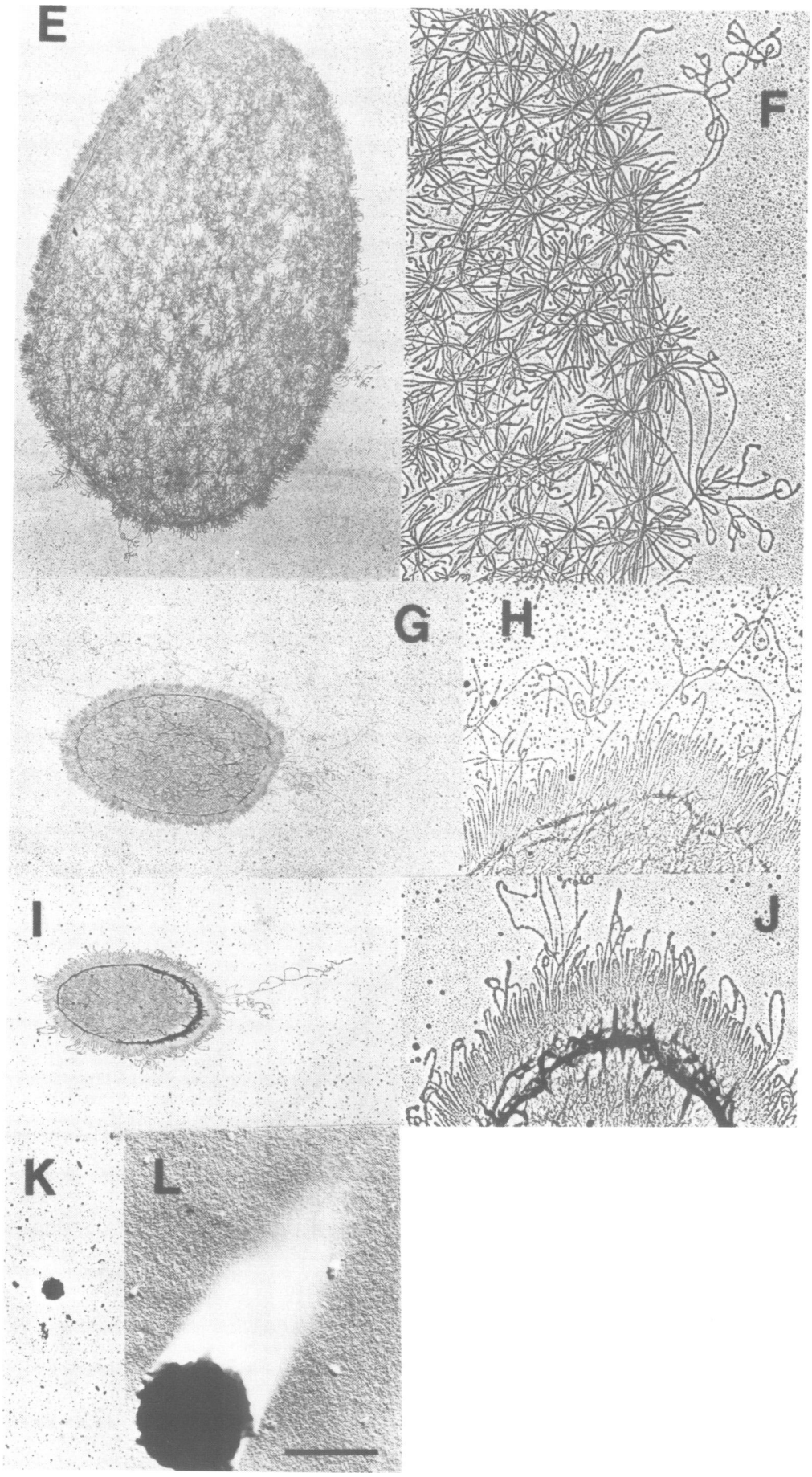


Fig. 5. Electron microscopy of kDNA networks in the presence of propidium diiodide. All of the networks are Form I (composed of covalently closed minicircles) isolated from stationary phase cells. Segments of the networks in panels A, C, E, G and I are also shown at higher magnification in panels B, D, F, H and J. **A** and **B**. No propidium diiodide. This network is cup shaped; therefore when collapsed on the grid, the region at the right in A or on the bottom in B is double thickness. **C** and **D**. 5 μg/ml dye. **E** and **F**. 10 μg/ml dye. **G** and **H**. 50 μg/ml dye. **I** and **J**. 100 μg/ml dye. Note maxicircles protruding from the network in panels G and I. **K** and **L**. 500 μg/ml dye. The network in panel L was single-angle shadowed. The scale bar in panels A, C, E, G, I and K is 4 μm. The scale bar in panels B, D, F, H and J is 1 μm. The scale bar in panel L is 0.5 μm.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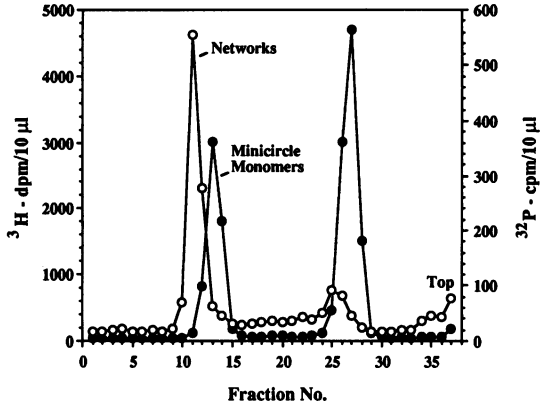

Fig. 6. Equilibrium centrifugation of kDNA networks and monomeric minicircles in a CsCl–propidium diiodide gradient. ^{32}P-labelled kDNA networks (9.5 µg, 12 500 c.p.m.) and ^{3}H-labelled monomeric minicircles (1.6 µg, 230 000 d.p.m.) were dissolved in a solution of 10 mM Tris–HCl pH 8.0, 1 mM EDTA containing 4.15 g CsCl and 2 mg propidium diiodide (final volume 5.7 ml; refractive index, 1.3855). The gradients were centrifuged at 70 000 r.p.m. in the NVT 90 rotor (Beckman) for 13 h at 20°C. The gradient was fractionated from the bottom and 10 µl aliquots were counted on the scintillation counter after TCA precipitation onto a glass fiber disk. Recoveries were 73% for the ^{32}P-labelled networks and ~100% for the ^{3}H-labelled minicircles. About 40% of the monomeric minicircles were covalently closed (peaking in fraction 13) and 60% were nicked (peaking in fraction 27). The small peak of ^{32}P-labelled DNA in fractions 24–27 could be contaminating nuclear DNA or newly replicated Form II networks containing nicked minicircles (a small amount of these could be present if the culture had not quite reached stationary phase). The ^{32}P-labelled networks contained 89% covalently-closed minicircles, as judged by agarose gel electrophoresis (in the presence of ethidium bromide) after decatenation by topoisomerase II; radioactivity in nicked and closed minicircles was assessed by Cerenkov counting of excised gel bands.

structure. In *L.tarentolae*, however, isolated networks banded at the same density as monomeric minicircles (Simpson and Berliner, 1974). The latter result may be due to the facts that minicircles in *L.tarentolae* networks are probably, on average, interlocked to fewer neighbors than those in *C.fasciculata* networks and are usually isolated in a fragmented form. The supercoiling of the *L.tarentolae* kDNA should be accommodated with a less dramatic change in network structure.

Why do kDNA minicircles lack supercoiling and what is the significance of the single interlock between network minicircles? It is likely that both of these factors are essential for the formation of networks. Supercoiling would probably prevent extensive catenation as shown in *in vitro* experiments with DNA gyrase and plasmids such as ColE1. Even under DNA aggregation conditions where the local DNA concentration is extremely high, this enzyme forms only small catenated oligomers if the plasmids are supercoiled, but giant networks if the plasmids are relaxed (Krasnow and Cozzarelli, 1982). Furthermore, the absence of supercoiling induced by network formation shows that catenation by one interlock has low enthalpic cost. Multiple interlocking between two DNA rings would not only require additional energy but also limit the ability of a single minicircle to interlock with several neighboring minicircles. The catenation products of DNA gyrase and phage T4 topoisomerase are also singly interlocked (Krasnow and Cozzarelli, 1982), and thus network formation can be envisioned as arising from a relatively simple process of random interlocking by a topoisomerase. We suggest that catenation of neighboring minicircles proceeds until the energetic cost of crowding in another ring becomes too high, and that minicircle supercoiling would diminish the space available for catenation. As shown in the CsCl-intercalating dye experiment (Figure 6), formation of the network increases the energetic cost of supercoiling.

Therefore, it seems likely that these parasites may have traded the advantages of supercoiling (Kanaar and Cozzarelli, 1992) for those of a network. The requirements of achieving negative supercoiling in eukaryotes are regular left-handed winding around histones and a topoisomerase to relax the compensating positive supercoils (Germond *et al.*, 1975). Since topoisomerases are abundant in *C.fasciculata* mitochondria (Shlomai and Zadok, 1983; Melendy and Ray, 1989), it is not surprising that minicircle-binding histones and histone-like proteins seem to be absent (Borst and Hoeijmakers, 1979). We conclude that trypanosomatids condense their kDNA by catenation rather than by supercoiling.

Materials and methods

Isolation of kDNA

C.fasciculata was grown at room temperature to stationary phase in brain heart infusion medium supplemented with hemin; in stationary phase networks, essentially all minicircles are covalently closed (Englund, 1978). Mid-log phase cells, in a 500 ml culture, were labelled by addition of 4 mCi of [^{3}H-methyl]thymidine (Amersham; 20 Ci/mmol) every 2 h for 12 h; the cells were then allowed to grow to stationary phase. For the kDNA used in the experiment in Figure 6, the medium contained 23 µCi/ml [^{32}P]orthophosphate or 23 µCi/ml [^{3}H]thymidine. kDNA networks were isolated as described previously by Hajduk *et al.* (1984) and monomeric minicircles were prepared by decatenation of networks with T4 phage topoisomerase II (a generous gift of K.Kreuzer) (Marini *et al.*, 1980). The minicircles were then extracted with phenol and ethanol precipitated.

Measurement of minicircle supercoiling

To measure supercoiling of the double helix induced by catenation, unlabelled networks (7.3 µg) were mixed with ^{3}H-labelled covalently-closed monomeric minicircles (25 ng; 98 000 d.p.m.) in 40 µl of 50 mM Tris–HCl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 µg/ml BSA and calf thymus topoisomerase I (a gift of A.Bodley). The quantity of topoisomerase I was sufficient to relax the networks and subsequently to fully relax pBR322 plasmid that was added to a portion of the reaction (see Results). After 30 min at 37°C, the reaction was quenched by addition of gel loading buffer (final concentrations, 5% Ficoll, 1% SDS, 10 mM EDTA and 0.01% bromophenol blue). Total minicircle supercoiling was measured under the same conditions except that topoisomerase I digestion was omitted.

The DNA was then sonicated briefly to release monomeric minicircles from the networks (Ultrasonics model W185 sonifier, setting 4, small probe, 3 s at room temperature). From this treatment, a few percent of the minicircles were recovered as covalently-closed circles; the remainder of the DNA was in the form of minicircle oligomers, nicked monomers and linear fragments.

The sonicate was then fractionated by two-dimensional gel electrophoresis to resolve the topoisomers of monomeric covalently closed minicircles. In the first dimension (50 V on a 24.5 cm 0.8% agarose gel at room temperature with buffer recirculation at 75 ml/h, in the dark), the TBE buffer contained 0.5 µM chloroquine phosphate; the chloroquine introduces sufficient positive supercoiling to maximally spread the topoisomer ladder. The gel was trimmed and turned 90° for the second dimension. Conditions were the same except the gel was equilibrated in TBE buffer containing 0.5 µg/ml ethidium bromide; the ethidium promotes separation of the covalently closed minicircles from the linearized and nicked molecules. Control fragments, run in separate lanes, included ^{32}P-end-labelled *Hpa*II fragments of ΦX174 DNA (to aid in alignment of photographs of ethidium bromide-stained gels and fluorographs of ^{3}H-labelled DNA; from T.Shapiro and K.Ryan) and ^{3}H-labelled fragments for calibrating the densitometer.

Gels were stained with 1 µg/ml ethidium bromide and then the DNA was nicked by 5 min exposure to UV light (260 nm) (Ultraviolet Products) before

additional final staining with ethidium bromide. After photography under UV light, the gels were destained in 0.5 M NaCl and then in 10 mM $MgSO_4$ (Maniatis *et al.*, 1982) and processed for fluorography by impregnating with En^{3}Hance (DuPont, NEN Research Products). The dried gel was then exposed to preflashed Kodak XAR5 film at −70°C.

Relative masses of DNA in topoisomer bands were determined by densitometry of photographic negatives or fluorographs using the LOATS Research Analysis System 1000 Image Analysis Program (Amersham Corporation). Bands were outlined using the polygon method and appropriate background subtractions were made for the slight inhomogeneities in background optical density. Control experiments showed that the densitometer response was linear with DNA mass in the range studied.

Electron microscopy of minicircle dimers

kDNA networks were partially digested with *Sst*II, which cleaves minicircles once, in a 0.6 ml reaction containing 5 μg kDNA networks, 40 units *Sst*II (New England Biolabs), 50 mM Tris−HCl pH 8.0, 50 mM NaCl and 10 mM $MgCl_2$. After 10 min at 37°C, the DNA was extracted with phenol and ether and precipitated with ethanol. Prior to electrophoresis, the DNA products were nicked by mild DNase I treatment in the presence of ethidium bromide to eliminate linking number topoisomers of covalently closed circles. The nicking solution, which contains 20 μg/ml DNA, 20 mM Tris−Cl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 300 μg/ml ethidium bromide and 10 μg/ml DNase I (Sigma), was incubated for 30 min at 30°C in the dark. After extracting with phenol and concentrating with a Centricon30 apparatus (Amicon), the DNA was fractionated by electrophoresis through an 0.8% agarose gel in 80 mM Tris−HCl pH 7.5, 5 mM sodium acetate, 1 mM EDTA and 0.03% SDS (Sundin and Varshavsky, 1980). Electrophoresis (24.5 cm gel at 40 V and 300 ml/h buffer recirculation) was for 30−40 h. The gel was exhaustively rinsed in distilled H_2O prior to staining with ethidium bromide (0.5 μg/ml) and photography. DNA was eluted from agarose using the Elutrap apparatus (Schleicher and Schuell). In this procedure, a gel fragment containing DNA was electrophoresed in TAE buffer (40 mM Tris−acetate and 1 mM EDTA with recirculation). The DNA migrates through the BT2 membrane but is trapped by the BT1 membrane. After concentrating the DNA by Centricon filtration, it was coated with RecA protein and examined by electron microscopy using the method of Thresher and Griffith (Thresher and Griffith, 1990).

Electron microscopy of networks

Networks were spread for microscopy using the formamide technique (Davis *et al.*, 1971). For spreading in the presence of propidium diiodide, the same dye concentration was used in the spreading solution and in the hypophase. For single angle shadowing, the angle was 8°. Magnifications were determined using a grating replica (2160 lines/mm).

Acknowledgements

We thank Dr Leroy Liu for valuable advice and Dr David Lynch for help with the Loats Image Analyzer. We thank Terry Shapiro for comments on the manuscript and Viiu Klein for providing valuable technical assistance. This research was supported by grants from NIH (GM27608 to P.T.E. and GM31657 to N.R.C.) and from the MacArthur Foundation to P.T.E. C.A.R. was supported by Medical Scientist Training Grant 5T32GM07309 and David Perez-Morga by a grant from the Rockefeller Foundation.

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Received on October 8, 1992