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Electron microscopic studies of the different topological forms of the cauliflower mosaic virus DNA: knotted encapsidated DNA and nuclear minichromosome

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Cauliflower mosaic virus (CaMV) DNA exists under different topological forms in infected plants. First, the population of encapsidated CaMV DNA molecules appears heterogeneous when analysed by gel electrophoresis. The electron microscopic study reported here reveals that CaMV virion DNA contains simple and multiple topological knots. Second, a supercoiled DNA form never found in virions exists as a chromatin-like nucleoprotein complex with nucleosome subunits in the nuclei of infected leaves. The compaction ratio of the minichromosomes is compatible with the nucleosomal structure, the number of nucleosomes (41.0 ± 2.5) is in keeping with the length of the viral genome.

Key words: CaMV/DNA plant virus/electron microscopy/knotted DNA/minichromosome

Introduction

Cauliflower mosaic virus (CaMV), the prototype of caulimoviruses, has been considered attractive as a vehicle for the expression of heterologous genes in plants (for reviews see Hull, 1979; Shepherd, 1979; Hohn *et al.*, 1981). Its genome consists of a single, relaxed circular DNA molecule with three discontinuities, a structure only described for caulimoviruses. By gel electrophoresis, the viral DNA appears heterogeneous with at least three additional DNA bands between the circular and linear forms (Volovitch *et al.*, 1978). We show here that these additional species are circular, knotted, full length molecules of CaMV DNA.

CaMV DNA is transcribed into two major RNAs. One is a 19S mRNA (Al Ani *et al.*, 1980; Odell and Howell, 1980; Covey *et al.*, 1981; Dudley *et al.*, 1982; Guilley *et al.*, 1982) which is translated into the viroplasm protein (Shockey *et al.*, 1980; Xiong *et al.*, 1982). The second is a full length 35S RNA (Howell, 1981; Covey and Hull, 1981; Dudley *et al.*, 1982; Guilley *et al.*, 1982) which so far has not been translated *in vitro*. This 35S RNA is probably not a transcript from the single-stranded interrupted DNA, but rather from a supercoiled DNA molecule (Ménissier *et al.*, 1982; Guilley *et al.*, 1982; Olszewski *et al.*, 1982). Olszewski *et al.* (1982) located such a viral DNA in infected turnip nuclei and suggested that this DNA, prior to deproteinization, could have existed in a nucleosome-like structure. The observed supercoiling of the deproteinized DNA would result from the removal of the histones.

We present here the first electron micrographs of the CaMV minichromosome and demonstrate its nucleosomal structure.

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Results

Separation of the different species of intracellular viral DNA

The CaMV minichromosome, extracted from infected plant nuclei, was purified by velocity sedimentation as described in Materials and methods. To monitor the amount and distribution of CaMV DNA in gradient fractions, an aliquot of each fraction of the gradient was observed in the electron microscope and the other part was deproteinised and analysed on a 1% agarose gel by electrophoresis and molecular hybridization. The autoradiogram of this gel (Figure 1) shows, in gradient fractions f and g, the presence of supercoiled DNA (Ménissier *et al.*, 1982) originating from the minichromosome. Contaminating viral particles sediment at the bottom of the gradient (Figure 1, slots i and j) as confirmed by electron microscopy of the gradient fractions (data not shown) and by their migration pattern, characteristic of virion DNA.

Topological forms of encapsidated DNA molecules

DNA extracted from virus particles appears heterogeneous when analysed by gel electrophoresis. This heterogeneity is not related to differences in length (Volovitch *et al.*, 1978). Native DNA preparations (Figure 2) contain a majority of

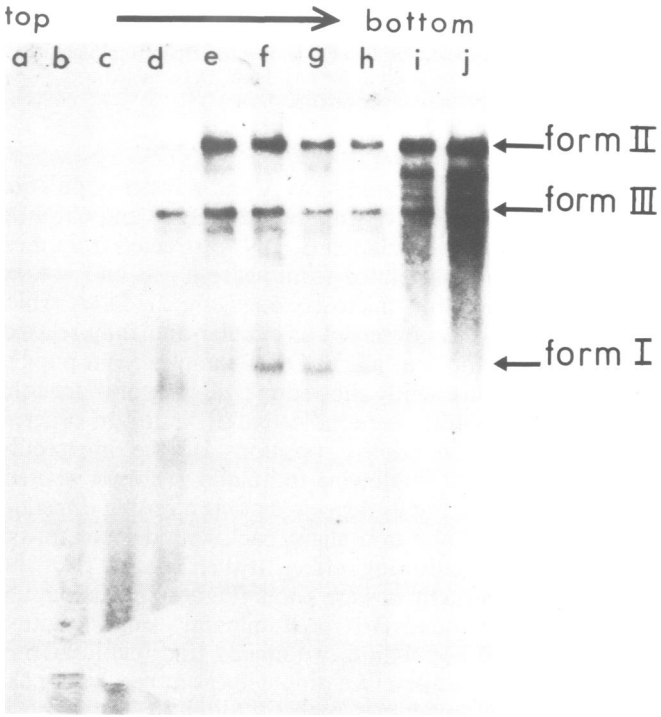

Fig. 1. Gel electrophoretic analysis of DNA in fractions from a minichromosome fractionation on sucrose gradient. 20 μl aliquots of the sucrose gradient fractions were analysed by electrophoresis on a 1% agarose gel. The gel was blotted on nitrocellulose (Schleicher and Schüll BA 85) and the DNA was hybridized with nick-translated CaMV DNA (sp. act. 2 x 10^8 c.p.m./μg). The arrows point to the positions of separately run markers of supercoiled DNA (form I), open circular DNA (form II) and linear DNA (form III).

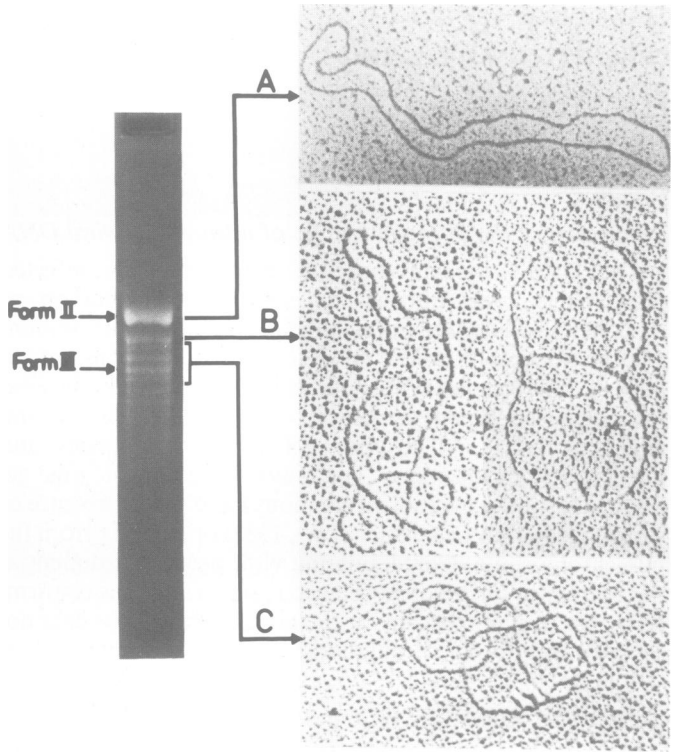

Fig. 2. 'Knotted' CaMV DNA. DNA (1 μg) purified from virion particles was electrophoresed on a 1% agarose gel at 30 V for 16 h. The gel was then stained with ethidium bromide (0.5 μg/ml) and observed under u.v. illumination. DNA in different sections of the gel was electroeluted and prepared for electron microscopy as described by Davis *et al.*, 1971. **Band A** corresponds to open circular DNA molecules. **Band B** corresponds to singly knotted DNA molecules. **Band C** mainly corresponds to highly knotted circular DNA. As explained in the text, linear DNA molecules, sometimes knotted, are also observed (not shown).

open circular DNA, referred to as A and a faster population referred to as B (a relatively abundant species) and C.Linear DNA is included in population C. DNA extracted from these three populations was eluted from agarose gels and was examined in the electron microscope (Figure 2). DNA which bands in A is always observed as circular and fully relaxed. When rotary shadowed, all the DNA samples from populations B and C consistently showed pretzel-like, or more complex structures, which were indicative of knots. In order to better visualize the relative positions of the intersecting strands, an unusual shadowing technique was used whereby platinium is preferentially deposited along a certain direction. This technique results in a slight background increase, but emphasizes the 'shadowing' effect. It then became clear that the pretzel-like structures were knots (Figure 2). The population of DNA in band B is predominantly singly knotted. Within the third population, we mainly find highly knotted circular DNA molecules. Occasional linear molecules are also present and they sometimes appear knotted. It is quite possible that linearization occurred during spreading. Indeed, when CaMV DNA is linearized by a single-cut restriction enzyme (*Sal*I, *Bst*EII) and analyzed by agarose gel electrophoresis, only one linear DNA band is found. It should be pointed out that the size of loops is variable in knotted circular molecules. This indicates that the knots are not due to any sort of cross-linking related to the specific single-strand interruptions of CaMV DNA.

Chromatin-like structure of the CaMV minichromosome

The CaMV minichromosome prepared by sucrose gradient sedimentation is sufficiently pure for electron microscopy. Figure 3 (A and B) gives typical micrographs obtained from fractions f and g of the gradient (Figure 1): they show circular nucleoprotein complexes containing globular particles or nucleosomes, connected by a thin filament of DNA. This structure is similar to that of cellular chromatin (Figure 3C). The examination of ~60 molecules of minichromosome yields a mean value of 41.0 nucleosomes per molecule with a standard deviation of 2.5. Figure 4 shows the histogram of the number of nucleosomes per molecule.

To determine the compaction ratio of the DNA in the nucleosomal structure, the contour length of 38 molecules of minichromosome was measured. The compaction ratio is calculated by dividing the theoretical length [8024 bp (Franck *et al.*, 1980) x 3.34 = 2.68 μm] of the DNA molecule by the contour length (0.93 μm) of the minichromosome. The mean value is 2.9 with a standard deviation of 0.2.

Discussion

Electron microscopic observations clearly show the presence of knots (multiple as well as single) (Figure 2), on some molecules of DNA extracted from virions. These observations explain the complexity of electrophoretic migration patterns of the DNA extracted from particles (Volovitch *et al.*, 1978). Richins and Shepherd (1983) have also shown that the encapsidated DNA of other caulimoviruses (dahlia mosaic virus and mirabilis mosaic virus) presents the same migration pattern in agarose gels. The topological multiplicity of the encapsidated DNA molecules would thus be a common characteristic of caulimoviruses. A similar phenomenon has been described in the DNA of P4, a satellite of bacteriophage P2 (Liu *et al.*, 1981). The transformation of a circular form of pBR322 DNA into knotted complex forms has been demonstrated *in vitro* by the action of the topoisomerase II (for review see Wang, 1982). The mechanism of knot formation would thus be similar to the mechanism of supercoiling and concatenate formation (Liu *et al.*, 1980). When the knotted DNA migration patterns observed by these authors are compared with the migration pattern of CaMV DNA (Figure 2) it is clear that there is considerable similarity in the intensity and distribution of the bands. It is thus possible that such an enzymatic activity is also expressed in CaMV-infected cells during viral morphogenesis. Recently Hohn (personal communication) has proposed another origin for the knotted DNA, namely that it would result from the synthesis of circular single strand DNA on a 35S RNA template that would be knotted as an artefact due to its length.

Our results demonstrate that the nuclei of cells infected with CaMV contain a chromatin-like nucleoprotein complex. This is the first structural description of such a structure in virus-infected plants. The structure is similar to those of SV40 DNA (Griffith, 1975; Bellard *et al.*, 1976) and polyoma minichromosomes (Cremisi *et al.*, 1976). Our results are in good agreement with those obtained by micrococcal nuclease digestion (Olszewski *et al.*, 1982). The compaction ratio of the DNA in the minichromosome (2.9 ± 0.2 at 5 mM NaCl) is in keeping with its nucleosomal structure. The mean value of 41.0 nucleosomes per molecule of minichromosome (8024 bp, Franck *et al.*, 1980) suggests a DNA repeat of 196 bp. This value is very close to the size of the DNA repeating subunit determined in the majority of chromatins from animals

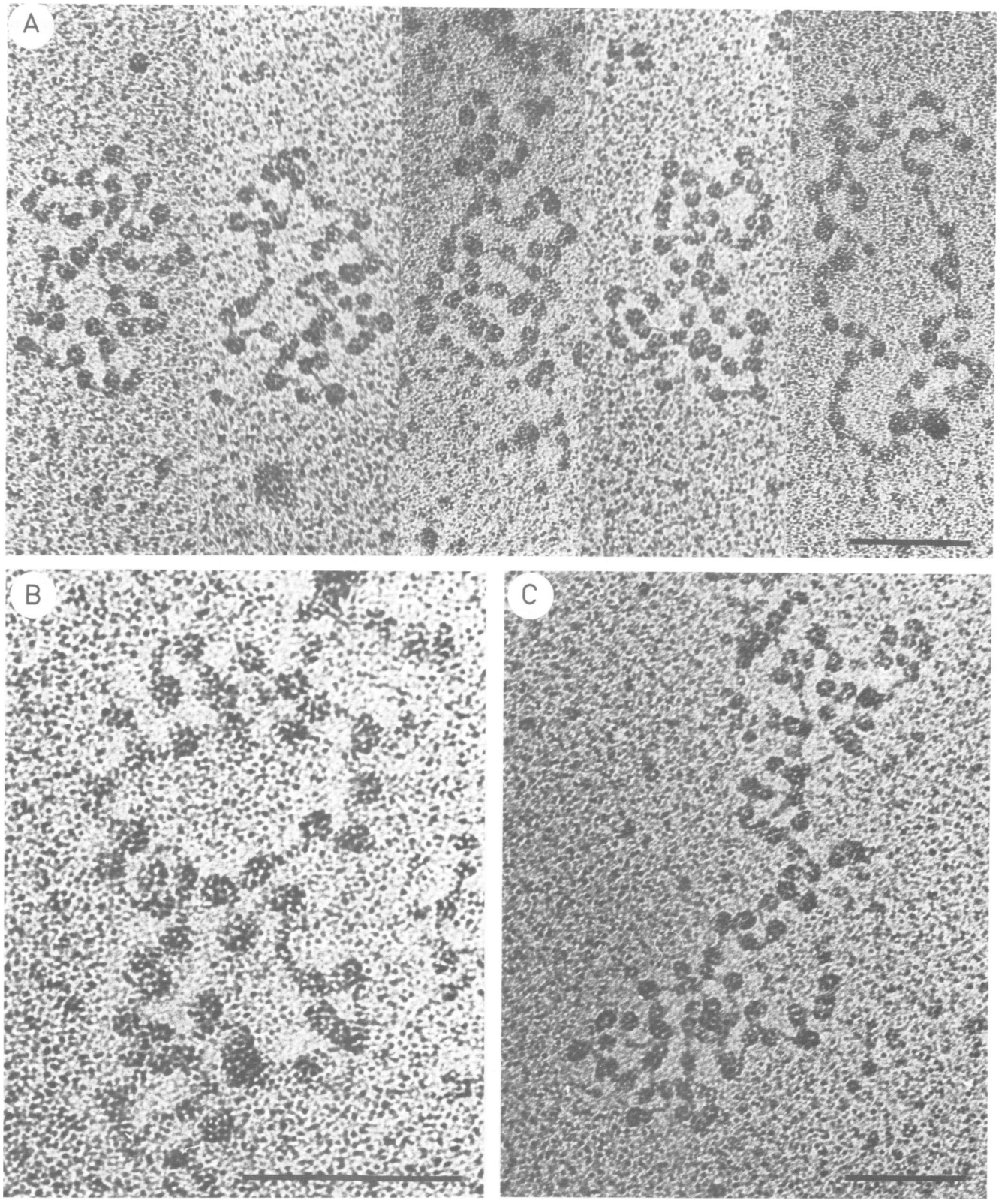

Fig. 3. Electron microscopy of CaMV minichromosome. Minichromosome-containing sucrose gradient fractions (f and g, Figure 1) were diluted 10-fold in TEA 5 mM pH 7.4, EDTA 0.5 mM and fixed with 0.1% glutaraldehyde for 1 h at 20°C as described in Materials and methods. Spreading was performed according to the method of Dubochet *et al.* (1971). The bar represents 100 nm. **A** and **B**: CaMV minichromosomes; **C**: turnip cellular chromatin.

(Compton *et al.*, 1976) and from higher plants (Philipps and Gigot, 1977) including turnip (Ménissier, unpublished data). We and others have suggested that the supercoiled DNA could be the template for the synthesis of full length 35S RNA (Ménissier *et al.*, 1982; Guilley *et al.*, 1982; Olszewski *et al.*, 1982). Furthermore, Guilley *et al.* (1983) proposed a model for CaMV genome replication whereby the 35S RNA could be reverse transcribed into cDNA, as observed for the retroviruses (see Weiss *et al.*, 1982); the newly synthesized cDNA would be copied into a complementary strand. The mini-

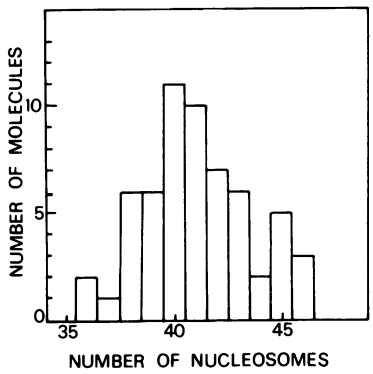

Fig. 4. Histogram of the number of nucleosomes per minichromosome molecule. The nucleosomes were counted on photographic enlargments. The mean value is 41 ± 2.5 nucleosomes.

chromosome could be used as a matrix for amplification of the 35S RNA. It would not be surprising, therefore, to find that a large proportion of the minichromosome behaves as active chromatin. Preliminary experiments are in favor of this hypothesis.

Materials and methods

Virus strain and propagation

CaMV Cabb-S was propagated in turnip leaves (*Brassica rapa* L. c.v. Just Right) as described by Hull *et al.*, 1976).

Encapsidated DNA extraction

Viral DNA was extracted from purified virus according to Hohn *et al.* (1980).

Isolation of turnip leaf nuclei

Nuclei were isolated from healthy uninfected and infected turnip leaves by a modification of the method of Gigot *et al.* (1976). 15 g of the two youngest fully expanded leaves from each plant were excised and cooled on ice. All the following operations were carried out in the cold. The midribs were removed and the remaining tissue immediately used for isolation of nuclei. The leaves were cut into small blades and ground for 2 min in a 'Virtis' 45 homogenizer in 100 ml of a pH 6.1 buffer (buffer A: 5 mM MES, 4 mM Mg acetate, 5 mM 2-mercaptoethanol, 10 mM Na bisulfite, 4% gum arabic, 350 mM sucrose and 0.5% Triton X-100). The homogenate was filtered through a nylon screen 10 μm in pore diameter. The suspension was centrifuged at 1700 *g* for 20 min. The pellets were rapidly resuspended in 40 ml of buffer A without gum arabic (buffer B) and 10 ml aliquots layered over a 10 ml of 1.25 M sucrose cushion prepared in buffer C (buffer B without Triton X-100) and centrifuged at 4000 *g* for 20 min. The pellets were then resuspended in 20 ml of buffer B and two aliquots layered over 15 ml of a 1.25 M sucrose cushion. Finally, the pellets were washed twice by resuspending in buffer C and pelleted to remove residual Triton X-100.

The typical yield of this purification is at least 2 x 10^5 nuclei/g of tissue. The purified nuclei were used immediately after the final resuspension in buffer C.

Partial purification of CaMV minichromosome

CaMV minichromosomes were selectively extracted from infected nuclei by the technique of hypotonic leaching described by Jakobovits and Aloni (1980) as modified by P.Pfeiffer (personal communication) for the extraction of the SV40 minichromosome.

10^6 nuclei were quickly washed in 0.5 ml hypotonic buffer (buffer D: 50 mM Tris pH 8, 1 mM $MgCl_2$ and 1 mM dithiothreitol), and resuspended in 0.5 ml of the same buffer. The diffusible chromatin was extracted for 1 h at 0°C. The nuclei were centrifuged at 1750 *g* for 5 min and the clear supernatant containing the CaMV chromosome was layered over a 10 – 40% (w/w) linear sucrose gradient prepared in buffer E [5 mM triethanolamine (TEA) pH 7.3, 0.2 mM EDTA] and centrifuged at 40 000 r.p.m. for 90 min in the Beckman SW 50.1 rotor at 10°C. Fractions of 0.5 ml were collected from the bottom of the centrifuge tube. The minichromosome was located by electron microscopy and was treated by SDS 1% and proteinase K 0.1 mg/ml, 30 min at 37°C to purify the DNA. The DNA was analysed on agarose gel and hybridized with a nick-translated CaMV DNA as described by Southern (1975).

Gel electrophoresis

Undigested DNA samples and preparative elution of the DNA were analysed on a 1% agarose gel in buffer F (40 mM Tris-HCl pH 8.1, 20 mM Na acetate, 2 mM EDTA). Electrophoresis was performed at 30 V for 16 h in buffer F. The preparative gels were stained for 15 min with 0.5 μg/ml of ethidium bromide and the DNA was visualized under u.v. illumination. The bands were excised from the gel and the excised sections were placed individually in a dialysis bag containing buffer F and electroeluted for 4 h at 80 V. DNA was then precipitated with ethanol.

Electron microscopy

All solutions were filtered through 250 Å Millipore filter. Carbon support films were prepared by direct evaporation onto freshly cleaved mica sheets in an Edwards evaporator. The films were floated off on redistilled water and were transferred onto 400 mesh copper-rhodium grids. The upper side of the carbon film was used.

Sucrose gradient fractions were diluted 10-fold in 5 mM TEA buffer, pH 7.4 containing 5 mM NaCl, 0.2 mM EDTA and fixed in 0.1% (v/v) glutaraldehyde for 1 h at 20°C. 50 μl of the sample were applied onto a carbon-coated grid, positively charged as described by Dubochet *et al.* (1971). After adsorption for 5 min the grid was transferred for 1 min into a 50 μl droplet of redistilled water, blotted dry on filter paper and finally rotary shadowed with carbon/platinum at an angle of 7° in an Edwards evaporator equipped with an electron gun (EVM 052, Balzers). The thickness of the metal deposition was monitored on a quartz thin crystal minotor (QSG 201 D Balzers). To better visualize knotted DNA, some of the sample, as mentioned in Figure legends, were spread onto carbon-coated grids by the cytochrome c technique (Davis *et al.*, 1971); shadowing was performed preferentially on one direction at an angle of 4°.

Specimens were examined in a Siemens Elmiskop 101 and in a Jeol 100 CX. The magnification was calibrated using a carbon grating replica (Fullam).

DNA length measurements and nucleosome counting were made on photographic enlargements (40 000 x 5) with a Hewlett-Packard digitizer/computer device (9874 A).

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