Incorporation of Exogenous Circular DNA into Large Catenated Networks in Isolated Nuclei

EVIDENCE FOR INVOLVEMENT OF THE NUCLEAR SCAFFOLD*

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Circular plasmid DNA was efficiently converted into huge catenated intranuclear networks by incubation with isolated nuclei in the presence of ATP. The network production is abolished by omission of ATP, strongly inhibited by etoposide (VP-16), but only slightly inhibited by antibody to topoisomerase I, indicating that the major enzyme responsible for catenation is DNA topoisomerase II. Under optimal conditions, a single nucleus incorporates about $4.2 \times 10^4$ DNA rings into its networks. Under the light microscope, networks retrieved from nuclei appear like spheres of various sizes. Sedimentation analysis showed that most of the networks are composed of thousands of catenated rings, which was confirmed by electron microscopy. Data from experiments that caused partial disruption of the networks were submitted to analysis based on probable models of catenane structure. The results suggest that the predominant pattern is a linear alignment of catenated rings. Similar networks are formed when the nuclear scaffold is incubated with circular DNA in the presence of nuclear extract containing topoisomerase II. Titration experiments showed that the scaffold binds a stoichiometric amount of the substrate and that a critical level of DNA is required for network formation. The results are consistent with the idea that DNA-binding sites are fixed on the scaffold and mediate catenation of bound DNA circles by holding them in close proximity to each other. We propose that catenation by the nuclear scaffold also occurs in intact nuclei, suggesting additional roles for the scaffold in vivo.

Catenanes consist of multiple DNA rings topologically interlocked. Many topological isomers exist even for catenated dimers, which are the simplest form of catenane (see Ref. 1 for a review on the topology of catenanes or knotted DNA and their biological significance). Multiply intertwined catenated dimers are known to be formed in the termination stages of circular DNA replication (2, 3). Catenated dimers are also produced through site-specific intramolecular recombinations (4). In addition to the intermediary occurrence of catenanes, larger catenanes can be formed from monomer DNA circles by the action of DNA topoisomerases, a class of enzyme which alters topological conformation of DNA. If each ring is covalently closed, only a type II topoisomerase which passes a segment of DNA through transient double-stranded breaks in substrate DNA can do the job. If some of the rings contain a nick or gap, a type I topoisomerase which introduces only single-stranded breaks would suffice (see Refs. 5–7 for review on topoisomerases). In some cases, catenanes can be as big as intracellular organelles. Mitochondrial DNA of trypanosomes, known as kinetoplast DNA, exists as a single massive network consisting of thousands of covalently closed DNA rings called minicircles and a few maxicircles interlocked together (8, 9).

While topoisomerases catalyze both catenation and decatenation of DNA circles, catenation reaction usually proceeds only in the presence of agents that aggregate, condense, or "crowd" DNA. Huge catenated networks are shown to be produced in vitro with purified topoisomerases and DNA-condensing agents such as polycationic compounds like spermidine (10–14), uncharged polymers like polyvinyl alcohol (15), and DNA-binding proteins including histone H1 (16), HMG17 (17), and a Mr 30,000 yeast protein (18). The DNA-binding proteins may serve as factors which regulate the course of reactions catalyzed by topoisomerases in vivo. Formation of oligomeric catenanes and large catenated networks has been demonstrated when circular but not linear DNA or chromatin is microinjected into nuclei of Xenopus laevis oocytes (19). It appears, however, that in vivo situation is more complex since the catenanes in oocyte nuclei appears only transiently and also decatenation reaction occurs when catenanes are injected.

We have shown recently that the nuclear scaffold exhibits at least two classes of DNA-binding sites available for DNA substrates added exogenously, one class being highly specific to supercoiled DNA in a sense that it does not bind relaxed or linear forms, and the other lacking this specificity (20). We speculated that these sites can interact with DNA in chromatin and thus participate in transcription or replication of chromosomal DNA which has been suggested to proceed in association with the nuclear scaffold (21–24). In the present study, we demonstrate that isolated nuclei incorporate exogenously added DNA rings into large catenated networks, and the DNA-binding sites on the nuclear scaffold promote this reaction, suggesting that these sites also contribute to in vivo processes that involve aggregation of DNA strands. The results provide additional implications for the role of the nuclear scaffold.

EXPERIMENTAL PROCEDURES

Isolation of Nuclei and the Nuclear Scaffold—Nuclei were isolated from brain tissue of 2-week-postnatal Sprague-Dawley rats as described previously (20) and stored at −20 °C in 50% glycerol contain-
ing 1.5 mM MgCl$_2$ (~5 x 10$^6$ nuclei/ml).

The nuclear scaffold was isolated from brain nuclei according to the published procedure (20). Briefly, nuclei were first treated with 0.5 mM CuSO$_4$, then extracted with 25 mM L1 in a low salt buffer (25). After washing several times with buffer A (0.05 mM spermine, 0.125 mM spermidine, 20 mM KCl, 70 mM NaCl, 10 mM MgCl$_2$, 0.01% digitonin, 0.2 mM phenylmethylsulfonyl fluoride, and 20 mM Tris-HCl, pH 7.6), the extracted nuclei were resuspended in buffer A and digested with micrococcal nuclease (100 units/mg of nuclear DNA) at 37°C for 60 min. The resulting nuclear scaffold was washed by centrifugation, finally suspended in 50% glycerol, 1.5 mM MgCl$_2$ and stored at -20°C. Concentrations for suspensions of the nuclear scaffold were determined by measuring the optical density at 260 nm. The spectrophotometric measurement was performed in a Beckman DU-90 spectrophotometer.

**DNA Substrates—** The de-pBR, pBR322 lacking the unique EcoRI site, was prepared by using the cutting-ligating procedure (20). The unique HindIII site of de-pBR was further deleted by a similar procedure to construct dEH-pBR. Supernucleated forms of the plasmid DNA were purified by ethidium bromide/cesium chloride equilibrium density gradient centrifugation. Calc thymus DNA, used as competitor DNA in some experiments, had been sonicated and treated with S1 nuclease (20).

**The standard reaction mixture (40 µl) contained 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM MgCl$_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 µg/ml bovine serum albumin, 1 µg of supercoiled de-pBR DNA, and 10$^6$ nuclei.** The mixture in a flat-bottomed Eppendorf tube was incubated at 30°C for 60 min with occasional stirring. Catenated networks formed inside nuclei were recovered together with the nuclei by centrifugation at 4000 × g for 1 min. The pellet was resuspended in 40 µl of buffer A and treated with 50 units of EcoRI at 37°C for 60 min to fragment nuclear DNA. In some cases, EcoRI was included directly into the catenation mixture. Samples were then treated with 500 µg/ml proteinase K and 1% SDS at 37°C for 60 min to digest protein.

When the catenation reaction was conducted with nuclear scaffold, nuclear extract obtained from approximately the same number of nuclei was added. To prepare the extract, nuclei were suspended at 10$^7$ nuclei/ml in 20 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 140 mM 2-mercaptoethanol, and 50 µg/ml bovine serum albumin, and incubated at 0°C for 30 min. After centrifugation at 9000 × g for 5 min, the supernatant (about 3 mg protein/ml) was used immediately for reaction. No EcoRI treatment was required after catenation when nuclear scaffold was used for the reaction.

**Electrophoretic Analysis of Catenated Networks—** Since catenated networks formed in the above reactions are a huge structure, quantitative recovery of the networks was possible by simply washing the product with TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) during repeated centrifugation. After the proteinase K treatment, samples were mixed with 10 µl of TE, centrifuged at 10,000 × g for 10 min, and the supernatant was removed carefully by aspiration with a thin-tipped Pasteur pipette. The pelletted network (not visible) was washed 3 times with 200 µl of TE by centrifuging each time at the same speed for 5 min. The network was resuspended in 10 µl of HindIII buffer (60 mM NaCl, 7 mM MgCl$_2$, 100 µg/ml bovine serum albumin, and 10 mM Tris-HCl, pH 8.0) and digested with 10 units of HindIII at 37°C for 60 min. Samples were mixed with 1/10 volume of loading buffer containing glycerol and bromophenol blue and subjected to electrophoresis on 0.8% agarose gels submerged in Tris-borate/EDTA buffer. The gels were stained with 0.5 µg/ml ethidium bromide and photographed under UV illumination. The de-pBR DNA incorporated into the networks is now visualized as a linearized product with TE.

**Fluorescence Microscopy of Catenated Networks—** Washed networks were mixed with 3 volumes of 5 µg/ml ethidium bromide solution containing 10% glycerol, 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1% agarose that had been kept molten at 65°C. The mixture was immediately transferred onto a slide glass and covered with a cover slip. The slices were observed with a fluorescence microscope (Olympus, model BHS-FPK) using a UV excitation mode with a cut filter (570 nm) inserted before the camera, then photographed at several exposures.

**Sedimentation Analysis of Catenated Networks—** To examine the size distribution of catenated networks, deproteinized samples were centrifuged through 5–20% sucrose linear gradients (4.5 ml) containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM NaCl, as originally described by Englund (26). The kinetoplast DNA network isolated from Crithidia fasciculata was centrifuged simultaneously in a separate tube as a size marker. After centrifugation at 7000 rpm in a RPS-65TA rotor (Hitachi), the sample was removed under water and photographed by increasing the degree of centrifugation (drops/fraction) from the bottom. The sedimentation pattern of kinetoplast DNA in the gradient was estimated by measuring absorbance at 260 nm. Catenation products in the gradient fractions were trapped on a nitrocellulose membrane using a dot-blotting apparatus (Bio-Rad) under negative pressure. The wells were washed 4 times with 300 µl each of the washing solution containing 1% SDS, 0.5 M NaCl, 20 mM EDTA, and 50 mM Tris-HCl, pH 8.0. After a brief rinsing, the filter was dried and treated with alkaline to denature the networks (20). Before the alkaline treatment, known amounts of pBR322 DNA were spotted on the filter as a standard. The filter was then baked at 80°C and processed for hybridization with 32P-labeled pBR322 DNA prepared by nick translation, followed by autoradiography at -70°C. The network DNA in the gradient fractions was quantitated by densitometric scanning of the film using the spotted standards.

**Partial Disruption Analysis of the Networks—** A statistical method was devised to deduce the topological structure of catenated networks. Let the network be composed of two different populations of DNA rings that are identical except that one is cleavable by a certain restriction endonuclease. In such cases, the number of rings constituting the network is large enough and equal numbers of rings are interlocked to each ring, the probability for a ring in the network to be released as a monomeric ring can be expressed as:

$$P(x) = x(1 - x)^m$$

where $x$ is the fraction of uncleavable rings ($0 < x < 1$) and $m$ is the number of rings interlocked to individual rings ($1 < m < \infty$). Experimentally, $P(x)$ is equivalent to the fraction of monomeric rings in the total restriction product of the networks. As cleavable and uncleavable rings would have the same behavior in the catenation reaction, the $P(x)$ be predetermined by varying the mixing ratio of cleavable rings to cleavable rings in the substrate mixture for the network formation.

Equation 1 is valid only when the cleavable rings are completely linearized by the restriction enzyme. We have observed, however, that this is not always the case in practice. The expression for $P(x)$ was then modified by substituting $x$, where $x = 0 < r < 1$, for the $x$ in Equation 1, and using the following equation for the actual analysis.

$$P(x) = x(1 - x)^m$$

In the present study, the networks were synthesized using mixed substrates with various ratios of de-pBR and deEH-pBR DNA. After digestion of the resulting networks with HindIII, samples were subjected to agarose gel electrophoresis, transferred to nitrocellulose membrane, and hybridized with 32P-pBR322 DNA. Autoradiographic films of the blots were scanned on a densitometer and band densities for the linear DNA (de-pBR) and the monomeric rings (deEH-pBR) were quantitated. The fraction of monomeric rings, $P(x)$, was plotted versus $x$ and a theoretical curve which is maximal deviation from the data points was obtained by assigning appropriate values for $m$ and $r$ in Equation 2. $P(x)$ was normalized against the linearized DNA $(1 - x')$, to cancel the experimental errors due to the variability in the network yield and the samples amounts loaded on the gel. By definition, the variable $m$ is an integer representing the degree of interlocking, and in simple networks it can determine the interlocking structure of the network by itself (for example, structures corresponding to $m = 1$ and $m = 2$ are shown in Fig. 7). Experimental values for $m$ are usually noninteger and in such cases $m$ approximates the average number of rings interlocked to individual rings in the network.
formed in a miniscale. The following three layers of solutions (100 μl each) were overlaid in Eppendorf tubes: from the bottom, CsCl (1.880 g/cm³), CsCl (1.377 g/cm³), and 60% sucrose each dissolved in TE. The lower CsCl solution contained 5 μg/ml ethidium bromide. After treatment with proteinase K/SDS or with other agents, samples (100 μl) were directly loaded onto the gradients, and the tubes were centrifuged in a swing rotor at 8000 × g for 25 min at room temperature. The tubes were illuminated with UV light from the bottom and photographed. Under these conditions, it is only the free networks that sediment through the middle CsCl layer and become fluorescent by ethidium diffused from the lower layer, whereas the networks associated with the scaffold remain on top of the gradient.

RESULTS

Exogenously Added Circular DNA Is Converted into Large Catenated Networks in Isolated Nuclei by Topoisomerase II—

Supercircular DNA substrates (form I) are readily relaxed to covalently closed circles (form I') by incubation with nuclear salt extracts which contain high levels of both type I and II topoisomerase activities (27). To see whether relaxation is the major consequence in the nuclear environment as well, supercoiled DNA (de-pBR) was directly incubated with isolated nuclei in the presence of ATP. After incubation nuclei were pelleted, treated with LIS to remove nuclear proteins mainly histones (26), digested with EcoRI, and the resulting fractions were analyzed by electrophoresis in agarose gels (Fig. 1A). In the extranuclear fluid, a portion of the substrate DNA was found relaxed and partially nicked (Fig. 1A, lane 4). While no substrate DNA was released by extracting the nuclei with LIS (lane 5), the residual nuclear fraction contained highly aggregated DNA which barely entered the gel (lane 6). When Cu²⁺ was included to inhibit both topoisomerase I and II, little DNA aggregate was detected at the top of the gel (lane 3). Most of the substrate was recovered as supercoiled and nicked forms in the LIS extract (lane 2) and in the extranuclear fluid (lane 1), whereas no relaxation product (form I') was noted. A significant proportion of the substrate was nicked by Cu²⁺ (lanes 1 and 2). No de-pBR DNA was detected in the region covered with smearing nuclear DNA (lanes 3 and 6) as confirmed by Southern hybridization (data not shown). These results indicate that essentially all the plasmid DNA associated with nuclei are incorporated into large DNA aggregates, but they remain in an extractable monomeric state when topoisomerases are inhibited. The DNA aggregates were recovered free from nuclear DNA fragments by simply washing the nuclear material digested with EcoRI and proteinase K (Fig. 1B, lane 1). Upon digestion with BamHI, they were completely converted into a linear form (form III) of unit length which is hybridizable to pBR probe on Southern blots, indicating that the aggregates are catenated networks composed of de-pBR DNA topologically interlocked.

The catenated networks appear to be formed and reside inside nuclei, but neither in the extranuclear space free from nuclei nor in association with nuclear periphery. This notion is supported by the experiment shown in Fig. 2. When the supercoiled substrate was incubated with nuclei in the absence of ATP, the substrate DNA was recovered as relaxed forms free in the supernatant (labeled S in Fig. 2A) and in another portion sedimented with nuclei (labeled P). Further incubation of the mixture with increasing amounts of BamHI efficiently converted the free substrate into the linear form depending on the enzyme concentration, whereas the plasmid DNA associated with nuclei was significantly resistant to the enzyme (Fig. 2A). These results imply that the nucleus-associated DNA is in a more protected environment, probably in an intranuclear compartment, and thus the internalization process does not require ATP. The internalized monomeric DNA appears to be the direct substrate for catenation since even after removal of the free substrate, the catenated networks were formed as long as ATP was supplemented (Fig.

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3 K. Tsutsumi, unpublished results.
The intranuclear occurrence of the resulting networks is consistent with observations such as: (i) the networks cosediment with nuclei at low speed centrifugation, (ii) nuclei need to be extensively digested with restriction endonucleases and proteinases in order to retrieve the networks, and (iii) very little DNA aggregates resembling the networks (shown below) are observed outside the nuclei when stained with ethidium bromide after the catenation reaction.

Nuclei were incubated with form I dE-pBR DNA under various conditions (Fig. 3). The network production increased with time of incubation at 30 °C up to 30 min and leveled off thereafter. The variation in DNA amount showed that a certain level of substrate concentration is required for the network formation as very few networks were formed with 0.25 μg of DNA. No increase in the network formation, however, was observed at substrate levels higher than 1 μg, suggesting that saturable sites are involved. Under optimal conditions, 10⁶ nuclei incorporate about 200 ng of the substrate into the catenated networks which comprise as much as 3% of total nuclear DNA. The catenation reaction was highly dependent on the salt concentration in the reaction medium. The networks were not formed if no KCl was added or if its concentration was higher than 0.12 M. The optimal KCl concentration was in the same range as that observed in a reconstituted system in which polyvinyl alcohol was used as a condensing agent (15).

Since small amounts of the nicked forms are always present in the DNA substrate and are also newly formed during incubation by endogenous nuclease activities, topoisomerase I can be responsible for the network formation observed in the nuclei. Extensive catenation of nicked or gapped DNA rings has been demonstrated with prokaryotic topoisomerase I which induces a single-stranded DNA break in these regions (28). To clarify this situation, the effects of ATP and topoisomerase inhibitors on the catenation reaction were examined (Fig. 4A). When ATP was omitted from the reaction mixture, no network was formed (lane 2, also in Fig. 2B). This result alone strongly suggests the involvement of topoisomerase II since this enzyme requires ATP for all the reactions that it catalyzes, including catenation. This was confirmed by the fact that VP-16, a specific inhibitor of topoisomerase II, inhibited the reaction as well, although somewhat partially (lane 4). Furthermore, anti-topoisomerase I IgG from an autoimmune patient did not seem to affect the network formation (lane 3). Inhibitory action of the antibody in the nuclear environment was confirmed by analyzing the topoisomerase I-dependent relaxation of the internalized substrate at various antibody doses (Fig. 5, left panels). The inhibition on relaxation was nearly complete at 20 μg of the antibody, as revealed by disappearance of form I and concomitant appearance of form I. In contrast, the network formation conducted under similar conditions in the presence of ATP was only slightly inhibited (81% of the networks, as determined by densitometry, were formed even at the highest antibody dose). The incomplete inhibition observed in the presence of both VP-16 and anti-topoisomerase I IgG added (lane 4, left panel) is due to the partial inhibition by VP-16. For some reason, relaxation of supercoiled substrate was also partially insensitive to the drug even at concentrations as high as 500 μM. From these experiments, we conclude that the major topoisomerase responsible for the network formation in situ is topoisomerase II.

As shown in Fig. 4, the networks were also produced when the nicked form (lane 6) but not the linear form (lane 7) was used as a substrate, supporting the idea that the networks are composed of extensively catenated DNA rings. The network formation from nicked circles was also insensitive to anti-topoisomerase I antibody (not shown).

Characterization of the Networks Formed in Nuclei—For structural analysis of the networks, we first observed them directly under a fluorescence microscope after ethidium bromide staining (micrographs not shown). The isolated networks appeared as spheres of heterogeneous sizes ranging from ~0.2 to 3.0 μm in diameter, while some were fused to larger particles. The size of the largest network was comparable to that of a nucleus. Morphological comparison between the networks formed inside the nuclei and those derived from kinetoplast revealed that the nuclear networks fluoresce brighter than the kinetoplast networks, and that the latter are mechanically more fragile than the former. While kine-
Topoplast DNA networks have been shown to be relatively resistant to shearing force (29), broken networks were frequently observed that were probably sheared by agarose fibers added to immobilize the structure. These observations are consistent with the idea that the networks from nuclei are tightly organized three-dimensional structures with spherical shape. In contrast, kinetoplast DNA is a two-dimensional network resembling a cap (9). When dEH-pBR, instead of dE-pBR, was used for the network formation and the resulting networks were treated with HindIII or BamHI, the networks disappeared only by the BamHI digestion, as expected. This experiment eliminates the possibility that the observed structure is composed of nuclear DNA, since if so, both enzymes should disrupt the structure to the same extent.

The catenated networks were next subjected to sedimentation through a sucrose density gradient to estimate their size distribution (data not shown). Conditions for the analysis were similar to those previously used for the analysis of kinetoplast DNA (26, 30). As had been predicted from the microscopic observation, the networks distributed broadly throughout the gradient, showing their size heterogeneity. Some of the networks were larger than kinetoplast DNA and some were too small to enter the gradient. The smallest populations are likely to be absent from the network preparations that are routinely obtained by repeated washing. The median of the distribution was calculated to be around 1900 S by using the mean value reported for kinetoplast DNA (26). If it is assumed that these networks of different origins have similar density under these conditions, this S value corresponds to the molecular mass of $3.4 \times 10^{10}$ dalton or 1200 molecules of dE-pBR DNA circles/individual network.

Analysis of the network structure in further detail is of interest since it is conceivable that the catenated networks formed inside the nuclei somehow reflect the topological organization of intranuclear space. When examined under an electron microscope, typical networks were identified as round or oval masses of densely packed DNA fibers (Fig. 6A). The network shown here is a “twin” network which occurred frequently also in fluorescence microscopy. DNA loops sticking out from the periphery of the network showed relaxed configuration with contour length comparable to that of pBR322 DNA (Fig. 6B). Although individual rings in the network are not discernible, it appears that the whole network is a patchwork of many small domains consisting of multiple DNA rings arranged in a rosette-like pattern.

As evident from Fig. 6, because of the network’s complexity, electron microscopic examination provides practically no information on the topological relationship among the constituent rings. We, therefore, introduce here a biochemical approach which provides more general information on the topological organization of the networks (the principle of the method is described under “Experimental Procedures”). For reference, the fractions of monomer rings released from two types of simple networks were calculated using Equation 1, and are plotted against the fraction of uncleavable rings (Fig. 7, left panel). The structure designated type A is a linear array of rings catenated each to neighboring ones, and the other one is a key-ring-like structure in which multiple rings are interlocked to a single ring (type B). When the networks were synthesized using dE-pBR and dEH-pBR mixed at various ratios and subsequently digested with HindIII, the released monomeric circles peaked at the fraction of 0.2 (Fig. 7, right panel).

![Fig. 6. Electron micrographs of catenated networks retrieved from nuclei.](image)

![Fig. 7. Release of monomer rings from partially disrupted networks.](image)
The bands that migrated between form II and III are probably topoisomer bands of form I which migrated faster in the presence of ethidium bromide (not shown). The slow-migrating bands are oligomeric catenanes whose structure was not determined. To estimate the mode of interlocking in the network, the released monomer rings were quantitated and plotted in Fig. 7 (see "Experimental Procedures" for detail). The variables $r$ and $m$ in Equation 2 were then evaluated by fitting the equation to the experimental points. The resulting $m (=1.9)$ indicates that the average number of interlocking in the network is close to 2, suggesting that catenanes similar to type A are the prevailing motif in the networks formed in nuclei.

DNA-binding Sites on the Nuclear Scaffold Are Involved in the Network Formation—Since catenation reaction requires agents that aggregate DNA, in addition to topoisomerases we next looked for nuclear component(s) serving as DNA-aggregating agents in situ. First, the ratio of substrate DNA to nuclei was altered as in Fig. 3, but this time increasing numbers of nuclei were added keeping the substrate concentration constant (Fig. 8A). Maximal incorporation of dE-pBR into the networks occurred at 10^6 nuclei (lane 3). At a lower nuclear dose, less incorporation was observed (lane 2), with a significant portion of the relaxed substrate remaining in the incubation supernatant (lane 8). At nuclear amounts higher than 1 x 10^6, there was no further increase in the incorporation (lanes 4–6) and unincorporated DNA disappeared from the supernatant (lanes 10–12). This is not due to degradation since no degradation fragments of the substrate were detected in the entire system. Increase in the substrate at these nuclear doses resulted in increased formation of the networks. These results suggest that a certain number of nuclei produce a stoichiometric amount of the network, and that the stoichiometry is determined by a fixed number of intranuclear sites involved in catenation. If such is the case, the network formation should be interfered by the presence of linear DNA which does not participate in the catenation but would compete for the sites. As shown in Fig. 8B, this does seem to be the case. Significantly less networks were formed when the competitor/substrate ratio exceeded unity (lane 5). Increasing amounts of the relaxed substrate were displaced from nuclei as form III DNA was increased (lanes 7–10), showing the presence of saturable binding sites available for exogenous DNA. That form III DNA does not inhibit topoisomerase II was confirmed by persistence of relaxation activity in the presence of antibody to topoisomerase I (data not shown).

Since the nuclear scaffold, a proteinaceous network in the nuclei, possesses DNA-binding sites that bind various topological forms of DNA (20), it is a likely candidate for the endogenous DNA-aggregating agents that participate in the network formation. To test this possibility, isolated nuclear scaffold was incubated with substrate DNA in the presence of nuclear extract that contained topoisomerase activity. A significant proportion of the substrate was incorporated into the highly catenated networks as has been demonstrated in nuclei. The requirement of this reaction and its susceptibility to inhibitors were similar to those for the nuclear system (Fig. 4B and Fig. 5, right panels), indicating that the network formation in the nuclear scaffold is also catalyzed by topoisomerase II. Although this enzyme has been immunologically located in the scaffold (31, 32), we failed to detect its activity in our preparations, and the addition of nuclear extracts was essential for the catenation to occur. Electron micrographs of the networks from the scaffold were indistinguishable from the ones formed in nuclei.

A titration experiment similar to the one shown in Fig. 8A was performed on the scaffold system (Fig. 9A). In the absence of the scaffold, no networks were formed (lane 1). For 1 μg of the substrate, maximal network formation was obtained when 6.8 x 10^6 of the scaffold was present (lane 3). As observed for nuclei, the unincorporated DNA residing in the supernatant disappeared when higher amounts of the scaffold were added (lanes 11–14). In contrast to the nuclear system, however, formation of the networks was completely suppressed at scaffold amounts greater than 1.4 x 10^10 (lanes 6 and 7), although the activity of topoisomerase II remained intact (not shown).
Additional extract did not restore the network synthesis. All the unincorporated DNA lost from the supernatant was bound to the scaffold as relaxed monomeric forms. The different titrational behavior of these systems does not seem to be trivial and will be discussed below (see “Discussion”). As shown in Fig. 9B, the network production was also susceptible to the competitive binding of linear DNA to the scaffold. These results are consistent with the presumption that the nuclear scaffold provides sites for DNA aggregation and thus promotes the catenation reaction. Interestingly, no catenated networks were formed, at any substrate/scaffold ratios, when nuclei were extracted with LIS and subsequently treated with EcoRI (this preparation was referred to as NS(Eco) in our previous report). We have shown in that study (20) that the NS(Eco) expresses only DNA-binding sites selective for supercoiled DNA. In NS(Eco), the other class of DNA-binding sites which lacks this specificity is masked by nuclear DNA fragments, whereas these sites are unmasked in scaffold preparations, like the one used in this study, that were obtained by extensive nuclease treatment. An important conclusion, therefore, is that the supercoil-specific DNA-binding sites on the scaffold are not involved in the network formation but the second class of sites are responsible. The latter sites are probably available for exogenous DNA in intact nuclei as well. Supercoiled DNA substrates are likely to be relaxed rapidly by topoisomerases and lose affinity for the supercoil-specific sites.

Since retrieval of the network from nuclei absolutely requires protease treatment, as well as fragmentation of nuclear DNA with restriction endonucleases, the networks formed in nuclei are likely to be tightly associated or “held” by a proteinaceous structure, probably the nuclear scaffold. The association between them could be a topological one since morphologically the scaffold itself has been claimed to be a three-dimensional network (33, 34). To examine this interesting possibility, we have searched for agents, other than proteases, that dissociate the scaffold into constituent proteins. A number of compounds including chelating agents, chaotropic salts, detergents, reducing agents, and their combinations were tested but thus far only the combination of SDS and reducing agents disrupted the structure completely (Fig. 10A, panel c). Although a striking swelling was induced by SDS alone (Fig. 10A, panel b), no change in morphology was observed with reducing agents (not shown). The internal network structure appears to be preserved in the scaffold treated with SDS. Essentially no proteins were released under these conditions, including the DNA-binding components identified previously (20). After the catenation reaction, the nuclear scaffolds containing the catenated networks were treated with these agents and subjected to a sedimentation analysis designed to separate these structures (Fig. 10B). When the scaffold was digested with proteinase K, the released networks sedimented rapidly and formed a clear band between the two CsCl layers (Fig. 10B, panel 5). Without any treatment, however, no DNA band was discernible, indicating the association of the network with a low density structure, namely the scaffold (Fig. 10B, panel 1). Note the presence of light-scattering material on top of the gradient, which was later confirmed to be the scaffold under a microscope. Incubation with a large excess of linear DNA, which is supposed to displace the network from DNA-binding sites, did not release the network (Fig. 10B, panel 2). Furthermore, despite the remarkable effect on morphology, the network was not released by SDS either (Fig. 10B, panel 3), whereas it was released in the presence of both SDS and 2-mercaptoethanol (Fig. 10B, panel 4). Thus, successful release of the network parallels complete disruption of the scaffold, supporting the idea that the association between these structures has a topological nature.

**DISCUSSION**

The pairs of reactions that are catalyzed by topoisomerases (such as supercoiling/relaxation, catenation/decatenation, and knotting/unknotting) are reciprocal reactions. What determines the type and direction of these reactions may not be topoisomerase itself in the nuclear environment. Although the reaction mechanisms of topoisomerases have been studied extensively with purified enzymes, the factors which regulate topoisomerases are still poorly understood. To define the topoisomerization reaction catalyzed by topoisomerases in situ, therefore, it is essential to investigate more complex experimental systems by using simple circular DNA substrates that can be retrieved and examined for changes in topology. Subsequent experiments with reconstituted components would enable identification of factors regulating topoisomerization. Despite its potential usefulness, however, this type of approach has not been carried out to any extent. The topological changes of exogenously introduced DNA rings may reflect the results of physiological processes modulating the topology of chromosomal DNA. The eukaryotic genome is believed to be organized into looped DNA domains demarcated by attachments to the nuclear skeletal structure called the nuclear matrix or nuclear scaffold (33, 35, 36). The DNA loops are thus topologically equivalent to small circular DNA. This pattern of organization may be constitutive throughout
Catenation in Nuclei and the Nuclear Scaffold

the cell cycle since DNA regions associated with the nuclear scaffold remain attached to the metaphase chromosomal scaffold (37). It is not known whether the DNA attachment or the scaffolding structure itself in interphase nuclei ever undergoes transient disruption during chromosomal condensation. It is likely, however, that these processes, including chromosomal segregation and decondensation, are prone to interference caused by entangling between the DNA loops and possibly between DNA and the scaffold. Therefore, they should involve catenation/decatenation-type transitions in DNA topology which might proceed on the nuclear scaffold. This idea is supported by the absolute requirement of topoisomerase II in the above processes (38-40).

We have shown in this study that circular DNA substrates, when incubated with isolated nuclei, are efficiently converted into huge catenated networks formed inside nuclei by the action of endogenous topoisomerase II. Production of catenated networks in vitro has been well documented by others using purified topoisomerases and DNA-aggregating agents (10-18). These studies suggested the existence of nuclear components that aggregate DNA. The presence of DNA-binding sites on isolated nuclear scaffold (20) prompted us to test whether these sites contribute to catenation by aggregating DNA. The titration experiments shown in Figs. 8 and 9 showed that in both nuclear and scaffold systems (i) stoichiometric amounts of the substrate DNA are bound to nuclei (scaffolds) and a significant portion of the bound DNA is incorporated into catenated networks, (ii) bound circular substrate is displaced by linear DNA and network formation is abolished when the linear competitor exceeds the substrate, and (iii) in the presence of excess substrate, the level of network production depends only on the numbers of nuclei (scaffolds) added to the reaction, and not on the amount of the topoisomerase. These results are consistent with the idea that the DNA-binding sites on the nuclear scaffold mediate catenation of bound circular DNA molecules by bringing them into close proximity to each other. The binding sites do not seem to turn over and the networks once formed need not dissociate from the sites. To assess the stoichiometry more quantitatively, the data shown in Figs. 8A and 9A were subjected to densitometry and plotted against the ratio of substrate (μg) to number of nuclei or scaffolds (results not shown). By extrapolating the free substrate in the incubation supernatant, the amounts of substrate required for saturating the DNA-binding sites in 10^6 nuclei (or scaffolds) were estimated to be 0.67 and 1.0 μg, respectively. Since these values should be roughly proportionate to the number of DNA-binding sites, the result suggests that some of the sites exposed in the scaffold are masked by endogenous DNA in nuclei. For maximal network formation, about 150% of the substrate level required for saturation has to be present in both systems. This implies the involvement of unbound substrates in catenation. In the nuclear scaffold, a critical level of the substrate was required for catenation. No networks were formed when the substrate level was less than 0.68 μg/10^6 scaffold. The result can be explained if it is assumed that the DNA-binding sites on the scaffold are fixed in place and that the topographical density of occupied sites becomes too low for catenation to occur when less than about 70% of the sites are occupied. This situation is somewhat different in nuclei since no critical substrate level was found. A significant amount of the network was formed even at 50% saturation. One may speculate that the DNA-binding sites in nuclei are mobile, and occupied sites are brought in proximity by some mechanism to facilitate the catenation. On the whole, the similarity in behavior of nuclei and the scaffold in network synthesis suggests that the scaffold is the major site for catenation in intact nuclei also. At present, however, we are unable to eliminate the possibility that alternative sites for DNA aggregation exist in other subnuclear structures such as chromatin. Some proteins associated with chromatin (histone H1 and HMG17) were shown to promote catenation (16, 17), although catenation of SV40 DNA in the presence of SV40 minichromosomes is highly dependent on spermidine (13).

We have demonstrated that the topoisomerase responsible for the production of catenated networks is topoisomerase II in both nuclei and the nuclear scaffold. The role of topoisomerase I seems to be negligible since catenation was relatively insensitive to antibody to topoisomerase I (Fig. 5). In catenation using polyvinyl alcohol as an aggregating agent and topoisomerases isolated from HeLa cell nuclei (15), it has also been shown that catenation is far less efficient with type I than with type II enzyme. However, the rate of catenation by topoisomerase II is greatly accelerated by the presence of a low level of topoisomerase I. This effect has been attributed to the fact that (i) topoisomerase II preferentially catenates relaxed DNA more than supercoiled DNA by a factor of 100, and (ii) topoisomerase I relaxes supercoiled DNA much faster than topoisomerase II. Therefore, one would expect that network formation in the nuclear system is sensitive to the inhibition of topoisomerase I. The discrepancy observed here may be due to (i) a higher relaxing ability of topoisomerase II in the nuclear environment, (ii) difference in the DNA-aggregating agents, and (iii) a high level of topoisomerase II activity in the nuclear preparations used in this study (27).

The catenated networks formed in the nuclei are likely to be a three-dimensional structure with spherical shape, so large in size that they could be easily visualized under light microscope. Also, they are produced in a large amount, around 200 ng/10^6 nuclei, which is equivalent to about 4.2 × 10^5 molecules of catenated rings for a single nucleus. A similar level of network production was observed in the scaffold system. Because of their heterogeneous sizes, it is difficult to estimate the number of networks formed inside a nucleus. If it is assumed that an average network is composed of 1.2 × 10^5 rings (this corresponds to the median of the size distribution determined by the sedimentation analysis), one nucleus would harbor about 35 networks. It is possible that the catenation reaction initiates around multiple foci in the nucleus and proceeds to form multiple networks. These estimations suggest that the networks occupy a considerable space in nuclei if they are not compacted. A reasonable assumption would be that the DNA rings of the network exist in association with the nuclear scaffold and their distribution in nuclei coincides with that of the scaffold, which is consistent with the three-dimensional organization of the network retrieved from nuclei.

It has been shown recently that the kinetoplast DNA network is packaged, in the presence of spermidine, into an ordered structure (41). The compacted network was suggested to be arranged as a double tier structure with the bent region of each minicircle directed toward the exterior of the network. The ordered packaging occurred with noncatenated minicircles as well but not with unrelated plasmid DNA, and required a circular conformation (either supercoiled or relaxed). It was proposed that the regular packaging of the network is directed by the bent region of the minicircles. In this case, therefore, the DNA-aggregating agent appears to have only a marginal role in the determination of the network's regularity. Whether spermidine is the compaction agent also in vivo is not known. In contrast, if the network formed inside nuclei represents some regularity in organization, it most likely reflects the
regularity in the arrangement of aggregation sites for DNA, including ones on the nuclear scaffold. By analyzing the structure of the network, it should be possible to deduce the topological nature of the scaffolding structure. Using catenated networks composed of cleavable and uncleavable rings, we have suggested that the major motif of the network formed in nuclei is a linear array of catenated rings (type A catenanes, Fig. 7). This type of experiment has been performed by others using circular DNAs of heterogeneous sizes and sequences at a fixed mixing ratio (10, 14, 16). Our result suggests a one-dimensional arrangement of DNA-binding sites on a three-dimensional framework. In order to obtain further information from this experiment, more quantitative measurements of released monomer rings and analysis of the oligomeric catenanes are necessary. The type B catenanes (key-ring structure) have been frequently observed under the electron microscope in a variety of systems using spermidine at a low salt concentration (10, 11, 13, 14). This can be explained by the fact that under these conditions topoisomerase II works exclusively in a processive manner (42). The enzyme stays on the central ring to which it catenates multiple rings. On the other hand, the conditions used in the present study appear to favor the distributive mechanism, although the difference in the resulting networks may not be entirely due to the difference in the reaction mechanism. The experimental strategies used in this study will be useful to probe the topological interconversion of nuclear DNA by topoisomerases in situ and to analyze the topographical distribution of the DNA-binding sites on the nuclear scaffold.

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REFERENCES