In Vivo Catenation and Decatenation of DNA†

JANET E. MERTZ* AND TIMOTHY J. MILLER‡

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

Received 8 June 1982/Accepted 1 October 1982

We have noted previously that when circular, but not linear, DNA or chromatin was injected into *Xenopus laevis* oocytes, much of it went through an intermediate form in which it did not readily enter an agarose gel; after a few hours, it reappeared as monomer DNA that had acquired its full complement of nucleosomes (T. J. Miller and J. E. Mertz, *Mol. Cell. Biol.* 2:1581-1593, 1982). We determined, using electron microscopy and a variety of biochemical techniques, the structure of this aggregated material. Most of it was oligomeric and multimeric catenanes of the injected sample. In addition, injection of DNA that had been catenated in vitro with DNA gyrase resulted in the conversion of most of it back to monomer circles. These findings demonstrate directly that both catenation and decatenation of DNA occur in vivo under physiological conditions. Whether these reactions play a crucial role in nucleosome formation, as well as in DNA replication and recombination, remains to be determined.

In procaryotic cells, DNA supercoiling has been clearly shown to play roles in DNA replication, transcription, and genetic recombination (see reference 6 for review). In eucaryotes, DNA possesses a negative superhelical density because it interacts with histone proteins to form nucleosomes (7). Linear DNA, being incapable of storing energy in the form of supertwists, fails to reconstitute into proper chromatin in vivo (13). These findings indicate that DNA supercoiling may also play an important role in properly arranging chromosomal proteins on DNA.

DNA topoisomerases are a class of enzymes that regulate the superhelical density of DNA (see references 3, 4, and 6 for recent reviews). In addition to removing and, in some cases, introducing supercoils into DNA, these enzymes form and resolve catenated (interlocked) and knotted DNA rings. Decatenation of DNA may be particularly important biologically to enable the separation of chromosomes after replication and genetic recombination.

The ability to both catenate and decatenate DNA in vitro has been shown to be associated with topoisomerases from a variety of sources (2, 8, 10, 11). The proposed mechanism by which topoisomerases catenate DNA rings predicts that the reaction should be reversible. However, whereas the catenation reaction requires low salt concentration and a polyvalent

cation such as spermidine, the decatenation reaction occurs only at higher salt concentration or in the absence of polyvalent cations.

While performing experiments concerned with the reconstitution into chromatin of circular simian virus 40 (SV40) DNA injected into the nuclei of *Xenopus laevis* oocytes, we noted that much of the DNA went through an intermediate form in which it did not readily enter a 1.4% agarose gel even though it had been deproteinized by treatment with both proteinase K and phenol (13, 14); after a few hours, it reappeared as monomer supercoiled DNA (see Fig. 5 of reference 14 for an example). Electrophoresis indicated that some of this aggregated material migrated in the positions of dimeric and trimeric length SV40 DNA molecules (see Fig. 4 of reference 13 for examples). This same phenomenon of aggregation followed by disaggregation was also observed when circular pBR322, ColE1, or *Drosophila melanogaster* H2A histone DNA was injected (T. J. Miller and J. E. Mertz, unpublished data). On the other hand, unit-length linear SV40 DNA failed to form this aggregated material when treated in an analogous manner (see Fig. 1 and 4 of reference 13 for examples). Therefore, this aggregated material was unlikely to be either recombinants or a trivial artifact of the experimental protocol.

In the experiments reported here, we show that: (i) most of this aggregated material is oligomeric and multimeric catenanes of the injected sample; and (ii) catenated DNA injected into *Xenopus* oocytes resolves back to monomer circles.

† This paper is dedicated to Norman Davidson, a man whose numerous contributions to chemistry and molecular biology made experiments of the type described here feasible, on the occasion of his 65th birthday.

‡ Present address: Norden Laboratory, Lincoln, NE 68521.

FIG. 1. Sucrose gradient analysis of SV40 DNA after incubation in *Xenopus* oocytes for 30 min. Forty oocytes were each injected intranuclearly with 5 ng of monomeric ^3H -labeled SV40 DNA at a concentration of 250 $\mu\text{g}/\text{ml}$ in injection medium. After incubation in HEPES-modified Barth solution for approximately 30 min at 19°C , the nuclei were manually dissected out of the oocytes as described previously (15) and homogenized in 2 ml of proteinase K buffer. The DNA was purified as described in the text, with greater than 95% of the input radioactivity being recovered. It was then sedimented (SW41 rotor; 39,500 rpm for 5 h at 4°C) in a 5 to 20% (wt/vol) gradient of sucrose containing 10 mM Tris-hydrochloride (pH 7.6)–1 mM EDTA–0.10 M NaCl layered on top of a 1.0-ml cushion of 60% sucrose. Fractions (0.40 ml) were collected from the bottom of the gradient. The amount of ^3H -labeled SV40 DNA present in one-tenth of each fraction was determined by scintillation spectroscopy. The approximate positions of monomer-length supercoiled (I) and nicked circular (II) SV40 DNAs were determined from uninjected ^3H -labeled SV40 DNA sedimented concurrently in a parallel gradient.

MATERIALS AND METHODS

DNA and enzymes. Supercoiled (form I) ^3H -labeled SV40 DNA (>99% monomers; specific activity, ca. 1.5×10^5 cpm/ μg) was isolated from virus-infected monkey cells and purified as described previously (14). Although initially containing less than 5% nicked circular molecules (form II), the preparation of DNA used in the experiments described here contained approximately 60% nicked molecules because of radiation damage and nonspecific endonucleolytic cleavages that occurred during prolonged storage at 4°C .

Catenated ^3H -labeled ColE1 DNA (specific activity, ca. 3×10^4 cpm/ μg) was the generous gift of N. Cozzarelli. It was prepared as described previously (9) by incubation at a concentration of 2 pmol/ml with *Escherichia coli* DNA gyrase in the presence of 5 mM spermidine. The oligomeric molecules were separated from the monomeric ones by sedimentation in a sucrose gradient.

Restriction endonucleases were obtained from commercial sources. They were used as recommended by the manufacturers.

Handling and injection of oocytes. Full-grown (early

TABLE 1. Summary of oligomeric DNAs produced in *Xenopus* oocytes^a

Structure	No. observed
Simple catenanes	
Dimer	32
Trimer	17
Tetramer	4
Pentamer	1
Hexamer	1
Other	
Noncatenated dimer	9
Monomer catenated with noncatenated dimer	1

^a The DNA present in fractions 7 through 13 of the sucrose gradient depicted in Fig. 1 was pooled and banded to equilibrium (type 50 rotor; 35,000 rpm for 47 h at 15°C) in a density gradient of CsCl (average density, 1.700) containing 10 mM Tris-hydrochloride (pH 7.6)–1 mM EDTA to reduce the level of contamination with endogenous oocyte nucleic acids. Afterwards, the material in the fractions containing most of the radioactivity was pooled and mounted for electron microscopy in 40% formamide essentially as described by Davis et al. (5). Randomly selected areas of platinum-shadowed grids were examined and photographed. The lengths of the molecules were determined; in all cases, each DNA ring measured within 10% of the length or twice the length of monomer SV40 DNA molecules present in the same photograph. Excluded from this table are the numerous monomeric and approximately trimeric length DNA molecules that were also observed. Although some of the latter may have been noncatenated trimers of SV40 DNA, most of them were probably *Xenopus* mitochondrial DNA that was present as a contaminant in the sample.

stage 6) oocytes were isolated from adult female *Xenopus laevis*, incubated at 19°C in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-modified Barth solution and injected intranuclearly with the indicated samples as described previously (15, 16).

Purification of radiolabeled DNAs from injected oocytes. After incubation at 19°C in HEPES-modified Barth solution for the indicated times, the reactions were terminated by homogenization of the oocytes or their nuclei directly in proteinase K buffer (50 mM Tris-hydrochloride [pH 7.4], 10 mM EDTA, 1% sodium dodecyl sulfate, 750 μg of proteinase K per ml, 50 μl per oocyte) and incubation at 37°C for 2 h. The homogenized mixtures were extracted twice with 0.1 M Tris-hydrochloride (pH 9.0)–saturated phenol:chloroform:isoamyl alcohol (25:25:1). The nucleic acids present in the samples were then precipitated with ethanol and suspended in 10 mM Tris-hydrochloride (pH 7.6)–1 mM EDTA–10 mM NaCl before sedimentation or electrophoresis.

RESULTS

***Xenopus* oocytes catenate circular DNA.** To characterize the aggregated material formed in oocytes, monomer circular ^3H -labeled SV40

FIG. 2. Electron micrographs of oocyte-produced catenanes of SV40 DNA. The experimental protocol was as described in the footnote to Table 1. All of the micrographs in this composite are at the same magnification. a, Monomer SV40 DNA molecules; b, a catenated dimer and a supercoiled monomer; c and d, dimers consisting of one supercoiled and one relaxed DNA molecule; e through h, trimer, tetramer, pentamer, and hexamer, respectively.

DNA was injected intranuclearly and reisolated 30 min later. The DNA was treated with proteinase K and sodium dodecyl sulfate and extracted with phenol. Afterwards, it was sedimented in a sucrose gradient. As shown in Fig. 1, 13% of the injected radiolabeled DNA was distributed among fractions 1 through 12 of the gradient. On the other hand, only 1% of the radioactivity was found in the corresponding fractions of a gradient run in parallel with an uninjected sample of the same DNA preparation (data not shown). Therefore, incubation in *Xenopus* oocytes results in some of the SV40 DNA undergoing changes that cause it to sediment at a variety of rates greater than that characteristic of either supercoiled or relaxed circular monomer SV40 DNA.

The radiolabeled SV40 DNA in fractions 1 through 6 and 7 through 13 of the sucrose gradient were pooled separately, concentrated by precipitation with ethanol, and purified further by banding to equilibrium in CsCl density

gradients (see footnote to Table 1 for protocol). All of the radioactivity banded at the density characteristic of pure SV40 DNA (data not shown). We therefore concluded that this fast-sedimenting material contains little or no *Xenopus* RNA or amplified ribosomal DNA, since the latter two both have significantly higher densities than SV40 DNA does.

At this stage, the only contaminants remaining in the viral DNA preparations should have been *Xenopus* chromosomal and mitochondrial DNAs. Since we injected 5 ng of SV40 DNA into a tetraploid cell that contained only 12 pg of chromosomal DNA, the first of these contaminants could be largely ignored. By reisolating the injected DNA from nuclei rather than from whole oocytes we succeeded in eliminating most, but not quite all, of the mitochondrial DNA. Therefore, when the purified DNA from fractions 7 through 13 of the sucrose gradient was examined by electron microscopy, many molecules approximately three times the length

FIG. 3. Electron micrograph of approximately one-third of a DNA network. The complex DNA structure shown here was obtained from fractions 1 through 6 of the sucrose gradient depicted in Fig. 1 and was purified and prepared for electron microscopy as described in the footnote to Table 1. The large spherical particles contaminating the sample were picked up accidentally during purification of the DNA and may represent globules of mineral oil.

of monomer SV40 DNA were seen. Besides these and monomer SV40 DNA, we also observed a variety of other structures (see Fig. 2 for examples and Table 1 for summary). Three things of note about these latter DNA molecules were: (i) they sometimes consisted of both supercoiled and relaxed DNA rings (Fig. 2c and d); (ii) most of the DNA rings within one structure were linked to a central "key" molecule; and (iii) most clearly appeared to cross another ring twice rather than once. These findings are all indicative of catenanes.

The DNA from fractions 1 through 6 of the sucrose gradient was also examined by electron microscopy. In this latter case, huge DNA networks were observed (Fig. 3). They contained centrally connecting fibers and were similar in appearance to the interlocked DNA networks seen when circular DNAs are catenated in vitro

with preparations of purified topoisomerases (see reference 10 for example).

To exclude further the possibility that these molecules were intermediates in DNA replication or recombination rather than catenanes, each of these DNA preparations was incubated with *Bam*HI, a restriction endonuclease that cleaves SV40 DNA at one site. The resultant molecules were then electrophoresed in a 1.4% agarose gel, along with appropriate size markers. Fluorographic analysis of the gel indicated that essentially all of the radioactivity migrated in the position of unit-length linear SV40 DNA (data not shown). We therefore conclude that most of the aggregated radiolabeled material produced when the ^3H -labeled SV40 DNA was injected into oocytes consisted of catenated oligomers and multimers of circular SV40 DNA molecules.

***Xenopus* oocytes decatenate interlocked DNA rings.** The catenanes that form when circular DNA is injected into oocytes disappear with prolonged incubation. However, since 20 to 40% of the injected molecules are degraded because of failure to hit the nucleus (13, 17), we could not directly exclude the possibility that the catenanes had also been degraded rather than resolved back to monomer circles.

To solve this problem, ^3H -labeled ColE1 DNA was catenated in vitro with purified DNA gyrase. The resulting oligomeric catenanes were purified and then injected into the nuclei of *Xenopus* oocytes; 21 h later, the reaction was terminated, and the radiolabeled DNA was purified and analyzed by electrophoresis in a 0.8% agarose gel (Fig. 4). The results indicate that, whereas <5% of the input DNA was monomeric in length, >95% of the product was. Furthermore, since 60 to 70% of the radioactivity was recovered, a majority of the catenated material was converted to supercoiled monomers. (The slight retardation in the mobilities of the supercoiled and linear monomeric DNAs present in lane 2 of the gel shown in Fig. 4 was probably the result of this sample containing a large amount of nonradiolabeled *Xenopus* oocyte nucleic acid.) We therefore conclude that *Xenopus* oocytes can decatenate, as well as catenate, circular DNA molecules.

DISCUSSION

The experiments reported here demonstrate directly that both catenation and decatenation of DNA occur in vivo under physiological conditions. Therefore, the finding by others that these reactions can be performed in vitro is unlikely to be a biochemical artifact. However, the reaction conditions used to generate catenanes in vitro were always different from those used to resolve them back to monomer rings. Recently, Krasnow and Cozzarelli (9) have shown that the role in vitro of polyvalent cations such as spermidine may be solely to enable aggregation of the substrate DNA, thereby facilitating the juxtaposition of DNA segments involved in the reaction. Since DNA normally exists in a compacted state in association with histone proteins, electrostatic repulsion between negatively charged DNA molecules would not be expected to hinder the catenation and decatenation reactions in vivo. In support of this hypothesis is our observation that SV40 minichromosomes (i.e., SV40 DNA isolated from virus-infected monkey cells in association with histone proteins) also form catenanes when injected into *Xenopus* oocytes (see Fig. 8 of reference 14).

One peculiar observation is the finding that this aggregated material is also produced when circular, but not linear, DNA is injected into the

FIG. 4. Decatenation of DNA in *Xenopus* oocytes. A 20-nl portion of catenated ^3H -labeled ColE1 DNA, prepared as described in the text, was injected intranuclearly into each of 40 oocytes at a DNA concentration of approximately 50 $\mu\text{g}/\text{ml}$. After incubation of 19°C for 21 h, the oocytes were homogenized in proteinase K buffer. The DNA in the sample was purified as described in the text and electrophoresed at 33 V for 16 h in a 0.8% agarose gel submerged in 4 $\times$ TA buffer (0.16 M Tris-acetate [pH 8.3], 0.08 M sodium acetate, 8 mM EDTA). Afterward, the gel was impregnated with En 3 Hance (New England Nuclear Corp.) and fluorographed at -70°C with Kodak XAR-5 film. The amount of DNA in each band was determined by densitometry as described previously (13). I and II indicate the positions of supercoiled and relaxed circular DNA molecules, respectively. Lane 1, approximately 0.8 μl of the catenated ColE1 DNA sample used in the injections; lane 2, the catenated ColE1 DNA after incubation in oocytes; lane 3, uninjected markers of ColE1 DNA.

cytoplasm of oocytes (see Fig. 1 of reference 13). Mattoccia et al. (12) have reported that complex DNA is only formed when nuclear and cytoplasmic extracts of *Xenopus* oocytes are included together in in vitro reactions. Therefore, a topoisomerase activity or a component needed for its functioning may exist in the cytoplasm of *Xenopus* oocytes.

Examination by electron microscopy of the fast-sedimenting material indicated that some of it was noncatenated dimers (Table 1). The origin of this material is unknown. Likely explanations are: (i) these molecules preexisted in the DNA preparation used in the experiment; and (ii) they were produced by recombination during the incubation in the oocytes. Consistent with

the latter hypothesis is the finding that these structures are also seen when SV40 DNA is incubated in a cell-free extract of *Xenopus* oocytes (1).

Lastly, the most interesting question raised by these studies is whether catenation and decatenation of DNA play a crucial role in nucleosome formation as well as in DNA replication and recombination. Findings consistent with an affirmative answer to this question include: (i) topoisomerases are needed to relieve the supertwists that are generated when DNA wraps around cores of histone proteins (7); (ii) a correlation exists between the appearance in oocytes of injected DNA molecules that are fully reconstituted into chromatin and the disappearance of DNA catenanes (14); and (iii) linear DNA neither catenates nor forms chromatin containing regularly spaced nucleosomes *in vivo* (13).

However, based upon our present knowledge about topoisomerases, an equally reasonable hypothesis is that these enzymes play a purely passive role, with their primary function being to resolve catenanes that form during DNA replication, recombination, and packaging. Looked at from this latter point of view, the observed catenation and decatenation of circular DNA injected into *Xenopus* oocytes may be a trivial consequence of the experimental conditions used. The high DNA concentration at which the sample was injected initially favored the catenation reaction; however, as the DNA molecules gradually diffused with time throughout the nucleus, decatenation became predominant. Further experiments will be needed to distinguish between these two hypotheses.

ACKNOWLEDGMENTS

We are indebted to Nicolas Cozzarelli for discussions and for supplying us with *in vitro* catenated ColE1 DNA. We also thank Catharine Conley for technical assistance and Richard Burgess, Bill Sugden, and Howard Temin for helpful comments on the manuscript.

This work was supported by Public Health Service research grants CA-07175 and CA-22443 from the National Cancer Institute. T.J.M. was supported by fellowship PF-1678 from the American Cancer Society.

LITERATURE CITED

1. Attardi, D. G., G. Martini, E. Mattoccia, and G. P. Tocchini-Valentini. 1976. Effect of *Xenopus laevis* oocyte extract on supercoiled simian virus 40 DNA: formation of complex DNA. *Proc. Natl. Acad. Sci. U.S.A.* 73:554-558.
2. Baldi, M. I., P. Benedetti, E. Mattoccia, and G. P. Tocchini-Valentini. 1980. *In vitro* catenation and decatenation of DNA and a novel eucaryotic ATP-dependent topoisomerase. *Cell* 20:461-467.
3. Cozzarelli, N. R. 1980. DNA gyrase and the supercoiling of DNA. *Science* 207:953-960.
4. Cozzarelli, N. R. 1980. DNA topoisomerases. *Cell* 22:327-328.
5. Davis, R. W., M. Simon, and N. Davidson. 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. *Methods Enzymol.* 21:413-428.
6. Gellert, M. 1981. DNA topoisomerases. *Annu. Rev. Biochem.* 50:879-910.
7. Germond, J. E., B. Hirt, P. Oudet, M. Gross-Bellard, P. Chambon. 1975. Folding of the DNA helix in chromatin-like structures from simian virus 40. *Proc. Natl. Acad. Sci. U.S.A.* 72:1843-1847.
8. Hsieh, T.-S., and D. Brutlag. 1980. ATP-dependent DNA topoisomerase from *D. melanogaster* reversibly catenates duplex DNA rings. *Cell* 21:115-125.
9. Krasnow, M. A., and N. R. Cozzarelli. 1982. Catenation of DNA rings by topoisomerases: mechanism of control by spermidine. *J. Biol. Chem.* 257:2687-2693.
10. Kreuzer, K. N., and N. R. Cozzarelli. 1980. Formation and resolution of DNA catenanes by DNA gyrase. *Cell* 20:245-254.
11. Liu, L. F., C.-C. Liu, and B. M. Alberts. 1980. Type II DNA topoisomerases: enzymes that can unknot a topologically knotted DNA molecule via a reversible double-strand break. *Cell* 19:697-707.
12. Mattoccia, E., D. Gandini Attardi, and G. P. Tocchini-Valentini. 1976. DNA-relaxing activity and endonuclease activity in *Xenopus laevis* oocytes. *Proc. Natl. Acad. Sci. U.S.A.* 73:4551-4554.
13. Mertz, J. E. 1982. Linear DNA does not form chromatin containing regularly spaced nucleosomes. *Mol. Cell. Biol.* 2:1608-1618.
14. Miller, T. J., and J. E. Mertz. 1982. Template structural requirements for transcription *in vivo* by RNA polymerase II. *Mol. Cell. Biol.* 2:1595-1607.
15. Miller, T. J., D. L. Stephens, and J. E. Mertz. 1982. Kinetics of accumulation and processing of simian virus 40 RNA in *Xenopus laevis* oocytes injected with simian virus 40 DNA. *Mol. Cell. Biol.* 2:1581-1594.
16. Stephens, D. L., T. J. Miller, L. Silver, D. Zipser, and J. E. Mertz. 1981. Easy-to-use equipment for the accurate microinjection of nanoliter volumes into the nuclei of amphibian oocytes. *Anal. Biochem.* 114:299-309.
17. Wyllie, A. H., R. A. Laskey, J. Finch, and J. B. Gurdon. 1978. Selective DNA conservation and chromatin assembly after injection of SV40 DNA into *Xenopus* oocytes. *Dev. Biol.* 64:178-188.