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DNA supercoiling by *Xenopus laevis* oocyte extracts: Requirement for a nuclear factor

(DNA-relaxing enzyme/histones/germinal vesicles factor)

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ABSTRACT **A purified system is described for the introduction of negative supercoils into simian virus 40 DNA. The system consists of histones H2A, H2B, H3, and H4, DNA-relaxing enzyme, and a purified factor from *Xenopus laevis* stage 6 oocyte nuclei. The nuclei are prepared *en masse* by the technique of F. Scalenghe, M. Buscaglia, C. Steinheil, and M. Crippa [(1978) *Chromosoma* 66, 299–308]. The supercoiled simian virus 40 DNA prepared by this method is indistinguishable from simian virus 40 supercoiled DNA prepared from infected monkey cells.**

DNA gyrase, an enzyme that introduces negative superhelical turns into double-stranded closed circular DNA, has recently been purified from *Escherichia coli* and *Micrococcus luteus* (1–3). It consists of two active components and requires ATP. Experiments with the drugs coumeromycin and novobiocin indicate that gyrase is involved in DNA replication and supercoiling in *E. coli* (4).

Xenopus laevis oocytes represent a promising eukaryotic system in which to study the mechanism of insertion of superhelical turns because relaxed circular simian virus 40 (SV40) DNA is promptly converted to the supercoiled form once injected into the oocyte nucleus (germinal vesicle) (5). In order to analyze the components of the reaction, we have fractionated nuclear extracts derived from stage 6 oocytes. In this paper we report that in addition to histones H2A, H2B, H3, and H4 and the DNA-relaxing enzyme (6), a nuclear factor is also required for the formation of supercoiled DNA.

MATERIALS AND METHODS

Materials. *N*-Ethylmaleimide was purchased from Sigma. Agarose powder was purchased from Bio-Rad.

Preparation of SV40 DNA. Confluent African green monkey CV-1 cells were infected at a multiplicity of 40 plaque-forming units per cell with plaque-purified SV40 virus and the DNA was extracted by the Hirt procedure (7) about 40 hr after infection. The viral DNA was purified from the Hirt supernatant by banding to equilibrium in a CsCl/ethidium bromide gradient. The fractions containing supercoiled DNA were pooled and ethidum bromide was removed with isopropanol. The DNA was precipitated with ethanol and redissolved in 10 mM Tris·HCl, pH 7.5/1 mM EDTA, and the supercoiled form was finally purified by sedimentation on a 5–20% neutral sucrose gradient. The fractions corresponding to the peak of material sedimenting at 21 S were pooled, precipitated with ethanol, dissolved in 10 mM Tris·HCl, pH 7.5/1 mM EDTA, and stored at −20°.

Isolation of Oocytes and Nuclei. Isolated stage 6 oocytes free of follicle cells were obtained from ovaries of *X. laevis* females (South African Snake Farm) by collagenase digestion (8). Nuclei from stage 6 oocytes were prepared according to Scalenghe *et al.* (9).

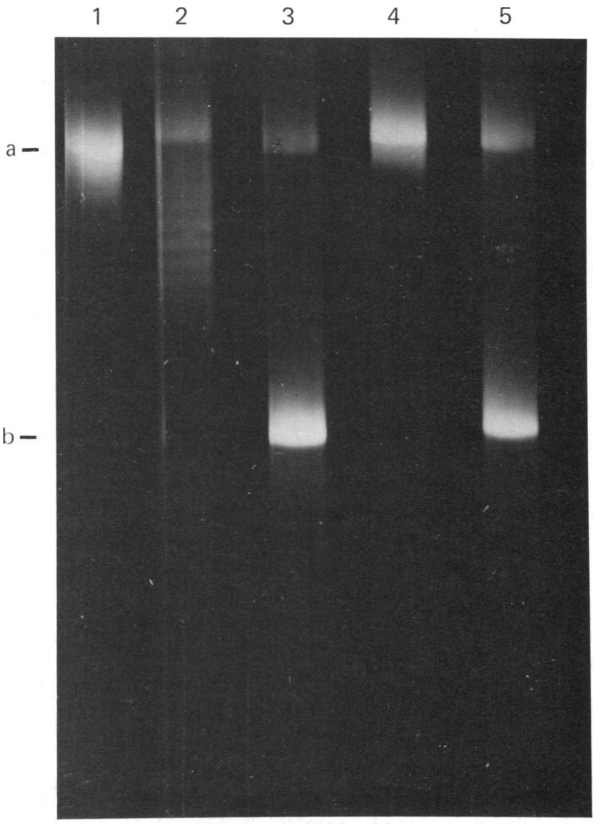

FIG. 1. Effect of added histones on the acquisition of superhelical turns in relaxed, covalently closed SV40 DNA. Agarose gel electrophoresis. a, Position of migration of nicked circular or fully relaxed DNA. b, Position of migration of supercoiled DNA. (Lane 1) Reaction mixture contained, in 15 μl: 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.6 mM dithiothreitol, 1 μg of SV40 supercoiled DNA, and 10 μl of purified *X. laevis* DNA-relaxing enzyme. Incubation was for 20 min at 30°. (Lanes 2–5) Reaction mixtures contained, in 60 μl: 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.6 mM dithiothreitol, 15 μl of a reaction mixture identical to the one described for lane 1, which had been preincubated for 20 min at 30°, and (lane 2) fraction II (40 μl); (lane 3) fraction II (40 μl) plus 2 μg of histones; (lane 4) fraction III (40 μl); (lane 5) fraction III (40 μl) plus 2 μg of histones. Fractions II or III and the histones were preincubated for 5 min at 30°. Incubation was for 60 min at 30°. Some residual DNA electrophoreses in the position of relaxed or nicked DNA. Electrophoresis in the presence of ethidium bromide (13) has demonstrated that the material in this position is nicked.

Preparation of DNA-Relaxing Enzyme, Histones, and Nuclear Extract. DNA-relaxing enzyme was prepared essen-

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Abbreviations: SV40, simian virus 40; TEMG buffer, 50 mM Tris·HCl, pH 7.5/1 mM EDTA/1.4 mM mercaptoethanol/20% (wt/vol) glycerol; $NaDodSO_4$, sodium dodecyl sulfate.

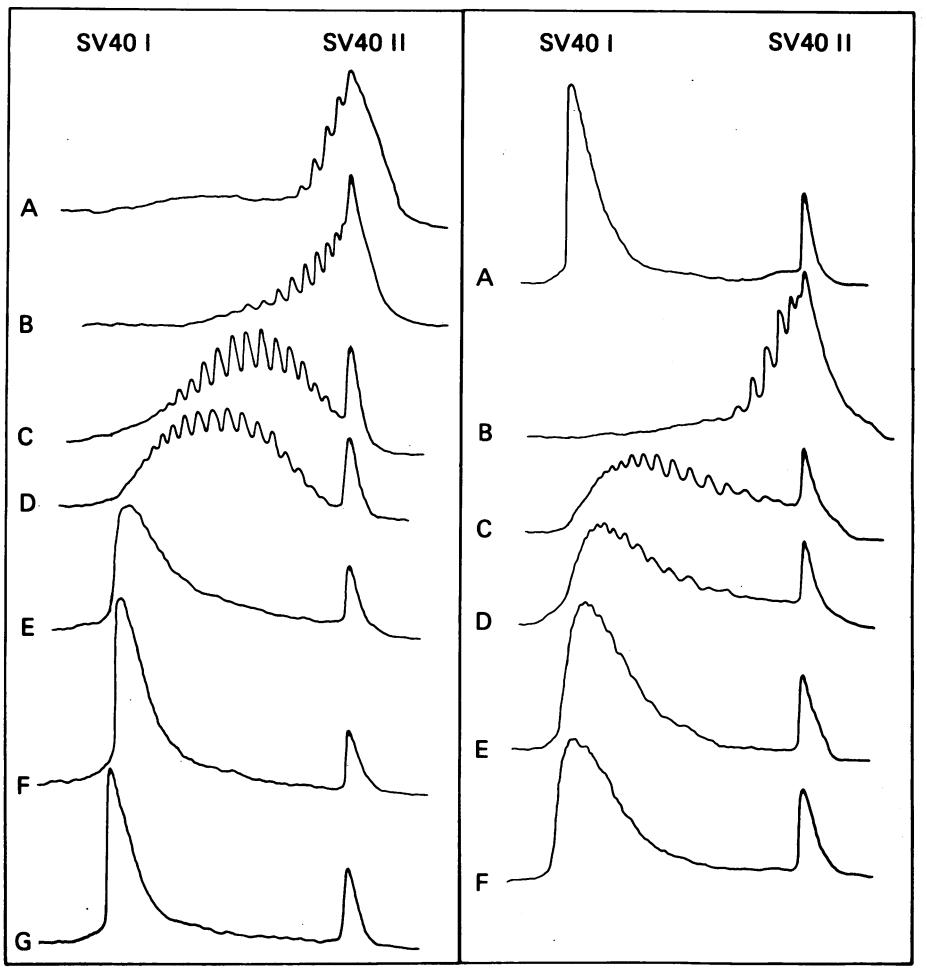

FIG. 2. Effect of increasing concentration of histones (*Left*) and of increasing concentration of "nuclear factor" (fraction III) (*Right*) on the supercoiling reaction. Densitometric tracings of agarose gel photographs. The reactions were carried out in two steps as indicated in the legend for Fig. 1. (*Left*) A, Fraction III and histones were omitted; B, histones were omitted. C–G, Histones added as follows: C, 0.5 μg; D, 1 μg; E, 1.5 μg; F, 2 μg; G, 2.5 μg. (*Right*) A, DNA-relaxing enzyme, fraction III, and histones were omitted; B, fraction III was omitted. C–F, Fraction III added as follows: C, 10 μl; D, 20 μl; E, 30 μl; F, 40 μl.

tially according to Mattoccia *et al.* (6). *X. laevis* erythrocyte histones were prepared according to Destrée *et al.* (10).

Nuclear extracts were prepared from 3 ml of packed nuclei. All operations were at 0°–4°. The nuclei were homogenized and the homogenate was centrifuged for 20 min at 12,000 × *g*. The supernatant was centrifuged in a Beckman 50.1 rotor at 38,000 rpm for 60 min. The high-speed supernatant (fraction I) was brought to 60% saturation with ammonium sulfate in the cold. The precipitate was collected by centrifugation, dissolved in 3 ml of TEMG buffer [50 mM Tris·HCl, pH 7.5/1 mM EDTA/1.4 mM mercaptoethanol/20% (wt/vol) glycerol], and dialyzed exhaustively against TEMG buffer. The dialyzate was centrifuged for 15 min at 12,000 × *g* to remove insoluble proteins (fraction II) and applied to a DEAE-cellulose column (Whatman DE-52) (1 × 15 cm) equilibrated with TEMG buffer. The column was washed with 3 vol of buffer and eluted with TEMG buffer containing 0.2 M KCl. The fractions containing high amounts of protein, as detected by absorbance at 280 nm, were pooled (5 ml) and dialyzed against TEMG buffer (fraction III). In a typical preparation, fraction I, fraction II, and fraction III contained, respectively, 7.0 mg, 4.5 mg, and 1.5 mg of protein per ml.

Assay of DNA Supercoiling. The reaction was carried out in two steps. In the first step the reaction mixture contained, in 15 μl: 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.6 mM dithiothreitol, 1 μg of SV40 supercoiled DNA, and 10 μl of purified *X. laevis* DNA-relaxing enzyme. Incubation was carried out for 20 min at 30°. In the second step the reaction mixture contained, in 60 μl: 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.6 mM dithiothreitol, 15 μl of the step one reaction mixture, 40 μl of fraction II or III, and 2 μg of histones.

Fractions II or III and the histones were preincubated for 5 min at 30°. Incubation was carried out for 60 min at 30°. The reaction was stopped by addition of sodium dodecyl sulfate ($NaDodSO_4$) and EDTA to final concentrations of 1% and 15 mM, respectively.

Gel Electrophoresis. DNA samples were analyzed on 1.2% agarose cylindrical gels. The electrophoresis buffer was 50 mM Tris/20 mM sodium acetate/20 mM NaCl/2 mM EDTA. The pH of the buffer was adjusted to pH 8.0 with acetic acid. The gels were subjected to electrophoresis at room temperature for 4 hr at 10 mA per tube. The DNA was photographed after being stained with ethidium bromide. The negatives were scanned with a Joyce–Loebl microdensitometer.

RESULTS

Extracts Derived from Nuclei Prepared *En Masse*. While investigating the intracellular distribution of components that influence the conformation of DNA in *X. laevis* oocytes, we found that extracts from manually prepared nuclei rapidly converted the supercoiled form of SV40 DNA to relaxed circles and that this end product was stable (6). In the experiments

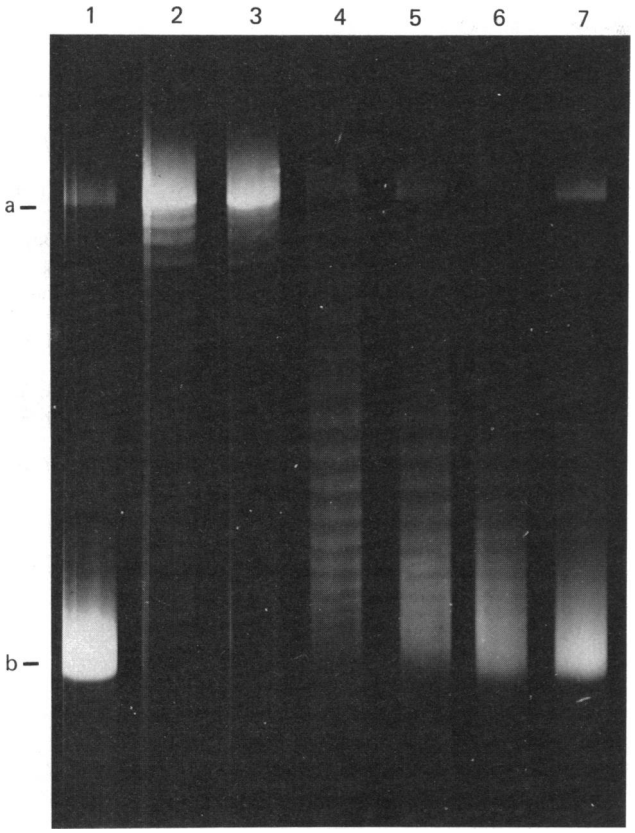

FIG. 3. Time course of insertion of superhelical turns. a, Position of migration of nicked circular or fully relaxed DNA. b, Position of migration of supercoiled DNA. Reactions were carried out in two steps as indicated for Fig. 1. (Lane 1) DNA-relaxing enzyme, fraction III, and histones were omitted. (Lane 2) Fraction III and histones were omitted. (Lanes 3–7) Incubation was for (lane 3) 0; (lane 4) 5; (lane 5) 10; (lane 6) 20; (lane 7) 60 min.

described in this paper we prepared nuclei by the procedure described by Scalenghe *et al.* (9). The procedure involves the use of Pronase, but if all the protease is removed, very stable vesicles can be obtained on a large scale. Under the dissection microscope, these vesicles are indistinguishable from nuclei isolated by hand and the nuclear envelope covering the nucleoplasm is visible. Both kinds of vesicles contain the same amount of proteins (~3 μg/nucleus), and an analysis of the polypeptide composition of nuclear extracts by $NaDodSO_4$/polyacrylamide gel electrophoresis reveals no significant difference.

An S-100 (fraction I) derived from nuclei prepared by this new method converted supercoiled DNA to the relaxed form (data not shown). The DNA-relaxing enzyme works optimally at 200 mM NaCl. Monovalent cation can be replaced by 6 mM $MgCl_2$, although the activity is considerably less than when 200 mM NaCl is used. The enzyme is completely inhibited in the presence of 0.3 mM *N*-ethylmaleimide. A striking feature of the nuclear extracts is the absence of detectable endonuclease, whereas extracts produced from anucleated oocytes convert supercoiled SV40 DNA to full-length linear, nicked, circular form and shorter fragments (6). This new method, then, allows the preparation of large amounts of nuclei free of cytoplasm, allowing fractionation of nuclear components.

Histones Are Required for DNA Supercoiling. In order to construct an *in vitro* system capable of inserting superhelical turns into relaxed DNA, we have fractionated the nuclear extract. The fractionation involved the preparation of a high-speed supernatant, precipitation with 60% ammonium sulfate (fraction II), and chromatography on DEAE-cellulose (fraction III). Fraction III appears totally deprived of histones when analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. This fact is particularly important since a large pool of histones (100 ng/cell) had been shown to exist in oocytes (11, 12). Fig. 1 shows that histones purified from *X. laevis* erythrocytes, a purified *X. laevis* DNA-relaxing enzyme, and either fraction II (Fig. 1, lanes 2 and 3) or fraction III (Fig. 1, lanes 4 and 5) converted relaxed SV40 DNA into a form that has a mean superhelical density equal to that of the supercoiled DNA extracted from infected monkey cells (14).

The experiments were performed in two stages: first supercoiled DNA was incubated with the DNA-relaxing enzyme for 20 min in order to produce relaxed closed circles; then a mixture of the histones and of the fraction derived from the nuclear extract, preincubated for 5 min, was added to the DNA, whereupon the incubation was continued for 60 min more. The reactions run in the presence of fraction III (Fig. 1, lanes 4 and 5) showed a complete dependence on added histones. This result allowed us to study the dependence of the reaction on the concentration of histones. Different concentrations of histones (Fig. 2 *left*, C–G) were incubated with relaxed DNA in the presence of both excess DNA-relaxing enzyme and fraction III up to histone/DNA weight ratio of approximately 2:1. Any increase over this limit of relative concentration of histones consistently resulted in inhibition of the reaction.

The histones used in all experiments described above were prepared according to Destrée *et al.* (10) and contained H1. However, in experiments with a preparation of the four histones (H2A, H2B, H3, and H4) free of H1, the same results were obtained; therefore the reaction described does not require H1.

Requirement of a "Nuclear Factor." Histones and the DNA-relaxing enzyme are not enough to promote the insertion of superhelical turns (Fig. 2 *right*, B). They cannot replace the requirement for a factor contained in the fractions derived from the nuclear extract; the reaction responsible for the conversion of the relaxed circles to the supercoiled form is dependent on the presence of a nuclear factor (Fig. 2 *right*, C–F). In this experiment increasing concentrations of fraction III were incubated with relaxed DNA in the presence of fixed amounts of the DNA-relaxing enzyme and histones. Using graded dialysis from 2 M NaCl, Germond *et al.* (15) have shown that in the presence of the DNA-relaxing enzyme the reassociation of relaxed SV40 DNA with histones H2A, H2B, H3, and H4 results in an increase in the number of superhelical turns. The process they described, however, requires several days. Fig. 3 shows a time course of our factor-dependent supercoiling reaction. The superhelical density of the template increases with incubation time, reaching a maximum at 60 min. Further incubation does not change the superhelical density.

DISCUSSION

It has been suggested that the bacterial DNA gyrase introduces superhelical twists by wrapping the DNA around the enzyme (3). In the reaction described here, the DNA is probably being wrapped around the nucleosome. It will be interesting to learn more about the role of the nuclear factor in this reaction. There are clearly differences in the two reactions. In addition to the use of histones, the *X. laevis* factor does not require ATP. In the experiments reported in this paper we used SV40 DNA, but we obtained the same results using pBR313 DNA (16) and a variety of other circular DNA molecules (17). Very probably, we are studying in an oocyte nuclear extract the same phenomenon that Laskey *et al.* (18) have described in extracts of *X. laevis* unfertilized eggs. The absence of nuclease activity in the extract

and the fact that complete histone dependence can be achieved after very simple purification steps are the main advantages of the system derived from oocyte nuclei.

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1. Gellert, M., Mizuuchi, K., O'Dea, M. H. & Nash, H. A. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3872–3876.
2. Sugino, A., Peebles, C. L., Kreuzer, K. N. & Cozzarelli, N. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4767–4771.
3. Liu, L. F. & Wang, J. C. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2098–2102.
4. Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T. & Tomizawa, J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4772–4776.
5. Wyllie, A. H., Gurdon, J. B. & Price, J. (1977) *Nature (London)* **268**, 150–152.
6. Mattoccia, E., Gandini Attardi, D. & Tocchini-Valentini, G. P. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4551–4554.
7. Hirt, B. (1967) *J. Mol. Biol.* **26**, 365–369.
8. Schorderet-Slatkine, S. (1972) *Cell Differ.* **1**, 179–181.
9. Scalenghe, F., Buscaglia, M., Steinheil, C. & Crippa, M. (1978) *Chromosoma* **66**, 299–308.
10. Destrée, O. H. J., D'Adelhart Toorop, H. A. & Charles, R. (1973) *Cell Differ.* **2**, 229–242.
11. Adamson, E. D. & Woodland, H. R. (1974) *J. Mol. Biol.* **88**, 263–285.
12. Woodland, H. R. & Adamson, E. D. (1977) *Dev. Biol.* **57**, 118–135.
13. Keller, W. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4876–4880.
14. Tegtmeyer, P. & Macasaet, F. (1972) *J. Virol.* **10**, 599–604.
15. Germond, J. E., Hirt, B., Oudet, P., Gross-Bellard, M. & Chambon, P. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1843–1847.
16. Bolivar, F., Rodriguez, R. L., Betlach, M. C. & Boyer, H. W. (1977) *Gene* **2**, 75–93.
17. Tocchini-Valentini, G. P., Baldi, M. I., Carrara, G., Gandini Attardi, D. & Mattoccia, E. (1978) *Cold Spring Harbor Symp.* **43**, in press.
18. Laskey, R. A., Mills, A. D. & Morris, N. R. (1977) *Cell* **10**, 237–243.