DNA-relaxing activity and endonuclease activity in Xenopus laevis oocytes

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ABSTRACT Complex simian virus 40 DNA produced by a soluble cell-free extract derived from stage 6 oocytes of Xenopus laevis consists of fully relaxed circles (i.e., with no superhelical turns). An endonuclease and a DNA-relaxing protein, either or both of which could be responsible for the relaxation of the complex DNA, have been purified from the extract. The endonuclease(s) produces nicked circles (having a single-strand scission) and linear full-size molecules. The DNA-relaxing protein is in the nucleus, has a molecular weight of approximately 70,000, and is able to remove both negative and positive superhelical turns.

A soluble cell-free extract derived from stage 6 oocytes of Xenopus laevis has recently been described (1). The extract, when challenged with supercoiled simian virus 40 (SV40) DNA, produces various forms of complex DNA. Complex DNA consists of a repertoire of structures such as: figure-8 dimers, catenated dimers, circular dimers, catenated trimers, Cairns' structures, complex multimers, and circular monomers with tails. The extract holds promise for the successful resolution of the steps involved in the formation of complex DNA. It is virtually without endogenous DNA and, since it is soluble, one can fractionate its various components and investigate their relative roles. Electron microscopy studies showed that the various forms of complex DNA produced by the extract were relaxed (i.e., not supercoiled). These relaxed molecules could be the product of the action of a nicking enzyme (a nick being a break in one strand of DNA), of a DNA-relaxing enzyme, or of both.

This paper describes the partial purification of a DNA-relaxing enzyme. The DNA-relaxing protein purified from Xenopus laevis oocytes presents essentially the same characteristics as do other DNA-relaxing proteins extracted from eukaryotic cells (2–5). In particular, in contrast to the Esherichia coli protein (6), the Xenopus laevis untwisting enzyme is capable of removing negative and positive superhelical turns.

A protein with endonuclease activity was also partially purified. The endonuclease activity (activities) produces nicked circles and linear full-size molecules from superhelical DNA. This activity was shown to produce the same kinds of products from a variety of supercoiled DNAs.

MATERIALS AND METHODS

Materials. Deoxynucleoside 5-triphosphates were obtained from Schwarz/Mann. Ribonucleoside 5-triphosphates were purchased from P-L Biochemicals. [Methyl-3H]thymidine (49 Ci/mmol) and [14C]thymidine (55 mCi/mmol) were purchased from New England Nuclear. 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. Labeled SV40 DNA was prepared by adding [3H]thymidine (10 µCi/ml) or [14C]thymidine (0.5 µCi/ml) to the infected cells between 24 and 40 hr after infection. The viral DNA was purified by the Hirt supernatant by banding to equilibrium in a CaCl2/ethidium bromide gradient. The fractions containing supercoiled DNA were pooled and ethidium bromide was removed with isopropanol. The DNA was precipitated with ethanol and redissolved in 10 mM Tris-HCl at pH 7.5.

Nuclear and Cytoplasmic Extract from Xenopus laevis Stage 6 Oocytes. The extracts were prepared as described in ref. 1.

Assay of DNA-Relaxing Activity. Reaction mixtures contained in a volume of 50 µl: 50 mM Tris-HCl at pH 7.5, 200 mM NaCl, 0.2 mM EDTA, 0.5 µg of SV40 DNA, and 5 µl of protein solution. After incubation for 30 min at 30° the reaction was stopped by the addition of sodium dodecyl sulfate at the final concentration of 1%.

Assay of Endonuclease Activity. Reaction mixtures contained in a volume of 50 µl: 50 mM Tris-HCl at pH 7.5, 6 mM MgCl2, 6 mM dithiothreitol, 0.5 µg of SV40 DNA, and 30 µl of protein solution. After incubation for 30 min at 30° the reaction was stopped by the addition of sodium dodecyl sulfate 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 sodium acetate, 20 mM NaCl, and 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.

Preparation of Endonuclease and of DNA-Relaxing Protein. Twenty milliliters of collagenase-treated stage 6 oocytes (8) (about 14,000 cells) were used. All operations were carried out at 0°–4°. The oocytes were homogenized in a Dounce homogenizer with 80 ml of TEMG buffer [50 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1.4 mM mercaptoethanol, 20% (wt/vol) glycerol]. The homogenate was centrifuged for 15 min at 8000 X g and the supernatant, after removal of the floating lipid layer, was centrifuged in a Beckman 50 Ti rotor at 40,000 rpm for 60 min. The high-speed supernatant was brought to 60% saturation with ammonium sulfate in the cold. The precipitate was collected by centrifugation, dissolved in 15–20 ml of TEMG buffer, and dialyzed exhaustively against TEMG. The dialyze was centrifuged 30 min at 30,000 X g to remove insoluble proteins and applied to a DEAE-cellulose column (Whatman DE-52) (3 X 20 cm) equilibrated with TEMG buffer. The column was washed with three volumes of buffer and eluted with
TEMG containing 0.2 M KCl. All the fractions containing proteins, as detected by absorbance at 280 nm, were pooled (50-60 ml) and applied to a phosphocellulose column (1 X 5 cm) equilibrated with TEMG containing 0.2 M KCl. The column was washed with three volumes of buffer and subsequently eluted with 40 ml of a linear gradient of 0.2-1.0 M KCl in TEMG containing 0.1 mg/ml of bovine serum albumin. Fractions (1.5 ml) were collected. Every fraction was dialyzed against TEMG buffer and assayed both for endonuclease activity and for DNA-relaxing activity.

One unit of DNA-relaxing activity is defined as the amount of protein that converts 1 µg of supercoiled SV40 DNA to the relaxed form in 30 min at 30°C.

The product of the final step in our preparation (i.e., the phosphocellulose fractions) contained, in a typical experiment, a total of about 1000 units. Our preparation started with approximately 14,000 cells; therefore, we have a minimum of 0.07 unit of DNA-relaxing activity per cell. The amount of DNA relaxed by the untwisting protein was determined by measuring its relative area on densitometric tracings.

RESULTS AND DISCUSSION

Analysis by agarose gel electrophoresis of the products of the cell-free system

The nuclei-cytoplasmic extract derived from *Xenopus laevis* oocytes, when challenged with SV40 supercoiled DNA, produces nicked circles, linear molecules of full unit size, shorter length fragments, and various forms of complex DNA (1). Our analysis of the products of the cell-free system involved sucrase gradient sedimentation and electron microscopy.

In this paper we report an analysis by agarose gel electrophoresis of the products of the extract. Fig. 1 shows that the cytoplasmic extract produces from supercoiled SV40 DNA material having the mobility corresponding to that of full-length linear SV40 DNA, material having the mobility corresponding to that of fully relaxed or nicked circular DNA, and shorter length fragments which migrate to the lower portion of the gel (not shown in the picture). When the products of the nuclear extract are analyzed, the gel pattern shows a series of bands migrating in the gel with mobilities intermediate between those of supercoiled DNA and fully relaxed circles. Since this pattern is characteristic of a population of DNA molecules having a decreasing number of superhelical turns (5), this finding suggests that an untwisting enzyme is present in our extract and that this enzyme is located in the nucleus. When the products of the combined nuclear and cytoplasmic extracts are analyzed in gel electrophoresis, a pattern is observed that includes all the same features observed in the products of the separate nuclear and cytoplasmic extracts. Moreover, in the upper portion of the gel, a series of slowly migrating bands is observed. This slowly migrating material, which requires for its formation the combined action of the nuclear and cytoplasmic extracts, corresponds to complex DNA. Slowly migrating bands are in fact observed when complex DNA, purified by sucrase gradient sedimentation (1), is examined by agarose gel.

The finding of DNA with reduced numbers of superhelical turns, nicked circles, and linear molecules prompted us to study DNA-relaxing activity and endonuclease activity in the extract.

Endonuclease activity and DNA-relaxing activity in *Xenopus laevis* oocytes

We made use of the method described by Keller (5) to assay for the DNA-relaxing protein in our cell-free system. Such a method is based on the observation that the superhelical DNA migrates in agarose gel electrophoresis more rapidly than the covalently closed DNA free of superhelical turns. Moreover, Keller has shown that superhelix density and electrophoretic mobility of closed circular DNA are correlated.

Since supercoiled DNA migrates more rapidly than linear full size molecules and nicked circles, the agarose gel electrophoresis method allows also for the assay of the endonuclease activity.

The fractionation of the extract involved (see Materials and Methods) the preparation of a high-speed supernatant, precipitation with 60% saturated ammonium sulfate, and chromatography on DEAE-cellulose and phosphocellulose. Fig. 2 shows the activities present in the phosphocellulose eluate. The phosphocellulose column was eluted with a 0.2-1.0 M KCl gradient and the diazoyed fractions were assayed for endonuclease activity and DNA-relaxing activity. Fractions 9 to 10 show an endonuclease activity eluting around 0.4 M KCl, and fractions 11 to 15 show a DNA-relaxing activity eluting around 0.7 M KCl.

The endonuclease activity (activities) converts SV40 supercoiled DNA to nicked circles and full-unit-size linear molecules.
The activity requires Mg\(^{2+}\) and is inhibited by 200 mM NaCl.

The endonuclease is active on polyoma DNA and on defective polyoma DNA (D-50), which consists of nucleotide sequences accounting for about 17% of the polyoma genome, tandemly repeated three times (9). With both polyoma DNA and D-50 DNA the nuclease produces nicked circles and full-size linear molecules.

Therefore, it appears that *Xenopus laevis* endonuclease cuts only once the supercoiled DNAs we have tested. However, the superhelical structure of the DNA is not required, since our enzyme cuts also relaxed and nicked circles of SV40 DNA.

It is possible that the nicked circles constitute an intermediate product of an enzyme producing linear molecules. Alternatively, we may be dealing with a mixture of two enzymes.

We assayed for the DNA-relaxing protein in the presence of 200 mM NaCl, a salt concentration at which optimal activity is observable. Fig. 3 shows a series of densitometric tracings of the reaction products of the DNA-relaxing activity assayed at 50 mM, 200 mM, and 400 mM NaCl. At 50 mM, a substantial amount of supercoiled DNA is not relaxed during incubation; at 400 mM the activity is completely inhibited. The monovalent cation can be replaced by 6 mM MgCl\(_2\), but the activity is considerably less than when 200 mM NaCl is used.

The *Xenopus laevis* DNA-relaxing activity is completely inhibited when assayed in the presence of 0.3 mM N-ethylmaleimide; at 0.15 mM N-ethylmaleimide already about 80% of the activity is inhibited.

Our standard assay was carried out at 30°, but the enzyme is active also at 0°, although at a reduced rate.

Fig. 2. Analysis by agarose gel electrophoresis of the activities present in the fractions of a phosphocellulose column eluted with a 0.2–1.0 M KCl gradient. The upper part of the figure represents the salt concentration gradient. In the lower part are shown agarose gels of supercoiled SV40 DNA incubated with aliquots of the dialyzed fractions (see Materials and Methods). Fractions 5 to 10 were assayed under standard conditions for endonuclease activity; fractions 11 to 15 were assayed under standard conditions for DNA-relaxing activity. X, supercoiled DNA incubated as control with the endonuclease mixture without protein; Y, supercoiled DNA incubated as control with the DNA-relaxing mixture without protein. B, C, D, as in Fig. 1.

Fig. 3. Densitometric tracings of agarose gel photographs of supercoiled SV40 DNA incubated with fraction 13 from the phosphocellulose gradient shown in Fig. 2, under standard conditions with different NaCl concentrations. (A) 200 mM NaCl, no enzyme; (B) 50 mM NaCl; (C) 200 mM NaCl; (D) 400 mM NaCl.

Fig. 4. Glycerol gradient centrifugation of purified DNA-relaxing activity. Fraction 13 (see Fig. 2) (0.3 ml), containing 60 units of DNA-relaxing activity, was layered on a 10–30% (wt/vol) glycerol gradient containing 50 mM Tris-HCl at pH 7.5, 300 mM KCl, 1.4 mM mercaptoethanol, and centrifuged in a Beckman SW 41 rotor at 38,000 rpm for 18 hr 30 min at 4°. DNA-relaxing activity was assayed under standard conditions. In parallel gradients immunoglobulin G (I), albumin (II), and myoglobin (III) were run as markers.
DNA containing negative superhelical turns (6); the human, mouse, and Drosophila proteins, in contrast, are active on DNA containing both negative and positive superhelical turns (2–5).

To test the effect of our DNA-relaxing activity on positive supercoiled DNA we added ethidium bromide to the reaction mixture, since no DNA with positive superhelical turns was available. The concentration of ethidium bromide required to induce such a change of conformation was estimated from the values given by LePecq and Paoletti (10). A control reaction was run in the absence of ethidium bromide. Fig. 5 shows that SV40 DNA treated with relaxing activity in the absence of ethidium bromide exhibited a greater buoyant density than marker DNA. Therefore the enzyme removed negative turns. When, on the other hand, the DNA initially contained positive turns, owing to the presence of ethidium bromide, it was converted by the enzyme to a species having lower density than marker DNA. Thus the relaxing activity removed positive superhelical turns. We conclude that our preparation is capable of acting on both negatively and positively supercoiled DNA.

Our extract contains, therefore, both a DNA-relaxing protein and an endonuclease activity that produces nicked circles. Either of the activities could be responsible for the fact that the circles present in complex DNA appear to be fully relaxed in the electron microscope. If the relaxation is due to an endonuclease, closure must follow the scission, since we have observed that by alkaline sucrose gradient sedimentation the majority of complex DNA molecules consists of closed circles. Closed monomeric circles sediment in alkaline gradients at 53 S and complex DNA sediments faster than 53 S (data not shown). A decision between the two different alternatives requires further fractionation of the extract.

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