Purification of a DNA supercoiling factor from the posterior silk gland of *Bombyx mori*

(DNA topoisomerase II/DNA gyrase/transcription)

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ABSTRACT   A protein factor with an estimated molecular mass of 50 kDa has been purified to homogeneity from the silk gland of *Bombyx mori*. In the presence of a molar excess of this factor and eukaryotic DNA topoisomerase II, relaxed circular DNA is converted to the negatively supercoiled form. Eukaryotic DNA topoisomerase I cannot substitute for eukaryotic DNA topoisomerase II in the supercoiling reaction. The reaction is dependent on ATP and is inhibited by VP-16, a specific inhibitor of eukaryotic DNA topoisomerase II. When DNA topoisomerase I is subsequently added to the supercoiling reaction mixture, the supercoiled DNA becomes relaxed. These results suggest that when both the 50-kDa protein and eukaryotic DNA topoisomerase II are present in excess, unconstrained negative supercoils are introduced into DNA.

In eubacteria, DNA gyrase has been shown to catalyze the negative supercoiling of DNA (for review, see refs. 1 and 2). In contrast, the eukaryotic counterpart of DNA gyrase, DNA topoisomerase II, catalyzes the relaxation of supercoiled DNA but not the reverse reaction (2). Nevertheless, there have been some inferences that a DNA supercoiling activity might also be present in eukaryotes (for review, see ref. 3). To test this possibility, we searched for a DNA gyrase-like activity in cell extracts from various eukaryotic sources. Among these, a posterior silk gland cell extract of *Bombyx mori* produced positive results (4). In this extract, covalently closed circular (ccc) DNA supported more efficient transcription of fibroin gene than did linear DNA, and a DNA supercoiling activity was present in the extract. The activity introduced negative supercoils into cccDNA (5) and the resulting supercoils were unconstrained (6). The reaction was inhibited by the drug VP-16, a specific inhibitor of eukaryotic DNA topoisomerase II, and thus the involvement of DNA topoisomerase II in the supercoiling reaction was implicated (6). Phosphocellulose fractionation of the extract yielded two fractions, a flow-through fraction and a 0.6 M KCl eluate fraction, both of which were required for DNA supercoiling. The 0.6 M KCl fraction had a DNA topoisomerase II activity, and the flow-through fraction contained a factor capable of generating negative supercoils in a relaxed DNA with the aid of DNA topoisomerase II (5).

In this work, we report the purification of the supercoiling factor to a homogeneous state and the reconstitution of the supercoiling activity from purified components. Liu and Wang (7) have proposed that transcription generates positively and negatively supercoiled domains in template DNA. Recently, evidence supporting the twin-supercoiled-domain model has been obtained in *Escherichia coli* and yeast (8, 9). The present study suggests that supercoiling of DNA can also occur in eukaryotic cells by a mechanism independent of transcription.

MATERIALS AND METHODS

Materials. Phosphocellulose (P11) was obtained from Whatman. DEAE-Sephacel, phenyl-Sepharose CL-4B, DNA-cellulose (native DNA), and DNase-free bovine serum albumin were obtained from Pharmacia LKB Biotechnology. VP-16 was a gift of A. Matsuda (Nippon Kayaku, Tokyo). Human DNA topoisomerase I was a gift of A. Kikuchi (Mitubishi-Kasei Institute, Tokyo). It gave two bands of 100 kDa and 70 kDa in a ratio of 2:1 upon SDS/PAGE. The latter component is thought to be a cleavage product of the intact polypeptide (100 kDa), as judged from its N-terminal amino acid sequence (A. Kikuchi, personal communication). Fission yeast DNA topoisomerase II was a gift of M. Yanagida (Kyoto University). It was purified from the yeast carrying the topoisomerase II gene on a multicopy plasmid and it gave a single polypeptide of 162 kDa upon SDS/PAGE. Preparation of DNA topoisomerase II from *B. mori* and HeLa cells has been described (10). The purified enzyme from *B. mori* showed a specific activity of 2×10^6 units/mg and gave a single band of 170 kDa upon SDS/PAGE. The HeLa cell enzyme had a specific activity of 2×10^6 units/mg and gave a predominant band of 172 kDa upon SDS/PAGE. The unit of topoisomerase II was defined as described by Hsieh (11).

Purification of the DNA Supercoiling Factor. All operations were done at 0–4°C unless otherwise specified. A posterior silk gland extract was prepared from 4000 silk-worm heads on the second day of the fifth instar according to a published procedure (12). The extract (1.9 g of protein) was loaded onto a 160-ml column of P11 equilibrated in buffer A [20 mM Hepes, pH 7.9/1 mM EDTA/20% (vol/vol) glycerol/1 mM dithiothreitol] containing 0.04 M KCl and the column was washed with 320 ml of the same buffer. The flow-through fraction contained the activity (fraction 1).

The KCl concentration of fraction I was adjusted to 0.1 M and the sample was applied to a 70-ml column of DEAE-Sephacel equilibrated in buffer A/0.1 M KCl. After washing with 140 ml of the same buffer, material was successively eluted with 210 ml of buffer A/0.2 M KCl and with 140 ml of buffer A/0.35 M KCl. The 0.35 M KCl eluate contained the activity (fraction II). Fraction II was heated at 80°C for 10 min and chilled in an ice-water bath. The heated sample was centrifuged at 25,000 rpm for 40 min in a Beckman type 50.2 Ti rotor and the supernatant was taken as fraction III.

Fraction III was dialyzed against 100 vol of buffer B (buffer A without glycerol) containing 2 M KCl for 2 hr with one change of buffer. The dialyzed sample was applied to a 3-ml column of phenyl-Sepharose CL-4B equilibrated in buffer B/2 M KCl. After washing with 15 ml of buffer B/1 M KCl, material was eluted with 30 ml of buffer A/0.04 M KCl. The activity formed a broad trailing peak. Fractions containing 70% of the input activity were pooled and dialyzed against

Abbreviation: ccc, covalently closed circular.
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100 vol of buffer A/0.02 M KCl for 2 hr with one change of the buffer (fraction IV).

Fraction IV was applied to a 0.75-ml column of DNA-cellulose (native DNA) equilibrated in buffer A/0.02 M KCl. The flow-through was collected and reapplied to the column, and this procedure was repeated once more. After washing with 4.5 ml of the same buffer, material was eluted with 4.5 ml of buffer A/0.35 M KCl and active fractions were pooled (fraction V). Fractions I–V were stable for at least 6 months when stored at −80°C.

Assay of the DNA Supercoiling Activity. The standard reaction mixture (12.5 μl) contained 12 mM Hepes (pH 7.9), 7.5 mM $MgCl_2$, 50 mM KCl, 1 mM ATP, 0.3 mM EDTA, 1.2 mM dithiothreitol, 10% glycerol, DNase-free bovine serum albumin (30 μg/ml), 8.5 ng (25 fmol as a homodimer) of *B. mori* DNA topoisomerase II, and 0.25 fmol (as a mol of polymer) of ^{32}P-labeled relaxed cccDNA of pFb205 (3.9 kilobases). After addition of the factor, the mixture was incubated at 30°C for 20 min. The reaction was stopped by the addition of SDS to 0.5% and proteinase K to 250 μg/ml. The mixture was incubated further at 37°C for 30 min. DNA was recovered by phenol treatment followed by ethanol precipitation and analyzed by two-dimensional electrophoresis as described (5). Autoradiograms were traced by a LKB Ultro-Scan XL laser densitometer and the extent of supercoiling was determined from the average of each distribution of topoisomers. One unit is defined as the amount of factor required to introduce a linking-number change of 10 under the reaction conditions described above.

^{32}P-labeled relaxed cccDNA was prepared as described (4). After treatment with DNA topoisomerase I, relaxed cccDNA was purified by electrophoresis through a 1% SeaPlaque agarose gel (FMC) containing ethidium bromide (0.5 μg/ml). The DNA was recovered from the melted gel slice by phenol treatment and concentrated by extraction with 2-butanol and subsequent ethanol precipitation. DNA of pFb205 carrying the fibroin gene segment (4) was routinely used as the substrate but essentially the same results were obtained with pBR322 DNA.

Other Methods. Protein concentrations were determined by the procedure of Bradford (13) with bovine serum albumin as the standard. SDS/PAGE was carried out according to the method of Laemmli (14). Recovery of protein from SDS gels was performed by a published procedure (15).

RESULTS

Purification of the DNA Supercoiling Factor. When the posterior silk gland extract was fractionated by phosphocellulose column chromatography, the flow-through fraction contained the DNA supercoiling factor, which introduces negative supercoils into cccDNA in conjunction with DNA topoisomerase II. Thus, supercoiled topoisomers appeared after incubation of relaxed cccDNA with the flow-through fraction and purified DNA topoisomerase II (Fig. 1). The diagonal line of trailing spots near the origin of electrophoresis (upper-left corners) were catenanes produced by stoichiometric amounts of DNA topoisomerase II. It has been reported that the enzyme itself can condense the DNA by bringing two DNA helices together to form catenanes (16, 17). The spot at the lower-right corner was a linear DNA that might be cleaved from a topoisomerase II–DNA complex (for the interpretation of the two-dimensional electrophoretogram, see refs. 5 and 10).

The supercoiling factor was purified to homogeneity by a combination of chromatographic procedures. The supercoiling factor is a heat-stable protein, and this stability facilitated its purification. Table 1 represents a summary of the purification. We are unable to reliably estimate the fold purification due to the presence of interfering substances in fractions I and II.

Fraction V gave a single polypeptide with the molecular mass of 50 kDa upon SDS/PAGE (Fig. 2A). When the 50-kDa region of a preparative SDS/polyacrylamide gel was cut out, protein recovered from the slice exhibited activity of the supercoiling factor (Fig. 2B). Analysis of the remaining portions of the gel showed that there was no activity above or below the 50-kDa region (data not shown). From these results, we conclude that the 50-kDa polypeptide is the supercoiling factor. The sedimentation coefficient obtained for the factor by glycerol gradient centrifugation was 2.3 S. The low value of its sedimentation coefficient suggests that the factor is an asymmetric monomer.

Reconstitution of the DNA Supercoiling Activity from Purified Components. The supercoiling activity was reconstituted from the purified factor and DNA topoisomerase II. The reaction is absolutely dependent on DNA topoisomerase II. Thus, no reaction occurred in the absence of the enzyme (Fig. 3A, sample 4). In the reaction mixtures containing 0.25 fmol of DNA and excess supercoiling factor (40 fmol), DNA supercoiling was not detectable when less than 10 fmol of
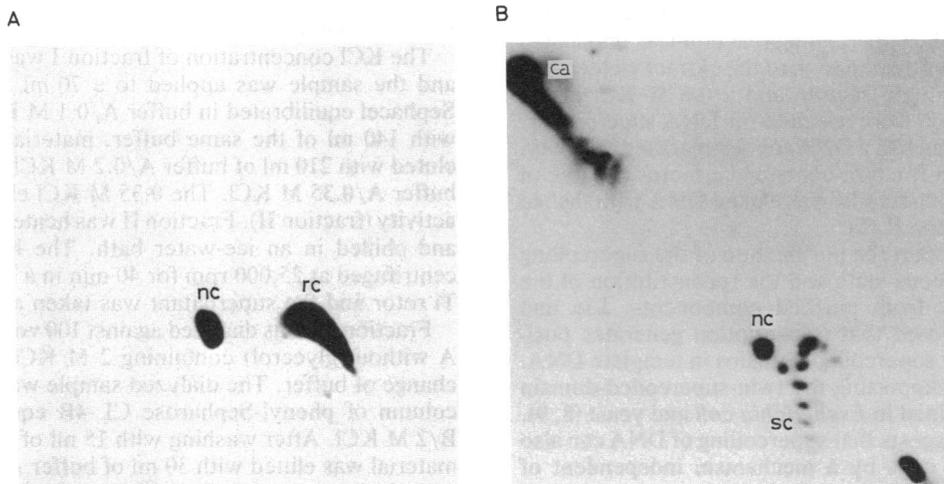

FIG. 1. Identification of DNA supercoiling factor. Radioactive relaxed cccDNA was analyzed by two-dimensional electrophoresis before (A) or after (B) incubation with 10 μg of the flow-through fraction from a phosphocellulose column in the standard reaction mixture. The first dimension was from the top to the bottom, and the second dimension was from the left to the right. rc, Relaxed cccDNA; nc, nicked circular DNA; sc, negatively supercoiled circular DNA with various degrees of coiling; ca, catenanes.

Biochemistry: Ohta and Hirose

Proc. Natl. Acad. Sci. USA 87 (1990) 5309

Table 1. Purification of DNA supercoiling factor

Fraction	Protein, mg/ml	Total activity ($\times 10^{-5}$), units	Specific activity, units/mg
I. Phosphocellulose	5.4	1.2	2.0×10^2
II. DEAE-Sephacel	2.5	1.0	1.0×10^3
III. Heat	1.6	3.7	6.3×10^3
IV. Phenyl-Sepharose	0.09	2.6	1.9×10^5
V. DNA-cellulose	0.3	1.7	5.8×10^5

DNA topoisomerase II was present (Fig. 3A, samples 2 and 3). The extent of supercoiling reached a plateau level at 25 fmol of topoisomerase (Fig. 3A, sample 1). *B. mori* DNA topoisomerase II was used in the standard assay. DNA topoisomerase II isolated from the fission yeast *Schizosaccharomyces pombe* or HeLa cells was also functional (data not shown). However, human DNA topoisomerase I could not substitute for DNA topoisomerase II.

The reaction is also dependent on the supercoiling factor. In the absence of the factor, no supercoiling occurred. In the presence of 0.25 fmol of DNA and an optimal amount (25 fmol) of DNA topoisomerase II, the activity was first detected at 5 fmol of the factor and more than 20 fmol was required for prominent supercoiling (Fig. 3B, samples 1–3).

We examined the effect of increasing concentrations of DNA on the supercoiling reaction in the presence of fixed amounts of proteins (25 fmol of DNA topoisomerase II and 25 fmol of the supercoiling factor). When the DNA concentration was raised by the addition of closed circular DNA, huge catetanes of DNA were formed and we could not analyze the supercoiling of DNA. Therefore, we added *Eco*RI-digested linear pFb205 DNA to the mixtures containing 0.25 fmol of radioactive relaxed circular DNA. DNA supercoiling became less prominent with increasing concentration of DNA and the linking number changes were barely detectable if the molar ratio of DNA topoisomerase II to DNA was less than 10 (data not shown). These results suggest that high molar ratios of protein to DNA are required for the supercoiling reaction.

Properties of the DNA Supercoiling Reaction. The supercoiling reaction required ATP. Neither adenosine 5'-[β,γ-imido]triphosphate nor adenosine 5'-[γ-thio]triphosphate could replace ATP (Fig. 4A). These results suggest that the reaction requires hydrolysis of ATP. The reaction is very sensitive to an inhibitor of DNA topoisomerase II, VP-16. Inhibition was clearly seen at 0.2 μg/ml (Fig. 4B, sample 2). No other reactions of DNA topoisomerase II, such as relaxation of supercoiled DNA under catalytic conditions, were
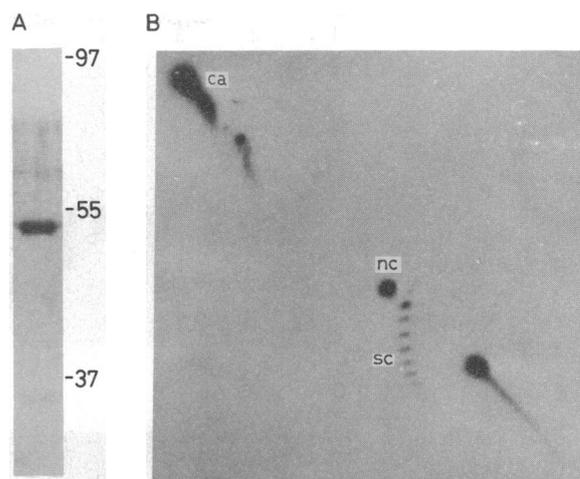
Fig. 2. DNA supercoiling factor is a 50-kDa protein. (A) A portion (0.1 μg) of fraction V was electrophoresed through a 12.5% polyacrylamide gel containing 0.1% SDS and the gel was subsequently stained with silver. The positions of molecular mass markers (in kDa) are indicated. (B) A larger portion (0.2 μg) of fraction V was electrophoresed in a parallel lane of the same gel. The 50-kDa region was excised from the nonstained lane. Polypeptides were recovered from the gel slice, renatured, and assayed for the DNA supercoiling activity. DNAs are identified as in Fig. 1.

inhibited at this concentration of the drug (data not shown). Indeed, catenation of cccDNA, as revealed by the appearance of a streak at the origin of electrophoresis, could be detected even at 2 μg/ml (Fig. 4B, sample 3). The reaction is also sensitive to ionic strength, being optimal in the range of 50–60 mM KCl (Fig. 4C). No activity could be seen at 120 mM KCl, at which the relaxation activity of DNA topoisomerase II was optimal.

Next, we examined whether the supercoils were constrained by protein–DNA interactions or not. Constrained supercoils, such as those in nucleosomes, should yield a supercoiled DNA product after DNA topoisomerase I treatment and subsequent deproteinization, whereas unconstrained supercoils, such as those in DNA gyrase products, should be relaxed by DNA topoisomerase I treatment. As shown in Fig. 4D, the negative supercoils produced by DNA topoisomerase II and the supercoiling factor (Fig. 4D, sample 1) were relaxed by further incubation with DNA topoisomerase I (Fig. 4D, samples 2 and 3). These results suggest that the supercoils are unconstrained. To confirm the above conclusion, we performed another experiment in which the reaction mixture contained the supercoiling factor and both
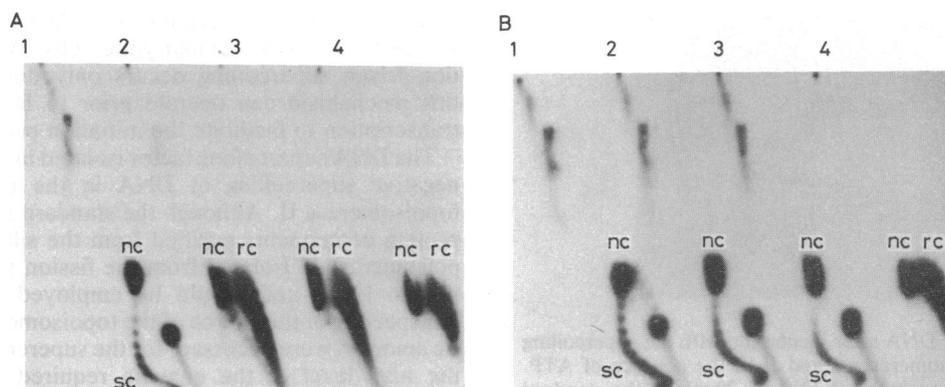
Fig. 3. DNA supercoiling activity depends on DNA topoisomerase II and supercoiling factor. (A) DNA topoisomerase II was omitted from the standard reaction mixture containing 2 ng (40 fmol) of the purified supercoiling factor and then 8.5, 3.5, 0.85, or 0 ng (25, 10, 2.5, or 0 fmol) of DNA topoisomerase II was added to samples 1–4, respectively. (B) The standard reaction mixtures received 4, 2, or 1 ng (80, 40, or 20 fmol) of the purified supercoiling factor in samples 1–3, respectively. Sample 4 received input DNA. DNAs are identified as in Fig. 1.
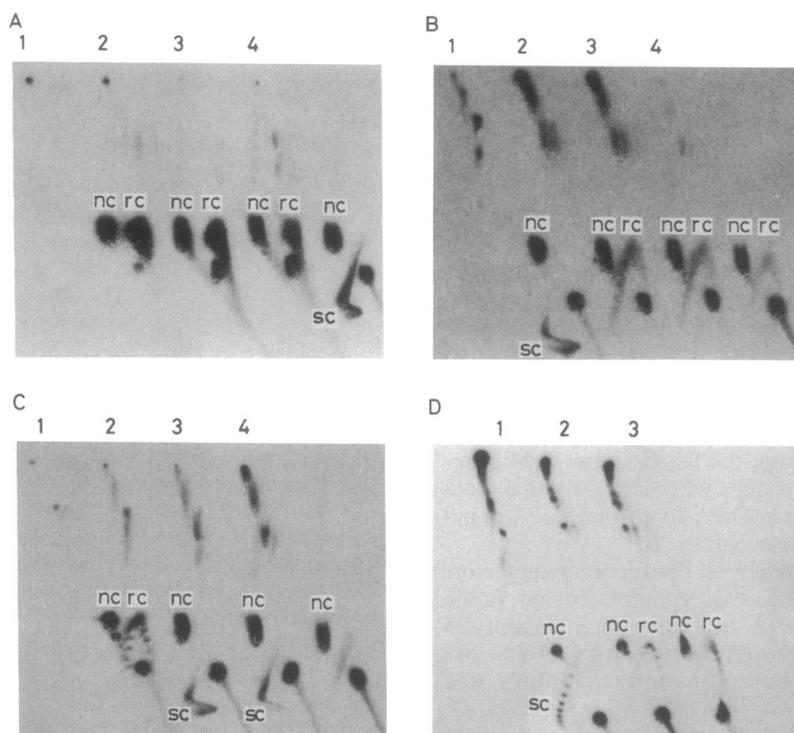
FIG. 4. Effect of ATP, VP-16, KCl, and DNA topoisomerase I on the supercoiling reaction. All reaction mixtures contained 2 ng (40 fmol) of the purified supercoiling factor. (A) ATP was omitted from the standard reaction mixture and then nothing, 1 mM adenosine 5'-[β,γ-imido]triphosphate, 1 mM adenosine 5'-[γ-thio]triphosphate, or 1 mM ATP was added to samples 1–4, respectively. (B) The standard reaction mixtures received VP-16 at 0, 0.2, 2, or 20 μg/ml in samples 1–4, respectively. (C) KCl was omitted from the standard reaction mixture and then 30 mM, 60 mM, 90 mM, or 120 mM KCl was added to samples 1–4, respectively. (D) The standard reaction mixtures were incubated at 30°C for 20 min and then 0, 2.8, or 14 pg of human DNA topoisomerase I was added to samples 1–3, respectively. The mixtures were incubated further at 30°C for 5 min. DNAs are identified as in Fig. 1.

DNA topoisomerases I and II but lacked ATP. If binding of DNA topoisomerase II, the supercoiling factor, or both to DNA constrains supercoils, then one should see supercoiling of DNA upon removal of the bound proteins. Though we detected a small decrease in the linking number under these
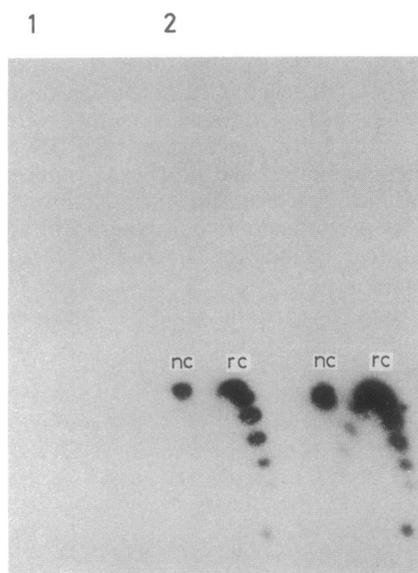

FIG. 5. Topology of DNA after incubation with the supercoiling factor and DNA topoisomerases I and II in the absence of ATP. Relaxed cccDNA was incubated at 30°C for 20 min in the standard reaction mixture containing 14 pg of human DNA topoisomerase I but lacking ATP with (sample 2) or without (sample 1) 40 fmol of the purified supercoiling factor and 25 fmol of DNA topoisomerase II. DNAs are identified as in Fig. 1.

conditions (Fig. 5, compare sample 2 with sample 1), we could not see extensive supercoiling of DNA as observed in standard reaction mixtures (e.g., Fig. 4B, sample 1). These results also suggest that most of the supercoils formed under standard conditions are unconstrained.

DISCUSSION

Though chromatin DNA in eukaryotic cells is topologically underwound, bulk DNA is not under supercoiling tension because the supercoils are constrained by histone–DNA interactions (18). This does not necessarily exclude the possibility that a small fraction of the chromatin has unconstrained supercoils. Indeed, transcription-driven supercoiling of DNA, as predicted by Liu and Wang (7), has been demonstrated in yeast (8, 9). The present study suggests that there is still another mechanism for the generation of unconstrained supercoils in eukaryotic cells. Although transcription-driven supercoiling occurs only during transcription, this mechanism can operate prior to DNA replication or transcription to facilitate the initiation process (6).

The DNA supercoiling factor isolated in this work achieves negative supercoiling of DNA in the presence of DNA topoisomerase II. Although the standard reaction contained protein components purified from the silk worm, DNA topoisomerase II isolated from the fission yeast *S. pombe* or human HeLa cells could be employed for the reaction. Irrespective of the source of the topoisomerase, stoichiometric amounts were necessary for the supercoiling. Considering the high level of the enzyme required for the reaction, contaminating proteins in the enzyme preparations, if any, could contribute to the reaction. However, the possibility seems to be unlikely because all DNA topoisomerases used were highly purified. At least in the preparations of *B. mori*

Biochemistry: Ohta and Hirose

Proc. Natl. Acad. Sci. USA 87 (1990)   5311

and fission yeast enzymes, we could not detect any contaminating proteins even after careful inspection by SDS/PAGE. We have detected a supercoiling factor activity in a partially purified fraction from a mouse tumor cell extract (unpublished results). We believe that the supercoiling activity is also present in eukaryotes other than the silk worm, although its detection in crude extract is hampered by a strong endonuclease activity and also by a high level of DNA topoisomerase I compared to DNA topoisomerase II. In this respect, the posterior silk gland extract has a great advantage. It contained a very low level of nicking activity and almost the same levels of type I and type II DNA topoisomerase activities. It should be noted that the posterior silk gland on the second day of the fifth instar is very active in both DNA replication and transcription.

The supercoiling reaction promoted by purified components showed two interesting features. (i) The reaction is markedly sensitive to the antitopoisomerase drug VP-16. The supercoiling activity is inhibited at drug concentrations of 0.2 μg/ml, whereas conventional reactions of DNA topoisomerase II, such as relaxation of supercoiled DNA under catalytic conditions, are inhibited at 20 μg/ml. We do not know the reason for the phenomenon, but it might be important in relation to the mechanism of drug resistance. (ii) The reaction is sensitive to ionic strength. The supercoiling reaction is optimal at 50–60 mM KCl and no activity was detectable at 120 mM KCl, at which the relaxation activity of DNA topoisomerase II reaches a maximum. Its dependency on ionic strength is thus more similar to the catenation than to the decatenation reaction of DNA topoisomerase II (19).

The supercoiling described here can be interpreted as resulting from a DNA gyrase-like reaction. DNA gyrase is capable of wrapping DNA in a positive sense (20, 21), which in turn generates a negative supercoil after the strand breakage and reunion event. On the contrary, eukaryotic DNA topoisomerase II does not seem to wrap DNA in a defined orientation (22). The supercoiling factor might dictate a coiling of DNA around DNA topoisomerase II with the appropriate handness. When relaxed cccDNA was incubated with the DNA supercoiling factor and both DNA topoisomerases I and II in the absence of ATP, we observed a small but significant decrease in the linking number. Moreover, preliminary experiments have shown that covalent closure of a nicked circular DNA in the presence of DNA topoisomerase II and the supercoiling factor but in the absence of ATP causes a small decrease in the linking number (unpublished observation), as above. These results suggest that the coiling is in a negative sense. The more extensive negative supercoiling of DNA in the presence of ATP might be achieved by ATP-driven repetitive coiling and uncoiling of the DNA around topoisomerase II. The coiling process might be inefficient and require high levels of both proteins. This type of interaction is not without precedence; a heat-stable acidic protein has been shown to direct coiling of DNA around histones in nucleosome assembly (23). An alternative interpretation is as follows. The supercoiling factor unwinds the DNA helix. The positive supercoils formed in the part of cccDNA outside the unwound region are relaxed by DNA topoisomerase II. Negative supercoils are generated when the unwound region rewinds. The unwinding step might require a large amount of DNA topoisomerase II. This mechanism seems less likely for the following reasons. (i) It is difficult to explain why human DNA topoisomerase I could not substitute for DNA topoisomerase II in the supercoiling reaction while both enzymes can relax positive supercoils. (ii) The supercoiling factor does not show a DNA-dependent ATPase activity that is accompanied with known DNA helicases (24, 25). Further work is needed to establish the mechanism of the supercoiling reaction promoted by DNA topoisomerase II and the 50-kDa supercoiling factor.

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