Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 718–722, February 1988
Biochemistry

In vitro transcription of eukaryotic genes is affected differently by the degree of DNA supercoiling

(supercoiling factor/DNA topoisomerase II/gene expression)

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Communicated by Motoo Kimura, October 1, 1987

ABSTRACT **In a posterior silk gland extract, covalently closed circular (ccc) DNA is in a superhelical state that supports more transcription of fibroin gene than does linear DNA. A HeLa cell extract showed neither the supercoiling activity nor the preference for the transcription of ccc DNA over linear DNA. These activities could be added to the HeLa cell extract. Phosphocellulose fractionation of the posterior silk gland extract yielded a flow-through fraction and a 0.6 M KCl eluate fraction that were required for the supercoiling and for the efficient transcription of the ccc template in the acceptor HeLa cell extract. The 0.6 M KCl fraction had a DNA topoisomerase II activity, and the flow-through fraction contained a supercoiling factor that, with the aid of topoisomerase II, introduced negative supercoils into ccc DNA. When both fractions were added to the posterior silk gland extract, more supercoiling occurred than with the extract alone. Several genes were optimally transcribed under various extents of supercoiling. The fibroin gene and adenovirus 2 major late promoter were fully transcribed as partially supercoiled templates. The sericin gene required more supercoiling for full transcription, whereas no preference for supercoiling was seen with the transcription of *hsp70*. These results suggest that DNA topology plays a role in the regulation of gene expression.**

Several lines of circumstantial evidence suggest the importance of DNA topology in eukaryotic gene expression (for reviews see refs. 1 and 2). Portions of chromatin in transcriptionally active states are hypersensitive to DNase I (3). Most DNase-I-hypersensitive regions contain S1-nuclease-sensitive sites (4) that are also found in supercoiled plasmids carrying the corresponding regions of DNA. These results are consistent with the hypothesis that chromatin DNA in the hypersensitive region is superhelical. DNase-hypersensitive regions contain binding sites for DNA topoisomerase II (5, 6). Topoisomerase II is found in the nuclear matrix or scaffold (7) to which chromatin loops are anchored (8, 9). These observations suggest that topoisomerase II might have an important role in the regulation of the topology of chromatin loops. Finally, when introduced into living cells, supercoiled DNA is more highly expressed than linear DNA (10–13). However, there is some evidence that topological tension is not important in eukaryotic gene expression. For example, the experimental results (14) suggesting that a transcriptionally active subportion of simian virus 40 minichromosomes has a torsional strain could not be reproduced by another group (15). Novobiocin alters gene expression (13, 16, 17), but the interpretation of these results is complicated by the nonspecific nature of the drug action (18). Worcel and colleagues (19–21) suggested that plasmid DNA carrying the *Xenopus* 5S RNA gene is assembled into a supercoiled, transcriptionally active chromatin in oocyte extracts. But their experiments could not be reproduced by another group (22).

To examine the possibility that a torsional stress on DNA has a direct effect on transcription, we have analyzed the *in vitro* transcription of the *Bombyx mori* fibroin gene in linear and covalently closed circular (ccc) forms. In a posterior silk gland (PSG) extract, ccc DNA takes on compact topological features and supports 3–10 times more transcription when compared to linear DNA (23). A correlation between this preferential transcription of ccc template and the extent of supercoiling could be observed in these PSG extracts (ref. 23; S.H., unpublished observations). Here we describe the fractionation of the PSG extract and the partial characterization of the activities that stimulate transcription of ccc templates. Two protein fractions are identified that are required to selectively stimulate transcription of ccc template in a HeLa cell extract and that constitute a DNA gyrase-like activity. By using these fractions, we have been able to manipulate the equilibrium between supercoiled and relaxed DNA and show that the fibroin gene and several type II genes were transcribed under various degrees of supercoiling. Interestingly, the rate of transcription of various genes responds differently to changes in template superhelicity. Thus DNA topology appears to play a role in the expression of some eukaryotic genes.

MATERIALS AND METHODS

Plasmids and Phages. The following plasmids were used: for the *B. mori* fibroin gene, pFb205 (23); for the *B. mori* sericin gene, pSr100 (24); and for the human adenovirus type 2 major late promoter (Ad2 MLP), pAd500 (24), and for the *Drosophila hsp70*, p56H8RIA (25). Single-stranded phage clones of the fibroin gene (f1Fb38) and the sericin gene (M13Sr1) have been described (23). M13Ad4 carrying the coding strand of the Ad2 MLP was obtained by inserting the small *Eco*RI–*Sal* I fragment of pAd500 between the *Eco*RI and *Sal* I sites of M13mp11. M13hsp70, which contains the coding strand of *hsp70*, was subcloned from p56H8RIA by inserting the 0.66-kilobase *Xba* I–*Bal* I fragment between the *Xba* I and *Hin*cII sites of M13mp11.

***In Vitro* Transcription.** Preparation of the silk gland cell extract (26) and the HeLa cell extract (27) have been described. A cell extract was also prepared from the *Antheraea* silkworm cell line Bm36 (28). *In vitro* transcription (12.5-μl reaction mixture) was performed as described (23) except that the ATP concentration was 600 μM. Supercoiled circular plasmid DNA was used as a template either directly or after it had been linearized with *Xho* I (pFb205), *Bam*HI

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Abbreviations: ccc, covalently closed circular; PSG, posterior silk gland; Ad2 MLP, adenovirus type 2 major late promoter.

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(pSr100), *Pvu* II (pAd500), or *Hin*dIII (p56H8RIA). The DNA concentration in the reaction mixture was 32 μg/ml. Protein concentrations of the PSG and HeLa cell extracts in the reaction mixtures were 7.5 mg/ml and 5 mg/ml, respectively.

Modified S1-Nuclease Assay. Amounts of the transcripts were measured by the modified S1-nuclease assay as described (23). The protected bands of faithful transcripts should have the following sizes: 510 base pairs (bp) (fibroin gene), 165 bp (sericin gene), 308 bp (Ad2 MLP), or 412 bp (*hsp70*).

Topology of Template DNA. ^{32}P-labeled relaxed ccc DNA was prepared as described (23). The labeling was done at the *Eco*RI (pFb205 and pSr100), *Sal* I (pAd500), or *Hin*dIII site (p56H8RIA). The labeled DNA (0.5 μg/ml) was incubated in a 12.5-μl reaction mixture under the conditions for transcription described above. Whenever the reaction mixture contained PSG or HeLa cell extract, poly(dI-dC)·poly(dI-dC) (8 μg/ml) was added to increase bulk DNA concentration. Protein concentrations of the PSG and HeLa cell extract in the reaction mixture were 7.5 mg/ml and 5 mg/ml, respectively. After incubation at 30°C for 60 min, 0.5% $NaDodSO_4$ and proteinase K at 250 μg/ml were added and further incubated at 37°C for 30 min. DNA was recovered by phenol treatment, ethanol precipitation, and electrophoresis through a 1% agarose gel. Two-dimensional electrophoresis was performed as described (29) except that ethidium bromide at 5 ng/ml was used instead of chloroquine. The gel was soaked in ethanol for 30 min, dried, and subjected to autoradiography. DNA topoisomers containing various linking numbers were prepared as described (29) and used as a marker in the two-dimensional electrophoresis.

Assay for DNA Topoisomerases. DNA topoisomerase I was assayed as described by Liu (30). Supercoiled circular pFb205 DNA was used as a substrate. To suppress DNA topoisomerase II, $MgCl_2$ was omitted from the reaction mixture, and EDTA was added to 2 mM. DNA topoisomerase II activity was measured by the "unknotting" of "knotted" P4 DNA (31). (Knotted DNA is an intramolecularly catenated DNA.)

Phosphocellulose Chromatography. PSG extract (≈400 mg of protein) was loaded onto a column (2.4 cm × 6.6 cm) of Whatman P11 equilibrated with buffer A [20 mM Hepes (pH 7.9), 1 mM EDTA, 20% (vol/vol) glycerol, and 1 mM dithiothreitol] containing 0.04 M KCl at a flow rate of ≈30 ml/hr. After washing with 60 ml of the same buffer (flow-through fraction A), the column was successively step-eluted with 90 ml of buffer A/0.35 M KCl (fraction B), 60 ml of buffer A/0.6 M KCl (fraction C), and 60 ml of buffer A/1 M KCl (fraction D). The fractions B–D were dialyzed against 100 vol of buffer A/0.1 M KCl for 2 hr with one change of the buffer and stored at −80°C. Typically, fractions A, B, C, and D contained 250 mg, 54 mg, 25 mg, and 3.8 mg of protein, respectively.

RESULTS

Fractionation of Activity That Stimulates Preferential Transcription of ccc Templates. In a PSG extract, the ccc form of the fibroin gene supported more efficient transcription than did the linear form of the gene (23). This efficient transcription was mediated by the sequence around the "TATA box" region. A HeLa cell extract also transcribed the fibroin gene but did not have a preference for the ccc form (ref. 23; see also Fig. 1, lanes 1 and 2). If the HeLa cell extract was mixed with the PSG extract, the preference for ccc DNA was still observed (data not shown). Thus the HeLa cell extract did not contain inhibitors. By using this observation, we designed a complementation assay for the purification of the stimulatory factors in PSG extract.

Four fractions were isolated from the PSG extract by phosphocellulose chromatography. When all four fractions (fractions A–D) were added to the HeLa cell extract, a preference for the transcription of ccc DNA over linear DNA was clearly seen (3-fold; Fig. 1, lanes 3 and 4). The omission of fraction B did not alter the result (3-fold; lanes 5 and 6). Omission of fractions B and D also did not change the preference for ccc DNA; in fact the stimulation was greater (5-fold; lanes 7 and 8). When either fraction A or fraction C was added to the HeLa cell extract, the preference for ccc DNA was diminished (1.5-fold for fraction A and 1.1-fold for fraction C, lanes 9–12). These results suggest that both fractions A and C are required for the preferential transcription of the ccc template in addition to the basic transcription machinery supplied from the HeLa cell extract.

DNA Topoisomerase Activities of Phosphocellulose Fractions. Since DNA topology is responsible for the efficient transcription of ccc DNA in the PSG extract (23), it is most likely that the phosphocellulose fractions A and C stimulate transcription by changing the DNA morphology in the complementation assay. Thus we analyzed DNA topoisomerase activities of the phosphocellulose fractions. Fig. 2*a* shows the results of an assay of topoisomerase I as measured by the relaxation of supercoiled DNA. Only fraction D contains topoisomerase I activity (lanes 8 and 9). Fig. 2*b* shows a specific assay of topoisomerase II that relies on the unknotting of knotted P4 DNA. The topoisomerase II activity was found in fraction C (lane 4). When relaxed ccc pFb205 DNA was incubated with the phosphocellulose fractions, topoisomers with higher mobilities appeared only in fraction C (Fig. 2*c*, lane 4). These compact forms of molecules can be due to two types of DNA topology; one is knotted and the other is supercoiled. To discriminate between these possibilities, the sample was analyzed by two-dimensional electrophoresis (Fig. 3). The starting material was a mixture of relaxed ccc pFb205 DNA and its nicked-circular form, which was produced by radioactive decay of ^{32}P during storage (Fig. 3*a Lower*). After incubation with fraction C, the radioactive spots (Fig. 3*b Lower*) did not correspond to the supercoiled marker DNA (Fig. 3*b Upper*). Instead, a mixture of knotted ccc DNA and knotted nicked-circular DNA of various degrees of knotting was formed. The knotted ccc forms were deduced by their conversion into knotted nicked circular form upon treatment with a small amount of DNase I (data not shown). These data suggest that the compact form of DNA found after incubation with fraction C is a knotted molecule produced by the action of topoisomerase II. The addition of fraction B or D to fraction C did not alter the result (data not shown). However, if relaxed ccc pFb205 DNA was incubated with both

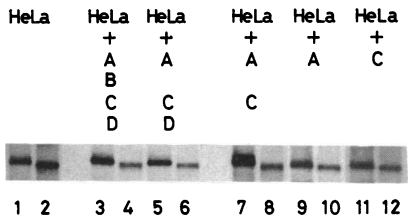

FIG. 1. Complementation assay for the activity that preferentially stimulates transcription of ccc DNA. Transcription of fibroin gene in a HeLa cell extract alone (lanes 1 and 2) or in the same extract supplemented with fractions A–D as indicated (lanes 3–12). Protein added was as follows: fraction A, 10 μg; fraction B, 5 μg; fraction C, 2 μg; and fraction D, 0.5 μg. Input pFb205 template DNA was in the supercoiled circular form (lanes 1, 3, 5, 7, 9, and 11) or in the linear form (lanes 2, 4, 6, 8, 10, and 12). The size of the protection bands produced from ccc templates was slightly larger than that from linear ones, because trimming of the unhybridized region of RNA by nuclease S1 would not reach completion.

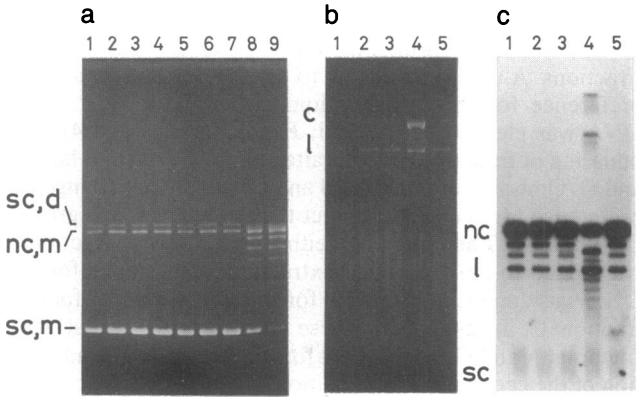

FIG. 2. DNA topoisomerase activities of the phosphocellulose fractions. (*a*) DNA topoisomerase I. Reaction mixtures (20 μl) contained the following fractions. Lanes: 1, none; 2, 2.5 μg of fraction A; 3, 10 μg of fraction A; 4, 1.25 μg of fraction B; 5, 5 μg of fraction B; 6, 0.5 μg of fraction C; 7, 2 μg of fraction C; 8, 0.13 μg of fraction D; 9, 0.5 μg of fraction D. SC, supercoiled circular DNA; nc, nicked circular DNA; m, monomer; d, dimer. (*b*) DNA topoisomerase II. Reaction mixtures (20 μl) contained the following fractions. Lanes: 1, none; 2, 10 μg of fraction A; 3, 5 μg of fraction B; 4, 2 μg of fraction C; 5, 0.5 μg of fraction D. Positions of unit length circular (c) and linear DNA (l) are indicated. (*c*) Relaxed ccc pFb205 DNA was incubated with the fraction, and the topology was analyzed. Fractions added were as follows. Lanes: 1, none; 2, 10 μg of fraction A; 3, 5 μg of fraction B; 4, 2 μg of fraction C; and 5, 0.5 μg of fraction D. Positions of supercoiled circular (sc), nicked circular (nc), and linear (l) DNA are indicated. The bands above nicked circular DNA are concatemers and their topoisomers.

fractions A and C, supercoiling as well as knotting occurred. Thus, there appeared a complex mixture of knotted and supercoiled DNA with various degrees of knotting and coiling (Fig. 3*c Lower*). In this reaction, fraction C could be replaced by purified topoisomerase II from *B. mori* (S.H. and H. Tabuchi, unpublished data). The supercoiling was probably not caused by restrained supercoils produced by chromatin assembly. (*i*) Neither the fractions A and C nor the PSG extract contains a detectable level of histones. (*ii*) ccc DNA incubated under the supercoiling conditions did not give the characteristic ladder of chromatin DNA upon micrococcal nuclease digestion (data not shown). (*iii*) Supercoils produced by fractions A and C could be relaxed by further addition of topoisomerase I (S.H. and H. Tabuchi, unpublished results). These results suggest that the DNA supercoiling factor in fraction A and purified topoisomerase II can introduce negative supercoils into ccc DNA.

DNA Supercoiling in HeLa Cell Extract Supplemented with Phosphocellulose Fractions. When relaxed ccc pFb205 DNA was incubated in the HeLa cell extract, knotting but not supercoiling occurred (Fig. 4*a*). However, if both fractions A and C were added to the HeLa cell extract, supercoiling as well as knotting occurred (Fig. 4*b*). In this case, simple supercoiling was evident, but knotted and supercoiled molecules were scarcely seen. When fraction A alone or fractions A, C, and D were added to the extract, the supercoiling was less prominent (data not shown). Though the HeLa cell extract contained a topoisomerase II activity, the addition of fraction C was required for the extensive supercoiling and efficient transcription of ccc DNA in the presence of fraction A. As described above, further addition of fraction D was inhibitory to the supercoiling and to the preferential transcription of ccc DNA. This is probably due to the topoisomerase I activity in fraction D that can relax supercoils. These results suggest that the supercoiling is responsible for the efficient transcription of ccc DNA in the HeLa cell extract supplemented with the fractions A and C (Fig. 1).

DNA Supercoiling in PSG Extract Supplemented with Phosphocellulose Fractions. It has been suggested that the topology of ccc DNA in the PSG extract is determined by an equilibrium between the relaxed and supercoiled DNA (23). Thus the addition of fractions A and C, which contain the supercoiling activity, to the PSG extract, should shift the equilibrium toward supercoiled DNA. When relaxed ccc pFb205 DNA was incubated with PSG extract alone, supercoiled pFb205 DNA was detected extending from knotted ccc DNA containing various degrees of knotting (Fig. 5*a*). Other PSG extracts with higher preferential activity for ccc templates had more prominent supercoiling (data not shown). When 5 μg of fraction A and 1 μg of fraction C were added to the reaction mixture containing the PSG extract, more supercoiling occurred than in the extract alone (Fig. 5*b*). If 10 μg of fraction A and 2 μg of fraction C were added, further supercoiling occurred (Fig. 5*c*). Note that most of the spots of knotted and supercoiled DNA exist below the array of knotted nicked circular DNA in the diagonal (Fig. 5*c*). Thus, by varying the amounts of fractions A and C, we changed the superhelicity of the template DNA. This effect was not specific to fibroin gene DNA. Similar results were obtained with *B. mori* sericin DNA, *Drosophila hsp70* DNA, and human Ad2 MLP DNA (data not shown).

Transcription Activity of Templates with Various Degrees of Supercoiling. We analyzed the effect of supercoiling on the *in vitro* transcription of fibroin gene and several other type II genes. Fig. 6*a* shows the results of the fibroin gene transcrip-

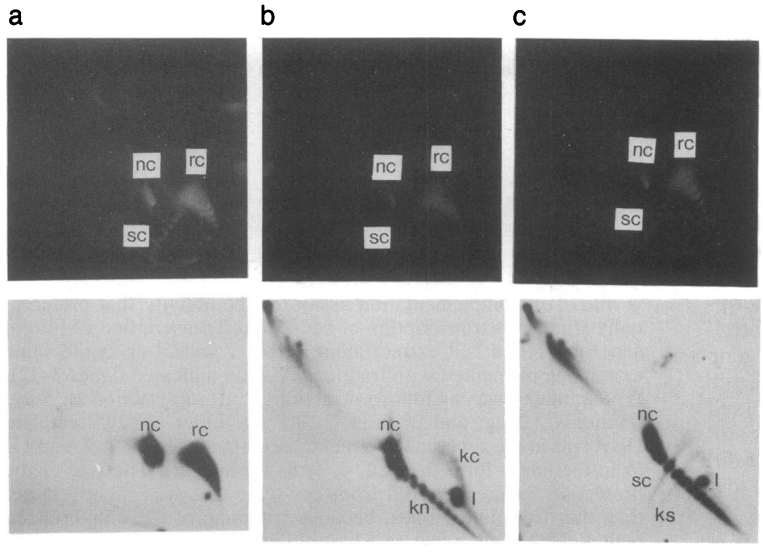

FIG. 3. Knotting and supercoiling by the phosphocellulose fractions. Radioactive relaxed ccc pFb205 DNA (*a*) was incubated with 2 μg of the fraction C (*b*) or a mixture of 10 μg of the fraction A and 2 μg of the fraction C (*c*), and topology was analyzed by two-dimensional electrophoresis after addition of the pFb205 marker DNA (1 μg) with various extents of supercoiling. The first dimension was from the top to the bottom, and the second dimension was from the left to the right. (*Upper*) Distribution of the marker DNA stained with ethidium bromide. (*Lower*) Autoradiograms. rc, Relaxed ccc DNA; kc, knotted ccc DNA with various degrees of knotting; nc, nicked circular DNA; kn, knotted nicked circular DNA with various degrees of knotting; sc, supercoiled circular DNA with various degrees of coiling; ks, knotted and supercoiled DNA with various degrees of knotting and coiling; l, linear DNA.

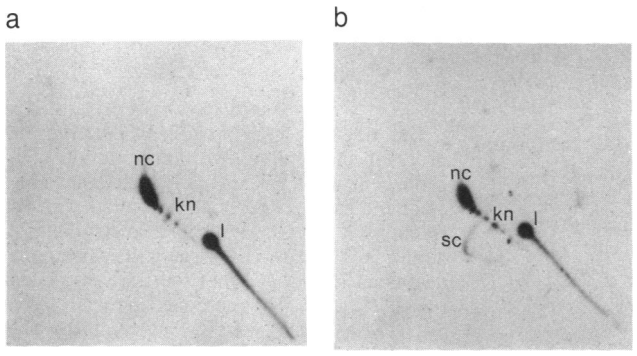

FIG. 4. Knotting and supercoiling in the HeLa cell extract supplemented with the phosphocellulose fractions. Radioactive relaxed ccc pFb205 DNA was incubated in the HeLa cell extract alone (*a*) or in the same extract supplemented with 10 μg of the fraction A and 2 μg of the fraction C (*b*), and topology was analyzed as in Fig. 3. Only autoradiograms are shown. DNAs are identified as in Fig. 3.

tion. In this PSG extract, fibroin gene transcription on ccc template was 4-fold higher than that on a linear template. When 5 μg of fraction A and 1 μg of fraction C were added to the PSG extract, more supercoiling (Fig. 5*b*) and more transcription (Fig. 6*a*, lane 4) occurred than in the extract alone (Figs. 5*a* and 6*a*, lane 3). However, further supercoiling by the addition of 10 μg of fraction A and 2 μg of fraction C (Fig. 5*c*) did not further increase the transcription (Fig. 6*a*, lane 5 versus lane 4). In the case of linear DNA, the addition of the fractions A and C did not give a significant change in the transcription activity (Fig. 6*a*, lane 2 versus lane 1). The overall stimulation of transcription by supercoiling was ≈20-fold (compare lane 4 with lane 2 of Fig. 6*a*). In a PSG extract, Ad2 MLP transcription is also more efficient on a ccc template (4-fold; Fig. 6*b*, lane 3 versus lane 1). When 5 μg of fraction A and 1 μg of fraction C were added to the transcription reaction, there was a 4-fold stimulation of transcription (compare Fig. 6*b* lane 4 with lane 3). No further increase of Ad2 MLP transcription was observed when 10 μg of fraction A and 2 μg of fraction C were used. Note that the transcription of Ad2 MLP on a linear template did not change significantly when 10 μg of fraction A and 2 μg of fraction C were supplemented to the PSG extract (lane 2 versus lane 1). Therefore, the transcription of Ad2 MLP is affected by the degree of DNA supercoiling in a manner similar to that of fibroin gene. In the case of sericin gene, both transcriptions on linear and ccc DNA were low and at almost the same level if incubated in the PSG extract alone (Fig. 6*c*, lane 1 versus lane 3; see also ref. 23). Upon addition of fractions A and C, transcription on ccc DNA increased concomitantly with the increase of negative supercoiling (lanes 4 and 5), whereas that on linear DNA was not affected (lane 2). Thus, the sericin gene requires more supercoiling for full activity than the fibroin gene or Ad2 MLP. In contrast to the results of these genes, no preference for supercoiled DNA was observed in *hsp70* transcription (Fig. 6*d*). From these results, we conclude that the *in vitro* transcription of eukaryotic genes is differentially affected by the degree of DNA supercoiling.

DISCUSSION

In a PSG extract, fibroin gene transcription is more efficient on a ccc template (23). This preferential transcription is not unique to the PSG extracts; a similar preference of ccc template can also be observed in a middle silk gland extract (23) as well as an extract from the cultured cell line Bm36 (S.H., unpublished data). However, in a HeLa whole cell extract, we could not observe such difference (23). The transcription of a human histone H2B gene (32) and a human immunoglobulin κ light-chain gene (33) has been found to be more efficient on a supercoiled template in HeLa cell nuclear extracts. In the case of the immunoglobulin gene, its cell-type-specific transcription in a B-cell extract is highly dependent on template topology (33). As reported here, the transcription of Ad2 MLP is also more efficient on a ccc template in a PSG extract. Therefore, we believe that the effect of DNA topology on transcription is a general phenomenon in most cell types. The lack of preference in our HeLa cell extract is most likely due to the loss of some crucial components during preparation. Here, by using a refractory HeLa cell extract as a basis for the *in vitro* transcription reaction, we have fractionated and identified the transcription stimulatory activities for ccc template from the PSG extract. Two protein fractions, the flow-through (fraction A) and the 0.6 M KCl eluate (fraction C), were required for the selective stimulation of fibroin gene transcription on ccc template. These two fractions were found to have a supercoiling factor and a DNA topoisomerase II activity, respectively. The supercoiling factor can introduce negative supercoils into ccc DNA with the aid of topoisomerase II. This negative supercoiling is not likely due to chromatin assembly, because, under our assay conditions, chromatin assembly does not take place and because the supercoils have torsional stress.

Our experiments strongly support the hypothesis that supercoiling of the fibroin gene template is necessary for enhanced transcription. (*i*) In PSG extracts, the preference of ccc template is correlated with the extent of supercoiling (ref. 23; S.H., unpublished data). In a HeLa cell extract, where the preference of ccc template is not observed, knotting but not supercoiling takes place (Fig. 4*a*). We have interpreted this knotting as a sign of supercoiling (see the discussion in ref. 23). (*ii*) The "rescue" of supercoiling of ccc templates in the HeLa cell extract by the addition of fractions A and C results in the preferential transcription of ccc fibroin genes. (*iii*) Increasing supercoiling in a PSG extract by the addition of fractions A and C further enhances the transcription of fibroin gene. This concomitant activation of transcription and supercoiling was also observed when a highly purified supercoiling factor and a purified topoisomerase II were used (S.H., unpublished data). Thus the suggestion that the stimulation of ccc template transcription was due to transcription factors in fractions A and C is

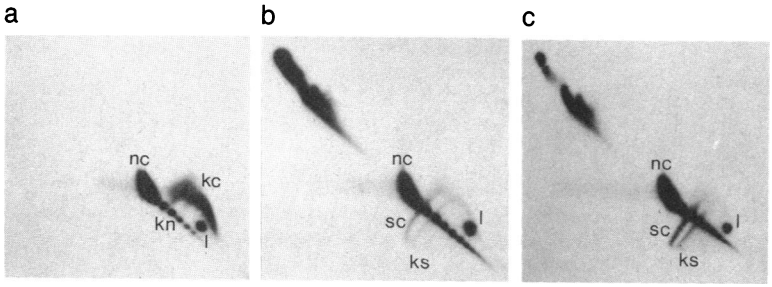

FIG. 5. Knotting and supercoiling in the PSG extract supplemented with the phosphocellulose fractions. Radioactive relaxed ccc pFb205 DNA was incubated in the PSG extract alone (*a*), the same extract supplemented with 5 μg of the fraction A and 1 μg of the fraction C (*b*), or the same extract supplemented with 10 μg of the fraction A and 2 μg of the fraction C (*c*). DNAs are identified as in Fig. 3.

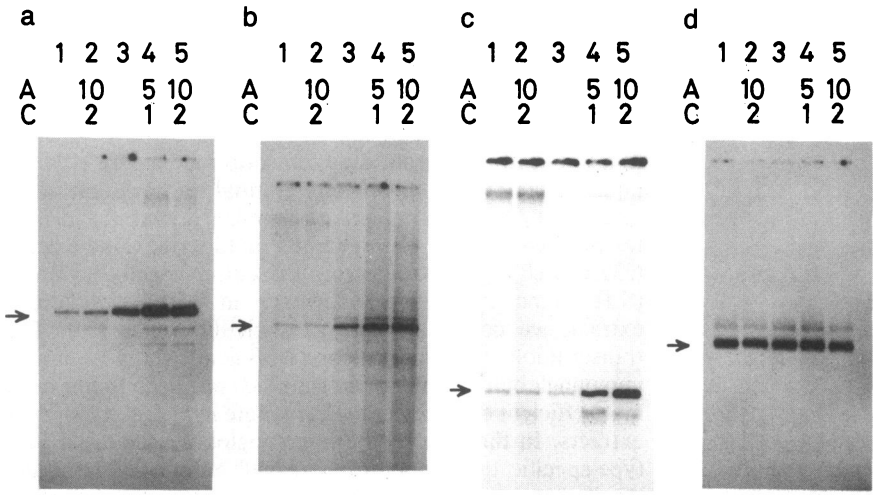

FIG. 6. Transcription under the various degrees of supercoiling. (*a*) Transcription of fibroin gene in the PSG extract alone (lanes 1 and 3) or the same extract supplemented with the fractions A and C as indicated (in μg) (lanes 2, 4, and 5). The extract used was the same batch described in Fig. 5. Input template was linear (lanes 1 and 2) or supercoiled circular (lanes 3–5) pFb205 DNA. Arrow indicates the protected bands of the faithful transcripts after S1-nuclease digestion. (*b–d*) Transcription of Ad2 MLP from pAd500 (*b*), sericin gene from pSr100 (*c*), or *hsp70* from p56H8RIA (*d*) is shown. Lanes 1–5 are as in *a* except for the plasmid used.

unlikely. Furthermore, there appears to be a tight linkage between supercoiling and transcription.

How does DNA supercoiling stimulate transcription? The effect of supercoiling might facilitate the formation of active template by increasing the affinity, stability, or both of some DNA–protein or protein–protein interactions (33, 34). *In vitro* as well as *in vivo*, the appropriate interaction of limiting factors with their binding sites is determined by these parameters. The addition of fraction D decreases the preferential transcription of the ccc form of the fibroin gene. In addition to topoisomerase I, which can interfere with supercoiling, fraction D contains high concentrations of factors that are crucial or rate-limiting for *in vitro* transcription (S. Takiya and Y.S., unpublished results). These observations suggest that DNA supercoiling probably helps to assemble the active template under rate-limiting conditions. We have analyzed the effect of DNA supercoiling on the *in vitro* transcription of the fibroin gene and several type II genes (Fig. 6). Partially supercoiled fibroin gene and Ad2 MLP are transcribed maximally, whereas maximal transcription of the sericin gene requires a more supercoiled state. These results are essentially the same when highly purified supercoiling factor and purified topoisomerase II are used instead. However, *hsp70* is actively transcribed regardless of whether the template is linear or ccc DNA. This implies that the topology of *hsp70* is not important for its transcription *in vitro*. Thus various promoters require various amounts of supercoiling for optimal function.

We thank Dr. S. Takiya and Mr. C. C. Hui for their comments on the manuscript, Dr. T. Enomoto for the gift of P4 DNA, and Mrs. E. Suzuki and M. Sasaki for their technical assistance. This research was partly supported by Grants-in-Aid for Special Research Projects and for Cancer Research from the Ministry of Education, Science, and Culture, and a Life Science Research Grant from the Institute of Physical and Chemical Research.

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