Negative supercoiling of DNA facilitates an interaction between transcription factor IID and the fibroin gene promoter

(DNA topoisomerase II/supercoiling factor/initiation complex)

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ABSTRACT Transcription of the fibroin gene can be reconstituted with partially purified components from HeLa cells. Transcription factors IID, IIB, and IIE and RNA polymerase II are required for accurate initiation of transcription. Linear and relaxed closed circular DNA show a similar level of template activity. However, transcription of closed circular DNA is stimulated when negative supercoils are introduced by the addition of DNA topoisomerase II and supercoiling factor purified from the posterior silk gland of Bombyx mori. Dissection of transcription into pre- and postinitiation steps by the use of Sarkosyl reveals that DNA supercoiling facilitates formation of a preinitiation complex. Furthermore, order of addition experiments suggest that DNA supercoiling facilitates a functional binding of transcription factor IID to the promoter.

The functional analysis of eukaryotic protein-coding genes has revealed a set of transcriptional control regions. These include core promoter elements, upstream elements, and enhancer elements (1, 2). The core promoter consists of the TATA box and its flanking sequence, which is required for fixing the start site as well as efficient transcription (1, 3). The upstream elements and enhancer elements serve as recognition sites for specific DNA-binding proteins (2, 4). These elements fulfill their function by modulating the utilization of the core promoter.

Besides being regulated by trans-acting factors, the activity of the core promoter is thought to be modulated independently by DNA supercoiling. In support of this notion, we have shown that transcription of certain eukaryotic genes in a posterior silk gland extract is activated by supercoiling of template DNA (5–7). The rate of transcription of various genes responds differently to changes in template superhelicity. For example, the Bombyx mori fibroin gene and the human adenovirus 2 major late promoter are fully transcribed on partially supercoiled templates, whereas the B. mori sercin gene requires more supercoiling for maximum transcription (6). When transcription of the fibroin gene in the posterior silk gland extract is separated into preinitiation and initiation/elongation steps on the basis of sensitivity to Sarkosyl, formation of a preinitiation complex on the promoter appears to be a slow process, which is accelerated by DNA supercoiling. In contrast, the subsequent steps are rapid and unaffected by DNA topology. From these results, we have proposed that DNA supercoiling enhances fibroin gene transcription by facilitating assembly of the preinitiation complex (8).

At least four components—transcription factors TFIIIB, TFIIID, and TFIIIE and RNA polymerase II—have been implicated in the formation of the preinitiation complex (9–12). TFIIID is the only one of these factors that has been shown to interact directly with the core promoter (13, 14). To examine which factor is responsible for rapid assembly of the complex on supercoiled templates, these components were partially purified from HeLa cell nuclear extract. We have also purified DNA topoisomerase II and supercoiling factor from the posterior silk gland of B. mori; these proteins are required for negative supercoiling of DNA (15). Analyses of the fibroin gene transcription with these components suggest that DNA supercoiling facilitates interaction between TFIIID and the promoter.

MATERIALS AND METHODS

DNA. The DNA used in the transcription and the supercoiling experiments was the plasmid pFB205, which carries the fibroin DNA segment HindIII-Pvu II (nucleotide positions −860 to +728) (5). Unlabeled and 32P-labeled relaxed closed circular DNA and Xho I-cut linear DNA were prepared as described (5).

Other Materials. Unlabeled nucleotides, Sarkosyl (N-lauroylsarcosine sodium salt), polyvinyl alcohol (average molecular weight 10,000), and S1 nuclease were purchased from Sigma. [α-32P]UTP was from Amersham. Heparin-Sepharose CL-6B, DEAE-Sepharose CL-6B, Mono S, and RNase/DNase-free bovine serum albumin were from Pharmacia–LKB Biotechnology. Phosphocellulose (P11) and DEAE-cellulose (DE52) were from Whatman.

Fractionation of Transcription Factors. HeLa cell nuclear extract was prepared according to the method of Dignam et al. (16). The nuclear extract from 40 liters of culture was chromatographed on a heparin-Sepharose column as described by Watanabe et al. (17). The 0.4 M KCl eluate fraction of the heparin-Sepharose column was further fractionated through a column of DEAE-Sepharose as described (17). The 0.1 M KCl eluate fraction of the DEAE-Sepharose column was used as TFIIIB.

A 1-ml column of P11 was made and equilibrated with buffer A [20 mM Hapes–NaOH, pH 7.9/1 mM EDTA/1 mM dithiothreitol/20% (vol/vol) glycerol] containing 0.1 M KCl. The column was successively washed with 1 ml of buffer A containing 0.1 M KCl and bovine serum albumin at 0.2 mg/ml, 5 ml of buffer A containing 1 M KCl, and 5 ml of buffer A containing 0.1 M KCl. The 0.225 M KCl eluate fraction of the DEAE-Sepharose column was dialyzed against buffer A containing 0.1 M KCl for 2 hr and applied to the P11 column. The column was washed with 3 ml of buffer A containing 0.1 M KCl and then proteins were eluted stepwise with 3 ml of buffer A containing 0.35 M, 0.6 M, and 1 M KCl. The 0.6 M and 1 M KCl eluate fractions were used as TFIIIE and TFIIID, respectively, after addition of bovine serum albumin to 0.2 mg/ml followed by dialysis against buffer A containing 0.1 M KCl for 2 hr. RNA polymerase II was prepared from HeLa cell nuclear pellets as described by

Abbreviation: TF, transcription factor.

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Reinberg and Roeder (18). All preparations were frozen in liquid nitrogen and stored at −80°C. The preparation of TFII E contained a DNA topoisomerase II activity. The preparations of TFII B, TFII D, and RNA polymerase II were virtually free from DNA topoisomerase activities. Yeast TFII D was expressed from the cloned gene in Escherichia coli and the flow-through fraction of DEAE-cellulose column was prepared from cell lysate as described (19). Starting from the DEAE flow-through, yeast TFII D was purified to homogeneity by gel filtration and chromatography on a Mono S column as described (20).

Transcription Reaction. Standard reaction mixtures (12.5 μl) contained 12 mM Hepes–NaOH at pH 7.9, 60 mM KCl, 0.6 mM EDTA, 7.5 mM MgCl2, 0.6 mM dithiothreitol, 12% glycerol, 2% polyvinyl alcohol, 0.6 mM each of ATP, CTP, and GTP, 10 μM [α-32P]UTP (8–16 × 10^4 cpm/pmol), 100 ng of DNA, 1.5 μl of TFII B, 1.5 μl of TFII D, 1.5 μl of TFII E, and 2 units (as defined in ref. 17) of RNA polymerase II. The reaction mixtures also contained 0.1 μg of purified B. mori DNA topoisomerase II (21) and 0.2 μg of purified DNA supercoiling factor (15) if indicated. After incubation at 30°C for 60 min, RNA was extracted and analyzed by the modified S1 nuclease assay as described previously (5).

Complex Formation Reaction. Two-stage reactions for transcription were carried out at 30°C. Standard preincubation mixtures (10 μl) contained 15 mM Hepes–NaOH at pH 7.9, 75 mM KCl, 0.75 mM EDTA, 9.4 mM MgCl2, 0.75 mM dithiothreitol, 15% glycerol, 2.5% polyvinyl alcohol, 0.25 mM ATP, 100 ng of DNA, 1.5 μl of TFII B, 1.5 μl of TFII D, 1.5 μl of TFII E, and 2 units (as defined in ref. 17) of RNA polymerase II. After preincubation for various times as described in the legends to figures, nucleotides and, if necessary, Sarkosyl in water were added in 2.5 μl. Standard mixtures for the second incubation (12.5 μl) contained 200 μM each of CTP and GTP, and 10 μM [α-32P]UTP (8–16 × 10^4 cpm/pmol) in addition to the ingredients carried from the preincubation. After incubation for 10 min, RNA was extracted and analyzed by the modified S1 nuclease assay as described (5). The specific transcripts were visualized by autoradiography and excised, and their radioactivities were measured.

Topology of DNA. [32P]-labeled relaxed closed circular DNA was incubated in the standard reaction mixtures for transcription except that unlabeled UTP was used in place of [α-32P]UTP. Total amount of DNA in the reaction was adjusted to 100 ng by the addition of unlabeled linear DNA. After incubation at 30°C for 60 min, DNA was extracted and analyzed by two-dimensional electrophoresis as described previously (6).

Protein. Protein concentration was determined as described by Bradford (22), with bovine serum albumin as a standard.

RESULTS

DNA Supercoiling Stimulates Transcription Reconstituted with Partially Purified Components. General transcription factors required for accurate initiation of transcription by RNA polymerase II were separated from a cell-free extract (Fig. 1A). We chose a nuclear extract of HeLa cells as a starting material for the following reasons. First, processes leading to transcription by HeLa cell factors are well characterized (9–12, 23, 24). Second, HeLa cell extracts contain enough factors to support a basal level of transcription from the fibroin gene promoter (ref. 25; M.M. and S.H., unpublished observation). Third, topology of DNA in the transcription reaction by HeLa cell factors should depend on the DNA supercoiling activity exogenously added because the activity is not detectable in HeLa cell extracts (6).

The first step of the fractionation was heparin-Sepharose chromatography. Transcription factors were eluted with 0.4 M KCl while a strong activity of DNA topoisomerase I was still retained on the column. It is essential to remove DNA topoisomerase I at an early step because the enzyme interferes with the DNA supercoiling activity (8, 15). The 0.4 M KCl eluate was further fractionated by a DEAE-Sepharose column. Both flow-through and the 0.225 M KCl eluate from this column are required for transcription. The active component in the flow-through fraction is thought to be TFII B from its chromatographic behavior (16). The 0.225 M KCl eluate was further fractionated by phosphocellulose chromatography. The component contained in the 0.6 M KCl eluate corresponds to TFII E (16). Complementation assay using a heat-treated nuclear extract (14) reveals the presence of TFII D in the 1 M KCl eluate. Components TFII B, TFII D, TFII E, and RNA polymerase II are essential for transcription from the fibroin gene promoter (Fig. 1B).

We analyzed the topology of DNA in the reconstituted transcription system by two-dimensional gel electrophoresis (Fig. 2A). In this gel, positively supercoiled DNA migrates faster than relaxed closed circular DNA, while negatively supercoiled DNA runs slower than relaxed closed circular DNA in the second dimension (6). When relaxed closed circular DNA was incubated with transcription components, little change was detected in the conformation of DNA except for the appearance of catenanes near the origin of electrophoresis (Fig. 2A, sample 1). Inclusion of B. mori DNA topoisomerase II did not alter the result (Fig. 2A, sample 2). However, negative supercoiling of DNA occurred upon the addition of purified DNA supercoiling factor (Fig. 2A, sample 3). The factor has been isolated from the posterior silk gland of B. mori and shown to introduce unconstrained negative supercoils into closed circular DNA in conjunction with eukaryotic DNA topoisomerase II (15). The observed supercoiling is most likely achieved by a combination of DNA topoisomerase II present in our preparation of TFII E and the exogenously added supercoiling factor. Further addition of DNA topoisomerase II increased the degree of supercoiling (Fig. 2A, sample 4), indicating that the amounts of endoge-
biochemistry, reaction analyzed by topology-affecting Sarkosyl coiling of relaxed closed circular the mixture fibroin gene 30 only Fig. ing coiling components transcription and relaxed closed transcription when was sion DNA nous DNA topoisomerase II are not enough to support maximum activity of supercoiling. In the presence of supercoiling factor and B. mori DNA topoisomerase II, supercoiling of DNA occurs rapidly. Thus, similar results as sample 4 of Fig. 2A were obtained when samples were incubated for only 30 s (data not shown). These results suggest that the topological state of DNA in the transcription reaction can be manipulated by using purified DNA topoisomerase II and supercoiling factor.

Next we examined the effect of DNA supercoiling on the fibroin gene transcription in the reconstituted system. Linear and relaxed closed circular DNA showed similar levels of transcription when the reaction mixtures contained only transcription components (Fig. 2B, lanes 1 and 2). However, transcription on closed circular DNA was enhanced if negative supercoils were introduced by the addition of DNA topoisomerase II and supercoiling factor (Fig. 2B, lane 3 versus lane 1). These topology-affecting components did not affect transcription on linear DNA (Fig. 2B, lane 4 versus lane 2). These results suggest that DNA supercoiling activates transcription of the fibroin gene in the reconstituted system.

DNA Superciling Accelerates Formation of a Preinitiation Complex. To separate transcription of the fibroin gene into preinitiation and initiation/elongation steps, we employed a two-stage reaction. First, DNA was preincubated with the partially purified transcription factors and RNA polymerase II. Then RNA synthesis was started by the addition of ribonucleoside triphosphates. Sarkosyl was added either before or after the preincubation stage. As shown in Fig. 3, 0.01% Sarkosyl abolished transcription when added before the preincubation (lane 2 versus lane 1) but only partially inhibited transcription when added after the preincubation (lane 4 versus lane 1). A higher concentration of Sarkosyl (0.05%) completely suppressed transcription even when added after the preincubation (lane 5 versus lane 1). The experiments shown in Fig. 3 were performed by using closed circular DNA as templates. But linear DNA gave essentially the same results (data not shown). These results suggest that the preincubation allows formation of a preinitiation complex which is resistant to 0.01% Sarkosyl but still sensitive to 0.05% Sarkosyl. The concentration of Sarkosyl required to suppress the formation of the preinitiation complex on the fibroin gene promoter was 0.01% in the reconstituted system and 0.025% in the unfractionated extract (8). A similar shift in the concentration of Sarkosyl has been reported for transcription from the adenovirus 2 major late promoter (10, 26).

We compared the time course for assembly of the preinitiation complex on linear and closed circular DNA (Fig. 4). DNA was incubated with partially purified transcription...

Fig. 2. DNA topology affects transcription. (A) Negative supercoiling of DNA with DNA topoisomerase II and supercoiling factor. 32P-labeled relaxed closed circular DNA was incubated in the standard reaction mixture for analysis of DNA topology (sample 1) or in the mixture containing B. mori DNA topoisomerase II (Topo II, sample 2), DNA supercoiling factor (SCF, sample 3), or both of these topology-affecting components (sample 4). The DNA was recovered and analyzed by two-dimensional electrophoresis. The first dimension was from the top to the bottom, and the second dimension was from the left to the right. rc, Relaxed closed circular DNA; nc, nicked circular DNA; sc, negatively supercoiled DNA; ca, catenanes. (B) Effect of DNA topology on transcription. Relaxed closed circular DNA (lanes 1 and 3) or linear DNA (lanes 2 and 4) was incubated in the standard reaction mixture for transcription (lanes 1 and 2) or in the mixture supplemented with B. mori DNA topoisomerase II and supercoiling factor (lanes 3 and 4).

Fig. 3. Detection of preinitiation complex. Relaxed closed circular DNA was incubated in the standard preincubation mixture containing B. mori DNA topoisomerase II and supercoiling factor for 40 min. Indicated concentrations of Sarkosyl were added before (lanes 2 and 3) or after (lanes 4 and 5) the preincubation. After addition of nucleotides, the second-stage incubation was carried out and the reaction products were analyzed. PolII, RNA polymerase II; TopoII, B. mori DNA topoisomerase II; SCF, DNA supercoiling factor; NTP, ribonucleoside triphosphate.

Fig. 4. Time course of formation of preinitiation complex. Relaxed closed circular DNA (○, ●) or linear DNA (△, ▲) was incubated in the standard preincubation mixture with (●, ▲) or without (○, △) B. mori DNA topoisomerase II and supercoiling factor. At the indicated times, Sarkosyl was added to 0.01%. Then nucleotides were added and the second-stage incubation was performed. After separation of the reaction products by electrophoresis, radioactivity in the specific transcript was measured.
factors and RNA polymerase II in the presence of ATP. Though ATP is known to destabilize the preinitiation complex once formed (27), it was included because it is indispensable for the supercoiling of DNA (15). At various times, 0.01% Sarkosyl was added to prevent further formation of the preinitiation complex. The extent of complex formation at each time was then measured by allowing a single round of transcription. Formation of the preinitiation complex proceeds slowly on linear and relaxed closed circular DNA. However, the process was markedly accelerated when closed circular DNA was formed the superhelical state by the addition of DNA topoisomerase II and supercoiling factor to the preincubation mixture. The addition of these topological components did not change the results in the case of linear DNA. When DNA was preincubated with the transcription factors and RNA polymerase II in the presence of ATP, UTP, and CTP, transcription became resistant to 0.05% Sarkosyl. Conversion of the preinitiation complex to an elongation complex, as inferred from the attainment of resistance to 0.05% Sarkosyl, and the subsequent chain elongation of RNA are not affected by DNA topology (data not shown). These results are in good agreement with the previous ones obtained with the unfraccionated extract (8) and further support the conclusion that DNA supercoiling enhances fibroin gene transcription by facilitating assembly of the preinitiation complex.

**TFIID Is Essential for Rapid Assembly of the Preinitiation Complex on Supercoiled Template.** To determine which transcription factor(s) are required for rapid assembly of the preinitiation complex on the supercoiled template, a subset of factors was incubated with relaxed closed circular DNA in the presence of DNA topoisomerase II, supercoiling factor, and ATP for 5 min. After addition of the remaining factor(s), the incubation was continued and 30 s later 0.01% Sarkosyl was added. The amounts of the preinitiation complex formed were measured by allowing a single round of transcription. As shown in Table 1, omission of TFIID from the first incubation almost eliminated transcription even though the factor was supplemented just before the addition of Sarkosyl (mixture no. 4). On the contrary, omission of other factor from the first incubation had little effect on the transcription.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Protein added at 0 time</th>
<th>UMP incorporated, fmol</th>
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<tbody>
<tr>
<td>1</td>
<td>Complete</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>Complete minus TFIID, plus yTFIID</td>
<td>53</td>
</tr>
<tr>
<td>3</td>
<td>Complete minus TFIIIB</td>
<td>47</td>
</tr>
<tr>
<td>4</td>
<td>Complete minus TFIID</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Complete minus Pol II</td>
<td>46</td>
</tr>
<tr>
<td>6</td>
<td>TFIIIB, Topo II, SCF</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>TFIID, Topo II, SCF</td>
<td>46</td>
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<tr>
<td>8</td>
<td>yTFIID, Topo II, SCF</td>
<td>48</td>
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<td>9</td>
<td>TFIIIE, Topo II, SCF</td>
<td>3</td>
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<tr>
<td>10</td>
<td>Pol II, Topo II, SCF</td>
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<tr>
<td>11</td>
<td>TFII, SCF</td>
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<tr>
<td>12</td>
<td>TFIIID, Topo II</td>
<td>11</td>
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Relaxed closed circular DNA was incubated in the standard preincubation mixture supplemented with B. mori DNA topoisomerase II and supercoiling factor (complete), or the mixture containing an indicated subset of the protein components for 5 min. Then the missing components were added and 30 s later Sarkosyl was added to 0.01%. After addition of nucleotides, the second-stage incubation was carried out. Amounts of UMP incorporated were calculated from radioactivity in the specific transcript and the specific radioactivity of [α-32P]UTP. Pol II, RNA polymerase II; Topo II, B. mori DNA topoisomerase II; SCF, DNA supercoiling factor; yTFIID, purified yeast TFIID.

as long as the relevant factor was added before quenching by Sarkosyl (mixtures 3, 5, and 6). When the first incubation mixture contained only TFIID as the transcription factor, the amounts of transcripts were almost the same as control where all factors were present during the first incubation (mixture no. 8 versus 1). Yeast TFIID, expressed in bacteria and purified to homogeneity, was as effective as partially purified TFIID from HeLa cells (mixture no. 9 versus 8). No significant level of transcripts was detectable when the first incubation mixture contained only TFIIIB, TFIIIE, or RNA polymerase II besides the topology-affecting components (mixtures 7, 10, and 11). These results suggest that an interaction between TFIID and the promoter is the rate-limiting step in the assembly of the preinitiation complex. In the above experiments, the template DNA is supercoiled because DNA topoisomerase II, supercoiling factor, and ATP were added at 0 time. Omission of either DNA topoisomerase II or supercoiling factor from the preincubation mixture containing TFIID reduced transcription (mixture 12 or 13 versus 8). These results suggest that DNA supercoiling facilitates the functional binding of TFIID to the promoter.

**DISCUSSION**

Transcription of the fibroin gene was reconstituted with partially purified components from HeLa cells. TFIIIB, TFIIID, TFIIE, and RNA polymerase II are essential for transcription. Our preparation of TFIIIE seems to be a mixture of TFIIE and TFIIIF (28). When the flow-through fraction of the heparin-Sepharose column was added to the standard reaction mixture, approximately 2-fold stimulation of transcription was observed. The stimulatory activity was recovered in a 0.35 M KCl eluate fraction where the heparin-Sepharose flow-through fraction was chromatographed on DEAE-Sepharose. It is most likely that the activity corresponds to TFIIA (16). However, we did not include it in the standard reaction mixture because the requirement is not absolute and the stimulation is small.

In this work, we have employed transcription assays to investigate the role of DNA supercoiling in the assembly of a functional preinitiation complex on the fibroin gene promoter. The results obtained suggest that the interaction between TFIID and the promoter is the rate-limiting step in the assembly of the preinitiation complex. A similar conclusion has been reported for the adenovirus 2 major late promoter (9, 11, 23). These findings indicate that the functional binding of TFIID to a promoter may be one of the major targets for the regulation of gene expression. Consistent with this idea, we show here that negative supercoiling of DNA stimulates fibroin gene transcription by facilitating the TFIID–promoter interaction. Other investigators also demonstrate that an adenovirus major late promoter-specific stimulation factor (USF), a cellular factor which stimulates transcription of the adenovirus E4 gene (ATF), and the pseudorabies virus immediate early protein execute their function through the TFIID–promoter interaction (13, 29, 30).

It has been established that the expression of a set of prokaryotic genes is strongly influenced by changes in DNA supercoiling (see ref. 31 and 32 for review). The processes leading to transcription by bacterial RNA polymerases have been studied extensively (see ref. 33 for review). First, RNA polymerase holoenzyme interacts with a promoter to form a closed complex. Then it is converted into an open complex. Because formation of the open complex is accompanied by local unwinding of the double helix within the promoter region, negative supercoiling of DNA facilitates the process in some cases. We have shown that transcription of certain eukaryotic genes is also stimulated by supercoiling of the template DNA (5–7). However, the present study reveals
that the component directly involved in the activation of transcription by DNA supercoiling is quite different between prokaryotic and eukaryotic systems. While RNA polymerase holoenzyme is responsible in the former, TFIIID is the relevant component in the latter. It should be noted that the TFIIID-promoter interaction is the first step for the initiation of transcription and occurs prior to the entry of RNA polymerase II (9, 11, 12, 23, 24).

What is the mechanism by which DNA supercoiling can affect the TFIIID-promoter interaction? As we argued previously (8), TFIIID might have a higher affinity for supercoiled DNA than relaxed DNA. Alternatively, the functional binding of TFIIID to a promoter might induce local melting of the DNA helix which is facilitated by DNA supercoiling. Formation of the TFIIID-promoter complex exhibits interesting properties. First, it is a slow process as shown in Fig. 4 (see also refs. 9, 11, and 24). It takes more than a few minutes, while a conventional protein–DNA interaction finishes within a minute. Next, the complex, once formed, is stable and resistant to challenges with another promoter element (9, 23, 24). These properties can be easily explained if the TFIIID-promoter interaction accompanies local melting of the DNA helix. In this case, DNA supercoiling exerts its effect on transcription at the same limiting step in both prokaryotes and eukaryotes: formation of the open complex.

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