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Difference between Supercoiled and Linear Deoxyribonucleic Acids in Preinitiation Complex Formation for Accurate Transcription *in Vitro*

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A nuclear extract of Ehrlich ascites tumor cells was separated into three fractions that were essential for accurate transcription initiation. Studies with these fractions showed that the efficiency of transcription of supercoiled deoxyribonucleic acid (DNA) was better than that of linear DNA. A clear difference was found in the rates of formation of a preinitiation complex with these two templates. Consistent with this difference, the results of gel shift assay showed that the complex of supercoiled DNA with transcription factors is much more complicated than that of linear DNA.

Keywords——supercoiled DNA; linear DNA; transcription factor; preinitiation complex; accurate transcription

Introduction

Transcription initiation is a key step in eukaryotic gene expression. There are two methods for study of the fundamental mechanism of transcription initiation. One is to identify cis-acting elements on the regulatory region of a specific gene, and the other is to find trans-acting factors interacting with these cis-acting elements or ribonucleic acid (RNA) polymerase II itself.[1] Along these lines, various general cis-acting elements such as the TATA box, CAAT box and GC box[2-4] and trans-acting factors specifically binding to these sequences have been identified.[5-8] In addition to these general cis-acting elements and trans-acting factors, there are also gene-specific regulatory sequences and transcription factors.[9-11]

Recently, Harland *et al.* reported that supercoiled deoxyribonucleic acid (DNA) was transcribed more actively than linear DNA when injected into *Xenopus* oocytes.[12] A requirement of negative supercoiling of the DNA template for initiation of certain promoters has been reported.[13-16] Thus, besides cis-acting elements and trans-acting factors, the conformation of DNA is likely to be important for efficient transcription. However, the mechanism by which supercoiled DNA is more actively transcribed remains to be elucidated. Previously we have reported that a supercoiled template is more efficiently transcribed in an unfractionated nuclear extract of Ehrlich ascites tumor cells.[17] This paper describes *in vitro* transcription of linear and supercoiled DNA in a reconstituted nuclear extract of Ehrlich ascites tumor cells. The results showed a difference between the interactions of the two templates with transcription factors during the preinitiation step of transcription. There may be a factor in the reconstituted transcription system that recognizes the supercoiled structure of template DNA.

Materials and Methods

Fractionation of Transcription Factors and *in Vitro* Transcription Assay——A nuclear extract of Ehrlich ascites

tumor cells was prepared by the method of Dignam *et al.*[18] The factors for accurate transcription by RNA polymerase II were fractionated essentially as described before.[6,19] Briefly, the nuclear extract was first applied to a column of phosphocellulose, and fractions P0.1, P0.5 and P1.0 were eluted with KCl concentrations of 0.1, 0.5 and 1.0 M, respectively. Fractions P0.1 and P1.0 were then applied to columns of diethylaminoethyl (DEAE)-cellulose and phosphocellulose, respectively, to obtain fractions PD0.3 (eluted from DEAE-cellulose with 0.3 M KCl) and PP0.7 (eluted from phosphocellulose with 0.7 M KCl). An accurate transcription system was reconstituted with P0.5, PD0.3 and PP0.7.[6]

To compare the transcriptions of supercoiled and linear DNAs, we used p*Sma*F as a supercoiled DNA, and *Sma*I-digested p*Sma*F as a linear DNA. There are two fragments of *Sma*I-digested p*Sma*F; one is a 2.3 kb fragment from adenovirus 2 DNA containing the major late promoter, and the other is an 8.9 kb fragment from vector pBR313. Transcription was measured by S1 nuclease assay. For this, RNA synthesized *in vitro* was extracted and hybridized with probe DNA (0.05 pmol, 3.5×10^5 cpm) in 15 μl of 80% formamide, 40 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (Pipes) (pH 6.4), 0.1 mM ethylenediaminetetra acetic acid (EDTA), 0.4 M NaCl at 50°C for 8 h. As a probe DNA, we used a 2.0 kb fragment of *Hind*III- and *Sma*I-digested p*Sma*F corresponding to 11.5—17.0 map unit of adenovirus 2 DNA, in which the 5' end of the *Hind*III site was labeled with ^{32}P. After hybridization, the resulting DNA : RNA hybrids were digested with S1 nuclease, and the undigested DNA fragment was analyzed by 7 M urea 5% polyacrylamide gel electrophoresis followed by autoradiography. Accurate transcripts from the major late promoter of adenovirus 2 protect 197 bases of DNA from S1 nuclease digestion. The amount of transcript was quantitated by scanning the autoradiogram with a densitometer.

Study of the Effect of Preincubation on Transcription——First, 0.4 μg of p*Sma*F or *Sma*I-digested p*Sma*F was preincubated with P0.5, PD0.3 and PP0.7 in 20 μl of reaction mixture containing 12 mM Pipes (pH 7.9), 0.12 mM EDTA, 60 mM KCl, 7.5 mM $MgCl_2$, 0.3 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride and 12% glycerol for appropriate periods at 30 or 4 °C. Then, 500 μM concentrations of 4 nucleoside triphosphates were added and RNA synthesis was carried out for 15 min at 30 °C. The amount of transcript was measured by S1 nuclease assay.

Agarose Gel Shift Assay——p*Sma*F or *Sma*I-digested p*Sma*F was incubated with test fractions under the same conditions as for preinitiation complex formation. Then the mixture was subjected to electrophoresis in 0.2% agarose gel. The electrode buffer used was 40 mM Tris–acetate (pH 5.0) containing 2 mM EDTA and 1 μg/ml of ethidium bromide.

Results

Difference in Template Activities of Supercoiled and Linear DNAs

Previously, we demonstrated that the template activity of supercoiled DNA was higher than that of linear DNA in an Ehrlich ascites tumor cell lysate.[17] Since an accurate transcription system was reconstituted from fractions P0.5, PD0.3 and PP0.7 of the nuclear extract of Ehrlich ascites tumor cells, we used this system to examine whether supercoiled DNA was a better template than linear DNA. The amount of transcript from the +1 site of adenovirus 2 major late gene was measured by S1 nuclease assay. A typical electrophoretic pattern of a 197-base DNA fragment due to accurate transcription is shown in Fig. 1 (arrow). The intensity of this fragment was clearly higher with supercoiled DNA than with linear

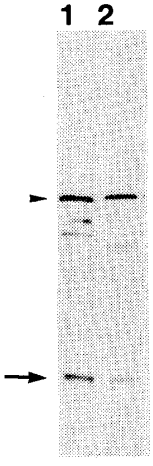

Fig. 1. Template Efficiencies of Supercoiled and Linear DNAs

p*Sma*I (lane 1) and *Sma*I-digested p*Sma*F (lane 2) were transcribed in the reconstituted transcription system. RNA synthesis was carried out at 30 °C for 60 min. The resulting accurate transcript was detected by S1 nuclease assay. The band of a 197-base protected DNA fragment formed by accurate transcription is shown by an arrow. The arrowhead shows the band of remaining probe DNA.

TABLE I. Relative Template Activities of Supercoiled and Linear DNAs in an Ehrlich Cell Nuclear Lysate and Reconstituted Transcription System

	Supercoiled DNA	Linear DNA
Ehrlich nuclear lysate	1.9	1.0
P0.5, PD0.3 and PP0.7	2.1	1.0

Relative amounts of transcripts from adenovirus 2 major late promoter were determined by densitometric scanning.

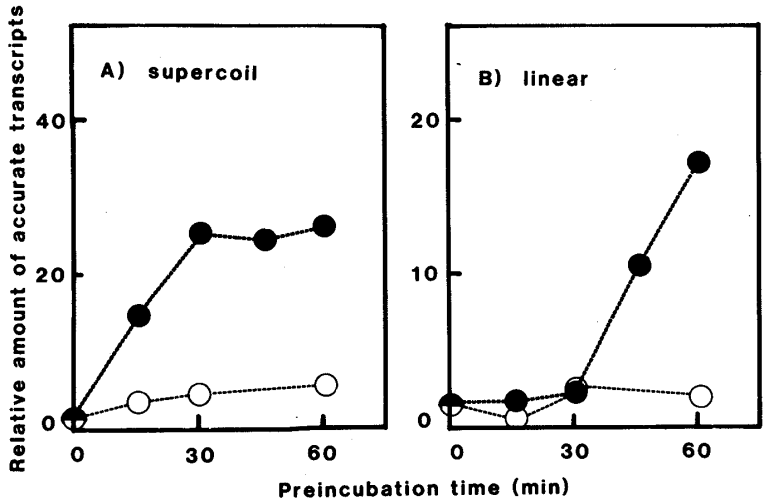

Fig. 2. Effects of Preincubation on Accurate Transcription of Supercoiled and Linear DNAs

Samples of 0.4 μg of p*Sma*F (A) or *Sma*I-digested p*Sma*F (B) were incubated with the P0.5, PD0.3 and PP0.7 fractions in the absence of nucleoside triphosphates for the indicated times. Then nucleoside triphosphates were added and RNA synthesis was carried out at 30 °C for 15 min. The resulting accurate transcript was detected by S1 nuclease assay, and the intensity of the band of the protected DNA fragment was scanned with a densitometer. The weight of paper corresponding to each peak of the densitometric chart was arbitrarily taken as the relative amount of transcript. Preincubation temperature: 0 °C (○), 30 °C (●).

DNA. The thick discrete band migrating more slowly than this fragment was that of the probe DNA used. We compared the template activities of these DNAs in a nuclear extract and in a reconstituted system, and found that the template activity of supercoiled DNA was about twice as high as summarized in Table I, indicating that P0.5, PD0.3 and PP0.7 contain all the factors necessary for accurate transcription on linear DNA and also contain a factor causing efficient transcription on supercoiled DNA.

We tried to separate the latter factor from the former factors by further fractionations of P0.5, PD0.3, and PP0.7, but the activity for accurate transcription on linear DNA and that for efficient transcription on supercoiled DNA behaved in the same way and could not be separated.

Difference in the Interactions of the Two Templates with Transcription Factors

Since the efficiency of transcription of supercoiled DNA was higher than that of linear DNA in the reconstituted system, we investigated the interactions of the P0.5, PD0.3 and PP0.7 fractions with the two structurally different templates. For this, we examined the effect of preincubation of templates with these fractions on subsequent RNA synthesis. p*Sma*F or *Sma*I-digested p*Sma*F was preincubated with a mixture of the P0.5, PD0.3 and PP0.7 fractions for 60 min at 30 °C. Then nucleoside triphosphates were added, RNA synthesis was

performed for 15 min, and RNA transcribed from the +1 site was measured by S1 nuclease assay. Without preincubation, the level of RNA synthesis after a 15 min incubation was very low with both templates. However, on preincubation, RNA synthesis increased more than 20-fold, indicating the formation of a preinitiation complex of the template and transcription factors. The effects of the time and temperature of preincubation were investigated using the two templates. As is evident from Fig. 2, preincubation at 0 °C did not increase RNA synthesis appreciably. Probably, no preinitiation complex is formed at this temperature. But, at 30 °C, RNA synthesis increased with increase in the preincubation time. Moreover, the relation between the preincubation time and RNA synthesis was different with the two templates: with supercoiled DNA, a 30 min preincubation at 30 °C was sufficient for maximum RNA synthesis, whereas with linear DNA, no appreciable preinitiation complex was formed in 30 min, a much longer preincubation time being needed for its formation. These results indicate that the structure of the template is important for the formation of the preinitiation complex formed in the absence of nucleoside triphosphates.

Interaction of Supercoiled DNA and a Factor in the Reconstituted Transcription System

We used a modified gel shift assay for direct examination of the difference between supercoiled and linear DNAs in preinitiation complex formation. p*Sma*F or *Sma*I-digested p*Sma*F was incubated with the P0.5, PD0.3 and PP0.7 fractions at 30 °C under the conditions for formation of the preinitiation complex. Samples were taken at intervals and analyzed by electrophoresis in 0.2% agarose gel to see the change in mobility of DNA. As shown in Fig. 3, the behavior of supercoiled DNA was quite different from that of linear DNA. Namely, supercoiled DNA became unable to enter the gel when incubated with these fractions (lanes 4—6), suggesting the formation of large complexes of DNA and proteins. The incubation time required to induce this complex formation coincided well with that required for formation of the preinitiation complex. Moreover, when incubated at 0 °C for 60 min, the

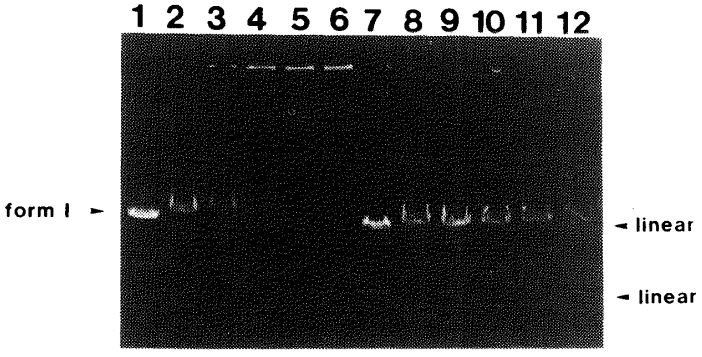

Fig. 3. Agarose Gel Shift Assay with Reconstituted Fractions

Samples of 0.4 μg of p*Sma*F (lanes 1—6) and *Sma*I-digested p*Sma*F (lanes 7—12) were incubated with the P0.5, PD0.3 and PP0.7 fractions at 0 °C for 60 min (lanes 2 and 8) or at 30 °C for 5 min (lanes 3 and 9), 15 min (lanes 4 and 10), 30 min (lanes 5 and 11) or 60 min (lanes 6 and 12). Then each mixture was subjected to 0.2% agarose gel electrophoresis. Lanes 1 and 7 contained the original DNA.

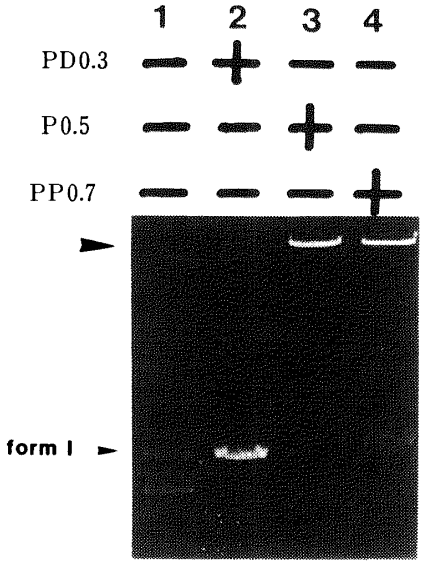

Fig. 4. Agarose Gel Shift Assay with Each Fraction

Samples of 0.4 μg of p*Sma*F were incubated for 10 min at 30 °C with the indicated fractions and then subjected to 0.2% agarose gel electrophoresis. The large arrowhead shows the origin of the gel.

mobility of DNA in the gel did not change appreciably. These results suggest that this change of the mobility of supercoiled DNA in the gel reflects the formation of the complicated preinitiation complex necessary for efficient transcription. When DNA was extracted from each complex with phenol, all the materials entered the gel after electrophoresis.

In contrast to supercoiled DNA, linear DNA showed no significant change in mobility under the same conditions, and the band of DNA tended to disappear on longer incubation. Thus, we extracted DNA from each complex and analyzed it by electrophoresis to see if disappearance of the band of DNA was due to selective degradation of linear DNA. Almost the same amount of DNA was recovered from each complex (data not shown). Thus, the disappearance of the band of linear DNA on prolonged incubation was probably due to dispersion into the gel of the various complexes formed during incubation. When pBR322 was analyzed under the same conditions, the results were similar to those with p*Sma*F. Therefore, some factor in the reconstituted transcription system probably interacts specifically with supercoiled DNA.

We tried to identify the fraction containing this factor by gel shift assay. As shown in Fig. 4, the P0.5 and PP0.7 fractions both affected the mobility of supercoiled DNA, indicating that these two fractions contain the factor. In contrast, the PD0.3 fraction did not appreciably affect the mobility of DNA. At present, we do not know whether the effects of the P0.5 and PP0.7 fractions are due to the same or different factors in these fractions. However, our results indicate the presence of some specific factor that causes a structural change of supercoiled DNA, and probably contributes to efficient transcription of supercoiled DNA.

Discussion

We tested the accurate transcription of supercoiled and linear DNAs in a reconstituted transcription system. For this, we first fractionated the nuclear extract of Ehrlich ascites tumor cells and obtained three fractions that were essential for *in vitro* transcription. Of these fractions, P0.5 may correspond to CB or a mixture of TFIIB, TFIIE and RNA polymerase II, PD0.3 to AB or TFIIA, and PP0.7 to DB or TFIID.[20-22]

The efficiency of transcription of supercoiled DNA was twice that of linear DNA in both a nuclear lysate and the reconstituted transcription system. To explain this difference, we investigated the interactions of supercoiled and linear template DNAs and factors in the three fractions, and found that these two template DNAs behave differently in preinitiation complex formation. Thus, it is possible that some factor specifically interacts with supercoiled DNA and contributes to efficient transcription. Probably the lag period of RNA synthesis observed on linear DNA (Fig. 2) is due to a low efficiency of the assembly of transcription factors on the template. At present, we do not know whether this factor is a new factor or one of the factors identified before, *i.e.*, TFIIA, TFIIB, TFIID or TFIIE.[19,20,23,24] The results of gel shift assay suggested that the P0.5 and PP0.7 fractions contain a factor that specifically alters the mobility of supercoiled DNA in agarose gel. It seems important to purify this factor and examine whether or not it increases the efficiency of transcription of supercoiled DNA.

Besides general transcription factors, proteins that change the structure of DNA are probably important in eukaryotic transcription. Proteins interacting with supercoiled DNA seem to be especially important, because, although chromosomal DNA is in general linear, it may have local regions with a supercoiled structure. For example, when DNA binds to the nucleo-skeleton at two sites, the region between these sites would be similar to a closed circular molecule and might have a partly supercoiled structure. A supercoiled structure may also be formed when histones constructing a nucleosome are released from the DNA chain, resulting in formation of a deoxyribonuclease I-hypersensitive site. Since supercoiled DNA is a better template than linear DNA *in vitro*, the supercoiled structure of DNA may also be

important *in vivo*, and proteins that interact specifically with supercoiled DNA may be transcription factors.

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