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Yeast RNA polymerase II transcription of circular DNA at different degrees of supercoiling

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ABSTRACT

Purified yeast RNA polymerase II was tested for transcriptional activity as a function of the degree of circular DNA supercoiling. Chimaeric plasmids P30 and P31 both containing inserts from the yeast transposable element TY1 cloned in pBR322 and the vector pBR322 were used as templates. For pBR322 the transcriptional activity increases about 4 fold from the fully relaxed covalently closed circles to the native supercoiled forms, further supercoiling having no effect on transcription. P30 shows a 5 fold increase of transcriptional activity reaching a plateau at the native supercoiled conformation. However, at an intermediate degree of supercoiling (σ = 0.024), transcription decreases to a value close to zero. P31 too exhibits a conformation (σ = 0.014) in which there is a drop of transcriptional activity. Furthermore, a 10 fold increase of transcription is obtained at the higher values of superhelix density. Both kinetic and autoradiographic experiments confirm the existence of DNA conformations that can inhibit "in vitro" transcription.

INTRODUCTION

The relationship between supercoiled DNA and the regulation of gene expression has been recently suggested and many papers on this topic have been published and reviewed (1,2). Many intriguing relations between supercoiling and transcription have been reported in prokaryotic systems,whereas the eukaryotic counterpart has not been so extensively studied. Calf thymus (3) and wheat germ (4) RNA polymerase transcriptional activity is greatly stimulated by supercoiling of the DNA template.

Yeast RNA polymerase II transcriptional activity on chimaeric plasmids bearing homologous DNA inserts has been recently investigated (5,6). It has been shown that initiation of transcription on supercoiled DNA is more efficient than on relaxed forms. These data were obtained using DNA templates prepared by CsCl-ethidium bromide gradients which yield circular DNA in the

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native supercoiled form. Methods for varying in vitro the degree of supercoiling of circular covalently closed DNA have been described (7). Using a topoisomerase I in presence of different amounts of ethidium bromide DNA conformation can be varied from relaxed to supercoiled also beyond the native one. According to this procedure we prepared an allomorphic series of circular DNA templates and studied the effect of DNA conformation on yeast RNA polymerase II in vitro transcription. We have chosen as templates two subclones (P30 and P31) of the yeast transposable element Tyl1D15 cloned in pBR322 and the vector pBR322.

MATERIALS AND METHODS

Templates

The two subclones P30 and P31 (kind gift from P.Philippsen) were derived from the original yeast clone Tyl1D15 (8) containing the transposable element Tyl and flanking chromosomal sequences. P30 contains the left part of Tyl1D15 (HindIII-EcoRI cut) and P31 the right part (EcoRI-HindIII cut) both cloned in pBR322 between EcoRI and HindIII sites. Native supercoiled DNAs were prepared by conventional CsCl-EtBr gradient procedure. The percentage of supercoiled versus nicked form was 90% (pBR322), 90% (P30) and 80% (P31) as judged by densitometric analysis of agarose gel pictures. Molecular weights of the templates used were $2.8 \cdot 10^6$ for pBR322 (10), $4.95 \cdot 10^6$ for P30 (9) and $6.27 \cdot 10^6$ for P31 (9).

RNA polymerase II

Yeast RNA polymerase II was purified by a procedure previously described (11). Electrophoretic analysis of the enzyme under denaturing conditions in sodium dodecyl sulfate polyacrylamide gel revealed a subunit composition identical to that already described (6).

For the transcriptional assay the reaction mixture contained, in a final volume of 0.1 ml, 20 mM Tris-HCl pH 7.9, 2mM $MnCl_2$, 0.2 mM Na_2EDTA, 50 mM $(NH_4)_2SO_4$, 0.3 mM each ATP, GTP, CTP and 1 μM ^{3}H UTP (2000 cpm per pmole), RNA polymerase II and DNA as indicated. Synthesis was carried out at 37°C for the indicated time. Trichloroacetic acid precipitable radioactivity was determined in a liquid scintillator system. For transcription in presence of topoisomerase

I the RNA polymerase transcription buffer was supplemented with 5 mM $MgCl_2$.

RNA transcripts analysis

The reaction mixture for the electrophoretic analysis on polyacrylamide gel of the RNA products was performed as described above, except triphosphates concentration (0.6 mM CTP, UTP, GTP and 25 μM ^{32}ATP (25 μCi). After 10 min of synthesis, the reaction was stopped with 2 μg/ml of α-amanitin (Sigma) and DNA was digested with 10 μg/ml Dnase RNase free (Worthington, further purified by two passages on Agarose 5'-(p-aminophenylphoshoryluridine 2' (3') phosphate from Myles). Samples were processed and loaded on 6% polyacrylamide 7 M urea as described (6).

Topoisomerase I extraction and topoisomers preparation

DNA relaxing enzyme (topoisomerase I) was prepared from chicken erythrocyte nuclei according to Bina-Stein et al. (12). The enzyme was stored frozen at -80°C at a protein concentration of 1 mg/ml in phosphate buffer 0.01 M pH 7.1, containing 0.4 M NaCl and bovine serum albumine at 1 mg/ml. In order to obtain circular covalently closed DNA at various degree of supercoiling we adopted the procedure described by Keller (7) with minor modification. 1 μg of enzyme preparation and 1 μg of DNA in a final volume of 50 μl were incubated for 60 min at 20°C in a buffer containing Tris-HCl 50 mM pH 7.8, NaCl 0.1 M, Na_2EDTA 1 mM, bovine serum albumine 1 mg/ml and EtBr from 0 to 8μM. DNA was extracted twice with phenol, once with chloroform-isoamylic alcohol (24:1), and finally with isoamylic alcohol. DNA was ethanol precipitated, the pellet washed with 70% ethanol and resuspended in Tris-HCl 10 mM pH 8, Na_2EDTA 1 mM buffer. The topoisomerase I extract used herein was checked for the absence of endogenous nucleic acids and of contaminating nuclease and RNA polymerase activities in the assay conditions described.

Agarose gel electrophoresis

We adopted essentially the procedure described by Keller (7) using agarose gel concentration of 1.4% (w/v) in Tris-HCl 40 mM pH 7.9, 5 mM sodium acetate, 1 mM Na_2 EDTA. Slabs (0.3x15x20 cm) were run at room temperature with recirculating buffer, at a constant voltage of 4 V/cm for times ranging from 18 to 40 h. For the complete resolution of topoisomers the gels and the buffer

contained EtBr in a concentration ranging from 0 to 0.06 μg/ml. After staining of the gel in electrophoresis buffer containing 1 μg/ml of EtBr for 1 h, the DNA bands were visualized under U.V. light. Quantitative analysis of the DNA and resolution of topoisomers were achieved by densitometric scanning.

RESULTS AND DISCUSSION

Allomorphic DNA templates characterization

The equation $\alpha = \beta + \tau$ (13) describes the topological properties of covalently closed circular duplex DNA. α, the "linking number" is the number of topological constraints of the two strands. The "twisting number" β is the number of helical turns of DNA in the B form with a pitch of 10.4 base pairs per turn (14). τ is the number of superhelical turns and is called the "writhing number". σ (13) is defined by the equation $\sigma = \frac{\tau}{\beta^0}$ where β^0 is the ratio between the base pairs number and 10.4. Using the procedure described in Materials and Methods, a series of circular DNA templates at different values of σ were prepared and analyzed by agarose gel electrophoresis.

In Fig. 1 A, C and E topoisomers of pBR322, P30 and P31, are shown. In lanes 1 (A, C and E) samples, incubated is absence of EtBr, yielded totally relaxed covalently closed DNA circles. In lanes 11 untreated native supercoiled DNAs are shown. Increasing amounts of EtBr during the DNA relaxing reaction, yielded, after the removing of the intercalating dye, the topoisomer distributions at increasing degree of supercoiling (lanes 2 to 10). The bands with the same mobility have the same value of τ and in each lane topoisomers assume a Gaussian distribution around an average value of τ, each band differing of one unit of τ (i.e. one superhelical turn). The resolution of topoisomer distributions, at each value of σ, has been obtained by agarose gel electrophoresis in the presence of EtBr (fig. 1B, D and F). In these conditions the DNA conformation and therefore its electrophoretic mobility varies, depending on the dye concentration and the initial σ value (7). Assuming $\tau = 0$ for the central band of fully relaxed DNA (lane 1), the τ values of topoisomers in adjacent lanes can be calculated. In order to obtain overlapping and resolution of topoisomers in adjacent lanes, it was necessary to run various gels at different EtBr concentrations. Fig. 1B, D and F shows an example of electro

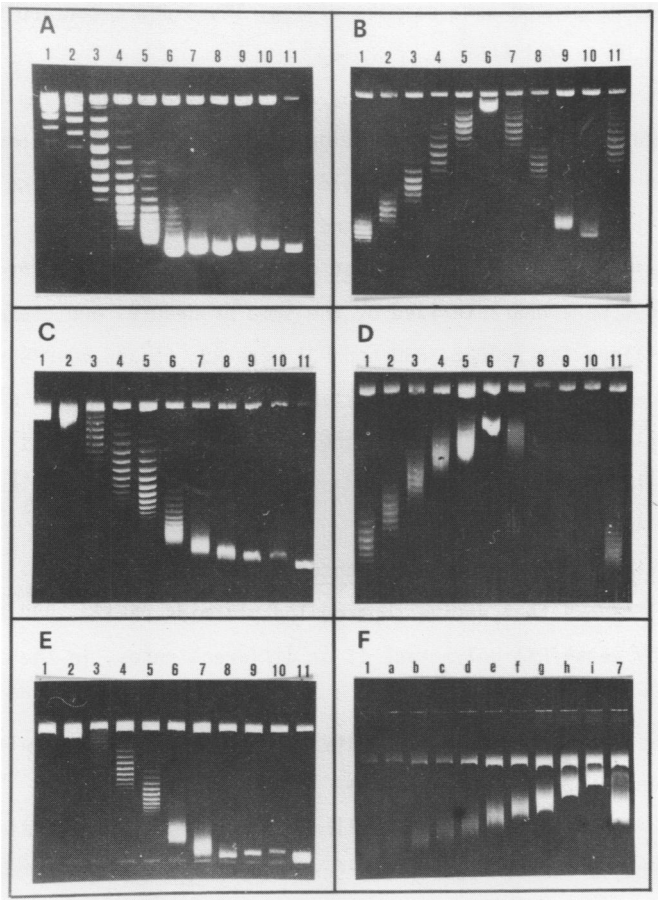

Figure 1. Topoisomers of pBR322 (A,B), P_{30}(C,D) and P_{31}(E,F)
A,C,E: agarose gels 1.4% without EtBr; B,D: agarose gels 1.4%, 0.02 μg of EtBr; F: agarose gel 1.6%, 0.02 μg EtBr. In A,B,C,D,E samples 1 to 10 were obtained using during DNA relaxing reaction respectively 0, 0.4, 0.8, 1.3, 1.7, 2.5, 3.4, 4.2, 6.3, 8.5 μM EtBr. Samples 11 are untreated native supercoiled DNAs. In F; the number of samples between lanes 1 to 7 of E has been increased to obtain a more suitable overlapping between adjacent lanes. Samples 1 and 7 are the same as in E, samples a,b,c,d,e,f,g,h and i were relaxed in presence of 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.0, 2.5 and 3 μM EtBr, respectively. In the different figures we note an increase of nicked circles and the appearance of linear forms due to the experimental procedure.

phoretic analysis of the same samples of fig. 1A, C and E carried out in the presence of 0.02 μg/ml EtBr.

The number of superhelical turns of the native supercoiled DNAs studied was calculated both by extrapolation of migration of topoisomers to that of the native supercoiled species (15,16) and by the band counting method(7). Both methods yielded for pBR322, P30 and P31 values of τ of 21 $\pm$ 1, 37 $\pm$ 3 and 47 $\pm$ 4 respectively. Topoisomers at degrees of supercoiling higher than the native ones were characterized by the same procedures and the maximal values of τ obtained for pBR322, P30 and P31 were 43, 65 and 80 respectively. At these values of supercoiling a substantial loss of covalently closed molecules takes place as demonstrated by the increase of nicked forms (fig. 1A, C and E, lanes 10). Therefore we limited our investigation to samples containing no more than 40% of nicked circles.

Characterization of RNA polymerase transcriptional activity

Fig. 2 shows that native supercoiled plasmids pBR322, P30 and P31 are transcribed by yeast RNA polymerase II at different rates, in the order P31 > P30 > pBR322.

A lower transcriptional activity towards prokaryotic templates has previously been described for yeast RNA polymerase II (6).

As shown in fig. 1, all the allomorphic DNA samples contain a fraction of nicked circular forms. However transcription is mainly due to supercoiled DNA, as poor transcription is supported by nicked circles generated by U.V. light exposure in the presence of EtBr of the native templates (data not shown).

Relationship between superhelix density and transcription

The relationship between superhelix density and *in vitro* transcription is shown in fig. 3, pBR322 is transcribed at maximal levels at conformations near the native supercoiled ones (σ = 0.05) higher degrees of supercoiling showing no further effects. A 4 fold increase of transcription is attained from the relaxed form. It has been reported (4) that wheat germ RNA polymerase II transcriptional activity on circular DNA of the bacterial virus PM2 is 30 fold stimulated passing from the relaxed to a supersupercoiled form. The different yeast RNA polymerase behaviour may reflect the request of this enzy

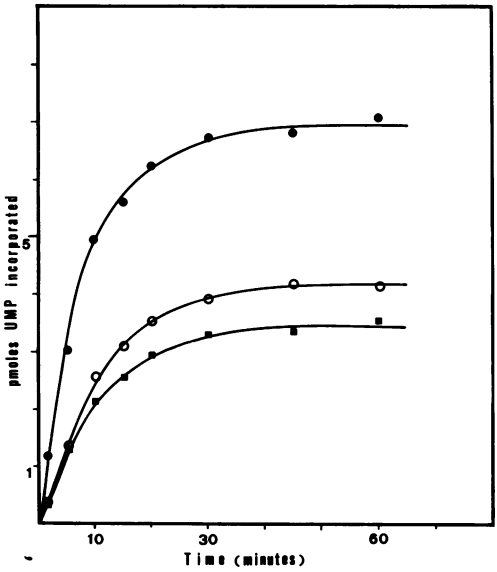

Fig. 2. Time course reaction of yeast RNA polymerase II on 1 μg of native supercoiled DNA templates: (■-■-■) pBR322, (o-o-o) P_{30} and (●-●-●) P_{31}. For reaction condition see Materials and Methods.

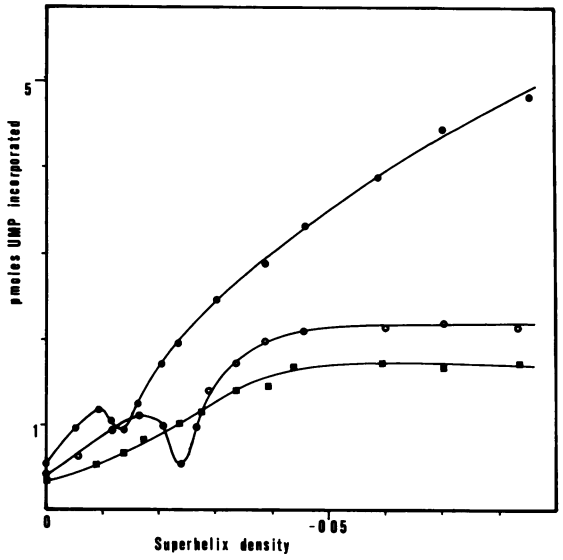

Fig. 3. Transcriptional activity of RNA polymerase II (1 μg) versus DNA at different degrees of supercoiling (■-■-■) pBR322 (o-o-o) P_{30} and (●-●-●) P_{31}: σ values of native forms are respectively -0.05 ±0.002, -0.0051 ± 0.004 and -0.051 ± 0.005.

me not only for conformations enhancing transcription (i.e. supercoiling) but also of specific homologous DNA sequences.

P30 and P31 show, at a supercoiling degree intermediate between the relaxed and supercoiled native form (respectively σ = -0.024 and σ = -0.014) a minimum in the transcription versus superhelix density curve. This unexpected result suggests that a regulatory mechanism may be operating in the DNA conformation.

P30, after a 5 fold increase of transcription from the relaxed to the native supercoiled form, reaches a plateau in the same fashion as pBR322. P31 on the contrary shows a continuous increase of transcription up to 10 fold.P30 and P31 thus differ in two features, the value of the minimum in transcriptional activity and quantitative increase related to supercoiling. The differences observed may be both attributed to the different sequences of the two plasmids.

It is known(17)that eukaryotic RNA polymerase II exhibit on nicked templates an RNA priming activity that leads to the incorporation of ribonucleotides covalently linked to DNA. Analysis of RNA transcripts after DNAse treatment of samples has been carried out by electrophoresis on polyacrylamide gel and autoradiography of the dried slabs. This procedure eliminates possible artifacts in transcription experiments when nicked DNA is present and provides an estimate of the size of the RNA transcripts.

Electrophoretic analysis of RNA transcripts at different values of superhelix density was carried out (fig. 4). The size of RNA transcripts generally increases with σ and, at the inhibition points, there is a lack of RNA polymerase II elongational activity. We have not tried to assign the origin of the RNA products by hybridization experiments because of the complexity of the patterns obtained and of the presence of large smearing between the bands. An exact correlation between the intensities in fig. 4 and the UMP incorporation in fig. 3 may not be expected due to the slightly different experimental conditions used in the two transcriptional assays (see materials and methods).

However the data obtained confirm the presence of inhibition points

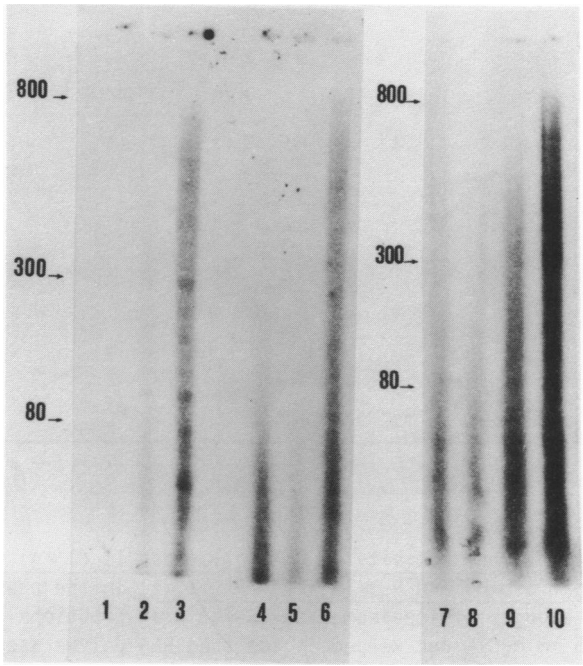

Fig. 4. Autoradiography of the electrophoresis analysis of RNA synthesized by RNA polymerase II on DNA template at various degree of supercoiling. pBR322 lanes: 1 σ = -0.01, 2 σ = -0.025, 3 =σ-0.06. P_{30} lanes: 4 σ = -0.016, 5 σ = -0.025, 6 σ = -0.06. P_{31} lanes: 7 σ = -0.01, 8 σ = -0.014, 9 σ = -0.026, 10 σ = -0.08. Arrows give the positions of the size marker migration (ϕ X174 digested by Hae III).

for P30 and P31 (fig. 4, lane 5 and 8). A further confirmation of this effect has been obtained by kinetic experiments in which synthesis of RNA was carried out during the action of the DNA relaxing enzyme on native templates. In fig. 5 the titrations with topoisomerase of DNA templates at fixed times of synthesys are shown. Increasing amount of enzyme drives the DNA to a more relaxed state and the transcriptional activity drops in a discontinous fashion. For P30 the effect is more pronounced and the minor amount of topoisomerase I needed for obtaining the inhibition point indicates, in agreement with the data of fig. 3, that P30 reaches the inhibition point at a supercoiling degree higher than P31. In fig. 6 are shown the time course reactions of RNA synthesis for P30 and P31 in presence of topoisomerase I. The curves exhibit

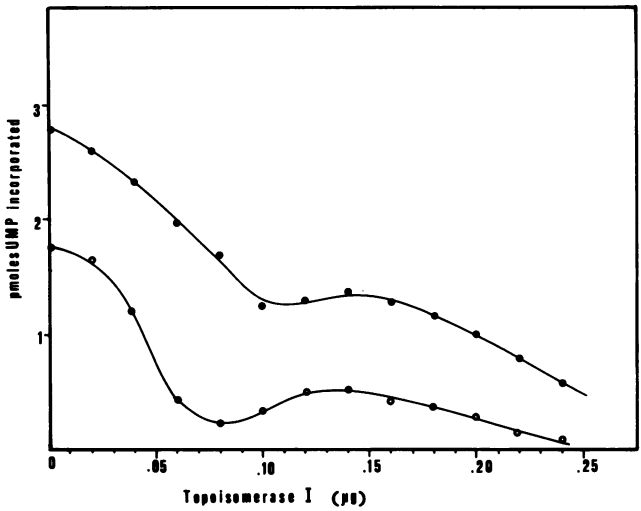

Fig. 5. Transcriptional activity of RNA polymerase II (1 μg) versus supercoiled P_{30} (o-o-o) and P_{31} (●-●-●) DNA (1 μg) in the presence of various amounts of topoisomerase I. The transcriptional mixture was made 5 mM $MgCl_2$ and keeped in ice then RNA polymerase and DNA relaxing enzyme were added. The mixture was incubated for 10 min and the incorporated radioactivity determined in the usual way.

an inflexion point before the saturation plateau and a comparison with the curves shown in fig. 2, obtained for the native supercoiled templates alone, suggests that during relaxation P30 and P31 assume conformations that can inhibit transcription.

CONCLUSIONS

It is now generally accepted that, in vivo, DNA is organized in circles or "quasi-circular" loops anchored to a nuclear matrix (18) and that DNA supercoiling strongly affects processes such as replication, recombination and transcription (2). It is shown that in eukaryotic systems transcription of certain DNA sequences can be drastically varied acting on DNA segments hundreds of base pairs from the expressed regions (19,20). This behaviour may suggest that the DNA elastic properties might be involved in the transmission at distance of local effects. Melting of A-T rich sequences (13), induction of hairpin-loops (16) and of Z DNA structure (21) are under the control of tor-

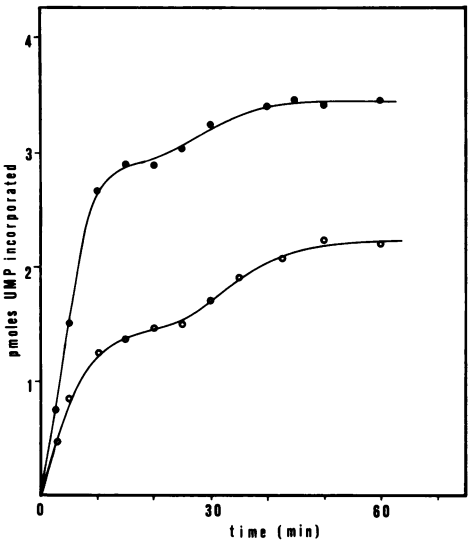

Fig. 6. Time course reaction of RNA synthesis in the presence of Topoisomerase I on P_{30} (o-o-o) and P_{31} (●-●-●) native supercoiled template(1 μg). Topoisomerase I 0.04 μg for P_{30} and 0.05 μg for P_{31} in a final volume of 0.1 ml.

sional strength stored in supercoiled DNA. Unfortunately little is known about the hydrodynamic behaviour of supercoiled DNA, i.e. the partition between twisting and writhing as a function of the sequence and the real conformation adopted between the possible ones.

The experiments described in this paper are in agreement with the hypothesis (1) that supercoiling may represent another level of regulating gene expression.

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