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Stimulation of Yeast RNA Polymerase II Transcription by Critical Values of Supercoiling†

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ABSTRACT: RNA chains of discrete length were obtained in vitro by yeast RNA polymerase II directed transcription of a supercoiled plasmid. On the basis of the amount and the molecular weight of the RNA chains synthesized in the absence of reinitiation events, the number of actively transcribing RNA polymerase molecules has been calculated. A stimulation of transcriptional activity was found to be related to the torsional strength of negative supercoiling of the template. The DNA unwinding angle measured in the complexes formed with the enzyme in the presence of three ribonucleoside triphosphates equals 485 ± 30°, marking a melting effect of 14 base pairs per bound enzyme molecule.

Supercoiling of the DNA template is now considered an essential requirement for the in vitro transcription by purified eukaryotic RNA polymerase II. In previous works (Ballario et al., 1981; Pedone et al., 1982), we have studied the in vitro expression of cloned yeast sequences such as the 2-μm DNA and the yeast transposable element Ty1. These sequences are known to be in vivo under the control of RNA polymerase II. We have shown that yeast RNA polymerase II prefers supercoiled templates to relaxed and linear ones and that a modulation of in vitro transcriptional activity is related to the degree of supercoiling of the template.

As reported by Lescure (Lescure, 1983), when the rNTP[1] concentration is lowered to submillimolar values, purified yeast RNA polymerase II can initiate and terminate transcription at specific points on a supercoiled template. We have adopted this protocol for a cloned fragment of the yeast transposable element Ty1 and obtained a reproducible pattern of RNA chains synthesized in vitro by yeast RNA polymerase II. The RNA synthesis was also performed in the presence of heparin to prevent reinitiation events so as to correlate the number of RNA chains produced with that of active RNA polymerase molecules.

To assess the effect of the degree of supercoiling of the DNA template on transcription, we have prepared an allomorphic series of the cloned circular DNA template at increasing values of negative superhelical densities. Yeast RNA polymerase II transcribes linear or relaxed circular molecules poorly; on the contrary, we observed a stimulation of transcription of supercoiled templates mainly at values of supercoiling close to those of the native conformation.

An attempt to measure the DNA unwinding angle induced by RNA polymerase on the template was made by following the strategy reported by Gamper & Hearst (1982) for the prokaryotic *Escherichia coli* RNA polymerase. Binary complexes between DNA and yeast RNA polymerase are not as stable as those with the prokaryotic enzyme, but the addition of three rNTPs enabled us to measure the extent of DNA unwinding induced by the enzyme.

Materials and Methods

Template. The subclone p30-δ (generous gift from P. Philippsen) was derived from the original yeast clone TyD15 (Cameron et al., 1979) and contains 750 base pairs of yeast insert, including 300 base pairs of one of the direct repeats of yeast transposable element Ty1. The insert is cloned in pBR322 between *Bgl*II and *Sal*I restriction sites. Native

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EB, ethidium bromide; PEI-cellulose, poly(ethylenimine)-cellulose; rNTP, ribonucleoside triphosphate; scDNA, supercoiled DNA; EDTA, ethylenediaminetetraacetic acid.

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supercoiled DNA was prepared by conventional CsCl–EB gradient procedure. The recovered p30-δ DNA contained 20% (w/w) nicked and linear molecules, and its molecular weight is 3.37×10^6.

Enzymes. Yeast RNA polymerase II was purified as previously described (Dezèlée & Sentenac, 1973). The RNA polymerase II was obtained 95% (w/w) pure and attributed a molecular weight of 586×10^3 (Dezèlée et al., 1976).

Topoisomerase I was prepared from chicken erythrocyte nuclei according to Bina-Stein et al. (1976). Topoisomer's preparation and characterization by agarose gel electrophoresis according to the band-counting method (Keller, 1975) has been described (Pedone et al., 1982).

RNA Synthesis. RNA synthesis was performed according to Lescure (1983). The standard reaction mixture (25 μL) contained 50 mM Tris-HCl, pH 8.0, 5 mM $(NH_4)_2SO_4$, 2.5 mM $MnCl_2$, and 0.5 mM dithiothreitol. Usually 1 μg of DNA template and 0.45 μg of yeast RNA polymerase II were added. Due to the low transcriptional activity of RNA polymerase on nicked and linear template, the enzyme to DNA molar ratios are referred to the amount of supercoiled DNA. The rNTP concentration was 5×10^{-6} M (0.04 μCi/pmol of [α-^{32}P]UTP commercially obtained from New England Nuclear) unless otherwise specified. After 10 min at 37 °C, the reaction was stopped with 2 μg/mL of α-amanitin (Sigma), and DNA was digested for 15 min at 37 °C with 10 μg/mL of DNase [RNase free, further purified by two passages on agarose–5′-[(*p*-aminophenyl)phospho]uridylyl(5′–3′)uridine 2′(3′)-phosphate, commercially obtained from Miles]. Samples were ethanol precipitated and resuspended in 10 μL of a buffer containing 80% (v/v) formamide, 1 mM EDTA, and 0.05% (w/v) xylene cyanol and bromophenol blue. Samples were prepared for electrophoresis by heating at 80 °C for 5 min. The electrophoretic analysis was conducted on a 6% polyacrylamide gel containing 7 M urea, 50 mM Tris-HCl, pH 8.2, and 1 mM EDTA. Autoradiography of the dried gel was performed on Kodak X-ray film with an intensifying screen at −70 °C. For the RNA synthesis in the absence of reinitiation, the reaction mixture was equilibrated at 37 °C for 2 min prior to the addition of UTP and heparin.

Determination of Superhelical Turns Removed by RNA Polymerase on p30-δ. The reaction mixture minus UTP was equilibrated for 2 min at 37 °C; then α-amanitin (2 μg/mL), $MgCl_2$, and topoisomerase I were added. The amount of enzyme was sufficient to relax the DNA completely, as judged from a preliminary calibration. After 20 min at 37 °C of topoisomerase I treatment, the reaction was stopped by addition of 1 volume of phenol. The aqueous phase was recovered and reextracted once with phenol, twice with chloroform–isoamyl alcohol (24:1, v/v), and finally with ethyl ether. A total of 2.5 volumes of ethanol was added, and the dried pellet was resuspended in 20 μL of a buffer containing 10 mM Tris-HCl, pH 8, 1 mM EDTA, 4% (w/v) Ficoll 400 (Pharmacia), and 0.05% (w/v) bromophenol blue. Samples were electrophoresed on 1.4% agarose gels in the presence of 0.01 μg/mL EB in 40 mM Tris-HCl, pH 8.5, 5 mM sodium acetate, and 1 mM EDTA. The DNA concentration was determined by densitometry of the negative pictures of the gels. The amount of labeled UMP incorporated was determined by Cerenkov counting of the excised bands. The amount of UDP and UMP contaminating the labeled UTP was monitored through PEI–cellulose thin-layer chromatography (Amersham).

Results and Discussion

Characterization of Allomorphic DNA Template. Figure 1 shows the pattern of p30-δ DNA topoisomers run in agarose gel in the absence (A) and in the presence (B) of 0.01 μg/mL EB. In Figure 1A, it is evident that topoisomers with values of superhelical densities lower than 0.009 and higher than 0.026 are not resolved. The resolution of these topoisomers is obtained in the gel of Figure 1B where the intercalating dye changes the conformation of each samples and, consequently, its electrophoretic mobility. Other runs with different concentrations of EB (data not shown) allowed the determination of the superhelical density of each sample. The mean superhelix density $\bar{\sigma}$ of each sample is calculated as the ratio τ/β, where τ is the number of negative supercoils of the DNA molecule and β is the molecular weight expressed in base pairs of p30-δ DNA divided by 10.4. The $\bar{\sigma}$ value obtained for the native supercoiled plasmid was -0.047 ± 0.002.

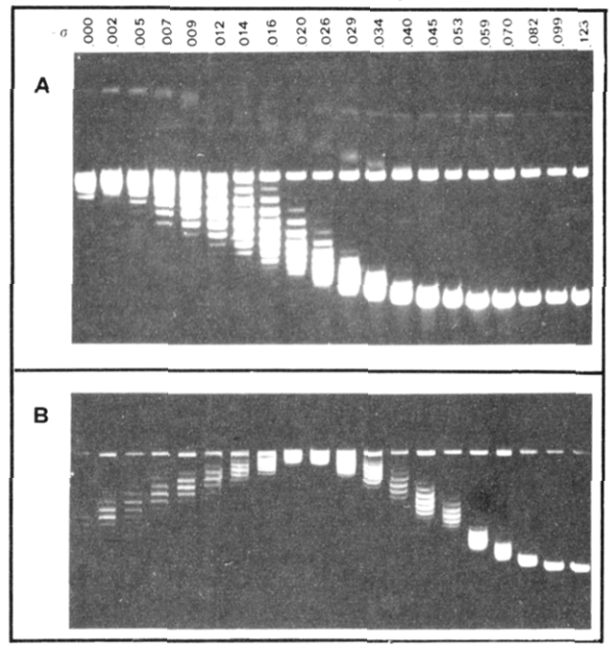

FIGURE 1: Topoisomers of p30-δ DNA. (A) 1.4% agarose gel electrophoresis of p30-δ DNA (1 μg) at increasing degree of supercoiling; negative superhelical densities ($-\sigma$) of each sample are indicated. (B) Agarose gel (1.4%) in the presence of 0.01 μg/mL EB of the same sample as in (A).

Transcription of p30-δ DNA at Different Degrees of Supercoiling. Figure 2A shows the electrophoretic analysis of the RNA transcripts of allomorphic templates. The amount of rNTP in the reaction mixture was 5×10^{-6} M; at this low substrate concentration, the RNA polymerase II initiates transcription at specific points on a supercoiled DNA template (Lescure, 1983). The molecular weight of the transcripts increases with the value of σ. A substantial increase of total transcriptional activity also becomes evident when σ values approach that of native supercoiled DNA. A plot of the percent of transcription vs. the superhelix density is reported in Figure 2B for three distinct enzyme to DNA molar ratios. A maximum differential increment of transcriptional activity is observed at critical values of superhelix density (indicated by arrows). From the same figure, it also appears that an increase in the enzyme to DNA molar ratio shifts the critical point toward higher values of σ. This result can be explained by assuming that a decrease of the original supercoiling value of the template is induced by RNA polymerase through the melting of the DNA double helix; additional supercoiling would then be necessary to stimulate transcription. From a rough calculation on the data of Figure 2B, the increase of one unit value of the enzyme to DNA molar ratio causes a

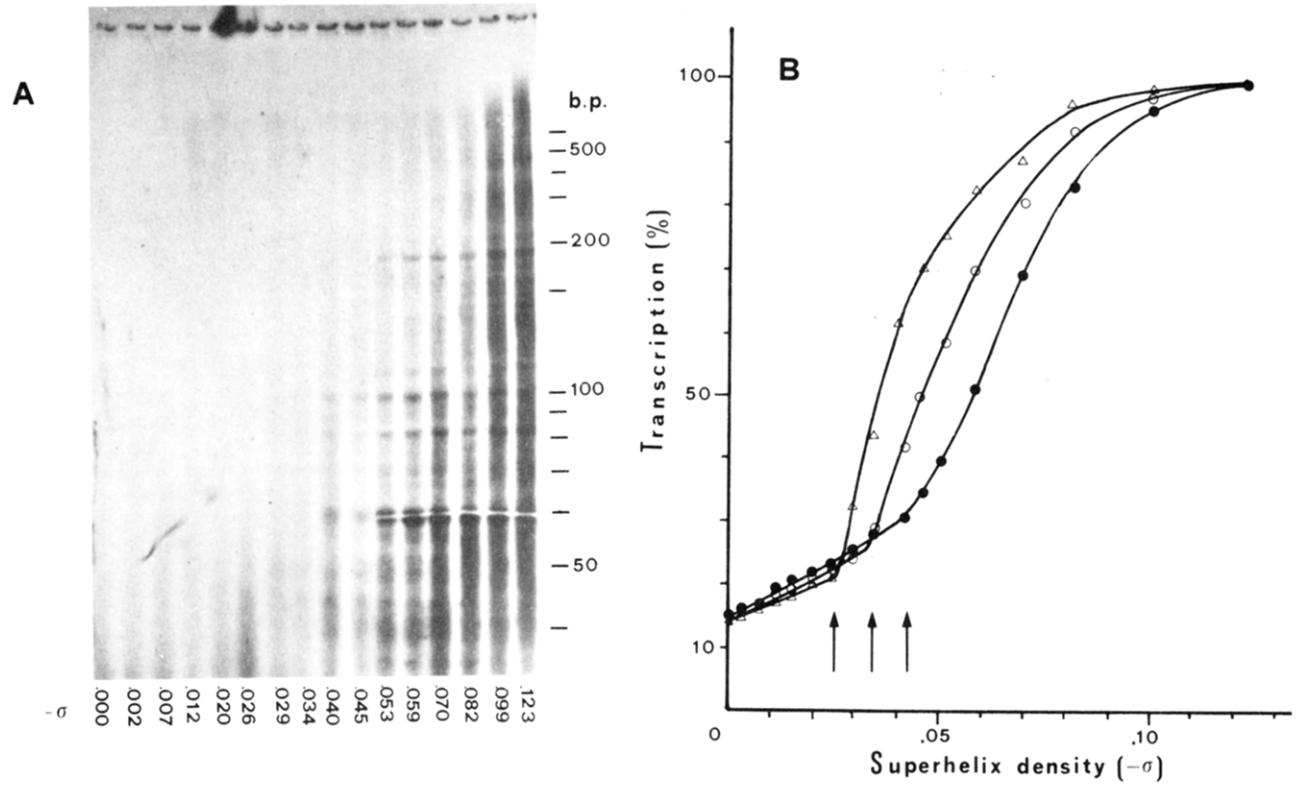

FIGURE 2: RNA transcripts. (A) Autoradiography of gel electrophoretic analysis of RNA synthesized by RNA polymerase II (0.45 μg) on p30-δ DNA (0.8 μg of scDNA) at various degrees of supercoiling. Enzyme to scDNA molar ratio = 3.2; [rNTP] = 5×10^{-6} M (size markers RFφX174 DNA digested by *Hae*III). (B) Plot of percent of transcription vs. superhelix density [as shown in (A)] at three enzyme to scDNA molar ratios: (△) E:scDNA = 2.1; (○) E:scDNA = 3.2; (●) E:scDNA = 4. [rNTP] = 5×10^{-6} M.

shift of the critical point of about 0.01 unit of σ. This corresponds for p30-δ DNA ($\beta = 511$) to the relaxation of about five supercoils per molecule of RNA polymerase added.

Gamper & Hearst (1982) have found a value of 1.6 supercoils for the *E. coli* RNA polymerase acting on SV40 genome. Our finding is probably overestimated due to the presence in our RNA polymerase preparations of an unknown fraction of inactive molecule. The observed relaxation could also be contributed for by RNA–DNA hybrids that are known to be formed during in vitro transcription of eukaryotic RNA polymerase II (Lescure et al., 1978).

In order to better estimate the DNA unwinding angle induced by yeast RNA polymerase II, we looked for experimental conditions of transcription that allow the calculation of active RNA polymerase II molecules and that prevent the formation of RNA–DNA hybrids.

Initiation Complexes of RNA Polymerase II on p30-δ DNA. Native supercoiled p30-δ DNA was transcribed by RNA polymerase II in the presence of the polyanion heparin, which inactivates free and loosely bound enzyme molecules (Ballario et al., 1980). Initiation complexes were formed upon incubation of the enzyme and DNA in the presence of only three rNTPs (UTP omitted); the reaction was then started by addition of the fourth rNTP and heparin. As shown in Figure 3A for representative autoradiograms, a drop in the amount of transcribed RNA chains is observed in these conditions; a quantitation of the transcripts is reported in Figure 3B. From such a plot, a concentration of 100 μg/mL heparin can safely be assumed to ensure one round of transcription without reinitiation. Nicked or linear templates support but a very low amount of synthesis (2–3%, lane 1 of Figure 3A) while a substantial number of low molecular weight RNA chains are avoided in the presence of heparin (compare in Figure 3A lane 2 with lanes 3–5).

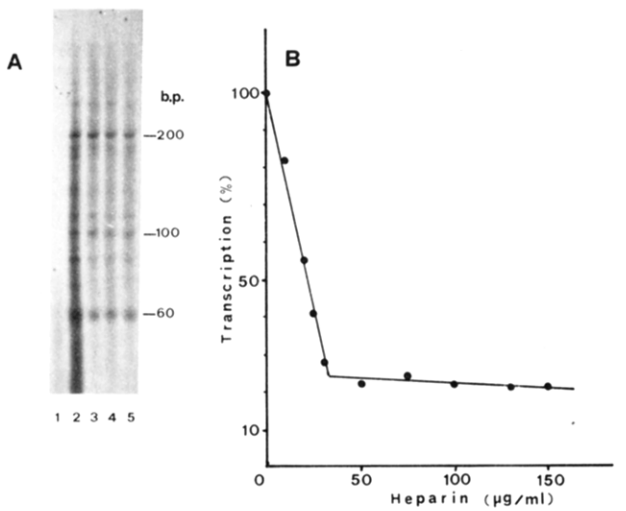

FIGURE 3: Residual transcription in the presence of heparin. (A) Autoradiography of synthesized RNA: (lane 1) p30-δ DNA template relaxed by UV light exposure in the presence of 1 μg/mL EB (see lane 2 of Figure 5A); (lanes 2–5) native supercoiled p30-δ DNA templates transcribed in the presence of 0, 50, 100, and 150 μg/mL heparin, respectively. Enzyme to scDNA molar ratio = 3.2; [rNTP] = 5×10^{-6} M. (B) Plot of percentual transcription vs. amount of heparin.

To calculate the number of active RNA polymerase molecules, RNA-labeled bands were excised from the dried gel and measured for Cerenkov effect. Various amounts of [α-^{32}P]-UTP were used as standards of radioactivity in the same conditions to quantitate, for each band, the amount of synthesized RNA. The sum of the ratios between these values and the molecular weights of the bands gives per se the number of active enzyme molecules in initiation complexes. In Figure 3A, lanes 3–5, smears between the bands are visible; the at-

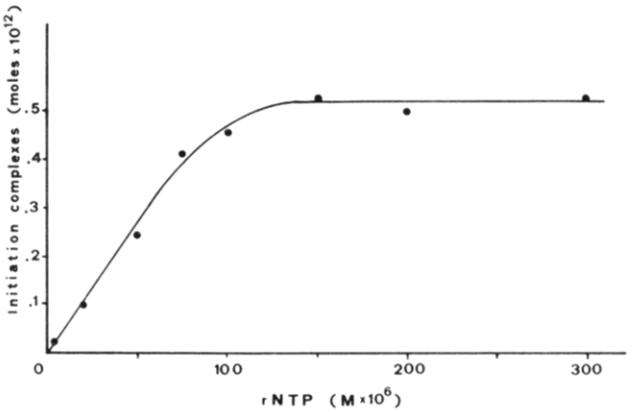

FIGURE 4: Initiation complexes formed by RNA polymerase II on native supercoiled p30-δ DNA as a function of amount of rNTPs. Enzyme to scDNA molar ratio = 3.2; [heparin] = 100 μg/mL.

tribution of mean molecular weights to such distributions of RNA chains of different length somehow reduces the accuracy of these determinations.

The number of active RNA polymerase molecules was also determined as a function of rNTP concentration. Figure 4 shows that, at a rNTP concentration above 150×10^{-6} M, all the enzyme added is active. The RNA patterns obtained at rNTP concentrations higher than 10^{-4} M exhibit more smearing that those obtained at lower values (data not shown), so the number of initiation complexes reported in Figure 4 must be taken with a confidence of $\pm 10\%$.

DNA Unwinding Angle Induced by RNA Polymerase II on Native p30-δ scDNA. We have adopted the strategy of Gamper & Hearst (1982) to calculate the unwinding angle induced by yeast RNA polymerase II on DNA. Complexes between the enzyme and native scDNA at various molar ratios were formed and then treated with an excess of topoisomerase I. The DNA relaxing enzyme removes all the superhelical turns present in the molecule except those preserved by the RNA polymerase. After the removal of the enzyme, different degrees of supercoiling can be visualized by agarose gel electrophoresis as a function of the enzyme fraction bound during relaxation. However, binary complexes between DNA and yeast RNA polymerase II are not stable during relaxation (data not shown), in contrast with the observation made with *E. coli* RNA polymerase (Gamper & Hearst, 1982). This was circumvented by the addition of three rNTPs (minus UTP); a stable formation of initiation complexes was thus ensured, as judged by the topoisomers pattern obtained after treatment with topoisomerase I (Figure 5A). Since it is known that each base of commercially available rNTPs preparations may be contaminated by the others, we have tested for hypothetical elongation in our experimental conditions. We have observed (data not shown) that transcription in the absence of UTP drops by a 10^3-fold factor; hence, the amount of RNA chains synthesized cannot detectably affect the DNA unwinding angle.

We have observed that heparin inhibits the topoisomerase I activity. On the other hand, in the absence of heparin, the RNA polymerase can form many more initiation complexes with DNA, as indicated by the appearance of a large fraction of low molecular weight RNA chains (Figure 3A, lane 2). Due to the uncertainty on the number of reinitiation events, we have not been able to calculate in this case the exact percent of active RNA polymerase molecules in the initiation complexes. The number of active RNA polymerase molecules per genome that we report in the plot of Figure 5B is, for the reasons described above, underestimated. The DNA unwinding angle

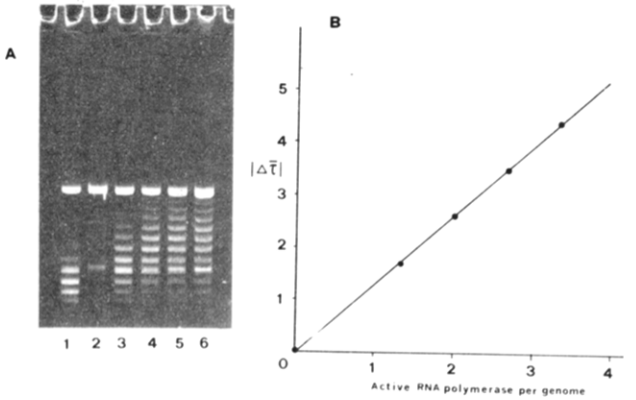

FIGURE 5: Determination of shift in τ induced by RNA polymerase II in initiation complexes (A) Agarose gel (1.4%) in the presence of 0.01 μg/mL EB. 0.75 μg of native supercoiled p30-δ DNA was relaxed by topoisomerase I in the presence of increasing concentrations of RNA polymerase II. Lanes 1 and 3–6 correspond respectively to 0, 1.3, 2, 2.7, and 3.4 active RNA polymerase molecules per genome. Lane 2 correspond to p30-δ DNA relaxed by UV light treatment as a marker of relaxed and linear forms. [ATP], [CTP], and [GTP] = 2×10^{-4} M. (B) Plot of shift in τ vs. active RNA polymerase per genome obtained by densitometry of the negative film of (A).

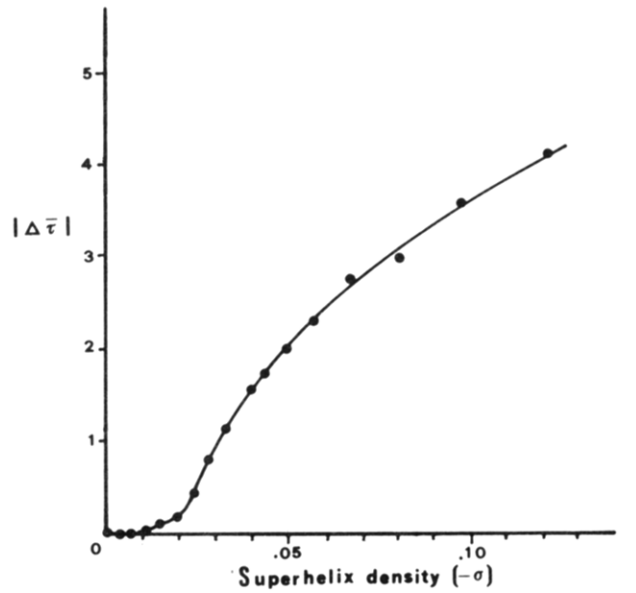

FIGURE 6: Shift in τ induced by RNA polymerase II on p30-δ DNA at different degrees of supercoiling. Enzyme to scDNA molar ratio = 3.2; [ATP], [CTP], and [GTP] = 2×10^{-4} M.

calculated by the data of Figure 5B is $485 \pm 30°$ (14 base pairs), slightly lower than the value of 580° determined for the *E. coli* RNA polymerase (Gamper & Hearst, 1982). We are aware that the unwinding angle value obtained for yeast RNA polymerase II is a rather rough approximation, but we hold it as a useful tool for relative measurements of RNA polymerase II binding to DNA.

Binding of RNA Polymerase II to p30-δ DNA at Different Degrees of Supercoiling. Initiation complexes were allowed to form between RNA polymerase II and p30-δ DNA at different degrees of supercoiling. The binding of RNA polymerase II to DNA is expressed in terms of variation in superhelical turns induced by the enzyme, as determined by agarose gel electrophoresis (Figure 6). At low values of δ, no binding of RNA polymerase seems to occur while, from data of Figure 2B, it is clear that the enzyme is transcribing though at a low rate. The curve of Figure 6 furthermore shows how, with increasing superhelix density, more enzyme molecules bind to DNA in a fashion that does not resemble the

drastic increase of transcriptional activity due to supercoiling as reported in Figure 2B. These results can be explained by assuming that a fraction of RNA polymerase molecules bound to DNA in initiation complexes is lost during the relaxation process. As a consequence, the number of active RNA polymerase II molecules considered in the calculation of the DNA unwinding angle is overestimated.

Conclusions

When the DNA double helix is subjected to the torsional strength induced by supercoiling, its conformational may vary in a discontinous fashion; melting of A-T-rich sequences, induction of hairpin-loop structures, and transition of DNA from the B to the Z form can occur at critical values of supercoiling. Here we have described the transcriptional behavior of yeast RNA polymerase II on circular DNA at various degrees of supercoiling and demonstrated that, in vitro the eukaryotic enzyme is sensitive to conformational variation of the template. The pattern of the RNA products analyzed in Figure 2 indicates that a critical value of supercoiling is required by RNA polymerase II for initiation and termination of in vitro transcription. Further increases of the supercoiling strongly affects the size and the amount of the RNA products.

We have set up a method for studying the binding of the enzyme to DNA through the measurement of the DNA unwinding angle. The values obtained must be considered relative ones due to the presence of systematic undetermined errors in their determination. Nevertheless, they have enabled us to show that the binding of RNA polymerase to DNA is enhanced by supercoiling and that the enzyme itself can lower the supercoiling degree of the template by melting the DNA double helix.

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Registry No. RNA polymerase, 9014-24-8.

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Complexation and Phase Transfer of Nucleic Acids by Gramicidin S†

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ABSTRACT: A novel interaction between gramicidin S (GrS) and nucleic acids is characterized which, like that between GrS and nucleotides, exploits both the dicationic and amphiphilic properties of the peptide. Complex formation between calf thymus DNA and GrS is demonstrated by (i) phase transfer to $CHCl_3$ of ultrasonically irradiated DNA and (ii) inhibition of phase transfer to $CHCl_3$ of adenosine 5′-triphosphate by either native or ultrasonically irradiated DNA. The stoichiometry of the interaction is 2:1 (DNA-P:GrS), which is consistent with a predominantly electrostatic mode of binding. The apparent affinity of GrS for DNA is considerably higher than it is for free nucleotides. The interaction of the monocationic derivative [2-N^{δ}-acetylornithyl]gramicidin S with calf thymus DNA is considerably weaker. DNA binding by GrS provides a rationale for the lag between germination and RNA synthesis exhibited by wild-type spores of producer strains of *Bacillus brevis* but not by GrS-negative mutants. On the basis of these results in vitro, a protective role is proposed for GrS in the dormant spore.

Very little is known of the physiological role of gramicidin S [GrS,[1] *cyclo*-(Val[1]-Orn[2]-Leu[3]-D-Phe[4]-Pro[5])$_2$] in the producer strains of *Bacillus brevis*, even though the mode of biosynthesis (Katz & Demain, 1977) and metabolic fate (Egorov et al., 1970) of the peptide are relatively well characterized. GrS is synthesized enzymatically at the end of logarithmic growth [at which time a GrS-dependent decrease in energy charge (Silaeva et al., 1965; Vostroknutova et al., 1981) and nucleotide efflux are observed (Glazer et al., 1966)] and is incorporated into the spore (Egorov et al., 1970; Nandi & Seddon, 1978). During germination and early vegetative growth, the GrS content of the culture decreases, and products of GrS hydrolysis are detected in the medium. It has also been

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[1] Abbreviations: GrS, gramicidin S; AcGrS, [2-N^{δ}-acetylornithyl]-gramicidin S; Me_6GrS, [2,2′-N^{δ}-trimethylornithyl]gramicidin S; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NAS, *N*-acetoxysuccinimide; Orn, ornithine; TLC, thin-layer chromatography; Me_2SO, dimethyl sulfoxide; UV, ultraviolet.

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