Volume 12 Number 20 1984 Nucleic Acids Research

Effect of superhelicity on the transcription from the tet promoter of pBR322. Abortive initiation and unwinding experiments

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Received 5 July 1984; Revised and Accepted 28 September 1984

ABSTRACT

Supercoiling of DNA is now known to have considerable effects on transcription in bacteria. By abortive initiation reaction (6) we have determined the binding constant K_B and the forward rate of isomerization k_2 as a function of temperature, pH and buffer for the tet promoter in a supercoiled plasmid. If the activation energy of isomerization is very similar to that obtained previously under the same conditions on a linearized plasmid (6) (respectively 21 ± 5 kcal/mole and 13 ± 5 kcal/mole) the supercoiling introduces very important and not well understood changes in the thermodynamic parameters of the association polymerase - promoter.
Using the technique of superhelical DNA relaxation by eukaryotic topoisomerase I, we have determined the specific unwinding by RNA polymerase of the tet promoter of pBR322 (430°). This unwinding differs only slightly from the mean value (470°) obtained for all the promoters of pBR322.

INTRODUCTION

One major point of gene expression regulation in prokaryotes is the specific interaction between RNA polymerase and promoters (1,2,3).
Especially the initiation of transcription appears as a finely controlled mechanism, with different regulation levels :
1) The frequency of initiation seems to be directly correlated with the interaction of polymerase with promoter DNA. This can well be pointed out by a comparison between the association of RNA polymerase to the "major" promoters (A_1, A_2 and A_3) and the "minor" ones (B, C, D and E) of bacteriophage T_7 (4).
A minimal model for the association of RNA polymerase with a promoter can be written as a two step mechanism

$$R + P \underset{K_B}{\rightleftharpoons} RP_C \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} RP_O$$

where R represents the RNA polymerase, P the promoter, RP_C a closed initial complex and RP_O the open complex. The binding constant K_B ($K_B = k_1/k_{-1}$) deter-

mines the fractional occupancy of RP_c, and k_2 and k_{-2} are the rates of isomerization between RP_c and RP_o.

In this paper we have used the technique of abortive initiation as described by Mc Clure (5) and adapted to a fluorescent detection by Bertrand et al. (6), for the study of the tet promoter in a supercoiled pBR322 to obtain the parameters K_B and k_2 as a function of temperature (from 25°C to 37°C) and pH (from 6 to 8.5). We have also compared our results to those obtained previously with a linear plasmid (6).

2) The degree of supercoiling of DNA is an important factor in transcription regulation.

In vivo the supercoiling of DNA is regulated by the activity of two antagonistic types of enzymes : the gyrases, creating superturns and stressing DNA and other topoisomerases eliminating them and relaxing DNA.When gyrase activity is blocked by chemical means, relaxation of cellular DNA leads to an important decrease of the transcriptional activity (7).

Direct effect of supercoiling on transcription was also tested by Botchan et al. (8), showing in vitro that increasing the number of superturns of the DNA generally favors the transcription. They found that total RNA synthesis from DNA was maximum for the template with -110 superhelical turns and that the regulatory role for superhelicity occurs probably during initiation of transcription. On the contrary, supercoiling seems to inhibit transcription of genes like the gyrase A and gyrase B genes (9, 10).

If it is clear that the unwinding of DNA during RNA polymerase-promoter complex formation, should occur more easily on negatively supercoiled DNA, the relation between transcription and DNA supercoiling is not well understood. In earlier studies Wang et al. (11) found with circular fd DNA an unwinding angle of 140° to 240° per promoter, depending on ionic strength, and concluded that half to one turn of helix is unwound . Subsequently Hsieh and Wang (12) using a T_7 DNA fragment and a different technique, estimated 10-12 bp unwound per promoter.

Recently Gamper and Hearst (13) found a unwinding angle of 580° for the early strand promoter on SV40 DNA.

In fact, the reported values are rather divergent. We have thus determined the unwinding of the tet promoter upon binding of the RNA polymerase by comparison of the unwinding of the integral pBR322 with a plasmid lacking a small DNA fragment which harbours the tet promoter.

MATERIALS AND METHODS

Preparation of enzymes

The RNA polymerase was extracted from the K-12 strain of E. coli, according to the classical method (Burgess and Jendrisak, (14)) and purified to electrophoretic homogeneity. The specific activity on calf thymus DNA was 920 units/mg, where 1 unit equals 1 nmol of UTP incorporated in 10 min. An estimation of σ content was at least 60% molar based on sodium dodecyl sulfate-polyacrylamide gel densitometry.

Purified topoisomerase I from chromatin extract of African green monkey kidney cell Vero P, was generously given to us by A. Kolb and H. Buc.

Preparation of DNA templates

Plasmid pBR322 was extracted from E. coli strain AB 1157. After growing cells at 37°C until $OD_{700} \simeq 0.6$, the plasmid was amplified by addition of chloramphenicol to the culture (200 mg/l culture) followed by an overnight incubation at 30°C. Total DNA was extracted by classical way and pBR322 DNA was separated from the nuclear one on a CsCl gradient.

Plasmid $pBREB_3$ was constructed as a shorter derivative (4171 bp) of pBR322. A 191 bp fragment, containing the whole promoter of the tetracycline gene,was deleted by digestion of pBR322 with Eco RI (Biolabs) and Eco RV (Biolabs) endonucleases at two single restriction sites.
To obtain blunt ends, the DNA was treated with T_4-DNA polymerase (Biolabs)(15). and religated with T_4-DNA ligase (Biolabs) in the presence of T_4-RNA ligase (Biolabs)(16).The resulting plasmid has lost the tetracycline resistance. As expected a single Eco RI site is restored by this procedure.

Nucleoside triphosphate

Unlabeled nucleosides triphosphate and dinucleosides monophosphate were purchased from Sigma and P.L. Biochemicals Inc, respectively.
ANS (1 Naphthylamin-5-sulfonic acid) was from Fluka AG, Bucks, S.G.
UTP-γ-ANS was prepared as described by Yarbrough et al. (17).

Fluorescence measurements

They were performed on a Jobin-Yvon JY 3 D spectrofluorimeter. Temperature inside the cell was kept constant by means of a thermostated cell holder connected to a circulation bath (Huber HS 40).
In order to record the first seconds of the initiation kinetics, we have adapted to the fluorescence cell a rapid mixing system initially used in a quenched-flow apparatus. The total volume which results from the mixing of the two solutions is 600 µl. The excitation wavelength was selected at 360 nm and the fluorescence emission was recorded at 430 nm.

The variation with time of the fluorescence intensity was recorded discontinously with a time between each measurement varying from 2 to 8 seconds. All the data were transferred to a Hewlett Packard 9815 A and then processed.

Abortive initiation assays

As reported previously (5,6), this technique leads to the production of short oligonucleotides.

In our case, using the dinucleoside GpU as starting nucleoside and UTP-γ-ANS as the elongating nucleotide, we obtained a tetranucleoside GpUpUpU for the tet promoter of pBR322. Standard reaction conditions were : 0.05 M Hepes or Tris-HCl (pH from 6 to 8.5), 0.4 mM phosphate buffer, 0.05 M KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM GpU, 0.1 mM UTP-γ-ANS as elongating nucleotide. The DNA template was 5.3 nM in plasmids and RNA polymerase concentration varied from 0.075 μM to 0.3 μM. After 10 min of equilibration at a given temperature, the two solutions were mixed directly in the fluorescence cell. Assuming that k_1 and k_{-1} are fast relative to k_2 (pre-equilibrium condition), that $k_2 >> k_{-2}$ and that the RNA polymerase is in large excess relative to the promoter, then the lag time τ is related to R the polymerase concentration as follows :

$$\tau = \frac{1}{k_2} + \frac{1}{K_B k_2 [R]}$$

and a plot of τ versus $1/[R]$ yields immediately k_2 (the rate of isomerization between RP_c and RP_o) and K_B (the affinity constant). Experimental data were recorded and treated as described previously (6).

Relaxation of supercoiled circular pBR322

16 nM of plasmid DNA were incubated at 37°C (or 25°C) in 80 μl of buffer containing 40 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT and 100 μg/ml bovine serum albumin. RNA polymerase was incubated with the DNA for 10 min before addition of 10 μl of purified topoisomerase (5-50 units). After 90 min (or in some cases after 180 min) of incubation, the reaction was stopped by addition of SDS (1% final) and EDTA (25 mM final) prewarmed to the same temperature. These DNAs were than extracted as described by Kolb and Buc (18) and dissolved in 30 μl of the following buffer (10 mM Tris-HCl pH 8, EDTA 1 mM, 6% Ficoll, 0.025% bromophenol blue and 0.025% xylenecyanol blue), before application of 10 μl to the agarose gel.

Electrophoresis was carried out at room temperature in a horizontal gel apparatus using 1.5% agarose gels (15 x 14.2 x 0.3 cm) equilibrated in the electrophoresis buffer (40 mM Tris-HCl pH 8, 20 mM sodium acetate, 5 mM EDTA and 0.015 μg/ml or 0.025 μg/ml ethidium bromide).

Table I - Variation of K_B and k_2 with temperature in a supercoiled plasmid pBR322 (on each value, the error is $\leq$ 10%).

Constant	Conditions	25°C	29°C	32°C	37°C
K_B x 10^{-7} M^{-1}	Tris	5.3	1.5	1.2	0.6
	Hepes	8.5	7.1	6.0	1.3
k_2 x 10^3 s^{-1}	Tris	4.1	10.9	13.8	20.9
	Hepes	5.4	7.8	10	22.1

A field of 3 V/cm was applied to the gel overnight. Each gel was stained with 1 µg/ml ethidium bromide and photographed with a polaroïd type 55 film. Negatives were traced by a transidyne 2955 scanning densitometer.

RESULTS

1. Kinetic analysis of the association between tet promoter of pBR322 in a supercoiled plasmid and RNA polymerase

Validity control of the abortive initiation assays

To be sure that non-specific transcription at any GpTpT sequence of pBR322 doesn't take place, we have tested the abortive initiation on the plasmid $pBREB_3$ (deleted for the tet promoter). In the most favorable conditions (37°C and 0.3 µM RNA polymerase) the observed increase of fluorescence with time was very slow with a lag time of more than 300 s.

A correction of the specific initiation by this background did'nt change the observed lag time by more than 5 s and was neglected.

Effect of temperature on K_B and k_2

The behaviour of the affinity constant K_B and of the rate of isomerization k_2 with temperature are opposite (table I) in a supercoiled plasmid.

k_2 increases with temperature. From an Arrhenius plot of these values (fig. 1), we can deduce an activation energy Ea = 21 ± 5 Kcal M^{-1}. The large uncertainties on Ea are due to the small temperature range studied.

K_B strongly decreases as temperature increases. These results are discussed in the next section.

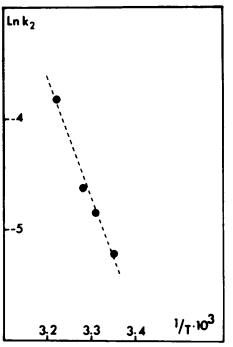

Figure 1 - Arrhenius plot of k_2 (the isomerization rate between "closed" and "open" complex) in a supercoiled plasmid (0.05 M Hepes pH 7.9, 0.4 mM phosphate buffer, 0.05 M KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol).

. Effect of pH on K_B and k_2

The values of τ are plotted as a function of the inverse of polymerase concentration for different pH values (fig. 2). The intercept ($1/k_2$) decreases when pH increases from 6 to 8.5, but the slopes remain constant. This means that the rate of isomerization k_2 increases while the association constant K_B decreases when pH increases. But the product $K_B k_2$ remains constant as shown in table II.

. Effect of the nature of the buffer

As can be observed in table I, the K_B values are lower in Tris-HCl buffer

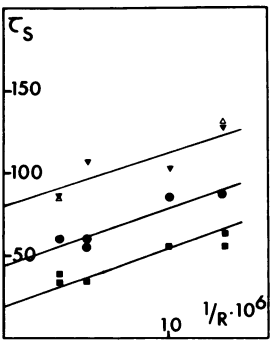

Figure 2 - τ plot for the tet promoter of pBR322 in a supercoiled plasmid DNA. The lag times observed from GpUpUpU synthesis are plotted versus the reciprocal of the RNA polymerase concentration at different pH values in Hepes buffer at 37°C. (△) pH 6, (▲) pH 7, (●) pH 8 and (■) pH 8.5.

Table II - Effect of pH on the values of K_B and k_2 (on each value, the error is $\leq$ 10%)

Constant	7	8	8.5
$K_B \times 10^{-8}\ M^{-1}$	0.25	0.13	0.06
$k_2 \times 10^{3}\ s^{-1}$	12.2	22	45
$K_B k_2 \times 10^{-6}\ M^{-1}\ s^{-1}$	0.3	0.3	0.27

than in Hepes buffer, indicating a role of the ion composition of the buffer in the association process and in the formation of the closed complex. The observed differences are too large to arise from the slight pH variation of Tris buffer with temperature. On the other hand, values of k_2, which correspond to the isomerization process, appear to be practically insensitive to the change of buffer.

2. Influence of supercoiling in transcription

Principle for measuring the unwinding of the DNA helix by gel electrophoresis

The topological properties of closed-circular duplex DNA are defined by the relationship $L_k = T_w + W_r$, where L_k represents the topological linking number, T_w the twist number and W_r the writhing number.

As described by Depew and Wang (19) relaxation of a circular DNA by topoisomerase I generates a family of topoisomers differing by the topological linking number L_k. These topoisomers can be resolved under appropriate conditions by electrophoresis. As shown in fig. 3a, a set of bands appears on the gel, and two adjacent bands differ by one superhelical turn.

Because of thermal fluctuations of the DNA helix at the time of ring closure, these topoisomers follow a Boltzmann distribution and the median of the gaussian curve can be easily determined (fig. 3b).

We have compared these distributions for DNA samples only treated with topoisomerase I and other samples first incubated with E. coli RNA polymerase holoenzyme in different conditions. After removal of RNA polymerase the median of the new distribution will be displaced correspondingly to the initial unwinding induced by RNA polymerase.

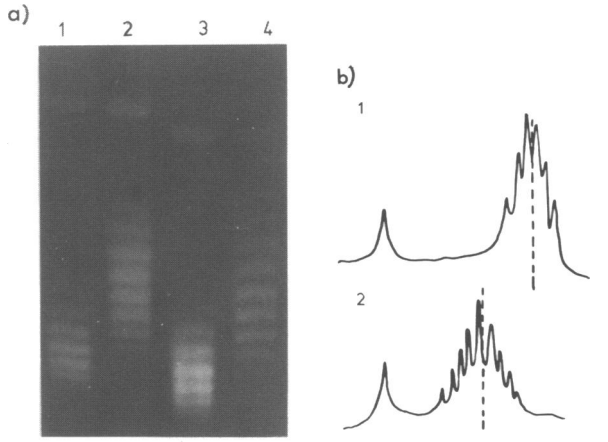

Figure 3 - a) 1.5% agarose gel run in presence of 0.025 µ g/ml ethidium bromide. The plasmids pBR322 and $pBREB_3$ relaxed with topoisomerase I alone are shown in lanes 1 and 3 respectively. In lanes 2 and 4 are displayed the migration pattern of the same plasmids relaxed in presence of RNA polymerase (RNA polymerase/DNA ratio = 6). b) Representative scans from fig. 3a, lanes 1 and 2 ; (---) median of the gaussian distribution of topoisomers.

Effect of temperature on the supercoiling of pBR322

To determine the importance of the topological variations of the circular DNA templates observed with temperature in the range studied for initiation, we have determined the variation of supercoiling of pBR322 between 25°C and 37°C. We have first confirmed that after 90 min of incubation of DNA with topoisomerase I (at 37°C or 25°C) the circular DNA gives the same repartition of topoisomers as after 3 h. This means that after 90 min of incubation either at 37°C or at 25°C the topoisomerase I unwinding is complete.
Under these conditions we have found a variation of 1.6 superturns between 25°C and 37°C. As pBR322 is a 4362 bp long plasmid, this means that the topological variation of pBR322 with temperature is about 0.011 degrees/base pair per °C value close to that already determined (19).

. Unwinding of the DNA helix by RNA polymerase

Figure 3b shows that in the presence of RNA polymerase the group of covalently closed species forms a gaussian distribution similar to the control sample, but the center of the distribution is displaced in comparison with the center of the control. This can be interpreted as an unwinding of circular DNA template by RNA polymerase. The agarose gels are run in the presence of ethidium bromide inducing positive superturns into all closed circular DNA species.

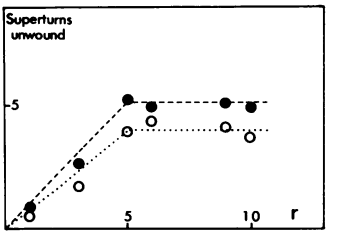

Figure 4 - Total unwinding of DNA template, as a function of r, the molar ratio of RNA polymerase to DNA.
(●) pBR322 (O) $pBREB_3$

Figure 4 depicts the total unwinding of pBR322 as a function of r, the molar ratio of RNA polymerase to DNA during reaction. We can note that the unwinding of pBR322 by RNA polymerase is very quickly saturated (when $r > 5$, unwinding of DNA is constant and corresponds to 5.2 superturns).
This result can be interpreted in terms of a specific binding of RNA polymerase to the promoters of pBR322 in our experimental conditions (40 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl). When $r < 5$, the occupation of the different promoters is progressive with probably a preferential order of occupation.

. Specific unwinding of the tet promoter by RNA polymerase

Applying the same analysis as previously to $pBREB_3$ (pBR322 lacking the 191 bp tet promoter fragment), we have obtained new gaussian distributions (fig. 4) differing from those obtained on pBR322 only by the effect of RNA polymerase on the 191 bp fragment.
The unwinding of $pBREB_3$ in presence of RNA polymerase (compared to $pBREB_3$ without RNA polymerase) is also limited to binding of the first molecules of RNA polymerase, but the total effect observed is only about 4 superturns. It seems clear that the difference (about 1.2 superturns) observed between pBR322 and $pBREB_3$ corresponds exactly to the unwinding by RNA-polymerase of the 191 bp fragment.

DISCUSSION AND CONCLUSION

Abortive initiation assays

Unlike the results previously obtained with the linearized pBR322 (6) in which the initial binding of RNA polymerase was stimulated by increasing temperature, we found an opposite behaviour in the case of a supercoiled template. The results for the linear plasmid could be easily explained as an entropy driven interaction. The situation is less clear in the case of a supercoiled plasmid DNA.

If, for example, the apparent changes of K_B with temperature are interpreted in terms of a Van't Hoff plot (data not shown) one obtains highly negative enthalpy and entropy changes. Such a result contradicts the general thermodynamic behaviour of protein-nucleic acid complexes, i.e. high positive ΔS values and small positive or negative ΔH values (20,21,22).

On the other hand, Van't Hoff calculation refers to the evolution with temperature of the association constant between two given species. It cannot be directly applied to a system in which one of the components undergoes structural modifications with temperature. As shown before, this is indeed the case for supercoiled DNA and therefore the RNA polymerase does not bind exactly to the same molecule at 25°C and 37°C.

If we assume the same positive ΔH value for the binding of RNA polymerase to either linear or supercoiled DNA since electrostatic interactions and hydrogen bonding are likely similar in both cases,and introducing the value of ΔH = 29 Kcal/mole as measured with linear DNA (6), we obtain decreasing values of ΔS with temperature (134 e.u. at 25°C and 126 e.u. at 37°C).

We have no explanation to this decrease of ΔS with temperature but it appears reasonably small. We have anyway to point out the difficulty of interpreting thermodynamic quantities in such a complex system. The definitive explanation of these results would probably come from the analysis of a more complex process as assumed by many works (25,26,27), and where environmental conditions like temperature, superhelicity and pH may act at different steps.

Results obtained by varying the pH shows the same features for the tet promoter in a supercoiled plasmid and in a linearized one : when the pH increases, the association constant K_B decreases while the isomerization rate constant k_2 increases. The main conclusion observed as a function of pH, is the constancy of the product $K_B k_2$ with or without supercoiling.

Unwinding of DNA helix by the RNA polymerase

During initiation of transcription the DNA double helix is unwound by E. coli RNA polymerase in the promoter region of a gene.

In our experimental conditions, the observed unwinding corresponds to a specific binding of RNA polymerase to promoter regions, since the total unwinding observed on pBR322 reaches a limiting value after the association of 5 polymerase molecules per plasmid.

As described previously there are 5 promoters on pBR322 but 2 of them (tet and anti-tet) are rather intricated. Moreover, the transcription of each promoter occurs in opposite directions (23). This exclude a simultaneous binding of the RNA polymerase to both of them (23).

We can thus consider that the observed unwinding corresponds to the specific binding of RNA polymerase to 4 promoters of pBR322 and determine a mean value of 470° unwinding per promoter. The difference of unwinding measured between pBR322 and $pBREB_3$ (430°) also corresponds to the binding of RNA polymerase to only one promoter, either tet or anti-tet, without any contribution of unwinding of the other promoter.

We have determined that the time τ necessary for the formation of the open complex of the anti-tet promoter is about three times longer than that of the tet promoter under the conditions used in the unwinding experiments (results not shown). This allows an estimation of the relative occupancy of the two promoters of about 75% for the tet promoter and 25% for the anti-tet promoter. This is in agreement with previous works (23,24) which have shown that the tet promoter is much stronger than the anti-tet one.

From these data we can reasonably deduce that the association of RNA polymerase with tet promoter is largely favored and that the difference of unwinding between pBR322 and $pBREB_3$ corresponds essentially to the tet promoter. In terms of base pair opening and assuming a value of 10.4 bp per helical turn in solution (28), the number of opened base pairs would be about 12.5 in the case of the tet promoter and 13.6 for the average of the promoters present in the pBR322 plasmid DNA. These two values compare favorably with that of 12 bp as estimated previously (29,30).

ACKNOWLEDGEMENTS

We thank Dr A. Kolb (Institut Pasteur) for the gift of topoisomerase I and Dr H. Buc (Institut Pasteur) for helpful discussions.

This work was supported by a grant n° 81.E.1225 of the former Délégation Générale à la Recherche Scientifique et Technique.

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