The main early and late promoters of *Bacillus subtilis* phage ϕ 29 form unstable open complexes with σ^A -RNA polymerase that are stabilized by DNA supercoiling

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ABSTRACT

Most *Escherichia coli* promoters studied so far form stable open complexes with σ^{70} -RNA polymerase which have relatively long half-lives and, therefore, are resistant to a competitor challenge. A few exceptions are nevertheless known. The analysis of a number of promoters in *Bacillus subtilis* has suggested that the instability of open complexes formed by the vegetative σ^A -RNA polymerase may be a more general phenomenon than in *Escherichia coli*. We show that the main early and late promoters from the *Bacillus subtilis* phage ϕ 29 form unstable open complexes that are stabilized either by the formation of the first phosphodiester bond between the initiating nucleoside triphosphates or by DNA supercoiling. The functional characteristics of these two strong promoters suggest that they are not optimized for a tight and stable RNA polymerase binding. Their high activity is probably the consequence of the efficiency of further steps leading to the formation of an elongation complex.

INTRODUCTION

The transcription initiation process in prokaryotes can be formally divided into four major steps: (a) binding of the RNA polymerase to the promoter to form a 'closed complex', (b) isomerization of the closed complex into a structure in which the DNA is melted in the initiation region, forming an 'open complex', (c) incorporation of the first nucleoside triphosphates (NTPs) to produce an initiated complex and, (d) transition into an elongation complex that leaves the promoter (1,2). A particularly relevant additional intermediate between the open and elongation complexes has been proposed, named the stressed intermediate, in which the RNA polymerase has started to produce short transcripts but still has not been able to clear the promoter (3). The overall strength and functional characteristics of a promoter will depend on the efficiency of the steps leading to the formation of each of these complexes, all of which can in principle act as a bottle-neck. It is now clear that not all strong promoters are optimized in the same way. Strong promoters that are negatively regulated by repression factors tend to bind RNA polymerase

far below maximal rates, the subsequent steps leading to the formation of an elongation complex being highly efficient. Other strong promoters are optimized for a tight RNA polymerase binding, the following steps leading to promoter clearance being less efficient (4–6). Most promoters analyzed in detail, which correspond to *Escherichia coli*, form open complexes with RNA polymerase that are essentially irreversible *in vitro*, in the sense that they have relatively long half-lives (in the range of several minutes to hours, refs. 3, 4, 6, 7), and are therefore resistant to the challenge of competitors such as heparin or heterologous DNA. There are however exceptions, of which the best known are the P_1 promoters of the *E.coli* *rrnB* and *rrnD* operons for rRNA. These promoters give rise to unstable open complexes that are sensitive to a short heparin challenge, and are stabilized either by the addition of the first two initiating NTPs (leading to the formation of an initiated complex), or by DNA supercoiling (8–10). Similarly, the study of some strong *Bacillus subtilis* promoters in linear templates has recently shown that many of them, though not all, form unstable open complexes with σ^A -RNA polymerase, being likely that this type of promoters is more frequent in *B.subtilis* than in *E.coli* (11). In line with this finding, we describe in this work that open complexes formed by σ^A -RNA polymerase at the main promoter for *B.subtilis* phage ϕ 29 early genes, named P_{A2b} , and at the late P_{A3} promoter responsible for the expression of all the viral late genes (12, 13), are unstable, being sensitive to heparin challenge. Both are strong and regulated promoters; the viral early protein p4 represses the early P_{A2b} promoter at late times of the infection (14) and simultaneously activates the late P_{A3} promoter (14, 15; see Figure 1). The heparin sensitivity of these two promoters led us to study their functional characteristics in comparison with other promoters known to form unstable open complexes.

METHODS

Template DNAs

The source of DNA was plasmid pFRC64 (14), which contains a 198 bp long *AccI*-*HindIII* DNA fragment from phage ϕ 29 genome, containing both P_{A2b} and P_{A3} promoters, cloned at the *SmaI* site of pUC19 Ω , so that transcription from P_{A2b} promoter faces the unique *EcoRI* site from the pUC polylinker sequence.

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In vitro transcription assays

Transcription reactions contained, in a final volume of 25 μ l, 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 20 mM NaCl, 4% glycerol, ammonium sulphate up to 48 mM (for P_{A2b} except in Figure 7, see legend) or 92 mM (for P_{A3}), and σ^A -RNA polymerase (35 nM final concentration except in Figure 5, see legend). 1 μ g of the regulatory protein p4 was also added when assaying P_{A3} activity or when analyzing its repression effect on P_{A2b}. The DNA templates used were either supercoiled pFRC64 plasmid (2 nM) or a 340 bp PvuI-BamHI fragment (2 nM), obtained from plasmid pFRC64, which gives rise to run-off transcripts 143 nt long from P_{A2b} and 79 nt long from P_{A3}. The initiating NTPs (GTP and UTP), the dinucleotide GpU, or GpU plus ATP, were also added when indicated at final concentrations of 40 μ M. Reaction mixtures were incubated for 10 min at 37°C to allow RNA polymerase to form the corresponding complexes with the promoters. Transcription was then started by the addition of 3 μ l of a preheated solution containing heparin and the four NTPs. The final concentration of heparin was 10 μ g/ml, and that of the NTPs, 200 μ M. Incubation was continued for 10 min, after which the reaction was stopped by the addition of 1 μ l of 0.5 M EDTA, 1 μ g of carrier tRNA, 3 μ l of 3 M potassium acetate and 70 μ l of ethanol. RNAs were precipitated and analyzed by primer extension as indicated below.

Primer extension of RNAs

The RNA was resuspended in a solution containing 40 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 50 mM NaCl, 10 units of RNasin ribonuclease inhibitor and 2–4 pmoles (a large excess) of oligonucleotides designed to hybridize 67 nt (for P_{A3}) or 98 nt (for P_{A2b}) downstream from the transcription start sites described in this work (see text and Figure 2). The mixture was heated for 5 min at 70°C and then allowed to cool slowly to 20°C. The solutions were then put on ice for 10 min and the RNA was precipitated by the addition of 4 μ l of 3 M potassium acetate and 3 volumes of ethanol. The RNA was resuspended in 5 μ l of water and the primer was extended in a solution containing 50 mM Tris-HCl (pH 7.5), 40 mM KCl, 7 mM MgAc₂, 2 mM DTT, 200 μ M each dNTP (except dATP which was 100 μ M), 2 μ Ci of α (³²P)dATP (400 Ci/mmol), 10 units of RNasin and 5 units of AMV-reverse transcriptase, in a total volume of 10 μ l. The reaction mixture was incubated at 42°C for 60 min, and then stopped by the addition of 0.5 μ l of 0.5 M EDTA and 30 μ l of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. The sample was filtered through a 1 ml Sephadex G-50 spun-column to eliminate the non-incorporated labelled nucleotide, and the eluted cDNA precipitated by the addition of 4 μ l of 3 M potassium acetate and 200 μ l of ethanol. The cDNA was analyzed by electrophoresis in 6% urea-polyacrylamide gels.

KMnO₄ footprinting

An end labelled DNA fragment containing P_{A2b} and P_{A3} promoters was incubated at 37°C for 20 min in a solution containing 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 2 μ g of poly(dI-dC), 0.5 μ g of σ^A -RNA polymerase (except in the control sample), in the absence (P_{A2b}) or presence (P_{A3}) of protein p4 (1 μ g), and with or without the initiating NTPs (GTP and UTP, 40 μ M each), in a total volume of 25 μ l. KMnO₄ was then added up to 4 mM and incubation continued for 30 seconds at 37°C. The reaction was stopped by the addition of 5 μ l of a solution containing 1 M β -mercaptoethanol and 1.5 M sodium

acetate. The DNA was precipitated, cleaved with piperidine as described (Maxam and Gilbert, 1980) and analyzed by electrophoresis in 6% urea-polyacrylamide gels.

Preparation of plasmids with different superhelical densities

Plasmid pFRC64 was relaxed with topoisomerase I in the presence of ethidium bromide concentrations ranging from 0 to 20 μ M, essentially as described (18). The superhelical densities of the samples obtained were calculated as described (19), from the linking number difference between the centre of the topoisomer distribution of a given sample and the centre of the topoisomer distribution corresponding to relaxed DNA. It is known that changes in temperature or ionic strength alter the average rotation angle between adjacent base pairs in the DNA helix which, in a closed-circular DNA, leads to a change in the superhelical density (20). Therefore, a DNA relaxed under the conditions optimum for topoisomerase I acquires certain superhelical density at the ionic conditions used in the transcription assays, which are higher. We have observed that plasmid pFRC64, relaxed at 100 mM KCl (optimum for topoisomerase I), acquires a superhelical density of -0.006 at the ionic conditions used in the transcription assays (not shown). We therefore took this data into consideration when calculating the superhelical densities of the different plasmid preparations. To obtain a relaxed template, we incubated the supercoiled plasmid with topoisomerase I under the same ionic strength conditions used in the transcription assays.

RESULTS**Determination of the precise start sites of the P_{A2b} and P_{A3} promoters**

The transcription start sites for the P_{A2b} and P_{A3} promoters analyzed by S1 mapping (13) indicated three contiguous possible start sites for each promoter that did not allow to determine which was the first nucleotide incorporated by the RNA polymerase, a problem frequently found with S1 mapping analysis. In an attempt to define the start site more precisely, a primer extension analysis of the transcripts produced *in vitro* from these promoters was carried out. Single transcription start sites were found very close to the previously described ones (Figure 2). RNAs produced *in vivo* in ϕ 29 infected cells gave the same results (not shown). A systematic search for the pair of NTPs that led to the formation of stable initiated complexes (to be shown and discussed below) indicated that only the simultaneous presence of GTP and UTP could give rise to stable complexes, indicating that these were

Figure 1. Arrangement of the main early (P_{A2b}) and late (P_{A3}) promoters of phage ϕ 29. Shaded boxes indicate the -10 and -35 regions and open boxes the binding sequences for the regulatory protein p4 (15, 16). Arrows indicate both the length and the orientation of the promoters.

the first two NTPs incorporated at both promoters. The start sites predicted by the primer extension analysis for both promoters agreed with GTP and UTP being the first NTPs incorporated (see Figure 2). Therefore, positions previously considered as +1 on the basis of S1 mapping assays, correspond indeed to -3 for P_{A2b} and -2 for P_{A3}. Similar discrepancies between the start sites predicted by S1 mapping and other methods have been noticed in other cases (see for example reference 21).

Stability of P_{A2b} and P_{A3} open and initiated complexes in linear templates

The addition of heparin to preformed σ^A -RNA polymerase-promoter complexes at 37°C in linear DNA fragments containing phage ϕ 29 P_{A2b} and P_{A3} promoters led to a drastic inhibition of the transcription reaction (not shown). Since most promoters can form stable open complexes under the conditions used, the heparin inhibition was interpreted to indicate that the two promoters were forming unstable complexes (presumably open complexes, since the incubation temperature was 37°C). To find conditions leading to the formation of stable complexes we investigated whether the addition in the incubation mixture of the first two initiating NTPs relieved the heparin

sensitivity, as it had been described for other promoters with similar characteristics (8).

It is known that the regulatory protein p4 efficiently displaces σ^A -RNA polymerase bound at P_{A2b} and directs it to the adjacent but divergently transcribed P_{A3} promoter (14), transcription from P_{A3} being negligible in the absence of p4 under the ionic conditions used (22). Therefore, protein p4 was always present when analyzing the P_{A3} promoter, while it was not added in the case of P_{A2b}. To analyze the stability of open complexes formed at these two promoters, a 339 bp DNA fragment containing both P_{A2b} and P_{A3} was incubated with σ^A -RNA polymerase at 37°C, with or without protein p4, and in the absence or presence of either the initiating NTPs GTP and UTP, the dinucleotide GpU, or GpU plus ATP. After 10 minutes incubation, heparin and the four NTPs were added and the transcripts produced analyzed by primer extension. Figure 3 shows that, using a linear template, a very reduced amount of transcripts was obtained when the initiating NTPs were omitted, whereas their addition led to the formation of initiated complexes that were resistant to the heparin challenge. Addition of combinations of two NTPs other than GTP plus UTP did not lead to complex-stabilization (not shown), corroborating that these were the two initiating NTPs for both promoters. The dinucleotide GpU could not stabilize the RNA polymerase-promoter complex formed at P_{A2b}, but produced a partial protection from heparin challenge in the case of P_{A3}. It is worth noting that in the presence of GpU+ATP, about 10% of the transcripts produced from P_{A3} started at position -3 instead of +1 (see Figures 2 and 3). The poor or null stabilizing effect of GpU indicated that, as it had been shown for the *E. coli* rrnB P₁ promoter (8), the appearance of a complex stable to heparin challenge requires the formation of at least the first phosphodiester bond. These results suggested that open complexes formed at the ϕ 29 P_{A2b} and P_{A3} promoters are

Figure 2. Transcription start sites for P_{A2b} and P_{A3} promoters. The horizontal arrows indicate the transcription start sites as deduced from a primer extension analysis of the corresponding transcripts. The asterisks show the start sites found by S1 mapping (13). The vertical arrows show the positions previously considered as +1 which, taking into account the start sites indicated by the primer extension analysis, correspond to -3 for P_{A2b} and -2 for P_{A3}.

Figure 3. Sensitivity to heparin challenge of open or initiated complexes formed at the ϕ 29 P_{A2b} and P_{A3} promoters. A linear or supercoiled DNA containing P_{A2b} and P_{A3} promoters was incubated at 37°C with σ^A -RNA polymerase, in the absence (P_{A2b}) or presence (P_{A3}) of protein p4, and with the NTP combinations indicated in each lane. After 10 min, a preheated mixture of heparin and the four NTPs was added and transcription allowed to proceed for 10 min at the same temperature. The reaction was then stopped, and the transcripts produced analyzed by primer extension and detected by polyacrylamide-urea gel electrophoresis and autoradiography. The transcription products were quantitated by densitometry; values obtained are indicated below each band relative to those obtained in the presence of the initiating NTPs, GTP and UTP, considered as 100%.

Figure 4. Analysis of open or initiated complexes by KMnO₄ footprinting. An end-labelled linear DNA fragment containing both P_{A2b} and P_{A3} promoters was incubated at 37°C with σ^A -RNA polymerase, in the absence (P_{A2b}) or presence (P_{A3}) of protein p4, with or without the initiating NTPs (GTP and UTP), and in the presence of an excess of non-specific DNA (see Methods, section for KMnO₄ footprinting). After 10 min, heparin was added up to 10 μ g/ml and incubation continued for either 15 seconds or 10 min (indicated in the figure as +h and +H, respectively). Open or initiated complexes were then probed by KMnO₄ footprinting, a reagent that cleaves the DNA preferentially in those positions in which the double helix is melted, single stranded thymine nucleotides being particularly sensitive. Arrows show nucleotides hyperreactive to the modifying agent in the template strand (P_{A2b}) or in the non-template strand (P_{A3}).

Figure 5. Activity of protein p4 on supercoiled DNA. The ability of protein p4 to activate P_{A3} and repress P_{A2b} on supercoiled templates was analyzed by *in vitro* transcription reactions performed as follows: a supercoiled DNA containing P_{A2b} and P_{A3} promoters was incubated at 37°C with σ^A -RNA polymerase, in the absence (P_{A2b}) or presence (P_{A3}) of protein p4, and with the four NTPs. The reaction was started by the addition of RNA polymerase (from 8.7 to 176 nM, final concentration); no heparin was added. After 10 minutes, the reactions were stopped and the transcripts produced analyzed by primer extension, polyacrylamide-urea gel electrophoresis and autoradiography. The activity is shown as x-fold activation on P_{A3} or x-fold repression on P_{A2b} .

unstable, and that initiated complexes are stable enough to resist the heparin challenge and give rise to elongation complexes. To confirm this idea, we analyzed by $KMnO_4$ footprinting the complexes formed. The $KMnO_4$ reagent cleaves the DNA preferentially at those positions in which the double helix is melted, single stranded thymines being particularly sensitive. Therefore, this method allows to detect the presence of open or initiated complexes, which appear as ssDNA regions, sensitive to $KMnO_4$, located around the +1 area of the promoter. In the absence of the initiating NTPs, open complexes were detectable at the P_{A3} promoter, but not at the P_{A2b} promoter (Figure 4). Under these conditions, however, the open complexes formed at P_{A3} were not resistant to a short heparin challenge. In the presence of the initiating NTPs, initiated complexes were detectable by $KMnO_4$ footprinting at both P_{A3} and P_{A2b} promoters, and were resistant (P_{A2b}) or partially resistant (P_{A3}) to a short heparin challenge. To get a rough idea of the half-life of the initiated complexes the heparin challenge was allowed to proceed for either 15 seconds or for 10 minutes before the addition of $KMnO_4$ (simultaneous addition of $KMnO_4$ and heparin was not possible since the chemical reagent oxidizes the heparin). The results suggested that the half-life of these complexes was short, in the range of a few minutes for P_{A2b} and of 10–20 seconds for P_{A3} , since the DNA-strand opening was undetectable at both promoters after a 10 minutes challenge and, in the case of P_{A3} , considerably reduced after a 15 seconds challenge (Figure 4).

DNA supercoiling stabilizes open complexes formed at P_{A2b} and P_{A3} promoters

DNA supercoiling has been shown to stabilize open complexes formed at several bacterial promoters (9, 23–27), and is known to influence either positively or negatively the activity of many other promoters (Pruss and Drlica, 1989). We therefore analyzed

Figure 6. Effect of DNA supercoiling on the stability of open complexes formed at the $\phi 29$ P_{A2b} and P_{A3} promoters. The stability of open complexes formed at P_{A2b} and P_{A3} promoters on plasmids with different superhelical densities (from $\sigma = 0$ to $\sigma = -0.071$) was analyzed as follows: supercoiled DNA containing P_{A2b} and P_{A3} promoters was incubated at 37°C with σ^A -RNA polymerase, in the absence (P_{A2b}) or presence (P_{A3}) of protein p4, and the absence of the initiating NTPs. After 10 min, a preheated mixture of heparin and the four NTPs was added and transcription allowed to proceed for 10 min at the same temperature. The reaction was then stopped, and the transcripts produced analyzed by primer extension and detected by polyacrylamide-urea gel electrophoresis and autoradiography. The superhelical densities indicated correspond to those observed under the ionic strength conditions used in the transcription assays (see Methods, section for preparation of plasmids with different superhelical densities). (*) nicked DNA, assayed only for P_{A3} .

Figure 7. Effect of the ionic strength on the stability of open or initiated complexes formed at the P_{A2b} promoter. Linear or supercoiled DNAs containing P_{A2b} and P_{A3} promoters were incubated at 37°C with σ^A -RNA polymerase, in the absence of protein p4, and at ammonium sulphate concentrations ranging from 20 to 112 mM. When analysing the stability of initiated complexes, the initiating NTPs were also included. After 10 min, a preheated mixture of heparin and the four NTPs was added and transcription allowed to proceed for 10 min at the same temperature. The reaction was then stopped, and the transcripts produced analyzed by primer extension and detected by polyacrylamide-urea gel electrophoresis and autoradiography. The transcription products were quantitated by densitometry; P_{A2b} activity is expressed in each case relative to that observed with supercoiled DNA at the lowest ionic strength assayed, which is considered as 100%. When linear DNA fragments were used, the stability of both open (filled circles) and initiated (triangles) complexes was analyzed. In the case of supercoiled templates (rectangles), only the stability of open complexes was studied (no initiating NTPs added).

its influence on P_{A2b} and P_{A3} promoters. By transcription assays in which the heparin challenge was omitted and the transcripts produced were detected by primer extension, it was shown that p4 is active on supercoiled DNA (Figure 5), being able to activate P_{A3} and to repress P_{A2b} . The p4 dependence of P_{A3} expression

diminished as the concentration of RNA polymerase increased, consistent with the results obtained in linear templates showing that the effect of p4 is to favour the binding of RNA polymerase to the promoter (28).

Interestingly, RNA polymerase–promoter complexes formed at these promoters in the absence of the initiating NTPs on supercoiled templates were resistant to heparin challenge (Figure 3), indicating that DNA supercoiling could stabilize open complexes at both P_{A2b} and P_{A3} promoters. These assays were performed with fully supercoiled plasmid DNA, purified from cells grown to stationary phase. It is known that, *in vivo*, half of the superhelical density is restrained by DNA-binding proteins, so that the free superhelical density has a value close to -0.025 (29, 30). Phage $\phi 29$ has a linear genome whose degree of supercoiling in the cell is not known, but it is probably lower than that of a plasmid. We therefore analyzed if the stabilizing effect on P_{A2b} and P_{A3} open complexes was maintained also at low superhelical densities. For this purpose, a plasmid containing both promoters was relaxed with topoisomerase I in the presence of increasing amounts of ethidium bromide, so obtaining a number of plasmid preparations with a superhelical density ranging from 0 (relaxed plasmid) to -0.071 (fully supercoiled, see methods). Open complexes formed at P_{A2b} were resistant to an heparin challenge at superhelical densities ranging from -0.071 to -0.016 (Figure 6), but became sensitive at lower superhelical densities (-0.006 or 0). Open complexes formed at P_{A3} were resistant to the heparin challenge at superhelical densities as low as -0.006 , although became sensitive when the plasmid was either relaxed by topoisomerase I or nicked by a mild treatment with DNase I (Figure 6).

Effect of ionic strength on the stability of open complexes formed at P_{A2b} promoter

If unstable complexes are in a rapid equilibrium with unbound RNA polymerase, as it has been suggested for the *E. coli* rrnD P_1 and for the *B. subtilis* citB promoters (10, 11), an increase in the ionic strength should impair promoter efficiency. Indeed, the *E. coli* rrnB and rrnD P_1 promoters for rRNAs are known to be salt-sensitive, being necessary not only the initiating NTPs but also low salt concentrations to obtain a stable complex in linear templates (8). The salt sensitivity of P_{A2b} was assayed in linear and supercoiled templates, and in the former case, both in the absence and presence of the initiating NTPs (Figure 7). Open complexes formed at P_{A2b} on linear templates were very sensitive to the salt concentration. Initiated complexes were significantly more stable, although the total amount of transcripts obtained decreased notably when the ionic strength was increased. Interestingly, open complexes formed on supercoiled templates in the absence of the initiating NTPs were considerably less salt-sensitive than initiated complexes formed on linear templates. The activity obtained at an intermediate ionic strength (70 mM ammonium sulphate) was about 80% of that observed at the lowest concentration used (20 mM), a situation in which the activity of P_{A2b} in linear templates was of about 10% if the initiating NTPs were present or even lower in their absence. Assuming that salt is acting as a competitor for the binding of the RNA polymerase to the promoter, these results suggest that open complexes formed at P_{A2b} on supercoiled templates are more stable than initiated complexes found on linear templates, open complexes formed on linear templates being the less stable ones. The salt sensitivity assays were not performed with the

P_{A3} promoter, since the regulatory protein p4 is known to stabilize RNA polymerase binding to the promoter at high salt concentrations (28).

DISCUSSION

The study of a number of promoters in *E. coli*, both natural and synthetic, has indicated that there are several ways in which a promoter can be optimized to be highly efficient (3, 6, 10, 31). If the promoter is optimized for RNA polymerase binding, the steps leading to promoter clearance are likely to be slow; on the other hand, a rapid formation of elongation complexes should be facilitated if RNA polymerase is not tightly bound to the promoter. Tight binding of *E. coli* RNA polymerase to the lac promoter was found to be correlated with its poor ability to escape from abortive cycling (32). The *E. coli* rrnB and rrnD P_1 promoters for rRNAs appear to be adapted to high productivity by having a rapid promoter clearance, something that is probably related to the characteristic instability of their open complexes (10). Our analysis of the main early and late promoters (P_{A2b} and P_{A3} , respectively) of *B. subtilis* phage $\phi 29$ has indicated that they share many of the functional characteristics of the *E. coli* rrnB and rrnD P_1 promoters. Namely, they form unstable open complexes with the vegetative RNA polymerase that are stabilized either by the first NTPs, that allow the formation of an initiated complex, or by DNA supercoiling. Open complexes formed at the *E. coli* rrnB and rrnD P_1 promoters decay within seconds in the presence of heparin (9,10), indicating that the complexes are unstable and equilibrate rapidly either with free enzyme (10) or with a closed complex that is sensitive to competitor challenge (33). The formation of a phosphodiester bond between the first two initiating NTPs gives rise to an initiated complex that is considerably more stable. Interestingly, an initiating dinucleotide does not have the same effect, suggesting that phosphodiester bond formation may be an important requirement in the process (8). The *E. coli* rRNA promoters are also salt sensitive (8), something that is also probably related with the instability of the open complexes and that is also characteristic of $\phi 29$ P_{A2b} promoter.

Despite the similarities of the two $\phi 29$ promoters analyzed, there are certain differences between them. In the case of P_{A2b} , we were unable to detect DNA-strand opening (open complexes) in linear templates if the initiating NTPs were omitted, while for P_{A3} DNA melting was clearly detectable in the absence of GTP and UTP. A behaviour similar to that of P_{A2b} has been described for the rrnB P_1 promoter on linear templates (33).

The RNA polymerase has been shown to bend and untwist the DNA upon promoter recognition at several promoters (34–36), and this is likely to be a general phenomenon required for the formation of transcription complexes. It has been proposed that DNA supercoiling would act to change the DNA structure at the promoter facilitating or inhibiting the formation of a stable RNA polymerase–promoter complex, in which the initiation region is melted, and that can proceed to the promoter clearance step (25). The stabilizing effect of DNA supercoiling on open complex formation is probably reflecting that the torsional stress is favouring the opening of the DNA strands driving the RNA polymerase–promoter complex into a configuration with an increased half-life. This is in agreement with the idea, proposed for *E. coli* rrn promoters (10) and for the *B. subtilis* tms and citB promoters (11), suggesting that heparin sensitivity occurs because

the open complexes in these promoters are unstable and equilibrate rapidly with free enzyme, which is then irreversibly bound by heparin. An alternative explanation has recently been proposed for the *E. coli* *rrnB* P₁ promoter (33), stating that DNA supercoiling would allow the RNA polymerase to establish more extended contacts with the DNA in the +1 region of the promoter, and that heparin would remove from DNA RNA polymerase molecules that are stably but not tightly bound to the promoter as closed complexes. Stabilization of open complexes by DNA supercoiling has been observed in other promoters (23–26). Nevertheless, it is important to distinguish between those promoters for which DNA supercoiling is essential for heparin resistance (e.g. *E. coli* *rrnB* P₁, ref. 9), from those in which the supercoiling stimulates the formation of a complex that is nevertheless considerably resistant to heparin challenge in linear templates (e.g. the *E. coli* *lac* promoter, ref. 25). It is interesting to note that the genome of phage ϕ 29 is linear, and its superhelicity is likely to arise mainly as a consequence of the proteins acting on the DNA, such as RNA polymerase (37) or the viral protein p6 (38). Protein p6 is known to participate in the replication of the ϕ 29 genome and to generate, at least at both ends of the genome, a nucleoprotein complex in which the DNA adopts a right handed superhelical structure (38). The degree of supercoiling imposed on the viral genome *in vivo* is not known, but our experiments show that a moderate superhelical density can exert an stabilizing effect on the open complexes formed at the P_{A2b} and P_{A3} promoters, which should be important in facilitating the transition into elongation complexes. It is reasonable to assume that a lytic phage as ϕ 29 should have its main promoters optimized for high productivity.

Instability of open complexes appears to be a frequent property of *Bacillus* promoters recognized by σ^A -RNA polymerase. Indeed, this behaviour has been described for the *B. subtilis* *tms* and *citB* promoters (11), for the *E. coli* *lac* UV5 promoter when recognized by *B. subtilis* σ^A -RNA polymerase (11), and for the phage ϕ 29 C1 promoter (also named A2 in ref. 39). In view of our results, ϕ 29 promoters A2b and A3 should be also included into this category. At least two *B. subtilis* promoters, however, have been shown to form stable open complexes with σ^A -RNA polymerase, namely the ϕ 29 C2 promoter (named G2 in ref. 39), and the *veg* promoter (11). The presence of the first two initiating NTPs could stabilize, at least to a partial extent, open complexes formed at the ϕ 29 A2b and A3 promoters (this work) and at the ϕ 29 C1 promoter (39). In the case of the *tms* and *citB* promoters, however, stable complexes were not detected until the nascent RNA had a few more nucleotides (11). Although the experimental approach used in each case was different, it is likely that the precise stage at which σ^A -RNA polymerase forms a stable complex is different in each promoter. Considering that σ^A -RNA polymerase can also form stable open complexes at some promoters, it seems that no general rule can be easily established, and that the characteristics of the different complexes should be expected to be a particular feature of each promoter. DNA supercoiling has been shown to stabilize the open complexes formed at the *E. coli* *rrnB* P₁ promoter (9) and at the *B. subtilis* phage ϕ 29 P_{A2b} and P_{A3} promoters (this work). Supercoiling also stimulates the formation of open complexes at several promoters in which these complexes have relatively long half-lives (24, 25). It would be interesting to see if DNA supercoiling can stabilize the open complexes formed at other *B. subtilis* promoters known to form unstable complexes, such as the *tms* and *citB* promoters.

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