Different thermal energy requirement for open complex formation by *Escherichia coli* RNA polymerase at two related promoters

Eileen Grimes, Stephen Busby and Stephen Minchin*
School of Biochemistry, University of Birmingham, PO Box 363, Birmingham B15 2TT, UK

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**ABSTRACT**

We have studied the effect of temperature on transcription initiation *in vitro* at two related promoters galP*<sub>con</sub>* and galP1, which have the same nucleotide sequence around the −10 region and transcription start site, but differ in upstream sequences. One of the promoters, galP<sub>con</sub>, carries the consensus −35 hexamer, 5′TTGACA 3′, whilst galP1 contains a block of ‘distortable’ upstream sequences that allow promoter function in the absence of a −35 region consensus sequence. RNA polymerase can form complexes with both promoters at a range of temperatures. However, the thermal energy requirement for open complex formation differs: open complexes can form at galP1 at low temperatures, whereas galP<sub>con</sub> requires higher temperatures. The thermal energy requirement for transcription from preformed open complexes is the same for both promoters.

**INTRODUCTION**

Transcription initiation by *Escherichia coli* RNA polymerase can be described by a number of discrete steps (1,2). First, RNA polymerase binds to promoter DNA to form a ‘closed’ complex. Subsequently, it extends contacts with the promoter to form an intermediate which then isomerises to the ‘open complex’, in which the DNA duplex around the transcription startpoint is unwound. Finally, several bases are incorporated into the nascent RNA chain before a stable elongation complex is formed. The frequency of transcription initiation by RNA polymerase varies greatly from one promoter to another, the primary determinant of promoter ‘strength’ being the DNA sequence (2). Sequence analysis shows that most *E.coli* promoters share sequence homology just around the −35 and −10 regions (3,4), suggesting that, to a first approximation, promoter activity is dependent solely on the −10 and −35 sequences. However, detailed genetic analyses at a number of promoters have provided clear evidence for the importance of other sequences outside of the −10 and −35 consensus regions (5–8).

Using both genetic and physical techniques, we have attempted to define sequences essential for RNA polymerase to recognise the P1 promoter in the *E.coli* galactose operon (galP1). This is an especially interesting promoter since, although the −10 region has strong homology with the consensus, the −35 region contains no sequence related to the consensus −35 hexamer, 5′TTGACA 3′ (Fig.1). Mutational analysis has shown that specific bases within the galP1 −35 region are not required for transcription initiation, but other sequences are needed, including the spacer sequence between the −10 and −35 regions (9–11). In particular, the ‘motif’ 5′TG 3′, located just upstream of the −10 hexamer at −15/−14, is necessary (but not sufficient) for the activity of galP1 and other promoters lacking consensus −35 sequences (7,12). Additionally, at galP1, bases upstream from −49 are important for optimal promoter activity: deletions replacing sequences upstream from −49 reduce transcription (10). Recently, we replaced the sequences upstream from the galP1 −10 region with a cassette containing a consensus −35 hexamer, producing a ‘consensus-like’ promoter, galP<sub>con</sub>, which is transcriptionally active (11). Thus the same galP1 −10 sequence can participate with different upstream sequences in promoting transcription initiation by RNA polymerase.

We have studied the binding of RNA polymerase to galP1 and galP<sub>con</sub> using a variety of probes (13): the interactions of RNA polymerase with the downstream part of both promoters appear to be identical. In particular, in both cases, the unwinding of DNA in open complexes, as judged by sensitivity to attack by potassium permanganate, is identical. In sharp contrast, the interactions of RNA polymerase with the upstream part of galP1 and galP<sub>con</sub> differ: the footprint of RNA polymerase at galP1 extends upstream from −49 and the DNA around the −35 region appears distorted. At galP<sub>con</sub>, the footprint of RNA polymerase only extends to −45 and there is little evidence for gross distortion of the promoter DNA (13). In this paper, we show that an important consequence of these differences is a striking variation in the temperature requirement for open complex formation. At galP1, RNA polymerase forms open complexes at low temperatures. In contrast, at galP<sub>con</sub>, RNA polymerase will only form a closed complex at low temperatures and higher temperatures are required for isomerisation to the transcriptionally-competent open complex.

* To whom correspondence should be addressed
MATERIALS AND METHODS

Promoters

The construction of EcoRI-HindIII fragments carrying the galP1 and galPcon promoters and cloning into plasmid pAA121 has been described previously (9,11). EcoRI-HindIII or PstI-HindIII fragments containing the promoters were prepared by polyclaylamide gel electrophoresis. Fragments for footprinting were 5' end-labelled with [γ-32P]ATP (Amersham) at the HindIII site on the bottom (template) strand.

Formation of binary complexes

DNA fragments (1—5 nM) containing promoters were incubated in 25 μl of transcription buffer (20 mM Tris.HCl pH8.0, 100 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 50 μg/ml BSA, 5% glycerol) at the appropriate temperature for 30 minutes to allow temperature equilibration. RNA polymerase (100 mM) (supplied by Pharmacia) was added and the mixture incubated for 30 minutes to allow open complex formation.

Potassium permanganate footprinting

Binary complexes were analysed by potassium permanganate footprinting at the appropriate temperature: 1μl of a freshly prepared 200 mM solution of potassium permanganate was added to the reaction containing the binary complex. The reaction was stopped after 4 minutes by the addition of 50 μl KMnO4 stop solution (3M Ammonium acetate, 0.1 mM EDTA, 1.5M β-mercaptoethanol). After phenol/chloroform extraction and ethanol precipitation the samples were cleaved by treatment with piperidine and analysed on a 6% sequencing gel.

In vitro transcription

Nucleotides and heparin (final concentration, 0.2 mM ATP, 0.2 mM CTP, 0.2 mM GTP, 0.05 mM [α-32P]UTP, 0.1 mg/ml heparin) were added to the binary complex at the appropriate temperature and the reaction was incubated for 30 minutes. The assay was stopped by the addition of 250 μl 10 mM EDTA and 300 μl of phenol/chloroform. After phenol/chloroform extraction and ethanol precipitation the samples were analysed on a calibrated 6% sequencing gel.

Temperature shift experiments

In experiments where binary complexes were formed at low temperature, heparin (final concentration, 0.1 mg/ml) was added before the binary complexes were transferred to a higher temperature for the transcription assay. In experiments where the binary complexes were formed at high temperature and then shifted down, the samples were incubated at the lower temperature for 30 minutes to allow temperature equilibration before addition of the nucleotides. Care was taken to avoid warming during transfer of samples from one bath to another.

DNAse I footprinting

Binary complexes were analysed by DNAse I footprinting at the appropriate temperature: 15 ng of DNAse I (Boehringer) was added to the binary complexes and the reaction incubated for 1 minute before the addition of 200 μl DNAse I stop (10 mM EDTA, 0.3 mM Sodium Acetate). The DNA was purified by phenol/chloroform extraction and ethanol precipitation before analysis on a calibrated 6% sequencing gel.

RESULTS

Promoters

The promoters galP1 and galPcon were cloned as EcoRI-HindIII fragments into the plasmid vector pAA121 (9) (Fig.1). The galP1 fragment was derived from E. coli gal operon DNA containing a mutation that inactivated the alternative promoter, galP2 (10). The galPcon fragment was derived from galP1 by replacing sequences upstream of base pair −12 (13) (Fig.1). Two types of fragment were used in this work: EcoRI-HindIII fragments containing just galP1 or galPcon, or longer PstI-HindIII fragments (Fig.1), carrying two extra promoters, the pBR322 bla promoter and a promoter of unknown function, Px (14), which were exploited as internal controls.

Probing polymerase-promoter complexes at different temperatures

We investigated the ability of RNA polymerase to form open complexes at low temperatures, by using potassium permanganate as a probe for single stranded DNA (15). The results, shown in Figs 2A and 2B, confirm that, at 37°C, open complexes form at both galPcon and galP1, and that unwinding at the two promoters is identical. Probing of the complexes formed at lower temperatures shows that, for galP1, strand separation occurs at 25°C and 14°C with some opening at 6°C. In contrast, with galPcon, the amount of opening decreases at 25°C and no

Figure 1. A) Nucleotide sequence of galP1 and galPcon. The E. coli promoter consensus sequence is shown below. The sequences are numbered with the transcription start as +1. The sequence of the −10 and −35 regions are in bold type. The * shows the position of the G to T transversion that inactivates the galP2 promoter. Sequence identity is indicated by vertical bars. B) Schematic representation of pAA121. Promoters are cloned into pAA121 as EcoRI to HindIII fragments. The position of restriction sites used in this work are shown, as are the promoters Phla and Px.
opening is evident at lower temperatures. At both promoters the relative intensities of the four bands due to reaction of non-base-paired thymines with permanganate is the same at 25°C and 37°C. In contrast, at galP1 at lower temperatures, the upstream T residue (at -11 on the ‘bottom’ strand) is preferentially attacked (Fig. 2B).

The experiments shown in Fig. 2 were performed with PstI-HindIII fragments containing the two additional promoters Px and Pbla (Fig. 1). Whilst signals due to unwinding at Px were weak, RNA polymerase clearly causes unwinding around the Pbla -10 region (Fig. 2A). Interestingly, at the bla promoter, signals are observed at 4°C, 6°C and 14°C, but they are reduced in intensity at 25°C and not seen at 37°C.

These results show that the temperature requirement for RNA polymerase-induced duplex opening can vary greatly from one promoter to another. Our results are especially striking, as the nucleotide sequence in the unwound region is the same at the two promoters. We repeated the study using shorter EcoRI-HindIII fragments (Fig. 1) carrying the gal promoters to check that our results were not affected by Px or Pbla: the results obtained were identical to those with the longer fragment (not shown).

The Tm for open complex formation was determined by potassium permanganate footprinting at different temperatures close to the estimated Tm. The potassium permanganate footprints were analysed by laser densitometry and the intensity of each band calculated as a percentage of the total intensities in each lane. A Tm was calculated using bands corresponding to all four T residues (fig. 3A), as well as a Tm for the individual T residues at -11 and +3 (fig. 3B & 3C). The Tm for open complex at galP1 is 9°C, whereas at galP_con it is 25°C. We have estimated the value of ΔH for open complex formation and then calculated ΔS. The values for ΔS, ΔH and Tm are given in Table 1. The results in this Table show that ΔH and ΔS are both lower for galP1. The difference in ΔH is seen as a lower thermal energy requirement for open complex formation at galP1 than at

Figure 2. Potassium permanganate footprints of galP1 and galP_con. PstI to HindIII fragments were used for the footprinting experiments in the presence or absence of RNA polymerase (RNAP) and at different temperatures as indicated. Lane M is a Maxam and Gilbert sequencing reaction specific for G residues. The reactive residues are indicated by the bold arrows. (A) Short exposure of autoradiograph. (B) Longer exposure of autoradiograph showing bands corresponding to galP1 and galP_con only.

Figure 3. Tm calculation. Potassium permanganate footprints were obtained at temperatures around the Tm for galP_con (Solid squares) and galP1 (Solid triangles). The autoradiographs were analysed using an LKB Ultrascan model XL densitometer. The intensities of individual bands were measured as a fraction of the total band intensity in that lane. The vertical axis represents the fraction of the maximum intensity for a particular band (or set of bands) plotted against temperature (horizontal axis). (A) Tm curve calculated using all four reactive T residues (see fig. 2B). (B) and (C) Tm curves for the T residues at -11 and +3 respectively.
The effect on AS indicates that the interactions between elongation with a mixture of P-labelled nucleoside 32 different temperatures with fragments carrying either promoter. We have also measured the stability of, at temperatures below the Tm, the galPl data. Interestingly, at residues at —11 and +3 correlates well with the combined during formation of the open complex. The data for the individual RNA polymerase and the DNA are different at the two promoters. We estimate the margin of error for the AH and AS values to be approximately 50%.

The half times for formation of the open complexes were measured using potassium permanganate footprinting at the temperatures indicated. The experiments were started by the addition of RNA polymerase (final conc. 150nM) and heparin (final conc. 0.15mg/ml) was added to prevent further polymerase binding, and the amount of the open complex assayed. The half-times for dissociation of open complexes were also measured using potassium permanganate footprinting. Open complexes were formed at 37°C, the assays were started by the addition of heparin (final conc. 0.15mg/ml) and incubated at the temperatures indicated. At different times aliquots were removed and assayed for the presence of the open complex. Laser densitometry was used to quantitate all permanganate footprints.

galP con. The effect on AS indicates that the interactions between RNA polymerase and the DNA are different at the two promoters during formation of the open complex. The data for the individual T residues at —11 and +3 correlates well with the combined data. Interestingly, at galPl, at temperatures below the Tm, the residue at —11 is preferentially attacked compared to the residue at +3 (figs 2B, 3B & 3C). However this has a minimal effect on the Tm (fig.3).

We have determined the effect of temperature on the rate of open complex formation at galPl and galP con, measuring the half-time for formation of the open complex (Table 1): the results show that temperature affects the rate of open complex formation at both promoters. We have also measured the stability of preformed open complexes at both promoters by monitoring reactivity to permanganate in the presence of heparin. According to this assay, open complexes at both galPl and galP con appear to be very stable at all temperature, there being little effect of temperature on the apparent dissociation rate.

**In vitro transcription**

Using run-off transcription assays, we checked whether RNA polymerase bound at galP con or galPl at different temperatures could make a transcript. RNA polymerase was preincubated at different temperatures with fragments carrying either promoter. Fig.4 shows gel analysis of run-off transcripts made after elongation with a mixture of 32P-labelled nucleoside triphosphates plus heparin. At the galP con promoter, RNA polymerase could initiate transcription at 25°C and 37°C: the amount of run-off transcript correlates well with duplex opening as judged by reaction with permanganate (Fig.2). In the experiments shown in Fig.4 the elongation step was performed at the same temperature as the preincubation: however, with galP con, the same result was obtained when the elongation step was performed at 37°C (not shown).

In contrast to galP con, RNA polymerase at galPl can make run-off transcripts at 14°C as well as 25°C and 37°C: again the capacity to make transcripts appears to correlate with opening of the duplex. The exception to this is at galPl after preincubation at 6°C, where no run-off transcripts were found, despite clear evidence from the permanganate experiment for some unwinding. Interestingly, transcripts from these complexes were detected when the temperature of the elongation was raised to 37°C.

**Notes:**

i) Tm and DH values were calculated from fig.3A using a derivative of the van t’Hoff equation F=1/(1+exp((AH/R)X(1/T)-(1/Tm)), F=Fraction maximum intensity, R=Gas constant (8.314 J K"mol"1) and T=Temperature in degrees Kelvin. ΔS was calculated since ΔG=0 at the Tm, and therefore ΔS=ΔH/T. We estimate the margin of error for the ΔH and ΔS values to be approximately 50%.

ii) The half times for formation of the open complexes were measured using potassium permanganate footprinting at the temperatures indicated. The experiments were started by the addition of RNA polymerase (final conc. 150nM) and heparin (final conc. 0.15mg/ml) was added to prevent further polymerase binding, and the amount of the open complex assayed. The half-times for dissociation of open complexes were also measured using potassium permanganate footprinting. Open complexes were formed at 37°C, the assays were started by the addition of heparin (final conc. 0.15mg/ml) and incubated at the temperatures indicated. At different times aliquots were removed and assayed for the presence of the open complex.

### Table 1. Thermodynamic and kinetic data for open complex formation at galP con and galPl.

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<tr>
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<th>galP con</th>
<th>galPl</th>
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<tr>
<td>Tm</td>
<td>398 ± 1 K</td>
<td>282 ± 1 K</td>
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<tr>
<td>DH</td>
<td>280 kJ mol⁻¹</td>
<td>160 kJ mol⁻¹</td>
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<tr>
<td>DS</td>
<td>930 J K⁻¹ mol⁻¹</td>
<td>580 J K⁻¹ mol⁻¹</td>
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<td>Half-time for formation of open complex</td>
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<td></td>
<td>80 sec at 27.5°C</td>
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<td>≥60 min at 14°C</td>
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<tr>
<td>Half-time for dissociation of open complex</td>
<td>≥60 min at 37°C</td>
<td>≥60 min at 37°C</td>
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<td>27.5°C and 14°C</td>
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**Figure 4. In vitro transcription assays of galPl and galP con.** EcoRl to Hindlll fragments were used as templates for in vitro transcription at the indicated temperatures. Lane M is a Maxam and Gilbert sequencing reaction specific for G residues. Horizontal arrows mark the position of transcripts initiating at +1. (A) Short exposure of autoradiograph. (B) Long exposure autoradiograph.

**Figure 5. In vitro transcription of preformed open complexes.** RNA polymerase was incubated with the EcoRl to Hindlll promoter fragments at 37°C. The complexes were then shifted to the indicated temperature for the transcription assay. Lane M is a Maxam and Gilbert sequencing reaction specific for G residues. The horizontal arrow marks the position of the transcripts.
immediately after addition of the mixture of nucleoside triphosphates and heparin (data not shown).

To be certain that lower temperatures were not blocking the elongation step in the synthesis of run-off transcripts, open complexes at both galP<sub>con</sub> and galP<sub>1</sub> were preformed at 37°C. The temperature was then shifted down to 6°C, 14°C or 25°C, and the cocktail of heparin and nucleoside triphosphates was then added to the assay. The results in Fig. 5 show that run-off transcripts are made at all temperatures at both promoters. Interestingly, run-off transcription at 6°C results in a unique length transcript rather than the doublet found at other temperatures. This experiment also confirms that functional open complexes are stable at low temperature.

DNase I footprinting was used to confirm the interaction of RNA polymerase with galP<sub>1</sub> and galP<sub>con</sub> at different temperatures: the results in Fig. 6 show that DNA sequence in the zone of strand separation is identical. This is clear evidence that sequences upstream from -12 have a vital role in determining the rate of open complex formation at a promoter. Interestingly, a similar though smaller effect was seen previously by Kirkegaard <em>et al.</em> (16), who monitored open complex formation as a function of temperature at the lacUV5 and tac promoters: although tac and lacUV5 have the same sequence downstream from -18, strand separation at tac could take place at lower temperatures than lacUV5. Surprisingly, in our work, it is the weaker promoter (at 37°C), galP<sub>1</sub>, that forms open complexes at lower temperatures, whilst in the case of lacUV5/tac it is tac that is the stronger promoter. Additionally, the results with galP<sub>1</sub> and galP<sub>con</sub> are more dramatic as there is a 16°C difference in the Tm for open complex formation whilst with lacUV5/tac the difference is 3–5°C.

We have shown that temperature affects the rate of open complex formation at these two promoters as seen by a difference in the forward rate. The lack of temperature effects on the dissociation rate is due to the assay which measures the appearance of heparin-sensitive complexes rather than the disappearance of unwinding. The ability of galP<sub>1</sub> to form open complexes at low temperature must be a function of the nucleotide sequence upstream from -12. In previous work, it has been shown that the sequence 5'TG 3', just upstream of the -10 hexamer, is essential for promoters that lack -35 region consensus sequences (7, 12). However, the 5'TG 3' sequence is not sufficient for open complex formation at low temperatures, since introduction of this sequence into galP<sub>con</sub> does not facilitate strand separation at low temperatures (E.G. and S.M., unpublished). A more likely reason to explain the formation of open complexes at galP<sub>1</sub> at low temperatures, is that the upstream transcription at this promoter: this is due to sequences in the spacer region, between -12 and -30, and sequences upstream from the -35 region that enable RNA polymerase to initiate transcription in the absence of a 'normal' -35 region (11).

In this work we have shown, using in vitro transcription assays, that RNA polymerase can initiate and elongate transcription at both the galP<sub>1</sub> and galP<sub>con</sub> promoters from preformed open complexes at temperatures as low as 6°C. However, RNA polymerase is unable initiate transcription de novo at 6°C. Thus, the thermally limiting process in transcription at both promoters must be a step in the pathway to the formation of the open complex. At galP<sub>con</sub>, DNase I footprints show that RNA polymerase can form a complex at 14°C, but extra thermal energy is required for strand separation. In contrast, at galP<sub>1</sub>, strand separation occurs at 6°C but this 'open' complex is not transcriptionally competent. However this is not a 'dead-end' complex, since transcription from the 6°C galP<sub>1</sub> 'open' complex is possible if the temperature is raised. Significantly, the relative reactivity of the four permanganate-sensitive T residues in the 6°C complex at galP<sub>1</sub> is different to that found at higher temperatures (Fig. 2 & 3). This suggests that the complex formed between RNA polymerase and galP<sub>1</sub> at 6°C is not the fully mature open complex but likely to be an intermediate open complex.

Our results show that intermediates on the way to open complex formation can be trapped by forming RNA polymerase-promoter complexes at different temperatures. This has also been demonstrated by several other groups working with different promoters (16–19). However the striking finding from this work is that the thermal energy requirements for open complex formation at galP<sub>1</sub> and galP<sub>con</sub> differ greatly, even though the DNA sequence in the zone of strand separation is identical. This is clear evidence that sequences upstream from -12 have a vital role in determining the rate of open complex formation at a promoter. Interestingly, a similar though smaller effect was seen previously by Kirkegaard <em>et al.</em> (16), who monitored open complex formation as a function of temperature at the lacUV5 and tac promoters: although tac and lacUV5 have the same sequence downstream from -18, strand separation at tac could take place at lower temperatures than lacUV5. Surprisingly, in our work, it is the weaker promoter (at 37°C), galP<sub>1</sub>, that forms open complexes at lower temperatures, whilst in the case of lacUV5/tac it is tac that is the stronger promoter. Additionally, the results with galP<sub>1</sub> and galP<sub>con</sub> are more dramatic as there is a 16°C difference in the Tm for open complex formation whilst with lacUV5/tac the difference is 3–5°C.

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sequence around −50 allows extended contacts that reduce the thermal energy requirement for DNA strand separation. It is well known that upstream sequences play a role in transcription initiation at many naturally-occurring and synthetic promoters (5,8,20−23) and many upstream sequences contain elements that can bend or distort (24,25). In some cases it is likely that the role of DNA distortion is to facilitate extended interactions between RNA polymerase and promoter sequences. Travers (26) postulated that the temperature of open complex formation is dependent upon the affinity of RNA polymerase for a promoter; the greater the affinity, the lower the temperature. Thus, extended contacts may increase the affinity of RNA polymerase for the promoter and perhaps reduce the thermal energy requirement for open complex formation. It has recently been shown that the introduction of a bend into DNA between −8 and +2 by the removal of a single base overcomes the temperature requirement for open complex formation at the A1 promoter (27).

One model suggests that open complex formation involves a stressed intermediate, the stress being partially relieved by the strand separation (28,29). This stressed intermediate may be formed because the −10 and −35 sequences are not on the same face of the DNA helix, and therefore RNA polymerase must distort the DNA so as to make close contacts with both regions. In the case of galP1, the interaction of RNA polymerase with the −35 region cannot be strong since this region has no homology with the consensus. However it is possible that interactions of RNA polymerase with upstream sequences similarly stabilise the RNA polymerase/promoter complex, enhancing the ability of RNA polymerase to distort the DNA prior to open complex. These extended contacts may anchor RNA polymerase such that a more stressed intermediate is formed, resulting in a reduced energy requirement for open complex formation. It has been observed that RNA polymerase can also form open complexes at the bla promoter at low temperatures: in fact, the thermal energy requirement is lower than that for galP1. Interestingly, the −35 region of pbla (5′TTCAAA 3′) has a four out of six match with the consensus but, surprisingly, the −50 regions of pbla and galP1 show some sequence similarities: both contain the motif, 5′TTTAT 3′, which has been implicated in the formation of DNA kinks (13,30). We suggest that contacts between RNA polymerase and the upstream region of the bla promoter are responsible for distortions that result in a lower thermal energy requirement for open complex formation. In our study we also found that open complex formation at Pbla was reduced at 25°C and suppressed at 37°C. This is consistent with the results of Duval-Valentin and co-workers (31) who showed that, at high RNA polymerase concentrations, and at temperatures above 25°C, a second RNA polymerase molecule binds to Pbla and inhibits transcription.

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