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Open complex formation during transcription initiation at the *Escherichia coli gal*P1 promoter: the role of the RNA polymerase α subunit at promoters lacking an UP-element

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ABSTRACT

We have studied the role of the C-terminal domain of the α subunit (αCTD) of *Escherichia coli* RNA polymerase during transcription initiation at promoters lacking an UP-element. The temperature requirement for open complex formation was used as an indication of the kinetics of this process. We have previously shown that αCTD is required for transcription initiation at low temperature at the *gal*P1 promoter, a promoter containing an UP-element. DNase I footprinting has been used to reveal the structure of open promoter complexes and the temperature requirement for open complex formation has been determined using potassium permanganate as a probe. In this work we show that, although αCTD is not absolutely required for transcription initiation at promoters lacking an UP-element, it does play a role during transcription initiation. This role is independent of the sequence of the promoter upstream from the –35 region and does not require stable αCTD–DNA interactions as determined by DNase I footprinting. The role of αCTD at promoters lacking an UP-element is discussed.

INTRODUCTION

Most promoters recognised by the major *Escherichia coli* RNA polymerase ($\alpha_2\beta\beta'\sigma^{70}$) contain two conserved sequence elements located 10 and 35 bp upstream from the transcription start site (1,2). The –10 and –35 hexamers provide contact points for the σ^{70} subunit of RNA polymerase. However, it is now clear that other regions of the promoter, including the –10/–35 spacer region and the sequence upstream of the –35 hexamer, also play an important role in transcription initiation (3,4).

The 5′-TG-3′motif, located 1 bp upstream of the –10 hexamer, is highly conserved in the promoters of a number of Gram-positive organisms and weakly conserved in *E.coli* promoters (3,5–8). Mutations within the 5′-TG-3′ motif have been shown to reduce gene expression, while mutations that generate a 5′-TG-3′ motif stimulate promoter activity (9–13). Furthermore, the absence of a recognisable –35 hexamer can be compensated for by the presence of the 5′-TG-3′motif, resulting in an ‘extended –10’ promoter (10,14,15). It has been shown that the extended –10 motif is recognised by region 2·5 of the σ^{70} subunit of RNA polymerase (16). Promoter activity can also be enhanced by the presence of an UP-element; an AT-rich region located upstream of the –35 sequence. The UP-element stimulates the rate of transcription initiation by interacting with the C-terminal domain of the RNA polymerase α subunit (αCTD) (4).

The *E.coli gal*P1 promoter is an example of an ‘extended –10’ promoter having a –10 hexamer with close homology to the consensus and the 5′-TG-3′ motif, but a –35 sequence with no homology to the consensus (11). The *gal*P1 promoter also requires additional sequences positioned around –50 for maximal activity. Footprinting analysis has shown that these sequences interact with wild-type σ^{70} RNA polymerase, resulting in extended upstream protection at the *gal*P1 promoter (17). In contrast, RNA polymerase reconstituted with truncated α subunits, containing only the N-terminal 256 amino acids (α-256 polymerase), gives an identical footprint around the transcript start but fails to protect sequences upstream of –40 (18). These data led to the proposal that the extended upstream footprint at *gal*P1 is due to the binding of the αCTD to an UP-element.

An unusual property of the *E.coli gal*P1 promoter is its ability to form an open complex at low temperatures, as shown *in vitro* and *in vivo* by probing with potassium permanganate (14,19,20). To investigate the mechanism of initiation at *gal*P1, a synthetic *gal*P1 derivative (*gal*PconTG6) was constructed by cloning a DNA sequence upstream from –12 which contained a 5′-TG-3′ motif and a consensus –35 hexamer (21). Introduction of a G→T transversion at position –14 in *gal*PconTG6, thereby mutating the 5′-TG-3′ motif, significantly increased the thermal energy requirements for opening (19). This observation led to the proposal that the 5′-TG-3′ motif reduces the thermodynamic barrier of melting during initiation. However, the *E.coli cysG* promoter, which also has an extended –10 region, is unable to form an open complex at low temperatures, demonstrating that the 5′-TG-3′ motif alone is insufficient for low temperature opening (20,22). In addition, Attey *et al*. (18), showed that reconstituted α-256 RNA polymerase was unable to form an open complex at *gal*P1 at low temperatures. Together, these data are

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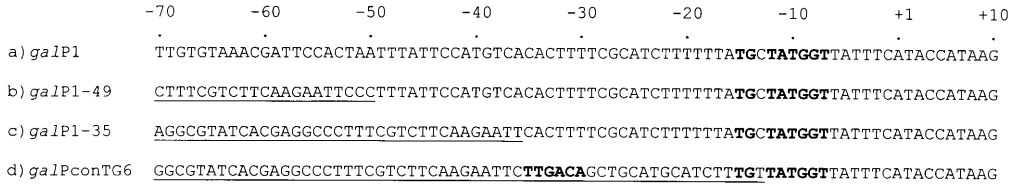

Figure 1. The nucleotide sequence of promoters used in this study. The extended –10 and –35 promoter elements are highlighted in bold. The nucleotides that replace 'wild-type' *gal*P1 are underlined.

consistent with a model in which function at low temperatures is a consequence of both the 5′-TG-3′ motif and extended upstream contacts. However, in opposition to this proposal, more recent evidence suggests that the *gal*P1 sequence upstream of –49 is dispensible for low temperature opening (20), raising questions concerning the nature of the upstream αCTD–DNA contact and its role in transcription initiation.

In this study we have examined the role of the RNA polymerase αCTD during open complex formation. Using truncated *gal*P1 promoter derivatives, we have shown that RNA polymerase makes a sequence-specific interaction with the upstream region of the *gal*P1 promoter. However, the *gal*P1 sequence upstream of –35 is dispensible for low temperature opening, demonstrating that specific upstream αCTD–DNA interactions are not required for strand separation at low temperature. In contrast, the C-terminal 73 amino acids of the α subunit are required for low temperature melting in both the presence and absence of an upstream α binding site (UP-element). We proposed that the αCTD has a role in transcription initiation which is independent of its ability to form a specific protein–DNA contact.

MATERIALS AND METHODS

Promoters

All promoters were carried as *Eco*RI–*Hin*dIII fragments in the *gal*K fusion vector pAA121 as described previously (23). Plasmids were isolated from the *E.coli* host, M182, by SDS lysis and then purified on a caesium chloride/ethidium bromide gradient (24). Unless stated otherwise, plasmids were linearised prior to potassium permanganate probing by digestion with the restriction endonuclease *Pst*I, which cuts all constructs once within the *bla* gene on pAA121. *Pst*I–*Bst*EII promoter fragments, prepared for DNase I footprinting, were isolated by PAGE and then 5′-end-labelled with [γ-^{32}P]ATP (Amersham) at the *Bst*EII site on the template strand.

The sequences of the promoters used in this work are shown in Figure 1. The *Eco*RI–*Hin*dIII *gal* promoter fragment encodes *gal* sequence from –92 to +45 with respect to the *gal*P1 transcript start site (25) and contains a G→T transversion at position –19 that completely inactivates the *gal*P2 promoter (11). *Gal*P1–49 and *gal*P1–35 were derived from *gal*P1 by introduction of an *Eco*RI linker upstream of –49 and –35, respectively (–49 and –35 denote the upstream limit of *gal*P1 sequence).

In vitro potassium permanganate probing

A 20 µl reaction mix, containing linearised or supercoiled template DNA (10 nM) in transcription buffer (5% v/v glycerol, 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50 µg/ml BSA), was incubated at the appropriate temperature for 30 min. RNA polymerase was added to a final concentration of 200 nM and the mixture incubated for a further 20 min to allow binary complex formation. Unless stated otherwise, RNA polymerase holoenzyme was supplied by Northumbria Biologicals Ltd. Reconstituted RNA polymerase holoenzyme containing either wild-type or C-terminally truncated α subunits (α-256) was prepared in Mishima from purified full-length or truncated RNA polymerase subunits (26). Single-stranded T residues were modified by adding 1 µl freshly prepared 200 mM potassium permanganate. After 4 min the reaction was quenched with 50 µl potassium permanganate stop solution (3 M ammonium acetate, 0.1 mM EDTA, 1.5 M β-mercaptoethanol). The modified DNA was purified by phenol/chloroform extraction and ethanol precipitation. The site of $KMnO_4$ modification was detected by primer extension analysis.

Primer extension analysis of modified DNA

This is a modified version of the method described by Sasse-Dwight and Gralla (27). An oligodeoxyribonucleotide primer (Alta Bioscience, University of Birmingham) complementary to the template strand upstream of the *Eco*RI site was 5′-end-labelled with [γ-^{32}P]ATP (Amersham). Labelled primer (20 nM), dNTPs (100 µM with respect to each) (Pharmacia), and Vent(exo$^-$) DNA polymerase (2 U) (New England Biolabs) or *Taq* DNA polymerase (2 U) (Boehringer Mannheim) were added to the modified DNA (3–4 nM) in a final volume of 50 µl. A mineral oil overlay (Sigma) was added and the extension reaction was started in a Biometra thermal cycler using the following programme: one cycle of 94°C for 180 s, 50°C for 120 s, 72°C for 90 s; followed by 15 cycles of 94°C for 60 s, 50°C for 120 s, 72°C for 90 s; then 94°C for 60 s, 50°C for 120 s, 72°C for 10 min. The aqueous layer was purified by phenol/chloroform extraction and ethanol precipitation and analysed on a calibrated 6% (w/v) denaturing polyacrylamide gel (Sequagel; National Diagnostics).

DNase I footprinting

RNA polymerase (3 µM) was incubated in the presence of 6 µM σ^{70} (kindly supplied by J. Bown, University of Birmingham) at 30°C for 15 min to increase core–σ association. A 20 µl reaction mix containing 5′-end-labelled DNA (7–8 nM) and RNA polymerase (300–400 nM) in binding buffer (20 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 50 mM potassium glutamate, 0.5 mg/ml BSA, 1 mM DTT) was incubated at 37°C for 20 min to allow binary complex formation. DNase I (5–15 ng) (Boehringer) was added to the binary complexes and the reaction allowed to proceed for 60 s at room temperature (~25°C). The reaction was quenched by the addition of 200 µl DNase I stop solution (10 mM EDTA, 0.3 M sodium acetate). After purification by phenol/

chloroform extraction and ethanol precipitation, the DNA was analysed on a calibrated 6% (w/v) denaturing polyacrylamide gel (Sequagel; National Diagnostics).

RESULTS

Investigation into the nature of the upstream contact at *gal*P1: specific or non-specific?

It has previously been shown that wild-type RNA polymerase forms an extended upstream contact at the *gal*P1 promoter. However, RNA polymerase reconstituted with truncated α subunits, containing only the N-terminal 256 amino acids (α-256 polymerase), fails to protect the *gal*P1 promoter upstream of –40 (18). This result led to the proposal that the extended upstream footprint at *gal*P1 is due to the binding of the αCTD to a putative UP-element. To further characterise this extended upstream contact, we have used DNase I footprinting to compare the interaction of wild-type RNA polymerase holoenzyme with the *gal*P1 promoter and two truncated derivatives, *gal*P1–49 and *gal*P1–35. The results (Fig. 2) clearly show that RNA polymerase binds to all three promoters. At *gal*P1, in accordance with previous data (21,28), RNA polymerase protects the DNA upstream to around –55 with three regions of hyper-reactivity at around –27, –38 and –49. The positioning and the periodicity of these hyper-reactive regions suggests increased attack of the minor groove due to curvature of the DNA helical axis. In contrast to the situation at *gal*P1, there is no clear protection in the –45 to –55 region at *gal*P1–49. However, the hyper-reactivity seen around –49 at this promoter has been attributed to polymerase-induced distortion of the DNA. The DNase I footprint of the *gal*P1–35 promoter provides compelling evidence that the upstream protection at *gal*P1 is sequence specific, since there is no evidence of any DNA–RNA polymerase interaction upstream of –45. The second region of protection observed at *gal*P1–35, upstream of –60, is attributed to polymerase binding at the upstream p*X* promoter (29). In conclusion, replacement of the *gal*P1 sequence upstream of –35 results in a loss of upstream protection by wild-type RNA polymerase. This observation is consistent with the proposal that the RNA polymerase αCTD makes a sequence-specific contact with the *gal*P1 promoter upstream of –45. However, it does not rule out the possibility that αCTD makes transient non-specific contacts with promoter DNA upstream from –35.

Investigation into the role of the upstream αCTD–DNA interaction in low temperature opening at the *gal*P1 promoter

In contrast to wild-type holoenzyme, reconstituted α-256 RNA polymerase is unable to form an open complex at *gal*P1 at low temperatures (18). To investigate the role of the upstream αCTD–DNA interaction on strand separation at the *gal*P1 promoter, open complex formation was monitored at the two truncated promoter derivatives *gal*P1–49 and *gal*P1–35. Figure 3 shows that substitution of the *gal*P1 sequence from –92 to –50 or –36 has no deleterious effect on the promoter's thermal energy requirements. The level of opening at 14°C is actually enhanced at *gal*P1–35 relative to *gal*P1, thus stable sequence-specific contacts with DNA upstream from –35 by αCTD are not required for low temperature opening at this derivative of *gal*P1. In view of the data presented by Attey *et al.* (18), this result raised the question as to the role of the αCTD in driving strand separation at low temperature.

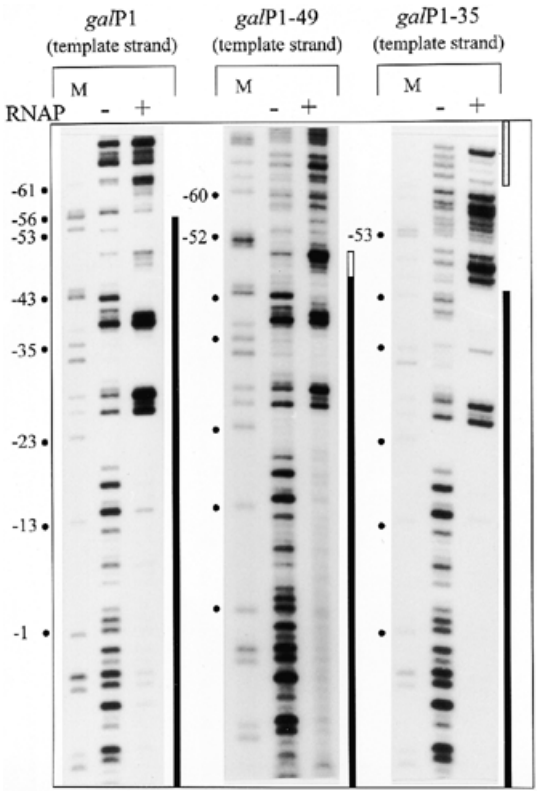

Figure 2. DNase I footprints of *gal*P1, *gal*P1–49 and *gal*P1–35. Regions of clear protection are indicated by a bar; solid bars represent protection due to polymerase binding at the *gal*P1 derivatives; the hatched bar indicates protection due to polymerase binding at the p*X* promoter. The open bar in the *gal*P1–49 lane indicates a region of hyper-reactivity. Lane M is a Maxam–Gilbert G reaction calibration ladder.

Investigation into the role of the αCTD in low temperature opening at a promoter which lacks an UP-element

At promoters lacking sequences with homology to the UP-element upstream from –35, the role of αCTD in activator-independent transcription is not fully understood. In order to gain insight into the role of αCTD at promoters lacking an UP-element, we monitored the ability of reconstituted α-256 RNA polymerase to form an open complex at the *gal*PconTG6 promoter. *gal*PconTG6, unlike *gal*P1, contains the consensus –35 hexamer but lacks an UP-element. The absence of an UP-element is consistent with DNase I footprinting data (Fig. 4) which shows that wild-type RNA polymerase does not form extended upstream contacts at *gal*PconTG6.

Potassium permanganate probing was then used to compare strand separation driven by wild-type and mutant RNA polymerase at the *gal*PconTG6 promoter (Fig. 5). Wild-type RNA polymerase (purified from *E.coli*) forms an open complex at 37 and 14°C, while reconstituted RNA polymerase, containing truncated α subunits, drives strand separation at 37°C alone. To eliminate the possibility that the reconstitution procedure is affecting enzyme function, the experiment was repeated using reconstituted wild-type enzyme. Figure 5b shows that, unlike α-256 RNA polymerase, wild-type reconstituted enzyme is clearly able to form an open complex at 14°C. Thus, αCTD has a functional role

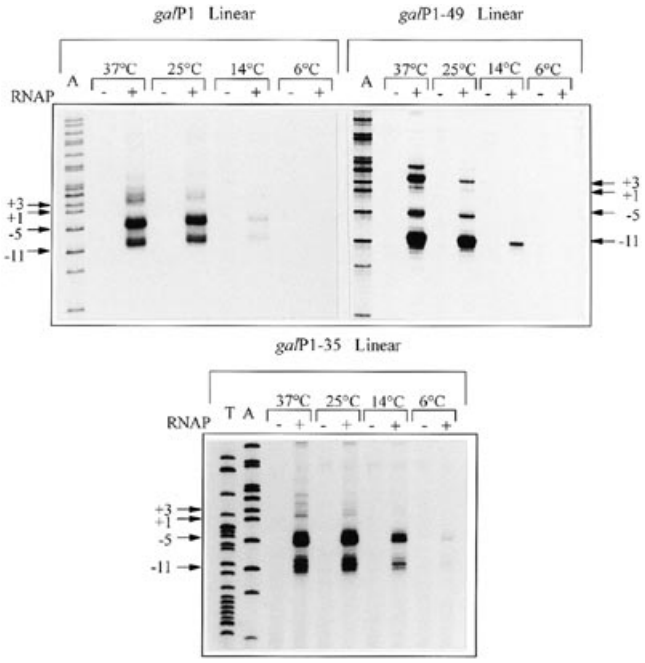

Figure 3. Potassium permanganate probing of *gal*P1, *gal*P1–49 and *gal*P1–35 on linear plasmid templates. Potassium permanganate was used to detect unpaired T residues present on melted DNA. The arrows indicate single-stranded T residues, numbered relative to the transcription start site +1. Lane A is a dideoxy sequencing ladder specific for A residues. The relative intensities of the bands obtained at different temperatures for each promoter are: for *gal*P1 at 37°C, 100%; 25°C, 77%; 14°C, 9%; 6°C, 0%; for *gal*P1–49 at 37°C, 100%; 25°C, 52%; 14°C, 10%; 6°C, 0%; for *gal*P1–35 at 37°C, 100%; 25°C, 98%; 14°C, 58%; 6°C, 10%.

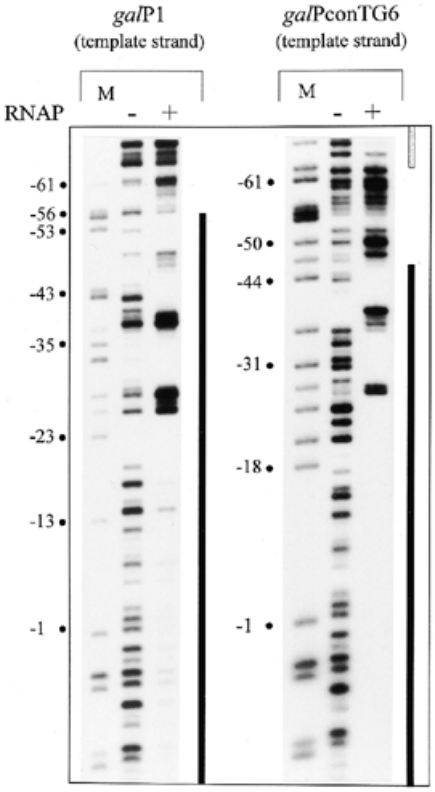

Figure 4. DNase I footprints of *gal*P1 and *gal*PconTG6. Regions of clear protection are indicated by a bar; solid bars represent protection due to polymerase binding at the *gal*P1 derivatives; the hatched bar indicates protection due to polymerase binding at the p*X* promoter (29). Lane M is a Maxam–Gilbert G reaction calibration ladder.

in promoting open complex formation at the *gal*PconTG6 promoter. However, since this role is not dependent on the formation of sequence-specific contacts between αCTD and DNA, the α subunit must facilitate duplex melting at this promoter by an alternative mechanism. The αCTD was also found to be important for open complex formation at the 'extended –10' promoter, *gal*P1–35, which lacks both an UP-element and a recognisable –35 hexamer. The results, shown in Figure 5c, resemble those obtained for *gal*PconTG6; both enzymes are capable of forming an open complex at 37°C, whereas at 14°C opening is observed with wild-type enzyme alone. Furthermore, the level of opening observed at 37°C is greatly reduced for the mutant enzyme compared to wild-type RNA polymerase at this promoter. This suggests that the αCTD is particularly important in transcription initiation in the absence of sequence-specific contacts between the –35 region and the σ subunit and this role is independent of an UP element.

The effect of supercoiling on open complex formation at the *gal*PconTG6 promoter with α-256 RNA polymerase

In the case of wild-type RNA polymerase, a lack of thermal energy needed to drive open complex formation can be compensated for by the introduction of negative supercoiling (19). Figure 6 shows open complex formation at *gal*PconTG6 carried on supercoiled (non-linearised) plasmid template. On supercoiled templates, in contrast to linear templates (Fig. 5b), opening is detected at 37, 25, 14 and 6°C for both wild-type and α-256 RNA polymerase. These data add support to the previous observation that supercoiling reduces the thermal energy requirements for opening. However, at low temperatures, the level of opening is reduced in the presence of α-256 RNA polymerase compared to the wild-type enzyme. Thus, the removal of the αCTD affects the kinetics of initiation on supercoiled templates. This may, in part, explain why *E.coli* cells containing only truncated α subunits are not viable (30).

DISCUSSION

It has previously been shown that *gal*P1, an extended –10 promoter, can form open complexes at low temperatures (19). In addition, Attey *et al.* (18) showed that reconstituted RNA polymerase containing C-terminally truncated α subunits (α-256) is unable to drive strand separation at *gal*P1 under limiting thermal energy conditions. These data suggest that sequence-specific contacts between region 2·5 of σ^{70} and the extended –10 motif and between αCTD and the *gal*P1 UP-element help determine the kinetics of open complex formation. However, the data presented here show that αCTD plays a role in determining the kinetics of transcription initiation in the absence of stable sequence-specific contacts with DNA. Requirement for the αCTD is most critical under low thermal energy conditions, where duplex opening is thought to be limiting. This suggests that αCTD acts to promote isomerisation of the closed to the open complex. It has previously been shown that αCTD is not absolutely required at activator-independent promoters lacking

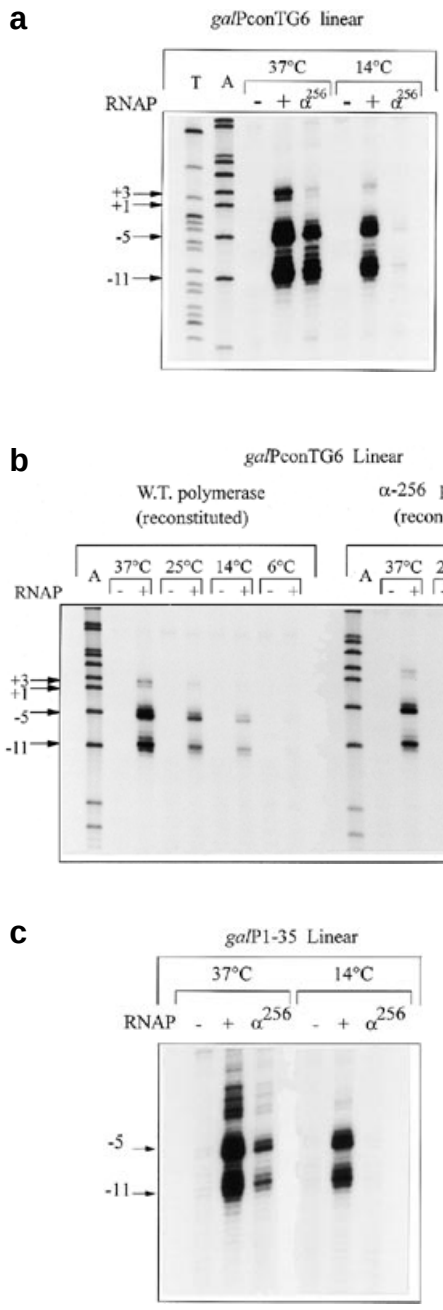

Figure 5. Temperature dependence of open complex formation by wild-type and α-256 RNA polymerase. (**a**) Potassium permanganate footprints of *gal*PconTG6 complexes formed at 37 and 14°C by wild-type RNA polymerase supplied by Northumbria Biologicals Ltd and reconstituted α-256 RNA polymerase. At 14°C the wild-type RNA polymerase and α-256 RNA polymerase drive 72 and 3% strand separation, respectively, relative to 37°C. (**b**) Potassium permanganate footprints of *gal*PconTG6 complexes formed at 37, 25, 14 and 6°C by reconstituted wild-type and α-256 RNA polymerase. The relative intensities of the bands obtained at different temperatures for the wild-type polymerase are: at 37°C, 100%; 25°C, 58%; 14°C, 29%; 6°C, 4%. The relative intensities of the bands obtained at different temperatures for α-256 RNA polymerase are: at 37°C, 100%; 25°C, 28%; 14°C, 3%; 6°C, 0%. (**c**) Potassium permanganate footprints of *gal*P1–35 complexes formed at 37 and 14°C by wild-type RNA polymerase supplied by Northumbria Biologicals Ltd and reconstituted α-256 RNA polymerase. Lanes A and T are dideoxy sequencing ladders. At 14°C the wild-type RNA polymerase and α-256 RNA polymerase drive 65 and 6% strand separation, respectively, relative to 37°C.

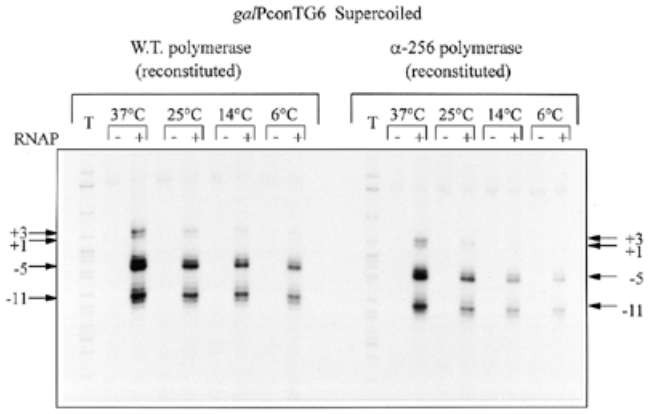

Figure 6. Open complex formation at *gal*PconTG6 on supercoiled plasmid templates with wild-type and α-256 RNA polymerase. Potassium permanganate footprints of *gal*PconTG6 complexes formed at 37, 25, 14 and 6°C by reconstituted wild-type and α-256 RNA polymerase. The arrows indicate single-stranded T residues, numbered relative to the transcription start +1. Lane T is a dideoxy sequencing ladder specific for T residues. The relative intensities of the bands obtained at different temperatures for the wild-type polymerase are: at 37°C, 100%; 25°C, 64%; 14°C, 52%; 6°C, 43%. The relative intensities of the bands obtained at different temperatures for α-256 RNA polymerase are: at 37°C, 100%; 25°C, 50%; 14°C, 30%; 6°C, 15%.

an UP-element (26). It is also tempting to speculate that, although not absolutely required, αCTD plays a role in transcription initiation at all promoters. There are two possible models to explain the role of αCTD in transcription initiation. In the first, since the αCTD does not form any detectable contact (as determined by DNase I footprinting) with promoters lacking an UP-element, the contribution of the αCTD to the kinetics of open complex formation depends on interactions between α and other RNA polymerase subunits. In the second model, αCTD contacts DNA at all promoters, but at promoters lacking an UP-element the interaction may be too transient to be detected by DNase I footprinting. The docking of αCTD on the DNA then positions it so that it can make 'constructive' contacts with the rest of RNA polymerase, these contacts helping to drive transcription initiation. Both models propose that αCTD increases transcription initiation by contacting other components of the transcription machinery. A number of different activator proteins increase transcription by interacting with region 4 of the σ subunit (31–34). The αCTD may facilitate open complex formation by interacting with this region of σ. This model is supported by the observation that αCTD can be cross-linked to σ^{70} (35) and is consistent with the proposal that the α subunit was once an independent transcription factor which has become incorporated into RNA polymerase (36). Attey *et al.* (18) also showed that cAMP–CRP can compensate for the αCTD truncation, driving open complex formation at the *gal*P1 promoter at low temperatures. It has been shown that cAMP–CRP can activate transcription by contacting σ^{70} (33). In the absence of the putative α–σ interaction, cAMP–CRP may therefore interact with σ to promote low temperature opening at *gal*P1.

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