Temperature-dependence of open-complex formation at two *Escherichia coli* promoters with extended –10 sequences

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We have studied the formation of open complexes between purified RNA polymerase from *Escherichia coli* and DNA fragments carrying the *galP1* promoter, a promoter with an extended –10 region. Unusually, these complexes are formed readily at low temperatures. This low-temperature opening is unaffected by deletions of either upstream or downstream promoter sequences. We conclude that low-temperature open-complex formation is due to specific base sequences in and just

upstream of the extended –10 region. In contrast, open complexes are not formed at low temperatures with DNA fragments carrying the *E. coli cysG* promoter, which also has an extended –10 region. This demonstrates that an extended –10 sequence alone is not sufficient for low-temperature opening. Additionally, we report the temperature dependence of a hybrid *galP1-cysG* promoter, the related *galP2* and *galP3* promoters and a derivative of *galP1* with an improved –10 hexamer sequence.

INTRODUCTION

Transcription initiation at promoters requires the binding of RNA polymerase to promoter DNA, followed by local unwinding of the DNA duplex around the transcript start site (to give the open complex) and RNA synthesis. Initiation at most activator-independent *Escherichia coli* promoters by *E. coli* holo RNA polymerase (RNAP) is contingent on the recognition of two hexamer elements known as the –10 and –35 hexamers, which are located around 10 and 35 bp respectively upstream of the transcript start and are separated by a spacer region [1]. However, *E. coli* also contains a small number of promoters where specific base sequences in the –35 region are not important; at these promoters RNAP recognizes an extended –10 sequence with the motif 5'-TG-3' located 1 bp upstream of the –10 hexamer [2].

In our previous studies we have made a detailed study of RNAP binding to one such 'extended –10 promoter', *galP1* [3,4]. Interestingly, in open RNAP-*galP1* complexes, bound RNAP makes extensive contacts upstream of the –35 region due to binding of the C-terminal domains of the α subunits of RNAP (α CTDs) [5]. A second curious feature of *galP1* emerged from *in vitro* studies of open-complex formation with purified DNA fragments and purified RNAP. We noticed [6] that these complexes can form readily at lower temperatures; this is especially interesting as most *E. coli* promoters are inactive at temperatures below 20 °C, most likely due to the thermal energy requirement for duplex unwinding [7]. In the present study we have investigated the sequence requirements for low-temperature opening at *galP1*, and the temperature dependence of opening of the related promoters *galP2* and *galP3*. We have found that low-temperature opening of *galP1* is unaffected by replacement of both upstream and downstream sequences, and must therefore be due to specific base sequences in and just upstream of the –10 region. However, we also show that low-temperature opening is not an intrinsic property of extended –10 sequences. The *E. coli cysG* promoter, which we previously showed to have an extended –10 region [8], is unable to open at low temperatures, but a hybrid promoter, carrying *galP1* sequences from upstream of base pair –15 (with

respect to the transcript start), has a reduced thermal energy requirement.

EXPERIMENTAL

Promoters and plasmids

Standard recombinant DNA methodology was used throughout this work [9]. The different promoters, listed in Figure 1, were constructed with an *EcoRI* linker upstream of the transcript start site and a *HindIII* linker downstream. By convention, promoter sequences are numbered with the transcript start site taken as +1, and with upstream and downstream sequences denoted by negative and positive coordinates respectively. All the promoters were cloned between the *EcoRI* and *HindIII* sites of plasmid pBR322. Plasmids carrying the different promoters were purified by caesium chloride gradient centrifugation and linearized with *PstI* prior to footprinting experiments. In the experiment shown in Figure 6, the linearization step was omitted and the plasmid DNA was used as purified in its supercoiled form.

The different promoter fragments studied were as follows. (1) *gal p19T*: this fragment, covering the *E. coli gal* operon regulatory region, carries the *p19T* substitution that inactivates *galP2*. This fragment carries *galP1* with an *EcoRI* linker at –92 and a *HindIII* linker at +45, as used in our previous work [3–6]. (2) *gal p19Tp45–48*: a derivative of *gal p19T* carrying 4 bp substitutions at base pairs –45 (45G), –46 (46C), –47 (47C) and –48 (48C) that disrupt an upstream TTATT motif. The derivative was made by oligonucleotide-directed mutagenesis using the Amersham mutagenesis kit (catalogue no. RPN 1523). (3) *gal p19TΔ49*: a derivative of *gal p19T* carrying deletion of *gal* sequences upstream of base pair –49. This derivative was constructed with an *EcoRI* linker at –49 as described by Busby et al. [10]. (4) *gal p19TΔ9*: a derivative of *gal p19T* carrying deletion of *gal* sequences from +8 to +16 inclusive, as described in [11]. (5) *gal p19TΔ14*: a derivative of *gal p19T* carrying deletion of *gal* sequences from +3 to +16 inclusive, as described in [11]. (6) *gal p14A*: this *gal* operon regulatory region fragment contains solely *galP2*, due to the *p14A* substitution that inactivates *galP1* [11,12]. The upstream and downstream *EcoRI* and

Abbreviations used: RNAP, *Escherichia coli* holo RNA polymerase; α CTD, C-terminal domain of the α subunit of RNAP.

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Figure 1 Base sequences of promoters used in this work

The top strand sequence of each of the promoters discussed in the text is shown. In each case the A at the transcript start site is doubly underlined, the -35 hexamer is singly underlined, the TG motif is doubly underlined and the -10 hexamer is singly underlined. Carets (v) denote the sites of RNAP-dependent base modification by permanganate, with the number of carets denoting the intensity of the band. The *gal p19T* and *pcysG* sequences are numbered with respect to the transcript start points (the *P1* start in the case of *gal p19T*). The upstream *EcoRI* site in the *gal p19TΔ49* sequence is in bold letters. Dots denote deleted bases in the *gal p19TΔ9* and *gal p19TΔ14* sequences. In the *pgal-cys* sequence, the upstream *gal* sequences are italicized.

HindIII linkers are the same as for the *gal p19T* fragment. (7) *gal p19Tp12C*: a derivative of *gal p19T* carrying the *p12C* substitution that inactivates *galP1* and unmasks *galP3*, which initiates transcription at position +14 with respect to the *galP1* start site [13]. (8) *pcysG*: this fragment, from the *E. coli nirC-cysG* region, carries the *cysG* promoter with an *EcoRI* linker at -135 and a *HindIII* linker at +248, as described in Figure 1 of [8]. (9) *pgal-cys*: a derivative of *pcys* with sequences from -16 and upstream replaced with *gal p19T* sequences. This fragment carries a hybrid of the *galP1* and *cysG* promoters. The hybrid promoter was made by a two-step PCR protocol. First, *pcysG* sequences were amplified using the primer 5' TTTTCGCATCTTTTATGGTATGCTACGCCGA 3' and a primer corresponding to sequences downstream of the pBR322 *HindIII* site. The product was purified and, together with a primer corresponding to sequences upstream of the pBR322 *EcoRI* site, used as a primer to amplify *gal p19T* sequences. The resulting PCR product was cut with *EcoRI* and *HindIII* and the resulting fragment was cloned into pBR322.

Potassium permanganate footprinting

Footprinting was performed using protocols based on those described by Gralla et al. [14]. Plasmids carrying different cloned promoters were incubated with RNAP at a range of temperatures.

Samples were then treated with potassium permanganate, which modifies thymine residues in single-stranded or distorted regions of the duplex. The positions of modified residues were then detected by primer extension using *Taq* DNA polymerase and a ^{32}P -labelled primer (5' AGGCGTATCACGAGGCCCT 3') that hybridizes just upstream of the *EcoRI* site (recall that the upstream end of each promoter fragment is cloned into this site). Since *Taq* polymerase is unable to copy from modified bases, modification of thymines on the bottom strand of the promoter will cause interruption of the extension of the labelled primer at the corresponding adenines on the top strand. The locations of the interruptions can be deduced by measuring the size of the interrupted extension products, most conveniently by using gels that are calibrated with dideoxy sequencing reactions run on unmodified template using the same primer.

In the experiments described here, we used 10–14 nM template DNA in buffer containing 20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl_2 , 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol and 50 $\mu\text{g/ml}$ BSA. Replicate samples of 20 μl were prewarmed, and open-complex formation was initiated by the addition of 150 nM RNAP (NBL Gene Sciences Ltd.) to one of the samples. Samples were incubated for 30 min at different temperatures ranging from 5 to 37 $^\circ\text{C}$ (we previously checked that 30 min was sufficient for equilibrium to be established; the RNAP becomes distributed between the cloned promoter and

non-specific sites on the vector DNA). After incubation, 1 μ l of freshly prepared 200 mM KMnO_4 was added to each tube and, after a further 4 min incubation, 50 μ l of stop mix (3 M ammonium acetate, 0.1 mM EDTA, 1.5 M β -mercaptoethanol) was added. The template DNA was then purified by phenol extraction and hybridized to the ^{32}P -labelled primer, and the primer was extended using a PCR cycle to amplify the signal. Extension products were analysed on the calibrated sequence gels shown in Figures 2–6. Gels were scanned using an LKB Ultrascan Model XL densitometer, and open-complex formation at different temperatures was assessed from quantification of the scans, with band intensity from the complexes formed at 37 °C taken as 100 %. Data were plotted and the approximate temperature range for 50 % promoter opening was estimated by extrapolation. All experiments were performed independently at least three times with good reproducibility; values of the temperature at which 50 % of the complexes were open were accurate to within 2 °C. In all cases values are quoted for the permanganate-reactive band at position -11 ; we noted that, in many cases, the reactivities of bands closer to the transcript start site were slightly more sensitive to decreases in the incubation temperature.

RESULTS AND DISCUSSION

Upstream and downstream sequences are not essential for open-complex formation at *galP1* at lower temperatures

The starting point of this work was the *gal p19T* fragment that carries the *galP1* promoter. In previous studies we showed that incubation of this fragment with RNAP leads to 1:1 transcriptionally competent open complexes in which the DNA duplex around the transcript start is unwound [3,10,12]. We found that, surprisingly, open complexes at *galP1* could form at temperatures

below 20 °C [6]. Figure 2 shows the results of an experiment to investigate whether upstream sequences are essential for this low-temperature opening. Opening of *galP1* was monitored by the pattern of permanganate modification after RNAP was incubated at different temperatures with *gal p19T*, *gal p19Tp45–48* and *gal p19T Δ 49* (recall that potassium permanganate modifies thymines only in single-stranded or distorted DNA). The results in Figure 2 show that bases at -11 , -5 and $+3$ are modified in the presence of RNAP, that they are modified at low temperatures, and that the temperature dependence of the modification is unaffected by the *p45–48* mutations or the $\Delta 49$ deletion. Quantification of this and other data shows that, under our conditions, the temperature at which 50 % of the template molecules form open complexes at *galP1* is 14–16 °C. The $\Delta 49$ deletion replaces α CTD-binding sequences known to be essential for optimal *galP1* activity [5,10], whereas the *p45–48* substitutions replace a 5' TTTATT 3' motif which has been proposed to introduce a flexible hinge in the promoter that permits upstream wrapping [3]. The results in Figure 2 show that neither sequence feature is necessary for open-complex formation at low temperatures.

Figures 3(A) and 3(B) show the results of a complementary experiment to investigate the role of base sequences just downstream of the *galP1* transcript start. The $\Delta 9$ and $\Delta 14$ derivatives of *gal p19T* carry deletions of 9 and 14 bp, but have little or no effect on the temperature dependence of *galP1*. We conclude that the low-temperature opening is independent of the nature of the promoter flanking sequences. Figure 3(C) shows an experiment in which the *galP1* -10 hexamer was altered from 5' TATGGT 3' to the consensus 5' TATAAT 3'. The results show that this change facilitates the low-temperature opening of *galP1*; the temperature at which 50 % of the template molecules form open complexes is decreased to 4–6 °C. Interestingly, the change of the

Figure 2 Open-complex formation at *galP1*: role of upstream sequences

Autoradiographs are shown of calibrated sequence gels on which primer extension products were analysed after treatment of different promoter DNAs with permanganate after incubation with or without RNAP. The promoter fragments were *gal p19T* (A), *gal p19Tp45–48* (B) and *gal p19T Δ 49* (C). For each promoter, lanes 1, 3, 5 and 7 were loaded with samples prepared without incubation with RNAP, whereas lanes 2, 4, 6 and 8 were loaded with samples prepared with incubation with RNAP. Pairs of samples, with and without RNAP, were incubated at a range of temperatures as indicated by the horizontal arrows at the bottom of the Figure: lanes 1 and 2, 37 °C; lanes 3 and 4, 20 °C; lanes 5 and 6, 12 °C; lanes 7 and 8, 5 °C. The gel in each panel is calibrated with Maxam–Gilbert G, T or A reactions as shown.

Figure 3 Open-complex formation at *galP1*: role of downstream sequences

Autoradiographs are shown of calibrated sequence gels on which primer extension products were analysed after treatment of different promoter DNAs with permanganate after incubation with or without RNAP. The promoter fragments were *gal p19TΔ9* (A), *gal p19TΔ14* (B) and *gal p19Tp8Ap9A* (C). For each promoter, lanes 1, 3, 5 and 7 were loaded with samples prepared without incubation with RNAP, whereas lanes 2, 4, 6 and 8 were loaded with samples prepared with incubation with RNAP. Pairs of samples, with and without RNAP, were incubated at a range of temperatures as indicated by the horizontal arrows at the bottom of the Figure: lanes 1 and 2, 37 °C; lanes 3 and 4, 20 °C; lanes 5 and 6, 12 °C; lanes 7 and 8, 5 °C. The gel in each panel is calibrated with a Maxam–Gilbert C reaction, as shown.

–10 hexamer from 5' TATGGT 3' to 5' TATAAT 3' increases the reactivity of the band at –11, but decreases the reactivity of the bands at –5 and +3.

The potassium permanganate footprints in Figures 2 and 3 reveal an additional unexpected feature: a strong RNAP-induced permanganate reactivity is seen downstream of the unwound region of the open complex in each case. In the case of *gal p19T*, this corresponds to modification of the lower-strand T at +19 (Figures 1 and 2A). The simplest interpretation of this signal is that it is due to a distortion of the promoter DNA at the downstream end of the open complex (most likely at the point where the DNA template separates from bound RNAP). Interestingly, with the *gal p19Tp45–48* and *gal p19TΔ49* templates, the reactive site moves 4 bases downstream to +23 (Figures 1, 2B and 2C). Thus the location of this apparent downstream distortion is flexible and, surprisingly, dependent on upstream sequences. With *gal p19TΔ9* and *gal p19TΔ14* the reactivity is found at +21 and +23 respectively (Figures 1, 3A and 3B), whereas with *gal p19Tp8Ap9A* reactivity is at +19 (Figures 1 and 3C). Note that these downstream reactive bands were not seen in our previous work [6,12] because we used DNA fragments labelled at the *Hind*III end, located at +45 with respect to the *galP1* transcript start. In the present experiments the labelled primer corresponds to sequence upstream of the promoter, the *Hind*III site is not cleaved and extension runs across this region.

Temperature dependence of the *galP1*, *galP2* and *galP3* promoters

The *E. coli gal* operon regulatory region contains two overlapping promoters, *P1* and *P2*. In the above experiments, to study *P1*, we

used the *p19T* substitution that inactivates *P2*, leaving *P1* as the sole promoter [11,12]. To investigate the temperature dependence of *P2* we used the *p14A* mutation that inactivates *P1*, leaving *P2* as the sole promoter [11,12]. We previously showed that the *p12C* substitution inactivates both *P1* and *P2* and unmasks a third promoter, *P3* [13]. We therefore compared the temperature dependence of *P1*, *P2* and *P3* by studying open-complex formation with the *gal p19T*, *p14A* and *p12C* fragments (Figure 1; *p19T* was combined with *p12C* to improve *galP3*). The results (Figure 4) show that open complexes at *P1* and *P2*, but not *P3*, form at lower temperatures. For *P1* and *P2*, the temperature at which 50% of the template molecules form open complexes is 14–16 °C, whereas for *P3* it is 24–26 °C. This result shows that low-temperature opening is not an intrinsic property of any promoter within the *gal* operon regulatory region. Interestingly, both *P1* and *P2*, but not *P3*, carry a 5' TG 3' motif located 1 bp upstream of the –10 hexamer (i.e. they both have an extended –10 region). Additionally, we note that neither open complex at *P2* or *P3* displays strong reactivity with permanganate downstream of the transcript start (in contrast to *P1*, where a clear band at +19 is seen). Since all three fragments carry the same sequence in this region, it is clear that the downstream reactivity is due to some special feature of the open complex at *galP1*.

Temperature dependence of the *E. coli cysG* promoter

From the preceding results, one might conclude that open-complex formation at lower temperatures is due to an extended –10 region. To check this, we examined opening of the *E. coli cysG* promoter. In previous work we showed that, as for *galP1*, the *cysG* promoter carries an extended –10 sequence, specific sequences in the –35 region are not essential for function, and

Figure 4 Open-complex formation at *galP1*, *galP2* and *galP3*

Autoradiographs are shown of calibrated sequence gels on which primer extension products were analysed after treatment of different promoter DNAs with permanganate after incubation with or without RNAP. The promoter fragments were *gal p19T* (*galP1*) (A), *gal p14A* (*galP2*) (B) and *gal p19Tp12C* (*galP3*) (C). For each promoter, lanes 1, 3, 5 and 7 were loaded with samples prepared without incubation with RNAP, whereas lanes 2, 4, 6 and 8 were loaded with samples prepared with incubation with RNAP. Pairs of samples, with and without RNAP, were incubated at a range of temperatures as indicated by the horizontal arrows at the bottom of the Figure: lanes 1 and 2, 37 °C; lanes 3 and 4, 20 °C; lanes 5 and 6, 12 °C; lanes 7 and 8, 5 °C. The gel in each panel is calibrated with Maxam–Gilbert T or C reactions as shown.

Figure 5 Open complexes at the *cysG* and *gal-cysG* promoters

Autoradiographs are shown of calibrated sequence gels on which primer extension products were analysed after treatment of promoter DNAs with permanganate after incubation with or without RNAP. The promoter fragments were *pcysG* (A) or the *pgal-cysG* hybrid (B). For each promoter, lanes 1, 3, 5 and 7 were loaded with samples prepared without incubation with RNAP, whereas lanes 2, 4, 6 and 8 were loaded with samples prepared with incubation with RNAP. Pairs of samples, with and without RNAP, were incubated at a range of temperatures as indicated by the horizontal arrows at the bottom of the Figure: lanes 1 and 2, 37 °C; lanes 3 and 4, 25 °C; lanes 5 and 6, 15 °C; lanes 7 and 8, 6 °C.

RNAP protects upstream sequences in open complexes [8]. Further, we noted that the two promoters share identical bases at 24 positions between base pairs +1 and –50. The data in Figure 5(A) confirm our previous observation [8] that RNAP-dependent unwinding around the transcript start at the *cysG* promoter can be detected using permanganate, and that a supplementary band is seen around –53 (which we previously ascribed to template distortion). However, the results show that open complexes at the *cysG* promoter, unlike those at *galP1*, are

not able to form at lower temperatures. From this result we conclude that an extended –10 sequence alone is insufficient for open-complex formation at low temperatures. To investigate this point further, we made a hybrid *galP1-cysG* promoter derivative by replacing *cysG* sequences upstream of position –15 with the corresponding sequences from *galP1*. The results in Figure 5(B) show that open complexes at the hybrid *galP1-cysG* promoter are clearly able to form at lower temperatures. Since the hybrid promoter contains the *cysG* extended –10 hexamer, we conclude

Figure 6 Open complexes on supercoiled templates

Autoradiographs are shown of calibrated sequence gels on which primer extension products were analysed after permanganate treatment of promoter DNAs, cloned in circular supercoiled plasmids, incubated at different temperatures either with or without RNAP. The promoter fragments were *gal p19T* (*galP1*) (A), *gal p14A* (*galP2*) (B), *gal p19Tp12C* (*galP3*) (C) and *pcysG* (D). For each promoter, lanes 1, 3, 5 and 7 were loaded with samples prepared without incubation with RNAP, whereas lanes 2, 4, 6 and 8 were loaded with samples prepared with incubation with RNAP. Pairs of samples, with and without RNAP, were incubated at a range of temperatures as indicated by the horizontal arrows at the bottom of the Figure: lanes 1 and 2, 37 °C; lanes 3 and 4, 20 °C; lanes 5 and 6, 12 °C; lanes 7 and 8, 5 °C. The gel in each panel is calibrated with Maxam–Gilbert C, T or A reactions as shown.

that bases upstream of this extended element confer the ability to open at lower temperatures.

Effects of supercoiling

In previous work we showed that the temperature requirement for open-complex formation *in vitro* at several promoters was reduced if the template was supercoiled [15]. Figure 6 shows an experiment to investigate the effect of supercoiling on the two promoters, *galP3* and *cysG*, that had failed to function at lower temperatures on linear fragments. The experiment shows that supercoiling permits both promoters to open at lower temperatures. The temperature at which 50% of the template molecules form open complexes is reduced to 8–10 °C. The experiments with the controls *galP1* and *galP2* confirmed our previous observation [15] that supercoiling lowers the thermal energy requirement for promoter opening; the temperature at which 50% of the template molecules form open complexes is reduced to 4–6 °C.

Conclusions

We previously showed that, at *galP1*, open complexes could form at temperatures below 20 °C [6,15]. This is in sharp contrast to most *E. coli* promoters, which are inactive at temperatures below 20 °C due to the thermal energy requirement for duplex unwinding. Interactions between *galP1* and RNAP must be sufficient to satisfy this energy requirement; our aim was to identify the important base sequences. From our results, we conclude that neither upstream nor downstream base sequences are important; thus the extended contacts between α CTD and

upstream sequences, while essential for optimal promoter activity, are not essential for low-temperature opening. Taken together, our results indicate that it must be base sequences upstream of the extended –10 element (i.e. upstream of –15) that permit open-complex formation at lower temperatures. In a very recent experiment, we found that low-temperature opening is unaffected by replacement of bases upstream of position –35 [15a], implicating the spacer region of *galP1*. Current efforts are directed at pinpointing the important sequences. We are also attempting to quantify the thermodynamic and kinetic parameters of the open complexes and their dependence on temperature; we are aware that the assays reported here give only a crude measure of open-complex formation, and that quantitative temperature-dependence studies can yield detailed information on conformational changes occurring during protein–DNA interactions [16,17].

Our footprint studies of open complexes at *galP1* unexpectedly revealed strong RNAP-induced permanganate reactivity downstream of the unwound region around the transcript start. We interpret this as a distortion in the promoter DNA at the downstream limit of bound RNAP of the open complex in each case. Although we have not proved this point, we note that flexibility and kinking of template DNA is supposed to play a role at a number of other promoters [18,19]. This downstream distortion is specific to *galP1* and is not found with *galP2*, *galP3* or the *cysG* promoter. Interestingly, the precise location of the downstream distortion is dependent on the upstream sequences, implying that there is interaction between the downstream and upstream ends of the open complex. This point also emerges from the study with the *galP1*–*cysG* hybrid promoter (Figure 5B). The presence of *galP1* upstream sequences clearly induces

the appearance of a reactive band at +25 (which we ascribe to DNA distortion in the open complex). Presumably, RNAP binding to the upstream *galP1* sequence affects the conformation of RNAP bound to the downstream *cysG* sequence. Two models could account for this: either conformational changes are passed through the RNAP, or the open complex is highly distorted, with the path of the DNA describing a U or an α , such that the upstream and downstream parts of the complex are adjacent to each other (a full discussion of possible distortions in bacterial promoter DNA during transcription initiation is given in [20]).

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