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The C-terminal domain of the *Escherichia coli* RNA polymerase α subunit plays a role in the CI-dependent activation of the bacteriophage λ p_M promoter

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ABSTRACT

The bacteriophage λ p_M promoter is required for maintenance of the λ prophage in *Escherichia coli*, as it facilitates transcription of the *cI* gene, encoding the λ repressor (CI). CI levels are maintained through a transcriptional feedback mechanism whereby CI can serve as an activator or a repressor of p_M. CI activates p_M through cooperative binding to the O_R1 and O_R2 sites within the O_R operator, with the O_R2-bound CI dimer making contact with domain 4 of the RNA polymerase σ subunit (σ_4). Here we demonstrate that the 261 and 287 determinants of the C-terminal domain of the RNA polymerase α subunit (αCTD), as well as the DNA-binding determinant, are important for CI-dependent activation of p_M. We also show that the location of αCTD at the p_M promoter changes in the presence of CI. Thus, in the absence of CI, one αCTD is located on the DNA at position −44 relative to the transcription start site, whereas in the presence of CI, αCTD is located at position −54, between the CI-binding sites at O_R1 and O_R2. These results suggest that contacts between CI and both αCTD and σ are required for efficient CI-dependent activation of p_M.

INTRODUCTION

Bacteriophage λ is a temperate phage which can enter one of two alternative developmental pathways, lytic or lysogenic, upon infection of its host, *Escherichia coli* (1,2). When the lysogenic pathway is chosen, phage DNA is incorporated into the *E. coli* genome, forming a prophage that can be maintained in this state for many cell generations. Stable maintenance of the prophage is achieved through the action of the phage-encoded repressor, the λ repressor (CI), which both represses the lytic promoters, p_L and p_R, and stimulates transcription of its own gene from the p_M promoter (3). The p_R and p_M promoters are divergently arranged with their start sites separated by only 82 bp. Both promoters are regulated by the binding of CI dimers to three related 17-bp sequences, O_R1, O_R2 and O_R3, located at −74 to −58, −50 to −34 and −27 to −11, respectively, with respect to the transcription start site at p_M. A CI dimer bound at the high-affinity operator, O_R1, acts as a repressor of the p_R promoter but also stabilizes the binding of a second CI dimer to a lower-affinity operator, O_R2, and the second dimer, in turn, interacts with RNA polymerase (RNAP) to stimulate transcription from p_M above basal levels (3,4). This stimulation occurs at the isomerization step (k_f) in the transcription initiation pathway that leads to open complex formation (5,6). At higher concentrations, CI also binds to O_R3, thereby repressing p_M (7).

Each CI monomer comprises an N-terminal DNA-binding domain (residues 1–92) and a C-terminal oligomerization domain (residues 132–236) connected by an interdomain linker known as the 'hinge' region (8). Detailed structural information is available for the isolated N-terminal and C-terminal domains (9–13). The oligomerization domain participates in dimerization of CI monomers and is also involved in weaker cooperative interactions between pairs of dimers bound to adjacent operator sites. The nature of both of these types of interaction have been elucidated by X-ray crystallography (12,13). It has also been shown that repressor tetramers (i.e. pairs of dimers) bound at O_R1–O_R2 and O_L1–O_L2 can interact through their oligomerization domains over a distance of ∼3 kb, forming an octamer that enhances

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repression of p_R (13–15). The N-terminal domain of CI contains a DNA-binding helix-turn-helix motif which is responsible for operator recognition. In addition, residues exposed on the first helix (specifically E34 and D38) generate a negatively charged patch which, in the case of the downstream subunit of the CI dimer bound to O_R2, is involved in interactions with positively charged residues (R588, K593 and R596) on the surface of domain 4 of the RNAP σ^{70} subunit (σ_4) during activation of p_M (6,16–23). For this reason, CI is classified as a Class II activator, along with other activators which bind to sites overlapping the −35 region and, in most cases, activate transcription by contacting σ_4 (22,24,25).

At many bacterial promoters, the C-terminal domain of the RNAP α subunit (αCTD) interacts with upstream promoter DNA, the RNAP σ^{70} subunit and/or transcription activator proteins (24,26). These interactions are mediated by determinants on the surface of αCTD and are facilitated by the presence of a flexible linker connecting αCTD to the N-terminal domain (27–29). For example, residue 265, and neigbouring residues, contribute to the 265 determinant, which is responsible for interactions with DNA (30–33). Similarly, residue 261 and neighbouring residues contribute to the 261 determinant, that can contact σ_4 (34–36), whereas the side chains of valine 287 and neighbouring residues form a surface-exposed patch, the 287 determinant, which interacts with an activatory surface, AR1, on CRP (cyclic AMP receptor protein) and with other activators (33,34,37,38).

Previously, we have shown that the *rpoA341* mutation, leading to substitution of glutamate for lysine at position 271 within αCTD, decreases λ prophage stability (39,40). This observation could be explained by a defective interaction between the mutant αCTD and the CI repressor at p_M. Therefore, the aim of this work was to determine whether αCTD plays a role in CI-dependent activation of p_M. Our results show that determinants on the surface of αCTD are required for fully efficient activation by CI. In addition, we demonstrate that the location of αCTD at p_M is shifted further upstream in the presence of CI. These observations suggest that CI makes direct contact with αCTD at p_M and that this interaction is important for transcription activation by CI.

MATERIALS AND METHODS

Bacterial strains

The *E.coli rpoA*$^+$ strain, WAM106 [*araD139*, Δ(*argF-lac*) *U169*, Δ(*his-gnd*), *thi*, *rpsL150*, *gltS*$_0$, *flbB5301*, *relA1*, *deoC1*, *rbsR*], and its otherwise isogenic *rpoA341* derivative (WAM105), bearing a chromosomal mutation that results in the K271E substitution in the RNAP α subunit (39), were used. Strains WAM140, WAM141 and WAM144, harbouring chromosomal *rpoA261*, *rpoA269* and *rpoA287* alleles, encoding α subunits with alanine substitutions at positions 261, 269 and 287, respectively, are otherwise isogenic with WAM106 and were isolated by a previously described procedure (34,41,42). Strain WAM142, bearing the chromosomal mutation *rpoA271*, which results in substitution K271A in α was isolated as a Cym$^+$ Mel$^+$ pseudorevertant of strain WAM105. The *E. coli* strain, TAP90 (*supE44*, *supF58*, *hsdR*, *pro*, *leuB*, *thi-1*, *rpsL*, *lacY*, *tonA1*, *recD1903*::mini*tet*) was used to titrate bacteriophage containing the *S7* amber allele (43).

Bacteriophage, plasmids and gene fusions

Bacteriophage λ*cI857S7* (44), which is unable to lyse *E.coli* cells unless the *supF* suppressor allele is present, was used for measuring prophage stability. For the expression of mutant *rpoA* alleles for the αCTD alanine scan analysis, derivatives of plasmid pHTf1α, encoding α mutants with alanine substitutions at positions 255–271 and 302, and pREIIα, encoding α mutants with alanine substitutions at the remaining positions in αCTD, were used (27,30,37,45–47). Plasmids pGW857 and pACλcI, both of which are p15A derivatives, were used to express the phage λ *cI* gene. pGW857 encodes the thermolabile CI_{857} protein under control of the *lac* promoter (48) and thereby allows for complete inactivation of repressor function by growth at 42°C. Plasmid pACλcI was used to overexpress the wild-type *cI* gene from the *lacUV5* promoter (49). For measuring the activity of the p_M promoter, two p_M–*lacZ* fusion plasmids were used: pAHA1, a pBR322-based replicon, and pTJSpM, an RK2-based replicon. To construct pAHA1, the wild-type p_M promoter region (248 bp) was amplified by PCR using the λ plasmid pKB2 (50) as a template, and the following primers: 5′-GCC GGA TCC CCA TCT TGT CTG C and 5′-TAT GCG TTG TTA GCT ATA GAC TCC TTA GTA C (35 cycles of the following program were performed: denaturation at 95°C for 30 s, annealing at 55.4°C for 30 s, extension at 72°C for 30 s). The product of the amplification was digested with BamHI and cloned between the BamHI and SmaI sites upstream of the *lacZ* gene of pHG86 (51). To construct pTJSpM, the EcoRI–HindIII fragment containing the p_M promoter was cut from plasmid pEM9-O_RP (52) and used to replace the BamHI–EcoRI fragment of pTJSpI containing p_I (53), following treatment of both the vector and the promoter fragment with T4 DNA polymerase. The p_M promoter present in pEM9-O_RP (and pTJSpM) contains the wild-type O_R1 and O_R2 operators, but O_R3 is inactivated by multiple mutations (TA<u>CAG</u>C<u>T</u>GCAAGGGATA). These changes (underlined) abolish CI binding but do not alter the −35 or −10 sequences of the p_M promoter. pJMH1 is a pSC101 derivative carrying the *lacI*^{q} and kanamycin resistance genes (39). pRLGpMmut was constructed by amplifying a DNA fragment containing the phage λ p_M promoter using primers 5′-GCC GAA TTC GTA CAT GCA ACC ATT ATC-3′ and 5′-TTG TAA GCT TAC GTT AAA TCT ATC ACC <u>A</u>CA AGG G-3′ (35 cycles of the following program were performed: denaturation at 95°C for 20 s, annealing at 50°C for 30 s, extension at 72°C for 60 s). This fragment was ligated between the HindIII and EcoRI sites of pRLG770 (54). The second primer introduces a G to A point mutation at −18 (underlined) which reduces binding of CI to O_R3 and consequent repression of p_M (55).

Measurement of the effect of mutant *rpoA* alleles on CI-dependent activation *in vivo*

For the alanine scanning experiment (merodipoid), expression of wild-type *cI* from pACλcI, and mutated *rpoA* alleles from pHTf1α and pREIIα derivatives, was simultaneously induced by addition of IPTG (0.1 mM final concentration) to cultures of WAM106 harbouring pJMH1 and pTJSpM growing at 37°C. The β-galactosidase activity was measured 1 h later. To assess the effect of haploid *rpoA* alleles on CI-dependent activation of p_M, strains harbouring chromosomal mutant *rpoA* alleles were transformed with pGW857 and pAHA1, and cultures were grown at 43°C to $OD_{575} = 0.2$ [the *cI857*(ts) gene product is inactive under these conditions and β-galactosidase activity is very similar to that measured in cells devoid of pGW857; data not shown] whereupon IPTG was added (0.05 mM final concentration) and the culture was immediately shifted to 30°C. Following incubation at this temperature for 1 h the β-galactosidase activity was measured. This induction regime minimizes problems due to CI occupancy of O_R3 present on pAHA1 (data not shown).

Measurement of β-galactosidase activity

The activity of β-galactosidase in bacterial cells was measured according to Miller (56). Since we used a multicopy *lacZ* fusion, the β-galactosidase activities were calculated per plasmid copy number, estimated as described previously (57), to compensate for any possible copy number variation between strains. For the alanine scanning experiment, bacteria were grown at 37°C to $OD_{578} = 0.2$, induced with 0.1 mM IPTG and, following further incubation for 1 h, β-galactosidase assays were performed. Results presented are averages of at least three independent experiments and are shown with standard deviations.

Measurement of the efficiency of prophage maintenance

λ prophage maintenance in lysogenic *E.coli* strains was estimated by measuring the efficiency of spontaneous induction of a λ*cI857S7* prophage as described previously (40). Briefly, samples (5 ml) of exponential phase cultures (OD_{578} 0.2–0.5) of bacteria lysogenic for bacteriophage λ*cI*857*S*7, growing at 30°C, were withdrawn and shaken vigorously with chloroform (0.5 ml) for 1 min to release progeny phage. Following centrifugation, liberated phages were titrated on the suppressor strain, TAP90, at 37°C. Other samples, withdrawn at the same time as those for phage titration, were centrifuged. Cell pellets were resuspended in 0.9% NaCl and used for titration of bacteria on LB agar at 30°C. Finally, the number of phages yielded per bacterial cell was calculated.

Protein purification and reconstitution of RNA polymerase

Plasmid pT7λcISa109His6 (21) was used for overproduction of C-terminally His-tagged CI protein, which was purified as described previously (21). For the reconstitution of RNAP, inclusion bodies of RNAP β, β′ and σ^{70} subunits from strains XL1-Blue (MKSe2), BL21(DE3)(pT7β′) and BL21(DE3)(pLHN12σ), respectively, were prepared as described previously (58). His-tagged RNAP α subunits were prepared using plasmid pHTT7f1NHα (58). Derivatives of pHTT7f1NHα carrying mutant *rpoA* alleles were constructed by replacing the HindIII–BamHI fragment, which encodes αCTD and the interdomain linker, with the corresponding fragments from derivatives of pHTf1α and pREIIα encoding the appropriate alanine-substituted α mutants (see above) or from pLAW2phs (encoding α containing the K271E substitution) (39). Overexpression of the α subunits in strain BL21(DE3), purification of α by Ni^{2+}-affinity chromatography and reconstitution into RNAP were performed essentially as described previously (30,58). Purification of α subunits with single cysteine residues, conjugation with Fe.BABE, and reconstitution into RNAP was performed as described by Lee *et al.* (59).

In vitro transcription

Single round *in vitro* transcription reactions were performed in a total volume of 20 µl in buffer containing 50 mM KCl, 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 100 µg/ml BSA and 30 ng linear template DNA. Template DNA containing the p_M promoter was prepared by isolating the 1260-bp NdeI–EcoRI fragment from plasmid pRLGpMmut. The 1313-bp NdeI–PstI fragment from the same plasmid, containing the RNA I gene, served as the internal control. The binding reaction of CI (80 ng) to the DNA (30 ng) was carried out at 37°C for 10 min, after which time *in vitro* reconstituted RNAP was added and the incubation continued for a further 10 min (this concentration of CI gave rise to ~4-fold activation of p_M in the presence of wild-type reconstituted RNAP (results not shown)). After the addition of nucleotides (CTP, GTP and ATP, each to a final concentration of 150 µM, UTP to 15 µM and 0.6 µCi [α-^{32}P]-UTP per reaction) and heparin to 50 µg/ml, the samples were incubated at 37°C for 15 min and the reactions were stopped by the addition of an equal volume of 95% formamide containing 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The samples were separated by electrophoresis in 6% polyacrylamide gels containing 46% urea in TBE buffer. The gel was dried, and RNA bands were visualized and quantified, following background subtraction, using a PhosphorImager (Bio-Rad). Concentrations of RNAP, calibrated to give the same amount of transcription from the activator-independent RNA-I promoter, were: 46 nM wild-type RNAP, 34 nM RNAP αK271E, 54 nM RNAP αK271A, 13 nM RNAP D258A, 28 nM RNAP αE261A, 35 nM RNAP αR265A, 34 nM RNAP αV287A.

Fe·BABE-mediated hydroxyl radical footprinting

A 150-bp DNA fragment containing the λ p_M promoter was amplified from bacteriophage λ DNA by PCR using primers 5′-GCT TTA AGC TTA CGT GCG TCC TCA AGC TGC-3′ and 5′-CCT GAA TTC ATG CAA CCA TTA TCA CCG-3′, cleaved with HindIII and EcoRI and cloned into the vector pSR (60). A 220-bp AatII–HindIII fragment was purified from the resultant plasmid (pSRpM) and labelled at the HindIII end with [γ-^{32}P]-ATP and T4 polynucleotide kinase. The Fe·BABE-mediated DNA cleavage reactions were carried out in a reaction volume of 25 µl (5 mM $MgCl_2$, 100 mM potassium glutamate, 40 mM HEPES pH 8.0, 50 µg/ml BSA, 10 µg/µl herring sperm DNA). Promoter DNA fragments were incubated with CI protein (250 nM final concentration) at 37°C for 10 min. After 10 min, RNAP holoenzyme was added (600 nM final concentration) and incubated at 37°C for 30 min. Complexes were then challenged with heparin (50 µg/ml final concentration) for 1 min at 37°C then DNA cleavage was initiated by the addition of 3 mM sodium ascorbate and 3 mM hydrogen peroxide. The reactions were incubated for 10 min before being stopped by the addition of thiourea and EDTA to final concentrations of 7 mM and 45 mM, respectively. DNA was then extracted with phenol/chloroform, precipitated with ethanol and analysed by electrophoresis in a 6% denaturing polyacrylamide gel. The gels were calibrated with Maxam–Gilbert G + A ladders and analysed using a PhosphorImager and Quantity One software (Bio-Rad).

RESULTS

Identification of αCTD determinants important for CI-dependent activation of p_M

To identify whether amino acid side chains on αCTD are important for activation by CI, we used an alanine scanning approach, exploiting a set of plasmids encoding the RNAP α subunit in which residues 255–329 were each changed individually to alanine. This approach has been used to identify αCTD residues important for transcription activation mediated by a number of different activator proteins (34,37,38,41,53,61–63). These plasmids were introduced into an *E.coli rpoA*$^+$ strain carrying a p_M–*lacZ* fusion plasmid and inducible CI function.

The results show that, under conditions promoting CI stimulation of p_M, alanine substitutions at residues R255, P256, D258, E261, S266, N268, C269, L270, V287 and S299 in αCTD most strongly impaired the activity of p_M (i.e. activity ≤80% of that afforded by plasmid-encoded wild-type α) (Figure 1A). The location of these residues in the αCTD structure is shown in Figure 1B (the residues at positions 266, 270 and 299 are buried in the αCTD structure and are therefore not included in this figure). Most of them are located on one side of αCTD and create a patch on the surface of the domain, whereas V287 is located on the opposite side of αCTD.

Effect of substitutions in αCTD on CI-dependent activation of p_M *in vitro*

To determine whether the effects of the alanine substitutions on *in vivo* p_M activity are direct, we measured the efficiency of CI-mediated stimulation of p_M *in vitro*, using run-off transcription assays. RNAP was reconstituted with the wild-type α subunit, and with some of the mutant α subunits giving rise to a significant decrease in p_M promoter activity *in vivo* (i.e. α containing the 258A, 261A and 287A substitutions). To confirm that R265, within the αCTD DNA-binding determinant, does not play an important role in CI-dependent activation of p_M, RNAP was also reconstituted with the R265A α subunit. In addition, due to our previous observation that the K271E substitution in α causes decreased prophage stability, we included RNAP reconstituted with the 271E and 271A α subunits in the analysis.

Our results are in general agreement with the *in vivo* results, i.e. the abundance of p_M-derived transcripts was significantly decreased when RNAP was reconstituted with α containing the 258A, 261A and 287A substitutions, whereas the efficiency of transcription obtained using RNAP reconstituted with α harbouring the 265A substitution was comparable to that of wild-type RNAP (Figure 2). Consistent with its effect on prophage stability, RNAP reconstituted with 271E α was significantly less active at the p_M promoter *in vitro*. This was also the case with 271A α, although alanine substitution at this position does not exert a negative effect at p_M *in vivo* (Figure 1A).

Effect of substitutions in αCTD determinants important for CI function *in vivo* in the absence of wild-type α

In vivo transcription assays. To investigate the full effect of amino acid substitution within αCTD on CI-dependent activation of the p_M promoter *in vivo*, we constructed *E. coli* mutant strains harbouring mutations within the chromosomal *rpoA* gene that result in alanine codon substitutions at positions 261, 269, 271 and 287 (*rpoA261*, *rpoA269*, *rpoA271* and *rpoA287*, respectively) [it was not possible to transfer to the *E.coli* chromosome alleles encoding substitutions at positions 265, 268 or 299 within the DNA-binding determinant (34; M.S.T., unpublished data)]. The mutant strains were transformed with a plasmid containing inducible CI function and a plasmid harbouring a p_M–*lacZ* fusion, and the effect of induction of *cI* expression on p_M activity was measured.

Under these conditions we observed ~5-fold activation of transcription from p_M in the *rpoA*$^+$ host (Table 1), which compares favourably with previously reported induction ratios (19,20). However, in strains harbouring the mutant *rpoA* alleles, CI-dependent activation of p_M was only 45–60% as efficient as in the wild-type strain, with the C269A substitution causing the most profound effect (Table 1). By way of comparison, the p_M activity in the strain harbouring the *rpoA341* allele, encoding the K271E substitution in α (39,40), was ~55% as efficient as in the wild-type strain (Table 1). These results confirm the important roles played by the 261 and 287 determinants and the DNA-binding region of αCTD in CI-dependent activation at p_M.

λ Prophage stability. As maintenance of a λ prophage only requires CI function, we investigated whether substitutions within αCTD which impair CI-dependent activation of the p_M promoter also impair λ prophage maintenance. To do this, we compared the efficiency of spontaneous induction of a λ*cI857S7* prophage in hosts harbouring wild-type or mutant *rpoA* alleles on the chromosome. As expected, we found that alanine substitution at positions 261, 269, 271 and 287 in α resulted in

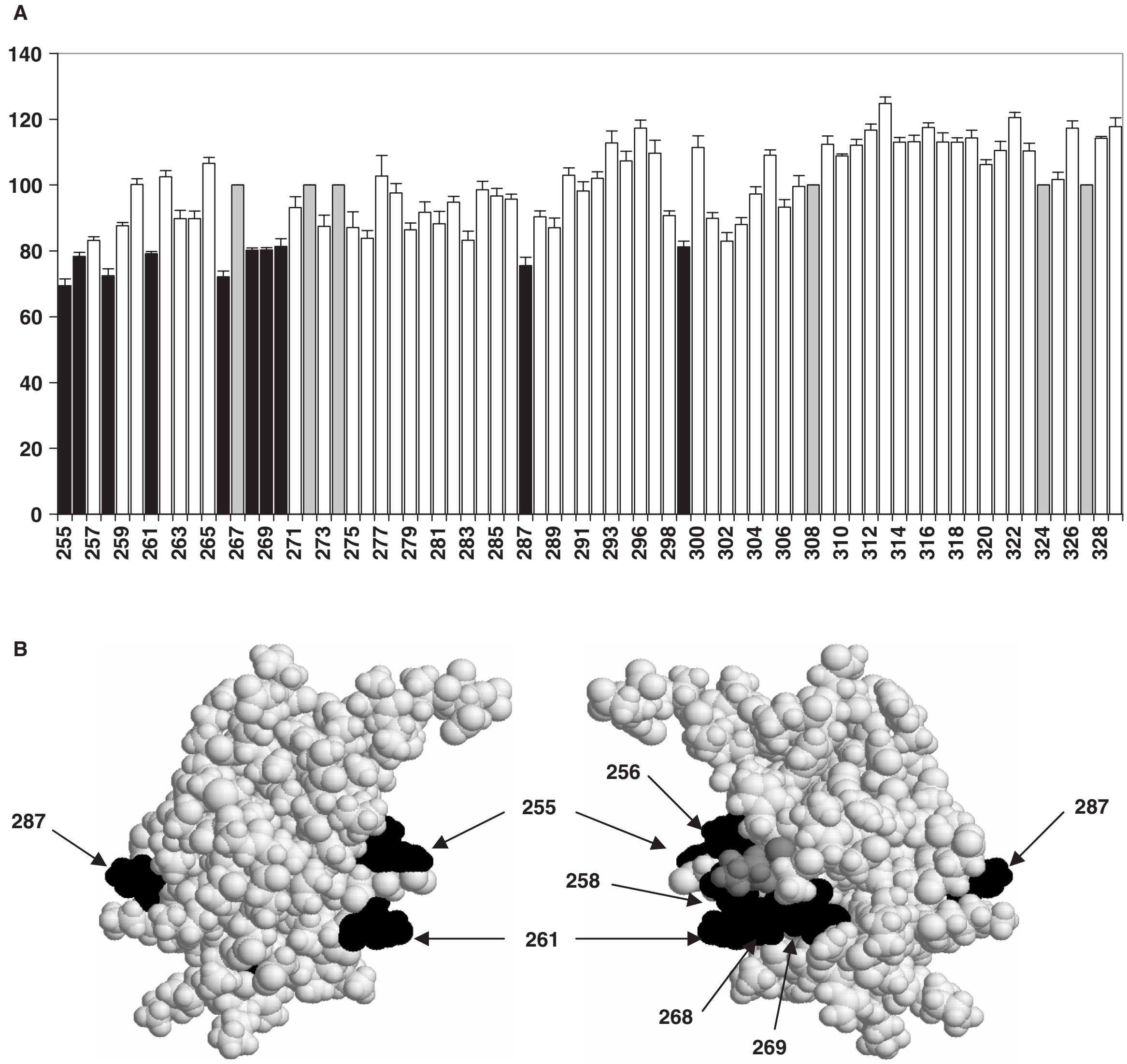

Figure 1. Identification of αCTD residues important for CI-dependent activation of p_M *in vivo*. (**A**) Strain WAM106, containing plasmids pTJSpM, pJMH1 and pACλcI, was transformed with each of a set of plasmids encoding the RNAP α subunit in which each residue of αCTD was changed individually to alanine. Cultures were grown at 37°C to OD≈0.2 in LB medium containing appropriate antibiotics, at which time IPTG was added to a final concentration of 0.1 mM. After 60 min induction of α and CI synthesis, the β-galactosidase activities were determined. The activities are presented relative to the activity of the strain harbouring plasmid pLAW2 encoding wild-type α (100% = 2300 Miller units) and are averages of at least three independent experiments. Grey bars indicate positions when alanine occurs naturally. Black bars correspond to the residues in which alanine substitution causes a decrease in activity of ≥20% compared to wild-type α. (**B**) Structure of αCTD, showing in black the residues that are important for CI-dependent activation of p_M. Residue K271 is highlighted in grey for reference.

a higher frequency of spontaneous induction of the λ prophage relative to the wild-type host (3–8-fold increase, depending on the position of the substitution) (Table 1). Consistent with the p_M promoter activity measurements, the prophage was most unstable in the host carrying the *rpoA269* allele. As shown previously, we measured a 5-fold increase in spontaneous induction of λ prophages in the *rpoA341* mutant relative to the wild-type (Table 1; 40). In support of the hypothesis that decreased prophage stability was due to decreased CI levels, overexpression of the *cI* gene from plasmid pACλcI resulted in equally efficient maintenance of the prophage in the wild-type and in all tested mutant strains (data not shown).

Location of the αCTD–DNA interactions at the p_M promoter

To determine the location of αCTD at the p_M promoter we exploited the DNA cleavage reagent, iron [S]-[*p*-bromoacetamidobenzyl] ethylenediaminetetraacetate (Fe·BABE), that can be attached to cysteine residues introduced at specific locations within αCTD (59,64,65). Thus, we derivatized αCTD with Fe·BABE by employing a functional α subunit in which cysteine was introduced at position 273, and used the derivatized product to reconstitute RNAP (53,59).

Analysis of DNA scission products following formation of the RNAP–Fe·BABE–p_M complex revealed that,

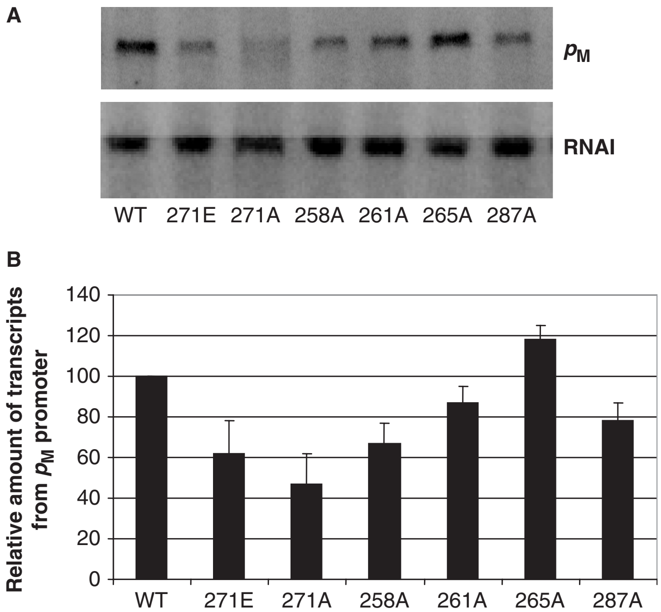

Figure 2. Identification of αCTD residues important for activation of p_M by CI *in vitro*. (**A**) The efficiency of transcription from p_M in the presence of reconstituted mutant RNAPs is shown in a typical transcription gel. Single-round *in vitro* transcription experiments were performed using linear template DNA containing p_M or specifying RNA-I, together with CI and RNAP reconstituted with hexahistidine-tagged α derivatives containing alanine substitutions at the positions indicated. The activities of purified RNAPs were normalized at the RNA-I promoter. The p_M-dserived *cI* run-off transcript is 141 nt in length and RNA-I is 108 nt. (**B**) The efficiency of CI-dependent transcription from p_M in the presence of each reconstituted mutant RNAP. The results are from three independent experiments. Values (with standard deviation) are expressed as percentages of the transcript yield obtained with wild-type RNAP.

Table 1. Effect of different chromosomal *rpoA* alleles on CI-dependent activation of p_M and on λ prophage stability

Chromosomal *rpoA* allele (α subunit)	Activation of p_M by CI[a]	Relative frequency of prophage induction[b]
rpoA$^+$ (α wild-type)	4.9	1
rpoA341 (α K271E)	2.7	4.9
rpoA271 (α K271A)	2.7	4.7
rpoA261 (α E261A)	2.9	2.7
rpoA269 (α C269A)	2.2	7.9
rpoA287 (α V287A)	3.0	5.5

[a]β-galactosidase activities were measured in cells harbouring pAHA1 and pGW857 at 43°C (basal p_M activity) and 1 h after IPTG addition (to 0.05 mM) and simultaneous shift to 30°C (CI-stimulated p_M activity), and calculated per single copy of pAHA1 per cell. The values presented in the table represent the induction ratios and were calculated by dividing the value for the stimulated p_M activity by the value for the basal activity. [b]Frequency of spontaneous induction of λ*cI857S7* prophage was estimated. Value = 1 corresponds to a yield of 1.25×10^{-5} phages (PFU) per cell. Presented values in both columns are mean results of three experiments. In all cases the standard deviation was below 15%.

in the absence of CI, cleavages occur in clusters separated by 10–11 bp, with the strongest signals occurring near position −44 relative to the transcription start site (Figure 3). This is consistent with the fact that p_M serves as a weak promoter in the absence of CI (66). The pattern of cleavages is similar to that found at other promoters that are active in the absence of transcription activators, such as *rrnB* P1 or *CC(−61.5)-p12T* (59), and suggests that one of the two α subunits binds to the first available minor groove upstream of the −35 region while the second αCTD binds to successive minor grooves (i.e. −54, −65 and −75, with −54 being the most favoured position) (Figure 3). This is in accordance with previously published results, which suggested that the α subunit contacts sequences upstream of p_M in a sequence non-specific manner (67). In the presence of CI, the strongest signals were observed near position −54, which is located in the minor groove between two CI dimers bound to major grooves within O_R2 (−34 to −50) and O_R1 (−58 to −74) (68) (Figure 3). Therefore, binding of CI results in re-positioning of αCTD at the p_M promoter.

DISCUSSION

The location of the stimulatory CI-binding site (O_R2) at p_M (see Figure 3B) suggests that CI activates this promoter by a Class II-type mechanism (22,24,69). Consistent with this, it has been shown that a negatively charged patch on the surface of the CI DNA-binding domain, located in helix 1 of the HTH motif, stimulates transcription from p_M through making contact with a positively charged patch on σ_4 (23). In this report, we have demonstrated that determinants on αCTD also contribute to CI-dependent activation of p_M. Alanine scanning analysis indicated that some of the surface-exposed residues on αCTD which are required for efficient CI-dependent activation are located within or near the previously identified 261 determinant (i.e. R255, P256, D258, E261 and K271) and the 287 determinant (V287). These determinants are located on opposite sides of αCTD and have been shown to play roles in activator-dependent transcription at other promoters. It is intriguing that the 261 determinant is implicated in CI-dependent activation, as it has previously been shown to play a role only at Class I CRP-dependent promoters and at some UP element-dependent promoters, where it interacts with σ_4 (34–36). At other Class II promoters, where αCTD is not in a position to interact with σ^{70}, the 261 determinant does not play a role in transcription activation (37). Our results with Fe·BABE-derivatized RNAP show that, in the presence of CI, αCTD is located close to position −54 at p_M, i.e. between O_R1 and O_R2, and therefore is also not in a position to contact σ. Therefore, the simplest explanation for our observations is that the 261 determinant is involved in contacts with CI.

The 287 determinant has been shown to interact with CRP at Class I and Class II CRP-dependent promoters and there is evidence that it interacts with MelR at the *pmelAB* promoter (34,37,38). Our results suggest that CI is another activator that utilizes this determinant. The involvement of residues on opposite sides of αCTD in CI-dependent activation could occur if αCTD is sandwiched between the two CI dimers, as demonstrated by the Fe·BABE analysis, with each determinant

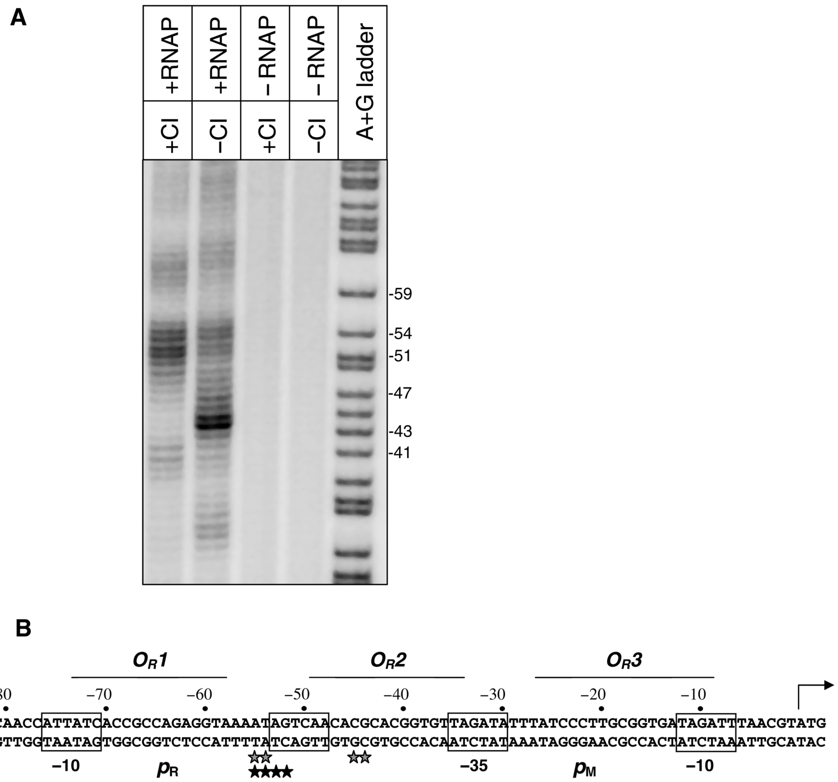

Figure 3. Location of αCTD–DNA interactions at the p_M promoter. (**A**) An autoradiogram of a polyacrylamide sequencing gel showing DNA cleavage resulting from attack triggered by RNAP reconstituted with Fe·BABE-derivatized α subunits. p_M promoter DNA was end-labelled on the template strand and incubated with or without CI and RNAP reconstituted with α derivatized with Fe·BABE at position 273. (**B**) DNA sequence of the divergently arranged p_M and p_R promoters, showing Fe·BABE-induced cleavage positions in the presence or absence of CI protein indicated by black or grey stars, respectively. Transcription startpoints (+1 position) are indicated by bent arrows. The −35 and −10 hexamer sequences of the p_M and p_R promoters are shown in boxes. Base pair co-ordinates in A and B are numbered with respect to the p_M transcription startpoint.

interacting with a different CI dimer. This is analogous to the situation at the artificial Class II promoter, *ML(−74.5)*, which contains tandem CRP sites centred at −41.5 and −74.5. At *ML(−74.5)*, one αCTD is recruited to the DNA between the two CRP-binding sites, whereas the other αCTD binds to DNA upstream of the CRP dimer bound at −74.5 (70). Furthermore, the 261 and 287 determinants of the αCTD sandwiched between the CRP dimers are likely to be aligned along the axis of the DNA, with the 287 determinant interacting with AR1 of the promoter-proximal CRP, as shown for the simple Class II CRP-dependent promoter *CC(−41.5)* (37,59). Although the location of the second αCTD at p_M was not addressed in this investigation, one intriguing possibility is that, in a situation where O_L (the CI operator overlapping the p_L promoter) is also present, the second αCTD binds O_L between the pair of CI dimers bound to the O_L1 and O_L2 sites.

Our results also revealed that alanine substitution of amino acids S266, N268, C269, L270 and S299 impaired CI-dependent activation. These residues are located within or near the DNA-binding surface of αCTD (33,71) (although L270 does not participate directly in DNA binding, the side chain is buried within the structure of αCTD and therefore substitution by alanine may cause a conformational change in the DNA-binding region). The DNA-binding determinant plays a role in UP element-dependent transcription initiation and at many activator-dependent promoters (24,30,34,37,53). Its involvement in CI-dependent transcription activation suggests that an interaction between αCTD and the promoter is important for CI-dependent activation. The results of the Fe.BABE analysis suggest that the important αCTD–DNA interaction is likely to be due to the αCTD positioned near −54. It is noteworthy that the side chain of R265, which plays an important role in DNA binding at many promoters, does not appear to be required for efficient CI-dependent activation. However, it has been shown previously that the contribution of this residue to DNA binding at some activator-dependent promoters is minimal (34). On the other hand, the broader Fe.BABE cleavage pattern that occurs at −54 in the presence of bound CI, in comparison to the more focussed cleavage at −44 in the absence of CI, may indicate that αCTD is not in intimate contact with the DNA when CI is present (i.e. αCTD may be interacting with CI 'off the DNA') or that the interaction of the DNA-binding determinant with the promoter is different to that which occurs at many other promoters. One possible reason for this is that, for steric reasons, αCTD may not be able to readily access the −54 region on the same side of the DNA as CI (Figure 4). Firstly, the diameter of αCTD (measured from the 261

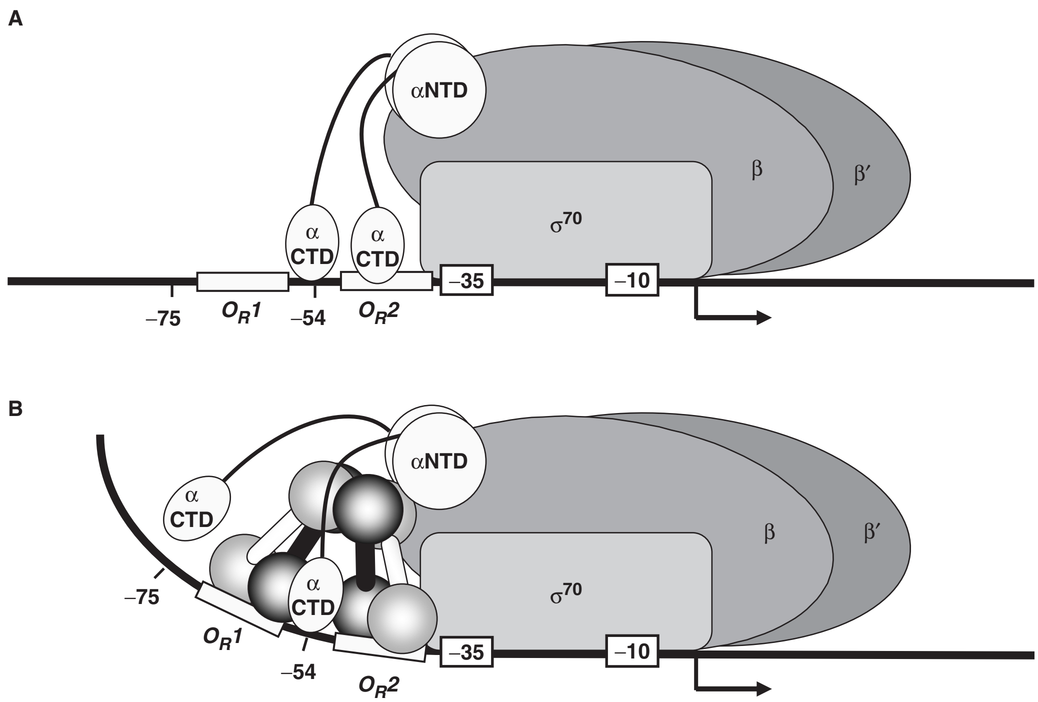

Figure 4. Model for activation of the λ p_M promoter by CI. (A) Proposed location of αCTD at p_M in the absence of CI based on Fe·BABE analysis. The most efficient cleavages induced by Fe.BABE tethered to αCTD occur around −44, with slightly less efficient cutting at −54. Thus, the promoter-proximal αCTD is 'parked' at O_R2. (B). Proposed location of αCTD at p_M in the presence of CI based on Fe·BABE analysis. In the presence of CI, αCTD bound at O_R2 is displaced to the −54 region, between O_R1 and O_R2, and is possibly sandwiched by the DNA-binding domains of the two CI dimers. Note that although αCTD is shown as contacting the DNA at −54 in the presence of CI, it is also possible that there is no direct contact between αCTD and this region of the DNA. The location of the other αCTD was not determined in this analysis.

determinant to the 287 determinant) is ~25 Å. Although the distance between the two operators, O_R1 and O_R2, is ~24 Å (based on a rise of a 3.4 Å per bp), the separation between the two CI dimers is likely to be less than this. This is due to the fact that the adenine tract between the two operator sites contains a static bend of the order of 18°, which becomes further bent by 15–18° upon binding CI, in a large part due to untwisting of the DNA (13,72–74). Access to the DNA between O_R1 and O_R2 may be further restricted by the cooperative interactions which occur between the C-terminal oligomerization domains of CI (12,13,73).

The other important observation from this investigation is that the location of αCTD at p_M is different in the presence and absence of CI. In the absence of CI, one αCTD is located adjacent to σ^{70} at a site that overlaps O_R2. In the presence of CI, O_R2 is occupied by CI and αCTD is relocated to a DNA site located between O_R1 and O_R2 (Figure 4). This observation, together with the analysis of α mutants, is consistent with a model in which activation of RNAP at p_M is mainly the result of the interaction between CI bound at O_R2 and σ^{70}, as previously proposed (19–22). The role of the αCTD–CI interaction may be to stabilize the interaction of αCTD with DNA upstream of O_R2, facilitating CI-dependent stimulation of the k_f step.

CI is not the only Class II transcription activator to make contact with αCTD in addition to σ_4. Both MelR and CRP (at the *galP1* promoter) also make a specific contact with αCTD, and this interaction contributes to the overall stimulatory activity of the regulatory protein (24,38,75,76). Other examples of so-called 'ambidextrous' activators include LuxR and the phage Mu Mor protein (76–79). In such cases, αCTD binds to the first available minor groove upstream of the activator binding site, with a preference for binding to the same face of the DNA as RNAP (38,53). In the case of p_M, the first available minor groove is located between the two CI dimers bound at O_R1 and O_R2.

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