Published online February 3, 2004

834–841 *Nucleic Acids Research, 2004, Vol. 32, No. 2*
DOI: 10.1093/nar/gkh230

Role of the RNA polymerase α subunits in CII-dependent activation of the bacteriophage λ p_E promoter: identification of important residues and positioning of the α C-terminal domains

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Received October 9, 2003; Revised November 28, 2003; Accepted December 22, 2003

ABSTRACT

The bacteriophage λ CII protein stimulates the activity of three phage promoters, p_E, p_I and p_{aQ}, upon binding to a site overlapping the –35 element at each promoter. Here we used preparations of RNA polymerase carrying a DNA cleavage reagent attached to specific residues in the C-terminal domain of the RNA polymerase α subunit (αCTD) to demonstrate that one αCTD binds near position –41 at p_E, whilst the other αCTD binds further upstream. The αCTD bound near position –41 is oriented such that its 261 determinant is in close proximity to σ^{70}. The location of αCTD in CII-dependent complexes at the p_E promoter is very similar to that found at many activator-independent promoters, and represents an alternative configuration for αCTD at promoters where activators bind sites overlapping the –35 region. We also used an *in vivo* alanine scan analysis to show that the DNA-binding determinant of αCTD is involved in stimulation of the p_E promoter by CII, and this was confirmed by *in vitro* transcription assays. We also show that whereas the K271E substitution in αCTD results in a drastic decrease in CII-dependent activation of p_E, the p_I and p_{aQ} promoters are less sensitive to this substitution, suggesting that the role of αCTD at the three lysogenic promoters may be different.

INTRODUCTION

The temperate bacteriophage, λ, is one of the simplest model organisms for the study of developmental regulation. The decision between lytic and lysogenic growth is based on the activity of two phage-encoded transcriptional activators, CI and CII, both of which are required for lysogenization. Establishment of lysogeny depends on the CII-dependent p_E promoter (also known as p_{RE}) that directs the expression of *cI* necessary for maintenance of the lysogenic state. CII also stimulates p_I, which directs expression of the λ *int* gene, and p_{aQ}, which directs synthesis of an antisense mRNA that regulates late gene expression [for a review, see Echols (1)].

The mechanism by which CII activates transcription is of special interest. The CII homotetramer regulates all three lysogenic promoters co-ordinately by binding to a tetrad repeat (TTGC) flanking the –35 element, and it was the first regulatory protein suggested to recognize direct repeats [for a review, see Ho *et al*. (2)]. The location of the CII-binding site at the λ lysogenic promoters is consistent with the idea that it is a class II activator (3). Class II activators bind to target sites that overlap the promoter –35 region and, in most cases, make contact with domain 4 of the RNA polymerase (RNAP) σ subunit, that is bound to the –35 element (4). However, CII binds on the opposite face of the DNA helix to RNAP, and thus, target promoter –35 elements are sandwiched between σ and the activator. Binding of CII to the direct repeats appears to distort the intervening –35 hexamer in some way, although it remains unclear whether this distortion facilitates recognition of the –35 region by σ^{70} or whether CII makes direct contact with σ (2).

At many bacterial promoters, the C-terminal domain of the α subunit (αCTD) interacts with upstream promoter DNA, the RNAP σ subunit and/or transcription activator proteins (5,6). These interactions are mediated by determinants on the surface of αCTD. For example, residue 265 and neigbouring residues contribute to the αCTD 265 determinant, which is responsible for interactions with DNA (7–10). Similarly, residue 261 and neighbouring residues contribute to the 261 determinant, that can contact σ (11–13). Previous experiments demonstrated that deletion of αCTD greatly reduces CII-dependent activation of p_E (14). We have shown that the *rpoA341* mutation, specifying the K271E substitution in αCTD, blocks lysogenization of *Escherichia coli* by phage λ (15). Analysis of reporter gene fusions revealed that this

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substitution abolishes activation of p_E by CII (16). At many class II or class II-like promoters, both αCTDs bind immediately upstream of the bound activator and, in some cases, one or both of them make interactions with the activator that contribute to activation. However, CII-dependent promoters present an interesting situation, as CII binds to the opposite face of the DNA to RNAP. Here we have carried out a genetic and biochemical analysis of the positioning and role of αCTD at the CII-dependent p_E promoter. Our results show that αCTD contacts the DNA immediately upstream of the –35 region at p_E, on the opposite face of the DNA helix to CII, and that these contacts are important for CII-dependent activation.

MATERIALS AND METHODS

Bacterial strains, plasmids and gene fusions

The *rpoA*$^+$ and *rpoA341* strains, WAM106 and WAM105, respectively, have been previously described (17). Derivatives of these strains containing single copy p_E–*lacZ*, p_I–*lacZ* and p_{aQ}–*lacZ* fusions were constructed by lysogenization with λ299 derivatives containing the respective fusions. The fusions were constructed according to Giladi *et al*. (18) and have been reported previously (16,19). Plasmid pJMH1 is a pSC101-based replicon carrying the *lacI*^{Q} and kanamycin resistance genes (17). Plasmid pMO23 is a p15A-derived replicon bearing a chloramphenicol resistance gene and the *cII* and *cIII* genes, each under the control of p_{tac} (16). Plasmid pTJSpE was constructed by cloning the EcoRI–DraI fragment of pHG86 (20), containing the p_E–*lacZ* promoter fusion, into the RK2 minireplicon, pTJS42, which harbours a tetracycline resistance gene (21). For the expression of mutant *rpoA* alleles for the alanine scan analysis, derivatives of plasmids pHTf1α, encoding alanine substitutions at position 255–271 and 302 in αCTD (7,22), pREIIα, encoding alanine substitutions at remaining positions in αCTD (7,22–25), or pLAW2phs, encoding the K271E substitution (17), were used. The control plasmid encoding wild-type *rpoA* was pLAW2 (26). All the plasmids which encode α and mutant derivatives thereof specify resistance to ampicillin.

Measurement of β-galactosidase activity.

β-Galactosidase assays were performed on mid-logarithmic phase cultures according to the method of Miller (27). Results presented are averages of at least three independent experiments and are shown with standard deviations.

Protein purification and reconstitution of RNA polymerase.

Plasmid pET-CII (28) was used for over-production of N-terminally His-tagged CII protein, which was purified as described previously (28). For the reconstitution of RNAP, inclusion bodies of RNAP β, β′ and σ subunits from strains XL1-Blue (pMKSe2), BL21(DE3)(pT7β′) and BL21(DE3) (pLHN12σ), respectively, were prepared as described previously (29). His-tagged RNAP α subunits were prepared using plasmid pHTT7f1NHα. Derivatives of pHTT7f1NHα carrying mutant *rpoA* alleles were constructed by replacing the HindIII–BamHI fragment, which encodes wild-type αCTD, with the corresponding fragments from plasmids pHTf1α (258A, 261A, 265A, 271A) (7,22,30) or pLAW2phs (271E) (17). Over-expression of the α subunits in strain BL21(DE3) and purification of α by Ni^{2+}-affinity chromatography and reconstitution into RNAP were performed essentially as described previously (7,29). Purification of α subunits with single cysteine residues at positions 273 and 302, conjugation with iron [*S*]-1-[*p*-bromoacetamidobenzyl] ethylenediamine-tetraacetate (Fe·BABE), and reconstitution into RNAP were performed as described in Lee *et al*. (31).

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 dithiothreitol (DTT), 100 μg/ml bovine serum albumin (BSA) and 150 ng of linear template DNA. Template was prepared by PCR amplification of λ DNA using primers 5′-TGG-CTGATGGTGCGATAGTC-3′ and 5′-ACGTGCGTCCTCA-AGCTG-3′, for 35 cycles of denaturation at 95°C for 30 s, annealing at 53.2°C for 30 s and extension at 72°C for 1 min. The resultant PCR product (1433 bp) was digested by BsuRI to obtain a fragment of 1172 bp, containing the λ p_E and p_{oop} promoters. The binding reaction of CII (40 ng) with the DNA (150 ng) was carried out at 37°C for 10 min, after which RNA polymerase was added and the incubation continued for a further 10 min. 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 of [α-^{32}P]UTP per reaction) and 50 μg heparin/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 (32). 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 p_{oop} promoter, were: 46 nM wild-type RNAP, 34 nM RNAP αK271E, 54 nM RNAP αK271A, 13 nM RNAP D258A, 28 nM RNAP αE261A and 35 nM RNAP αR265A. Transcriptional activities were calculated from at least three independent experiments and are presented as a percentage (with standard deviation) of transcripts obtained with wild-type RNAP.

Fe·BABE-mediated hydroxyl radical footprinting

A 275 bp DNA fragment containing the λ p_E promoter was amplified from bacteriophage λ DNA by PCR using primers 5′-GCGAAGCTTCCACACCTATGGTGTATGC-3′ and 5′-GCCGAATTCCATGTCGTCGTCAACGACCC-3′, cleaved with EcoRI and HindIII restriction enzymes and cloned into the vector pSR (33). A 355 bp AatII–HindIII fragment was purified from the resultant plasmid (pSRpE) and labelled at the HindIII end with either [γ-^{32}P]ATP and T4 polynucleotide kinase (for the template strand) or [α-^{32}P]ATP and *E.coli* DNA polymerase Klenow fragment (for the non-template strand). 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 CII protein (3 μM final concentration) at 37°C for 10 min. After 10 min, RNAP

holoenzyme was added (200 nM final concentration) and incubated at 37°C for 15 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 at least 2 min before being stopped by the addition of thiourea and EDTA to final concentrations of 7 and 45 mM, respectively. DNA was then extracted with phenol/chloroform, precipitated with ethanol and analysed by electrophoresis on a 6% polyacrylamide gel. The gels were calibrated with Maxam–Gilbert G + A ladders and analysed using a PhosphorImager and Quantity One software (Bio-Rad).

RESULTS

Location of αCTD–DNA interactions at the p_E promoter

To determine the location of αCTD at the p_E promoter, we exploited the DNA cleavage reagent Fe·BABE that can be attached to specific locations in RNAP α subunits (34,35). Thus, RNAP was reconstituted with purified α subunits that had been covalently modified with Fe·BABE, CII-dependent open complexes were formed at p_E, and the DNA cleaving ability of Fe·BABE was triggered by the addition of ascorbate and hydrogen peroxide. In these experiments, the Fe·BABE reagent was tethered either to position 273 or to position 302, located on opposite faces of αCTD (Fig. 1A) (31). Figure 1B shows the patterns of DNA cleavage by Fe·BABE-tagged RNAP, revealed by gel electrophoresis and phosphorimager analysis. These cleavages occur in small clusters, separated by 10–11 bp, suggesting that αCTD binds to successive minor grooves on one face of the promoter DNA [see Murakami *et al.* (34,35)]. The strongest DNA cleavage on both strands is found near position –41, and we interpret this as due to the binding of one of the two αCTDs. For both strands, cleavage due to Fe·BABE conjugated to residue 302 occurs 4–5 bp downstream of the sites of cleavage due to Fe·BABE conjugated to residue 273. The locations of the different cleavage sites on the two strands are illustrated in Figure 1C and D. The simplest interpretation of these data, based on the model shown in Figure 1A, is that this αCTD binds to the minor groove near position –41, and is oriented such that the 261 determinant points downstream. The 261 determinant would thus be well placed to interact with region 4 (domain σ_4) of the RNAP σ subunit, in agreement with results from previous authors (11–13). The results in Figure 1B show that αCTD can also bind to promoter DNA near positions –51 and –61. The signals near these positions are weaker than those near position –41, but it is clear that the cuts, at least around the –51 position, are also staggered. Therefore, we suggest that the position of the second αCTD is not fixed, and that the orientation of this αCTD, bound at –51, is the same as that of αCTD bound at –41.

Determinants in αCTD important for CII-dependent activation of p_E *in vivo*

To determine whether the αCTD–DNA interactions at p_E are important for CII-dependent activation, and to identify other amino acid side chains in αCTD that are important for CII-mediated activation, we used an alanine scanning approach.

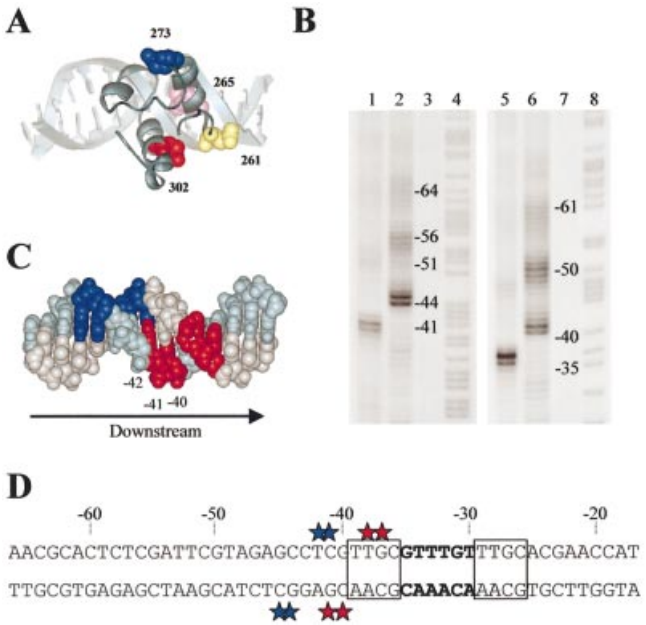

Figure 1. Location and orientation of αCTD at the p_E promoter. (**A**) Model of αCTD bound to DNA to show the relative location of amino acid residues pertinent to this study. The model of αCTD is adapted from Benoff *et al.* (10). R265, located within the DNA-binding determinant, is shown in pink; residue 261, which participates in interactions with σ, is shown in yellow; and residues 273 and 302, which were derivatized with Fe·BABE, are shown in blue and red, respectively. The orientation shows the 261 determinant facing downstream, as occurs at p_E. (**B**) Cleavage of p_E promoter DNA by Fe·BABE-labelled RNAP. Phosphorimager scan of a polyacrylamide sequencing gel showing DNA cleavage at the p_E promoter resulting from attack by RNAP reconstituted with Fe·BABE-derivatized α in the presence of CII. Lanes 1–4 show results for the template strand, and lanes 5–8 show results for the non-template strand. Lanes 1 and 5, RNAP containing Fe·BABE-derivatized E302C α; lanes 2 and 6, RNAP containing Fe·BABE-derivatized E273C α; lanes 3 and 7, no proteins; lanes 4 and 8, Maxam–Gilbert A + G ladder. (**C**) Model of DNA showing the minor groove locations of DNA cleavage at p_e due to RNAP reconstituted with α subunits derivatized with Fe·BABE at position 273 (blue) or 302 (red). The template strand is shown in pale blue and the complementary strand in grey. The arrow indicates the direction of transcription. (**D**) DNA sequence of the p_E promoter upstream region showing sites of Fe·BABE-induced cleavage indicated by coloured stars (colour coded as in A). The –35 region is in bold and the tetrad repeats recognized by CII are boxed.

To do this, we exploited a set of plasmids encoding the RNAP α subunit in which residues 255–329 were each changed individually to alanine. These plasmids were introduced into a host strain carrying the p_E–*lacZ* fusion plasmid (pTJSpE) and plasmids specifying inducible CII function (pMO23 and pJMH1). In addition, the host strain carried the *rpoA341* mutant allele that encodes α subunits with the K271E substitution. Since this substitution greatly reduces expression from p_E, CII-induced *lacZ* expression is low in the absence of plasmid-encoded wild-type α. This provides a simple system to measure the effects of different alanine substitutions in plasmid-encoded αCTD on CII-dependent activation of p_E. In this experiment, the *cII* gene was expressed from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter (on pMO23) and, due to the toxicity of overproduced CII (36), induction was allowed to proceed only for 75 min. In our conditions, sufficient incorporation of plasmid-encoded α into RNAP was achieved to assay the effects of the alanine

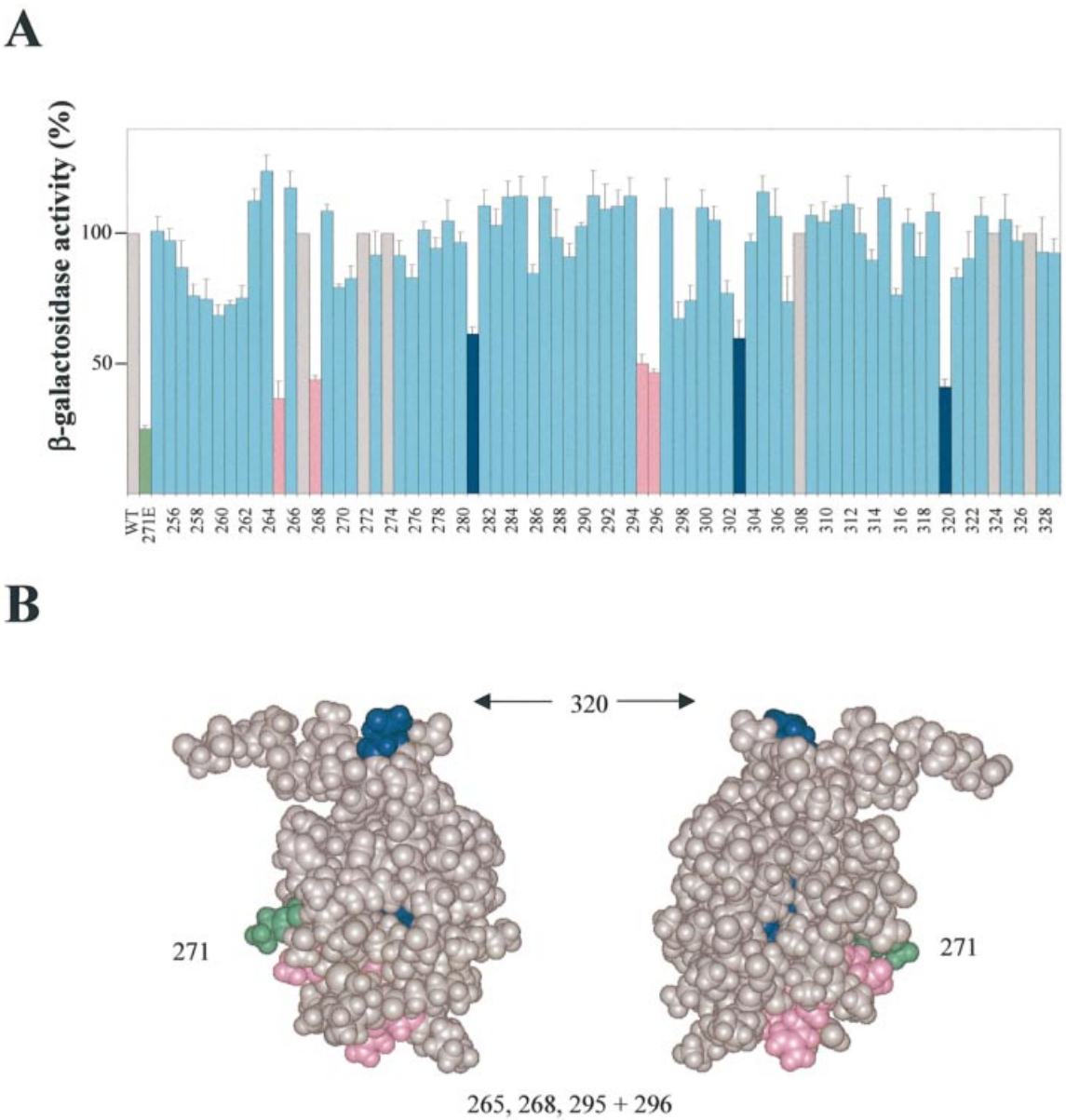

Figure 2. Identification of αCTD residues important for CII-dependent activation of p_E *in vivo*. (**A**) Strain WAM105, containing plasmids pTJSpE, pJMH1 and pMO23, was transformed with one of a set of plasmids encoding the RNAP α subunit in which each residue of αCTD (positions 255–329) was changed individually to alanine. Cultures were grown in LB medium containing appropriate antibiotics at 37°C until OD ~0.2, whereupon IPTG was added to a final concentration of 0.1 mM. After 75 min induction, the β-galactosidase activities were determined. The activities are presented relative to the activity of the strain harbouring pLAW2, encoding wild-type (WT) α (100% = 18 293 Miller units), and are averages of at least three independent experiments. The green bar shows the activity for the strain harbouring pLAW2phs (specifying α K271E). Blue bars and pink bars indicate positions within αCTD at which alanine substitution causes a decrease in activity of ⩾40% compared with wild-type α, with the pink bars corresponding to residues within the DNA-binding determinant (7,8). Grey bars indicate positions where alanine occurs naturally (i.e. same as WT α). (**B**) Structure of αCTD (37), showing amino acid residues that are important for CII-dependent activation of p_E. Residues comprising part of the DNA-binding determinant are shown in magenta; other residues are shown in blue (residues 281 and 303 are buried within the αCTD structure and so are not visible in this representation). Lys271 is also highlighted (green).

substitutions, i.e. plasmid-directed synthesis of wild-type α allowed for a 4- to 5-fold stimulation of p_E activity relative to plasmid-directed synthesis of α containing the K271E substitution (Fig. 2A).

The results show that alanine substitutions at residues 265, 268, 281, 295, 296, 303 and 320 in αCTD most strongly impaired CII-dependent activation of p_E [due to the higher sensitivity of our system in comparison with previous alanine scan experiments with αCTD, which required dominant-negative effects of plasmid-encoded α (11), we regard substitutions that give rise to ⩽60% of the activity afforded by plasmid-encoded wild-type α as exerting strong inhibitory effects on p_E] (Fig. 2A). The location of these residues on the αCTD structure is shown in Figure 2B. Residues 265, 268, 295 and 296 fall within the 265 DNA-binding determinant (7,8), suggesting that DNA binding of

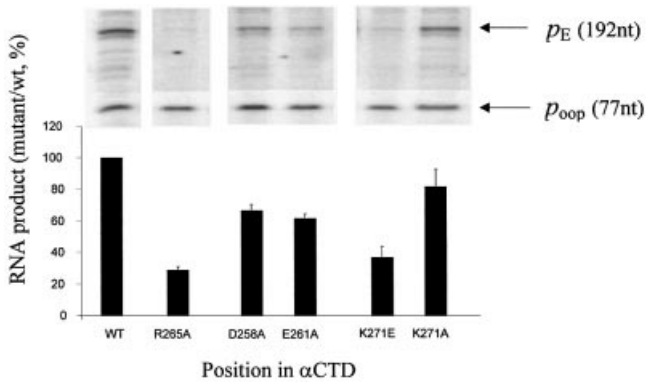

Figure 3. Identification of αCTD residues important for activation of p_E by CII *in vitro*. Single-round *in vitro* transcription experiments were performed using linear template DNA containing p_E and p_{oop}, CII, and RNAP reconstituted with hexahistidine-tagged α derivatives containing alanine substitutions at the positions indicated. The activities of purified RNAPs were normalized at the p_{oop} promoter. The efficiency of transcription from p_E in the presence of each reconstituted mutant RNAP is shown below the corresponding lane from a typical transcription gel. Values (with standard deviation) are expressed as percentages of the transcript yield obtained with wild-type RNAP.

αCTD is important for CII-dependent activation of p_E. Interestingly, in contrast to the *rpoA341*-encoded K271E substitution, substitution of K271 by alanine exerted only a small inhibitory effect on activation of p_E by CII. Concerning the effects of alanine substitutions at residues 281, 303 and 320, these could be due to either direct or indirect effects. Residues 281 and 303 are buried within αCTD and so the latter possibility appears more likely. Regarding residue 320, the side chain is surface exposed but is located some distance from the other important residues (Fig. 2B).

Determinants in αCTD important for CII-dependent activation of p_E *in vitro*

In the next set of experiments, we reconstituted RNAP containing wild-type or mutant α subunits and used run-off transcription assays to measure its activity at p_E. Our primary aim was to quantify the effect of disrupting DNA binding by αCTD, and thus we compared wild-type RNAP with RNAP carrying α subunits harbouring the R265A substitution. Results presented in Figure 3 show that CII-dependent activation of p_E is greatly reduced by the R265A substitution (while transcription from the control *oop* promoter is unaffected). Since our analysis with Fe·BABE suggested that one αCTD was positioned such that it could contact the RNAP σ subunit, we also reconstituted RNAP with α subunits carrying the D258A and E261A substitutions in the 261 determinant, known to be involved in αCTD–σ interactions. Results in Figure 3 show that these preparations of RNAP were also impaired for CII-dependent activation, although to a lesser degree than with the R265A substitution. Finally, we also measured CII-dependent activation at p_E with RNAP containing α subunits with the K271E or K271A substitutions. Consistent with the *in vivo* analysis (Fig. 2A), RNAP reconstituted with K271Eα was severely impaired for CII-mediated activation of p_E, whereas RNAP reconstituted with K271Aα supported efficient activation (Fig. 3).

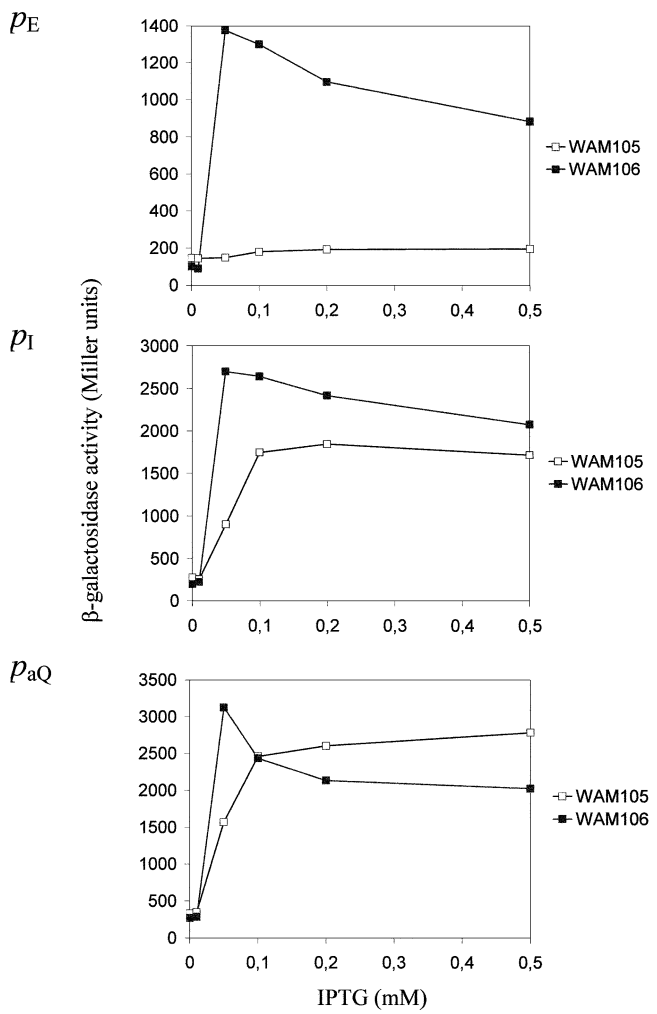

Figure 4. CII-dependent and CII-independent activities of the phage λ p_E (**A**), p_I (**B**) and p_{aQ} (**C**) promoters in *rpoA*$^+$ (WAM106) and *rpoA341* (WAM105) hosts. Each host strain (*rpoA*$^+$ and *rpoA341*), carrying a single copy p_E–*lacZ*, p_I–*lacZ* or p_{aQ}–*lacZ* fusion together with plasmids pMO23 and pJMH1, was grown in LB medium containing chloramphenicol (34 μg/ml) and kanamycin (50 μg/ml) at 37°C to OD ~0.2. IPTG was added to the indicated final concentrations and the activities of β-galactosidase (in Miller units) were measured 60 min later.

Effect of the *rpoA341* mutation on CII-mediated transcription activation at p_I and p_{aQ}

To determine whether the K271Eα substitution affects the other CII-dependent promoters, p_I and p_{aQ}, reporter gene fusions to all three CII-dependent promoters were constructed in single copy using the same genetic system, and promoter activity was measured in the presence and absence of CII. For this experiment, levels of induced CII production were varied by using different concentrations of IPTG. We found that the maximum induced activities of the three lysogenic promoters in the *rpoA*$^+$ background occurred following induction with 0.05 mM IPTG (Fig. 4). With this level of CII synthesis, the degree of activation afforded by CII at each promoter was similar, i.e. 12.5- to 14-fold. Consistent with previous observations, in the *rpoA341* mutant, the activity of the p_E promoter was approximately equal to that observed in the wild-type strain in the absence of CII synthesis, i.e. CII

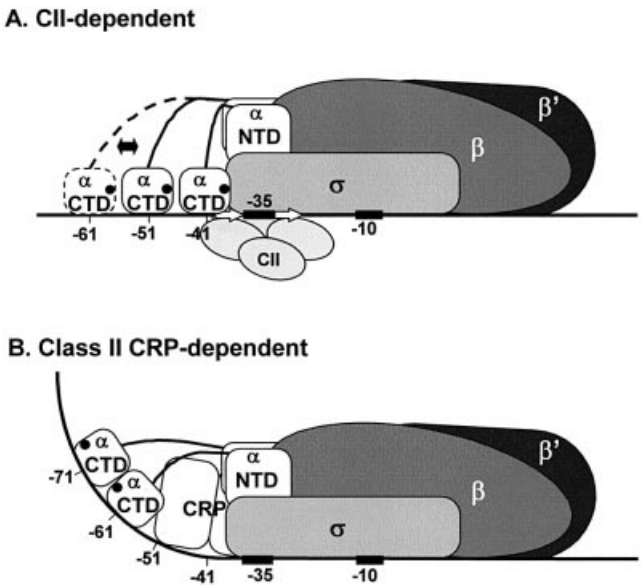

Figure 5. Model for activation of the λ p_E promoter by CII. (**A**) Location and orientation of αCTD at p_E as determined in this work. The approximate location of the 261 determinant in αCTD is shown as a filled circle. (**B**) For comparison, a model for class II CRP-dependent transcription activation is also shown (5).

activation of p_E was negligible (Fig. 4A) (16). The p_I promoter was ~30% as active in the *rpoA341* mutant in comparison with the wild-type strain when CII synthesis was induced by 0.05 mM IPTG. However, this difference became less pronounced with higher levels of CII induction (Fig. 4B). The efficiency of CII-mediated stimulation of p_{aQ} was least affected by the *rpoA341* mutation (Fig. 4C). Only when CII synthesis was induced with 0.05 mM IPTG was there a significant difference between p_{aQ} activity in the wild-type and mutant strains, suggesting that it may be slightly less sensitive to CII in the mutant background. These results suggest differences in the role of αCTD at each of these promoters.

DISCUSSION

Previous biochemical and genetic studies have suggested a role for αCTD in CII-dependent activation of the λ p_E promoter (14,15). Using a DNA cleavage reagent that was tagged to two different locations in αCTD, we deduced the location and orientation of αCTD in RNAP–promoter open complexes at p_E. These experiments showed that one αCTD binds near position –41 and the other αCTD binds further upstream. The αCTD near position –41 must be bound on the opposite face of the DNA helix to CII (Fig. 5A), in accord with previous investigators who showed that CII binds to the opposite face of the DNA helix to σ (38). The results of the Fe·BABE analysis are also consistent with previous DMS protection studies (38) showing that protection occurs at positions –40 and –41 at p_E in the presence of CII and RNAP. Our results show that the αCTD near position –41 is bound with its 261 determinant pointing downstream such that it could interact with region 4 of the RNAP σ subunit bound to the –35 element (Fig. 5A). Our observation that substitutions in the 261 determinant slightly impair CII-dependent activation of p_E is consistent with such an interaction, which may be required for full activation of p_E by CII. In this regard, it is noteworthy that, at certain activator-independent promoters such as *rrnB* P1, where αCTD binds at the same location, functional interactions occur between two residues within the 261 determinant, D259 and E261, and the side chain of R603 in region 4.2 of σ (12). Also, at the class I CRP-dependent *lac* promoter, where αCTD binds to the –42/–43 region, the 261 determinant also interacts with σ and this interaction is required for activation by CRP (10,11,13).

The location of αCTD in CII-dependent open complexes at p_E is very similar to that found at factor-independent promoters such as *rrnB* P1 and *lacUV5* (39–41). This contrasts sharply with the situation at most class II activator-dependent promoters, where the activator is positioned on the same side of the DNA helix as σ, and αCTD is displaced to a site upstream of the bound activator (Fig. 5B). An exception is found at the *Bordetella pertussis fha* promoter, where three dimers of the activator, BvgA, occupy a region extending from positions –35 to –100 on the same face of the DNA helix as σ. At this promoter, αCTD binds to the same segment of DNA as BvgA, but to a different face of the DNA helix (42). One common pattern which is emerging from studies of class II or class II-like promoters is that, at this class of promoter, αCTD appears to bind to the nearest 'vacant' segment of upstream DNA to the promoter, with a preference for binding to the same face of the DNA as RNAP (43).

The alanine scanning analysis identified four residues in the αCTD DNA-binding determinant where alanine substitution causes at least a 40% decrease in activation in our assays, suggesting that DNA binding by αCTD is important for CII-dependent activation of p_E. This conclusion was supported by run-off transcription assays *in vitro*. However, the mechanism of activation of the p_E promoter by CII still remains unclear. In particular, it is not apparent whether there is any direct contact between CII and αCTD at this promoter. The alanine scanning analysis showed that substitutions of residues at positions 281, 303 and 320 caused large decreases in activation, but, for reasons discussed above, we think it is unlikely that L281 and I303 contact CII. We are unable to conclude whether N320 is involved in contacts with CII. However, due to its location on αCTD, this residue would not be expected to contact an activator bound to the opposite face of the DNA. Interestingly, although the K271E substitution severely inhibits CII-mediated activation of p_E, alanine substitution at position 271 had very little effect. This suggests that the effect of the K271E substitution might be to create an interaction (or 'clash') which impairs CII function. In the last part of our study, we showed that each of the three CII-dependent λ promoters is affected differently by the K271E substitution. This suggests that, although CII binds to identical sequences at identical locations at these promoters, the mechanisms of transcription activation, particularly with respect to the role of αCTD, are distinct. This is consistent with previous observations that the initial binding and isomerization steps in the transcription initiation pathway are differentially affected by CII at each of these promoters (2,44). Thus, the K_B step is stimulated 10^3- to 10^4-fold at p_I and p_{aQ}, whereas at p_E, which is distinguished by having an 'extended' –10 sequence, K_B is increased by only 15-fold (44–46).

ACKNOWLEDGEMENTS

We are grateful to Wenmao Meng, Tamara Belyaeva and Lars Westblade for their help. This work was supported by the Polish State Committee for Scientific Research (project no. 3 P04A 049 24) and by WT project grants 050794 (to M.S.T. and S.J.W.B.) and 059718 (to S.J.W.B). G.W. also acknowledges financial support from the Foundation for Polish Science (subsidy 14/2000). B.K. acknowledges support from EMBO (scholarship ASTF 9531) and FEBS for visits to the UK.

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