Repression of *deo*P2 in *Escherichia coli* by CytR: conversion of a transcription activator into a repressor

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In the *deo*P2 promoter of *Escherichia coli*, a transcription activator, cAMP–CRP, binds at two sites, centered at –41.5 and –93.5 from the start site of transcription, while a repressor, CytR, binds to a space between the two cAMP–CRP complexes. The mechanisms for the cAMP–CRP-mediated transcription activation and CytR-mediated transcription repression were investigated *in vitro* using purified components. We classified the *deo*P2 promoter as a class II cAMP–CRP-dependent promoter, primarily by the action of cAMP–CRP at the downstream site. Interestingly, we also found that *deo*P2 carries an 'UP-element' immediately upstream of the downstream cAMP–CRP site. The UP-element overlaps with the DNA site for CytR. However, it was observed that CytR functions with the RNA polymerase devoid of the C-terminal domain of the α-subunit as well as with intact RNA polymerase. The mechanism of repression by CytR proposed in this study is that the cAMP–CRP bound at –41.5 undergoes an allosteric change upon direct interaction with CytR such that it no longer maintains a productive interaction with the N-terminal domain of α, but instead acts as a repressor to interfere with RNA polymerase acting on *deo*P2.
Keywords: *in vitro* DNA–protein interaction/prokaryotic transcription initiation/transcription activator/ transcription repressor

Introduction

Bacterial transcription initiation is often modulated by the action of either the transcription activator or repressor. It has been well established that transcription activation involves a direct protein–protein interaction between RNA polymerase (RNP) and transcription activator, which binds to a DNA site in the vicinity of a promoter (Ishihama, 1993; Busby and Ebright, 1997, 1999; Ptashne and Gann, 1997; Hochschild and Dove, 1998). On the other hand, transcription repression is attributed to a mechanism of steric hindrance, in which repressor binding physically interferes with RNP binding. However, several lines of evidence support the notion that a protein–protein contact between repressors and RNP could also lead to transcription repression (Choy *et al*., 1995; Monsalve *et al*., 1996).

Transcription activation at CRP (cAMP-receptor protein)-dependent promoters provides a paradigm for elucidating the mechanism by which an activator makes multiple interactions with RNP to enhance transcription initiation. CRP bears two activating regions (AR1 and AR2) that recognize different determinants within the RNP α-subunit (Figure 1) (reviewed in Busby and Ebright, 1999). This subunit consists of an N-terminal domain (α-NTD) containing determinants for interaction with the remainder of RNP and with CRP, a C-terminal domain (α-CTD) containing determinants for interaction with DNA and with various activators, and an unstructured/ flexible linker connecting the two subdomains (Blatter *et al*., 1994; Negishi *et al*., 1995; Jeon *et al*., 1997; Meng *et al*., 2000). At promoters that have a DNA site (approximately –60) for CRP upstream of the DNA site for RNP (class I CRP-dependent promoters), AR1 of the promoter-proximal subunit of CRP makes direct contact with α-CTD, which results in α-CTD–DNA interaction (Figure 1A) (Bell *et al*., 1990; Eschenlauer and Reznikoff, 1991; Zou *et al*., 1993a,b; Niu *et al*., 1994; Tang *et al*., 1994; Gaal *et al*., 1996; Murakami *et al*., 1996). This protein–protein interaction increases the affinity of RNP for promoter DNA (K_B) and thereby increases transcription initiation (Malan *et al*., 1984; Dove *et al*., 1997; Busby and Ebright, 1999). Consistent with this model, some strong bacterial promoters with intrinsically high K_B, such as rRNA promoters, carry an AT-rich sequence between –40 and –60, termed the 'UP-element', which is recognized and bound by α-CTD in the absence of transcription activator (Ross *et al*., 1993, 1998; Rao *et al*., 1994; Czarniecki *et al*., 1997; Estrem *et al*., 1998; Noel and Reznikoff, 1998; Tagami and Aiba, 1999; Ozoline *et al*., 2000). At class II CRP-dependent promoters (the DNA site for CRP is centered near or at –41, overlapping the –35 region), CRP utilizes both AR1 and AR2 to contact α-CTD and α-NTD, respectively, thereby stimulating both closed promoter complex formation (K_B) and open complex formation (k_i) (Figure 1B) (Bell and Busby, 1990; Williams *et al*., 1991, 1996; West *et al*., 1993; Zhou *et al*., 1994; Niu *et al*., 1996; Savery *et al*., 1998; Busby and Ebright, 1999). AR1 is presented as functional by the promoter-distal subunit, and AR2 is presented as functional by the promoter-proximal CRP subunit. At class II promoters, in contrast to class I promoters, the AR1 interaction does not provide direct activation but appears to overcome an inhibitory effect of α-CTD (Niu *et al*., 1996; Busby and Ebright, 1999). Thus, removal of α-CTD has no negative effect on class II CRP-dependent transcription and eliminates the requirement for a functional AR1 region. Finally, two properly positioned CRP

molecules can work synergistically. At such compound class I/class II CRP-dependent promoters, CRP activates transcription through the set of interactions described for class I and class II CRP-dependent promoters (i.e. three sets of interactions) (Busby and Ebright, 1999).

The *Escherichia coli deo*P2 promoter contains two DNA sites for cAMP–CRP, an upstream site centered at position –93.5 (CRP1) and a downstream site centered at position –41.5 (CRP2), and a DNA site for CytR, centered at position –65 (Figure 1C). Under conditions in which CytR is inactive (i.e. in the presence of cytidine), CRP interacts with RNP to form transcriptionally active CRP–RNP–promoter complexes, whereas under conditions in which CytR is active (in the absence of cytidine), two CRPs interact with CytR to form transcriptionally inactive (CRP)$_2$–CytR–*deo*P2 complexes (Søgaard-Andersen *et al.*, 1990a,b, 1991a,b; Søgaard-Andersen and Valentin-Hansen, 1993; Meibom *et al.*, 1999). The formation of these complexes involves both CytR–DNA interaction and CytR–CRP interaction. The action of CytR *in vivo* is absolutely dependent on the presence of cAMP–CRP, and therefore CRP interacts alternatively, and mutually exclusively, with CytR or RNP. Based on these observations, it has been suggested that CytR and RNP are rivals that compete for binding with CRP to *deo*P2, and that CytR acts as an anti-activator, nullifying the activation by cAMP–CRP (Søgaard-Andersen *et al.*, 1990a,b; Møllegaard *et al.*, 1993; Kristensen *et al.*, 1997; Meibom *et al.*, 2000).

In this paper, we address how the formation of CytR–CRP promoter complexes at *deo*P2 might result in transcription repression. Recent work has shown that only one subunit of the CRP dimer, the subunit proximal to CytR, functionally interacts with CytR in (CRP)$_2$–CytR–promoter complexes (Figure 1C), ruling out the proposal that masking of the activation region of CRP (AR2) is responsible for the transcriptional inactivity of the complexes (Meibom *et al.*, 2000). Therefore two, not mutually exclusive, repression models seem possible: (i) CytR may interfere with interactions between AR1 of CRP and α-CTD of RNP (by occupying the DNA segment adjacent to CRP, preventing access of α-CTD to this DNA segment) (Valentin-Hansen *et al.*, 1996); and (ii) CytR may interfere with interaction between RNP and the core promoter. A prediction of the first model is that the formation of CytR–CRP promoter complexes should not result in repression if transcription is conducted by RNP derivatives lacking α-CTD (Igarashi and Ishihama, 1991). To test this idea, we have investigated CRP and CytR regulation of transcription initiation at *deo*P2 with reconstituted wild-type RNP and RNP derivatives lacking α-CTD.

Results

cAMP–CRP activation of deoP2

We constructed pdeoP2 to examine the regulation of *deo*P2 *in vitro*. This plasmid is a derivative of the well characterized transcription vector pSA508 (Choy and Adhya, 1993), and carries all the *deo*P2 sequence information (from –116 to +25) required for cAMP–CRP activation and CytR repression, followed by a strong factor-independent transcription terminator. When
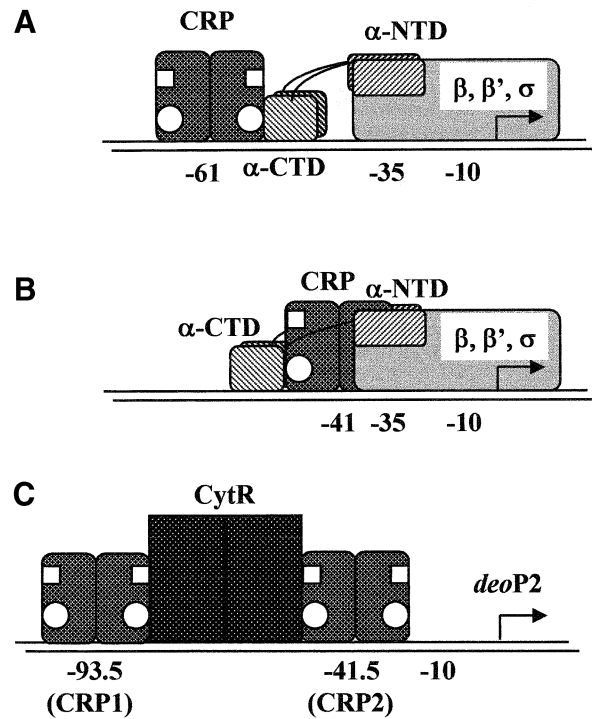

Fig. 1. Schematic model of CRP–RNP interaction at class I (**A**) and class II CRP (**B**) dependent promoters. White circles and squares on CRP represent AR1 and AR2, respectively (see text). (**C**) A schematic model of two CRPs and CytR bound on *deo*P2.

supercoiled pdeoP2 DNA was used as a template in transcription assays, two small transcripts were generated: a 65 nucleotide *deo*P2 transcript and a 108 nucleotide RNA-I transcript originating from the plasmid native RNA-I promoter. The RNA-I transcript served as an internal control for the various transcription assays shown below.

In the first set of experiments, activation of *deo*P2 by cAMP–CRP was examined. Transcription was performed at increasing cAMP concentrations with constant CRP and wild-type RNP using supercoiled pdeoP2 as the DNA template (Figure 2A). Addition of cAMP had a marked effect on *deo*P2 transcription, but no significant effects on transcription initiation at the RNA-I promoter. The lower panel (Figure 2B) shows the quantification of *deo*P2 RNA as a function of cAMP concentration. The cAMP concentration needed for maximal transcription from *deo*P2 was ~100 μM. At this concentration of cAMP, transcription was activated ~4-fold.

In the next set of experiments we examined activation of *deo*P2 transcription using RNP reconstituted from purified subunits. Three α-subunits were employed: α-wild type and two different derivatives lacking αCTD and part of the flexible linker (α-235 and α-256) (Igarashi and Ishihama, 1991). In these experiments, a pSA508 derivative carrying the *lac* promoter (placZP) was included in all reactions. *lac*ZP acted as a control promoter, which is activated by cAMP–CRP only when transcribed by RNP carrying an intact α-subunit (Igarashi and Ishihama, 1991). The results of *in vitro* transcription experiments with the reconstituted RNPs are presented in Figure 3. The transcriptional activity of the reconstituted RNPs appeared to be very
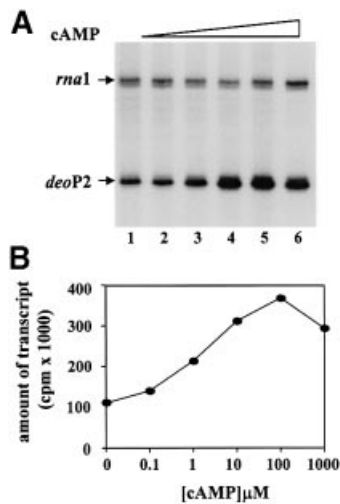
Fig. 2. cAMP was titrated in the presence of 0.1 μM CRP using *deo*P2 DNA template. (**A**) The transcription products were resolved in denaturing 8% polyacrylamide gel (see Materials and methods). The *rna*1 and *deo*P2 RNAs were labeled as such. The cAMP concentration in each lane was as follows: 0, 0.1, 1, 10, 100 and 1000 μM for lanes 1–6. (**B**) *deo*P2 RNAs were quantified with a β-scanner (PhosphorImager; Molecular Dynamics, CA) and the total c.p.m. were plotted as a function of cAMP concentration.

Fig. 3. cAMP–CRP activation of *deo*P2 with RNP carrying intact α (α-WT) (37 nM), α with 256 amino acids (α-256) (150 nM) or with 235 amino acids (α-235) (150 nM). In the same reaction tube was also added 2 nM *lac*Zp DNA. cAMP was present at 0.1 mM in all lanes and CRP (0.1 μM) was present only in the even-numbered lanes.

similar: a constant amount of the RNA-I transcript as well as a constant amount of factor-independent *lac*ZP transcript were generated. As expected, only when transcribed by RNP containing the intact α-subunit (α-WT; Figure 3, lane 2) could the cAMP–CRP activate transcription at *lac*ZP. By contrast, cAMP–CRP stimulated the transcription at *deo*P2 when transcribed by RNP reconstituted with truncated α-subunits as well as intact α-subunits. A similar activation pattern has been described at the *E.coli gal*P1 promoter, the prototype class II cAMP–CRP-dependent promoter (Igarashi and Ishihama, 1991; West *et al.*, 1993; Busby and Ebright, 1999). These results are consistent with the previous reports (Søgaard-Andersen *et al.*, 1990a) that CRP-2 plays no role, or a very small one, in cAMP–CRP activation of transcription at *deo*P2. However, it was also noted that removal of α-CTD from RNP had a pronounced effect on the basal level *deo*P2 promoter strength (Figure 3, lanes 3 and 5). Therefore, the fold activation of *deo*P2 by cAMP–CRP when transcribed by the RNP reconstituted with the truncated α-subunit appeared to be much greater than that obtained with the RNP reconstituted with intact α, although the actual activity is less. This feature of *deo*P2 transcription suggested the possibility that the promoter bears an UP-element within the *deo*P2 promoter, which enhances transcription initiation by wild-type RNP but not by RNP derivatives lacking α-CTD. To clarify this possibility we performed a gel-shift experiment with a DNA fragment carrying an intact *deo*P2 promoter. The results are presented in Figure 4. Addition of purified α-subunit (>273 nM) resulted in a complete retardation of *deo*P2 DNA probe (Figure 4A). When a DNA probe containing a sequence within *deo*C was used, only a partial retardation was observed even at 820 nM α-subunit (Figure 4B). In this experiment, *E.coli mal*T promoter DNA known to carry the UP-element was included as a control (Tagami
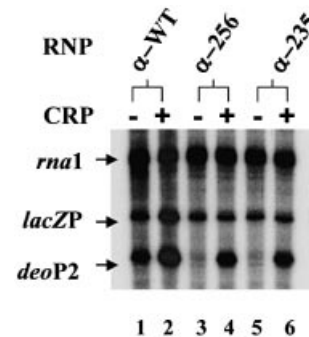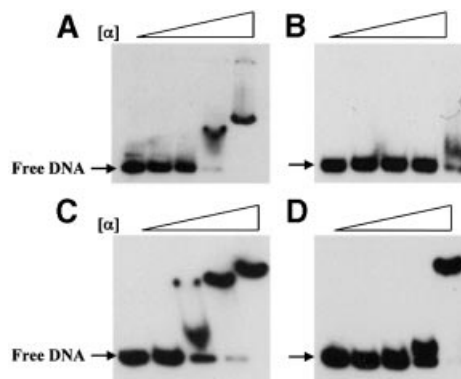
Fig. 4. A mobility shift assay with purified α and a DNA fragment carrying *deo*P2 [–116 to +25; (**A**)], a structural part of *deo*C [+58 to +193; (**B**)], wild-type *mal*T promoter [–121 to +18; (**C**)] and a mutant *mal*T promoter [–121 to +18; (**D**)] (see text for details). The mutant *mal*T promoter carries GCGTCGCGA instead of ATAAAAAAA between –53 and –43 of the wild-type promoter (Tagami and Aiba, 1999). The α concentration in each lane is 0, 30.3, 91, 273.8 and 820 nM from left to right.

and Aiba, 1999). The DNA probe carrying wild-type *mal*T promoter was shifted partially with 91 nM α-subunit and completely with 273 nM α-subunit (Figure 4C). However, a complete retardation of a mutant *mal*T promoter DNA deleted of the UP-element was observed with 820 nM α-subunit (Figure 4D). Taken together, our results show that *deo*P2 bears an UP-element, and that the interaction between this element and α-CTD contributes significantly to the basal and activated level of *deo*P2 activity.

CytR–CRP-mediated repression at deoP2

To assess CytR regulation at *deo*P2 *in vitro*, we first examined transcription with intact RNP in the presence of CytR, CytR and cAMP–CRP, and CytR, cAMP–CRP and cytidine. The results are presented in Figure 5. In the absence of cAMP–CRP, addition of CytR had no effect on *deo*P2 transcription (Figure 5, left panel). Addition of increasing amounts of CytR to transcription assays containing cAMP–CRP gradually decreased transcription (Figure 5, middle panel). This repressive effect of CytR was abolished by the addition of cytidine, an allosteric effector for CytR (Figure 5, right panel). The regulatory patterns were as expected. However, the finding that the
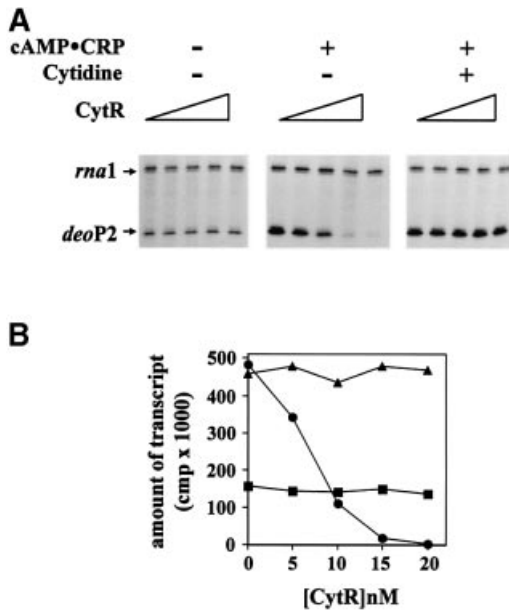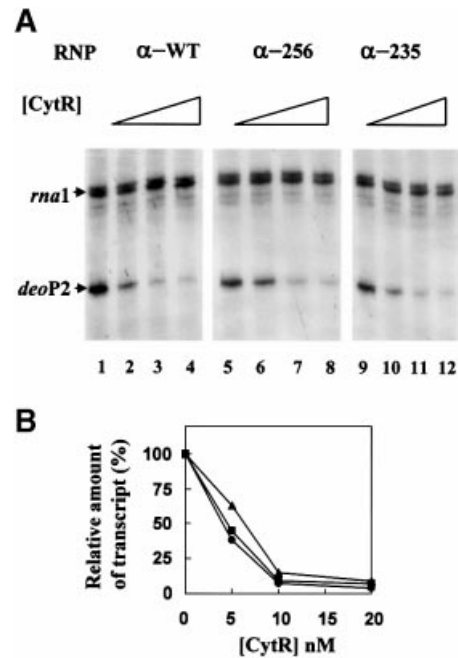
Fig. 5. CytR titration in the absence (left panel) and presence (middle and right panels) of cAMP–CRP. The right panel is in the presence of 20 mM cytidine in addition to cAMP–CRP. The concentration of CytR in each lane for all three panels was as follows: 0, 5, 10, 15 and 20 nM from left to right. (**A**) The transcription products were resolved in denaturing 8% polyacrylamide gel. (**B**) *deo*P2 RNAs were quantified with a β-scanner and the total c.p.m. were plotted as a function of CytR concentration.

Fig. 6. Repression of *deo*P2 by CytR with RNP carrying intact α (α-WT) (37 nM), α with 256 amino acids (α-256) (150 nM) or α with 235 amino acids (α-235) (150 nM). The CytR concentration used in each lane was as follows: 0 for lanes 1, 5 and 9; 5 nM for lanes 2, 6 and 10; 10 nM for lanes 3, 7 and 11; 20 nM for lanes 4, 8 and 12. (**A**) The transcription products were resolved on denaturing 8% polyacrylamide gel. (**B**) *deo*P2 RNAs were quantified with a β-scanner and the total c.p.m. in the presence of CytR relative to that in the absence of CytR (100%) were plotted as a function of CytR concentration.

formation of (CRP)$_2$–CytR–promoter complexes resulted in a complete repression of *deo*P2 transcription (i.e. to a promoter activity that was considerably lower than that in the absence of cAMP–CRP) was unexpected. Thus, it appears that CytR–CRP communication at *deo*P2 also affected the cAMP–CRP-independent transcription.

In an attempt to determine the role of α-CTD in CytR/CRP-mediated repression at *deo*P2, we next investigated transcription by the reconstituted RNP derivatives in the presence of a constant concentration of cAMP–CRP and varying amounts of CytR. The results are presented in Figure 6A, and the quantification of *deo*P2 transcripts is given in Figure 6B. Clearly, the formation of repression complexes at *deo*P2 turned off transcription by the RNP derivatives lacking α-CTD in a fashion quite similar to that seen for intact RNP. This observation is inconsistent with the previously proposed 'anti-activation' model, in which CytR interferes with the interaction between α-CTD of RNP and the DNA site immediately upstream of CRP1 (Valentin-Hansen *et al.*, 1996). Rather, the *in vitro* results shown in Figures 5 and 6 suggest that an important component of the mechanism for CytR-mediated repression should involve interference with interactions between RNP and the core promoter.

Subsequently, we examined the DNase I footprint of cAMP–CRP and CytR bound to *deo*P2. The DNase I footprint was obtained by a primer extension method following DNase I treatment of DNA–protein complexes since supercoiled DNA was used as a template (Figure 7). Figure 7A shows cAMP–CRP footprints where CRP was present at 0.1 μM (lane 2) or 1 μM (lane 3) in the presence of a saturating concentration of cAMP (0.1 mM). With CRP at 0.1 μM, the cAMP–CRP footprint was barely

detectable only at the upstream site (centered at –93) with a hyper-reactive T (–99, vertical arrows), even though the activation by cAMP–CRP, as determined by transcription assay (see Figure 2), was maximal at this concentration. cAMP–CRP footprint at the downstream site (centered at –41) was detected only in the presence of 1 μM CRP (Figure 7A, lane 3). Figure 7B shows the cAMP–CRP footprint at 0.1 μM CRP and 0.1 mM cAMP in the absence (lane 2) or presence of CytR (20 nM) (lane 3). CytR binding shows a marked disappearance of hyper-reactive A (–65, open triangle) together with the bands extended from the downstream CRP site to the upstream CRP site. The cAMP–CRP-specific hyper-reactive T at –99 was enhanced further. Taken together, the CytR (20 nM) binding significantly enhanced the cAMP–CRP binding, particularly at the downstream site, suggesting that a protein–protein interaction between two proteins resulted in increased cAMP–CRP binding to the DNA sites.

Discussion

Activation of deoP2

We propose here that *deo*P2 should be categorized in the class II CRP-dependent promoters, based on the observations that (i) a deletion of upstream CRP site (centered at –93.5) did not affect the cAMP–CRP activation of *deo*P2 *in vivo* (Søgaard-Andersen *et al.*, 1990a) and (ii) cAMP–CRP activated *deo*P2 *in vitro* with RNP reconstituted either with truncated α at CTD or intact α equally well (Figures 2 and 3). Transcription activation at class II
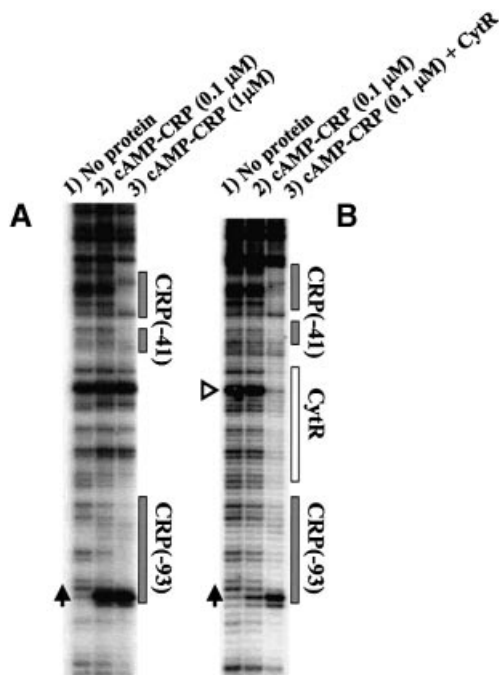
Fig. 7. cAMP–CRP binding to *deo*P2 DNA as probed with DNase I. The DNase I footprint was revealed by a primer extension method since a supercoiled DNA template (pdeoP2) was used (see Materials and methods). (**A**) Different concentrations of CRP were reacted with *deo*P2 in the presence of 0.1 mM cAMP. (**B**) cAMP (0.1 mM) and CRP (0.1 µM) were reacted with pdeoP2 in the presence or absence of CytR (15 nM). The cAMP–CRP binding site is shown as a shaded box and CytR as an open box. Vertical arrows show the hyper-reactive T at –99, and the open triangle in (B) a hyper-reactive A (–65) in the middle of the CytR binding site (see text). The *deo*P2 promoter sequence (–105 to –25) is 5′-AATTAT<u>TTGAACCAGATCGCA</u>TTA-CAGTGATGCAACTTGTAAGTAGATTTCCTTAAT<u>TGTGATGTG-TATCGAA</u>GTGTGTTG-3′, in which the two cAMP–CRP sites are underlined.

CRP-dependent promoters has two components: a first component involving anti-inhibition and a second component involving direct activation. Anti-inhibition is through an interaction between the AR1 of CRP and α-CTD, which overcomes an inhibitory effect of α-CTD (Figure 1); thereby, the CRP activation of class II CRP-dependent promoter is unaltered by the mutant RNP devoid of α-CTD (Attey *et al.*, 1994; Zhou *et al.*, 1994; Belyaeva *et al.*, 1996). Direct activation is through an interaction of α-NTD with AR2 of cAMP–CRP (see Figure 1B) (Attey, 1994; Belyaeva *et al.*, 1996; Murakami *et al.*, 1997; Rhodius *et al.*, 1997; Savery, 1998; Busby and Ebright, 1999). Therefore, in the case of *deo*P2, the cAMP–CRP at –41.5 should be primarily responsible for the activation. It has been shown that at some artificial CRP-dependent promoters, cAMP–CRP centered near position –103, –93 or –83 could synergistically activate transcription with a cAMP–CRP centered near position –42, a class III CRP-dependent promoter (Belyaeva *et al.*, 1996, 1998; Murakami *et al.*, 1997; Busby and Ebright, 1999). However, the observation with *deo*P2 suggests that the synergistic activation by two or more cAMP–CRP should depend on the context of the promoter.

At least three lines of evidence support the view that there is an UP-element in *deo*P2, presumably between two cAMP–CRP sites: (i) a gel mobility shift assay showed purified α binding to *deo*P2 promoter DNA; (ii) the basal level *deo*P2 promoter activity is significantly impaired with the RNP carrying truncated α at CTD; (iii) there is AT-rich sequence (25/36) immediately upstream of the CRP1 site centered at –41.5 (Figures 3, 4 and 6). Previously, DNase I and uranyl footprinting of RNP complex on *deo*P2 revealed that there was a contact at this site in addition to those in the core promoter region (Møllegaard *et al.*, 1993), which is presumably ascribed to the interaction between α-CTD and the UP-element. Moreover, transcription activation is sensitive to replace-ments in the A/T-rich sequence present upstream of the CRP1 site (Kristensen *et al.*, 1997). Thus, all available data are consistent with the proposal that *deo*P2 is a class II CRP-dependent promoter which is equipped with a supplementary promoter element. It has been proposed that transcription activation of class II CRP-dependent promoters is established by positioning α-CTD to the DNA site immediately upstream of the cAMP–CRP site in addition to direct activation (Attey *et al.*, 1994; Belyaeva *et al.*, 1996, 1998; Niu *et al.*, 1996; Busby and Ebright, 1999). This DNA sequence-independent interaction between the DNA adjacent to cAMP–CRP and α-CTD of RNP has been implicated as playing a role in class II cAMP–CRP-dependent activation (Savery *et al.*, 1995, 1998; Lloyd *et al.*, 1998). In the case of *deo*P2, however, there is an UP-element having a natural affinity for α-CTD. It has been shown that placement of an UP-element (from *rrn*B P1) immediately upstream in the *E.coli mel*R promoter, a class II CRP-dependent promoter, increased the promoter activity without altering its dependence on CRP (Savery *et al.*, 1995). Therefore, the contributions of α-CTD–UP-element interactions and α-CTD–CRP interactions to promoter activity were sug-gested to be independent and additive. Nevertheless, in the case of *deo*P2, if α-CTD–UP-element interactions and α-CTD–CRP interactions both recruit α-CTD to the DNA site, the activation system appears to be redundant. It is possible, however, that the above two interactions, although both with α-CTD, may affect different steps in transcription initiation. It should be noted that there is an UP-element binding protein in *E.coli*, methylated Ada protein, which binds to an UP-like element sequence in *ada* and *aid*B promoters, and stimulates transcription by modifying the nature of the RNP–promoter interaction (Landini and Volkert, 1995).

Repression of deoP2

Repression of *deo*P2 by CytR was only observed in the presence of cAMP–CRP, in agreement with the *in vivo* observation that CytR binding requires cAMP–CRP (Søgaard-Andersen *et al.*, 1990a,b). It was therefore proposed that CytR acts as an anti-activator, especially since CytR binding centered at around –65 is too far from the promoter to interfere with RNP directly (Søgaard Andersen *et al.*, 1990a,b; Møllegaard *et al.*, 1993; Valentin-Hansen *et al.*, 1996; Kristensen *et al.*, 1997; Meibom *et al.*, 2000). In this model, CytR–CRP and CytR–DNA interactions block transcription activation by CRP, by sterically blocking the functional AR1 of CRP and by sterically preventing α-CTD from interacting with the DNA segment immediately upstream of the CRP dimer at the CRP1 site. We observed in this study that CytR

A

B
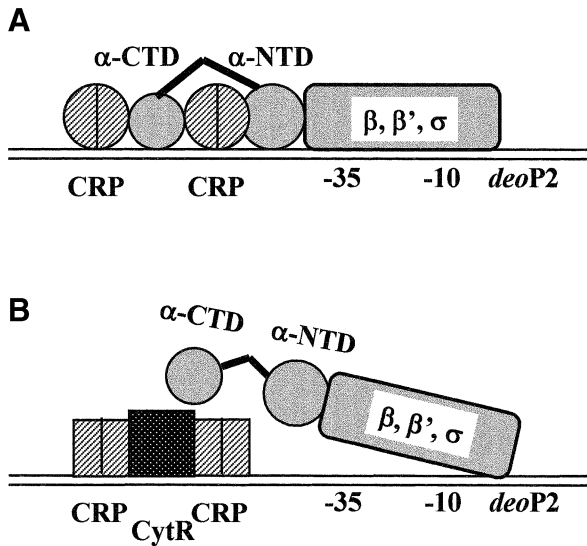
Fig. 8. Schematic model. The interaction between α-NTD and cAMP–CRP at –41 (CRP1 site) should, therefore, be important for activation of *deo*P2, class II CRP-dependent promoter (**A**). In the presence of CytR (**B**), the cAMP–CRP, especially that bound at the promoter-proximal site, undergoes an allosteric change such that it no longer interacts with α-NTD positively, but instead negatively. In this model, we speculate that CytR converts cAMP–CRP, especially at the promoter-proximal site, from an activator into a repressor to interfere with the RNP acting on *deo*P2.

could block transcription *in vitro* to a level that is significantly lower than that observed in the absence of CRP (Figure 5). Moreover, CytR inhibits *deo*P2 transcription conducted by RNP reconstituted with α-subunits lacking α-CTD (Figure 6). These results suggest that CytR repression also involves interference with interactions between RNP and the core promoter ('direct repression'). 'Oriented heterodimer' analysis showed that only one subunit of the CRP dimer, the subunit proximal to CytR, functionally interacts with CytR in the repression complexes (Meibom *et al.*, 2000). We therefore envisage that CytR contact with the promoter upstream subunit of the CRP dimer at the CRP1 DNA site may induce a conformational change in the AR2 region of the promoter downstream subunit that renders it incompetent to interact properly with RNP. Consistently, it was noted that cAMP–CRP binding was greatly enhanced in the presence of CytR, especially at the CRP1 site, suggesting a protein–protein interaction between two proteins (Figure 7). Taking together the results presented in this study, we propose a simple mechanism to account for the repression by CytR: the cAMP–CRP complex at the CRP1 site, upon interaction with CytR, undergoes allosteric changes such that its binding to the DNA site is improved but its interaction with α-NTD is no longer productive, but rather destructive, leading to transcription repression (Figure 8). This is a likely possibility since cAMP–CRP activation of a CRP II-dependent promoter should require precise interaction between those amino acid determinants on CRP and α-NTD, and therefore a subtle change in protein conformation could interfere with RNP and core promoter interaction. In this context, cAMP–CRP, an

activator, is converted through an interaction with CytR to a repressor that interferes with RNP acting at *deo*P2.

Materials and methods

Plasmids
The pdeoP2 and placZP plasmids were constructed by cloning a 141 bp fragment of the *deo*P2 promoter (–116 to +25) and a 201 bp fragment of the *lac*Z promoter (–124 to +76), respectively, into the *Eco*RI and *Pst*I sites of the transcriptional vector pSA508 (Choy and Adhya, 1993).

Proteins
CRP was purified from an *E.coli* strain carrying the *crp*+ gene on a multicopy plasmid, pHA5, by fast protein liquid chromatography (S.Ryu, Seoul National University, Korea). CytR was a generous gift from C.Jorgenson (Odense University, Denmark). Wild-type RNP polymerase was purchased from Pharmacia LKB. Reconstituted RNP composed of wild-type subunits or of C-truncated α-subunits was as used by Igarashi and Ishihama (1991).

Transcription assay
Transcription reactions were carried out using the procedure described by Choy and Adhya (1993). Briefly, 2 nM DNA template, 1 mM ATP, 0.1 mM GTP, 0.1 mM CTP, 0.01 mM UTP and 10–20 μCi of [α-^{32}P]UTP were pre-incubated in buffer [20 mM Tris–acetate pH 7.8, 10 mM magnesium acetate, 100 mM potassium glutamate, 1 mM dithiothreitol (DTT)] at 37°C for 5 min. When present, a repressor was included in the pre-incubation mix at concentrations described in the figure legends. Transcription was initiated by the addition of RNP (20 nM) in a total volume of 20 μl, and terminated after 10 min at 37°C by the addition of an equal volume (20 μl) of RNA loading buffer [80% (v/v) deionized formamide, 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), 0.025% bromophenol blue, 0.025% xylene cyanol]. The mixture was heated at 90°C for 2 min and electrophoresed in 8 M urea–8% polyacrylamide sequencing gels (40 cm × 0.4 mm). The RNA transcripts were quantified as counts per minute (c.p.m.) as determined with an Ambis β-scanner.

DNase I experiments
The experiments were conducted essentially as described by Brenowitz *et al.* (1986). The reaction conditions (in 20 μl) were the same as in *in vitro* transcription reactions, except that the nucleotides were omitted. After incubation at 37°C for 10 min, probing with DNase I was performed by adding 1 ng of the enzyme to the reaction buffer. The incubation was continued at 37°C for 5 min and stopped by addition of 20 μl of 40 mM EDTA. Modified DNA was purified using a PCR clean-up kit (Qiagen).

Primer extension analysis
Primer extension analysis used the alkaline denaturation procedure described in Rostock *et al.* (2000). The primer was a 33mer carrying the top strand sequence immediately upstream of multiple cloning sites in pSA508. Briefly, 1 μl of ^{32}P-labeled primer (1 pmol) and 4 μl of 10 mM NaOH were added to 35 μl of modified DNA and incubated for 2 min at 80°C, which was followed by a quick chilling in ice (for 5 min). To the denatured DNA were added 5 μl of hybridization buffer (0.5 M Tris–HCl pH 7.2, 100 mM MgSO$_4$, 2 mM DTT) with incubation for 3 min at 45°C, followed by a quick chilling in ice (for 5 min). Primer extension was carried out for 10 min at 50°C in the presence of dNTP (5 μl of 5 mM stock) and Klenow (0.5–1 U; Pharmacia). The reaction was stopped with 35 μl of 5 M NH$_4$CH$_3$COO, and the DNA was precipitated with ethanol and dissolved in formamide loading buffer. The mixture was heated for 2 min at 90°C and electrophoresed on 6% polyacrylamide DNA sequencing gels containing 8 M urea (40 cm × 0.4 mm).

Gel mobility shift assay
Gel mobility assays were performed with PCR fragments of *deo*P2 promoter (–116 to +25), *deo*C structural part (+58 to +193), wild-type *mal*T promoter (–121 to +18) and an A/T deleted mutant *mal*T promoter (–121 to +18) (see the legend to Figure 4 for DNA sequence alteration). For the mutant *mal*T promoter DNA fragment, pMT203(–A/T) (Tagami and Aiba, 1999), kindly provided by H.Aiba (Nagoya University, Japan), was used as the DNA template for PCR. The PCR fragment was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). The reaction mixture containing 2 nM end-labeled *deo*P2 DNA fragments and different concentrations of purified α-subunit was incubated at 37°C in

transcription buffer (50 mM Tris–HCl pH 7.9, 50 mM NaCl, 3 mM DTT, 5% glycerol) for 5 min. The gel running buffer was 1× Tris/borate/EDTA containing 5% glycerol, and the reaction mixtures were run on 5% acrylamide gels (acrylamide:bis-acrylamide = 50:1) containing 1× Tris/borate/EDTA and 5% glycerol.

Acknowledgements

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