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Dual-function regulators: The cAMP receptor protein and the CytR regulator can act either to repress or to activate transcription depending on the context

(combinatorial regulation/protein–protein interaction/nucleoprotein complex formation)

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ABSTRACT **Studies of gene regulation have revealed that several transcriptional regulators can switch between activator and repressor depending upon both the promoter and the cellular context. A relatively simple prokaryotic example is illustrated by the *Escherichia coli* CytR regulon. In this system, the cAMP receptor protein (CRP) assists the binding of RNA polymerase as well as a specific negative regulator, CytR. Thus, CRP functions either as an activator or as a corepressor. Here we show that, depending on promoter architecture, the CRP/CytR nucleoprotein complex has opposite effects on transcription. When acting from a site close to the DNA target for RNA polymerase, CytR interacts with CRP to repress transcription, whereas an interaction with CRP from appropriately positioned upstream binding sites can result in formation of a huge preinitiation complex and transcriptional activation. Based on recent results about CRP-mediated regulation of transcription initiation and the finding that CRP possesses discrete surface-exposed patches for protein–protein interaction with RNA polymerase and CytR, a molecular model for this dual regulation is discussed.**

The *Escherichia coli* cAMP receptor protein (CRP) has proven to be a remarkable regulator that in analogy with certain key regulators in higher organisms can function as a transcriptional activator, a repressor, a coactivator, or a corepressor. CRP appears to accomplish this diversity of functions by the differential use of its DNA-binding and -bending capacities and its ability to make specific contacts to other proteins. Evidence that CRP interacts with other proteins is available from the action of CRP as an activator and as a corepressor of transcription (for review, see refs. 1 and 2).

The best-characterized cAMP-CRP-dependent promoter, the *lac* promoter, provides the most clear example of the role of direct protein–protein contact between CRP and RNA polymerase in transcriptional activation. In this promoter, CRP is the only activator and binds to a site located immediately upstream (around −61.5) from the binding site for RNA polymerase. Activation requires a surface exposed loop of CRP (designated activating region 1, see Fig. 1) that interacts with the transcription machinery (3–5). Several lines of evidence have established that this contact involves a specific region in the C-terminal domain of the RNA polymerase alpha subunit (6, 7).

In the CytR regulon, CRP plays a dual role: it functions both as an activator and as a corepressor. On its own, the specific regulator of this system, the CytR repressor, lacks the ability to efficiently recognize its binding sites. When present together, however, CytR and CRP bind in a highly cooperative fashion. The synergistic binding relies on direct protein–protein interaction on the DNA helix (8). Part of the evidence for this was the isolation of a special class of CRP mutants (called *nc* for negative control) that abolish regulation by CytR without altering CRP activation (9). These mutant proteins contain substitutions in a surface-exposed epitope situated on the face opposite to the DNA-binding domain. Also, this epitope is physically separated from the activating region 1 of CRP (Fig. 1). The CytR/CRP regulatory system is highly flexible. Two types of repression complexes can be formed and nucleoprotein complex formation can in some of the promoters trigger a repositioning of CRP on the DNA helix (10, 11).

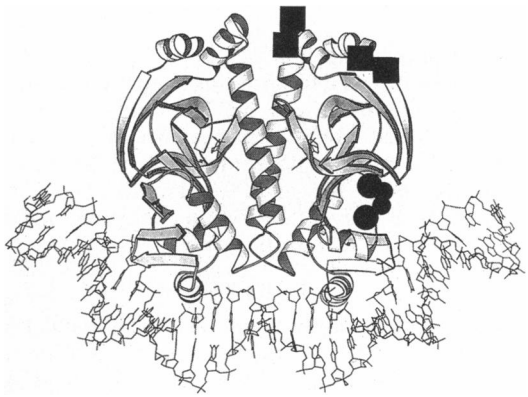

FIG. 1. Structure of the CRP-DNA complex showing the repressing and activating domains. The His-17, Cys-18, Val-108, and Pro-110 residues, sites of negative control mutations, are marked in one of the subunits of CRP by solid squares. The amino acids that are essential for transcription activation (amino acids 156, 158, 159, and 162) are indicated by solid circles (called the activating region 1).

In all the natural promoters studied so far, the CytR repressor binds to operators overlapping the DNA target for RNA polymerase and interferes with the formation of a transcription complex (12). Here we show that CytR and CRP function synergistically to activate transcription from recombinant *lac* promoters carrying appropriately positioned upstream targets for the two proteins. Thus, CytR can act in concert with CRP either to repress or to activate transcription depending on promoter architecture.

MATERIALS AND METHODS

Bacterial Strains. All strains were *E. coli* K-12 derivatives: SØ928 (Δ*deo* Δ*lac* *cytR*$^+$); SØ929 (as SØ928 but *cytR*$^-$); SØ2928 (as SØ928 but Δ*crp*) (9). Strain HB101 containing pBR322 with a 203-bp *lac* insert in the *Eco*RI site was a gift of A. Kolb (Institut Pasteur, Paris; ref. 13). The 203-bp

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Abbreviations: CRP, cAMP receptor protein; wt, wild type.

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fragment carries either the wild-type (wt) *lac* promoter-operator region or the mutant derivatives, *L8* or *L29* (14).

Plasmids. The two *cdd* promoter derivatives used (pB and pC, see Fig. 2) were derived from p13-184(96A, 97A) and p13-188Δ79 (10); the *Eco*RI–*Nsi*I fragments containing the mutant promoters were cloned into the *Eco*RI and *Pst*I sites of pBR322. To create *cdd-lacp* recombinant promoters, the *Ase*I–*Ase*I promoter fragments of pA*WT*, pA*L8*, and pA*L29* were excised (see Fig. 2) and cloned into *Ase*I-restricted pB and pC plasmids, respectively. The *Eco*RI–*Eco*RI fragments carrying the recombinant promoters and the *lac* promoter derivatives were subsequently cloned into the *Eco*RI site of the single copy *lacZYA* fusion vector pJEL150.

β-Galactosidase Assays. Growth of cells and *in vivo* assay of promoter activity were done as described (15). Specific activities of β-galactosidase are expressed as OD_{420}/OD_{450} per minute.

DNase I Footprinting. Purification of proteins, ^{32}P-end-labeling of fragments, and footprinting studies were carried out as described previously (12). *E. coli* RNA polymerase holoenzyme was purchased from Boehringer Mannheim. The proteins were added to final concentrations of 200 nM RNA-polymerase, 160 nM CRP, and 20 nM CytR. The final concentration of the B-*cdd-lacL8* promoter fragment and cAMP were 0.3 nM and 100 μM, respectively.

RESULTS

Construction of Promoters and Transcriptional Fusions. The objective of our experiments was to determine how variations in the position of CytR- and CRP-binding sites within a promoter sequence affect promoter activity. To this end, we constructed a set of *E. coli lac* promoter derivatives bearing upstream DNA elements derived from the CytR-controlled *cdd* promoter.

The starting points for this work were the *lac* promoter and two *cddp* derivatives that are regulated negatively by the concerted action of CRP and CytR (see Fig. 2). Detailed investigation has shown that CytR and CRP can form two types of nucleoprotein complexes at *cddp* (10). In the pB promoter derivative, a repression complex is formed in which CytR is sandwiched between tandem DNA-bound cAMP-CRP complexes. In this complex, CRP occupies a high affinity site (CRP-1) and a low affinity site (CRP-3), centered at positions −41.5 and −93.5, respectively. *In vivo* initiation of transcription from pB is strongly repressed in the presence of CRP and CytR (60-fold). In *cdd* promoters deleted for the CRP-3 site (Fig. 2, pC), a complex consisting of one molecule of each of the two regulators is formed. Such truncated promoters are partially regulated by CytR (10-fold).

Based on the three plasmids illustrated in Fig. 2, two sets of *cdd-lacp* recombinant promoter were constructed in which the promoter upstream *Ase*I–*Eco*RI fragment of *lacp* was replaced by the promoter upstream *Ase*I–*Eco*RI fragments of the two *cddp* derivatives (Fig. 3). Note that this strategy causes a displacement of the CRP/CytR-binding sites from *cddp* of two helical turns, relative to the transcription start site. However, the location of the CytR operator with respect to the CRP-binding site(s) in the two hybrid promoters is analogous to the arrangements in the *cdd* promoter (10).

To weaken binding of CRP to the *lac* binding site centered around −61.5, we also created recombinant promoters carrying the strong down mutations *L8* or *L29* (14). These single mutations within the *lac* recognition site for CRP prevent stable CRP binding. Finally, the resulting two sets of hybrid promoters, together with the parental *lac* promoters (*wt*, *L8*, and *L29*; see Fig. 3), were cloned in pJEL250, a low copy number transcriptional *lacZ* fusion vector.

Activity of Promoters in the Presence and the Absence of CytR. The low copy number fusion plasmids were transformed into the Δ*lac* strain SØ928 and the Δ*lac cytR*$^-$ strain SØ929; the resulting cells were grown in minimal glycerol medium. Table 1 shows the specific activity of β-galactosidase expressed from the nine promoters. As observed earlier, the *L8* and *L29* mutations severely reduce CRP activation of *lacp* transcription (about 25-fold; A promoters). The presence of CytR has little or no effect on transcription from the three *lac* promoters (Table 1, compare lines 1 and 2). Also, the sequences in *lacp* upstream from the −74 position may be substituted with *cdd* sequences without affecting promoter strength when present in strain SØ929 (*cytR*$^-$) (Table 1, compare lines 2 and 4). However, a remarkable difference is seen with the B-type *cdd-lac* promoters in the presence of the CytR regulator. CytR stimulates expression levels from the *L8*-template 11-fold and from the *L29*-template 9-fold (Table 1, line 3, and Fig. 3). This activation appears to be completely dependent on the synergistically binding of CRP and CytR. First, the same basal level

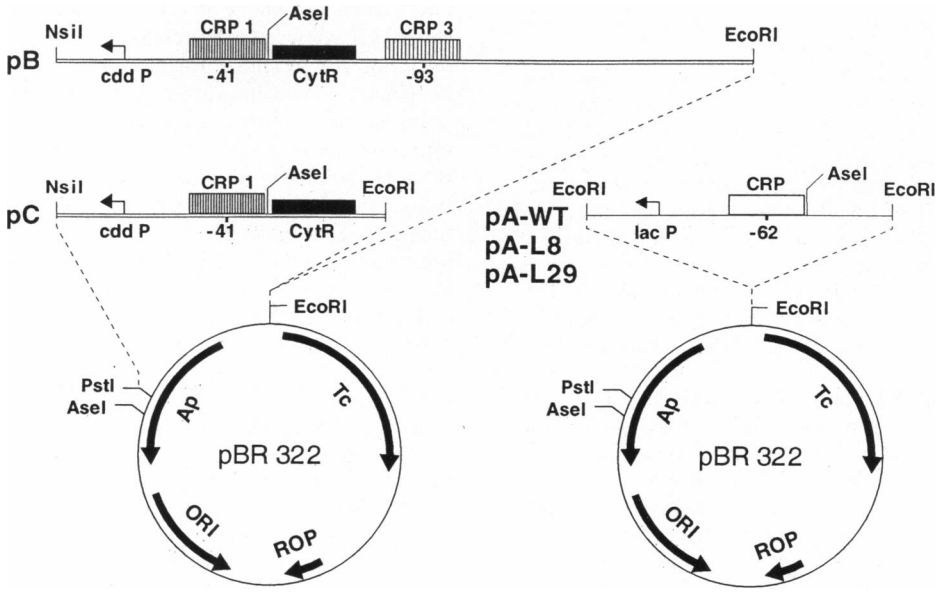

FIG. 2. Strategy for the construction of recombinant *cdd-lac* promoters. A schematic map of the pBR322 derivatives used for the construction of low copy number *lacZ* gene fusions is shown. Details of the cloning strategy are given in the text. Relevant restriction sites and binding sites for CytR and cAMP-CRP are indicated. Start sites for transcription are shown by an arrow.

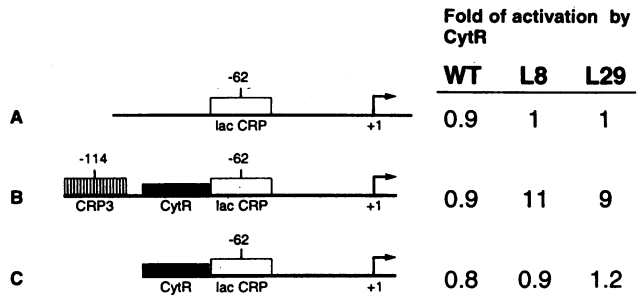

FIG. 3. Schematic map of the *lac* promoters (*A*) and the two recombinant promoters (*B* and *C*) carrying the wild type (WT), the *L8*, or the *L29 lac* CRP site. Fold of CytR activation of the three classes of promoters is indicated to the right.

of β-galactosidase is expressed from all nine templates in a Δ*crp* strain (see legend to Table 1). Second, a deletion of the distal CRP-3 site eliminates CytR-dependent activation as observed in the C-type hybrid promoters (Fig. 3). In this case, the cobinding of CytR and CRP is strongly impaired by the *L8* and *L29* mutations (see below). Third, CytR/CRP-binding sites would not be expected to mediate an increase in transcription when placed upstream of an already saturated promoter-proximal CRP target, if cooperative activation in our system solely relies on cooperative DNA binding. Accordingly, CytR does not enhance transcription of templates bearing the near-consensus wild-type *lac* CRP-binding site (likely to be saturated in the conditions used here). Finally, primer extension was used to show that the transcription start site at the hybrid promoters are unchanged from the *wt-lac* promoter (data not shown), ruling out the possibility that the CytR-dependent activation of the two B-type promoters is caused by creation of new promoters.

Footprinting of Nucleoprotein Complexes. The *in vivo* results strongly point to the fact that the CytR protein may participate in the formation of a higher order transcription initiation complex in which multiple protein–protein and protein–DNA interactions take place. To address this question directly, the binding of CytR, cAMP-CRP, and RNA polymerase to a linear substrate of the B-type *cdd-lacL8* promoter was probed by DNase I cleavage protection assays (Fig. 4). Addition of RNA polymerase (lane 8) and cAMP-CRP (lane 3) alone and in combination (lane 6) gives no significant protection or enhancement of DNase I cleavage as compared with the control in which no protein was added (lane 1). Similarly, no protection is observed in the presence of CytR alone (lane 2). Addition of both CytR and cAMP-CRP, however, results in the formation of a nucleoprotein complex that covers the region from approximately −50 to −120 (lane 4). Furthermore, a huge complex that covers the entire promoter region is formed when CytR, cAMP-CRP, and RNA polymerase are combined in a single binding reaction (lane 5). The CytR- and CRP-induced changes in the digestion pattern resemble those obtained in well-characterized CytR-controlled promoters (8, 10, 11). In particular, the region between the two CRP targets (occupied by CytR) is almost completely protected from DNase I digestion and, due to bending of the DNA by CRP, DNase I sensitive sites are present in each of the two CRP targets.

Table 1. Specific activities of β-galactosidase in the presence and absence of CytR

Promoter	*lac* CRP-binding site wt	*L8*	*L29*	Strain
A	30.0	1.3	1.2	*cytR*$^+$
	34.3	1.2	1.2	*cytR*$^-$
B	30.8	21.0	16.3	*cytR*$^+$
	34.3	1.9	1.8	*cytR*$^-$
C	29.6	1.8	1.8	*cytR*$^+$
	35.0	2.1	1.5	*cytR*$^-$

Enzyme levels were measured during exponential growth at 35°C in minimal medium using glycerol as carbon source. Samples were taken for enzyme assays between OD_{450} = 0.2 and 0.6. Specific activities are expressed as OD_{420}/OD_{450} per minute. The values are the average of three independent experiments (the observed variations do not exceed 10%). The same low activity of β-galactosidase (0.2) is expressed from all nine promoters when present in strain SØ2928 (Δ*crp* Δ*lac*).

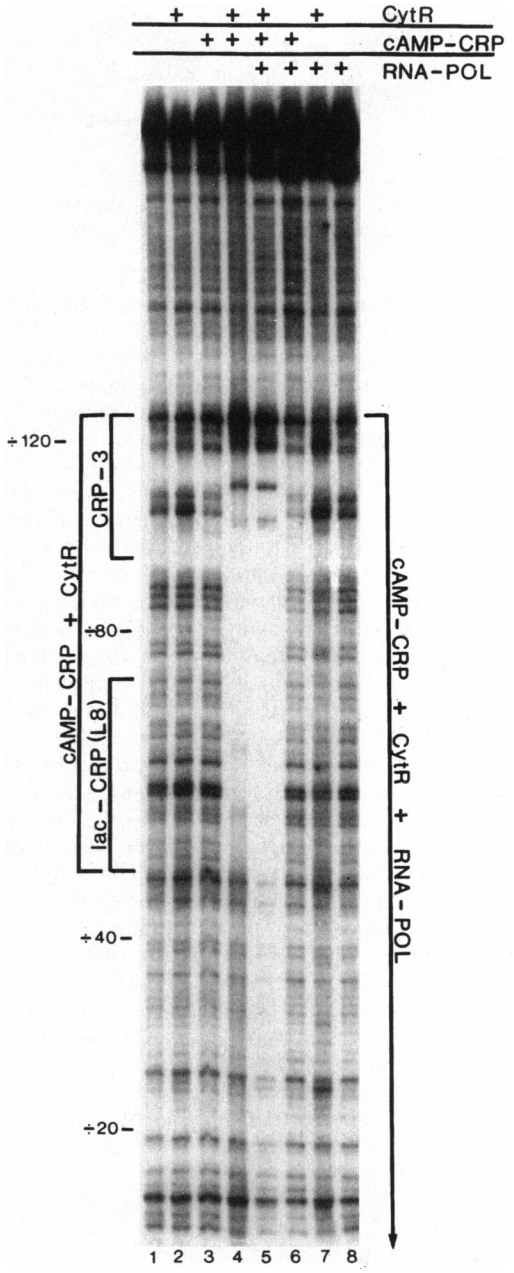

FIG. 4. DNase I footprinting analysis of complex formation in the B-*cdd-lacL8* promoter. The proteins were added according to the scheme above the autoradiogram; lane 1, no protein was added. The limits of the regions protected by CRP; CytR and CRP; and RNA polymerase, CRP and CytR, respectively, are indicated. The distance in base pairs from the start of transcription (+1) is indicated to the left.

These results demonstrate that CytR, CRP, and RNA polymerase bind in a highly organized and cooperative fashion to the B-type promoter and emphasize that CytR constitutes an authentic component of the activating apparatus. Moreover, they provide a model for the higher order transcription

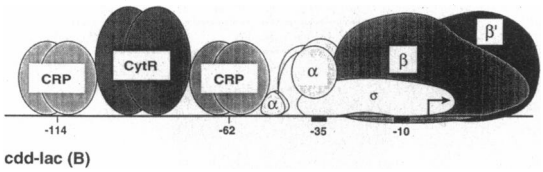

FIG. 5. Schematic representation of the cAMP-CRP/CytR/cAMP-CRP/RNA polymerase initiation complex at the B-*cdd-lac* promoters.

initiation complex (Fig. 5). In a similar analysis, with the B promoter fragment bearing the *wt-lac* CRP target, only the cAMP-CRP complex is required for stable promoter recognition by polymerase. However, the multicomponent initiation complex is also formed in this case when all three proteins are present simultaneously (data not shown).

DISCUSSION

The location and context of binding sites for regulatory proteins have been found to be a critical determinant of promoter function. Thus, one protein can activate some genes and repress others in the same cell (for reviews, see refs. 16–18). Another feature of many transcription factors, especially the eukaryotic ones, is that they show no or only a modest degree of DNA binding specificity. Such regulators are guided to their site of action in promoter complexes by specific interactions with other DNA-binding factors (19).

To explore in more detail (*i*) the interaction between DNA-bound proteins, (*ii*) the consequences of changes in the location of DNA binding sites, and (*iii*) the regulatory capacities of a heterologous multiprotein complex, we have analyzed a relatively simple bacterial model system. This consists of a set of *lac* promoter derivatives that bear either a strong or a weak CRP-binding site at position −61.5. In addition, the promoters have been equipped with *cdd*− sequences that support CytR binding just upstream of the *lac* CRP target (Fig. 3). The artificial promoters differ from previously studied CytR-regulated promoters in that the regulator-binding sites are positioned upstream of the DNA site for RNA polymerase.

We show that the CytR/CRP regulatory system is highly adaptable and can switch activity dependent on the location of binding sites in the promoter. Thus, a displacement of CRP and CytR targets to a more distal location in a promoter region is accompanied by interconversion of the repression complex to an activating complex. Several lines of experimental evidence are consistent with the idea that the concerted activation is due solely to cooperative DNA binding that leads to an increased occupancy of the proximal CRP target.

(*i*) CytR and CRP are both absolutely required to activate the B-type hybrid promoters with the *lacL8* or *L29* targets. Moreover, the introduction of a upstream CRP target in the *lac* promoters does not affect the promoter strength in the absence of CytR (Table 1). Hence, there is no reason to invoke a direct role for the upstream CRP-binding site in transcription activation. In this context, we note that synergy between appropriately spaced CRP molecules in transcription activation has been observed for natural CytR-regulated promoters and for semisynthetic *E. coli* promoters (10, 11, 20, 21).

(*ii*) Previous studies have established that proper binding of CRP to its binding sites is a prerequisite both for CRP-mediated activation of *lacp* and for CytR repression. By introducing point mutations in the *lac* CRP site of the C-type hybrid promoter, the cobinding of RNA polymerase, CytR and CRP *in vivo*, is impaired to such an extent that no stable transcription initiation complex can be formed. However, this problem can be solved either by extended cooperative binding of CytR and CRP (B-type promoters) or by increasing the intracellular concentration of CytR. Thus, activation of the C-type hybrid *L8* or *L29* promoters can be partially reestablished *in vivo* when CytR is overexpressed (data not shown).

(*iii*) Our *in vitro* experiments demonstrate that CytR triggers the formation of a huge initiation complex, consisting of RNA polymerase attached to a nucleoprotein complex in which CytR is sandwiched between tandem cAMP-CRP complexes (Fig. 5)

Models for Activation and Repression. Previous studies on transcription activation by CRP, promoter recognition by RNA polymerase, and organization of CytR-regulated promoters provide a framework for understanding how CytR-CRP nucleoprotein complexes can switch between activator and repressor. CRP-dependent promoters, in which CRP acts as the sole activator, can be grouped into two classes (for review, see ref. 22). In class I promoters, the DNA site for CRP is upstream of the DNA site for RNA polymerase (i.e., centered at or near position −61.5, position −72.5, position −82.5, or position −92.5). In class II promoters, the DNA site for CRP overlaps the DNA site for RNA polymerase (centered near or at position −41.5). Most strikingly, it has been established that transcription activation requires the activating region 1 (see Fig. 1) of only one subunit of the CRP dimer: the downstream, promoter-proximal subunit at class I promoters and the upstream, promoter-distal subunit at class II promoters (23, 24). Moreover, the target for activating region 1 is located in the C-terminal domains of the RNA polymerase alpha subunits (6, 7, 22, 24). These findings suggest a simple model for CytR/CRP-dependent activation at the *cdd-lacL8* and *cdd-lacL29* promoters, in which CRP bound at position −61.5 participates in protein–protein contacts both with the a-subunit of RNA polymerase and with CytR (see Fig. 5). The model envisages that CytR contacts the subunit of CRP that is adjacent to the CytR DNA site, thereby leaving the downstream subunit of CRP at the *lac* DNA site accessible for RNA polymerase (Fig. 6, lines 1 and 2). Also, it is implied in the model that the physiological role of CytR, when acting as a coactivator, is to recruit the principal activator (CRP) to its site of action.

Promoters that are negatively regulated by CytR can be separated into three groups based on the number of DNA sites for CRP (Fig. 6; for review, see ref. 26). Group 1 promoters contain just one site for CRP (exemplified by *cytRp* and *cdd* Δ79, lines 3 and 4; the DNA sites for CRP and CytR at *cytRp* are centered at positions −64.5 and −43.5, respectively). Group 2 promoters possess tandem targets for CRP that flank the CytR operator (exemplified by *deop2*, line 5; DNA sites for CRP centered at positions −40.5 and −93.5; DNA site for CytR centered at position −70.5). Transcription activation of these promoters primarily depends on the downstream CRP target. Members of the third group of promoters contain three functional CRP sites, and the formation of activation and repression complexes involves different subsets of these DNA sites (10, 11). At *cddp*, the two CRP-binding sites required for full activation are centered at −41.5 (CRP-1) and −91.5 (CRP-2), respectively (line 7). Binding of CytR provokes a repositioning of the distal CRP molecule to a lower affinity site (CRP-3) located 2 bp further upstream (line 6; DNA site for CytR centered at position −63.5).

In considering how CytR might turn off transcription initiation of the three distinct groups of promoters, it is important to stress the following points. (*i*) CytR is a specific antagonist of cAMP-CRP-dependent initiations. (*ii*) To turn off transcription, CytR must bind to a DNA site that is close to or overlaps the recognition elements for RNA polymerase and its binding requires protein–protein interactions with one or two DNA bound CRP molecules. (*iii*) CRP and RNA polymerase, as well as CRP and CytR, interact on the same face of the DNA helix (8, 11, 12, 27). (*iv*) The subunit of CRP that is involved in transcription activation is located adjacent to CytR in the repression complexes formed in the CytR regulon (see Fig. 6, lines 3–6). (*v*) In the presence of CRP, CytR prevents the binding of RNA polymerase to the *deop2* promoter. It is likely

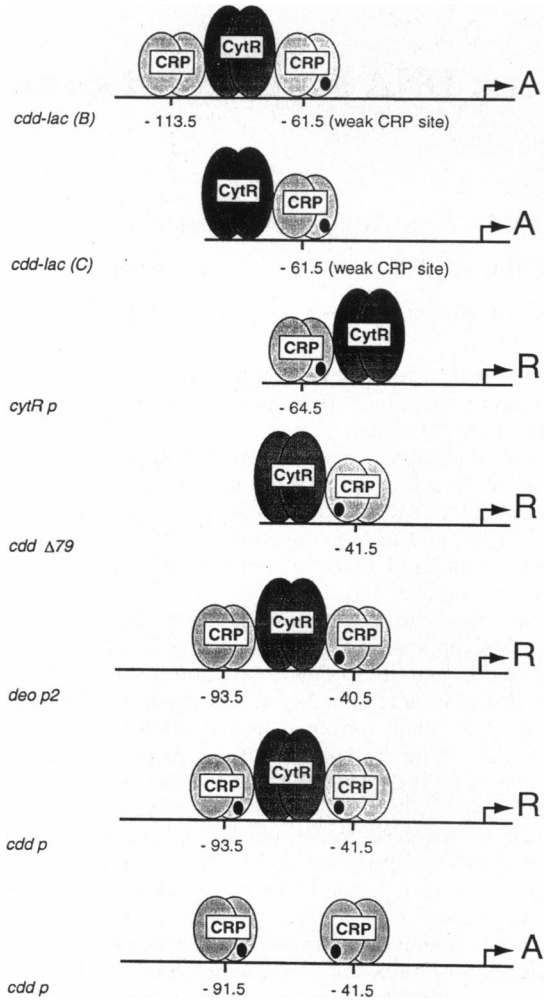

FIG. 6. Schematic representation of the architectures of CytR- and cAMP-CRP-regulated promoters. The contexts leading to repression or activation are marked by R and A, respectively. The location of CRP-binding sites relative to the start site for transcription is indicated. The solid circle denotes the activating region 1 of CRP. At class I promoters, where the bound CRP is located upstream of bound RNA polymerase, a functional activating region 1 is essential in the downstream CRP subunit, but not in the upstream subunit (22). At class II promoters, activating region 1 is functional in the upstream subunit of the bound CRP dimer, but not in the downstream subunit (23). Notably, a supplementary activating region on CRP, located adjacent to the patch recognized by CytR, seems to be involved in activation of Class II promoters (for review, see ref. 25). Activation at a number of promoters is dependent on binding of two CRP molecules (e.g., *cddp*). In these cases, it is likely that both bound CRP dimers contact RNA polymerase via activating region 1 as outlined in line 7 (21, 25).

that *deop2* bears a third binding element for RNA polymerase that partially overlaps the CytR operator, designated "Up-element" and recognized by the α-subunit of RNA polymerase (28). Thus, footprinting studies have shown that RNA polymerase in open complexes and CytR in repression complexes make very similar contacts to the DNA backbone in an A+T rich region just upstream of position -60 (12).

The simplest interpretation of these findings is that CytR and RNA polymerase compete for cooperative binding with CRP to the promoter regions. In principle, CytR could prevent binding of RNA polymerase at the group 1 and 2 promoters by competitive DNA binding or by masking the activating region 1 of the subunit of CRP that is involved in transcription activation (see Fig. 6). Additionally, at group 3 promoters, CytR can induce the repositioning of CRP to alternative sites that are suboptimum (e.g., *cddp*) or nonfunctional in CRP activation (V. Nielsen and P.V.-H., unpublished data). It is emphasized that the proposed models are not mutually exclusive, and it is possible that more than one will turn out to be required for complete repression by CytR.

Taken together, our results have revealed a particular clear example of how dual-function regulators can be recruited to the promoter, and then depending on the context act as coactivators or corepressors. As this regulatory case exhibits remarkable similarities to certain eukaryotic gene regulatory systems (17, 18), further studies on the well-characterized CRP and CytR regulators should reveal important insights into mechanistic models that will probably be applicable to a wide variety of promoters. Moreover, our data raise the possibility that CytR-dependent activation occurs at natural promoters.

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