The EMBO Journal vol.11 no.10 pp.3635-3643, 1992

The cAMP-CRP/CytR nucleoprotein complex in *Escherichia coli*: two pairs of closely linked binding sites for the cAMP-CRP activator complex are involved in combinatorial regulation of the *cdd* promoter

B.Holst, L.Søgaard-Andersen, H.Pedersen and P.Valentin-Hansen[1]

Department of Molecular Biology, Odense University, Campusvej 55, DK-5230 Odense M, Denmark

Communicated by H.Buc

[1]Corresponding author

Transcription initiation at CytR regulated promoters in *Escherichia coli* is controlled by a combinatorial regulatory system in which the cAMP receptor protein (CRP) functions as both an activator and a co-repressor. By combining genetic studies and footprinting analyses, we demonstrate that regulated expression of the CytR controlled *cdd* promoter requires three CRP-binding sites: a high affinity site (CRP-1) and two overlapping low affinity sites (CRP-2 and CRP-3) centred at positions −41, −91 and −93, respectively. In the absence of CytR, cAMP-CRP interacts at one set of sites (CRP-1 and CRP-2) and both of these binding sites are required for full promoter activation. In the presence of CytR, however, the two regulators bind cooperatively to *cddP* forming a nucleoprotein complex in which cAMP-CRP binds to CRP-1 and CRP-3 and CytR occupies the sequence between these sites. Thus, association of the two regulators involves a repositioning of the cAMP-CRP complex. Moreover, mutant *cdd* promoters in which CRP-2 and CRP-3 have been deleted are partially regulated by CytR, and cAMP-CRP and CytR still bind cooperatively to these promoters. These findings provide clues to an understanding of how cAMP-CRP and CytR interact at a structurally diverse set of promoters.

Key words: cAMP-CRP regulation/cooperative binding/nucleoprotein complexes/protein-DNA interaction/repositioning of regulators

Introduction

Selective repression of transcriptional initiation by DNA binding proteins is a widely used strategy for gene regulation both in prokaryotes and eukaryotes. It has generally been assumed that the classical bacterial repressors contain all the structural features required for efficient interaction with their DNA targets. However, the recent identification of a new class of gene regulatory proteins that rely on interactions with other DNA-binding proteins has made it clear that the repression apparatus can be more complex (for review see Struhl, 1989; Keleher *et al.*, 1988; Søgaard-Andersen *et al.*, 1991b).

The CytR regulon in *Escherichia coli* constitutes a combinatorial regulatory system in which the cyclic AMP receptor protein (CRP) functions both as an independent transcriptional activator and as an 'adaptor' for a specific repressor, CytR (Gerlach *et al.*, 1991; Pedersen *et al.*, 1991; Søgaard-Andersen *et al.*, 1991b). This regulon comprises at least eight operons that code for proteins involved in the transport or metabolism of deoxy- and ribonucleosides (for review see Hammer-Jespersen, 1983). Detailed investigation of the *deo* operon has shown that cAMP-CRP and CytR bind cooperatively to the *deoP2* promoter forming a nucleoprotein complex in which CytR is sandwiched between two DNA-bound cAMP-CRP complexes. *In vitro*, CytR exhibits a low affinity for the sequence located between the two CRP sites; however, its binding to *deoP2* can be stimulated as much as 1000-fold by cAMP-CRP (Søgaard-Andersen *et al.*, 1991b; Pedersen *et al.*, 1991). This ability of cAMP-CRP to stimulate the binding of CytR cooperatively requires a set of amino acids that are located in a specific region on the surface of cAMP-CRP (Pedersen *et al.*, 1991; Søgaard-Andersen *et al.*, 1991a).

An interesting aspect of the CytR regulon is the great structural diversity of the regulatory regions of the different genes or operons. The only common feature observed so far is the presence of two binding sites for cAMP-CRP (Valentin-Hansen, 1982; Valentin-Hansen *et al.*, 1989; Gerlach *et al.*, 1990, 1991). Most strikingly, these sites are not found at the same positions relative to the transcription start site in all the promoters. This structural complexity, together with the fact that CytR repression at *deoP2* has a strict requirement for a spacing of 52 or 53 bp between the two CRP binding sites (Søgaard-Andersen *et al.*, 1990b, 1991b), has hampered the proposal of a unifying binding model for CytR.

This study of the regulatory region of the *cdd* gene which encodes cytidine deaminase has brought forward a number of new aspects of cAMP-CRP/CytR regulated promoters. We show that the CytR repressor can trigger a repositioning of the cAMP-CRP complex on the DNA helix and that the two regulators can still interact cooperatively and repress transcription in promoters containing only a single binding site for each of the two proteins. Also, the present data reveal that full cAMP-CRP activation of *cddP* requires two properly spaced binding sites for the activator.

Results

Deletion mapping of cddP

Expression of *cdd* is strongly regulated by CytR and is completely dependent on cAMP-CRP for activity (Josephsen and Hammer-Jespersen, 1981); moreover, two binding sites (CRP-1 and CRP-2) for cAMP-CRP have been identified in *cddP* in DNase I footprinting experiments (Figure 1) (Valentin-Hansen *et al.*, 1989). To define the sequence information required for regulated expression of the *cdd* promoter, we first constructed two low copy number *cdd'-lacZ'* protein fusion plasmids, pB184 and pB188, encoding *cdd* sequences from −184 to +254 and from −184 to +188, respectively (Figure 1). As shown in Table IA, the regulatory patterns of *lacZ* expression from these two

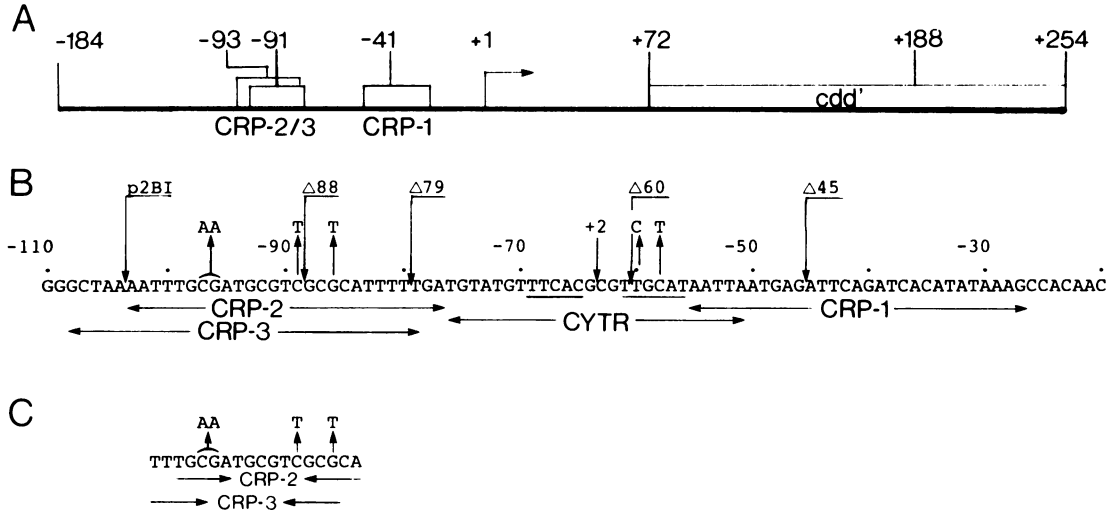


Fig. 1. Schematic map of *cddP*. (**A**) Coordinates are in base pairs; +1 refers to the start site of transcription as indicated by the arrow. Boxes labelled CRP-1 and CRP-2/3 indicate regions protected by cAMP–CRP in DNase I footprinting experiments; the centre of each CRP site is indicated. The box labelled *cdd'* indicates the 5'-end of *cdd*. (**B**) Nucleotide sequence of *cddP*. The regions protected by cAMP–CRP (CRP-1, CRP-2 and CRP-3) and CytR in DNase I footprinting experiments are indicated by arrows. The sequence motifs that may be involved in sequence specific binding of CytR are underlined. The upstream endpoints of the five deletions, the 2 bp insertion at position −63 (+2) and the substitutions in the point mutation derivatives are indicated above the sequence. (**C**) Nucleotide sequence of the central part of CRP-2/3. Arrows indicate regions of homology with the consensus CRP site 5'-TGTGAN$_6$TCACA (de Crombrugghe *et al.*, 1984). The mutations introduced in this region are indicated. The EMBL database accession number of *cdd-P* is X16419.

plasmids are identical and closely parallel the regulatory pattern of the chromosomal *cdd* gene (Josephsen and Hammer-Jespersen, 1981).

Starting with pB188, a set of upstream deletions was generated as outlined in Figure 1 and Table IA. The deletion that ends at position −103 and removes the upstream flanking sequences of CRP-2 exhibited a 30% reduction in both promoter activity and CytR regulation. Removal of CRP-2 resulted in a further reduction of promoter activity; however, these promoters are still regulated 10-fold by CytR. In the deletion that ends at position −60, CytR regulation is almost absent. Finally, removal of CRP-1 completely inactivated the promoter. In Δ*cya* strains, the promoter activity remained unaffected by the deletions, and S1 mapping analyses confirmed that transcription starts at the same position in all constructs (Figure 2, lanes 4–8).

To ensure that the reduced CytR regulation in the truncated promoters is not a consequence of reduced promoter activity, an *in vivo* repressor titration experiment was performed (Søgaard-Andersen *et al.*, 1990b). In this test, the competence of the *cddP* derivatives to bind CytR is measured by their ability to alleviate CytR regulation when cloned in a high copy number plasmid, pUC13. The strain employed in this analysis, S01316/F'*lacI*^{q}, is CytR$^+$ and contains a *cdd–lacZ* fusion on the chromosome; the *lacI*^{q} allele has been included to repress the *lac* promoter in the pUC13 derivatives (Søgaard-Andersen *et al.*, 1990b). The results of this experiment are shown in Table II and a clear correlation is observed between CytR regulation of the truncated promoters (Table IA) and their competence to bind CytR. Thus, these data strongly suggest that the reduced CytR regulation is due to reduced CytR binding.

Isolation of cddP mutants with reduced CytR regulation

To define more precisely the sequences involved in CytR regulation, mutants of *cddP* with a reduced regulatory response to CytR were isolated. The plasmids p2BI, pB188 and pB184 (Table IB) were mutagenized by passage through an *E.coli mutD5* strain. Following screening for plasmids with an elevated *lacZ* expression in a CytR$^+$ strain, four mutant *cdd* promoters were identified containing single base pair mutations at positions −58, −60, −86 and −89, respectively (Figure 1B). In all the mutant promoters, transcription was found to initiate at position +1 (Figure 2, lanes 1–2 and 12–13), ruling out the possibility that the reduced CytR regulation was caused by the introduction of a new promoter.

The regulatory features of the four mutant promoters are shown in Table IB. All mutations resulted in a marked decrease in negative regulation of *cddP*. Promoter strength and cAMP–CRP activation, however, remained unaffected by the mutations. The mutations immediately upstream of CRP-1 (−58T and −60C) nearly knock out CytR regulation, whereas negative regulation of the promoters with substitutions in the CRP-2 region (p2BI-86T; p2BI-89T) is similar to that obtained with the mutant promoters deleted for the CRP-2 region (10-fold; Table IA Δ88 and Δ79). Taken together, these results clearly demonstrate the importance of two distinct domains in *cddP* for CytR regulation.

Binding of cAMP–CRP and CytR to wild-type and mutant promoters

In order to investigate the interaction of cAMP–CRP and CytR with *cddP* and to define the role of the different protein binding sites, we performed DNase I footprinting experiments on ^{32}P-end-labelled fragments in the presence of purified proteins. As previously reported (Valentin-Hansen *et al.*, 1989), CRP-1 is a high affinity target and CRP-2 is a low affinity target for cAMP–CRP (Figure 3A, lanes 2–3 and 8–9). Addition of CytR protein to binding reactions containing cAMP–CRP resulted in protection of an 84 bp region spanning from CRP-1 to around position −110. Several striking features of this combined footprint are apparent. Firstly, the cleavage pattern in CRP-1 is

Table I. Activity of β-galactosidase[a] expressed from *cdd–lacZ* fusions

Plasmid	Fusion	CytR$^+$	CytR$^-$	Fold of CytR regulation[b]
A				
pB184	-184 CRP-2 CRP-1 +1 +264	0.12	6.82	57
pB188	+188	0.05	3.01	60
p2BI	-103	0.05	2.04	40
pB188Δ88	-88	0.08	0.80	10
pB188Δ79	-79	0.08	0.81	10
pB188Δ60	-60	0.20	0.34	1.5
pB188Δ45	-45	<0.02	<0.02	1
B				
pB184-58T	C-T	1.60	7.21	5
pB188-60C	T-C	1.61	3.20	2
p2BI-86T	G-T	0.21	2.07	10
p2BI-89T	C-T	0.21	2.02	10
C				
pB184+2	+CG	0.05	3.50	70
pB188-60C+2	+CG T-C	0.15	2.05	14
pB188Δ79+2	-79 +CG	0.08	0.82	10
pB184-96A97A	C-A,G-A	0.04	2.24	56

[a]Enzyme levels were measured during exponential growth in minimal medium using glycerol as a carbon source at 30°C as described (Miller, 1972). The activity of β-galactosidase is expressed as OD_{420}/OD_{450}/ml/min (Dandanell *et al.*, 1987). The activity of β-galactosidase in a Cya$^-$ strain was <0.02 in all constructs. The values are the average of three independent experiments in which the observed variation did not exceed 10%.
[b]The fold of CytR regulation was calculated as the activity of β-galactosidase in a CytR$^-$ strain divided by the activity in a CytR$^+$ strain.

identical to that obtained in the presence of saturating concentrations of cAMP–CRP alone (Figure 3A, compare lanes 3–4 with 5–6 and 9–10 with 11–12) indicating that cAMP–CRP also occupies this target in the presence of CytR. Secondly, cAMP–CRP binding to CRP-1 is clearly enhanced by CytR (Figure 3B, compare lanes 4–5 with 6–10). However, the DNase I digestion pattern at CRP-2 resembles that obtained by cAMP–CRP alone, but the position of the footprint is displaced 2 bp upstream (Figure 3A, compare lanes 4 and 10 with 6 and 12). Furthermore, the protection of this DNA segment is enhanced in the presence of CytR.

DNase I footprinting experiments performed in the presence of increasing concentrations of CytR (Figure 3B, lanes 12–13) revealed that CytR interacts with the region located between the two CRP sites. On addition of cAMP–CRP, however, a >1000-fold lower CytR concentration is required to give half protection of this DNA segment (Figure 3B, compare lanes 8–10 with 12–13). Hence, cAMP–CRP and CytR bind cooperatively to *cddP*. Moreover, the protection pattern conferred by the activator and repressor, alone and in combination, is compatible with the formation of a nucleoprotein complex in which CytR is flanked by a cAMP–CRP complex on each side.

The effect(s) of the different promoter mutations on CytR and/or cAMP–CRP binding was monitored with *cddP* fragments containing the various mutations. The mutations at position −58 and −60 resulted in a 3- to 4-fold reduced affinity of CytR for its binding site (Figure 4). On the other hand, cAMP–CRP binding to the wild-type (wt) and the two mutant promoters was indistinguishable, and also, the combined cAMP–CRP/CytR footprint on both wt and mutant promoters was identical when CytR was present at high concentrations (data not shown). Hence, the mutations at positions −58 and −60 affect CytR regulation by interfering with its direct interaction with DNA.

In a promoter with CRP-2 deleted, the independent binding of cAMP–CRP to CRP-1 and CytR to the sequence upstream of CRP-1 is similar to that observed with the intact promoter (Figure 5). cAMP–CRP and CytR bind cooperatively to this promoter and the combined footprint is the sum of the independent footprints. However, at least a 2-fold higher CytR concentration is required to obtain complete protection compared with the intact promoter.

Table II. *In vivo* titration assay in SO1316/F'*lacI*^{q}

Plasmid	Activity of β-galactosidase[a]	Fold of derepression[b]
pUC16[c]	0.12	
p13-188	6.52	54
p13-2BI	4.51	38
p13-188Δ88	2.20	18
p13-188Δ79	2.04	17
p13-188Δ60	0.21	2
p13-188Δ45	0.15	1

[a]The activity was measured in the presence of the indicated plasmid. Otherwise refer to Table I.
[b]The fold of derepression was calculated as the activity of β-galactosidase in the presence of a pUC13 derivative divided by the activity in the presence of pUC16.
[c]pUC16 is a pUC13 derivative in which the α-*lacZ* gene has been inactivated, otherwise the α-acceptor would complement the α-acceptor encoded by the F plasmid. The plasmids are named according to the *cddP* derivative cloned (refer to Figure 1 and Table I).

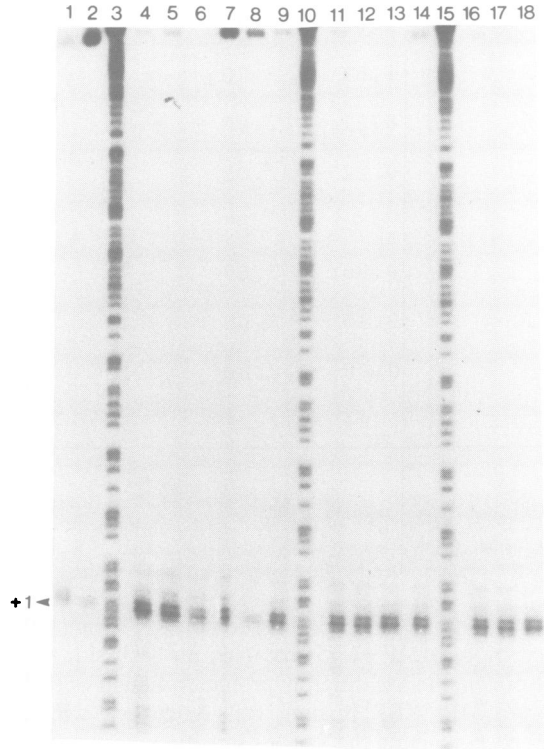
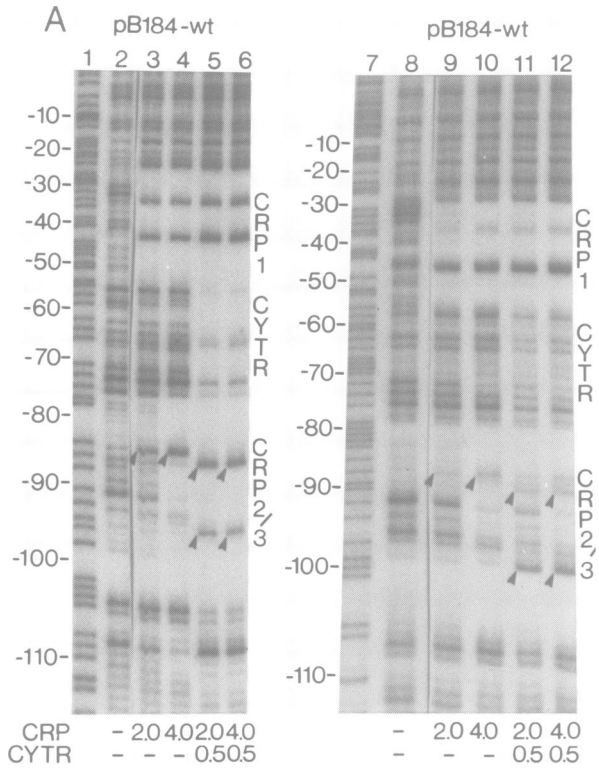


Fig. 2. S1 nuclease mapping of the start site of transcription in *cddP* derivatives. The probe is an *Eco*RI−*Sca*I fragment isolated from pB184-wt and labelled at the 5′ end of the *Sca*I site (*Sca*I restricts *cddP* at position +107). Lanes 3, 10 and 15 are the A/G sequence of the hybridization probe. Lane 1, RNA isolated from SO929 harbouring p2BI-86T; lane 2, p2BI-89T; lane 4, pB188; lane 5, p2BI; lane 6, pB188Δ88; lane 7, pB188Δ79; lane 8, pB188Δ60; lane 9, pB188Δ79+2; lane 11, pB184; lane 12, pB184-58T; lane 13, pB188-60C; lane 14, pB184+2; lane 16, pB188-60C+2; lane 17, pB184-96A97A.

cAMP−CRP and CytR independently interact at the two promoters containing mutations in the CRP-2 region as efficiently as with the analogous p2BI promoter (Figure 6, compare lanes 4−6 with 10−12 and 16−18; data not shown). Strikingly, however, no protection of the DNA region encompassing the CRP-2 site was observed in the



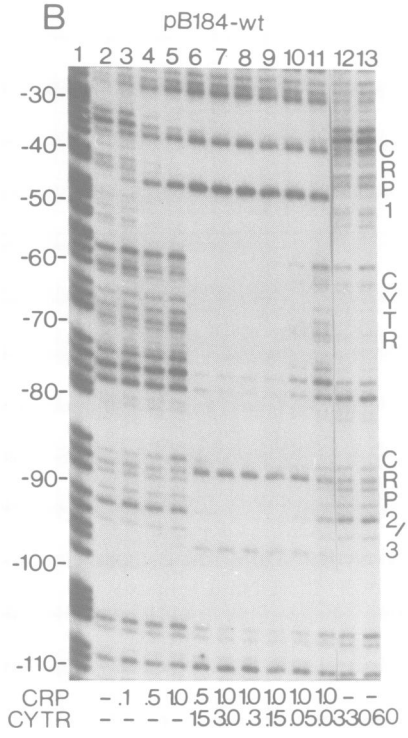


Fig. 3. Binding of cAMP−CRP and CytR to wt *cddP*. The fragment employed is an *Eco*RI−*Bam*HI fragment isolated from pB184-wt and ^{32}P-end-labelled at the upper strand at the 5′-end of the *Eco*RI site (left panel in A and panel B) or at the lower strand at the 3′-end of the *Eco*RI site (right panel in A). The final concentrations of CRP and CytR in ng/μl are indicated below each lane. The regions protected by the proteins are indicated. The sequence is numbered with respect to the start site for transcription. The first lane in all panels is the A/G sequence of the template. The arrows in panel A show the enhanced bands in the cAMP−CRP footprint in the CRP-2/3 region in the absence (lanes 3 and 4) and presence (lanes 5 and 6) of CytR.

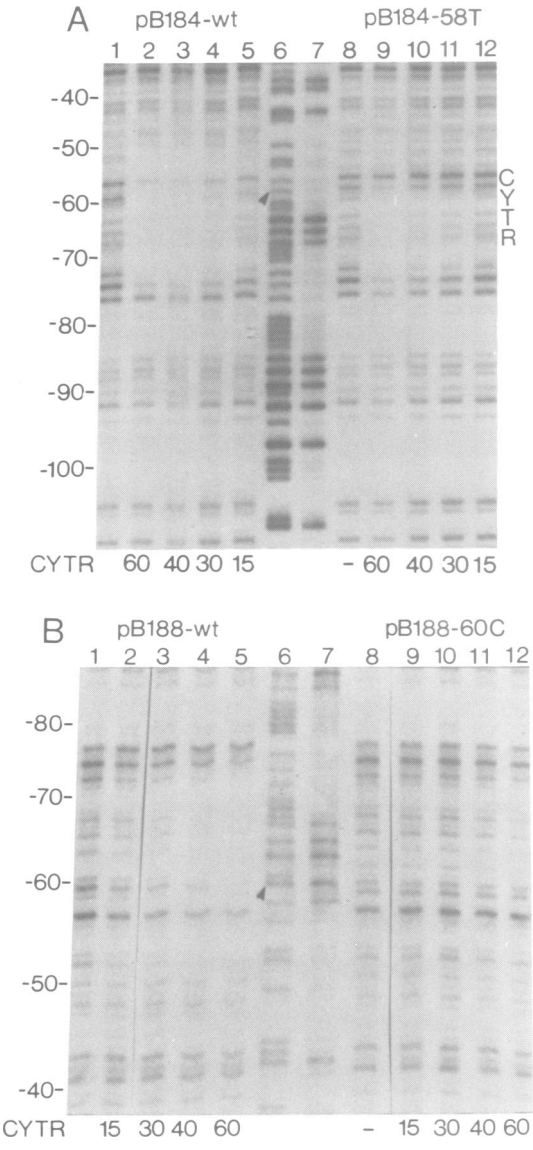


Fig. 4. Binding of CytR to wt and point mutation derivatives of *cddP*. The fragments used are: (**A**), *Eco*RI–*Bam*HI fragments ^{32}P-end-labelled at the upper strand at the 5′-end of the *Eco*RI site and (**B**), *Pvu*II–*Nsi*I fragments ^{32}P-end-labelled at the upper strand at the 3′-end of the *Nsi*I site (*Nsi*I restricts *cddP* at position +26). The final concentrations of CytR are indicated in ng/μl. The regions protected by CytR are indicated. Lanes 6 and 7 in both panels are the C/T and C reactions, respectively, of the probes. The arrows indicate the positions of the mutations.

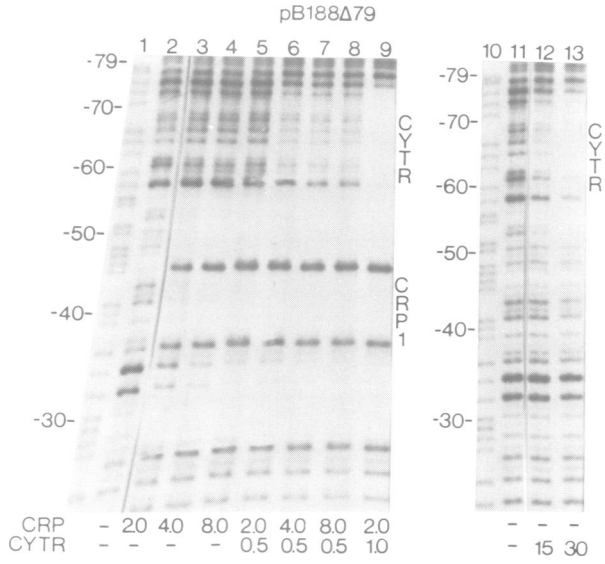


Fig. 5. Binding of cAMP–CRP and CytR to a *cddP* derivative with CRP2/3 deleted. A *Pvu*II–*Nsi*I fragment was employed and labelled as described in Figure 4. Final concentrations of CRP and CytR are indicated in ng/μl. The regions protected by the proteins are indicated. Lanes 1 and 10 are the A/G sequence of the probe.

combined footprint in the two mutant promoters (Figure 6, compare lanes 3, 9 and 15). In fact, the combined cAMP–CRP/CytR complex formed at these two promoters closely resembles that observed for the promoter in which the CRP-2 region has been deleted (Figure 5). This result, together with the CytR-induced change of the protection pattern in the CRP-2 region of the wt promoter (Figure 3), clearly shows that the simultaneous binding of the activator and repressor proteins at *cddP* is rather intricate.

Close inspection of the DNA sequence around the CRP-2 site reveals that this region might contain two overlapping CRP sites, CRP-2 and CRP-3, (Figure 1B and C) that are displaced by 2 bp. This view of the architecture of *cddP* is attractive because it provides a simple explanation of the properties of the two promoters containing mutations in the CRP-2 region, i.e. these mutations could specifically inactivate the CRP-3 binding site. Based on the consensus sequence of CRP sites (TGTGA.N6.TCACA; de Crombrugghe *et al.*, 1984) in particular the −89 mutant would be expected to decrease cAMP–CRP binding to CRP-3 seriously without affecting its binding to CRP-2. Thus, in the CRP-3 site a replacement of cytosine by thymine at position −89 corresponds to the strong L29 mutation in the CRP target of *lac* (Reznikoff and Abelson, 1978). In the CRP-2 site, however, position −89 is located in the non-conserved 6 bp spacer (see Figure 1C). The simplest interpretation of our results is, therefore, that *cddP* contains two overlapping upstream targets for cAMP–CRP, and CRP-2 has a higher affinity for CRP than CRP-3. Furthermore, we propose that different combinations of these CRP sites are used during activation and repression: activation of *cddP* involves CRP-1 and CRP-2 whereas cAMP–CRP binding in the presence of CytR involves CRP-1 and CRP-3, i.e. the cooperative binding of cAMP–CRP and CytR induces a 2 bp repositioning of cAMP–CRP from CRP-2 to CRP-3.

Evidence for CytR induced repositioning of cAMP – CRP

Several predictions follow from this model. Increasing the distance between CRP-1 and CRP-2 by 2 bp in the wt promoter and in the promoter containing the point mutation at position −60 would be expected to result in decreased activation and increased CytR regulation. Insertion of 2 bp between positions −64 and −63 (Figure 1B) resulted in a 50% reduction in *cddP* activity in the wt promoter and a 40% reduction in the O^c mutant (Table IC). Negative regulation by CytR is, however, clearly enhanced in both cases. DNase I footprinting experiments with the modified promoters, as illustrated with the wt+2 promoter in Figure 7, revealed a higher degree of cooperative DNA binding of the two regulators and, as predicted, no rearranged binding of cAMP–CRP in the upstream region

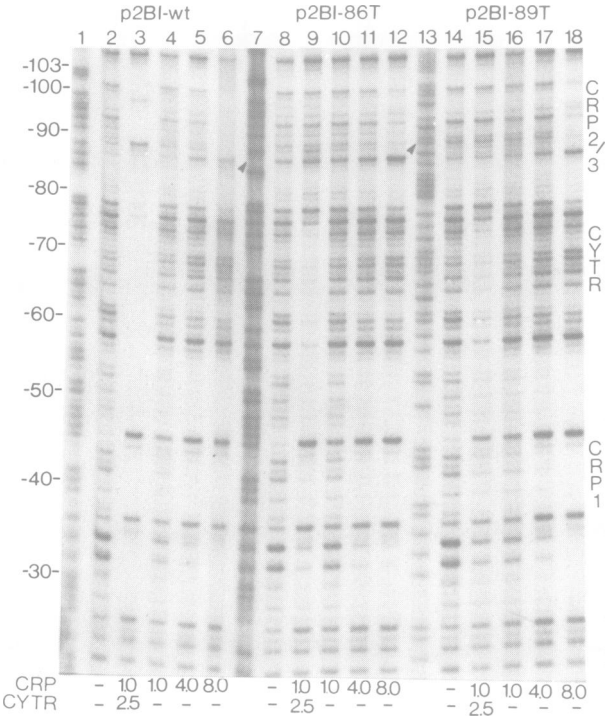


Fig. 6. Binding of cAMP−CRP and CytR to *cddP* derivatives containing point mutations in CRP2/3. *Pvu*II−*Nsi*I fragments were employed and labelled as in Figure 4. Final concentrations of CRP and CytR are indicated in ng/μl. Regions protected by the proteins are indicated. Lanes 1 and 7 are the A/G sequences of the probes and lane 13 is the C/T sequence of the probe. Arrows indicate positions of the mutations.

(Figure 7B, compare lanes 4−6 with 10−12). Furthermore, the affinity of cAMP−CRP (Figure 7A) and CytR (data not shown), when present alone, was similar to that observed with the wt promoter. As a 2 bp insertion in the deletion mutant pB188Δ79 lacking the CRP-2/CRP-3 sites affected neither promoter efficiency nor negative regulation *in vivo* (Table IA and C), the changed regulatory properties of the two new constructs are most probably a consequence of the altered position of the two upstream CRP sites.

In view of these results, we investigated the functional importance of CRP-2 by site-directed mutagenesis. Substitution of the G at position −96 with an A would be expected to decrease cAMP−CRP binding to CRP-2 drastically (Figure 1C) but only affect CRP-3 minimally. Moreover, the primary effect of a replacement of C at position −97 with A would be expected to increase binding of cAMP−CRP to CRP-3 marginally (de Crombrugghe *et al.*, 1984). Hence, two As were introduced at positions −96 and −97 to inactivate CRP-2 specifically and to increase the homology of CRP-3 with the consensus. *In vivo*, this mutated promoter was highly regulated by CytR (56-fold) whereas promoter activity was three times lower than for the wt promoter (Table IC). The footprint obtained with cAMP−CRP clearly indicated that the same targets (CRP-1 and CRP-3) were occupied both in the presence and in the absence of CytR (Figure 8, compare lanes 4 and 5). Finally, in all these constructs the start site for transcription, as determined by S1 mapping, was identical to that of wt *cddP* (Figure 2, lanes 14, 16 and 17). Taken together, these results confirm the existence of three functional CRP sites in *cddP*, and that a change between activated and repressed states in *cddP* involves a switch in the combinations of CRP sites used.

Discussion

The cAMP−CRP complex plays an important role in the regulation of many promoters in *E.coli*. In most cases studied, it acts as an independent activator by binding to a single site that is located at well defined distances from the −10 sequence (Gaston *et al.*, 1990; Ushida and Aiba, 1990; Valentin-Hansen *et al.*, 1991). However, cAMP−CRP also acts in concert with other regulators in more complex promoters and in these cases multiple CRP targets may be required for proper regulation, e.g. in the divergent $malE_P$−K_P promoter region cAMP−CRP interacts at three adjacent binding sites and functions as a co-activator with MalT (Vidal-Ingigliardi and Raibaud, 1991). In CytR regulated promoters, cAMP−CRP constitutes an essential part of the repression apparatus and the available data suggest that two appropriately positioned CRP binding sites are a prerequisite for negative regulation by CytR (Søgaard-Andersen *et al.*, 1990a,b). It has consistently been shown that CytR and cAMP−CRP bind cooperatively to *deoP2* forming a nucleoprotein complex in which the repressor protein bridges the tandem DNA-bound cAMP−CRP complexes (Pedersen *et al.*, 1991; Søgaard-Andersen *et al.*, 1991a,b). The present study has brought forward a number of new aspects of the cAMP−CRP/CytR regulatory system. Below we discuss these aspects and their importance for the understanding of combinatorial regulation in other cAMP−CRP/CytR regulated promoters.

cAMP−CRP activation of cddP

We have distinguished two functional regions within *cddP* that are involved in cAMP−CRP activation of transcription. In the absence of the CytR repressor, cAMP−CRP binds to two targets (CRP-1 and CRP-2; Figure 1) and it is evident from our analysis of deletion mutants that the high affinity site, CRP-1, is the essential site for promoter activation (Table IA). However, full activation of *cddP* requires in addition an intact and precisely positioned CRP-2 target. Thus, mutant promoters in which CRP-2 has been deleted, inactivated by point mutations or rotated away from the original orientation all display a 2- to 3-fold reduced activity in the presence of the cAMP−CRP complex (Table I).

Two mechanisms have been proposed to underlie cAMP−CRP activation in promoters where cAMP−CRP is the only activator: direct protein−protein interaction between cAMP−CRP and the RNA polymerase, or DNA bending induced by cAMP−CRP (Schultz *et al.*, 1991). A unifying model for activation is difficult to reconcile with the ability of cAMP−CRP to activate transcription from sites lying at different positions within the various promoters (Gaston *et al.*, 1990; Ushida and Aiba, 1990; Valentin-Hansen *et al.*, 1991). Interestingly, several lines of evidence suggest that both types of mechanism may be involved in activation. On the one hand, the recent isolation of CRP mutants that cannot activate transcription but still bend the DNA like wt CRP point to the importance of direct interactions between cAMP−CRP and RNA polymerase (Bell *et al.*, 1990; Eschenlauer and Reznikoff, 1991). On the other hand, the observation that the CRP sites in *gal* and *lac* can be replaced by properly phased intrinsically bent

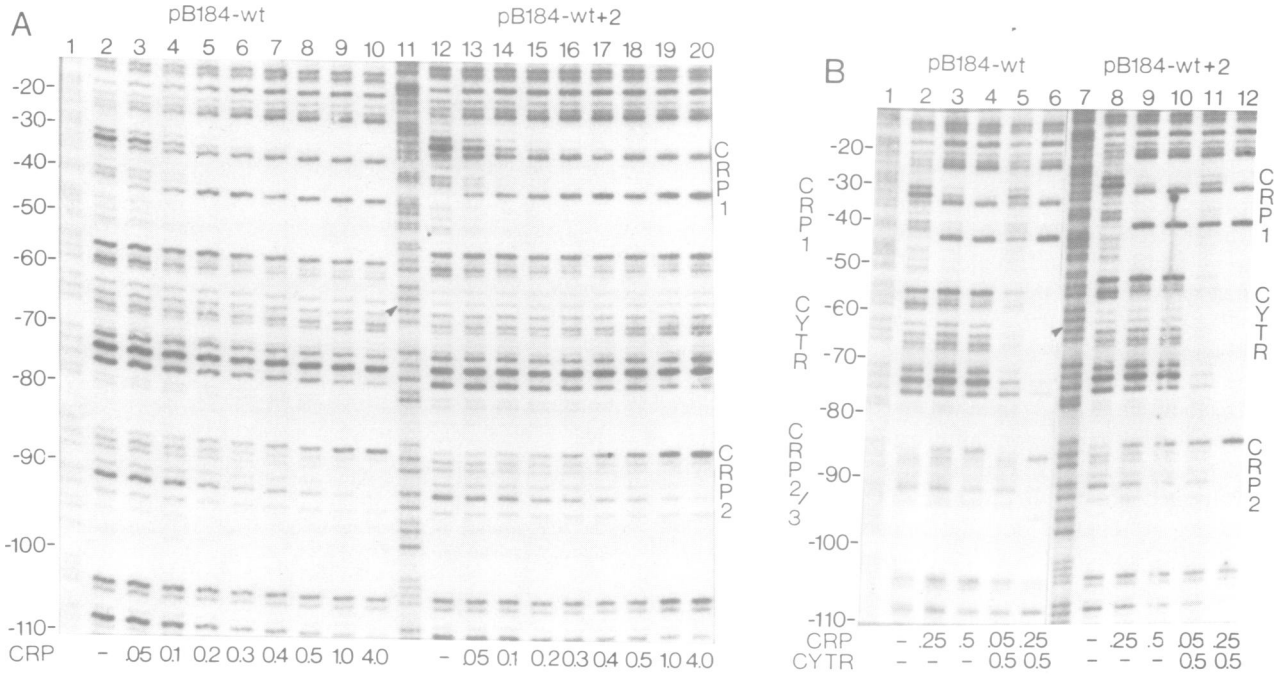


Fig. 7. Binding of cAMP–CRP and CytR to a *cddP* derivative containing 2 bp inserted between CRP-1 and CRP-2/3. *Eco*RI–*Bam*HI fragments labelled at the upper strand as described in Figure 3 were employed. Final concentrations of CRP and CytR are indicated in ng/μl. Regions protected by the proteins are indicated. Lanes 1 and 11 in A and lanes 1 and 7 in B are the A/G sequences of the probes. The arrow indicates the position of the 2 bp insertion. Note that the DNase I digestion pattern in the CRP2/3 region in the presence of CytR only changes in the wt promoter (panel B).

DNA sequences points to the importance of the cAMP–CRP induced DNA bend in the activation process (Bracco *et al.*, 1989; Gartenberg and Crothers, 1991). In *cddP*, CRP-1 is centred 41 bp upstream of the start site for transcription, a position that is optimal for activation in several promoters (Gaston *et al.*, 1990; Ushida and Aiba, 1990; Valentin-Hansen *et al.*, 1991). CRP-2 is centred around position −91, a location at which cAMP–CRP is unable to activate transcription as the sole activator in semi-synthetic promoters (Ushida and Aiba, 1990). In light of the results discussed above, it therefore seems reasonable to propose that cAMP–CRP bound at CRP-1 may act in the activation process by a combination of bending the DNA and by directly contacting the RNA polymerase whereas cAMP–CRP bound at CRP-2 may act to facilitate transcription initiation solely by bending the promoter DNA.

The structure of the repression complex

From the analysis of *deoP2* it has been proposed that the pentamer 5′-TGCAA plays an important role in sequence specific DNA binding of CytR (Pedersen *et al.*, 1991). This sequence motif is also present in the region protected by CytR in *cddP* (Figure 1B). The importance of this motif is strongly emphasized in this analysis as two of the mutations that result in decreased CytR regulation of *cddP* both map in the 5′-TGCAA box and in both cases, independent DNA binding by CytR is perturbed *in vitro*.

In addition to its role in activation, cAMP–CRP also plays a crucial role in negative regulation of *cddP*. cAMP–CRP and CytR bind cooperatively to *cddP* forming a nucleoprotein complex that covers 84 bp. These results are similar to those reported for *deoP2* (Pedersen *et al.*, 1991) and the *in vitro* analyses strongly indicate that the same type of repression complex, consisting of CytR sandwiched between tandem DNA-bound cAMP–CRP complexes, is involved in

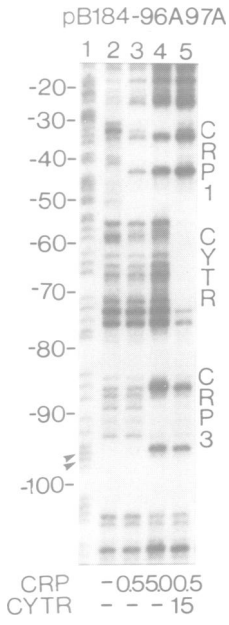


Fig. 8. Binding of cAMP–CRP and CytR to a *cddP* derivative containing a double mutation in the CRP2/3 region. The *Eco*RI–*Bam*HI fragment used was labelled at the upper strand as described in Figure 3. Final concentrations of CRP and CytR are indicated in ng/μl. Regions protected by the proteins are indicated. The two arrows indicate the position of the mutations. Lane 1 is the A/G sequence of the probe.

negative regulation of these two promoters. Despite these similarities there are significant structural and regulatory differences between *deoP2* and *cddP* that may have implications for the regulation of other cAMP–CRP/CytR controlled promoters. Firstly, the uppermost binding site, CRP-2, is indispensable for repression of *deoP2* (Søgaard-

Andersen *et al.*, 1990a), whereas upstream truncated *cddP* promoters are still regulated 10-fold by CytR (Table IA). Secondly, in *cddP* the CRP-1 target is the high affinity binding site whereas in *deoP2*, CRP-2 has the highest affinity for cAMP−CRP (Valentin-Hansen, 1982). As the cooperative binding of cAMP−CRP and CytR relies on both protein−DNA and protein−protein interactions and is dramatically reduced by mutations that affect these parameters (Søgaard-Andersen *et al.*, 1990a,b, 1991a; Søgaard-Andersen and Valentin-Hansen, 1991), this regulatory difference might be explained by the relative affinity of cAMP−CRP for its binding sites in the two promoters.

The third important observation was the existence of a third binding site, CRP-3, for cAMP−CRP that is absolutely required for full repression but dispensable for activation. *In vitro*, CRP-3 is only occupied in the presence of CytR and is located at a position relative to CRP-1 that has been shown to be optimal for the cooperative binding of cAMP−CRP and CytR in *deoP2* (Søgaard-Andersen *et al.*, 1991b). On the other hand, cAMP−CRP occupies CRP-2 in the absence of CytR *in vitro*. The simplest explanation for the CytR-induced repositioning of cAMP−CRP is that the formation of the complete repression complex involves an intermediate complex (which is similar to the complex observed in the promoter deleted for the CRP-2 region, pB188Δ79) in which the CRP-2/CRP-3 sites are unoccupied. In this complex, the presence of CytR forces cAMP−CRP to interact at CRP-3 by means of protein−protein interactions. These observation also imply that the organization of the different CRP sites in *cddP* makes it possible for the promoter to achieve both maximal activation and repression.

General considerations

The CytR regulated promoters exhibit a high level of structural diversity. The present description clearly shows that regulatory systems that involve synergistic action of multiple regulators can exhibit a high degree of flexibility, and the modes by which cAMP−CRP and CytR interact at *cddP* may illustrate how the repression complexes are formed at a set of promoters with different architecture (Gerlach *et al.*, 1990, 1991).

Repositioning of regulators on the DNA helix has only recently been recognized as an important feature in transcriptional regulation. In the divergent $malE_P-K_P$ promoter region, cAMP−CRP triggers a repositioning of MalT protein at $malK_P$ (Richet *et al.*, 1991). Interestingly, the binding of a low molecular weight effector to a regulator can also induce repositioning, as has recently been shown for AraC in the *araBAD* promoter (Lobell and Schleif, 1990). Finally, protein repositioning also plays a prominent role in Xis-induced inactivation of the intasome formed at *attP* in the lambda phage (Moitoso de Vargas and Landy, 1991). This ability of regulators to interact and induce binding to alternative targets may thus be a general mechanism to direct the formation of alternative nucleoprotein complexes.

Materials and methods

All enzymes used for DNA manipulations were purchased from Boehringer, Mannheim. ^{32}P-labelled nucleotides were obtained from NEN and Amersham. Isolation of plasmid DNA, cloning, transformation of *E.coli* and gel analyses of recombinant plasmids were performed as described by Maniatis *et al.* (1982). S1 nuclease mapping of start sites for transcription and purification and sequencing of ^{32}P-labelled fragments were performed as described (Valentin-Hansen *et al.*, 1984). Site-directed mutagenesis was performed according to Taylor *et al.* (1985)

Bacterial strains

All strains are *E.coli* K12 derivatives: SO928(Δ*deo*, Δ*lac*, *cytR*$^+$), SO929 (as SO928 but *cytR*$^-$) (Valentin-Hansen *et al.*, 1978), SO1316/F′*lacI*^{q} (*araD139*, *lacU169*, *cod*, *thi*, *rpsL*, *cdd*::ΔMu::λpl(209)/F′*proA*$^+$*B*$^+$, *lacI*^{q}*ZΔM15*::Tn*10*)(Søgaard-Andersen *et al.*, 1990b), RM1036(Δ(*lac-pro*), *thi*, *rpsL*, *supE*, *endA*, *sbcB*, *r*$^-$*m*$^-$, *mutD5*, *zaf13*::Tn*10*/F′*traD36*, *proAB*, *lacI*^{q}, *lacM15*) (isolated by R.Maurer and obtained through E.Bremer).

Plasmids

pB184: An *Nhe*I−*Bss*HII fragment extending from −184 to +214 in *cdd* was isolated from pcddΔBal (Valentin-Hansen *et al.* 1989), the 5′ overhangs were filled in with Klenow and cloned in the *Sma*I site of the low copy number protein fusion vector pJEL122 (Valentin-Hansen *et al.*, 1986). *pB188*: This was constructed as for pB184 except that an *Nhe*I−*Hga*I fragment extending from −184 to +188 was used. In all the fusion plasmids, the *cdd* sequence is flanked by unique *Eco*RI and *Bam*HI sites. *pUC13 derivatives:* The *Eco*RI−*Bam*HI *cddP* fragments from the fusion plasmids were cloned in the corresponding sites of pUC13 (Vieira and Messing, 1982). *pB184+2:* p13-184 was cut with *Mlu*I (which cleaves *cdd* at position −66), the 5′ overhangs were filled in with Klenow and ligated. The resulting plasmid was restricted with *Bss*HII (which cleaves *cdd* in the filled in *Mlu*I site) and *Eco*RI. The large *Bss*HII−*Eco*RI fragment was purified and ligated to the small *Eco*RI−*Mlu*I fragment from p13-184. From the resulting plasmid, p13-184+2, the *Eco*RI−*Bam*HI fragment was cloned in pJEL122 to give pB184+2. pB188-60C+2 was constructed as for pB184+2 except that p13-188-60C was the starting plasmid. The sequences of all new constructs were confirmed by DNA sequencing.

Isolation of deletion mutants

pB188 was restricted with *Eco*RI and subjected to S1 nuclease treatment at 37°C. At 5, 10 and 20 min, aliquots were collected and extracted with phenol. Following restriction with *Bam*HI the deleted fragments were cloned in the *Sma*I−*Bam*HI sites of the fusion vector pNM480 (Minton, 1984). The *Eco*RI−*Bam*HI fragment from each of these constructs was cloned in pJEL122. Using this strategy all deletions have the same sequence (5′-GAATTCCC-3′) upstream of the *cdd* DNA. The plasmids are named according to the endpoint of the deletion (Figure 1 and Table I). p2BI has been described previously (Valentin-Hansen *et al.*, 1989).

DNase I footprinting

Footprinting experiments were carried out as described by Galas and Schmidt (1978) with the changes described by Petersen *et al.* (1991). CRP and CytR were purified as described (Ghosaini *et al.*, 1988; Petersen *et al.*, 1991).

Acknowledgements

We thank Dorthe Dolleriis Pedersen for excellent technical assistance. This work was supported by The Danish Center for Microbiology and The Carlsberg Foundation. L.S.-A. was supported by a post-doctoral fellowship from the Danish Natural Science Research Council.

References

Bell,A., Gaston,K., Willams,R., Chapman,K., Kolb,A., Buc,H., Minchin,S., Willams,J. and Busby,S. (1990) *Nucleic Acids Res.*, **18**, 7243−7250.

Bracco,L., Kotlarz,D., Kolb,A., Diekmann,S. and Buc,H. (1989) *EMBO J.*, **8**, 4289−4296.

Dandanell,G., Valentin-Hansen,P., Løve-Larsen,J.E. and Hammer-Jespersen,K. (1987) *Nature*, **325**, 823−826.

de Crombrugghe,B., Busby,S. and Buc,H. (1984) *Science*, **224**, 831−838.

Eschenlauer,A.C. and Reznikoff,W.S. (1991) *J. Bacteriol.*, **173**, 5024−5029.

Galas,D.J. and Schmitz,A. (1978) *Nucleic Acids Res.*, **5**, 3157−3170.

Gartenberg,M.R. and Crothers,D.M. (1991) *J. Mol. Biol.*, **219**, 217−230.

Gaston,K., Bell,A., Kolb,A., Buc,H. and Busby,S. (1990) *Cell*, **62**, 733−743.

Gerlach,P., Valentin-Hansen,P. and Bremer,E. (1990) *Mol. Microbiol.*, **4**, 479−488.

Gerlach,P., Søgaard-Andersen,L., Pedersen,H., Martinussen,J., Valentin-Hansen,P. and Bremer,E. (1991) *J. Bacteriol.*, **173**, 5419–5430.
Ghosaini,L.R., Brown,A.M., and Sturtevant,J.M. (1988) *Biochemistry*, **27**, 5257–5261.
Hammer-Jespersen,K. (1983) In Munch-Petersen,A. (ed.), *Metabolism of Nucleotides, Nucleosides and Nucleobases in Microorganisms*, Academic Press, London, pp. 203–258.
Josephsen,J. and Hammer-Jespersen,K. (1981) *Mol. Gen. Genet.*, **182**, 154–158.
Keleher,C.A., Goutte,C. and Johnson,A.D. (1988) *Cell*, **53**, 927–936.
Lobell,R.B. and Schleif,R.F. (1990) *Science*, **250**, 528–532.
Maniatis,T., Fritsch,E.T. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Miller,H.J. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Minton,N.P. (1984) *Gene*, **31**, 269–273.
Moitoso de Vargas,L. and Landy,A. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 588–592.
Pedersen,H., Søgaard-Andersen,L., Holst,B. and Valentin-Hansen,P. (1991) *J. Biol. Chem.*, **266**, 17804–17808.
Reznikoff,W.S. and Abelson,J.N. (1978) In Miller,J.H. and Reznikoff,W.S. (eds), *The Operon*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 221–243.
Richet,E., Vidal-Ingigliardi,D. and Raibaud, O. (1991) *Cell*, **66**, 1185–1195.
Schultz,S.C., Shields,G.C. and Steitz,T.A. (1991) *Science*, **253**, 1001–1007.
Struhl,K. (1989) *Annu. Rev. Biochem.*, **58**, 1051–1077.
Søgaard-Andersen,L. and Valentin-Hansen,P. (1991) *Mol. Gen. Genet.*, **231**, 76–80.
Søgaard-Andersen,L., Martinussen,J., Møllgaard, N.E., Douthwaite,S.R. and Valentin-Hansen,P. (1990a) *J. Bacteriol.*, **172** 5706–5713.
Søgaard-Andersen,L., Møllegard,N.E., Douthwaite,S.R. and Valentin-Hansen,P. (1990b) *Mol. Microbiol.*, **4**, 1595–1601.
Søgaard-Andersen,L., Mironov,A.S., Pedersen,H., Sukhodelets,V.V. and Valentin-Hansen,P. (1991a) *Proc. Natl. Acad. Sci. USA*, **88**, 4921–4925.
Søgaard-Andersen,L., Pedersen,H., Holst,B. and Valentin-Hansen,P. (1991b) *Mol. Microbiol.*, **5**, 969–975.
Taylor,J.W., Ott,J. and Eckstein,F. (1985) *Nucleic Acids Res.*, **13**, 8765–8785.
Ushida,C. and Aiba,H. (1990) *Nucleic Acids Res.*, **18**, 6325–6330.
Valentin-Hansen,P. (1982) *EMBO J.*, **9**, 1049–1054.
Valentin-Hansen,P., Svenningsen,A., Munch-Pedersen,A. and Hammer-Jespersen,K. (1978) *Mol. Gen. Genet.*, **159**, 191–202.
Valentin-Hansen,P., Hammer-Jespersen,K., Boetius,F. and Svendsen,I. (1984) *EMBO J.*, **3**, 179–183.
Valentin-Hansen,P., Albrechtsen,B. and Løve-Larsen,J.E. (1986) *EMBO J.*, **5**, 2015–2021.
Valentin-Hansen,P., Holst,B., Josephsen,J., Hammer,K. and Albrechtsen,B. (1989) *Mol. Microbiol.*, **3**, 1385–1390.
Valentin-Hansen,P., Holst,B., Søgaard-Andersen,L., Martinussen,J., Nesvera,J. and Douthwaite,S.R. (1991) *Mol. Microbiol.*, **5**, 433–437.
Vidal-Ingigliardi,D. and Raibaud,O. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 229–233.
Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259–268.

Received on April 30, 1992; revised on June 12, 1992