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CRP interacts with promoter-bound σ^{54} RNA polymerase and blocks transcriptional activation of the *dctA* promoter

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The cAMP receptor protein (CRP) is an activator of σ^{70}-dependent transcription. Analysis of the σ^{54}-dependent *dctA* promoter reveals a novel negative regulatory function for CRP. CRP can bind to two distant sites of the *dctA* promoter, sites which overlap the upstream activator sequences for the DctD activator. CRP interacts with Eσ^{54} bound at the *dctA* promoter via DNA loop formation. When the CRP-binding sites are deleted, CRP still interacts in a cAMP-dependent manner with the stable Eσ^{54} closed complex via protein–protein contacts. CRP is able to repress activation of the *dctA* promoter, even in the absence of specific CRP-binding sites. CRP affects both the final level and the kinetics of activation. The establishment of the repression and its release by the NtrC activator proceed via slow processes. The kinetics suggest that CRP favours a new form of closed complex which interconverts slowly with the classical closed intermediate. Only the latter is capable of interacting with an activator to form an open promoter complex. Thus, Eσ^{54} promoters are responsive to CRP, a protein unrelated to σ^{54} activators, and the repression exerted is the direct result of an interaction between Eσ^{54} and the CRP–cAMP complex.

Keywords: cAMP–CRP repression/Eσ^{54} holoenzyme-regulated promoters/loop formation/protein–protein interactions/regulation in *E.coli*

Introduction

Transcription initiation is a major point at which gene expression is regulated. The *Escherichia coli* cAMP receptor protein (CRP) was identified initially as an activator of σ^{70}-dependent transcription at promoters for catabolic operons. It was later found that CRP participates in much wider regulatory networks. An ever increasing number of CRP-related transcription factors has been identified from different micro-organisms (for a review, see Kolb *et al.*, 1993). This evolutionary duplication and divergence highlights the importance of understanding the mechanisms of CRP-mediated regulation and, in particular, its possible role in transcription mediated by RNA polymerase containing sigma factors other than σ^{70}.

When the dimeric CRP protein binds to its DNA target sites, it can act as the sole transcriptional activator of σ^{70}-dependent promoters. In such cases, it normally binds to a site centred at either four, six, seven or eight helical turns upstream from the transcription start site (Gaston *et al.*, 1990; Ushida and Aiba, 1990; Valentin-Hansen *et al.*, 1991; Déthiollaz *et al.*, 1996). In the absence of CRP, the promoter often displays a low affinity for σ^{70} RNA polymerase holoenzyme (Eσ^{70}). In the presence of CRP, CRP and Eσ^{70} bind cooperatively to the promoter. The magnitude of this synergistic effect generally reflects the extent of activation. It involves direct contact between site-bound CRP and Eσ^{70} (Heyduk *et al.*, 1993). CRP mutants defective in activation, but still proficient in DNA binding, have been located on a surface-exposed loop of CRP, called 'activating region 1' (ARI) (Bell *et al.*, 1990; Eschenlauer and Reznikoff, 1991; Zhou *et al.*, 1993). This region contacts a patch on the C-terminal region of an α subunit of Eσ^{70} (Tang *et al.*, 1994; Murakami *et al.*, 1996; for reviews, see Ishihama, 1992; Ebright and Busby, 1995). When the CRP-binding site is centred around position –40, activation by CRP then requires an additional interaction between a second, promoter class-specific activating region of CRP (activating region 2) (ARII) and the N-terminal domain of an α subunit of Eσ^{70} (Niu *et al.*, 1996; for a review, see Busby and Ebright, 1997).

The *Rhizobium meliloti dctA* gene encodes the permease of the C_4-dicarboxylic acid transport system, essential for symbiotic nitrogen fixation. The *dctA* promoter is σ^{54} dependent. This sigma factor is genetically widely divergent from the σ^{70} family proteins (Merrick, 1993). Promoter sequences recognized by Eσ^{54} are well conserved (TGGCAC N5 TTGCa/t situated between –26 and –11 bp) and distinct from classical –35, –10 σ^{70}-type consensus promoters (Merrick, 1993). Eσ^{54} is the only known form of eubacterial RNA polymerase which shows some functional resemblance to eukaryotic RNA polymerases (Merrick, 1993). The Eσ^{54} polymerase binds to its cognate promoters as a transcriptionally inactive closed complex. Transcriptional activation of the *dctA* promoter usually depends on phosphorylated DctD. This protein is a member of the NtrC and NifA transcriptional activator protein family (Morett and Segovia, 1993; North *et al.*, 1993). Activators of σ^{54}-dependent transcription are able to catalyse the isomerization of closed complexes formed between Eσ^{54} and a promoter, in a reaction requiring hydrolysis of ATP (or other NTPs) on the activator. The activators bind to upstream activator sequences (UASs), typically located $>$100 bp away from the transcription start site of σ^{54}-dependent promoters. For instance, the *dctA* UAS contains two DctD-binding sites, centred at positions –110.5 and –143.5 bp, respectively (Ledebur *et al.*, 1990). Experiments

with NifA- and NtrC-regulated promoters indicate that their UASs are enhancer-like elements which can work up to at least 1 kb away from the promoter, in either orientation (Buck *et al.*, 1986; Reitzer and Magasanik, 1986). Activation of transcription is often face-of-the-helix dependent, suggesting that the upstream 'enhancer'-bound activator contacts promoter-bound Eσ^{54} via formation of a DNA loop (Buck *et al.*, 1987; Minchin *et al.*, 1989; Perez-Martin *et al.*, 1994b; for a review, see Perez-Martin *et al.*, 1994a). This DNA loop has been visualized directly by electron microscopy in the case of the *glnA*P2 promoter (Su *et al.*, 1990). Protein cross-linking studies suggest that DctD interacts with σ^{54} and the β subunit of Eσ^{54} (Lee and Hoover, 1995). Activation of the *dctA* promoter can also be observed both *in vivo* and *in vitro* with NtrC (Allaway *et al.*, 1995; Y.-P.Wang, unpublished results). This activator may interact with the Eσ^{54}-closed complex either directly from solution or when bound to a non-specific site on DNA (see Huala and Ausubel, 1989; Huala *et al.*, 1992; Allaway *et al.*, 1995; Jovanovic *et al.*, 1996; for *in vitro* evidence, see Berger *et al.*, 1994; North and Kustu, 1997). Activators of Eσ^{54} share a strongly conserved central domain of 238 amino acids which is believed to interact directly with Eσ^{54} and thus stimulate transcription from σ^{54}-dependent promoters. However, the Eσ^{54} activator NtrC fails to activate σ^{70}-dependent transcription at *lac* (Ray *et al.*, 1990).

It was observed previously that CRP repressed the *dctA* promoter when the *R.meliloti dct* system was reconstituted in *E.coli* (Wang *et al.*, 1993). A direct interaction of CRP with the *dctA* promoter was demonstrated by gel mobility shift assays (Wang *et al.*, 1993). Sequence analysis suggested that one mechanism by which CRP could repress the *dctA* promoter was through competition with DctD for occupancy of the UAS (Wang *et al.*, 1993). In order to establish the protein and DNA interaction patterns occurring in this system, we have performed a series of *in vitro* footprinting experiments on the *dctA* promoter with CRP, DctD and Eσ^{54} proteins. Experiments were designed to investigate the role of the putative CRP-binding sites and the mechanism of the repression exhibited by the CRP–cAMP complex *in vitro*. Our results, consistent with our *in vivo* data, reveal surprising features in this unorthodox partnership. CRP is able to interact *in cis* from remote sites and *in trans* with the Eσ^{54} closed complex, an interaction kinetically linked to its repression effect.

Results

CRP binds to the UAS of the dctA promoter with low affinity in a non-cooperative manner

We first identified CRP-binding sites on the *dctA* promoter using DNase I footprinting techniques. Based on the statistical analysis of known CRP-binding sites developed by Berg and von Hippel (1988), several putative CRP-binding sites were proposed for the *dctA* promoter DNA region from bp –178 to +42 (Wang *et al.*, 1993). DNase I footprinting revealed two binding sites separated by 50 bp, centred at positions –160.5 (CRP-site 1) and –110.5 (CRP-site 2) (Figure 1). These two remote sites are the best candidates from theoretical analysis (Wang *et al.*, 1993). Both sites cover ~25 bp. Phosphate bonds

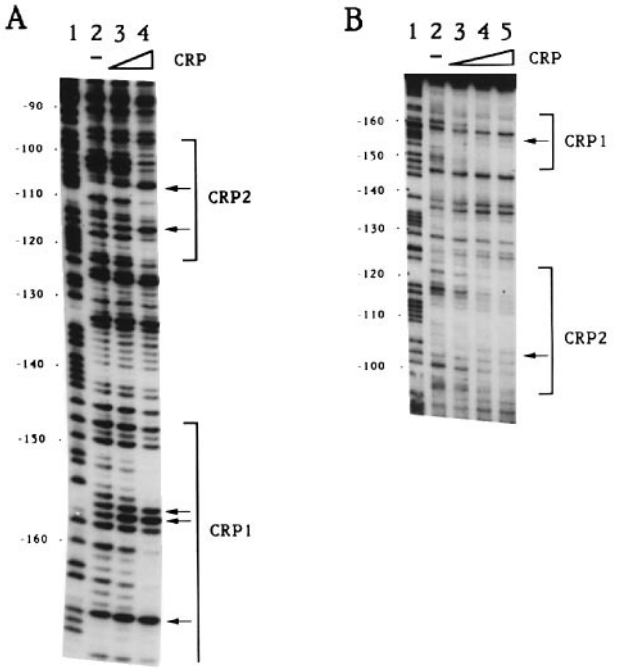

Fig. 1. Binding of CRP–cAMP in the regulatory region located upstream from the *dctA* promoter. 3′ end-labelled DNA fragment was mixed with increasing concentrations of CRP in the presence of 0.2 mM cAMP. After incubation for 20 min at 30°C, the reaction mix was treated with DNase I as described in Materials and methods. Samples were analysed on a denaturing 7.5% (w/v) polyacrylamide gel, which was calibrated using a Maxam and Gilbert (1980) sequencing reaction for G+A (lane 1 of A and B). The *dctA* promoter region is numbered with respect to the consensus TGGCAC N5 TTGCa/t from –26 to –11 (Merrick, 1993). The brackets show the location of the two CRP-binding sites, and the arrows indicate the hypersensitive DNase I bands. (**A**) Bottom strand. The concentrations of CRP were 0, 50 and 400 nM in lanes 2, 3 and 4 respectively. (**B**) Top strand. The reactions were performed with 0, 50, 150 and 400 nM of CRP in lanes 2, 3, 4 and 5, respectively.

hypersensitive to DNase I are evident within the CRP-protected region. For instance, on the bottom strand (Figure 1A), in each binding site the hypersensitive bonds are spaced by 9–10 bp. On the top strand (Figure 1B), some hypersensitive bonds are also detected displaced by 2–3 bp in the 3′ direction with respect to the bottom strand. These hypersensitive sites are due to minor groove widening caused by CRP-induced kinks and are found in all known CRP-binding sites (Schultz *et al.*, 1991). At the *dctA* promoter, both sites exhibit a low affinity for CRP, ~2-fold lower than the mutant *lac* L8 site (Fried and Crothers, 1983; Kolb *et al.*, 1983). As the CRP concentration is raised, the two sites are equally and independently populated (see Figure 1), showing no evidence of synergistic effects.

It has been proposed previously that CRP represses the *dctA* promoter through competition with the transcriptional activator DctD for binding to their sites. We have confirmed the location of the DctD-binding sites centred at –110.5 and –143.5 (Ledebur *et al.*, 1990; see Figure 4) by DNase I footprinting and have effectively shown that CRP bound at sites 1 and 2 could be competed away by increasing the concentration of unphosphorylated DctD (data not shown). As DctD binds cooperatively and strongly to its two adjacent sites (Ledebur and Nixon, 1992; Scholl and Nixon, 1996), it seems doubtful that the competition

between CRP and DctD should favour CRP binding rather than DctD occupancy *in vivo* unless a strong synergy could stabilize CRP binding.

Long distance interactions between CRP and Eσ^{54} on the dctA promoter enhance CRP binding to its target sites

Both *in vivo* and *in vitro* Eσ^{54} forms a closed complex as it occupies its specific promoter (Reitzer *et al.*, 1987; Sasse-Dwight and Gralla, 1988; Morett and Buck, 1989; Popham *et al.*, 1989). Because CRP was bound at unusual distances from the polymerase-binding site, but at upstream locations where activators of Eσ^{54} do bind, we investigated whether Eσ^{54} could stabilize CRP binding.

The binding of Eσ^{54} on the *dctA* promoter fragment was established with DNase I footprinting experiments (Figure 2). The results confirm that Eσ^{54} binds to the *dctA* promoter sequence (–12, –24 region, Figure 2). The protected phosphate region encompasses 30–35 bp (from –36.5 to –4.5 on the top strand and from –39.5 to –5.5 on the bottom strand). The leader-transcribed DNA region is not protected against DNase I attack. This is consistent with the structure of closed complexes observed at other σ^{54}-dependent promoters (see, for example, Popham *et al.*, 1989; Buck and Cannon, 1992). By contrast, Eσ^{70} does not bind the *dctA* promoter. Also, under our experimental conditions, σ^{54} alone does not bind to the *dctA* promoter sequence even at very high concentrations (800 nM, data not shown). This may be due to the absence of, within the *dctA* promoter, specific DNA sequences required for core polymerase-independent binding of σ^{54} (Buck and Cannon, 1992). In short, these experiments demonstrated that Eσ^{54}, but neither σ^{54} nor Eσ^{70}, binds to the *dctA* promoter.

The CRP–cAMP complex was then included in the reaction mixture. DNase I footprinting patterns showed that the binding of Eσ^{54} plus CRP–cAMP to the *dctA* promoter induces striking changes when compared with patterns obtained with each protein alone. A periodic DNase I protection and hypersensitivity is now observed in the intervening DNA between the UAS and the promoter sequences (Figure 2A, compare lanes 3, 6 and 7). The same pattern is observed regardless of which protein, Eσ^{54} or CRP, is added to the reaction mixture first. Hypersensitive bands lie every 10–11 bp, i.e. one helical turn apart and therefore on the same face of DNA. The enhanced and diminished cleavages are separated by 5 bp. They reflect the consecutive widenings and narrowings of the minor groove on the outside and inside of a bent DNA structure as typically detected by DNase I when a DNA loop is formed between two proteins bound to DNA (see Figure 2; Hochschild and Ptashne, 1986). Such periodic changes in DNase I cutting strongly suggest here the formation of a DNA loop between the upstream-bound CRP molecules and the promoter-bound Eσ^{54}. Direct interactions between CRP and Eσ^{54} on the *dctA* promoter are likely to provide the energy for the formation and stabilization of such a loop. Contacts between CRP and Eσ^{54} are also supported by the presence of additional hypersensitive bands appearing in the intervening DNA between the two CRP-binding sites (Figure 2B and C). Furthermore, quantitative scans of DNase I footprints reveal that the binding affinity of CRP at its target sites is clearly increased by the presence of Eσ^{54} (Figure 2D and data not shown). We estimate that the increase in CRP-binding affinity is between 5- and 10-fold at the CRP-site 2. The quantification of binding at the distal CRP-site 1 is less accurate due to the proximity of the *Eco*RI terminus; however, we observe at least a 3-fold enhancement in the affinity of CRP for this site. Thus, the long distance interactions between Eσ^{54} and CRP result in a marked stabilization of CRP binding at both of its specific upstream target sites.

We confirmed that an interaction was indeed taking place between DNA-bound Eσ^{54} and CRP. Since some interactions between DNA-bound CRP and Eσ^{70} can occur (Heyduk *et al.*, 1993), we first ruled out a slight σ^{70} contamination in our core RNA polymerase preparation. When 1 μg of anti-σ^{70} polyclonal IgG was added to the reaction mixture together with 50 nM Eσ^{54} and 50 nM CRP, the footprinting patterns were not altered. We also established that the looping and stabilization effect required the presence of Eσ^{54} holoenzyme. No effect could be observed when Eσ^{54} was replaced by σ^{54} alone. Also, when Δσ^{54}, a C-terminal deleted version of σ^{54} (Cannon *et al.*, 1995), was reconstituted with the core enzyme, the resulting polymerase does not bind *per se* to the promoter and, subsequently, the looping and stabilization of CRP binding is abolished (data not shown). A significant and specific interaction does, therefore, occur between DNA-bound Eσ^{54} and CRP at the *dctA* promoter, resulting in an increased CRP affinity for its two target sites, and very probably in the formation of a DNA loop.

CRP can interact directly with promoter-bound Eσ^{54} in the absence of its target sites and in a cAMP-dependent fashion

In principle, the *cis* interactions described above could include an activity functional in *trans*. We examined whether CRP binding to its target sites is essential for CRP–Eσ^{54} interaction on the *dctA* promoter. We have conducted gel mobility shift assays under conditions similar to those used for footprinting assays. The experiment was carried on the 'core' *dctA* promoter, which is a 94 bp DNA fragment containing from –40 to +42 of the *dctA* promoter and some multiple cloning sites from plasmid pUC18, and thus lacking the CRP-binding sites [both the two identified from this study and the seven others suggested from previous statistical analysis (Wang *et al.*, 1993)]. The results indicate that first, in the absence of CRP, Eσ^{54} alone can form stable closed complexes on the 'core' *dctA* promoter (Figure 3A, lane 2, and B, lanes 2–4). In contrast, σ^{54} alone or core RNA polymerase alone does not give any distinct complexes. These results are consistent with results obtained with DNase I footprinting assays. Secondly, although CRP alone cannot bind to the DNA fragment, even in the presence of 200 μM cAMP, and although no additional complexes stable under the gel shift conditions can be observed between CRP, 200 μM cAMP and the closed Eσ^{54}–DNA complex, the presence of CRP and cAMP enhances stable complex formation between Eσ^{54} and the 'core' promoter 3- to 5-fold. The stabilization effect can be demonstrated either by varying the CRP concentration (Figure 3A, lane 2, Eσ^{54} alone versus lanes 3–5, Eσ^{54} with increasing concentrations of CRP), or through titration of Eσ^{54} (Figure 3B,

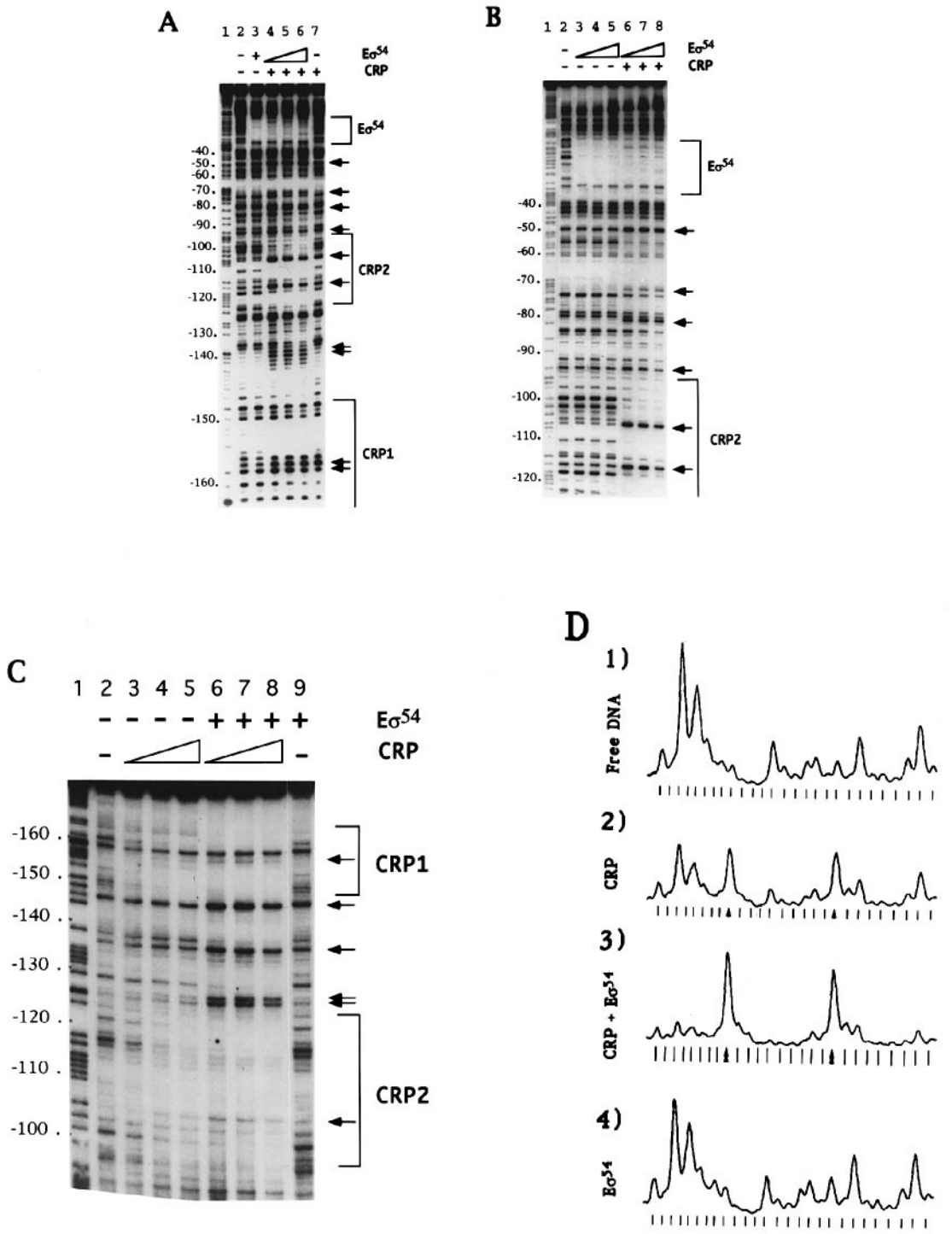

Fig. 2. Interactions between CRP–cAMP bound at the UAS region and Eσ^{54} bound at the *dctA* promoter as probed with DNase I footprinting: evidence for DNA loop formation in the intervening region of DNA. The experimental conditions are the same as described in Figure 1, except here the CRP final concentration was 50 nM when added. Lane 1, G+A reaction. Lane 2, no protein added. The CRP-binding sites are bracketed and the arrows point to DNase I-hypersensitive bands induced by CRP with or without Eσ^{54}. (**A**) Bottom strand: overall footprint from positions –170 to +20. Eσ^{54} was added at various concentrations (lane 2, 0 nM; lane 3, 150 nM; lane 4, 25 nM; lane 5, 75 nM; lane 6, 150 nM; lane 7, 0 nM) in the absence or presence of 50 nM CRP (lanes 4–7). Note the presence of DNase I-hypersensitive bands every 10–11 bp at positions –53.5, –73.5, –83.5, –94.5, –106.5, –116.5, –135.5, –136.5, –147.5, –157.5, –158.5 and –166.5. The gel also illustrates the increase in occupancy of both CRP sites in the presence of Eσ^{54} (compare lanes 6 and 7). (**B**) Bottom strand: extended autoradiogram showing Eσ^{54} binding and CRP-binding sites. Titration with increasing concentrations of Eσ^{54} (lanes 3 and 6, 25 nM; lanes 4 and 7, 75 nM; lanes 5 and 8, 150 nM) were performed in the absence (lanes 3–5) or presence of 50 nM CRP (lanes 6–8). The appearance of the hypersensitive bands at positions –53.5, –73.5 and –83.5 is only seen in the presence of both Eσ^{54} and CRP. (**C**) Top strand: CRP interacts with Eσ^{54}. Labelled DNA was incubated with increasing concentrations of CRP (lanes 3 and 6, 50 nM; lanes 4 and 7, 150 nM; lanes 5 and 8, 400 nM). Eσ^{54}, when added, was present at a final concentration of 150 nM. (**D**) Scans of the CRP2-binding site footprints under various conditions. Lanes 2, 3, 6 and 7 of the autoradiogram shown in (A) were analysed with a PhosphorImager. Arrows correspond to DNase I-hypersensitive bands induced by CRP with or without Eσ^{54}.

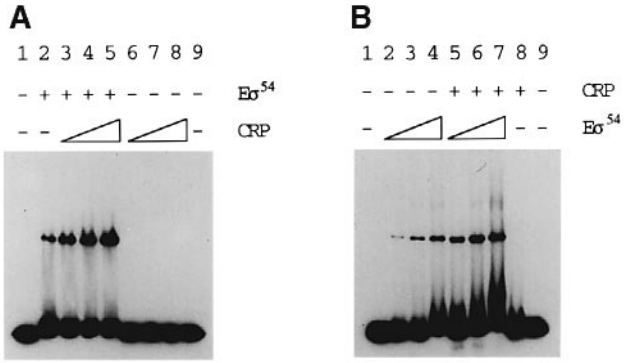

Fig. 3. Direct interactions between CRP–cAMP and Eσ^{54} bound at the 'core' *dctA* promoter as probed with gel mobility shift assays: evidence for the CRP–cAMP-mediated enhancement of stable complex formation between Eσ^{54} and the 'core' *dctA* promoter. Gel mobility shift assays were performed as described in Materials and methods. (**A**) Demonstration of the CRP–cAMP-mediated effect on stable complex formation between Eσ^{54} and the 'core' *dctA* promoter through titration of CRP. Eσ^{54}, when added, was present at a concentration of 70 nM. CRP concentrations were at 37.5 nM for lanes 3 and 6; 75 nM for lanes 4 and 7; and 150 nM for lanes 5 and 8. The DNA concentration was at 3 nM. (**B**) Demonstration of the CRP–cAMP-mediated effect on stable complex formation between Eσ^{54} and the 'core' *dctA* promoter through titration of Eσ^{54}. CRP, when added, was present at a concentration of 75 nM. Eσ^{54} concentrations were at 4 nM for lanes 2 and 5; 12 nM for lanes 3 and 6; and 36 nM for lanes 4 and 7. The DNA concentration was at 0.1 nM.

compare respectively lanes 2–4, Eσ^{54} alone, with lanes 5–7, in the presence of CRP). A stabilization of similar magnitude is observed in either case. A measure of the residence time of the enzyme within the closed complex confirms the synergy. Challenge with a polynucleotide, poly[d(I–C)] at a concentration of 100 μg/ml, was performed in the presence or absence of CRP for various times. The half-life of the retarded complex was found to be ~30 min in the presence of CRP and 12 min in its absence. Since the addition of poly[d(I–C)] rapidly removes CRP molecules bound in a non-specific manner to a double-stranded DNA sequence, non-specific contacts between CRP and the upstream-deleted 'core' *dctA* promoter provide only a marginal energetic contribution to the stability of the CRP–Eσ^{54}–promoter DNA complex.

The Eσ^{54}–CRP interaction specifically requires a promoter-bound Eσ^{54}. Under conditions similar to the gel shift, but in the absence of the promoter DNA, with up to 2 μM CRP, specific complexes between CRP, cAMP and Eσ^{54} and/or any changes of the Eσ^{54} protein band were not detected on native protein gels. Similarly, negative results were obtained when σ^{54}, or core polymerase, was used in the assay with CRP and the promoter DNA fragment. We conclude that CRP is able to interact specifically with promoter-bound Eσ^{54}, and stabilizes the complex formed between Eσ^{54} and the promoter, without being itself bound to its upstream DNA target sites. Direct protein–protein interactions best explain the synergy.

All the above experiments were performed in the presence of 200 μM cAMP since it was thought that the CRP–cAMP complex was in fact mediating this interaction. This point was investigated further. Firstly, cAMP was omitted from the running buffer of the gel retardation assay described in Figure 3. This resulted in the loss of the CRP-dependent stabilization of the Eσ^{54}–DNA complex. Secondly, at a fixed concentration of CRP, the concentration of cAMP in the reaction mixture was decreased gradually from 200 to 2 μM. An electrophoresis performed under conditions similar to those in Figure 3 showed that the enhancement of closed complex formation by CRP was gradually abolished. Half saturation of the closed complex stabilization occurred at 10 μM cAMP and 75 nM CRP, a concentration similar to the one required to observe binding of the CRP–cAMP complex to its target site at the *gal*P1 promoter under the same conditions (results not shown).

CRP can repress the dctA promoter in vitro

In order to study the influence of CRP and its DNA-binding sites on the enzymatic activity of Eσ^{54} at the *dctA* promoter, a transcription assay system was constructed. Into plasmid pOM90, which contains strong transcription terminators on both sides of an *Eco*RI restriction site, the entire *dctA* promoter sequence from position –178 to +42 was inserted in both orientations (designated as pYP101 and pYP102 respectively). The sequence upstream from –40 of the *dctA* promoter was then deleted from pYP102 (designated as pYP102ΔU) (for details see Materials and methods).

Supercoiled plasmids were prepared and used for *in vitro* single round transcriptional assays (detailed in Materials and methods). As expected, no transcript could be detected with Eσ^{54} alone or in the presence of CRP. The constitutive S160F mutant NtrC protein was used as a transcriptional activator (Popham *et al.*, 1989; Weglenski *et al.*, 1989; Dixon *et al.*, 1991). The *dctA* promoter lacks any obvious NtrC-binding site and, consistently, DNase I footprinting experiments show that the NtrC^{S160F} protein does not bind detectably to the *dctA* promoter fragment (data not shown). In a first series of transcriptional assays, the proteins were incubated for a fixed amount of time (10 min). In the absence of CRP, insert-dependent transcripts with the expected size were detected. Depending on the orientation of the *dctA* promoter, they are either 111/112 nucleotides long (for pYP101) or 167/168 nucleotides long (for pYP102) (Figure 4A, lanes 3 and 4). No differences could be detected in terms of the size or amount of transcript when pYP102ΔU was compared with its parent plasmid pYP102.

CRP-mediated repression was then examined using pYP102 and its derivative pYP102ΔU. When NtrC^{S160F} is first allowed to form open complexes with Eσ^{54} and the *dctA* promoter DNA, a subsequent addition of CRP for 10 min has little influence on the efficiency of transcription, regardless of whether the upstream sequences are present or not (Figure 4B). However, when CRP is first incubated with Eσ^{54} and DNA, a reduced level of transcription is observed on subsequent addition of NtrC^{S160F} for a fixed amount of time (10 min) (Figure 4C). The repression is ~10-fold for pYP102, which contains the wild-type *dctA* promoter, and 5-fold for pYP102ΔU, where the upstream region of the *dctA* promoter has been deleted. Repression was again found to be cAMP dependent, in agreement with the previous observations on the stability of the polymerase–DNA complex.

In order to show that this effect was not specific for a given activator, NtrC^{S160F} was replaced by a different transcriptional activator, PSPFΔHTH. Repression was

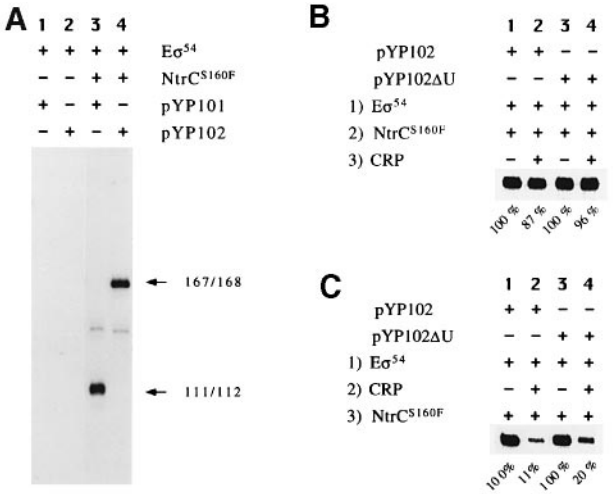

Fig. 4. *In vitro* single round transcriptional assays of the *dctA* promoter. Run-off assays were performed as described in Materials and methods: in each case, the template DNA concentration was at 1 nM. cAMP, ATP, Eσ^{54} and NtrC^{S160F}, when added, were present at a final concentration of 0.2 mM, 2 mM, 25 nM and 100 nM respectively. Reaction mixtures were incubated for 10 min at 30°C prior to addition of a mixture containing heparin and nucleoside triphosphates containing [α-^{32}P]UTP. Incubation was continued for another 10 min to allow synthesis of transcripts. The samples were ethanol precipitated and loaded onto a 7.5% polyacrylamide sequencing gel. (**A**) NtrC^{S160F}-dependent *in vitro* transcriptional activation of the *dctA* promoter. Plasmids pYP101 and pYP102 are supercoiled DNA templates. They are derivatives of pOM90 in which the *dctA* promoter has been cloned in different orientations between two divergent terminators. The size of the *dctA* transcripts from both constructs is indicated by arrows. Note the presence of a faint σ^{54}-dependent transcript from the pOM90 vector (see lanes 3 and 4). (**B**) CRP, when added after NtrC^{S160F}, has little effect on *dctA* expression. Incubation of pYP102, for lanes 1 and 2, or pYP102ΔU, for lanes 3 and 4, Eσ^{54}, NtrC^{S160F}, ATP and cAMP was for 10 min at 30°C before addition of CRP (final concentrations of 150 nM for lanes 2 and 4). The reaction mixture was incubated for another 10 min before addition of heparin and nucleoside triphosphates. The numbers beneath each lane are arbitrary units of transcription obtained by scanning bands corresponding to full-length transcripts and normalizing the data relative to the level of transcription without CRP. (**C**) CRP, when added before NtrC^{S160F}, represses the *dctA* promoter. Same as in (B) except for the order of addition of the reactants: template, Eσ^{54}, ATP and cAMP, with or without CRP were pre-incubated for 10 min at 30°C before addition of NtrC^{S160F}. The reaction mixture was incubated for another 10 min before run-off transcription was allowed to proceed.

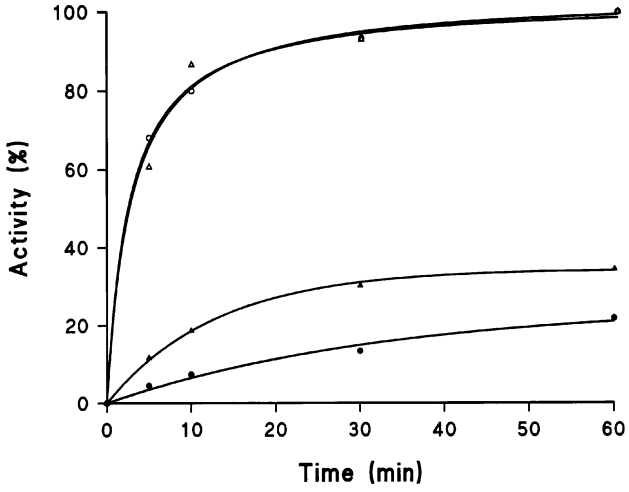

Fig. 5. CRP effect on the kinetics of activation by NtrC^{S160F} of the *dctA* promoter. The conditions were the same as in Figure 4C, except Eσ^{54}, ATP and cAMP, with or without CRP, were pre-incubated for 30 min at 30°C before addition of NtrC^{S160F}. The reaction mixture was then incubated for various times (as indicated in the figure) before run-off transcription was allowed to proceed. The activity, *A*, was characterized by the amount of corresponding transcript and normalized with respect to a common standard (promoter activity for an incubation time of 90 or 60 min with the activator, called here A_∞). A is plotted here as a function of the incubation time *t*. Upper curves: activation profile observed with the intact (○) or the upstream deleted (△) *dctA* promoter in the absence of CRP. Curves are better fitted by a hyperbola of the type $A/(A_\infty - A) = k\mathrm{Cot}$ (*k* second order rate constant, Co concentration of the activator) than by an exponential. $A_\infty = 100$ in both cases. The half-times $\tau_- = (k\mathrm{Co})^{-1}$ are equal respectively to 2.9 min when the assay is performed on the upstream deleted promoter and to 2.7 min on the intact promoter. Lower curves: activation profiles observed after incubation with CRP on the intact (•) or on the upstream deleted (▲) promoter. Points are fitted, within experimental error, by an exponential response of the type $A = A_\infty (1 - e^{-t/\tau+})$ For the upstream deleted promoter, $A_\infty = 34$, $\tau_+ = 13$ min. For the intact promoter, $A_\infty = 25$, $\tau_+ = 30$ min. When activation reaches a plateau, the extent of inhibition is therefore 3-fold for the upstream-deleted promoter and 4-fold for the intact promoter. Under initial velocity conditions, the extent of inhibition is measured by the relative slopes of the response or $(A_\infty/\tau_-)/(A_\infty/\tau_+)$, giving factors of inhibition of 13 for the upstream-deleted promoter, and of 51 for the intact promoter.

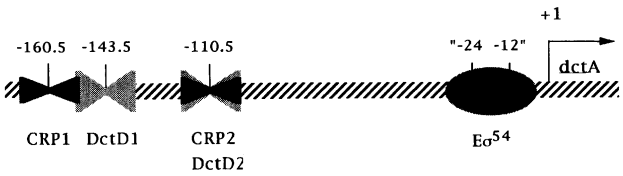

Fig. 6. Organization of the *dctA* promoter. The sequence is numbered as described in Figure 1; the position +1 indicates the start of *dctA* transcription. The Eσ^{54} site is represented as a shaded oval. CRP and DctD sites are represented by dark and light box-shaped symbols, respectively, centred at positions –160.5 and –110.5 for the former, and at positions –143.5 and –110.5 for the latter. Note the perfect overlap of CRP2 and DctD2 sites.

again observed, though at a reduced level (25% residual activity at 150 nM CRP). PSPFΔHTH is fully defective in DNA binding (Jovanovic *et al.*, 1996). The closed complex between Eσ^{54} and the minimal *dctA* promoter can therefore be either activated (by PSPFΔHTH and NtrC), or repressed (by the cAMP–CRP complex) through protein–protein interactions.

Because the extent of repression was strongly dependent on the order of addition of the factors, experiments were conducted to follow NtrC activation of the closed complex as a function of time in the absence or presence of CRP. As shown in Figure 5, in the absence of CRP, NtrC requires a rather long time to establish its full level of activation. The kinetics are better fitted with a hyperbolic response, representative of a second order process [NtrC is known to form an oligomer as it activates (Wyman *et al.*, 1997)]. These profiles are similar for the intact and upstream-deleted promoters (with a half-time of 2.7 min for the intact, and 2.9 min for the upstream-deleted promoter, respectively).

NtrC was then incubated first with the closed complex for 30 min, and CRP added second for various times before performing the run-off experiments. In both cases, times longer than 30 min were required to see the establishment of a significant inhibition by CRP (data not

shown). Lastly, the promoters were incubated with $E\sigma^{54}$ and CRP for 30 min and then with NtrC for various times. In both cases, only a partial restoration of activity was observed (as shown in Figure 5). For the upstream-deleted 'core' promoter, the final activity corresponds to one-third of the level observed in the absence of CRP. Activity is also established with a kinetic profile compatible with a slow reorganization of the complex (exponential kinetic with a characteristic time of 13 min instead of 2.9 min for the control). These two characteristics were accentuated when the intact promoter was used. The plateau value, corresponding to 25% of the control, is now established with a characteristic time of 30 min (see legend of Figure 5).

Therefore, a close parallel is observed between the CRP-binding assays and the transcription experiments. The gel retardation experiments demonstrate that CRP can interact directly with promoter-bound $E\sigma^{54}$ in the absence of its target sites (*trans* effect, Figure 3). This interaction '*in trans*' also results in the lengthening of the residence time of the enzyme within the closed complex. The *in vitro* transcription experiments reveal in parallel a partial inhibition with the promoter devoid of its CRP-binding sites (Figures 4 and 5). When the specific sites are reintroduced *in cis*, the DNase I footprinting experiments indicate an enhancement of CRP binding to its target sites in the presence of the enzyme, as well as the establishment of a DNase I cutting pattern consistent with the formation of a DNA loop (Figure 2). Run-off assays performed from this intact promoter show that the level of activation in the presence of CRP is reduced further, and takes place even more slowly (Figures 4 and 5). These experiments also indicate that activation by NtrC as well as repression by CRP are always established very slowly. As a result, the extent of repression monitored after a given period of incubation generally depends on the order of addition of the two proteins. We conclude that the mode of CRP repression on the *dctA* promoter is not solely a consequence of the occlusion of a DNA site by the CRP protein, but rather involves direct contacts between CRP and $E\sigma^{54}$. We suggest that this inactive complex is then locked in a form which interconverts slowly with the classical closed intermediate. Only the latter will be capable of interacting with NtrC to form the open promoter complex.

Discussion

A single regulatory factor interacts with more than one form of RNA polymerase

Transcription factors can be divided into a number of groups. In prokaryotes, each group of factors is thought to be responsible for the primary regulation of one specific form of RNA polymerase. In eukaryotic systems, single transcriptional regulatory factors may regulate the activity of various RNA polymerases via protein–protein contacts (Lescure *et al.*, 1992). However, due to the complexity of eukaryotic transcription systems, it may not be easy to analyse the elements common to two regulatory mechanisms at the molecular level. Recent studies indicate that CRP can activate promoters recognized not only by $E\sigma^{70}$, but also by another form of RNA polymerase $E\sigma^{38}$, in which σ^{38} belongs to the σ^{70} family (Kolb *et al.*, 1995; Tanaka *et al.*, 1995). Similarly, the ribosomal *rrnB*P1 can be activated by the Fis protein with both $E\sigma^{70}$ and $E\sigma^{32}$ (Newlands *et al.*, 1993). The precise mechanisms involved in the response of two different RNA polymerases to the same factor are not understood. It was of interest, therefore, to study the molecular mechanism behind a previous *in vivo* observation that CRP had a potential role in the regulation of the σ^{54}-dependent *dctA* promoter (Wang *et al.*, 1993). In this study, we have shown, for the first time, that CRP can interact with one form of RNA polymerase $E\sigma^{54}$, where the sigma factor is non-homologous and functionally distinct from σ^{70} (Merrick, 1993).

CRP does not need a specific binding site to exert most of its effect

CRP displays synergy with $E\sigma^{54}$ in the closed complex. CRP does not need a specific binding site to exert the synergistic effect, functioning *in trans* as well as *in cis*. This property is atypical for the classical prokaryotic transcriptional regulator CRP. However, it is familiar for $E\sigma^{54}$ and eukaryotic related regulatory systems, where transcription factors can function *in trans*, but are normally presented *in cis* from enhancer elements.

The fact that CRP can interact with promoter-bound $E\sigma^{54}$ without specific CRP target sites on DNA suggests that for the synergy between CRP and $E\sigma^{54}$ to occur, protein 'patches' or domains from CRP and $E\sigma^{54}$ are involved. At this moment, it is not yet known how many 'patches' of CRP and $E\sigma^{54}$ are participating in such an event. The CRP ARI is responsible for interaction with the α subunit of the $E\sigma^{70}$ on the σ^{70}-dependent *lac* promoter (and other type I promoters). When ARI mutants were used in footprinting experiments with the *dctA* promoter containing the two CRP upstream binding sites, they could still interact with $E\sigma^{54}$ bound on the promoter, as evidence of formation of a DNA loop. When tested in gel mobility shift assays together with the 'core' *dctA* promoter, they were still found to exert a smaller stabilizing effect on the closed complexes (data not shown). This is consistent with previous results showing that the repression effect of a mutant in ARI (H159L) on *dctA* is diminished, but not abolished, *in vivo* (Wang *et al.*, 1993). Therefore, the above data suggest that ARI of CRP is involved in but is not essential for the interaction and repression observed. Some N-terminal truncated forms of σ^{54} protein (Cannon *et al.*, 1995) which lack sequences needed for activation of $E\sigma^{54}$ were used for reconstitution with the core RNA polymerase, and tested for DNA loop formation. Results show that they all behave similarly to the wild-type. These negative results suggest that the N-terminal domain of σ^{54} is not essential for the interaction with CRP.

The regulatory role of CRP on the σ^{54}-dependent dctA promoter

Activation of the *dctA* promoter was achieved *in vitro* by addition of NtrC and ATP which converted the closed complex to an open complex. Activation *in vivo* has also been demonstrated by inducing phosphorylation of the wild-type NtrC protein under low ammonium conditions in various *E.coli* strains (Allaway *et al.*, 1995). In contrast, CRP, when added to the $E\sigma^{54}$–*dctA* promoter complex, was unable to trigger transcription initiation. Activators of $E\sigma^{54}$ are specialized in that they must be able to engage

the polymerase, probably through protein–protein contact, and also to hydrolyse ATP or other NTPs (Kustu *et al.*, 1991; Weiss *et al.*, 1991). CRP is unable to fulfil all or some of these functions and fails to activate Eσ^{54}.

However, CRP is able to repress activation of the *dctA* promoter (Wang *et al.*, 1993). *In vitro*, we activated the *dctA* promoter by using either the S160F constitutive mutant of NtrC, or PSPFΔHTH. Repression of those activations by CRP could be due to a direct interference between Eσ^{54} and NtrC or PSPFΔHTH contacts; in this case, the contact region of CRP occludes activator access to the polymerase. [Similarly, the negative control protein NifL is thought to block access of the activated NifA to the Eσ^{54} (Berger *et al.*, 1994; Hill *et al.*, 1996).] However, the very slow reversibility of the inhibition process, when the order of addition of CRP and NtrC is exchanged, and the very slow establishment of a residual activation profile (a half-time of 13 min for the upstream-deleted 'core' promoter and 30 min for the intact *dctA* promoter) indicate that a new rate-limiting step is established when CRP is added before NtrC. In parallel, the residence time of the closed complex is markedly increased. Both observations suggest that in the presence of CRP and cAMP a new form of the closed complex is generated. This explanation also accounts for the observation that efficient repression requires that CRP be added before NtrC. If NtrC was added first with ATP, open complex formation would proceed before CRP is present. Repression would only have a chance to occur when the long-lived open complex returns to the closed intermediate, a result consistent with our observations.

We have identified two upstream CRP-binding sites on the *dctA* promoter. A direct link exists between DNA loop formation, enhancement of CRP binding to its upstream sites and the upstream sites-bound CRP interacting with downstream promoter-bound Eσ^{54}. After 10 min of incubation with the activator, the CRP repression effect is enhanced 2-fold, when the upstream CRP-binding sites are present compared with when they are deleted (Figure 4). Also, a slower activation profile was observed (Figure 5). It is likely that binding of CRP to its target upstream DNA sites stabilizes the interaction between CRP and promoter-bound Eσ^{54}, which in turn enhances CRP-mediated repression.

The mode of repression exerted by CRP at the *dctA* promoter appears quite distinct from those previously reported in *E.coli*. Firstly, there is no previous example in the literature of a CRP-dependent regulation which does not require a CRP-binding site. When the site is out of phase, regulation is always abolished (see, for example, Gaston *et al.*, 1990). We have checked this point specifically in a control experiment performed with Eσ^{70}. We have used a *gal*P1 promoter where the CRP-binding site was eliminated and replaced by a stretch of A tracts (see Bracco *et al.*, 1989). Addition of CRP (150 nM) and cAMP (200 μM) in a run-off assay has strictly no effect on open complex formation at this promoter. Secondly, in general, CRP sterically blocks RNA polymerase access to the promoter as, for example, at the *lac*P2 or *gal*P2 promoters; in some rare cases, CRP can exclude activator binding in the upstream region of a promoter (see Collado-Vides *et al.*, 1991; Botsford and Harman, 1992; Kolb *et al.*, 1993). Strikingly, CRP interacts with the Eσ^{54} polymerase bound at the *dctA* promoter, repressing transcription by a mechanism involving protein–protein interactions. This phenomenon is reminiscent of CRP repression at the CytR-regulated promoters, where CRP and CytR form a stable multimeric heterologous protein complex stabilized by protein–protein contacts, which is, in most cases, unable to perform activation (Valentin-Hansen *et al.*, 1996; for exceptions, see Rasmussen *et al.*, 1996).

The potential role of CRP on σ^{54}-dependent promoters

The significance of our observations are several fold: primarily, our results demonstrate that CRP can act as a modulator of Eσ^{54}. The generality of CRP repression, or repression by other members of the CRP family of proteins, on σ^{54}-dependent promoters has yet to be established (Sze *et al.*, 1996). However, since CRP can exert an effect on a 'core' promoter, which lacks a specific CRP-binding site, this effect might be general, with potential relevance to other σ^{54}-dependent promoters. In the present case, CRP locks the closed complex in a form refractory to the effect of two widely different activators, NtrC and PSPFΔHTH. The CRP–cAMP complex, or an analogue, could, therefore, potentially modulate the action of many regulators of σ^{54}-dependent promoters. In particular, transcription of *glnA* in *E.coli*, the gene responsible for the synthesis of glutamine synthetase, is controlled by a CRP-activated σ^{70} promoter (*glnA*p1) which is located upstream of the σ^{54}-dependent *glnA*p2 promoter, an arrangement consistent with a potential role for CRP in negatively controlling *glnA*p2 via interaction with σ^{54} RNA polymerase (for a review, see Magasanik, 1996). More generally, the direct action of the CRP–cAMP complex as a modulator of Eσ^{54} could provide a novel regulatory linkage between carbon and nitrogen metabolism. This coordination has been studied for decades, and the function of several key elements has been partially clarified in recent years (Merrick *et al.*, 1995; Du *et al.*, 1996; Reizer *et al.*, 1992, 1996; for a review, see Charbit, 1996). It is well known that preferential utilization of carbon sources in *E.coli* and related bacteria is regulated by the phosphoenolpyruvate-dependent phosphotransferase system (PTS), in which the phosphorylated state of IIA^{Glucose} is believed to activate adenylate cyclase allosterically (Saier and Feucht, 1975). More recently, it has been reported that in various eubacteria the *rpoN* operon codes for both σ^{N} and for the IIA^{Ntr} protein, which is homologous to the fructose and mannitol enzyme IIA proteins of the PTS system. The IIA^{Ntr} protein can be phosphorylated by the PTS (Powell *et al.*, 1995), and this provides a formal link between nitrogen utilization and carbon metabolism. Furthermore, in *Klebsiella pneumoniae ptsN*, the gene coding for IIA^{Ntr} is known to down-regulate some σ^{54}-dependent promoters. An attractive hypothesis would be that the PTS might modulate the activity of its nitrogen counterpart, IIA^{Ntr}, which in turn might exert its repression effect on σ^{54}-dependent promoters. The finding of the present report, a cAMP-dependent repression of a σ^{54}-dependent promoter via CRP, could now suggest that at least part of the PTS action could proceed via the increase in the level of cAMP. In a similar manner, other regulators of the CRP family might in turn interact with Eσ^{54} to adjust the expression

of the corresponding promoters to other metabolic states, in *E.coli* as well as in other organisms.

Materials and methods

Chemicals

[α-^{32}P]dATP was purchased from Amersham and restriction enzymes from New England Biolabs or Boehringer-Mannheim.

DNA fragments

Large-scale preparations of plasmid DNA (pCU699-1 from *E.coli* MC1061) were performed using standard methods (Sambrook *et al.*, 1989). For the wild-type *dctA* promoter fragment, a 3 mg aliquot of plasmid DNA was restricted with *Eco*RI and *Hin*dIII restriction endonucleases, and the restriction mixture was separated by electrophoresis on 7.5% polyacrylamide, 10% glycerol gels. The concentration of the purified 245 bp *Eco*RI–*Hin*dIII wild-type *dctA* promoter fragment was determined using spectrophotometric methods. Similarly, the same plasmid DNA was restricted with *Eco*RI and *Stu*I restriction endonucleases, and the 94 bp *Eco*RI–*Stu*I 'core' *dctA* promoter fragment, containing from –40 to +42 of the *dctA* promoter and some multiple cloning sites from plasmid pUC18, was purified as described above.

Footprinting studies

The 245 bp *Eco*RI–*Hin*dIII *dctA* promoter fragment was labelled with [α-^{32}P]dATP using Klenow fragment. The labelled fragment was restricted with either *Sph*I or *Sma*I to obtain DNA fragments with labelling at either the top or bottom strand. Binding of CRP–cAMP to the labelled DNA was performed in HEPES-Mg-glutamate buffer (20 mM HEPES pH 8, 5 mM $MgCl_2$, 50 mM K glutamate) containing 0.5 mg/ml bovine serum albumin (BSA) and 200 μM cAMP. Template DNA (1 nM) was incubated with CRP in the presence or absence of Eσ^{54} for 15 min at 30°C in a total volume of 20 μl (before polymerase addition, core and σ^{54} at a stoichiometry of 1:2 were previously combined and warmed at 30°C for at least 5 min). After complex formation, 3 μl of DNase I solution (0.5 μg/ml in 15 mM Tris–HCl pH 8, 7.5 mM $MgCl_2$, 5 mM $CaCl_2$, 25 mM K glutamate and 62 mM KCl) were added and incubated for 12 s with naked DNA, 15 s with CRP–DNA complexes and 17 s with polymerase–DNA complexes. The reaction was stopped with 40 μl of phenol and 200 μl of a solution containing 0.4 M sodium acetate, 2.5 mM EDTA and 50 μg/ml calf thymus DNA. After phenol extraction, the samples were precipitated with ethanol and analysed on a 7.5% denaturing polyacrylamide gel. The positions of protected and hypersensitive bands were identified by comparison with the migration of the same fragment treated for the G+A sequencing reaction. The intensities of the bands in the binding sites for CRP or RNA polymerase were quantified in the absence and presence of protein with a PhosphoImager (Molecular Dynamics), and the occupancy of the site was determined from the decrease in reactivity of the protected bonds.

Gel mobility shift assays

The 94 bp *Eco*RI–*Stu*I 'core' *dctA* promoter fragment was labelled with [α-^{32}P]dATP using Klenow fragment. DNA–protein binding reactions were carried in HEPES-Mg-glutamate buffer (20 mM HEPES pH 8, 10 mM $MgCl_2$, 50 mM K glutamate) containing 0.5 mg/ml BSA, 8% glycerol and 200 μM cAMP at 30°C. Complexes were formed by mixing appropriate labelled and unlabelled DNA fragments with CRP in the presence or absence of Eσ^{54} (or with Eσ^{54} in the presence or absence of CRP) (before polymerase addition, core and σ^{54} at a stoichiometry of 1:1 were previously combined and warmed at 30°C for at least 30 min). Mixtures were equilibrated for 60 min in a total volume of 10 μl and loaded directly on a 4.5% polyacrylamide gel. Electrophoresis was performed in Tris–glycine buffer (25 mM Tris and 192 mM glycine pH 8.3), with addition of 200 μM cAMP in the cathode reservoir, at 8 V/cm for 2 h. The amount of the complexes and the free DNA for each sample were quantified with a PhosphoImager (Molecular Dynamics).

In vitro transcription assays

Transcription assays were performed at 30°C in the similar buffer to that used in gel mobility shift assays, with the exception that 4 mM ATP is also present. Template DNA (1 nM) was pre-mixed with Eσ^{54} and then aliquoted for each sample before addition of other proteins (NtrC^{S160F} alone, or CRP then NtrC^{S160F}, or NtrC^{S160F} then CRP as indicated in the figure legends). A single round of transcription was then initiated by addition of ribonucleoside triphosphates ATP, CTP and GTP, to 400 μM, [α-^{32}P]UTP (100 Bq/pmol) to 10 μM and heparin to 100 μg/ml. After 10 min incubation, the reaction was terminated by adding an equal volume of formamide-blue-EDTA (0.1% xylenecyanol blue, 15 mM EDTA). The labelled RNAs were analysed on a 7% (w/v) polyacrylamide sequencing gel, calibrated with sequencing reactions.

For the kinetic experiments, 15 μl of the solution containing Eσ^{54}–promoter complexes with NtrC^{S160F} alone, or CRP and NtrC^{S160F} (added in different orders) were aliquoted, at different time intervals, into 5 μl of a mixture containing heparin (400 μg/ml), 40 μM [α-^{32}P]UTP (100 Bq/pmol) and ATP, CTP and GTP (1.6 mM), and processed as described above.

Genetic manipulations

Plasmid pCU699-1 carries the *dctA* promoter region (from –178 to +42) as a 220 bp *Pst*I–*Sma*I fragment. This plasmid was constructed through subcloning of pCU699 (Wang *et al.*, 1993) by *Sma*I digestion and religation.

Plasmid pOM90 (Richet and Søgaard-Andersen, 1994) contains two divergent sets of transcription terminators on both sides of the *Eco*RI restriction site, respectively (a *rpo*C terminator at its upstream side, and four tandem repeats of the *rrnB*T_1 terminators at its downstream side) which can be used as a vector for *in vitro* transcriptional assays. The 245 bp *Eco*RI–*Hin*dIII fragment containing the *dctA* promoter region was isolated from pCU699-1 and inserted in both orientations into the *Eco*RI site of pOM90, using Klenow fragment and T4 DNA ligase. The constructs were designated pYP101 where *dctA* transcription stops at the *rrnB*T_1 terminators, and pYP102 where *dctA* transcription stops at the *rpoC* terminator. The upstream sequences of the *dctA* promoter were removed from pYP102 with restriction endonucleases through utilizing a unique *Stu*I site located at –40 of the *dctA* promoter and a unique *Bam*HI site located in pOM90, and plasmid pYP102ΔU was constructed using Klenow fragment and T4 DNA ligase.

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