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Molecular mechanism of negative autoregulation of *Escherichia coli crp* gene

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ABSTRACT

Transcription of the *Escherichia coli crp* gene encoding cAMP receptor protein (CRP) is negatively regulated by CRP-cAMP complex that binds to a specific site located downstream from the transcription start site. The binding of CRP-cAMP to this site activates transcription from a second divergent overlapping promoter. The mechanism of this negative autoregulation of the *crp* gene has been investigated by *in vitro* transcription, gel shift, DNase I footprinting, and exonuclease III protection assays. We demonstrated that the *crp* and divergent promoters are reciprocally and coordinately regulated by CRP-cAMP. The abortive initiation assay revealed that the divergent RNA itself is not required for the inhibition of *crp* transcription. Detailed binding studies revealed that CRP-cAMP stimulates the binding of RNA polymerase to the divergent promoter and thus blocks the occupation of the *crp* promoter by RNA polymerase.

INTRODUCTION

The cyclic AMP receptor protein (CRP or CAP) of *Escherichia coli* is a sequence-specific DNA binding protein which regulates transcription of a number of genes in response to the carbon source (1–3). The protein is composed of two identical subunits containing 209 amino acids (4–6). When complexed with its allosteric effector cAMP, CRP undergoes a conformational transition (7, 8) and binds to 22-bp target sites (9–12) within or near promoters of catabolite-sensitive operons to activate their transcription.

While CRP-cAMP activates transcription of many genes, the protein acts as a negative effector in some cases. For example, CRP-cAMP represses the transcription from one of the two overlapping *gal* promoters (13, 14). The transcription of *crp* (15–18), *cya* (19–22), and *spf* (23) genes has been shown to be negatively regulated by CRP-cAMP. Thus the existence of negative regulation by CRP-cAMP is well documented, however the mechanism by which CRP-cAMP inhibits transcription is not fully understood. In the case of the *crp* gene, we found that the binding of CRP-cAMP to the specific site located downstream from the *crp* promoter is responsible for the inhibition of *crp* transcription (15). It was noteworthy that the CRP binding site in the *crp* regulatory region is clearly separated from the RNA polymerase binding site. This raised a question of how the binding of CRP-cAMP to this site inhibits the *crp* transcription. Okamoto *et al.* (17, 18) found a second divergent promoter in the *crp* promoter region that is activated by CRP-cAMP. They proposed a model where RNA from the divergent promoter is directly involved in the inhibition of the *crp* transcription by forming a RNA-RNA hybrid with the *crp* RNA.

In this paper, we investigated the molecular mechanism of the negative autoregulation of the *crp* gene by *in vitro* transcription, gel shift, DNase I footprinting, and exonuclease III protection assays. Our data indicate that the inhibition of the *crp* transcription by CRP-cAMP could occur in the absence of the divergent RNA. We showed that CRP-cAMP stimulates the binding of RNA polymerase to the divergent promoter and this binding of RNA polymerase interferes the occupation of RNA polymerase at the *crp* promoter.

MATERIALS AND METHODS

DNA and proteins

DNA fragments containing the *crp* promoter were generated from plasmid pHA5 carrying the entire *crp* gene (5). The 238-bp *Mlu*I-*Hin*dIII and 950-bp *Hin*dIII-*Hin*dIII fragments (Figure 1) were used in this study. The 5′ ends of the fragment were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. RNA polymerase was purified from *Escherichia coli* strain W3350 essentially according to the method of Fukuda *et al.* (24). CRP was purified from *Escherichia coli* strain PP47 harboring the *crp* plasmid pHA7 by the procedure of Eilen *et al.* (25).

In vitro transcription

Run-off transcription assays were performed in a total volume of 30 μl of transcription buffer (50 mM Tris-HCl, pH7.9, 50 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, and 100 μg/ml bovine serum albumin) containing 0.1 mM cAMP. The 238-bp *Mlu*I-*Hin*dIII fragment of a final concentration of 0.6 nM was first incubated with CRP (0–90 nM) for 3 min at 37°C and then with RNA polymerase (8 nM) for 5 min. For a single round transcription, the mixtures were treated with 1.5 μl of heparin

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(2 mg/ml) for 5 min to inactivate free RNA polymerase. The transcription was started by adding 3 μl of a solution containing 0.1 mM [α-^{32}P]UTP (5 μCi) and 1 mM each of ATP, GTP, and CTP. After 15 min of incubation at 37°C, the reaction was terminated by adding 60 μl of phenol, 30 μl of 0.6 M sodium acetate (pH 5.5), 20 mM EDTA, and 200 μg/ml of tRNA. The products were precipitated with ethanol, dissolved in urea loading buffer (8 M urea, 0.025% bromphenol blue, and 0.025% xylene cyanol in TBE) and fractionated by electrophoresis on an 8% polyacrylamide gel containing 8 M urea.

In the abortive initiation assay (26), the *Mlu*I-*Hin*dIII fragment (2 nM) in 30 μl of transcription buffer containing 0.1 mM cAMP was incubated with RNA polymerase (26 nM) in the presence and the absence of CRP (150 nM) as mentioned above. The transcription was started by adding 3 μl of a solution containing 2 mM GpA and 1 mM [α-^{32}P]UTP (10 μCi). Following the incubation at 37°C, the products were precipitated with ethanol and analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea.

RNA sequencing

The divergent RNA was synthesized *in vitro* by using the *Mlu*I-*Hin*dIII fragment as a DNA template. The reaction mixture contained 60 nM DNA, 0.1 mM cAMP, 120 nM CRP, 120 nM RNA polymerase, 200 μM each of the four NTPs in 200 μl of the transcription buffer. After 30 min of incubation at 37°C, the reaction was terminated by adding 200 μl of phenol. The RNA products were precipitated with ethanol and separated by electrophoresis in a 6% polyacrylamide gel containing 8 M urea. The divergent RNA was extracted from the gel. The purified RNA was treated with alkaline phosphatase then labeled with with [γ-^{32}P]ATP at its 5′ end by T4 polynucleotide kinase. The labeled RNA was purified again by electrophoresis and sequenced by enzymatic methods using the RNA sequencing kit containing RNAses T1, U2, Phy M, and *B.cereus* from Pharmacia. The partial digestions were performed under the conditions specified by the supplier.

Gel shift assay

The *Mlu*I-*Hin*dIII fragment labeled at its 5′ *Hin*dIII end (0.6 nM) was incubated first with CRP (0−120 nM) for 3 min at 37°C in 30 μl of transcription buffer containing 0.1 mM cAMP and then with RNA polymerase (8 nM) for 5 min. Then the mixture was treated with 1.5 ml of heparin (2 mg/ml) for 5 min. After adding 5 μl of 40% glycerol, 0.1% bromphenol blue, 0.1% xylene cyanol, the mixture was electrophoresed on a native 5% polyacrylamide gel containing 0.1 mM cAMP in 1/2×TBE at room temperature.

DNase I footprinting in gel

DNase I footprinting in gel slice was performed as described by Straney *et al.* (27). The *Mlu*I-*Hin*dIII fragment labeled at its 5′ *Hin*dIII end (3.6 nM) were incubated with RNA polymerase (20 nM) in the presence or absence of CRP (90 nM) in 50 μl of transcription buffer containing 0.1 mM cAMP. After treating with 3 μl of heparin (2 mg/ml) for 5 min, the mixture was separated on a 6% gel. The bands of free DNA, the complex formed in the absence of CRP-cAMP, and the complex formed in the presence of CRP-cAMP were cut out from the gel. A solution (3 μl) containing 0.2 μg/ml DNase I, 10 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 5% glycerol, 0.5 mg/ml bovine serum albumin was spread on the gel slice (about 30 μl) in an Eppendorf tube, which was then incubated for 45 min at room temperature. The DNase I cleavage reaction was started by adding 3 μl of a solution of 50 mM $MgCl_2$, 50 mM $CaCl_2$. After 3 min incubation at 37°C, the reaction was terminated by adding 15 μml of 0. 1 M EDTA, 0.15% SDS. The DNA was extracted from gel, precipitated with ethanol, dissolved in urea loading buffer, and fractionated by electrophoresis on an 8% polyacrylamide gel containing 8 M urea.

Exonuclease III protection

The 3′ ends of the 950-bp *Hin*dIII-*Hin*dIII fragment ^{32}P-labeled at its 5′ ends were filled in with Klenow enzyme by using dCTP-α-S, dATP, dGTP, and dTTP. Then the fragment was cleaved with *Mlu*I to isolate the *Mlu*I-*Hin*dIII promoter fragment. The *Hin*dIII 3′ end of this fragment is resistant to exonuclease III digestion. The fragment (0.6 nM) was incubated in 30 μl of transcription buffer containing 0.1 mM cAMP in the presence or absence of CRP (90 nM) and RNA polymerase (8 nM) for 5 min at 37°C. The mixtures were competed with 2 μl of 1 mg/ml poly(dA-dT) for 5 min at 37°C and treated with 2 μl of 24 units/μl exonuclease III for 5 min at 37°C. After phenol extraction the reaction products were precipitated with ethanol, dissolved in 8 M urea loading buffer, and fractionated by electrophoresis on an 8% polyacrylamide gel containing 8 M urea.

RESULTS

The *crp* and divergent promoters are regulated coordinately by CRP-cAMP

Figure 1 represents the diagram and the nucleotide sequence of the *crp* promoter region. The transcription initiation site of *crp* was shown to be 167 bp upstream from the translation start codon by the analysis of 5′ terminus of *crp* mRNA made *in vitro* and *in vivo* (15). Based on *in vitro* transcription assay of DNA fragments containing the *crp* promoter region, we demonstrated that the transcription of the *crp* is specifically inhibited by CRP-cAMP complex (15). In addition, a DNase I footprinting analysis revealed that CRP-cAMP binds to a specific site located about 40 bp downstream from the transcription start site. We also identified another CRP binding site located about 60 bp upstream from the *crp* start site. Subsequently, Okamoto *et al.* (17) identified a second promoter, from which transcription proceeds to the opposite direction to the *crp*, within the *crp* promoter region. Transcription from this promoter was shown to be completely dependent on CRP-cAMP.

In order to learn more about the regulation of two overlapping promoters in the *crp* regulatory region by CRP-cAMP, we conducted *in vitro* transcription experiments by using the 238 bp *Mlu*I-*Hin*dIII fragment containing the *crp* and divergent promoters. As expected, transcription of this fragment produced a *crp* mRNA of 146 nucleotides in the absence of CRP-cAMP (Figure 2A, lane 2). When increasing concentrations of CRP-cAMP were added to the reaction mixture, the transcription of *crp* decreased while another RNA (divergent RNA) of 95 nucleotides appeared correspondingly (Figure 2A, lanes 3−7), as reported by Okamoto *et al.* (17). The decrease in *crp* RNA and the increase in divergent RNA follow the similar CRP concentration dependence. This suggests that the two promoters are coordinately regulated by CRP-cAMP.

Determination of the start site for the divergent RNA

Okamoto *et al.* (17) reported, based on the S1 mapping assay, that the transcription of the divergent RNA initiates from a

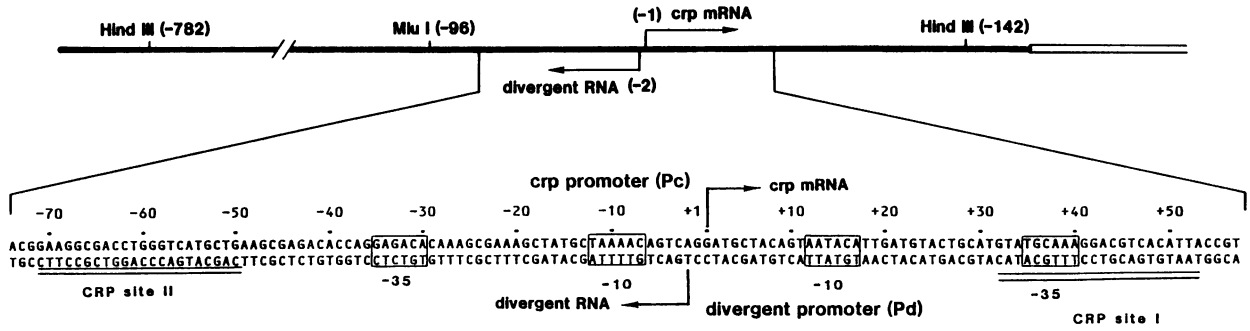

Figure 1. Diagram and nucleotide sequence of the *crp* promoter region. The relevant restriction sites are shown on the top. Arrows indicate the start and direction of transcription. The start site of *crp* promoter (Pc) is numbered as +1. The transcription of divergent promoter (Pd) starts at −2 (see Figure 3). The CRP coding region is shown by an open bar. The nucleotide sequence is taken from Aiba *et al.* (5). The −35 and −10 sequences for two promoters are boxed. The CRP binding sites are double-underlined.

guanine residue at position −3 with respect to +1 of the *crp* start site. To test this conclusion we performed *in vitro* transcription assay with the *Mlu*I-*Hin*dIII fragment by using [γ-^{32}P] GTP. While the *crp* RNA whose transcription starts with G (15) was labeled with ^{32}P as expected, no radioactivity incorporated into the divergent RNA (data not shown). This suggests that the 5′ endpoint of the divergent RNA is not the G at position −3. To determine directly the initiation site for the divergent RNA, ^{32}P-end-labeled RNA was subjected to the enzymatic RNA sequencing analysis. The sequence of the RNA was shown to be 5′-UGACUGUU--- (Figure 3). This indicates that the divergent RNA initiates from the T located 2 bp upstream from the start of the *crp* RNA.

The divergent RNA is not required for the inhibition of *crp* transcription

Concerning the mechanism of the inhibition of *crp* transcription by CRP-cAMP, Okamoto *et al.* (17) proposed a model where the divergent RNA is directly involved in the inhibition by forming a RNA-RNA hybrid. This model was based on the observations the 5′ portions of two RNAs are partially complementary and that the addition of purified 5′ segment of the divergent RNA specifically inhibited *crp* transcription *in vitro*. If this model were correct, one might expect that the inhibition of the *crp* transcription is largely reduced in a single round transcription reaction. The result of this experiment showed that the *crp* transcription was strongly inhibited by CRP-cAMP in the single round assay (Figure 2B) as in the case of multiple rounds reaction (Figure 2A), suggesting that the divergent RNA itself is not necessary for the inhibition of *crp* transcription.

The abortive initiation reaction shown in Figure 4 directly revealed that the inhibition of *crp* transcription by CRP-cAMP occurs in the absence of the divergent RNA. For this experiment the transcription reaction was carried out by using the dinucleotide GpA and [α-^{32}P]UTP. Under this condition a trinucleotide GpApU is the only transcription product that corresponds to the 5′ portion of the *crp* RNA. While a significant amount of GpApU was made from the *crp* promoter in the absence of CRP-cAMP (Figure 4, lanes 1 and 3), the production of this trinucleotide was greatly reduced in the presence of CRP-cAMP (Figure 4, lanes 2 and 4) This result clearly indicates that the divergent RNA is not involved in the inhibition of *crp* transcription by CRP-cAMP.

Analysis of protein-DNA complexes on a polyacrylamide gel

To study further the molecular mechanism of the negative autoregulation of the *crp*, we examined the binding of RNA

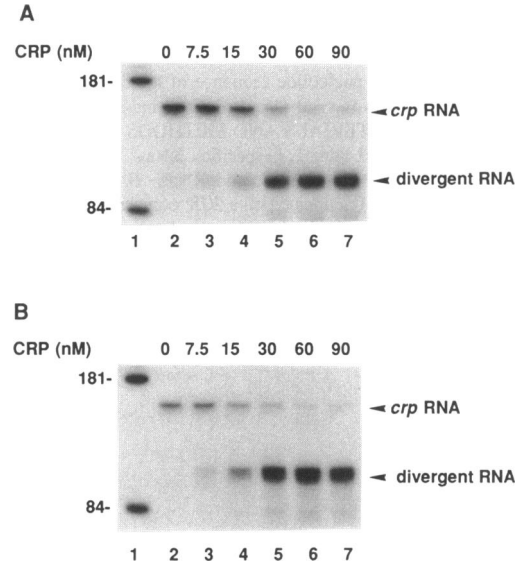

Figure 2. Effect of CRP-cAMP on transcription of DNA fragment containing the *crp* promoter region. The 238-bp *Mlu*I-*Hin*dIII fragment was used for multiple rounds (A) and a single round (B) transcription *in vitro*. In each reaction 0.6 nM template, 8 nM RNA polymerase, and 0−90 nM CRP were used. Run-off transcription assays were performed as described in MATERIALS AND METHODS. For a single round transcription, the mixtures were treated with heparin to inactivate free RNA polymerase prior to the addition of ribonucleotide solution. Lanes 2−7, RNA transcripts made in the presence of indicated concentrations of CRP. Lane 1, DNA size markers.

polymerase to the *crp* promoter in the presence and absence of CRP-cAMP by a gel shift assay using the *Mlu*I-*Hin*dIII fragment ^{32}P-labeled at its *Hin*dIII 5′ end. As shown in Figure 5, lane 2, RNA polymerase without CRP formed an open complex (Pc complex) that is resistant to heparin. Although the addition of CRP-cAMP did not affect the mobility of the open complex, the amount of the complex increased with increasing concentrations of CRP-cAMP in the reaction mixture (Figure 5, lanes 3−6). In other words, CRP-cAMP stimulates the binding of RNA polymerase to the promoter fragment. It is reasonable to assume that the open complex formed in the presence of CRP-cAMP (Pd complex) is different from that in the absence CRP, since the Pc complex exclusively transcribes the *crp* while the Pd complex predominantly transcribes the divergent gene.

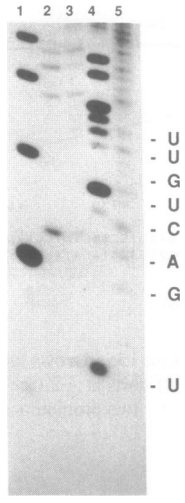

Figure 3. Determination of nucleotide sequence of the 5′ end of the divergent RNA. The divergent RNA was synthesized *in vitro* and its 5′ end was labeled with ^{32}P as described in MATERIALS AND METHODS. The end-labeled RNA partially digested by RNase T1 (lane 1, G specific), RNase U2 (lane 2, A specific), RNase Phy M (lane 3, A+U specific), and RNase *B.cereus* (lane 4, C+U specific). The products were fractionated on a 20% polyacrylamide gel containing 8 M urea. Lane 5 represents the hydroxide base ladder.

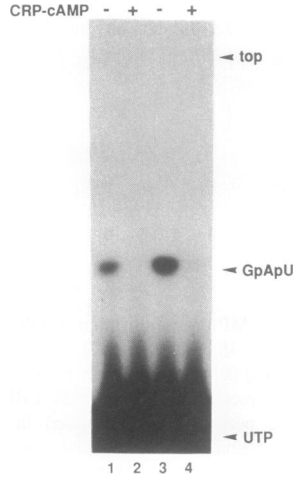

Figure 4. Effect of CRP-cAMP on the abortive initiation of the *crp* transcription. The *Mlu*I-*Hin*dIII fragment (2 nM) in 30 μl of transcription buffer containing 0.1 mM cAMP was incubated in the presence (lanes 2 and 4) and the absence (lanes 1 and 3) of CRP (150 nM) for 3 min at 37°C and then with RNA polymerase (26 nM) for 5 min. The transcription was started with the addition of GpA and [α-^{32}P]UTP to final concentrations of 0.2 mM and 0.1 mM, respectively. The reaction mixtures were incubated for 10 min (lanes 1 and 2) and 30 min (lanes 3 and 4). The products were analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea.

CRP-cAMP changes the binding mode of RNA polymerase at two overlapping promoters

To determine the binding mode of RNA polymerase and CRP to the *crp* promoter region in two open complexes, we carried out a DNase I footprinting in the gel slice as developed by Straney *et al.* (27). The gel slices corresponding to the open complexes were cut out and treated with DNase I. The products were extracted from the gel and analyzed by electrophoresis on an

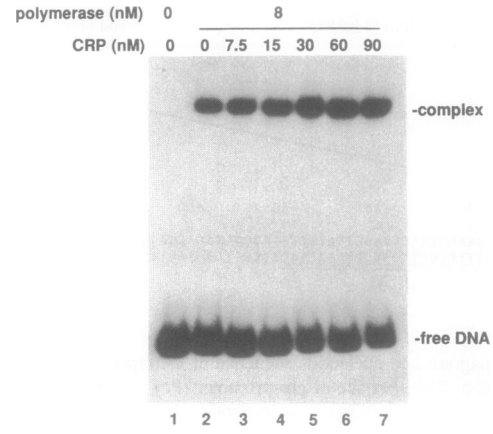

Figure 5. Effect of CRP-cAMP on the binding of RNA polymerase to the *crp* promoter fragment. The *end-labeled Mlu*I-*Hin*dIII fragment with ^{32}P (0.6 nM) was incubated with indicated concentrations of CRP and RNA polymerase in 30 μl of transcription buffer containing 0.1 mM cAMP as described in MATERIALS AND METHODS. The mixtures were analyzed by electrophoresis in a 5% polyacrylamide gel containing cAMP.

acrylamide gel. The protection pattern of these complexes was compared to that of free DNA. As shown in Figure 6, lane 3, in Pc complex RNA polymerase protected from DNase I attack a region between −39 and +20 on the lower strand of the *Mlu*I-*Hin*dIII fragment. This protection pattern in a gel slice is essentially the same as that in a solution (15). A different protection pattern, a protection between −24 and +63 along with a strong enhanced cleavage at +22, was observed for the open complex formed with CRP-cAMP (Figure 6, lane 4). This implies that the binding mode of RNA polymerase in Pd complex is different from that in Pc complex. In the previous DNase I footprinting experiment we showed that CRP-cAMP alone protects a region between +30 and +53 (15). It is interesting to note that the upstream boundary of the RNA polymerase protected region moves approximately 15 bp downstream (from −39 to −24) by the addition of CRP-cAMP. This clearly indicates that CRP-cAMP causes a conversion of RNA polymerase occupancy in the *crp* regulatory region.

To examine further the change of RNA polymerase occupancy at overlapping promoters, we have performed an exonuclease III protection experiment using the *Mlu*I-*Hin*dIII fragment labeled at its *Hin*dIII 5′ end. The labeled fragment was subjected to a limited digestion with exonuclease III in the presence and absence of RNA polymerase and CRP. This enzyme catalyzes the stepwise release of nucleotides from the 3′ end of duplex DNA. The protein bound to the fragment should halt the progress of exonuclease III. As shown in Figure 7, lane 3, a DNA band was observed at −33 in the presence of RNA polymerase alone. This position is thought to correspond to the upstream boundary of RNA polymerase bound at the *crp* promoter. In the presence of RNA polymerase and CRP-cAMP the DNA band at −33 was no longer observed and the new band at −17 was produced (Figure 7, lane 4). This result is consistent with that of DNase I footprinting, indicating again a change in the occupancy of RNA polymerase.

Taken together we conclude that RNA polymerase binds to the *crp* promoter in the absence of CRP-cAMP while RNA polymerase preferentially binds to the divergent promoter in the presence of CRP-cAMP.

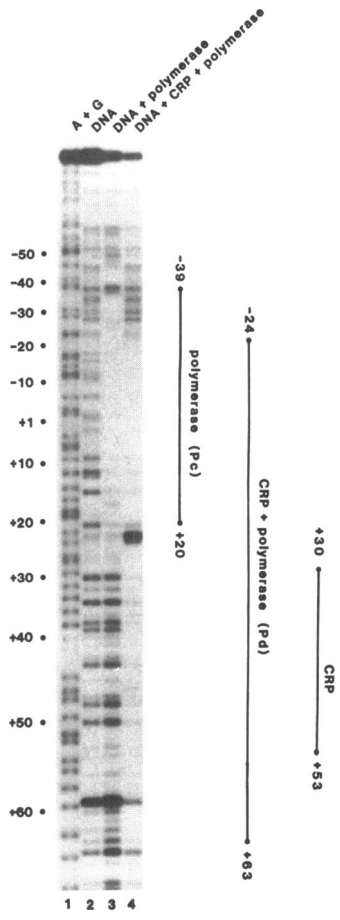

Figure 6. Footprint patterns of gel-purified protein-DNA complexes. DNase I footprinting of protein-DNA complexes in the gel was performed as described in MATERIALS AND METHODS by using the *Mlu*I-*Hin*dIII fragment ^{32}P-labeled at 5′ *Hin*dIII end. Lane 2, ıfree DNA; lane 3, polymerase-DNA complex in the absence of CRP-cAMP (Pc complex); lane 4, polymerase-DNA complex in the presence of CRP-cAMP (Pd complex). Lane 1 is the products of A+G chemical reaction. The regions protected from DNase I attack are shown by vertical lines. RNA polymerase in Pc complex protects a region between −39 and +20. A region between −24 and +63 was protected in Pd complex. The region (+30 to +53) protected by CRP-cAMP alone was taken from Aiba (5).

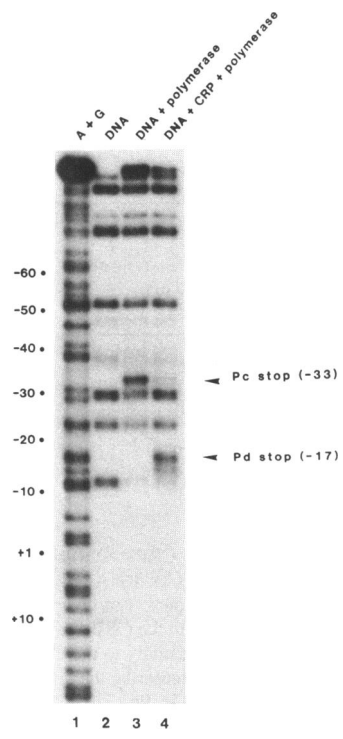

Figure 7. Exonuclease III protection of protein-DNA complexes. Exonuclease III protection assay was performed as described in MATERIALS AND METHODS by using the *Mlu*I-*Hin*dIII fragment ^{32}P-labeled at 5′ *Hin*dIII end. Lane 2, DNA alone; lane 3, RNA polymerase added (Pc complex); lane 4, RNA polymerase and CRP-cAMP added (Pd complex). Lane 1 is the products of A+G chemical reaction.

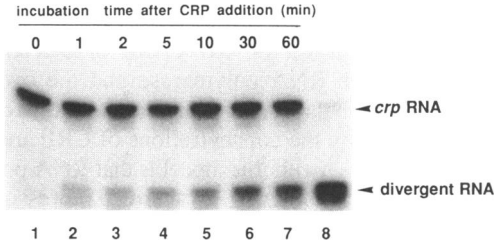

Figure 8. Effect of CRP-cAMP on prebound open complex at the *crp* promoter. The *Mlu*I-*Hin*dIII fragment (0.6 nM) in 30 μl of transcription buffer was first incubated with RNA polymerase (8 nM) for 5 min at 37°C in the presence of 0.1 mM cAMP and then with CRP (90 nM) for periods indicated (lanes 1−7). The transcription was started by adding a ribonucleotide mixture and the products were analyzed by electrophoresis in an 8% polyacrylamide gel containing 8 M urea. Lane 8, CRP added before polymerase.

CRP-cAMP can not displace RNA polymerase bound at the *crp* promoter

In the previous experiments the DNA template was incubated with CRP-cAMP prior to the addition of RNA polymerase. We examined next the effect of CRP-cAMP on preformed open complex at the *crp* promoter. For this the DNA template was incubated with RNA polymerase first to form Pc complex and then CRP-cAMP was added. Following incubation for various times, transcription was started by adding ribonucleotides. As shown in Figure 8, even 60 min incubation with CRP-cAMP caused no significant inhibition of *crp* transcription. This means that preformed open complex at the *crp* promoter is quite stable and CRP-cAMP can not displace RNA polymerase bound at the *crp* promoter.

DISCUSSION

CRP-cAMP regulates negatively the *crp* promoter while it regulates positively the divergent overlapping promoter. We have shown that the two promoters are reciprocally and coordinately regulated by CRP-cAMP *in vitro*. Although the negative autoregulation of the *crp* is due to the activation by CRP-cAMP of the divergent promoter, our data do not support the model of Okamoto *et al.* (17) which involves the inhibition of *crp* transcription by the divergent RNA as a trans-acting regulatory element.

The data presented in this paper are consistent with the following model for the mechanism of the negative autoregulation of the *crp* gene. In the absence of CRP-cAMP, Pc is the only functional promoter and RNA polymerase exclusively occupies Pc to transcribe the *crp* gene (Figure 9A). As the concentration of CRP-cAMP increases, its binding to the CRP site increases. The binding of CRP-cAMP to the CRP site allows RNA

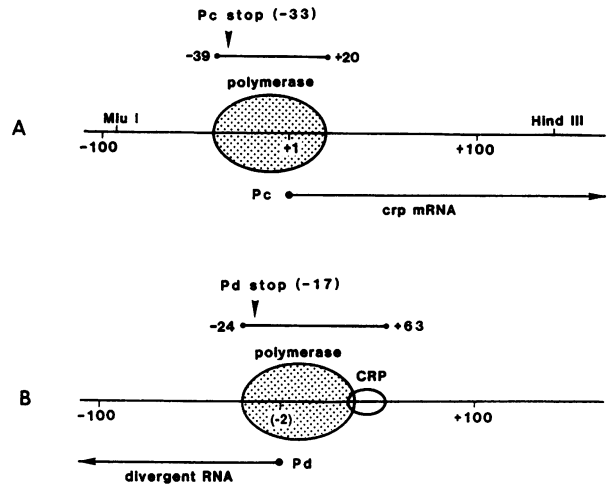

Figure 9. Schematic representation of CRP-cAMP action at the *crp* promoter region. In the absence of CRP-cAMP (A), RNA polymerase binds exclusively to the *crp* promoter (Pc) to form Pc complex. This complex is capable to transcribe the *crp* gene. When CRP-cAMP binds to the CRP site located downstream from Pc (B), RNA polymerase binds preferentially to the divergent promoter (Pd) to form Pd complex which is capable to transcribe the divergent gene. At this state RNA polymerase can no longer bind to Pc. Thus the transcription of *crp* is inhibited. Open bars indicate the region of DNase I footprint. Arrowheads represent the position of Exo III stop.

polymerase to bind predominantly at Pd and the divergent gene is actively transcribed (Figure 9B). The occupancy of Pd by RNA polymerase excludes RNA polymerase occupancy of Pc resulting in the inhibition of the *crp* transcription. On the other hand RNA polymerase prebound at Pc prevents the Pd occupancy of RNA polymerase directed by CRP-cAMP (Figure 8). Thus two RNA polymerase molecules interacting with Pc and Pd are mutually exclusive. The principal role of CRP-cAMP is to determine the binding mode between RNA polymerase and the overlapping promoters. The final balance between Pc and Pd occupancies would be determined by the concentrations of CRP and cAMP. Another important aspect with this model is that RNA polymerase bound at one promoter is acting as a direct repressor for the transcription from the other promoter. Consistent with this view, Okamoto *et al.* (17) found that the insertional mutation between the −10 and −35 regions of the divergent promoter eliminates the inhibition of *crp* transcription by CRP-cAMP without affecting the binding of CRP-cAMP to the CRP site. In other words the binding itself of CRP-cAMP to the CRP site is not sufficient to inhibit the *crp* transcription. This means that the elongation of transcription is not significantly affected by CRP-cAMP on the template DNA. In fact we showed previously that CRP-cAMP bound to the consensus CRP site placed far downstream of the transcription start site of a test promoter only weakly blocks enlongating RNA polymerase, while CRP-cAMP bound to the CRP site placed close to the promoter strongly inhibits the transcription by excluding RNA polymerase from the promoter (9).

The presence of two overlapping promoters and their selection by CRP-cAMP in the *crp* regulatory region are analogous to those in the *gal* (13, 14) and *lac* (28, 29) operons, although two overlapping promoters are on the same strand in the *gal* and *lac* systems. In these operons CRP-cAMP activates one promoter (P1) while it represses another promoter (P2) located 5 bp or 22 bp upstream from P1, respectively.

Recent studies on several gene regulatory proteins revealed that the organization and expression of their genes are strikingly similar to those of the *crp* gene (30−33). For example, a family of regulatory proteins such as OxyR, LysR, CysB, NodD, and MetR, that act as positive regulator for other genes, were shown to negatively regulate their own expression (30, 32). Furthermore this negative regulation seems to be due to the activation of a divergent overlapping promoter by the respective activator protein just as in the case of *crp* gene. Thus, the negative autoregulation involving activation of a divergent overlapping gene seem to be a common mechanism among several activator genes in bacteria.

We do not know presently the physiological significance of the negative autoregulation of the *crp* gene. In general, negative autoregulation is thought to provide the cells with a regulatory circuit that ensures a controlled production of a regulatory protein. However, the regulation of the *crp* gene seems to be more complicated, since cells carrying a multicopy *crp* plasmid still overproduces CRP protein (5). In this connection we have observed that the binding of CRP-cAMP to the second CRP site activates transcription in certain conditions (manuscript in preparation). Furthermore little is known about the divergent gene except that the activation of this gene is involved in the inhibition of the *crp* transcription *in vitro* and the gene is also expressed in intact cells. The following questions arise immediately. How long is the divergent RNA? Does the divergent RNA encode a protein? If so, what is the role of the protein? Elucidation of these questions may contribute to further understanding of the regulation of the *crp* gene.

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