A common role of CRP in transcription activation: CRP acts transiently to stimulate events leading to open complex formation at a diverse set of promoters

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We have shown previously that the cyclic AMP receptor protein (CRP) is not required after the formation of the open complex at the *lac* promoter (Tagami and Aiba, 1995, *Nucleic Acids Res.*, 19, 6705–6712). In this paper, we investigate the role of CRP in transcription activation at the *malT* and *gal* promoters. At the *malT* promoter, RNA polymerase (RNAP) forms a nonproductive RNAP–promoter binary complex in the absence of CRP and a productive CRP–RNAP–promoter ternary complex in the presence of CRP. CRP can be removed from the *malT* ternary complex by a moderate concentration of heparin. The resulting binary complex is functionally identical to the ternary complex. At the *gal* promoter, RNAP predominantly forms a binary complex at the *P2* promoter in the absence of CRP and a ternary complex at the *P1* promoter in the presence of CRP. A very high concentration of heparin is able to dissociate CRP from the *galP1* ternary complex without changing the properties of the complex. These data indicate that CRP is not required for the maintenance of the ternary complex and plays no role in the subsequent steps, irrespective of the promoter. We conclude that the common role of CRP in the activation of transcription is to stimulate events leading to the formation of a productive open complex at a diverse set of CRP-dependent promoters. We suggest that the interaction between CRP and RNAP is needed only transiently for the activation of transcription.

Keywords: activation of transcription/CRP action/CRP-dependent promoters/heparin/transcription complex

Introduction

The initiation of transcription is assumed to be made up of three major steps (McClure, 1985; Levine *et al.*, 1987). Firstly, RNA polymerase (RNAP) binds reversibly to promoter DNA to form a closed complex that is transcriptionally inactive. Secondly, the closed complex is converted to an open complex in which a short region of DNA around the transcription start site is unwound. The open complex is able to abortively produce a series of short RNAs in the presence of ribonucleotides. The third step is the promoter clearance in which RNAP escapes from the promoter with a loss of a σ factor to form a stable elongation complex. The efficiency of each of these

steps may be subject to regulation by a transcriptional activator or repressor proteins.

The cyclic AMP receptor protein (CRP; also known as CAP, catabolite gene activator protein) of *Escherichia coli* is involved in regulating the transcription of a large number of genes, either positively or negatively, in response to carbon nutrient conditions (Botsford and Harman, 1992; Kolb *et al.*, 1993). The protein is a dimer of two identical subunits composed of 209 amino acids (Aiba *et al.*, 1982). When complexed with its allosteric effector cAMP, CRP undergoes a conformational transition and binds to a specific sequence of 22 bp located within or near target promoters in order to regulate transcription (Botsford and Harman, 1992; Kolb *et al.*, 1993). The CRP binding sites lie at different locations relative to the transcription start site of various promoters. Promoters in which CRP alone is sufficient for activation can be divided into two groups: type I (also referred to as Class I) promoters, where the CRP binding site is located upstream of the –35 region; and type II (Class II) promoters, where the CRP binding site overlaps the –35 region (Aiba *et al.*, 1989; Ebright, 1993). The *lac* and *malT* promoters are examples of type I promoter, with a CRP binding site at positions –61.5 and –70.5, respectively. The *galP1* promoter is a prototype type II promoter with a CRP binding site at position –41.5.

The mechanism by which CRP activates transcription has been extensively studied as a paradigm for understanding how a single activator may act. Numerous studies indicate that the interaction between CRP and RNAP plays a pivotal role in the activation of transcription in CRP-dependent promoters (Kolb *et al.*, 1993; Busby and Ebright, 1994, 1997). In particular, recent genetic and biochemical studies of CRP and RNAP have identified particular sites or regions located at the surface of both proteins which are involved in protein–protein interaction and thereby responsible for the activation of transcription. The isolation and characterization of CRP mutants that bind normally to target DNA but fail to activate transcription have established that a surface exposed loop of CRP, of around 156–164 amino acids, constitutes a region essential for the activation of transcription at both type I and type II promoters (Bell *et al.*, 1990; Eschenlauer and Reznikoff, 1991; Zhou *et al.*, 1993). Studies of RNAP α -subunit mutants have revealed that the C-terminal domain of the α -subunit is involved in the interaction with the activating region of CRP (Igarashi and Ishihama, 1991). An additional contact between the N-terminal domain of CRP and the N-terminal domain of the RNAP α -subunit is involved in the activation of transcription at type II promoters (Niu *et al.*, 1996). CRP is also known to induce a sharp bend in DNA which may also play a role in the activation of transcription (Wu and Crothers, 1984; Crothers and Steitz, 1992; Déthiollaz *et al.*, 1996).

Despite remarkable advances in the understanding of

the action of CRP, several important questions remain to be resolved. For example, it is not well understood how the interaction between CRP and RNAP can lead to transcription activation. The related question is which transcription initiation step(s) is modulated by the CRP–RNAP interaction. It is believed that CRP can affect different steps depending on the relative location of the CRP binding site in the promoter. Kinetic studies have claimed that CRP enhances the initial binding of RNAP at the *lac* promoter (Malan *et al.*, 1984), stimulates both initial binding and isomerization to the open complex at the *gal* promoter (Herbert *et al.*, 1986; Lavigne *et al.*, 1992), and accelerates the rate of promoter clearance at the *malT* promoter (Menendez *et al.*, 1987; Eichenberger *et al.*, 1996, 1997). It should be noted, however, that in another study (Straney *et al.*, 1989) CRP was inferred as affecting predominantly the isomerization step at the *lac* promoter. This suggests that our understanding of which step(s) in the initiation of transcription is affected by CRP is not yet certain. In particular, much less is known about the role of the CRP–RNAP interaction in the steps following the formation of the open complex.

We recently demonstrated that removal of CRP from the *lac* ternary open complex by a high concentration of heparin did not alter the structural and functional properties of the complex (Tagami and Aiba, 1995). This finding clearly established that the CRP–RNAP interaction is not necessary for the maintenance of the open complex or for any of the steps following its formation. In this paper, we performed the heparin-challenge experiment on the *malT* and *gal* promoters to examine the role of CRP in the activation of transcription at a diverse set of CRP-dependent promoters. We show evidence that the ternary open complex, containing CRP at two promoters, can be converted to its respective binary complex although the concentration of heparin required for the removal of CRP is substantially different from that required at the *lac* promoter. The resulting binary complexes retain the same properties as the ternary complexes, indicating that CRP has no role in the maintenance of the open complex or in promoter clearance at the *malT* and *galP1* promoters. We conclude that the major target of CRP action is restricted to the formation of a productive open complex irrespective of the location of the CRP binding site. We suggest that the CRP–RNAP interaction is needed only transiently for the activation of transcription.

Results

Transcription complexes at the *malT* promoter

In order to examine the role of CRP in the activation of transcription at the *malT* promoter, a DNA fragment containing the *malT* promoter was incubated with RNAP in the presence and the absence of CRP. The protein–DNA complexes were treated with increasing concentrations of heparin and the products were analyzed by a gel mobility shift assay (Figure 1A). RNAP formed a binary complex, which was resistant to heparin, even in the absence of CRP (lanes 3–7). The formation of a CRP-independent complex at the *malT* promoter has been reported previously (Eichenberger *et al.*, 1996, 1997). When the *malT* promoter was incubated with RNAP and CRP without heparin treatment, an extremely retarded complex was formed

Fig. 1. Effect of increasing concentrations of heparin on transcription complexes at the *malT* promoter. (A) Gel mobility shift analysis. The 247 bp *EcoRI*–*HindIII* fragment containing the *malT* promoter was incubated with or without CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with the indicated concentrations of heparin for 3 min and was analyzed by electrophoresis in a 5% polyacrylamide gel containing cAMP. (B) Immunoblot analysis using an anti-CRP antibody. The gel shown in (A) was used directly for Western blotting. The arrow indicates the existence of CRP in the protein–DNA complexes. (C) Stability of transcription complexes. The DNA fragment containing the *malT* promoter was incubated with CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with 2 μg/ml (lanes 1–3) or 50 μg/ml (lanes 4–6) of heparin for the times indicated and was analyzed by electrophoresis in a 5% polyacrylamide gel containing cAMP.

Fig. 2. DNase I footprinting analyses of the *malT* promoter fragments. The 247 bp *EcoRI*–*HindIII* fragment ^{32}P -labeled at its 3' *HindIII* end (coding strand) (A) and 230 bp *SacI*–*HindIII* fragment ^{32}P -labeled at its 5' *HindIII* end (template strand) (B) were used. Lane 1 shows the products of the A + G chemical reaction. The regions protected/enhanced by CRP–cAMP and/or RNA polymerase are shown by vertical lines and brackets, respectively. Arrowheads with numbers on the right mark the sites where a significant difference is observed between the CRP-independent binary complex (A, lane 6 and B, lane 5) and CRP-dependent binary complex (A, lane 9 and B, lane 7). Numbers on the left indicate the positions relative to the transcription start site of the *malT* promoter.

which presumably corresponded to an aggregate (lane 8). In the presence of a low concentration (2 $\mu\text{g/ml}$) of heparin, the aggregate decreased and two new complexes appeared (Figure 1A, lane 9). Increasing the concentration of heparin reduced the amount of the upper band and increased the amount of the lower band (lanes 9–12). We assumed that the upper band corresponded to a ternary complex containing both RNAP and CRP while the lower one was a binary complex without CRP. This assumption was examined directly using Western blot analysis of the gel. As shown in Figure 1B, CRP was detected only in the upper complex, indicating that CRP was dissociated from the *malT* ternary complex by heparin treatment. To examine the effect of CRP on the stability of the complex, we changed the incubation time after the addition of heparin. Although increasing incubation times reduced the amount of each complex, no significant difference in the decay rate was observed between the binary and ternary complexes (Figure 1C). In other words, removal of CRP did not affect the stability of the CRP-dependent open complex. The concentration of heparin required for the complete dissociation of CRP from the *malT* ternary complex was 50 $\mu\text{g/ml}$ which is ~ 10 -fold less than that required at the *lac* promoter (Tagami and Aiba, 1995). It should be noted that the presence of CRP did not increase the amount of the heparin-resistant complex itself. An important observation is that the CRP-dependent binary

complex, derived from the ternary complex, exhibited a decreased mobility compared with the CRP-independent binary complex. This indicates that the two binary complexes are structurally different from each other.

Protein–DNA interaction in the complexes at the *malT* promoter

To characterize the *malT* complexes further, we performed a DNase I footprinting experiment. Figure 2A and B shows the footprinting patterns of the coding and template strands, respectively. CRP binds to a region between -60 and -80 in the presence of cAMP as reported by Chapon and Kolb (1983). When RNAP alone was incubated with the *malT* DNA, a region between $+20$ and -60 was protected against DNase I digestion. Several sites in both the coding and template strands exhibited an enhanced cleavage (Figure 2A and B, lane 4). The presence of heparin caused little change in the protection/enhancement pattern (Figure 2A, lanes 5 and 6; Figure 2B, lane 5). In the presence of both CRP and RNAP, protection was extended to the CRP site at both strands (Figure 2A, lane 7; Figure 2B, lane 6). On the one hand, the protection/enhancement pattern at the CRP binding site was almost lost in the presence of 50 $\mu\text{g/ml}$ of heparin (Figure 2A, lane 9; Figure 2B, lane 7). On the other hand, the binding of RNAP to the *malT* promoter was essentially retained after the heparin treatment although the overall protection

Fig. 3. Transcriptional activities of complexes at the *malT* promoter. (A) *In vitro* run-off transcription assay. The 247 bp *EcoRI*–*HindIII* fragment containing the *malT* promoter was incubated with (lanes 6–10) or without (lanes 1–5) CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with the indicated concentrations of heparin for 3 min. The transcription reaction was started by the addition of ribonucleotides containing [α - ^{32} P]UTP. The products were analyzed by electrophoresis on an 8% polyacrylamide gel containing 8 M urea. (B) *In vitro* run-off transcription assay in the presence of an internal control promoter. Both the 247 bp *EcoRI*–*HindIII* fragment containing the *malT* promoter and the 205 bp *EcoRI* fragment containing the *lacL8UV5* promoter (Backman *et al.*, 1976) were incubated together with (lanes 3 and 4) or without (lanes 1 and 2) CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with the indicated concentrations of heparin for 3 min. The transcription reaction was started by the addition of ribonucleotides containing [α - ^{32} P]UTP. The products were analyzed by electrophoresis on an 8% polyacrylamide gel containing 8 M urea. (C) Abortive initiation assay. The transcription complexes were formed under the same conditions as in (A). The abortive reaction was started by the addition of ApU and [α - ^{32} P]UTP to final concentrations of 0.2 mM and 0.1 mM, respectively. The reaction mixture was incubated for 15 min and the products were analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea.

signal was reduced due to an increase in the amount of free DNA. It should be noted that the protection/enhancement pattern in the CRP-dependent binary complex is not the same as that in the CRP-independent complex, although RNAP appears to occupy the same DNA region in two complexes (Figure 2A, lanes 6 and 9; Figure 2B, lanes 5 and 7). The difference is particularly significant in the region between –20 and –60. For example, the signals at positions –21, –32, –33, –42, –43, –56, –60 and –61 in the template strand are clearly stronger in the CRP-independent complex than in the CRP-dependent binary complex in the presence of heparin (Figure 2B, lanes 5 and 7). It has been reported that the extent of DNA melting around the transcription start site in the CRP-dependent complex is greater than that in the complex formed in the absence of CRP (Eichenberger *et al.*, 1996). We observed a significant DNA melting even in the CRP-independent complex along with a slight difference in the pattern of the KMnO₄ footprint between two complexes (unpublished result).

Transcriptional activity of the complexes at the *malT* promoter

It has been thought that a major role of CRP at the *malT* promoter is to facilitate the escape of RNAP from the promoter (Menendez *et al.*, 1987; Eichenberger *et al.*, 1996, 1997). If this were the case, then removal of CRP from the ternary complex would reduce the amount of the run-off transcript and increase the number of abortive transcripts. To examine this, both CRP-independent and CRP-dependent transcription complexes were formed under the conditions of the gel mobility shift assay mentioned above. After treating the complexes with increasing concentrations of heparin, a run-off transcription reaction was started by adding ribonucleotides. The CRP-independent complex has little ability to make the

run-off transcript (Figure 3A, lanes 1–5) while the CRP-dependent ternary complex produced a significant amount of the run-off transcript (lanes 5 and 6). When CRP was completely removed from the ternary complex using increasing concentrations of heparin, the amount of the run-off transcript was unaffected (lanes 7–10). The presence of an internal control promoter (*lacL8UV5*) in the reaction mixture did not affect the result (Figure 3B). We also observed that the rate of production of the run-off transcript was not affected by the presence of CRP (unpublished result). These results clearly indicate that CRP has no role in transcription activation in any of the steps following the formation of open complex at the *malT* promoter.

Since the complex at the *malT* promoter formed in the absence of CRP is reported to have a significant activity in abortive initiation assays (Menendez *et al.*, 1987), we also performed the abortive initiation assay with the *malT* complexes. The RNAP–promoter complexes were formed both in the presence and absence of CRP and the abortive synthesis was started by adding a dinucleotide ApU and [α - ^{32} P]UTP. The synthesis of the trinucleotide ApUpU, which corresponds to a transcript originated from the *malT* promoter, was analyzed. As shown in Figure 3C, the abortive product was clearly produced both in the presence and absence of CRP. This result is consistent with that of the previous report. Thus, the CRP-independent heparin-resistant complex is able to produce abortive RNAs but not to make the run-off transcript. We conclude that the major role of CRP in the activation of transcription at the *malT* promoter is to stimulate events leading to the formation of a productive transcription complex and to prevent the formation of a nonproductive one. Thus, the promoter clearance step *per se* can not be the major target for CRP action.

Fig. 4. Effect of increasing concentrations of heparin on transcription complexes at the *gal* promoter. (A) Gel mobility shift analysis. The 167 bp *HhaI*–*EcoRI* fragment containing the *gal* promoter was incubated with or without CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with the indicated concentrations of heparin for 3 min and then analyzed by electrophoresis in a 5% polyacrylamide gel containing cAMP. (B) Immunoblot analysis using an anti-CRP antibody. The gel shown in (A) was used directly for Western blotting. The arrow indicates the existence of CRP in the *galP1* open complex.

Transcription complexes at the *gal* promoter

Next we investigated the role of CRP at the *gal* promoter which is a prototype type II CRP-dependent promoter. Figure 4A shows a gel mobility shift assay of transcription complexes formed at the *gal* promoter with increasing concentrations of heparin. It is well known that RNAP binds preferentially to the *galP2* promoter in the absence of CRP while binding to the *galP1* promoter in the presence of CRP (DiLauro *et al.*, 1979; Aiba *et al.*, 1981). Although RNAP formed the complex more efficiently in the presence of CRP, no difference in the mobility between the CRP-dependent (*P1*) and CRP-independent (*P2*) complexes was observed (Figure 4A). The increasing heparin concentration affected neither the mobility nor the amount of the two complexes. Western blot analysis revealed that the content of CRP in the *galP1* complex was reduced by the addition of increasing concentrations of heparin (Figure 4B). However, a significant amount of CRP was still detected in the complex even when 1.6 mg/ml of heparin was used. Approximately 6 mg/ml of heparin was required to remove most of the CRP from the ternary complex. Thus, CRP can be selectively removed from the ternary complex at the *galP1* promoter. That the increasing concentrations of heparin affected neither the mobility nor the amount of the complex suggests that removal of CRP does not drastically alter the overall structure and the stability of the open complex.

Transcriptional activity of the complexes at the *gal* promoter

To examine the transcriptional activity of the *gal* complexes, a run-off transcription assay was performed. As shown in Figure 5, two run-off transcripts were produced in the absence of CRP. As is well known, the upper and

Fig. 5. *In vitro* transcription assay of complexes at the *gal* promoter. The 167 bp *HhaI*–*EcoRI* fragment containing the *gal* promoter was incubated with or without CRP in the presence of cAMP and then with RNA polymerase. The mixture was treated with increasing concentrations of heparin (0.1, 0.4, 1.6 and 6.4 mg/ml) for 3 min. The transcription reaction was started by the addition of ribonucleotides containing [α - ^{32} P]UTP. The products were analyzed by electrophoresis on a 20% polyacrylamide sequence gel containing 8 M urea. Run-off and abortive RNAs are shown by arrow heads and a bracket, respectively.

lower bands correspond to the *P2* and *P1* transcripts, respectively (Aiba *et al.*, 1981). In addition, a large number of short RNAs were produced in the absence of CRP (lanes 1–4). As expected, CRP stimulated *P1* transcription while it inhibited *P2* transcription (lanes 5–8). The presence of high concentrations of heparin did not affect the transcription profile although it did nonspecifically affect the mobility of the transcripts when 6.4 mg/ml heparin was used (lanes 4 and 8). Thus, the binary complex without CRP, which was produced from the *galP1* ternary complex with the heparin treatment, retained the ability to produce the *P1* run-off transcript. In addition, neither the run-off *P2* transcript nor *P2*-specific short RNAs were produced in the CRP-dependent binary complex. In other words, the *P1* complex was not converted to the *P2* complex by the removal of CRP. This means that once formed the *P1* open complex no longer needs CRP for any subsequent steps as in the case of the *lac* and *malT* promoters.

Discussion

CRP can activate transcription at different locations relative to the transcription start point when a correct helical phasing is maintained between CRP and RNAP (Gaston *et al.*, 1990; Ushida and Aiba, 1990). The positions around –41, –61 and –71 are favorable for CRP action and correspond with those at naturally occurring promoters

represented by the *gal*, *lac* and *malT* promoters, respectively. We have shown previously, based on a heparin-challenge experiment, that CRP is no longer required for the activation of transcription following the formation of the open complex (Tagami and Aiba, 1995). The major advantage of this approach is that one can examine directly the role of CRP and CRP–RNAP interaction in steps following open complex formation. Using this approach, we investigated the role of CRP and CRP–RNAP interaction in the activation of transcription at the *malT* and *gal* promoters in which CRP is reported to act by mechanisms different from those in the *lac* promoter.

Several studies have suggested that CRP may increase the rate of escape from the *malT* promoter (Menendez *et al.*, 1987; Eichenberger *et al.*, 1996, 1997). It has also been suggested that the mechanism of transcription activation at the *malT* promoter may be similar to that at the *lac* promoter because the same activating region of CRP and the C-terminal domain of the α -subunit are involved in CRP–RNAP interaction at the two promoters (Ebright, 1993; Busby and Ebright, 1994). In this paper we show that CRP can be removed from the ternary *malT* open complex by heparin and that the resulting binary complex is functionally equivalent to the ternary complex (Figure 3). This clearly establishes that CRP is no longer necessary for transcription activation after the formation of CRP-dependent open complex at the *malT* promoter. Thus, we conclude that the promoter clearance step can not be the major target of CRP action. It should be noted that a moderate concentration of heparin was sufficient to remove CRP from the ternary complex. This suggests that CRP is loosely bound into the ternary complex at the *malT* promoter compared with the *lac* promoter.

How is our conclusion reconciled with the previous report by Menendez *et al.* (1987)? They found that open complexes were formed at the *malT* promoter with an equivalent efficiency whether or not CRP was present in the abortive initiation assays, while the yield of the full-length of transcript in the run-off transcription assay was markedly enhanced in the presence of CRP. We also observed that RNAP is able to bind to the *malT* promoter to form a stable heparin-resistant complex that has the ability to make an abortive transcript but not the run-off transcript in the absence of CRP. An important finding is that the CRP-independent complex is structurally and functionally different from the CRP-dependent binary complex. The identification of two different binary complexes can easily explain the apparent discrepancy between the two studies. Our results clearly indicate that a major role of CRP at the *malT* promoter is to stimulate events leading to the formation of the productive open complex and there is no further role for CRP once the productive open complex is formed.

How does CRP lead to the formation of the productive open complex at the *malT* promoter? Since RNAP can form a stable heparin-resistant complex even in the absence of CRP and this CRP-independent complex binds to the same *malT* promoter region as the CRP-dependent complex, the initial binding of RNAP to the promoter may not be the major target for CRP action. The *malT* promoter has an A/T-rich sequence between the –35 region and the CRP binding site which is similar to the UP-element found in the *rrnBP1* promoter (Ross *et al.*, 1993).

In fact, it has recently been shown that the interaction between the C-terminal domain of the α -subunit and the A/T-rich region at the *malT* promoter may play a role in the formation of the CRP-independent transcription complex (Déthiollaz *et al.*, 1996; Eichenberger *et al.*, 1996). Taken together, we suggest that CRP stimulates the formation of the productive open complex by modulating the RNAP–promoter interaction at the *malT* promoter. It is likely that the role of CRP is to reposition the C-terminal domain of the α -subunit from one location to another. Although we observed that the CRP-independent nonproductive complex can be converted to the productive complex by the addition of CRP (unpublished results), it is not yet clear whether the nonproductive complex is an intermediate for the productive ternary complex or it is a product in a branched pathway in the early stage of transcription (Kubori and Shimamoto, 1996). In any case, further characterization of the CRP-independent binary complex will be needed to understand what exactly CRP is doing at the *malT* promoter.

We also investigated the role of the CRP–RNAP interaction at the *gal* promoter as a representative of type II CRP-dependent promoter. The role of CRP in the transcription of the *gal* operon is to activate one promoter, *P1* and to repress the other, *P2* (DiLauro *et al.*, 1979; Aiba *et al.*, 1981). The major effect of CRP at the *galP1* appears to be to accelerate the rate of isomerization to the open complex (Herbert *et al.*, 1986). However, little is known about whether CRP plays any other role after the formation of open complex. CRP appears to be tightly bound into the open complex through the multiple interactions with RNAP (Niu *et al.*, 1996). We succeeded in selectively removing CRP from the *galP1* open complex by using a very high concentration of heparin, 10-fold higher than that used at the *lacP1* promoters. The removal of CRP from the *galP1* open complex caused no change in its mobility or amount. We conclude that the CRP–RNAP interaction does not contribute to the maintenance of the *galP1* open complex. In addition, the resulting binary complex retained the ability to initiate and elongate the *galP1* transcription, indicating that the interaction between CRP and RNAP is not required for any subsequent steps after the formation of open complex at the *gal* promoter.

The present study is summarized in Figure 6. It has been suggested that the RNAP–promoter open complex forms a nucleosome-like structure in which the promoter DNA is wrapped around RNAP (see references in Tagami and Aiba, 1995). The architecture of ternary open complexes at the *malT*, *lac* and *gal* promoters must differ with respect to the CRP–RNAP interaction. This is because the concentration of heparin needed to dissociate CRP from the ternary complex decreases markedly with increasing distance between the CRP site and the transcription start site. This indicates that the stability of the CRP–RNAP–DNA interaction in the ternary complex at the *malT*, *lac* and *gal* promoters increases in that order. Thus, the location of the CRP binding site certainly determines the structural property of the ternary open complex. However, we would like to point out the striking similarities among the transcription complexes formed at three representative CRP-dependent promoters. They are: (i) in the absence of CRP, RNAP forms a CRP-independent binary complex that is either nonproductive (*malT*) or less active (*lac* and

Fig. 6. Common properties of transcription complexes at *malT*, *lac* and *gal* promoters. RNAP forms a CRP-independent binary complex in the absence of CRP while it forms a CRP-dependent ternary complex in the presence of CRP at three representative CRP-dependent promoters. The CRP-independent binary complex is inactive (at the *malT* promoter) or less active (at the *lac* and *gal* promoters). The ternary complex is fully competent for transcription at three promoters. CRP can be removed from the ternary complex by heparin, resulting in an active binary complex that is functionally equivalent to the parent ternary complex. The concentration of heparin needed to dissociate CRP varies depending on the promoters. Models for the geometry of CRP-dependent ternary and binary complexes are depicted. The transition from the CRP-dependent binary complex to the CRP-independent binary complex does not occur. In contrast, the CRP-independent binary complex can be converted to the ternary complex. A common major role of CRP in the activation of transcription at three promoters is to stimulate events leading to the formation of the transcriptionally active ternary complex.

Fig. 7. Promoter fragments used in this study. The upper and lower lines represent the *malT* and *gal* promoter regions derived from plasmid pMT100 and pGAL100, respectively. The thick lines correspond to the native promoter regions and the thin lines are derived from the vectors. The relevant restriction sites are shown. The transcription start sites for *malT* and *galP1* are numbered as +1. The numbers in parentheses represent the distance in base pairs relative to the transcription start site. The hatched boxes represent the CRP binding site.

gal); (ii) in the presence of CRP, RNAP forms a CRP-dependent complex that is fully competent for transcription; (iii) CRP can convert the CRP-independent binary complex to the ternary complex; (iv) removal of CRP from the ternary complex results in a CRP-dependent binary complex that is functionally equivalent to the ternary complex; (v) the CRP-dependent binary complex does not change to the CRP-independent binary complex.

An important general conclusion from the present study is that a major common role of CRP at three representative CRP-dependent promoters is to stimulate events leading to the formation of the open complex. In other words, although CRP is present in the ternary open complex, the role of the CRP–RNAP interaction is already completed

when the productive open complex has been formed at these promoters. We propose that the CRP–RNAP interaction is needed only transiently for transcription activation at CRP-dependent promoters, irrespective of the location of the CRP binding site. A recruitment model was formulated recently as a common mechanism for the activation of transcription in bacteria and yeast (Ptashne and Gann, 1997). Our model is analogous to the recruitment model although the latter did not address the role of activator after the formation of open complex. It would be interesting to test whether our conclusion would be generally applicable to a variety of transcriptional activators both in bacteria and eukaryotes. The heparin-challenge experiment would be useful in answering this

important question, at least in bacteria. Furthermore, how the interaction between activator(s) and RNAP is able to facilitate the formation of productive open complex is the most challenging problem for future work.

Materials and methods

DNA and proteins

The DNA fragments used in this study are shown in Figure 7. The *malT* promoter region was amplified by PCR from the chromosomal DNA of wild-type *E.coli* strain PP6 (Aiba *et al.*, 1981) using two synthetic primers: 5'-TATCCAGTGTGCTCCATCTC-3' and 5'-GCTCAGCAAC-CACGGTATGG-3'. The amplified DNA was digested with *Sau*3AI and *Hpa*II. The resulting 208 bp *Sau*3AI-*Hpa*II fragment containing the *malT* promoter region was cloned between the *Bam*HI and *Acc*I sites of pUC19 to construct pMT100. The 247 bp *Eco*RI-*Hind*III fragment containing the *malT* promoter region was prepared from pMT100 and used for most experiments. The 230 bp *Sac*I-*Hind*III *malT* fragment was used only for the DNase I footprinting experiment. The 240 bp *Hpa*II fragment containing the *gal* promoter region derived from plasmid pBdC1 (DiLauro *et al.*, 1979) was cloned into the *Hinc*II site of pUC119 to construct pGAL100. The 167 bp *Hha*I-*Eco*RI fragment carrying the *gal* promoter region was prepared from pGAL100. CRP was purified from cells harboring pHA7 (Aiba *et al.*, 1982) using the procedure of Eilen *et al.* (1978). RNA polymerase was purified from strain W3350 according to the method of Fukuda *et al.* (1974).

Gel shift assay

The gel mobility shift assays were performed in a total volume of 30 μ l of transcription buffer (20 mM Tris-HCl, pH 7.9, 100 mM NaCl, 3 mM MgCl₂, 0.1 mM EDTA and 50 μ g/ml bovine serum albumin) containing 50 μ M of cAMP and 5% glycerol. The DNA fragments, at a final concentration of 5 nM, were incubated first with CRP (0 or 50 nM) for 5 min at 37°C and then with RNA polymerase (30 nM) for 30 min. The mixture was then treated with 3 μ l of heparin (final concentration 2–6400 μ g/ml) for 3 min. The mixture (5 μ l) was fractionated using electrophoresis on a native 5% polyacrylamide gel containing 0.1 mM cAMP in 1/2 TBE (45 mM Tris-borate, pH 8.3, 1 mM EDTA) at room temperature. The gel was stained with 5 μ g/ml ethidium bromide solution and exposed to a UV lamp. Reversed photographs were used for the figures.

Western blotting

The gels used were subjected to Western blotting as described previously (Tagami and Aiba, 1995). Polyclonal anti-CRP antibody (Ishizuka *et al.*, 1993) was used to probe CRP on the membrane.

DNase I footprinting

Reactions were performed in a total volume of 100 μ l of a transcription buffer containing 5 mM CaCl₂, 50 μ M cAMP and 5% glycerol. The ³²P-end-labeled DNA fragments, at a final concentration of 1 nM, were incubated with CRP (0 or 110 nM) for 5 min and then incubated with RNA polymerase (0 or 110 nM) for 30 min at 37°C. The mixtures were treated with heparin (0–50 μ g/ml) for 3 min at 37°C and incubated at 25°C for 5 min. DNase I was added at a concentration of 50 ng/100 μ l and incubation was continued for 1 min at 25°C. Following the addition of 25 μ l of 1.5 M sodium acetate, 20 mM EDTA, 100 μ g/ml tRNA, the mixture was treated with phenol and precipitated with ethanol, dissolved in 8 M urea loading buffer (0.025% bromophenol blue and 0.025% xylene cyanol in TBE). The products were analyzed on an 8% polyacrylamide-8 M urea gel.

In vitro transcription

The run-off transcription assays were performed in a total volume of 30 μ l of transcription buffer containing 50 μ M cAMP and 5% glycerol. The open complexes were formed under the same conditions as in the gel shift assays. Transcription was started by the addition of 3 μ l of a substrate solution of 0.5 mM 4NTPs containing [α -³²P] UTP (5 μ Ci). After 15 min of incubation at 37°C, the reaction was terminated by the addition of 60 μ l of phenol, 30 μ l of 0.6 M sodium acetate (pH 5.5), 20 mM EDTA and 200 μ g/ml tRNA. The products were precipitated with ethanol and fractionated by electrophoresis on 8 or 20% polyacrylamide gels containing 8 M urea. For the abortive initiation assay, the *Eco*RI-*Hind*III fragment containing the *malT* promoter (5 nM in 30 μ l of transcription buffer containing 50 μ M cAMP and 5% glycerol) was

incubated with RNA polymerase (30 nM) in the absence and presence of CRP (50 nM) as mentioned above. The reaction was started by adding 3 μ l of a solution containing 2 mM ApU and 1 mM [α -³²P]UTP (10 μ Ci). Following incubation at 37°C, the products were precipitated with ethanol and analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea.

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