Coactivation of the RpoS-Dependent proP P2 Promoter by Fis and Cyclic AMP Receptor Protein

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The Escherichia coli proP P2 promoter, which directs the expression of an integral membrane transporter of proline, glycine betaine, and other osmoprotecting compounds, is induced upon entry into stationary phase to protect cells from osmotic shock. Transcription from the P2 promoter is completely dependent on RpoS (σ38) and Fis. Fis activates transcription by binding to a site centered at −41, which overlaps the promoter, where it makes a specific contact with the C-terminal domain of the α subunit of RNA polymerase (α-CTD). We show here that Fis and cyclic AMP (cAMP) receptor protein (CRP)-cAMP collaborate to activate transcription synergistically in vitro. Coactivation both in vivo and in vitro is dependent on CRP binding to a site centered at −121.5, but CRP without Fis provides little activation. The contribution by CRP requires the correct helical phasing of the CRP site and a functional activation region 1 on CRP. We provide evidence that coactivation is achieved by Fis and CRP independently contacting each of the two α-CTDs. Efficient transcription in vitro requires that both activators must be preincubated with the DNA prior to addition of RNA polymerase.

During the transition from rapid vegetative growth to stationary phase, a set of genes is induced which is involved in long-term survival under environmental stress. One such gene in Escherichia coli is proP, which encodes a transporter of proline, glycine betaine, and other osmoprotecting compounds (12, 23, 32). While the upstream P1 promoter of proP is transiently induced as part of a specific stress response to osmotic shock, the downstream P2 promoter is expressed for a brief period as cells are about to enter stationary phase, presumably as a safeguard from potential osmotic stress (39, 56).

Expression from the proP P2 promoter has an unusually high dependence both in vivo and in vitro on the Fis protein and the stationary-phase sigma factor, σ38, encoded by rpoS (57). Fis belongs to the general family of nucleoid-associated factors and is a global regulator of transcription, acting as a repressor at some promoters and an activator at others (13, 19, 21, 24, 35, 43, 48, 54, 55). Under rapid growth conditions, Fis is the most abundant transcriptional regulator in the cell (1, 3). However, Fis levels decline dramatically in late exponential phase and become undetectable in stationary phase. In contrast, σ38 levels do not begin to accumulate until late exponential phase (34). This results in a narrow window of P2 expression due to declining Fis levels and a rising σ38 population shortly before cells enter stationary phase.

We have previously investigated transcriptional activation by Fis at the proP P2 promoter. As shown in Fig. 1A, there are two specific Fis binding sites, located at −41 (site I) and −81 (site II) nucleotides from the start of transcription (56). Activation at P2 is mediated by Fis at site I, which overlaps the −35 binding element for the sigma subunit of RNA polymerase, dictating that Fis is acting as a class II transcriptional activator (57). Fis binding to the weaker Site II does not significantly affect transcription (57). An essential activation patch on Fis has been localized to a four-amino-acid region spanning the loop between helices B and C adjacent to the DNA binding domain of one subunit of the Fis homodimer (7, 38). This region on the upstream subunit of Fis is believed to directly contact the C-terminal domain of the α subunit of RNA polymerase (α-CTD) (38).

In addition to Fis activation, there is an upstream cyclic AMP (cAMP) receptor protein (CRP) binding site located at −121.5 relative to the start of P2 transcription (57). The CRP protein represses the P1 promoter under low osmotic growth conditions; however, the effect of CRP on P2 activation has not been reported (33, 58). In this report, we further investigate the regulation of proP by examining the effects of CRP on transcriptional activation of the P2 promoter. We find that while CRP alone is a very weak activator of P2 transcription, CRP and Fis coactivate transcription synergistically. The properties of CRP and Fis coactivation are explored.

MATERIALS AND METHODS

Plasmids. Plasmid pRJ4069 was used as a template for most of the single-round in vitro transcription assays (57). It contains a segment of the proP sequence from +109 to −200 with respect to the P2 transcription initiation site upstream of the rmdB terminators (T1+T2) in the vector pKK223-3 (Pharmacia). The DNA template with the mutation that prevents CRP binding (pRJ4070) was derived from pRJ4069 (Fig. 1) (58). pRJ677 (+5 insertion in Fis site II) was created from pRJ4069 by a two-step PCR method using an oligonucleotide ranging from −66 to −100 nucleotides that contained a 5-bp insertion between nucleotides −81 and −82 as shown in Fig. 1. For multiple-round transcription reactions, the plasmids used were pRJ4039 (wild type), pRJ4045 (mutations in Fis site II), and pRJ4051 (mutations in Fis site I) (57). All of these plasmids are proP-1043I-lacZ fusion derivatives of the lacZ protein fusion vector pRS414 (50). The sequence changes in these mutations are as noted in Fig. 1 and have been shown previously to strongly reduce or abolish Fis or CRP binding to their respective sites (56, 58).

The in vivo β-galactosidase assays were performed using two sets of plasmids containing CRP, pZ2CRP was the parent of the activation region 1 (AR1) mutations, which were obtained from R. Ebright, Rutgers University. The AR2 mutation (H19YK101E) was derived from pZCRP+, which was provided by S. Bubay, University of Birmingham.

Proteins. Fis wild-type and mutant proteins were purified as described elsewhere (7, 22, 45). CRP was obtained from J. Krakow, Hunter College. The core RNA polymerase enzyme was isolated by the protocol of Burgess and Jendrisak (9) and Lowe et al. (36) as previously described (57). The σ38 protein was overproduced from plasmid pET21 and purified as described elsewhere (51). To purify the α-CTD, extracts containing overproduced levels of the α-CTD peptide
RESULTS

Fis and CRP activate transcription synergistically in vitro at proP P2. A high-affinity CRP site is centered −121.5 nucleotides from the start of transcription at the proP P2 promoter (33, 58). To test the effect of CRP on proP P2 regulation, single-round in vitro transcription reactions were performed with CRP-cAMP and RNA polymerase complexed with σ70 (Es38). As shown in Fig. 2, addition of CRP-cAMP had only a small effect, stimulating transcription about twofold over the basal level (no added activator). Fis typically activated transcription 20- to 30-fold. However, when both Fis and CRP-cAMP were added to the in vitro transcription reaction, the level of activated transcription ranged from 75- to 125-fold. This level of activation is greater than the multiplicative effect of each activator alone. Transcripts from each lane were normalized to the levels of m11 from the vector promoter, which were unaffected by the addition of saturating amounts of CRP-cAMP or Fis. CRP coactivation was dependent on the addition of cAMP, as expected (data not shown). These data show that Fis and CRP activate transcription synergistically at proP P2.

The conditions that promote maximal coactivation by Fis and CRP in vitro were examined. Coactivation was strongly stimulated by a supercoiled template, as was observed with Fis and CRP.
Transcripts originating from contained mutations that reduce Fis binding to site II and site I, as indicated. The DNA templates were wild type (WT), and plasmids that multiple-round in vitro transcription reactions performed in the presence of Fis binding site mutations. Gels show primer extension products generated after but not Fis site II. Fis site II, located at most favorable for coactivation with CRP. The conditions which best promote activated transcription by Fis alone are the most favorable for coactivation with CRP.

Coactivation requires the CRP site at −121.5 and Fis site I but not Fis site II. Fis site II, located at −81, has previously been shown to have little affect on Fis-dependent transcription at proP P2 (57). Since Fis site II is positioned between the essential Fis binding site I at −41 and the CRP binding site at −121.5, it seemed possible that it could influence coactivation with CRP. To test this hypothesis, transcription reactions on the wild-type proP P2 promoter and a template containing a mutation that prevents Fis from binding at site II (56) were compared (Fig. 4). The site II mutant template was able to support efficient synergistic activation with Fis and CRP, indicating that Fis binding to site II is not important for coactivation. As expected, Fis binding to site I was essential for coactivation of proP P2 (Fig. 4). We have shown previously that the site I mutation virtually eliminates binding whereas the site II mutation abolishes binding but creates a weak binding site centered 7 bp upstream of site II (56). The site II mutant was shown to have no effect on Fis activation (57). Likewise, when the CRP site was mutated to prevent binding (58), little coactivation was obtained in the presence of Fis (Fig. 5). Therefore, synergistic activation of proP P2 requires CRP binding at −121.5 and Fis binding at site I but not site II.

Coactivation is dependent on the phasing between CRP and Fis at site I. To determine if the relative helical orientation between Fis and CRP was important for activation, a 5-bp insertion was created between nucleotides −81 and −82 in the Fis site II binding site which would shift the orientation of CRP by a half helical turn of DNA. As shown in Fig. 5, this change in the helical position of the CRP dimer abolished any contribution by CRP on proP P2 activation. As expected, Fis still activated transcription on this template in both the presence and absence of CRP. Thus, coactivation by Fis and CRP is absolutely dependent on the helical phasing between CRP and Fis bound at site I within the promoter.

CRP coactivates proP P2 in vivo. Transcriptional activation by CRP in vivo was also examined. Figure 6A shows proP P2 transcripts visualized by primer extension of total mRNA isolated from wild-type and Δcrp cells. A sharp peak of P2 transcript at 3 h after subculture, typical of proP P2 transcription, was seen only in the wild-type cells, suggesting that CRP is important for optimal P2 expression. However, the growth defect inherent to the Δcrp strain raised the possibility that the CRP effect on P2 transcription might be amplified in this assay. For example, Fis and/or RpoS levels may be altered in the Δcrp cells. Therefore, β-galactosidase assays were also performed using two different proP-lacZ fusions carried on a λ prophage as reporters. Both reporters contain a...
P1(–12) mutation that abolishes transcriptional initiation from the P1 promoter of proP (39, 56). One construct has an otherwise wild-type proP promoter region, while the other carries a mutation that strongly reduces CRP binding to the promoter in vitro as well as in vivo (Fig. 1) (58). With these constructs, the effect of CRP on proP P2 transcription can be evaluated without using cells lacking CRP. Figure 6B shows that in the presence of wild-type CRP (pZYCRP) and a functional CRP binding site, transcription at proP P2 was stimulated 2.5-fold in vivo when the cells were grown in Luria broth. The CRP site mutant had 126 U of β-galactosidase, while the natural promoter produced 391 U. Up to a sevenfold increase in β-galactosidase activity by CRP binding has been measured in M9 glycerol medium, although the overall level of expression was considerably lower (80 U of β-galactosidase).

The stimulation by CRP in vivo was completely dependent on the presence of Fis; cells lacking Fis had <5 U of β-galactosidase (data not shown).

**CRP activates proP P2 through AR1.** Two regions on the CRP protein, AR1 and AR2, have been found to be critical for transcriptional activation at other promoters (10). Depending on the position of the CRP binding site, either AR1 or both regions are required for efficient activation. CRP mutants containing substitutions in both AR1 and AR2 were tested for the ability to activate transcription in vivo at proP P2 by measuring β-galactosidase activity generated by the reporter constructs described above. Wild-type and mutant crp genes were supplied on a plasmid in a Δcrp strain. Figure 6B shows that the CRP mutants T158A and H159L, which contain mutations in AR1, had similar levels of activity regardless of the presence of the CRP binding site, indicating that they were unable to stimulate P2 transcriptional activation. The transcriptional activation was reduced to 106 and 82 U, respectively, from the 391 U of β-galactosidase produced by the wild-type control. Immunoblotting of cell extracts indicated that the CRP AR1 mutants did not alter the temporal expression of Fis (data not shown). In contrast to the AR1 mutants, CRP containing the substitutions H19Y K101E located in AR2 had no detectable effect on P2 transcription (Fig. 6C). The twofold stimulation of transcription in the presence of a functional CRP binding site was maintained in the AR2-CRP mutant, although overall transcription in both reporters was elevated. The reason for these higher levels of transcription with the AR2 mutant is not known. Thus, AR1, but not AR2, is required for CRP activation of proP P2.

**The α-CTD of RNA polymerase binds to the DNA adjacent to CRP.** Due to the remote position of the CRP binding site at proP, CRP is most likely contacting Eσ through the α-CTD of polymerase, which is attached to the rest of polymerase through a flexible linker (6, 29). As shown above, AR1 of CRP, a region that has been shown to contact α-CTD at other promoters (10), is necessary for CRP activation of P2. To deter-
mine if CRP is contacting the α-CTD at proP, we mapped the binding position on the proP promoter of a truncated α subunit containing only the last 85 amino acids comprising the C-terminal domain (20) by DNase I footprinting. The α-CTD peptide was used for these experiments since Es38 binds poorly to a linearized proP P2 template, consistent with the supercoiling requirement for transcription (57). The α-CTD peptide caused a modest reduction in DNase I cleavages throughout the lanes. However, in the presence of CRP, specific protection of DNase I cleavage was observed immediately adjacent to the downstream side of CRP at concentrations of α-CTD used to detect binding at the rrnB P1 promoter containing a strong UP element (6). This protection extends the CRP protected region at least 9 bp on the top strand (Fig. 7) and 7 bp on the bottom strand, as indicated in Fig. 1. The precise boundaries at the CRP and α-CTD junctions are not possible to determine because of the lack of DNase I reactivity within this A/T-rich segment. Since the α-CTD preferentially binds to A/T-rich regions in the minor groove (18), the binding site for the α-CTD may overlap the highly A/T-rich segment present at the downstream end of the CRP protected region. No CRP-dependent protection by the α-CTD was observed upstream of the CRP site. This footprinting data is consistent with CRP stimulating transcription by the promoter-proximal subunit of CRP directly contacting the α-CTD.

Coactivation by CRP and mutant Fis proteins. We have previously shown that a small region on one subunit of Fis, the B-C loop, is required for activation of proP P2 by Fis alone. The important amino acids within this region include residues Gln 68, Arg 71, Gly 72, and Gln 74. Arginine 71 is believed to directly contact RNA polymerase because this residue strongly affects cooperative binding with Es38, and most substitutions at Arg 71 strongly reduce transcriptional activation (38). To determine the effect of this region on coactivation with CRP, in vitro transcription reactions were performed with CRP together with Fis mutants that by themselves are strongly defective in P2 activation. A gel of in vitro transcription reactions showing coactivation by CRP and representative Fis mutants containing substitutions at residue 71 and 72 is shown in Fig. 8. These Fis mutants promoted little to no activated transcription. However, when these mutants were combined with CRP, transcript levels were considerably increased. Coactivation ranged up to 30-fold, depending on the Fis mutant, even though these mutants only weakly potentiated P2 transcription on their own. The full level of activation that is seen with CRP and wild-type Fis is not achieved; however, it is evident that the transcriptionally impaired Fis mutants are still competent for coactivation with CRP.

The order of addition of activators is important for synergistic activation at proP P2. In the previous transcription reactions, Fis and CRP were allowed to bind to the DNA prior to Es38 addition. To determine whether this order of addition was important for efficient activation, we incubated one acti-
DISCUSSION

Previously it has been shown that proP P2 expression is completely dependent on Fis and the stationary-phase sigma factor, σ^38 (39, 56, 57). The 20- to 30-fold activation by Fis is absolutely dependent on the α-CTD of RNA polymerase that contacts the B-C loop region of one of the subunits of the Fis homodimer (7, 38). In this report, we show that the CRP protein acts synergistically with Fis to mediate even higher levels of transcription. Binding of CRP at a site centered 121.5 bp upstream of the P2 promoter gives little (<2-fold) activation by itself. In combination with Fis binding at site I, which overlaps the promoter, CRP mediates a 75- to 125-fold stimulation of transcription at proP P2.

While transcriptional synergy by multiple activators is often found in eukaryotic organisms, there have been relatively few reports of synergy in prokaryotes (26, 46). Examples of synergistic activation include the ansB promoters: in E. coli the coactivation is dependent on both CRP and FNR, and in Salmonella enterica serovar Typhimurium coactivation is dependent on two CRP dimers (49). Synergy has been clearly demonstrated in artificial bacterial promoters between CRP bound upstream of the promoter and either another CRP or lambda cI protein bound to a site overlapping the −35 element (11, 30, 31). Transcriptional activation at proP P2 is the first example of synergy involving the Fis protein, though the reports of Fis as a coregulator of transcription appear to be increasing (14, 16, 25, 28, 37, 54). To our knowledge, a direct role in activation by CRP binding this far upstream of the promoter has not been previously reported.

Coactivation by CRP at proP P2. To provide evidence that CRP binding at −121.5 directly contacts Er^38 at proP P2, CRP mutants containing substitutions at residues that disrupt polymerase-CRP interactions at either class I or class II promoters were tested. The CRP AR1 mutants were found to strongly reduce activation, while the AR2 mutants had no effect on activation of transcription at proP P2. This is not surprising because CRP positioned upstream of the promoter usually does not mediate transcriptional activation through AR2. These results for CRP activation at proP through AR1 are similar to those found where CRP is acting as an upstream activator alone or in conjunction with either another molecule of CRP, FNR, or the λ cI protein bound in an analogous position to Fis site I at proP P2 (11, 30, 31). However, 121 bp upstream of the transcriptional start site is an unusually large distance for direct activation by CRP.

Because AR1 of CRP has been shown to stimulate transcription at other promoters by contacting the α-CTD of RNA polymerase, it is highly likely that CRP is contacting the α-CTD of Er^38 at proP P2 (17, 27). In support of this, DNase I footprinting analysis with purified α-CTD revealed binding of α-CTD to the DNA just downstream of the CRP binding site. This specific association of the α-CTD to the DNA is dependent on CRP binding, suggesting that the CRP protein, together with the sequence of the DNA on the downstream side, is directing the positioning of the α-CTD. Murakami et al. measured the position of both α-CTDs of RNA polymerase at promoters containing two CRP binding sites by affinity cleavage of α-CTD-EDTA·Fe conjugates (41). They found that the α-CTD was unable to contact the DNA when the binding site was at −113.5, even when another CRP dimer was bound at −41.5. Similar results have also been obtained by hydroxyl radical footprinting of CRP-RNA polymerase complexes (5). These findings differ from what we observe at proP P2, where the α-CTD contacts the CRP protein bound even further upstream at −121.5. While the localization of α-CTD by DNase I footprinting performed here is with the α-CTD not tethered to RNA polymerase, in contrast to the previously mentioned experiments, CRP bound at −121.5 nonetheless functions to activate transcription of proP P2 in a manner dependent on AR1. In addition to the sequence of the intervening DNA, an obvious difference between these situations is that at proP the Fis protein is bound at −41 instead of a second CRP molecule. However, it is not expected that Fis would induce a greater degree of DNA bending or a significantly different trajectory of the DNA from CRP bound at the same position (38, 45).

Coactivation by Fis at proP P2. An essential activation region on Fis has previously been localized to the B-C loop...
region (38). In transcription reactions performed in vitro with Fis as the sole activator, B-C loop mutants stimulate little, if any, activated transcription. However, together with CRP, these mutants are able to synergistically activate transcription. Therefore, with CRP bound at the promoter, the dependence on the B-C loop of Fis appears to be alleviated. A competent B-C loop region is necessary to achieve the full level of transcription seen with CRP and wild-type Fis, implying that the B-C loop is still playing a role in coactivation. It is possible that CRP may stabilize the polymerase sufficiently to partially overcome a weakened interaction by the Fis B-C loop mutants with the α-CTD. In addition, it is possible, although we consider it unlikely, that DNA bending promoted by the B-C loop of Fis mutants is sufficient to stimulate transcription when CRP is present.

An alternative explanation for coactivation between CRP and the Fis B-C loop mutants is that a second determinant other than the B-C loop region on the Fis dimer mediates the residual coactivation with CRP. Without a competent B-C loop region, this potential second transcriptional activation region is unable to stimulate significant transcription when Fis is the solitary activator (38). This postulated second patch has two possible targets. One is CRP protein bound at −121.5, but we have not observed any cooperative binding between Fis and CRP at proP that could support such a model. Another explanation is that a second patch on Fis could contact polymerase in a manner different from the previously discovered contact between the B-C loop of Fis and α-CTD of RNA polymerase. Because Fis binding site I overlaps the −35 sigma subunit recognition element, a Fis-σ70-protein-protein contact is possible. Other transcriptional activators such as CRP, FNR, and the Mu Mor protein that bind to an analogous position have been shown to make multiple contacts with RNA polymerase (2, 4, 44, 53). Most of these activators contact the α-CTD through their upstream side and also make a contact on the downstream side with either the σ70 subunit or the N-terminal domain of the alpha subunit. An α-CTD-independent stimulation of rrnB transcription has been noted by Bokal et al. (7). Muskheilishvili et al. have also reported cooperative DNA binding between Fis and σ70 at the tyrT promoter (42).

**A model of synergistic activation at proP P2.** In our model for synergistic activation of proP P2, Fis minimally contacts one α-CTD domain, and the second α-CTD domain is bound to the CRP subunit oriented proximal to the promoter (Fig. 10). The lack of coactivation by CRP on a DNA template with a 5-bp insertion is consistent with the requirement for looping of the intervening DNA. One notable aspect of the coactivation seen at proP is that it is most efficient when both activators are bound to the DNA prior to Err38. This observation implies that once a complex is formed between the first activator and Err38, the second activator is no longer fully capable of stimulating transcription. This could be due to the formation of one of a variety of specific protein-DNA architectures which limit accessibility to the second activator or, in the case of CRP alone, the targeting of Err38 to a nonproductive location. As mentioned previously, an alternative model is that Fis and CRP bound to their respective sites must first interact. In order to achieve maximal coactivation, this interaction may be required to colocalize the two activators close to the promoter via DNA looping prior to the binding of Err38.

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