JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 530–537 Vol. 179, No. 2
0021-9193/97/$04.00+0

Function of the C-Terminal Domain of the Alpha Subunit of *Escherichia coli* RNA Polymerase in Basal Expression and Integration Host Factor-Mediated Activation of the Early Promoter of Bacteriophage Mu

PETER VAN ULSEN,[1] MARCEL HILLEBRAND,[1] MARK KAINZ,[2] ROMAIN COLLARD,[1] LAURENCE ZULIANELLO,[1] PIETER VAN DE PUTTE,[1] RICHARD L. GOURSE,[2] AND NORA GOOSEN[1]*

Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, 2300 RA Leiden, The Netherlands,[1] and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706[2]

Received 31 July 1996/Accepted 7 November 1996

Integration host factor (IHF) can activate transcription from the early promoter (Pe) of bacteriophage Mu both directly and indirectly. Indirect activation occurs through alleviation of H-NS-mediated repression of the Pe promoter (P. Van Ulsen, M. Hillebrand, L. Zulianello, P. Van de Putte, and N. Goosen, Mol. Microbiol. 21:567–578, 1996). The direct activation involves the C-terminal domain of the alpha subunit (αCTD) of RNA polymerase. We investigated which residues in the αCTD are important for IHF-mediated activation of the Pe promoter. Initial in vivo screening, using a set of substitution mutants derived from an alanine scan (T. Gaal, W. Ross, E. E. Blatter, T. Tang, X. Jia, V. V. Krishnan, N. Assa-Munt, R. Ebright, and R. L. Gourse, Genes Dev. 10:16–26, 1996; H. Tang, K. Severinov, A. Goldfarb, D. Fenyo, B. Chait, and R. H. Ebright, Genes Dev. 8:3058–3067, 1994), indicated that the residues, which are required for transcription activation by the UP element of the *rrnB* P1 promoter (T. Gaal, W. Ross, E. E. Blatter, T. Tang, X. Jia, V. V. Krishnan, N. Assa-Munt, R. Ebright, and R. L. Gourse, Genes Dev. 10:16–26, 1996), are also important for Pe expression in the presence of IHF. Two of the RNA polymerase mutants, αR265A and αG296A, that affected Pe expression most in vivo were subsequently tested in in vitro transcription experiments. Mutant RNA polymerase with αR265A showed no IHF-mediated activation and a severely reduced basal level of transcription from the Pe promoter. Mutant RNA polymerase with αG296A resulted in a slightly reduced transcription from the Pe promoter in the absence of IHF but could still be activated by IHF. These results indicate that interaction of the αCTD with DNA is involved not only in the IHF-mediated activation of Pe transcription but also in maintaining the basal level of transcription from this promoter. Mutational analysis of the upstream region of the Pe promoter identified a sequence, positioned from −39 to −51 with respect to the transcription start site, that is important for basal Pe expression, presumably through binding of the αCTD. The role of the αCTD in IHF-mediated stimulation of transcription from the Pe promoter is discussed.

Transcription initiation in *Escherichia coli* is influenced by promoter architecture and by the presence of regulatory proteins, which can either stimulate or repress this process (for a review, see reference 6). A classical *E. coli* promoter contains the −35 and −10 elements; both the sequence and the spacing of these elements are important determinants for promoter strength. Sequences upstream of the −35 element can increase promoter activity (2, 5). It was shown in the *rrnB* P1 promoter that sequences between −40 and −60 (the so-called UP element) increase transcription by interacting with the alpha subunit of RNA polymerase (12, 33). Mutant RNA polymerase, lacking the C-terminal domain of the alpha subunit (αCTD), was unable to contact the UP element, and subsequently, transcription from the *rrnB* P1 promoter was less efficiently initiated (33). The αCTD is a distinct domain of 64 amino acids which can dimerize and bind the DNA (3). The structure of this domain has been solved by nuclear magnetic resonance (21). By using the alanine scan method, in which every amino acid of two regions of the αCTD one by one was exchanged for an alanine, the residues at positions 265, 268, 269, 296, 298, and 299 were identified as important for UP-element binding (12).

A number of bacterial activators which generally bind to a site close to the promoter and which stimulate the initiation of transcription have been identified (20). Of these activators, the cyclic AMP receptor protein (CRP) is the best studied (reviewed in reference 23). Two classes of promoters stimulated by CRP can be distinguished (20). For class I promoters, with the CRP binding site located upstream of the −35 element, most frequently around position −61, contacts have been found between the activator and the alpha subunit of the RNA polymerase complex. Mutation and cross-linking studies revealed that the target for CRP interaction also resides in the αCTD (7, 47, 48). An alanine scan identified glutamic acid 261 as one of the most important residues for this contact (37). In class II promoters, the CRP binding site is located around position −41 where it overlaps with the classical −35 promoter element. Although CRP bound at such a site makes contact with the alpha subunit, mutant RNA polymerase lacking the αCTD is still activated by CRP (1, 45, 47). For these class II

* Corresponding author. Mailing address: Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, Einsteinweg 55, 2300 RA Leiden, The Netherlands. Phone: (71) 5274773. Fax: (71) 5274537. E-mail: GOOSEN_N@RULGCA.LEIDENUNIV.NL.

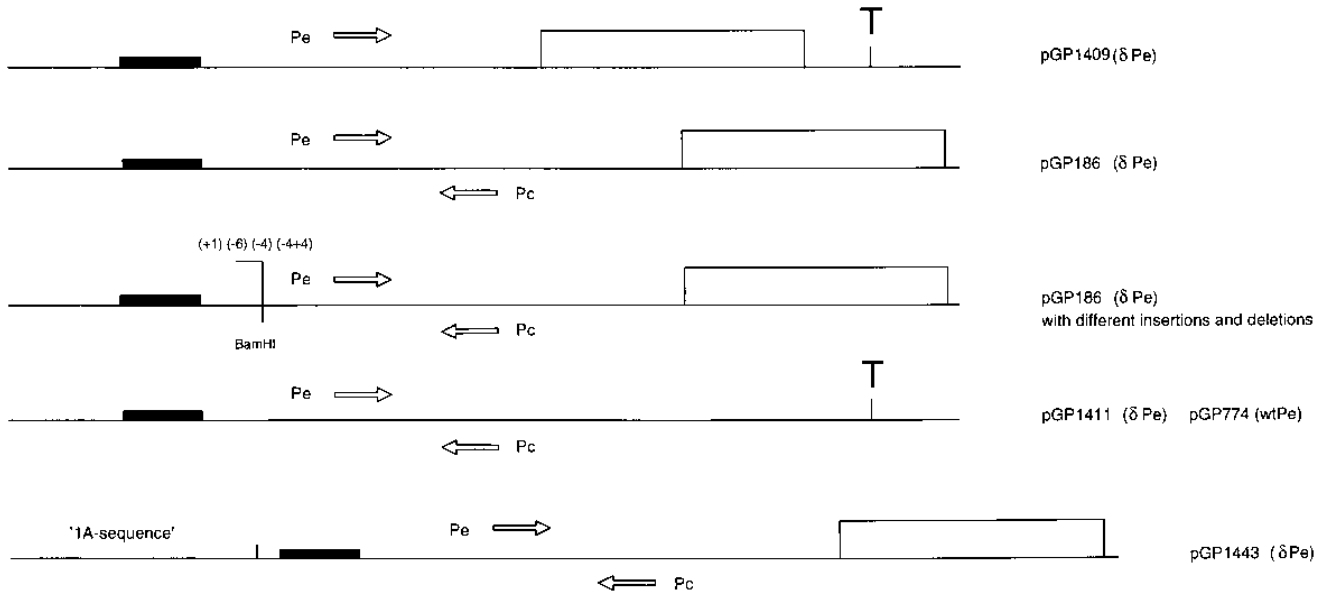

FIG. 1. Schematic representation of Pe promoter constructs used in this paper. The promoters are indicated by arrows; the *ihf* site is indicated by a filled box. The open boxes represent the *galK* reporter gene. T indicates the position of the transcription terminator fragment. Plasmid pGP1409 (δPe) contains the Mu sequence from coordinates 821 to 1063. Plasmids pGP186 (δPe), pGP774 (wtPe), and pGP1411 (δPe) contain the Mu sequence from coordinates 821 to 1243. PGP186 derivatives carry different insertions or deletions between the *ihf* site and the δPe promoter that change the spacing between the two with the number of base pairs indicated. Plasmid pGP1443 contains a 1A sequence upstream of the *ihf* binding site, replacing Mu coordinates 821 to 930.

promoters, contacts between the CRP and the σ subunit of the RNA polymerase complex seem to be most important for stimulation (22, 25).

For a number of other bacterial activators, among them the CRP-like protein FNR, PhoB, AraC, OxyR, and integration host factor (IHF), it has been shown that they mediate their effect via the αCTD (20). For this reason, they were classified as class I activators, although it is not known whether all these proteins indeed function by directly contacting the αCTD. IHF belongs to the family of histone-like proteins in *E. coli*, which also includes proteins like HU, Fis, and H-NS (reviewed in reference 36). These proteins bind and bend the DNA and are believed to be involved in compacting the bacterial chromosome, in analogy to the eukaryotic histones. IHF is a dimer consisting of two homologous subunits, IHF α and IHF β, which recognizes a specific 13-bp consensus sequence (WAT CAANNNNTTR). This consensus is located in one half of the DNA region that IHF protects in a DNase I footprint (8, 16, 46). The stable complex of IHF and its binding site results in a strong bending of the DNA.

IHF is involved in many cellular processes (reviewed in reference 11), including site-specific recombination, DNA replication, transposition, phage packaging, and gene expression. In most processes, the role of IHF is architectural, by helping the formation of nucleoprotein complexes through bending of the DNA. IHF can influence gene expression both positively and negatively (reviewed in reference 18). Activation of transcription by the protein can be achieved via three distinct mechanisms. First, its role can be purely architectural, as for instance is the case for a number of σ^{54} promoters (19, 35). Binding of IHF to its recognition site located between an activator binding site and the promoter induces a bend in the DNA that facilitates interaction between the activator and RNA polymerase. A second mechanism of regulation was recently discovered for the divergent Pe and Pc promoters of bacteriophage Mu and involves the histone-like protein H-NS (43). IHF counteracts inhibition of transcription by H-NS at these promoters, through interference with the formation of a repressive H-NS–DNA complex. Finally, IHF can stimulate transcription directly, without the involvement of other proteins. This direct stimulation of transcription was shown in vivo and in vitro for the bacteriophage λ pL1 promoter (13, 15), the *ilvGMEDA* P_G2 promoter (30), and the bacteriophage Mu Pe promoter (17, 24). In these promoters, the *ihf* site is located around position −85, or −95 in the upstream Pe promoter region. Since transcription from the λ pL1 and Mu Pe promoters could no longer be stimulated by IHF in vitro, when the αCTD was deleted (14, 43), IHF was classified as a class I activator. In this paper, we searched for amino acid residues in the αCTD involved in IHF-mediated activation of the Pe promoter, by using the same set of alanine substitutions used for identification of the targets of the CRP (37) and the UP element (12). The results show that the same amino acids of the αCTD that are important for binding to the UP element of the *rrnB* P1 promoter affect also the basal expression from the Pe promoter in the absence of IHF. Furthermore, mutant RNA polymerase with substitution R265A in the αCTD, which showed the lowest level of basal expression, could no longer be activated by IHF. These results suggest that the αCTD binds to a UP-element-like sequence in the Pe promoter region and that IHF activates transcription by improving the binding of the αCTD to the DNA. Possible αCTD binding sequences in the Pe promoter region are investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains used in this paper are AB1157 (28), pp3294 (*thy rpsL galK*), pp3295 (*thy rpsL galK ihfB*/*hip157*), pp3296 (*thy rpsL galK hns*::Tn*10*), and pp3297 (*thy rpsL galK ihfB*/*hip157 hns*::Tn*10*). Construction of the last four strains has been described previously (43).

The plasmids carrying the mutated alpha subunit genes generated by the alanine scan method have been described elsewhere (12, 37). The gene coding for the alpha subunit lacking the αCTD was constructed by filling in a *Hin*dIII site with the Klenow fragment of DNA polymerase I, followed by religation of the blunt ends. The filling-in was checked by sequencing and results in a frame-

shift that leads to a truncated alpha subunit of 230 amino acids to which three additional amino acids (SFR) are fused.

All Pe promoter constructs used in this study are depicted in Fig. 1. The δPe promoter has a 1-bp deletion between the −35 and −10 elements, resulting in a reduced activity of this promoter (42). For the construction of the δPe-*galK* fusion in the low-copy-number plasmid pACYC184, a *Cla*I-*Nde*I fragment, containing the δPe-*galK* fusion of plasmid pGP188 (42), was cloned into the *Acc*I-*Nde*I sites of the polylinker of pUC21 (44). Next, a *Bam*HI-*Xba*I fragment containing the transcription terminators of the *rrnB* gene was inserted downstream of the *galK* gene, resulting in plasmid pGP1407. Finally, the *Sph*I-*Xba*I fragment of pGP1407, containing the whole construct, was ligated in the *Sph*I-*Xba*I sites of plasmid pACYC184, resulting in plasmid pGP1409.

The plasmids used for the in vitro transcription studies (pGP774 and pGP1411) have been described before (42, 43).

Plasmids pGP186, pGP186+1, pGP186−6, and pGP186−4 used in the in vivo experiments have been described by Van Rijn et al. (42). The plasmid pGP186−4+4 was constructed by filling in the *Bam*HI site, with the Klenow fragment of DNA polymerase I followed by the religation of the blunt ends. The results were checked by sequencing.

For the construction of plasmid pGP1443, in which Mu-derived sequences upstream of the *ihf* binding site are replaced by a sequence which was shown (9) to contain no intrinsic bending (1A sequence), first the Mu-derived sequences (coordinates 821 to 930) upstream of the *ihf* binding site of plasmid pGP186+1 were eliminated, by inserting a synthetic fragment

*Cla*I, 5′-CGATTACTGATTTAAACTCCTTATT**TATCAACGCGTTA**ATCAGGG-3′
3′-TAATGACTAAATTTGAGGAATAA**ATAGTTGCGCAA**TTAGTCCCCTAG-5′ *Bam*HI.

(the *ihf* consensus sequence is given in boldface; the *Mlu*I restriction site is underlined) in the *Cla*I-*Bam*HI sites of this construct, resulting in plasmid pGP1424. Next, the *Bam*HI site was removed by making use of the *Mlu*I sites in the *ihf* consensus sequence and the *galK* gene. The *Mlu*I fragment of plasmid pGP1434 was exchanged for the *Mlu*I fragment of wild-type (wt) plasmid pGP186. Finally a *Cla*I-*Acc*I fragment from pUC21, containing the 1A sequence (9), was cloned in the *Cla*I site, resulting in plasmid pGP1441. For the exact sequence, see Fig. 3B.

Proteins. IHF was purified as described by Zulianello et al. (50). The commercially purchased *E. coli* RNA polymerase was from Pharmacia. Reconstitution of wt and mutant RNA polymerases has been described elsewhere (38). IHF was diluted in buffer B (50 mM Tris [pH 7.5], 1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol). The RNA polymerases were diluted in in vitro transcription reaction buffer (see "In vitro transcription assay"), omitting the nucleotides.

GalK assay. Galactokinase (GalK) activities were determined as described elsewhere (27). GalK activities (the average of three to six experiments) are expressed as nanomoles of galactose phosphorylated per minute per 10^8 bacteria at 32°C.

In vitro transcription assay. In vitro transcription reactions were carried out essentially according to the work of Ross et al. (34) in a total volume of 10 μl containing 10 fmol of supercoiled plasmid DNA, 10 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 500 μM ATP, 50 μM GTP, 50 μM CTP, 10 μM UTP, and 40 μM [α-^{32}P]UTP (Amersham, 3,000 Ci/mmol). Different amounts of IHF were preincubated with pGP774 or pGP1411 DNA for 10 min at room temperature. When no protein was added, buffer B was added instead. Next, the transcription was initiated by adding 10 fmol of reconstituted wt or αG296A RNA polymerase or 50 fmol of reconstituted Δ235 or αR265A RNA polymerase. These concentrations of reconstituted RNA polymerases gave rise to the same amount of RNA I transcript (expressed from the same pGP774 or pGP1411 plasmids) in the absence of IHF. After incubation at room temperature for 30 min, the reactions were terminated by addition of 10 μl of stop solution (7 M urea, 1% sodium dodecyl sulfate, 10 mM EDTA, 0.04% bromophenol blue). A 6-μl aliquot of the incubation mixture was electrophoresed on a 6% acrylamide–7 M urea gel, and the transcripts were visualized by autoradiography. For quantification of the transcripts, the gels were counted in a β-scope radiographic imager (Betagen Inc.).

RESULTS

Effect of mutant alpha subunits on expression from the Pe promoter in the presence of IHF in vivo. The early (Pe) promoter of bacteriophage Mu controls the expression of the genes needed for transposition (17). The stimulation of transcription from this promoter by IHF was shown in vivo, with *galK* fusion plasmids (41, 42). Furthermore, in vitro studies indicated that this stimulation is mediated via the αCTD of the RNA polymerase (43). To locate the target of IHF-mediated activation in the αCTD more precisely, two sets of alanine scan mutants isolated by Tang et al. (37) and Gaal et al. (12) were tested in vivo for their activities in Pe expression in the presence of IHF. For this purpose, strain AB1157 was cotransformed with a high-copy-number plasmid carrying a gene coding for one of the mutated alpha subunits and the low-copy-number Pe-*galK* construct pGP1409 (see Materials and Methods). Expression of the mutated *rpoA* gene from the high-copy-number plasmid results in a greater part of the RNA polymerase complexes being mutant. The Pe-*galK* construct contains a down-mutation in the Pe promoter (δPe [42]), with a lower activity than that of the wt. The IHF-mediated stimulation of this δPe promoter is more readily observed. The alanine substitutions in the αCTD that were tested are located in the regions from residues 256 to 270 and 291 to 301 (Table 1). Plasmids expressing either the wild type or a truncated alpha subunit, with a deleted αCTD (see Materials and Methods), were used as controls.

TABLE 1. GalK activity of pGP1409 in the presence of different mutant alpha subunits[a]

Subunit	Activity of strain AB1157 (pGP1409)	% of wt
α-wt	14.2 ± 3.0	
Deletion-α	5.7 ± 1.3	40
αP256A	14.1 ± 0.9	99
αV257A	10.7 ± 1.7	75
αD258A	11.7 ± 1.6	82
αD259A	10.5 ± 1.2	74
αL260A	9.6 ± 1.1	68
αE261A	11.7 ± 1.7	82
αL262A	10.0 ± 1.3	70
αT263A	10.0 ± 1.3	70
αV264A	13.5 ± 1.7	95
αR265A	4.9 ± 1.6	35
αS266A	11.9 ± 2.2	84
αA267	Not determined	
αN268A	7.9 ± 1.4	56
αC269A	10.5 ± 2.2	74
αL270A	11.2 ± 1.2	79
αK291A	15.3 ± 1.1	107
αT292A	16.1 ± 1.6	113
αP293A	12.4 ± 2.2	87
αN294A	13.4 ± 0.7	94
αL295A	12.1 ± 0.9	85
αG296A	7.6 ± 0.3	54
αK297A	15.3 ± 1.8	108
αK298A	8.4 ± 0.9	58
αS299a	7.6 ± 0.6	54
αL300A	15.4 ± 1.2	108
αT301A	16.0 ± 1.9	113

[a] GalK activities are expressed in nanomoles of galactose phosphorylated per minute per 10^8 cells at 32°C.

Deletion of the αCTD results in lower *galK* expression from the δPe promoter, as was expected (Table 1). Within the region 255 to 270, substitution R265A strongly reduces expression of the δPe-*galK* fusion, while substitution N268A has a moderate effect. Substitution of R265A in the αCTD results in an RNA polymerase that is even more impaired in *galK* expression than mutant RNA polymerase lacking the αCTD. This could be the result of a reduced stability of the truncated α subunit, since construction of the mutant gene encoding this subunit results in the addition of 3 amino acids, which are normally not present in this protein (see Materials and Methods). Such unstable truncated alpha subunits would lead to a higher level of wt RNA polymerase with respect to the mutant RNA polymerase in the cell. Within the region 291 to 301, substitutions G296A, K298A, and S299A lead to a moderately

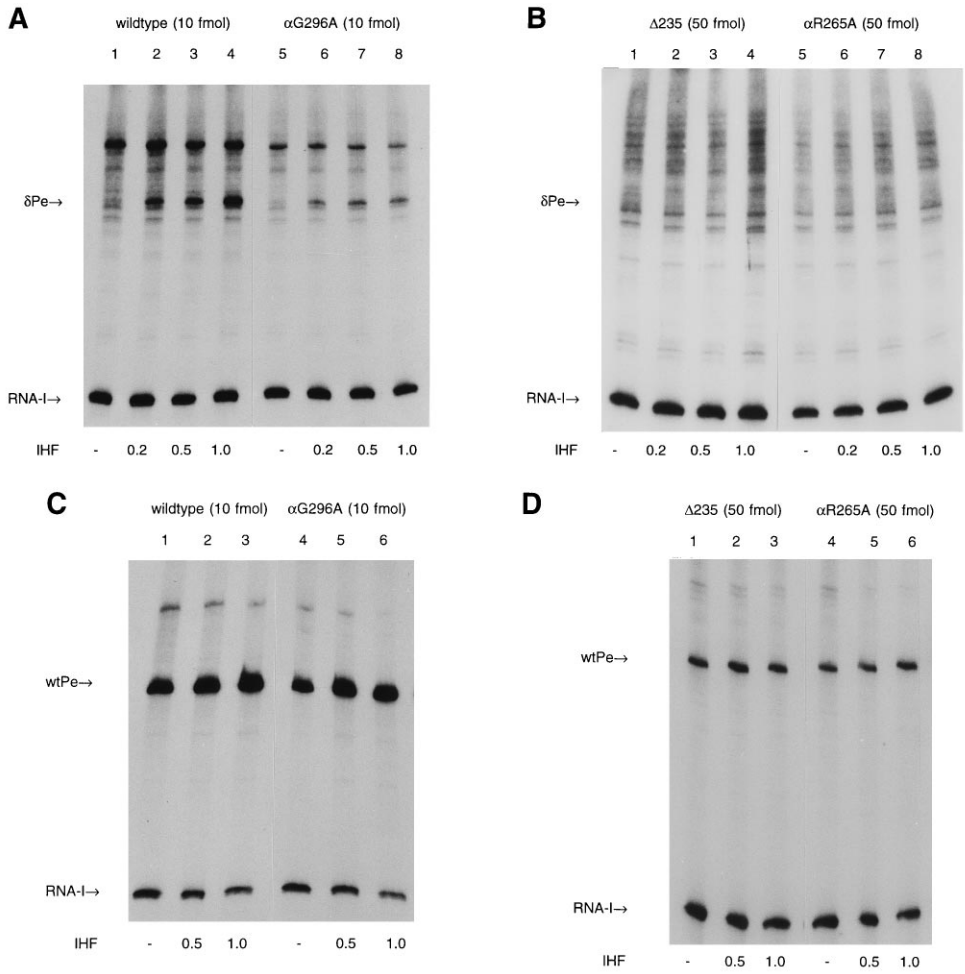

FIG. 2. In vitro transcription assays with plasmids pGP1411 (δPe) (A and B) and pGP774 (wtPe), (C and D) in the absence and presence of the IHF protein. The reconstituted RNA polymerases used are wt RNA polymerase and αG296A RNA polymerase (10 fmol) (A and C) and Δ235 RNA polymerase and αR265A RNA polymerase (50 fmol) (B and D). Amounts of IHF are in picomoles. The positions of the Pe and RNA I transcripts are indicated. The amounts of wt Pe transcripts were quantified in a β-scope radiographic imager. From these quantifications, the factor of IHF-mediated activation at 1 pmol of IHF was calculated to be 2.2 for wt RNA polymerase, 3.6 for αG296A RNA polymerase, and 1.0 for Δ235 RNA polymerase and αR265A RNA polymerase.

reduced *galK* expression, comparable to that of the αN268A mutant.

All the mutations that result in lower Pe expression have also been shown to lead to reduced binding of the alpha subunit to the UP element of the *rrnB* P1 promoter (12). This suggests that, for optimal expression from the Pe promoter, the αCTD has to contact the DNA, as was found for the *rrnB* P1 promoter.

Effects of mutant alpha subunits on transcription from the Pe promoter in vitro. To investigate whether the results with the alpha mutants obtained in vivo indeed reflect a direct effect of these mutants on Pe expression, we performed in vitro transcription experiments with reconstituted RNA polymerases, carrying wt or mutant alpha subunits. Two single-substitution mutants, αR265A and αG296A, were chosen, one from each region, mutants which strongly affected Pe expression in our in vivo assay. In addition, RNA polymerase reconstituted with an alpha subunit lacking the αCTD (Δ235 RNA polymerase) and a reconstituted wt RNA polymerase were included in our assays as controls. The reconstitution of the RNA polymerase complexes has been described previously (38). In the in vitro transcription assay, supercoiled plasmid pGP774 (wt Pe) or pGP1411 (δPe) is incubated with reconstituted wt and mutant RNA polymerases in the absence and presence of purified IHF (see Materials and Methods). When the reconstituted wt RNA polymerase is compared with the commercially obtained RNA polymerase, twice the amount of reconstituted polymerase has to be used to obtain a comparable level of the RNA I transcript, which serves as a reference in our assays (results not shown). Both the δPe and the wt Pe promoters are stimulated by IHF when the reconstituted wt RNA polymerase is used (Fig. 2A and C). To obtain comparable levels of RNA I transcript, five times more of the Δ235 RNA polymerase than of the reconstituted wt RNA polymerase has to be used (Fig. 2B and D). This might be due to a reduced stability of the mutant RNA polymerase complex or to an overall reduced level of transcription initiation by this mutant RNA polymerase. Transcription from the Pe promoter is even more reduced with the Δ235RNA polymerase, since the relative amount of wt Pe-derived transcript, compared to the RNA I transcript, is much lower than when the reconstituted wt RNA polymerase is used (Fig. 2, compare C and D); the δPe transcript cannot even be detected (Fig. 2B).

When the αR265A RNA polymerase is used (Fig. 2B and

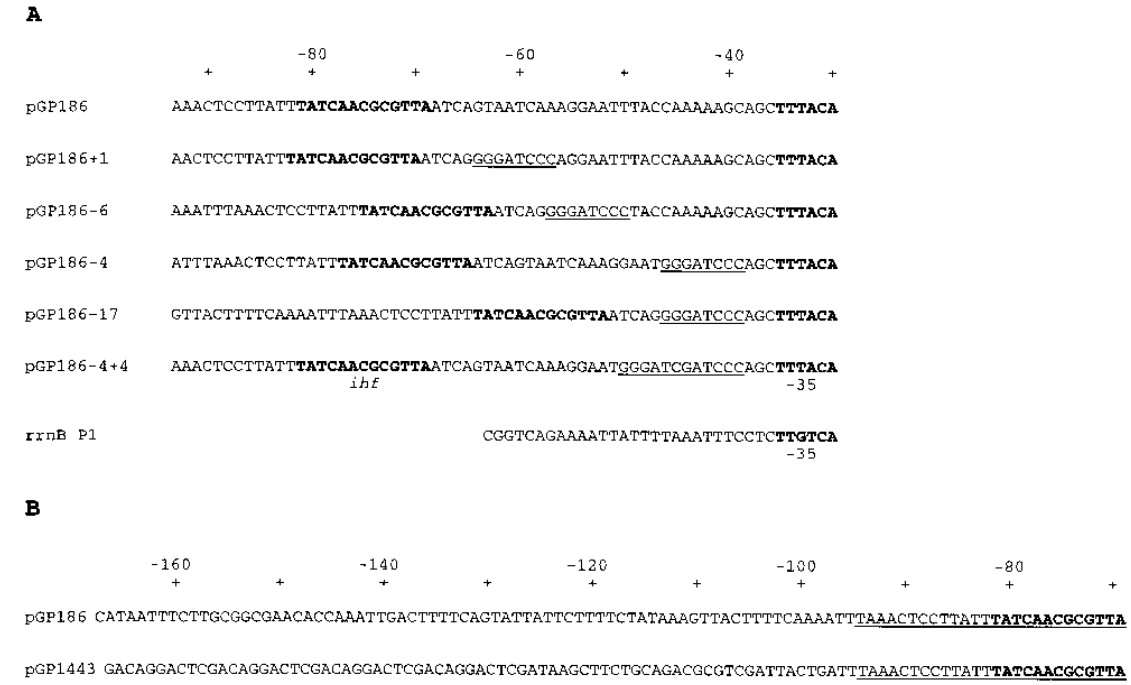

FIG. 3. Sequences of the upstream region of the Pe promoter variants used in this paper. (A) Sequences of plasmids used for identification of a UP-element-like sequence upstream of the Pe promoter. The *ihf* consensus sequence and the −35 promoter element are given in boldface. The *Bam*HI linker sequences are underlined. Positions are indicated with respect to the transcription start site. The upstream region of the *rrnB* P1 promoter, containing the UP element, is also shown (31). (B) Sequences of plasmids used for exchanging the Mu-derived sequence upstream of the *ihf* binding site. Positions are indicated with respect to the transcription start site. The *ihf* consensus is given in boldface; the *ihf* binding site is underlined.

D), overall transcription is as low as with the Δ235RNA polymerase, as judged by the amount of mutant RNA polymerase required. In addition, the αR265A RNA polymerase is specifically impaired in transcription from the Pe promoter, resulting in a reduced amount of wt Pe transcript and no detectable δPe transcript. The αG296A RNA polymerase seems to be less affected in overall transcription compared to the Δ235 and the αR265A mutants, since a normal amount of RNA I transcript was found (Fig. 2A and C). Nevertheless, the rate of transcription initiation at the wt Pe promoter appears to be slightly affected, as the relative amount of wt Pe transcript, compared to the amount of RNA I transcript, is somewhat lower than with wt RNA polymerase (about 60% when measured). These results show that mutations in the αCTD can lead to a reduced basal expression from the Pe promoter, presumably because this domain needs to interact with the DNA for optimal Pe promoter activity.

Addition of purified IHF to the in vitro transcription reaction results in stimulation of transcription from both Pe promoters when the reconstituted wt RNA polymerase is used (Fig. 2A and C). As expected, IHF does not stimulate transcription with the Δ235 RNA polymerase, since the αCTD has been shown to be essential for IHF-mediated activation of Pe (Fig. 2B and D). Addition of IHF does not stimulate when the mutant αR265A RNA polymerase is used (Fig. 2B and D). Apparently, R-265 is not only involved in basal expression from the Pe promoter but is also involved in the IHF-mediated activation. This could mean that R-265, which for basal transcription is contacting the DNA, is now contacting IHF. More likely, since the 265-amino-acid moiety has been shown to be involved in DNA contacts (12, 21), the interaction of the αCTD with DNA that enhances the level of basal expression could be further promoted by IHF. IHF can normally activate transcription from the Pe promoter when the αG296A RNA polymerase is used (Fig. 2A and C). Apparently, a mutation in the alpha subunit that leads to a partial reduction of basal Pe expression can still be activated by IHF.

Sequences between the *ihf* site and the −35 promoter element are important for basal Pe expression. The in vitro transcription experiments indicate that binding of the αCTD to the promoter region is required for optimal basal Pe expression. In the *rrnB* P1 promoter, the αCTD contacts the UP element located from −40 to −60 with respect to the transcription start site (12, 33). Therefore, a UP-element-like sequence could be present in the region between the −35 promoter element and the *ihf* recognition sequence. The sequence of this region is A/T rich, like the sequence of the *rrnB* UP element (Fig. 3). We investigated this hypothesis by using a set of δPe-*galK* constructs with *Bam*HI linkers inserted at different positions in the DNA region upstream of the −35 promoter element (Fig. 3). Since expression from the Pe promoter is influenced by IHF and H-NS (43), the basal expression from this promoter has to be studied in a strain lacking both proteins (pp3297 [see Materials and Methods]). The constructs that were tested are listed in Fig. 3. For plasmids pGP186+1 and pGP186−6, with the *Bam*HI linker inserted at positions −57 and −50, respectively, the same basal expression is found as for the wt construct pGP186 (Table 2). The insertion of a *Bam*HI linker changes the relative position of all sequences located further upstream with respect to the promoter (Fig. 3). Therefore, our results show that sequences upstream of position −49 are not important for the basal expression. Also, other plasmids with the *Bam*HI linker inserted at the same positions but fused to different sequences upstream gave comparable levels of GalK expression (results not shown). The basal expression of the pGP186−4 plasmid, with the *Bam*HI linker inserted at posi-

TABLE 2. GalK activities of δPe promoter constructs with *Bam*HI linkers inserted at different locations[a]

Construct	Activity in pp3297 (*ihf*/*hns*)
pGP186 (no *Bam*HI)	201 ± 44
pGP186+1 (*Bam*HI at −57)	197 ± 35
pGP186−6 (*Bam*HI at −50)	236 ± 38
pGP186−4 (*Bam*HI at −39)	61 ± 6
pGP186−17 (*Bam*HI at −39)	62 ± 6

[a] Activities were measured in an *ihf*/*hns* strain. GalK activities are expressed in nanomoles of galactose phosphorylated per minute per 10^8 cells at 32°C.

TABLE 3. GalK activities of δPe promoter constructs with mutations in the putative UP-element-like sequence[a]

Strain	pGP186	pGP186−4	pGP186−4+4
pp3294 (wt)	460 ± 70	14 ± 3	184 ± 26
pp3295 (*ihf*)	21 ± 3	10 ± 3	22 ± 2
pp3296 (*hns*)	430 ± 75	24 ± 4	287 ± 47
pp3297 (*ihf*/*hns*)	201 ± 44	61 ± 6	154 ± 27

[a] Activities were measured in wt, *ihf*, *hns*, and *ihf*/*hns* strains. GalK activities are expressed in nanomoles of galactose phosphorylated per minute per 10^8 cells at 32°C.

tion −39, however, is considerably reduced (Table 2). This suggests that the sequence upstream of −39 (inserting the *Bam*HI linker did not change the C at position −39) but downstream of position −50 influences basal expression, probably by binding the αCTD, in analogy to the *rrnB* UP element. Also, construct pGP186−17, with the *Bam*HI linker at the same position, but with a different fusion to the upstream sequences, shows a reduced basal Pe expression (Table 2).

It has been shown that the purified alpha subunit specifically binds to the UP element of the *rrnB* P1 promoter (33). We did not observe binding of purified alpha to the Pe upstream region, however, although the concentrations that we use (up to 1 μM) may not have been high enough to observe complete occupancy. Thus, although there might be sequences upstream of the Pe −35 promoter element that show a higher affinity for the αCTD, the binding of the alpha subunit to this region seems less stable compared to the binding to the *rrnB* UP element.

The in vitro transcription experiments show that residue R-265 is important for both basal Pe expression and IHF-mediated activation. This residue has also been described as important for DNA binding (12) and thus possibly for interaction with the −39 to −51 region. IHF might therefore activate Pe expression by helping the αCTD to bind to this upstream Pe promoter region. To test this hypothesis, we investigated whether impaired basal Pe expression, as a result of the disrupted UP-element-like sequence, influences IHF-mediated activation. The *ihf* site in the pGP186−4 construct, however, is not properly positioned on the DNA helix, since it is located 4 bp closer to the −35 promoter element compared to the normal situation, and as a consequence, IHF is no longer able to stimulate Pe transcription (42, 43). We therefore restored the phasing of the *ihf* recognition sequence, by filling in the receding ends of the *Bam*HI site, resulting in plasmid pGP186−4+4 (see Materials and Methods). As a consequence, the sequence between positions −39 and −51 of plasmid pGP186−4+4 remains different from the wt sequence, but it also differs from the sequence of plasmid pGP186−4 (Fig. 3). Expression from the Pe promoter was measured in the absence and presence of IHF and H-NS (Table 3). The basal Pe expression of the pGP186−4+4 construct (in the absence of IHF and H-NS) appears to be higher than the basal expression of pGP186−4 but is still reduced compared to the normal basal Pe expression. Apparently, the additional 4 bp partially restore the binding of the αCTD to this region, although they do not restore the original sequence of the region. IHF is activating Pe expression in pGP186−4+4 (compare pp3296 and pp3297), but the level is lower than in the wt construct pGP186. This shows that IHF does not fully compensate for the reduced binding of the αCTD in the −39 to −51 region, suggesting that also in the presence of IHF the αCTD must remain in contact with this region for optimal Pe expression. The Pe promoter in the pGP186−4+4 construct is repressed by H-NS (pp3295), and this repression is alleviated by IHF (pp3294). This is in agreement with previous results, which showed that the αCTD is not involved in this process (43).

Replacement of sequences upstream of the *ihf* site has no effect on IHF stimulation. Our experiments suggest that the αCTD binds to a UP-element-like region in the Pe promoter both in the absence and in the presence of IHF. IHF might activate transcription by improving the binding of the αCTD to this upstream Pe promoter region. Since, in the RNA polymerase complex, two alpha subunits are present, it seems also possible that IHF facilitates the binding of the second αCTD to DNA sequences upstream of the *ihf* binding site, which are brought in the vicinity of the RNA polymerase-promoter complex by the bending introduced by IHF. The most straightforward way to test this would be to use linear promoter constructs lacking the sequences upstream of the *ihf* site in the transcription assay. These experiments, however, are not feasible, since the activity of the Pe promoter on linear DNA is too low to detect (our unpublished observations). Instead, we constructed a δPe-*galK* fusion plasmid (pGP1443) in which we replaced sequences upstream of the *ihf* binding site (Mu coordinates 821 to 930) of plasmid pGP186 with other DNA sequences (see Materials and Methods). The DNA that was chosen for this purpose is a 1A sequence fragment consisting of eight repeats of CAGGACTCGA (9), flanked by sequences of the polylinker of pUC21 (see Materials and Methods). Unlike the original Mu-derived sequences, this DNA is not A/T rich and contains no sequence-directed bends, which have been proposed to influence transcription initiation (4, 26, 49). Expression was measured both in the absence and in the presence of IHF and H-NS (Table 4). Although the levels of *galK* expression of pGP1443 differ slightly from levels obtained with the wt construct pGP186, IHF is able to activate Pe expression from this plasmid, both in the absence and in the presence of H-NS. Apparently, transcription activation by IHF is not mediated via sequence-specific binding of the αCTD to the DNA upstream of the *ihf* site, although it cannot be excluded that nonspecific contacts are facilitated.

TABLE 4. GalK activities of δPe promoter constructs with or without deletion of the DNA sequences upstream of the *ihf* binding site[a]

Strain	pGP186	pGP1443
pp3294 (wt)	460 ± 70	361 ± 65
pp3295 (*ihf*)	21 ± 3	50 ± 7
pp3296 (*hns*)	430 ± 75	421 ± 65
pp3297 (*ihf*/*hns*)	201 ± 44	281 ± 43

[a] Activities were measured in wt, *ihf*, *hns*, and *ihf*/*hns* strains. GalK activities are expressed in nanomoles of galactose phosphorylated per minute per 10^8 cells at 32°C.

DISCUSSION

Besides being involved in the activation of RNA polymerase by many transcription factors (6, 20), the αCTD of the RNA polymerase complex plays an important role in the basal expression of a promoter, when this promoter contains an αCTD binding site, such as the UP element in the *rrnB* P1 promoter (12, 33). The experiments described in this paper indicate that the early promoter (Pe) of bacteriophage Mu requires for efficient basal expression the binding of the αCTD upstream of the −35 promoter element. A mutant RNA polymerase that lacks the αCTD is specifically impaired in transcription from Pe in an in vitro transcription assay. Two mutant RNA polymerases, carrying alpha subunits with Ala substitutions at position 265 or 296, also showed reduced transcription from the Pe promoter. These two mutations impair the binding of the αCTD to the UP element of the *rrnB* P1 promoter (12), suggesting that for efficient Pe expression the αCTD has to bind a UP-element-like sequence upstream of the Pe promoter. Such a sequence could be identified in the region from −39 to −51, with respect to the transcription start site. When this sequence is changed by inserting a *Bam*HI linker, the basal Pe expression decreases about fourfold. This reduction, however, is less pronounced compared to the 30-fold reduction that was observed when the *rrnB* UP element was deleted (32). Furthermore, unlike the *rrnB* UP element, no specific binding of purified alpha subunit to the Pe UP-element-like region could be shown. The affinity of the αCTD for the upstream Pe promoter region appears to be much lower than the affinity for the UP element. There are no obvious homologies between the *rrnB* UP element and the upstream region of the Pe promoter, but both DNA sequences are relatively A/T rich (Fig. 3).

The sequence of the Pe UP-element-like region contains a tract of adenines. For a number of different promoters, it has been shown that the cloning of A/T-rich sequences upstream of the promoter increases transcription (4, 26, 31, 40, 49). These results are commonly explained by the intrinsic curvature of these sequences, but an enhanced binding of the αCTD of the RNA polymerase complex to these A/T-rich sequences could also explain their stimulating effect. It has also been shown that A/T-rich sequences upstream of a promoter can inhibit the clearance of this promoter by RNA polymerase, due to the increased stability of the RNA polymerase-promoter complex (10). Such an enhanced stability can possibly also be explained by binding of the αCTD to the A/T-rich DNA.

IHF activates Pe expression via two distinct mechanisms, indirectly via counteracting the H-NS-mediated repression of the Pe promoter and directly without the involvement of other proteins (43). The indirect transcription activation does not involve the αCTD, since repression of transcription from the Pe promoter by H-NS is also relieved by IHF, when a mutant RNA polymerase lacking the αCTD is used. The results presented in this paper confirm this observation. The Pe promoter constructs that give a reduced basal expression in vivo, as a result of mutations in the UP-element-like sequence, are still normally repressed by H-NS, and this repression is alleviated by IHF. Furthermore, when mutant RNA polymerases containing the αR265A or the αG296A are used, the Pe promoter is still subject to H-NS-mediated repression, which IHF can counteract (results not shown).

For both the λ pL promoter and the Mu Pe promoter, it has been shown that the presence of the αCTD is required for direct activation (14, 43). The results described here show that the same amino acid (R-265) that is involved in binding of the αCTD to DNA is also important for the IHF-mediated activation. Our results suggest furthermore that both in the presence and in the absence of IHF the αCTD binds to the UP-element-like region upstream of the Pe promoter. In the RNA polymerase complex, there are two alpha subunits and it appears that the αCTDs can dimerize (3). Nevertheless, it has not been shown unambiguously that both CTDs are involved in contacting DNA. If they do, the most straightforward model for the IHF-mediated activation would be that IHF stabilizes the binding of the two αCTD's to the UP-element-like region between the *ihf* site and the Pe promoter, either by inducing conformational changes in the DNA or by contacting the αCTD domain. Residues of the αCTD involved in such a contact might well be located in a different region of this domain, which has not been explored in the collection of alanine substitution mutants presented in this study.

Alternatively, if only one αCTD interacts with the DNA upstream of the −35 region, the second αCTD would be available for making contacts with DNA sequences located further upstream, i.e., on the other side of the *ihf* site. The activating function of IHF would then be to facilitate this contact by bringing the upstream sequences closer to the RNA polymerase complex through bending of the DNA. Such an interaction of the αCTD with sequences upstream of the *ihf* site would have to be independent of the base composition, since fusion of the IHF site to different non-A/T-rich sequences did not alter the IHF-mediated activation.

For the activation of class I promoters by the CRP, residue R-265 of the αCTD did not seem to be important, at least in vivo (37). Recently, however, it was found that substitution of R-265 did affect the CRP-mediated activation in vitro (29). The reason for this discrepancy is not clear, but since R-265 has also been found to be essential for the response to other class I activators, like OxyR (39), it is conceivable that the class I activators function in a common way, by stabilizing the binding of αCTD to the DNA.

ACKNOWLEDGMENTS

We gratefully acknowledge Richard Ebright for the gift of plasmids carrying mutated alpha subunit genes and Stephan Diekman for providing the plasmid containing the 1A sequence.

The work was supported by NIH grants GM37048 (to R. L. Gourse) and GM16543 (to M. Kainz).

REFERENCES

1. **Attey, A., T. Belyeava, N. Savery, J. Hogget, N. Fujita, A. Ishihama, and S. Busby.** 1994. Interactions between the cyclic AMP receptor protein and the alpha subunit of RNA polymerase at the *Escherichia coli* galactose operon *P1* promoter. Nucleic Acids Res. **22:**4375–4380.
2. **Banner, C. D. B., C. P. Moran, Jr., and R. Losick.** 1983. Deletion analysis of a complex promoter for a developmentally regulated gene from *Bacillus subtilis*. J. Mol. Biol. **168:**351–365.
3. **Blatter, E. E., W. Ross, H. Tang, R. L. Gourse, and R. H. Ebright.** 1994. Domain organization of RNA polymerase α subunit: C-terminal 85 amino acids constitute a domain capable of dimerization and DNA binding. Cell **78:**889–896.
4. **Bracco, L., D. Kotlatz, A. Kolb, S. Diekmann, and H. Buc.** 1989. Synthetic curved DNA sequences can act as transcriptional activators in *Escherichia coli*. EMBO J. **8:**4289–4296.
5. **Bujard, H., M. Brenner, U. Deuschle, W. Kammerer, and R. Knaus.** 1987. Structure-function relationship of *Escherichia coli* promoters, p. 93–103. *In* W. S. Reznikoff et al. (ed.), RNA polymerase and the regulation of transcription. Elsevier, New York, N.Y.
6. **Busby, S., and R. H. Ebright.** 1994. Promoter structure, promoter recognition, and transcription activation in prokaryotes. Cell **79:**743–746.
7. **Chen, Y., Y. W. Ebright, and R. H. Ebright.** 1994. Identification of the target of a transcription activator by protein-protein crosslinking. Science **265:**90–92.
8. **Craig, M. L., and H. A. Nash.** 1984. *Escherichia coli* integration host factor binds to specific sites in DNA. Cell **39:**707–716.
9. **Diekmann, S.** 1987. Temperature and salt dependence of the gel migration anomaly of curved DNA fragments. Nucleic Acids Res. **15:**247–265.
10. **Ellinger, T., D. Behnke, R. Knaus, H. Bujard, and J. D. Gralla.** 1994.

Context-dependent effects of upstream A-tracts. Stimulation or inhibition of *Escherichia coli* promoter function. J. Mol. Biol. **239:**466–475.
11. **Friedman, D. I.** 1988. Integration host factor: a protein for all reasons. Cell **55:**545–554.
12. **Gaal, T., W. Ross, E. E. Blatter, T. Tang, X. Jia, V. V. Krishnan, N. Assa-Munt, R. Ebright, and R. L. Gourse.** 1996. DNA-binding determinants of the α subunit of RNA polymerase: novel DNA-binding domain architecture. Genes Dev. **10:**16–26.
13. **Giladi, H., M. Gottesman, and A. B. Oppenheim.** 1990. Integration host factor stimulates the phage Lambda pL promoter. J. Mol. Biol. **213:**109–121.
14. **Giladi, H., K. Igarashi, A. Ishihama, and A. B. Oppenheim.** 1992. Stimulation of the phage λ pL promoter by integration host factor requires the carboxy terminus of the α-subunit of RNA-polymerase. J. Mol. Biol. **227:** 985–990.
15. **Giladi, H., S. Koby, M. E. Gottesman, and A. B. Oppenheim.** 1992. Supercoiling, integration host factor, and a dual promoter system participate in the control of the bacteriophage λ pL promoter. J. Mol. Biol. **224:**937–948.
16. **Goodrich, J. A., M. L. Schwartz, and W. R. McClure.** 1990. Searching for and predicting the activity of sites for DNA binding proteins: compilation and analysis of the binding sites of *Escherichia coli* integration host factor (IHF). Nucleic Acids Res. **18:**4993–5000.
17. **Goosen, N., and P. Van de Putte.** 1984. I. Localization of the presumed recognition sites for HimD and Ner functions controlling bacteriophage Mu transcription. Gene **30:**41–46.
18. **Goosen, N., and P. Van de Putte.** 1995. The regulation of transcription initiation by integration host factor. Mol. Microbiol. **16:**1–7.
19. **Hoover, T. R., E. Santero, S. Porter, and S. Kustu.** 1990. The integration host factor stimulates interaction of RNA polymerase with NIFA, the transcriptional activator for nitrogen fixation operons. Cell **63:**11–22.
20. **Ishihama, A.** 1992. Role of the RNA polymerase α subunit in transcription activation. Mol. Microbiol. **6:**3283–3288.
21. **Jeon, Y. H., T. Negishi, M. Shirakawa, T. Yamazaki, N. Fujita, I. Ishihama, and Y. Kyoguku.** 1995. Solution structure of the activator contact domain of the RNA polymerase α subunit. Science **270:**1495–1497.
22. **Jin, R., K. A. Sharif, and J. S. Krakow.** 1995. Evidence for contact between the cyclic AMP receptor protein and the σ^{70} subunit of *Escherichia coli* RNA polymerase. J. Biol. Chem. **270:**19213–19216.
23. **Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya.** 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. **62:**749–795.
24. **Krause, H. M., and N. P. Higgins.** 1986. Positive and negative regulation of the Mu operator by Mu repressor and *Escherichia coli* integration host factor. J. Biol. Chem. **261:**3744–3752.
25. **Kumar, A., B. Grimes, N. Fujita, K. Makino, R. A. Malloch, R. S. Hayward, and A. Ishihama.** 1994. Role of the sigma^{70} subunit of *Escherichia coli* RNA polymerase in transcription activation. J. Mol. Biol. **235:**405–413.
26. **McAllister, C. F., and E. C. Achberger.** 1988. Effect of polyadenine-containing curved DNA on promoter utilization in *Bacillus subtilis*. J. Biol. Chem. **263:**11743–11749.
27. **McKenny, K., H. Shimatake, D. Court, U. Schmeissner, C. Brady, and M. Rosenberg.** 1981. A system to study promoter and terminator signals recognized by *Escherichia coli* RNA polymerase, p. 383–415. *In* J. G. Chirikjian and T. S. Papas (ed.), Gene amplification and analysis, vol. 2. Elsevier North Holland, New York, N.Y.
28. **Miller, H. I., A. Kikuchi, H. A. Nash, R. A. Weisberg, and D. I. Friedman.** 1979. Site specific recombination of bacteriophage λ: the role of host gene products. Cold Spring Harbor Symp. Quant. Biol. **43:**1121–1126.
29. **Murakami, K., N. Fujita, and A. Ishihama.** 1996. Transcription factor recognition surface on the RNA polymerase α subunit is involved in contact with the DNA enhancer element. EMBO J. **15:**4358–4367.
30. **Pagel, J. M., and G. W. Hatfield.** 1991. Integration host factor-mediated expression of the *ilvGMEDA* operon of *Escherichia coli*. J. Biol. Chem. **266:**1985–1996.
31. **Pérez-Martin, J., and M. Espinosa.** 1994. Correlation between DNA bending and transcriptional activation at a plasmid promoter. J. Mol. Biol. **241:** 7–17.
32. **Rao, L., W. Ross, J. A. Appleman, T. Gaal, S. Leirmo, P. Schlax, M. T. Record, Jr., and R. L. Gourse.** 1994. Factor independent activation of *rrnB* P1. An "extended" promoter with an upstream element that dramatically increases promoter strength. J. Mol. Biol. **235:**1421–1435.
33. **Ross, W., K. K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. L. Gourse.** 1993. A third recognition element in bacterial promoters: DNA binding by the α subunit of RNA polymerase. Science **262:**1407–1413.
34. **Ross, W., J. F. Thompson, J. T. Newlands, and R. L. Gourse.** 1990. *E. coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. EMBO J. **9:**3733–3742.
35. **Santero, E., T. R. Hoover, A. K. North, D. K. Berger, S. C. Porter, and S. Kustu.** 1992. Role of IHF in stimulating transcription from the σ^{54}-dependent *nifH* promoter. J. Mol. Biol. **227:**602–620.
36. **Schmid, M. B.** 1990. More than just 'histon-like' proteins. Cell **63:**451–453.
37. **Tang, H., K. Severinov, A. Goldfarb, D. Fenyo, B. Chait, and R. H. Ebright.** 1994. Location, structure and function of the target of a transcriptional activator protein. Genes Dev. **8:**3058–3067.
38. **Tang, H., K. Severinov, A. Goldfarb, and R. H. Ebright.** 1995. Rapid RNA polymerase genetics: one-day, no-column preparation of reconstituted recombinant *Escherichia coli* RNA polymerase. Proc. Natl. Acad. Sci. USA **92:**4902–4906.
39. **Tao, K., C. Zou, N. Fujita, and A. Ishihama.** 1995. Mapping of the OxyR protein contact site in the C-terminal region of RNA polymerase α subunit. J. Bacteriol. **177:**6740–6744.
40. **Travers, A. A.** 1990. Why bend DNA? Cell **60:**177–180.
41. **Van Rijn, P. A., N. Goosen, and P. Van de Putte.** 1988. Integration host factor of *Escherichia coli* regulates early- and repressor transcription of bacteriophage Mu by two different mechanisms. Nucleic Acids Res. **16:**4595–4605.
42. **Van Rijn, P. A., N. Goosen, S. C. H. J. Turk, and P. Van de Putte.** 1989. Regulation of phage Mu repressor transcription by IHF depends on the level of the early transcription. Nucleic Acids Res. **17:**10203–10212.
43. **Van Ulsen, P., M. Hillebrand, L. Zulianello, P. Van de Putte, and N. Goosen.** 1996. Integration host factor alleviates the H-NS-mediated repression of the early promoter of bacteriophage Mu. Mol. Microbiol. **21:**567–578.
44. **Vieira, J., and J. Messing.** 1991. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene **100:**189–194.
45. **West, D., R. Williams, V. Rhodius, A. Bell, N. Sharma, C. Zou, N. Fujita, A. Ishihama, and S. Busby.** 1993. Interactions between the *Escherichia coli* cyclic AMP receptor protein and RNA polymerase at class II promoters. Mol. Microbiol. **10:**789–797.
46. **Yang, C., and H. A. Nash.** 1989. The interaction of *E. coli* IHF protein with its specific binding site. Cell **57:**869–880.
47. **Zhou, Y., T. J. Merkel, and R. H. Ebright.** 1994. Characterization of the activating region of *Escherichia coli* catabolite gene activator protein (CAP). II. Role at class I and class II CAP-dependent promoters. J. Mol. Biol. **243:**603–610.
48. **Zou, C., N. Fujita, K. Igarashi, and A. Ishihama.** 1992. Mapping of the cAMP receptor protein contact site on the α subunit of *Escherichia coli* RNA polymerase. Mol. Microbiol. **6:**2599–2605.
49. **Zuber, F., D. Kotlarz, S. Rimsky, and H. Buc.** 1994. Modulated expression of promoters containing upstream curved DNA sequences by the *Escherichia coli* nucleoid protein H-NS. Mol. Microbiol. **12:**231–240.
50. **Zulianello, L., E. De la Gorgue de Rosny, P. Van Ulsen, P. Van de Putte, and N. Goosen.** 1994. The HimA and HimD subunits of integration host factor can specifically bind to DNA as homodimers. EMBO J. **13:**1534–1540.