Volume 13 Number 13 1985 Nucleic Acids Research

Positive regulation of the colicin E1 gene by cyclic AMP and cyclic AMP receptor protein

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Received 18 April 1985; Revised and Accepted 12 June 1985

SUMMARY

In previous experiments, we showed that the in vivo transcription of the colicin E1 gene was dependent on cyclic AMP in adenylate cyclase-defective mutant cells of *Escherichia coli* (Ebina, Y. and Nakazawa, A (1983) J. Biol. Chem. 258, 7072-7078). We now show that cyclic AMP and cyclic AMP receptor protein stimulated the in vitro transcription of the gene in the presence of spermidine. As determined in DNase I protection experiments, two binding sites for the complex of cyclic AMP and the receptor protein were identified about 60 base pairs (CRP-1) and 110 base pairs (CRP-2) upstream from the transcription initiation site of the colicin E1 gene. CRP-1 had a higher affinity for the complex than that of CRP-2. Substituting an unrelated DNA sequence for CRP-2 reduced the efficiency of in vitro stimulation of the gene by cyclic AMP and the receptor protein. These potential binding sites for the cyclic AMP-cyclic AMP receptor protein complex probably participate in the stimulation of the colicin E1 gene transcription.

INTRODUCTION

Colicin E1, an antibiotic protein, is encoded by the colicin E1 gene on the colicin E1 plasmid (ColE1). The synthesis of the protein is induced on treatment with DNA-damaging agents or inhibitors of DNA replication. We previously reported that the transcription of the colicin E1 gene is repressed by LexA protein in vivo (1) and in vitro (2), and that LexA protein is the sole repressor of the gene (3). Thus the expression of the colicin E1 gene is considered to be an "SOS response," which is controlled coordinately by RecA and LexA proteins (4, 5). In the presence of RecA protein that has been activated by signals occurring after SOS-inducing treatments, LexA protein, a common repressor of the "SOS genes" is proteolytically inactivated, and the repression is released.

Among the SOS genes, the colicin E1 gene is unique in that it is positively regulated by cAMP (6, 7). We previously demonstrated that cAMP stimulated the expression of the colicin E1 gene in vivo at the transcriptional level (8). The in vitro transcription of the colicin E1

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gene, however, was not significantly stimulated by cAMP and cAMP receptor protein (CRP) under the conditions we employed (9). We also suggested that the superhelical structure of the template molecule was related to the cAMP-dependent expression of the gene in vivo (3).

This paper describes conditions under which cAMP and CRP stimulate the in vitro transcription. As determined in DNase I protection experiments, the CRP-cAMP complex bound to two DNA segments upstream from the promoter of the colicin E1 gene. The binding sites contained a sequence homologous to that of other CRP binding sites. These potential binding sites for the CRP-cAMP complex probably participate in the stimulation of the colicin E1 gene transcription.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonuclases, T4 polynucleotide kinase and T4 DNA polymerase were purchased from Takara Shuzo (Kyoto). Pancreatic DNase I was obtained from Boehringer Mannheim. [γ-^{32}P]ATP (5400 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were products of Amersham. [γ-^{32}P]GTP (about 600 Ci/mmol) was prepared by the method of Walseth and Johnson (10). RNA polymerase was purified by a modification of the method of Chamberlin and Berg (11). Purified CRP was provided by Dr. H. Aiba (Kyoto University Faculty of Medicine). LexA protein was purified as described previously (2). Plasmids ColE1 and pACYC177 were described previously (3).

DNA fragments

The method for purification of plasmids and DNA fragments were described previously (8). DNA fragments used in this study are summarized in Fig. 1. Fragments A, B, and C were used for transcription analysis, while fragments D and E were for DNase I protection experiments. Fragment C was obtained as follows. The *Hha*I-*Sma*I fragment (fragment B) was inserted in the unique *Sma*I site in the kanamycin resistance gene of pACYC177 after changing the *Hha*I end to a blunt end with T4 DNA polymerase. From the ampicillin-resistant and kanamycin-sensitive transformants of *E. coli* W3110, a recombinant plasmid was isolated, from which the 629-base pairs (bp) *Hin*dIII-*Sma*I fragment (fragment C) was excised. DNA was measured by the fluorometric assay (12).

In vitro transcription

The standard reaction mixture of RNA synthesis (20 µl) contained 120 mM KCl, 4 mM $MgCl_2$, 40 mM Tris-HCl (pH 8.0), 4 mM spermidine, 50 mM

2-mercaptoethanol, 2 mM each ATP, CTP, and UTP, 0.5 mM [γ-^{32}P]GTP (50 μCi), 25 nM DNA fragment, and 0.15 μM RNA polymerase. When the effect of cAMP was examined, 0.5 mM cAMP and 0.2 μM or 2 μM CRP were preincubated in the reaction mixture without RNA polymerase for 10 min at 37°C, and then RNA polymerase was added to initiate the transcription. Incubation was carried out for 10 min at 37°C, and the reaction was terminated by shaking with phenol. After adding 10 μg of tRNA to the aquaous phase, transcripts were precipitated with ethanol and subjected to electrophoresis on an 8% polyacrylamide slab gel containing 8 M urea. The products were visualized autoradiographically, and the amounts were estimated by scanning bands on the film by a densitometer.

DNase I protection experiments

The DNase I protection experiments ("footprinting") were done by the procedure of Johnson et al. (13). The reaction mixture (20 μl) containing 10 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM NaCl, 1 mM dithiothreitol, 0.1 or 0.2 nM ^{32}P-DNA fragment, and various amounts of DNA binding proteins was incubated for 15 min at 22°C. After adding 2 ng/ml of DNase I to the mixture, the incubation was continued for 10 min at 22°C. The reaction was stopped with 5 μl of a cold mixture of 8 M ammonium acetate and tRNA at 0.3 mg/ml. The DNA was precipitated with ethanol and analyzed by electrophoresis on an 8% polyacrylamide gel containing 8 M urea, followed by autoradiography. The 5' end of *Dde*I site of fragment D was labeled with [γ-^{32}P]ATP and polynucleotide kinase, while the 3' end of *Dde*I site was labeled with [α-^{32}P]dCTP and T4 DNA polymerase as described previously (14). The specific radioactivities of ^{32}P-DNA fragments were from 0.3 to 1.2 μCi/pmol.

RESULTS

Selection of two promoters by RNA polymerase

In the previous analysis of in vitro transcripts using fragment A which contained the promoter region of the colicin E1 gene, two products, mRNA-1 and mRNA-2, were detected (9). The synthesis of mRNA-2 was 4 times more efficient than that of mRNA-1. The mRNA-1 and mRNA-2 started, respectively, 75 bp upstream and 10 bp downstream from the start codon of the colicin E1 gene (Fig. 1). In contrast, in the S1 mapping analysis of in vivo transcripts, only mRNA-1 was detected (8). Therefore, the synthesis of mRNA-2 was considered to be somehow masked in vivo. We previously showed that the major in vitro transcript from the closed

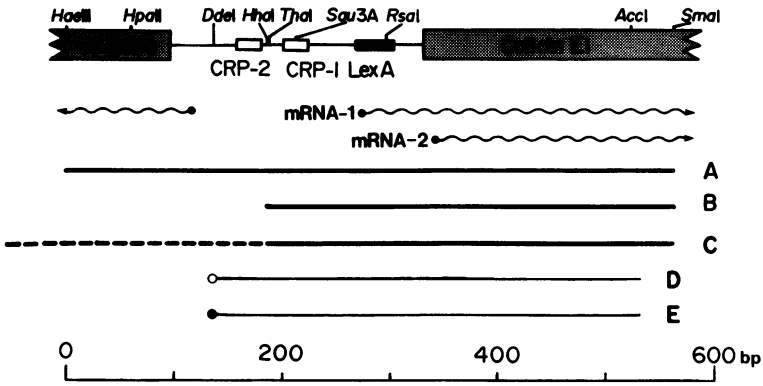

Fig. 1. The promoter-operator region of the colicin E1 gene and DNA fragments used in this study. The structural gene for colicin E1 and the coding region for the *X* gene (see "Discussion") are shown by stippled boxes. The positions of the restriction enzyme cleavage sites used in this study are shown on the top. Open boxes represent the CRP-cAMP binding sites (CRP-1 and CRP-2), and a filled box shows the LexA protein binding site (3). Wavy lines below the map are the transcripts from the genes. The DNA fragments used in the in vitro transcription are indicated by thick lines: A, the 571-bp *Hae*III-*Sma*I fragment; B, the 381-bp *Hha*I-*Sma*I fragment; C, the 629-bp *Hin*dIII-*Sma*I fragment. Fragment C was obtained from a recombinant plasmid between pACYC177 and fragment B (see "Experimental Procedures"). The dashed line indicates a DNA portion derived from pACYC177. The left end of fragment C corresponds to the unique *Hin*dIII site of pACYC177. The fragments used in the footprinting experiments are presented as thin lines: D and E, the 390-bp *Dde*I-*Acc*I fragment. An open circle at the end of line indicates the ^{32}P-labeled 5'-end, and a filled circle at the end of line is the ^{32}P-labeled 3'-end of the fragment. Distances are given in bp from the *Hae*III site.

circular DNA template was mRNA-1 (8). Thus, the superhelicity of the template molecule should be one of the factors involved in selection of the promoters in vivo.

We attempted to search for other in vitro conditions under which the transcription initiation would occur in a manner similar to that in vivo. Using the fragment A as a template and [γ-^{32}P]GTP as a tracer, two factors, KCl and $MgCl_2$ concentrations, were found to affect the efficiency of in vitro transcription from these promoters. At 90 mM KCl, the amount of mRNA-2 synthesized was 4 times more than that of mRNA-1 (Fig. 2a, lane 1). However, at KCl concentrations higher than 100 mM, the synthesis of mRNA-2 was severely inhibited, whereas the mRNA-1 synthesis did not drastically change up to 120 mM KCl (Fig. 2a, lanes 2, 3, and 4). The $MgCl_2$ concentration, which we previously used for the in vitro

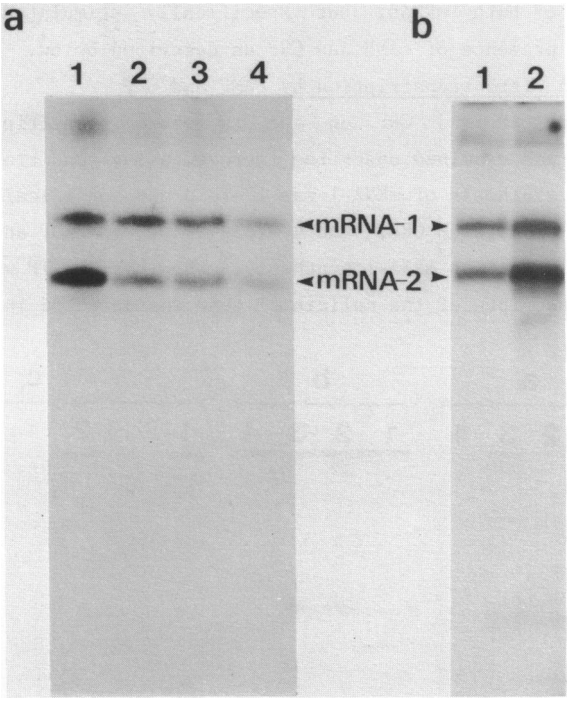

Fig. 2. Effect of salt concentrations on in vitro transcription of the colicin E1 gene. mRNA-1 (289 nucleotide) and mRNA-2 (205 nucleotide) transcribed from fragment A (Fig. 1) were analyzed on polyacrylamide-urea gels. Incubation conditions were described under "Experimental Procedures" except for concentrations of KCl in a, and $MgCl_2$ in b. a, KCl concentration, 90 mM (lane 1), 100 mM (lane 2), 120 mM (lane 3), and 140 mM (lane 4); b, $MgCl_2$ concentration, 4 mM (lane 1) and 10 mM (lane 2). The decrease in the transcription products at higher KCl concentrations was not due to poor recovery during processing and electrophoresis of the products. ^{32}P-RNA-1 (about 5 nCi, 6.6 fmol) that had been transcribed from pA03 (15) was recovered equally well after electrophoresis from the mixtures containing the KCl concentrations used in these experiments. Under the standard conditions as described in "Experimental Procedures," the amounts of mRNA-1 and mRNA-2 synthesized from fragment A were estimated from the radioactivities to be 3.3 and 2.0 fmol per 10 min, respectively.

transcription, was 10 mM. When the concentration was reduced to 4 mM, both the mRNA syntheses were inhibited, but the degree of inhibition in mRNA-2 was greater than in mRNA-1 (Fig. 2b). Thus, at 120 mM KCl and 4 mM $MgCl_2$, the amount of mRNA-2 synthesized in vitro decreased to almost the same level as that of mRNA-1. Therefore, we used these salt concentrations for the following in vitro experiments. In addition, spermidine at 4 mM was included in the reaction mixture, because it did not affect the efficiency

of the syntheses of both mRNAs, but specifically stimulated the mRNA-1 synthesis in the presence of cAMP and CRP as described below.

Stimulation of in vitro transcription by cAMP and CRP

The effect of cAMP and CRP on the in vitro transcription of the colicin E1 gene was examined under the improved assay conditions. As shown in Fig. 3a, the synthesis of mRNA-1 was 17-fold and specifically stimulated by 0.5 mM cAMP and 0.2 μM CRP, and 40-fold by 0.5 mM cAMP and 2 μM CRP. CRP alone had no effect on mRNA-1 synthesis. When [γ-^{32}P]ATP was used as a tracer, no transcript of the colicin E1 gene was detected in the presence

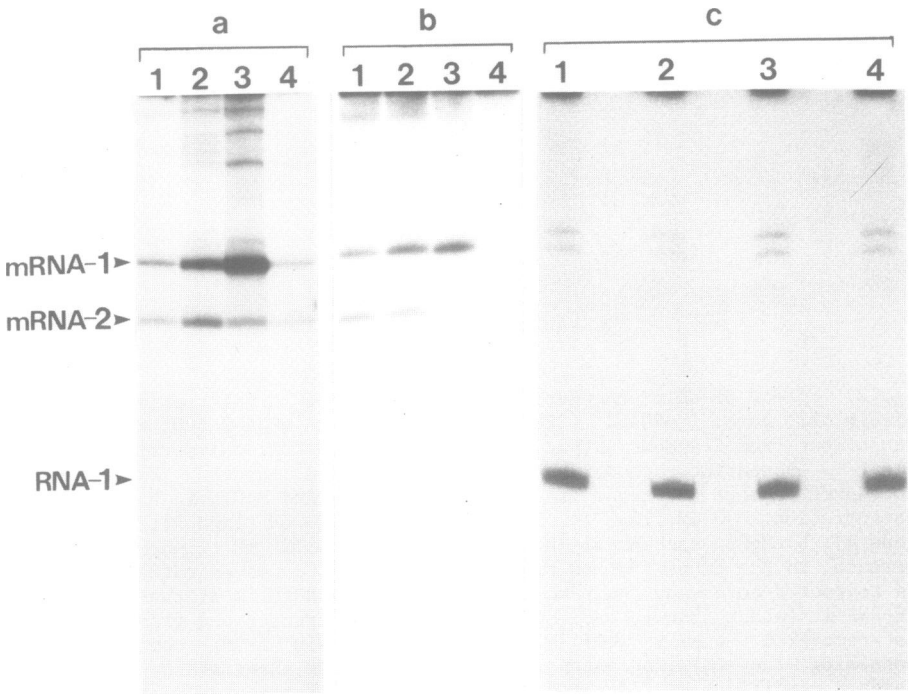

Fig. 3. Spermidine-dependent stimulation by cAMP and CRP of the in vitro transcription of the colicin E1 gene. mRNA-1 (289 nucleotide) and mRNA-2 (205 nucleotide) transcribed from fragment A (Fig. 1) were analyzed on polyacrylamide urea gels. Incubations were carried out as described under "Experimental Procedures" in the presence (a) and absence (b) of 4 mM spermidine. As a control experiment (c), RNA-1 (108 nucleotide) transcription was done using pAO3 (15) as a template. Incubations were made as described under "Experimental Procedures" except that [γ-^{32}P]ATP was used as a tracer instead of [γ-^{32}P]GTP. Lane 1, no cAMP and no CRP; lane 2, 0.5 mM cAMP and 0.2 μM CRP; lane 3, 0.5 mM cAMP and 2 μM CRP; lane 4, no cAMP and 2 μM CRP. Under these conditions, the amount of mRNA-1 synthesized corresponded to about 1 to 10% of that of the template molecule (0.5 pmol).

of cAMP and CRP, indicating that these factors did not switch the start point from G to neighboring A (data not shown). In a control experiment, cAMP and CRP did not stimulate the synthesis of RNA-1, a regulatory RNA for the ColE1 plasmid replication, the synthesis of which is known to be independent of cAMP and CRP (Fig. 3c). When spermidine was omitted, cAMP and CRP stimulated the transcription of mRNA-1 by only 1.5 fold and rather suppressed the synthesis of mRNA-2 (Fig. 3b). Other polyamines such as spermine or putrescine at 4 mM also supported the cAMP-dependent transcription of the colicin E1 gene (data not shown). Thus the presence of a polyamine was important to establish the cAMP-dependent transcription of the colicin E1 gene in vitro.

Two binding sites for the CRP-cAMP complex

The binding site for the CRP-cAMP complex was analyzed by the DNase I footprinting technique. When DNA fragments D and E were limitedly digested with DNase I in the presence of 0.5 mM cAMP and 2 μM CRP under conditions described in "Experimental Procedures," two DNA segments were protected in the region upstream from the colicin E1 promoter (Fig. 4a; lane 4, Fig. 4b; lane 5). The proximal segment, designated as CRP-1, was located at about 50- to 80-bp upstream from the initiation of transcription, while the distal one (CRP-2) was located at 100- to 125-bp upstream from the initiation. Some bands in the protected segments were intensified on binding of the CRP-cAMP complex, as was often observed in the footprinting experiments for CRP binding sites. When the CRP concentration was reduced to 0.2 μM, only CRP-1 was detected (Fig. 4a; lane 3, Fig. 4b; lane 4). The *Kd* value for CRP-1 was estimated to be 0.1 μM, and the value for CRP-2 was thought to be between 0.2 and 2 μM, according to the method of Johnson et al. (13). At a high salt concentration (100 mM KCl), the binding of the CRP-cAMP complex to CRP-2 was not detected, but the binding was restored on addition of RNA polymerase (data not shown).

Role of CRP-1 and CRP-2 in in vitro transcription

To examine whether CRP-2, a weak binding site for the CRP-cAMP complex, has any effect on transcription of the colicin E1 gene, fragments A, B, and C were subjected to transcription in the presence of [γ-^{32}P]GTP. Fragment A contained both CRP-1 and CRP-2. CRP-2 was removed in fragment B, while the site was replaced by another unrelated fragment in fragment C. As shown in Fig. 5, the addition of CRP and cAMP stimulated the synthesis of mRNA-1 8 times with fragment A, whereas it enhanced the synthesis 2.5 times with fragments B and C. These results suggested that CRP-2 was

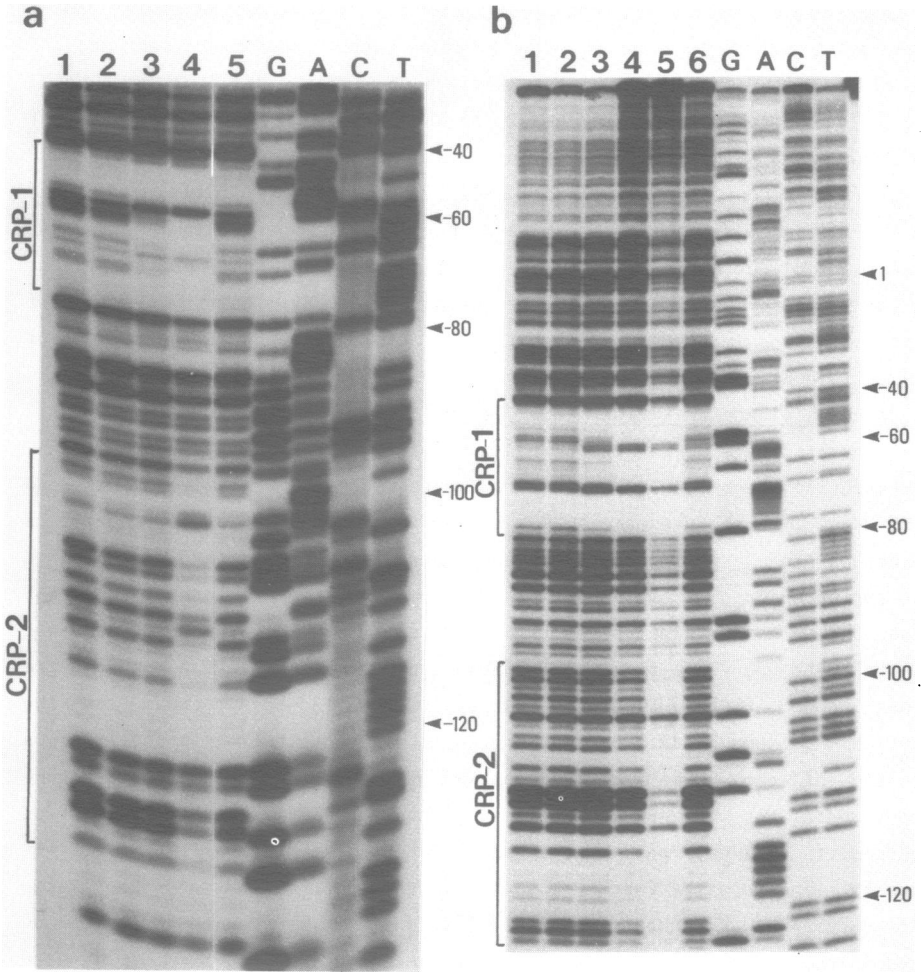

Fig. 4. Binding of the CRP-cAMP complex to the colicin E1 control region. Footprinting was done as described under "Experimental Procedures." The chemical sequencing was carried out by the method of Maxam and Gilbert (lanes G, A, C and T) (24). a, about 0.2 nM fragment D (labeled with ^{32}P on the antisense strand, Fig. 1) was used. Lane 1, no cAMP and no CRP; lane 2, no cAMP and 0.2 μM CRP; lane 3, 0.5 mM cAMP and 0.2 μM CRP; lane 4, 0.5 mM cAMP and 2 μM CRP; lane 5, no cAMP and 2 μM CRP; b, about 0.1 nM fragment E (labeled with ^{32}P on the sense strand, Fig. 1) was used. Lane 1, no cAMP and no CRP; lane 2, no cAMP and 0.2 μM CRP; lane 3, 0.5 mM cAMP and 0.1 μM CRP; lane 4, 0.5 mM cAMP and 0.2 μM CRP; lane 5, 0.5 mM cAMP and 2 μM CRP; lane 6, no cAMP and 2 μM CRP. The bases were numbered relative to the initiation site of colicin E1 mRNA-1. Brackets indicate the region that were protected by CRP from digestion with DNase I. CRP-1 and CRP-2 were assigned from five independent experiments with the antisense strand and two independent experiments with the sense strand.

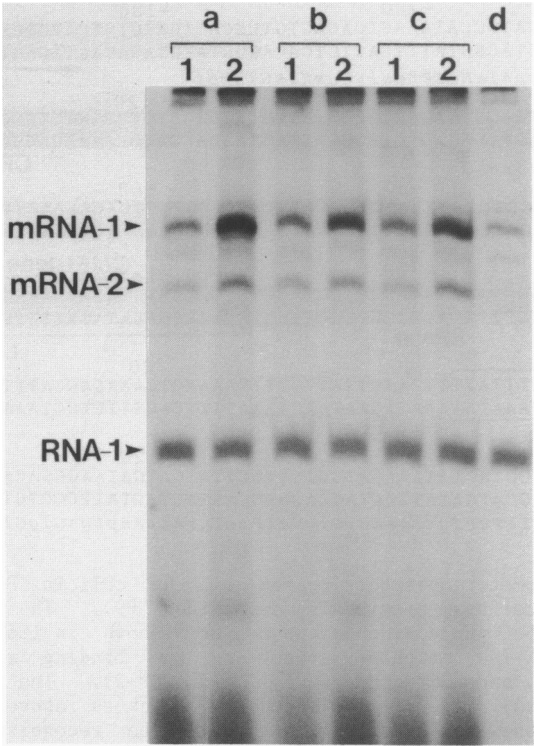

Fig. 5. Effect of CRP-2 on the activation by cAMP and CRP of the in vitro transcription of the colicin E1 gene. Transcription in vitro was done as described under "Experimental Procedures" using fragment A (a), fragment B (b), fragment C (c) as templates. RNA polymerase and [γ-^{32}P]GTP used in these experiments were the products of New England Biolabs and Amersham (14.9 Ci/mmol), respectively. Lane 1, no cAMP and no CRP; lane 2, 0.5 mM cAMP and 2 μM CRP. In a separate experiment, pAO3 was transcribed using [γ-^{32}P]ATP (10 μCi) as a tracer. An aliquot of the transcription product from pAO3 was mixed with each of the transcription products from the colicin E1 gene, and the mixture was subjected to electrophoretic analysis. The aliquot of the transcription product from pAO3 was electrophoresed in d.

necessary for full activation of the colicin E1 gene by cAMP and CRP in vitro.

DISCUSSION

The colicin E1 gene is one of the SOS genes. The gene expression is controlled negatively by LexA protein and positively by cAMP and CRP. The nucleotide sequence of the promoter-operator region of the gene (9) is shown in Fig. 6. Three proteins bind to this region as determined from the

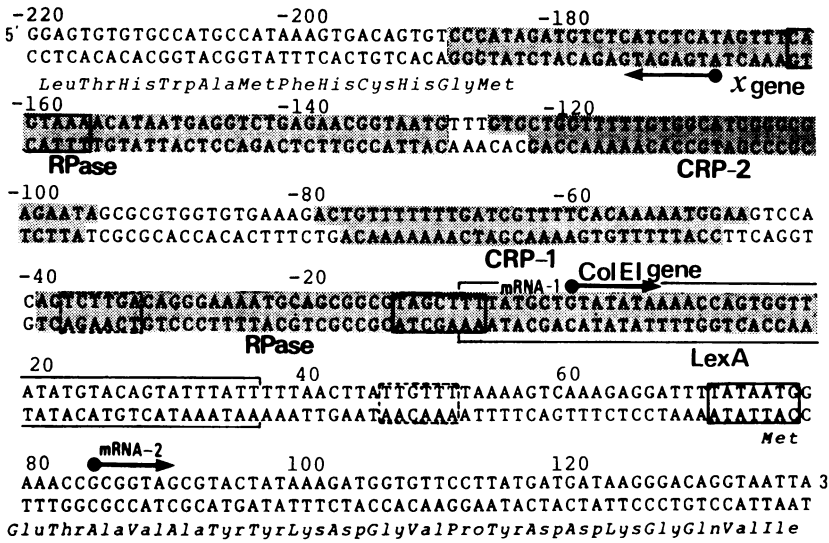

Fig. 6. The promoter-operator region of the colicin E1 gene. The nucleotide sequence was previously determined (9). The amino acids predicted from the nucleotide sequence are shown in italics under the coding sequence. The stippled areas are the binding sites for RNA polymerase (RPase), and CRP-cAMP (CRP-1 and CRP-2). The LexA protein binding site (LexA) (2) is indicated by brackets above and below the sequence. The Pribnow box and the RNA polymerase recognition site are boxed with solid and dashed lines, respectively. The arrows above or below the sequences show the direction of transcription for the colicin E1 and the *X* genes. The predominant initiation site of each mRNA is indicated by a filled circle. Numbers above the sequence are counted from the position of the initiation site for colicin E1 mRNA-1.

DNase I protection experiments. At least two RNA polymerase binding sites have been identified; one is for the colicin E1 promoter, and the other is for the promoter of a putative gene (*X* gene) which we have recently found (K. Shirabe et al., to be published). [The transcription of the *X* gene starts from the site 179 bp upstream from the transcription initiation of the colicin E1 gene and proceeds in the opposite direction. In the downstream region, there is a reading frame of 145 amino acids. The transcription in vivo is induced by mitomycin C and stimulated by cAMP.] We have demonstrated two potential binding sites for the CRP-cAMP complex, CRP-1 and CRP-2, between the two RNA polymerase binding sites. The affinity for CRP-1 is higher than that for CRP-2. The LexA protein binding site is located between the Pribnow box and the start codon of the colicin E1 gene and composed of two overlapping "SOS boxes" (2). Two transcripts, mRNA-1

and mRNA-2 were detected in vitro, but only mRNA-1 was observed in vivo (8). We obtained evidence indicating that the superhelical structure of the DNA template directs the use of a proper promoter (8). The ionic circumstances such as KCl and $MgCl_2$ concentrations around the promoter region has been shown in the present study to be another factor for the selection of the promoters.

The colicin E1 gene is unique among catabolite-regulated genes with respect to the requirement of polyamine for cAMP-dependent expression in vitro. Spermidine is known to interact with DNA (16) and stimulate the reaction of RNA polymerase of *E. coli* (17). However, spermidine per se had no effect on transcription of the colicin E1 gene in the absence of cAMP and CRP. The binding of the CRP-cAMP complex occurred in the absence of spermidine, but did not lead to activate the in vitro transcription. Therefore, it is suggested that spermidine activates the transcription of the colicin E1 gene after formation of the CRP-cAMP-DNA complex.

The potential binding sites for the CRP-cAMP complex identified upstream from the colicin E1 gene are arranged in the direction opposite to each other and separated by about 20 bp. Recent analysis of several CRP binding sites proposed the existence of the highly conserved 5'TGTGA3' sequence followed after 6 bp by the less conserved sequence 5'TCA(C or A)A3' (18). Both the sequences are related by a 2-fold axis of symmetry. In the case of the colicin E1 gene, CRP-1 has the sequence 5'TGTGAAAACGATCAAA3', and CRP-2 has the sequence 5'TGTGGCATCGGGCGAG3'. Underlined bases are homologous to the proposed consensus sequnece. It is obvious that CRP-1 shows higher homology than CRP-2. Lower affinity for CRP-2 to CRP-cAMP may be attributed to the less homologous sequence.

Among the catabolite-regulated genes which had been investigated, several genes had the second site with lower affinity for the CRP-cAMP complex, as was shown here for the colicin E1 gene (19-23). Footprinting experiments presented in Fig. 4 were carried out at a low salt concentration (5 mM NaCl), because the affinity of CRP-2 was so low that the binding could not be demonstrated at higher salt concentrations which were used for the in vitro transcription experiments. However, when RNA polymerase bound to either the colicin E1 promoter or the *X* gene promoter, or both of them, CRP-cAMP could bind to CRP-2 even at 100 mM KCl. Therefore, it is conceivable that during in vitro transcription experiments, the CRP-cAMP complex does bind to both CRP-1 and CRP-2. On the other hand, when DNA fragments containing only CRP-1 were used as

templates, stimulation by cAMP and CRP of transcription of the colicin E1 gene was greatly reduced. The above results suggest that both of the potential binding sites participate in the activation of the colicin E1 gene. Analysis of the operator mutant for CRP would bring about clearer understanding about the relationship between the binding of CRP-cAMP to these sites and the stimulation of the transcription initiation.

ACKNOWLEDGMENTS

We thank Dr. H. Aiba for providing purified CRP. This work was supported by Grants-in-Aid for Scientific Research from Ministry of Education, Science and Culture, Japan.

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REFERENCES

1. Ebina, Y., Kishi, F., and Nakazawa, A. (1982) J. Bacteriol. 150, 1479-1481.
2. Ebina, Y., Takahara, Y., Kishi, F., Nakazawa, A, and Brent, R. (1983) J. Biol. Chem. 285, 13258-13261.
3. Ebina, Y., Takahara, Y., Shirabe, K., Yamada, M., Nakazawa, T., and Nakazawa, A. (1983) J. Bacteriol. 156, 487-492.
4. Little, J. W., and Mount, D. W. (1982) Cell, 29, 11-22.
5. Walker, G. C. (1984) Microbiol. Rev. 48, 60-93.
6. Nakazawa, A., and Tamada, T. (1972) Biochem. Biophys. Res. Commun. 46, 1004-1010.
7. Katz, L., Kingsbury, D. D., and Helinski, D. R. (1973) J. Bacteriol. 114, 577-591.
8. Ebina, Y., and Nakazawa, A. (1983) J. Biol. Chem. 258, 7072-7078.
9. Ebina, Y., Kishi, F., Miki, T., Kagamiyama, H., Nakazawa, T., and Nakazawa, A. (1981) Gene (Amst.) 15, 119-126.
10. Walseth, T. F., and Johnson, R. A. (1979) Biochim. Biophys. Acta 526, 11-31.
11. Chamberlin, M., and Berg, P. (1962) Proc. Natl. Acad. Sci. U. S. A. 48, 81-94.
12. Hinegardner, P. T. (1971) Anal. Biochem. 39, 197-201.
13. Johnson, A. D., Meyer, B. J., and Ptashne, M. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5061-5065.
14. Miki, T., Shirabe, K., Ebina, Y., and Nakazawa, A. (1984) Nucleic Acids Res. 12, 1203-1217.
15. Morita, M., and Oka, A. (1979) Eur. J. Biochem. 97, 435-443.
16. Tabor, C. W., and Tabor, H. (1984) Ann. Rev. Biochem. 53, 749-790.
17. Abraham, K. A. (1968) Eur. J. Biochem. 5, 143-146.
18. de Crombrugghe, B., Busby, S., and Buc, H. (1983) Science 224, 831-838.
19. Schmitz, A. (1981) Nucleic Acids Res. 9, 277-292.
20. Shanblatt, S. H., and Revzin, A. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1594-1598.
21. Le Grice, S. F. J., Matzura, H., Marcoli, R., Iida, S., and Bickle, T. A. (1982) J. Bacteriol. 150, 312-318.
22. Valentin-Hansen, P. (1982) EMBO J. 1, 1049-1054.
23. Ogden, S., Haggerty, D., Stoner, C. M., Kolodrubetz, D., and Schleif, R. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3346-3350.
24. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499-560.