C. EcoO109I, a regulatory protein for production of EcoO109I restriction endonuclease, specifically binds to and bends DNA upstream of its translational start site

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ABSTRACT
The EcoO109I restriction-modification system, which recognizes $5'-(A/G)GGNCC(C/T)-3'$, has been cloned, and contains convergently transcribed endonuclease and methylase. The role and action mechanism of the gene product, C. EcoO109I, of a small open reading frame located upstream of ecoO109IR were investigated in vivo and in vitro. The results of deletion analysis suggested that C. EcoO109I acts as a positive regulator of ecoO109IR expression but has little effect on ecoO109IM expression. Assaying of promoter activity showed that the expression of ecoO109IC was regulated by its own gene product, C. EcoO109I. C. EcoO109I was overproduced as a His-tag fusion protein in recombinant Escherichia coli HB101 and purified to homogeneity. C. EcoO109I exists as a homodimer, and recognizes and binds to the DNA sequence 5'-CTAAAG(N)₂CTTAG-3' upstream of the ecoO109IC translational start site. It was also shown that C. EcoO109I bent the target DNA by 54 ± 4°.

INTRODUCTION
A type II restriction endonuclease, R. EcoO109I, which recognizes $5'-(A/G)GG\downarrow GNCC(C/T)-3'$, has been isolated from Escherichia coli H709c, an anti-genetic tester strain of E. coli O109 (1). A DNA fragment carrying the genes for the EcoO109I restriction-modification (R-M) system has been cloned from chromosomal DNA, and the complete nucleotide sequence was determined (2). The R. EcoO109I and M. EcoO109I genes showed convergent alignment and were each expected to be regulated by their own autogeneous promoter. More than 3000 type II R-M systems have been identified and the nucleotide sequences have been determined for about 150 systems (3). In several R-M systems such as BamHI (4) and PvuII (5), a small protein is produced and acts in trans to stimulate the expression of R endonuclease. This protein has been named C (for controller), and its gene generally precedes and in some cases partially overlaps the R endonuclease gene. A small open reading frame (ORF) is located upstream of the R. EcoO109I gene and partially overlaps the gene. Although the deduced amino acid sequence of C. EcoO109I shows slight homology to those of the other control elements, which are associated with several type II R-M systems, the molecular mass (11 455 Da) was in good agreement with the predicted sizes of other control elements. The product of the ORF might be involved in regulation of the EcoO109I R-M system. In accordance with the accepted nomenclature (5), the putative protein was designated as C. EcoO109I. A conserved DNA sequence element termed a ‘C box’ was found immediately upstream of the C gene (6), and it was shown that the PvuII C box sequence was at least one target of C. PvuII binding (7). No ‘C box’ like sequence was found upstream of the ecoO109IC translational start site.

Because some R-M systems have been indicated to behave as mobile genetic elements, regulation of an R-M system is important for (i) establishment of the system, when it is transferred via a plasmid or phage to a new host with unprotected DNA by cognate or other appropriate methyltransferases, or (ii) maintenance of the system as a ‘selfish gene’, when competitive R-M systems invade and lead to competitive exclusion of the native one (8,9). We have obtained evidence that the EcoO109I R-M system was horizontally transferred to E. coli chromosomal DNA by the P4 phage, based on the nucleotide sequence surrounding the R-M gene. A cell’s DNA must be completely protected by M. EcoO109I before R. EcoO109I can act on the invading DNA, in other words, C. EcoO109I might generate a timing delay, allowing M. EcoO109I to appear before R. EcoO109I in a new host cell, e.g. E. coli H709c.

In this paper, we describe the regulation of both ecoO109IR and ecoO109IM by C. EcoO109I, and characterize C. EcoO109I as a DNA-binding protein.

MATERIALS AND METHODS
Plasmids and strains
Escherichia coli HB101 (10) and JM109 (11) were used as host strains. Plasmids pACYC184 and pBluescriptII SK were from our collection.

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Plasmids p184CRM, p184dCRM and p184intCRM were constructed as follows: a 3.2-kb XbaI–AvII fragment that was carried the genes for R.EcoO109I, M.EcoO109I, and a putative regulatory protein (C.EcoO109I) was excised from pKF3-1 (2), and then inserted into the XbaI and HincII sites of pACYC184 to construct p184CRM. A 0.5-kb Smal–HpaI fragment was deleted from p184CRM, followed by self-ligation to construct p184dCRM. Oligonucleotides 5'-AATT-TGATCCTTAA-3' and 5'-TTTAACTACATCTGTTATCATGAC-3', which was flanked by a BamHI site, were used to subclone the ecoO109IC gene. Plasmid pKDF3-1 was used as the template for PCR. The profile (30 s at 94°C, 1 min at 60°C and 1 min at 72°C) was repeated for 30 cycles. The PCR-generated DNA fragment was digested with XbaI and BamHI, ligated into pBluescriptSK SK cleaved with XbaI and BamHI, and then transformed into E.coli JM109. pBSCeoO109I–Hixs6 was constructed as follows: primers C109N and C109C (5'-TTTTAAGTATAGGATCCTGCTTATTCATGAC-3'), which was flanked by a BamHI site, were used to subclone the ecoO109IC–Hixs6 gene. Plasmid pBSCeoO109I was used as the template for PCR. The profile (30 s at 94°C, 1 min at 55°C and 1 min at 72°C) was repeated for 30 cycles. The PCR-generated DNA fragment was digested with XbaI and BamHI, ligated into pBluescriptSK SK cleaved with XbaI and BamHI, and then transformed into E.coli JM109.

The culture medium for recombinant E.coli was LB broth comprising 1% Bacto tryptone peptone, 0.5% Bacto yeast extract and 1% NaCl, pH 7.0. When necessary, ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml) were added to the medium.

**Purification of C.EcoO109I–Hixs6**

For purification of C.EcoO109I–Hixs6, cells of E.coli HB101 carrying pBSCeoO109I–Hixs6 were grown at 37°C in 500 ml of LB broth containing 100 µg/ml ampicillin and 1 mM IPTG for 20 h. Cells were harvested, washed with 0.15 M NaCl in 10 mM Tris–HCl (pH 8.0), and then stored at −20°C. Frozen cells were suspended in 20 ml of a buffer comprising 20 mM Tris–HCl (pH 8.0), 5 mM imidazole and 0.5 M NaCl. The cells were disrupted by sonication and cell debris was removed by centrifugation. The supernatant was applied to a His-Bind column (Novagen), washed with 20 mM Tris–HCl (pH 8.0), 12 mM imidazole, 0.5 M NaCl, and then eluted with a linear 0.012–1 M imidazole gradient with a simultaneous linear decrease in NaCl concentration from 0.5 to 0.25 M in 20 mM Tris–HCl (pH 8.0). The fraction containing C.EcoO109I was stored at −20°C. The molecular weight of C.EcoO109I–Hixs6 was measured on HiPrep 16/60 Sephacryl S-100 (Amersham Pharmacia Biotech) using 20 mM Tris–HCl (pH 8.0), 0.325 M imidazole and 0.5 M NaCl as the elution buffer.

**Measurement of promoter activity**

Promoter assay vector pMCLTerAR was constructed as follows: to amplify the aldehyde reductase (AR1) gene from Sporobolomyces salmonicolor AKU4429 (12), two primers, primer ARN (5'-GGTACCGAGGATATTAGGTCGG-CAC-3'), which contained a Shine–Dalgarno sequence (underlined nucleotides) (13) and a KpnI site, and primer ARC (5'-GGATCCTACTTATCCTACCGGTTCTTTG-3'), which was flanked by a KpnI site, were used. Plasmid pKAR (12) was used as the template for PCR. The profile (30 s at 94°C, 1 min at 60°C and 2 min at 72°C) was repeated for 30 cycles. The PCR-generated DNA fragment was digested with KpnI, ligated into pMCL210 (14) cleaved with KpnI, and then transformed into E.coli JM109. Several clones were picked up and plasmid DNA was examined by restriction analysis to select the pMCLAR in which the AR1 gene was inserted in the same direction as the chloramphenicol acetyltransferase gene. The 0.5-kb fragment encoding rnmBT1T2 was excised from pKK232-8 (Amersham Pharmacia Biotech) with PvuII and EaeI, and then ligated to pMCLAR digested with NotI and BsrI1107I to construct pMCLTerAR.

The DNA fragment to be assayed for promoter activity was amplified using PCR with selected primers and pKDF3-1 as the template. The PCR products were ligated into the pGEM-T vector (Promega). The DNA fragments were excised with SalI and SpeI, and then ligated into Apal–SpeI-digested pMCLTerAR.

The activity of AR1 was determined at 37°C by measuring the rate of the decrease in the absorbance at 340 nm (12). The standard reaction mixture (1.0 ml) comprised 0.2 mM 4-chloro-3-oxobutanoate ethyl ester (4-COBE), 0.2 mM NADPH and 200 mM potassium phosphate buffer (pH 7.0). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 µmol of NADPH per minute.

**DNA-binding assay**

The DNA fragments isolated from a plasmid and synthetic oligonucleotides were labeled with [γ-32P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase, and then used as substrates. The binding activity was assayed by incubation for 30 min at 37°C in 10 µl of a reaction mixture comprising 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM DTT and 50 nM substrate DNA. Immediately after the incubation for 30 min at 37°C, 0.2 mM 4-chloro-3-oxobutanoate ethyl ester (4-COBE), 0.2 mM NADPH and 200 mM potassium phosphate buffer (pH 7.0). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 µmol of NADPH per minute.

**DNA-binding assay**

The synthetic oligonucleotides encoding the C.EcoO109I-binding site were ligated into the XbaI–SalI site of pBend2 (15) to construct pBend109. Different DNA fragments containing a single C.EcoO109I-binding site were isolated by digesting pBend109 with proper sets of restriction enzymes, and the resulting DNA fragments were isolated by gel electrophoresis and then labeled with T4 polynucleotide ligase.
kinase and [γ-32P]ATP. Binding of \textit{C.EcoO109I}–Hisx6 to DNA was performed as described above.

Other methods

\textit{C.EcoO109I}–Hisx6 from \textit{E.coli} HB101 carrying pBSCEcoO109I–Hisx6 was blotted from an SDS–polyacrylamide gel to a polyvinylidene difluoride membrane by the method of Matsudaira (16), and then subjected to N-terminal amino acid sequence analysis. Antibodies were raised against either \textit{R.EcoO109I} or \textit{M.EcoO109I}, which were purified almost homogeneously as described elsewhere (2,17). The western blots were probed first with a purified antibody and then antirabbit immunoglobulin G coupled to alkaline phosphatase. Blocking and probing with antibodies were performed as described by Sambrook \textit{et al.} (18). Ni-NTA AP conjugates (Qiagen) were used for detection of the Hisx6-tagged protein according to the manufacturer’s instructions. Protein was assayed by the method of Bradford (19) using a kit from Bio-Rad with BSA as the standard. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (20).

RESULTS AND DISCUSSION

Effect of \textit{C.EcoO109I} on production of \textit{R.EcoO109I} and \textit{M.EcoO109I}

The effect of \textit{C.EcoO109I} on the production of \textit{R.EcoO109I} and \textit{M.EcoO109I} was investigated by constructing a deletion mutant of the 5’-untranslated region and a frame-shift mutant of \textit{ecoO109IC}. A DNA fragment carrying the complete \textit{EcoO109I} R-M system was subcloned into pACYC184, p184CRM being generated, and then mutations were introduced into \textit{ecoO109IC}: one (p184dCRM) by deleting the 0.5-kb DNA fragment carrying the gene encoding the N-terminal and 5’-untranslated regions of \textit{ecoO109IC}, and the other (p184intCRM) by inserting a nonsense mutation at the \textit{HpaI} site. As shown in Figure 1, we also put \textit{ecoO109IC} under the control of the lac promoter of pBluescriptII SK to supply \textit{C.EcoO109I} in \textit{trans}.

Cotransformants of \textit{E.coli} HB101, carrying the plasmids derived from p184CRM and pBSCEcoO109I, were grown, and the \textit{R.EcoO109I} and \textit{M.EcoO109I} produced in cell-free extracts were examined by western blot analysis. Figure 2 shows that the production of \textit{M.EcoO109I} appears to be unchanged whether \textit{ecoO109IC} is mutated or not, or \textit{C.EcoO109I} is provided in \textit{trans} or not. It can be concluded that \textit{C.EcoO109I} does not function as a specific regulator of \textit{ecoO109IM}.

In contrast to \textit{M.EcoO109I}, the production of \textit{R.EcoO109I} decreased to below the limit of detection when \textit{ecoO109IC}
was disrupted, but returned to a normal level when \( \text{ecoO109IC} \) was supplied in \text{trans}. When a DNA fragment upstream of \( \text{ecoO109IC} \) was deleted, no restoration of production was observed on supplying \( \text{ecoO109IC} \) in \text{trans}. These results suggest that \( \text{ecoO109IC} \) and \( \text{ecoO109IR} \) are cotranscribed under the control of the \( \text{ecoO109IC} \) promoter, and that \( \text{C.EcoO109I} \) acts as a positive regulator of both \( \text{ecoO109IC} \) and \( \text{ecoO109IR} \) expression. In other words, the accumulation of \( \text{C.EcoO109I} \) in a cell is required for the production of \( \text{R.EcoO109I} \).

Based on the nucleotide sequence surrounding the R-M gene, we have assumed that the \( \text{R-EcoO109I} \) R-M system is horizontally transferred to \( \text{E.coli} \) chromosomal DNA via P4 phage integration. Regulation of the R-M system is important, especially when the system is transferred to a new host with unprotected DNA, that is, the cellular DNA must be completely protected by the methylase before the endonuclease can act on invading DNA. It is assumed from our results that just after the \( \text{R.EcoO109I} \) R-M system is transferred to \( \text{E.coli} \) H709c, \( \text{R.EcoO109I} \) is synthesized but \( \text{R.EcoO109I} \) is not, because of the absence of \( \text{C.EcoO109I} \); after a while, \( \text{C.EcoO109I} \) accumulates in the cell, and then triggers the synthesis of \( \text{R.EcoO109I} \).

A protein that has been shown to play an important role in regulation of the R-M system has been discovered in some R-M systems, and is named C (5). Its gene generally precedes and in some cases partially overlaps the R gene. We demonstrated that in the \( \text{R.EcoO109I} \) R-M system as well as in the \( \text{BamHI} \) (4), \( \text{PvuII} \) (5) and \( \text{EcoRV} \) (9) systems, C protein acts in \text{trans} and is required for the production of cognate restriction endonuclease. A conserved DNA sequence element termed a ‘C box’ has been identified immediately upstream of \( \text{bamHIC} \) and \( \text{pvuIIIC} \) (6), however, it was not found upstream of \( \text{ecoO109IC} \) or \( \text{ecoO109IM} \). Moreover, no significant homology has been detected between \( \text{C.EcoO109I} \) and other C proteins. We assumed that the site of action of \( \text{C.EcoO109I} \) is distinct from those of \( \text{C.BamHI} \) and \( \text{C.PvuII} \), and investigated the mechanism of \( \text{C.EcoO109I} \) action in \text{vivo} and in \text{vitro}.

Promoter activity of various DNA fragments upstream of \( \text{ecoO109IC} \) and \( \text{ecoO109IM} \)

To determine the location and \( \text{C.EcoO109I} \) responsiveness of \( \text{C.EcoO109I} \) promoters, we cloned the putative promoter region for each of the genes upstream of the promoterless NADPH-dependent aldehyde reductase (\( \text{AR1} \)) (EC 1.1.1.2) gene in plasmid pMCLTerAR (Fig. 3A). Fragments to be assayed for promoter activity were PCR-amplified with selected oligonucleotide primers, and DNA products were inserted between the \( \text{SpeI} \) and \( \text{ApuI} \) sites of the promoter screening vector, pMCLTerAR, in both orientations. Bacterial cell extracts were prepared and enzyme assays were carried out using 4-COBE as a substrate (Fig. 3B).

In the absence of \( \text{C.EcoO109I} \), the promoter activity of a 143-bp fragment upstream of \( \text{ecoO109IM} \) was high enough, but that of a 457-bp fragment upstream of \( \text{ecoO109IC} \) was below the limit of detection. However, in the presence of \( \text{C.EcoO109I} \), the promoter activity of the 143-bp fragment upstream of \( \text{ecoO109IM} \) increased to twice as much as in the absence of \( \text{C.EcoO109I} \), and that of the 457-bp fragment upstream of \( \text{ecoO109IC} \) became comparable with the \( \text{ecoO109IM} \) promoter activity. We do not have any ideas to explain why \( \text{C.EcoO109I} \) induces a 2–3-fold increase of reporter gene expression when a DNA fragment which is originally located upstream of \( \text{ecoO109IM} \) is inserted in both orientations upstream of the reporter gene. Activation of the \( \text{ecoO109IC} \) promoter by \( \text{C.EcoO109I} \) required at least 75 bp upstream of the translational start site of \( \text{ecoO109IC} \).

Purification of \( \text{C.EcoO109I} \)-His6

In order to test the ability of \( \text{C.EcoO109I} \) to bind to DNA in a sequence-specific manner, we constructed a plasmid which expresses \( \text{C.EcoO109I} \)-His6, a fusion protein consisting of \( \text{C.EcoO109I} \) and a hexahistidine affinity domain (His6) at the C-terminus, and purified the protein from recombinant \( \text{E.coli} \). Escherichia coli cells harboring pBSCEcoO109I–His6 and pMCLTerAR457, which carries the 457-bp DNA upstream of \( \text{ecoO109IC} \) in the same direction as the \( \text{AR1} \) gene, showed
70% AR1 activity compared with those carrying pBSCEcoO109I and pMCLTerAR457 (data not shown). This indicates that the addition of His6-tag to the C-terminus does not significantly affect the action of C.EcoO109I in vivo. As shown in Figure 4, two major proteins were eluted from an Ni(II)-charged column with 0.3–0.35 M imidazole. After the solution had been frozen at −20°C followed by melting on ice, a protein with a molecular mass of 12.4 kDa (Fig. 4, lane 2) was recovered in the supernatant fraction on removal of a finely dispersed precipitate. The protein was blotted onto a membrane and then subjected to N-terminal amino acid sequence analysis. The first 10 amino acids of the protein were MLPIRLKKAR, which was identical to the sequence deduced from the nucleotide sequence. The presence of the His6-tag was confirmed by western blot analysis using anti-His6-tag antibodies (data not shown). The predicted mass of the fusion protein, 12 277, was close enough to the value estimated by SDS–PAGE. These results strongly suggest that the purified protein was C.EcoO109I–His6. In contrast, the first 10 amino acids of a protein with a molecular mass of 15.6 kDa (Fig. 4, lane 1) were TMITPSAQLT, which was identical to the N-terminal amino acid sequence of β-galactosidase in pBluescriptII (21). In plasmid pBSCEcoO109I–His6, the lac promoter directs transcription of the N-terminus of lacZ gene followed by an in-frame C.EcoO109I–His6 gene. Consequently, induction of the lac promoter results in the production of two proteins, C.EcoO109I–His6 and the fusion protein of β-galactosidase and C.EcoO109I–His6.

Attempts to remove NaCl and imidazole from the protein solution by gel filtration or dialysis failed, because of the poor solubility of the protein in a low ionic strength solution. When purified C.EcoO109I–His6 was stored at 4°C in the elution buffer used for Ni(II)-charged column chromatography for 5 months, neither precipitation nor loss of DNA-binding activity was observed. The molecular mass of the native protein, which was measured by HiTrap Sephacryl S-100 gel filtration using 20 mM Tris–HCl (pH 8.0), 0.5 M NaCl and 0.325 M imidazole as the elution buffer, was estimated to be 35 kDa. Although the determined molecular mass exceeds the deduced one three times, we assume that C.EcoO109I dimerizes but does not form a globular structure, and binds to a palindromic sequence.

Figure 4. SDS–PAGE of the C.EcoO109I–His6 obtained from E.coli HB101. Fractions containing C.EcoO109I–His6 eluted from the His-Bind column were collected and stored at −20°C. A finely dispersed precipitate appearing on melting was removed by centrifugation, and the resulting clear solution (lane 2) and eluted fractions (lane 1) were separated on a 0.1% SDS–15% polyacrylamide gel. The positions of molecular mass standards (Mr) are indicated (in kDa) on the left.

DNA-binding activity of C.EcoO109I–His6

We have examined whether or not C.EcoO109I–His6 binds to the DNA fragments which were examined for promoter activity by gel mobility shift assay. DNA fragments I–V in Figure 3 were excised from each plasmid by digestion with Sphi and ApaI, and then incubated with C.EcoO109I–His6. As shown in Figure 5, C.EcoO109I–His6 bound to the DNA segments of longer than 75 bp but not to that of 37 bp upstream of ecoO109IC. In contrast, C.EcoO109I–His6 did not bind to the 143-bp DNA segment upstream of ecoO109IM. These results indicate that the binding of C.EcoO109I–His6 is correlated with stimulation of the promoter activity of ecoO109IC, and it is suggested that C.EcoO109I–His6 binds to the −75 to −38 region upstream of the ecoO109IC translational start site. As indicated in Figure 6A, the −70 to −46 region consists of a 2-fold symmetrical sequence with an axis of symmetry at −58 and is expected to be the binding site of C.EcoO109I–His6.

Oligonucleotides of various lengths with the DNA sequence upstream of ecoO109IC were synthesized and used for binding assays. C.EcoO109I–His6 bound to oligoduplexes (C11–12) covering −76 to −39 but not to oligoduplexes (C09–10) covering −38 to −1, oligoduplexes (C13–14) covering −76 to −56, or oligoduplexes (C15–16) covering −60 to −39 (Fig. 6C). These results strongly suggested that C.EcoO109I–His6 binds to the −75 to −38 region upstream of the ecoO109IC sequence. In order to determine the minimum length to which C.EcoO109I–His6 binds, substrate oligoduplexes containing 25–17 bp were synthesized and used for binding assays. C.EcoO109I–His6 bound to oligoduplexes of longer than 21 bp but did not bind to oligoduplexes of shorter than 19 bp. This indicates that the outer two AT base pairs in the 25-bp symmetrical sequence shown in Figure 6A were not indispensable for binding to C.EcoO109I–His6.

In order to identify the base pairs that play a control role in recognition of C.EcoO109I oligoduplexes bearing base-pair substitutions at nine positions of the 25-bp symmetrical sequence shown in Figure 7A were examined for binding to C.EcoO109I–His6. As expected from the results shown in Figure 6, substitution at the second position did not affect the binding. Substitutions at the third and ninth positions slightly decreased the C.EcoO109I binding, but ones at the fourth, fifth, sixth, seventh and eighth positions completely abolished the C.EcoO109I binding. We constructed a series of mutant oligoduplexes with different spacer lengths between the inverted repeat, and examined for binding of C.EcoO109I.
As seen in Figure 8, C.EcoO109I–Hisx6 bound to neither the oligoduplexes with a 1-, 3- or 5-bp deletion nor those with a 1-, 3- or 5-bp insertion. These results demonstrate that C.EcoO109I recognizes and binds to the sequence 5'−CTAAG(N)5CTTAG−3', composed of 5-bp inverted repeats at 5-bp intervals. DNA-binding activity has been examined for C.PvuII using 22-bp oligoduplexes containing the originally proposed PvuII C box sequence located −54 to −30 upstream of the pvuIIC translational start site, and the dyad consensus sequence GACT(N)3AGTC is at least one target of C.PvuII binding (7). The target sequence of C.EcoO109I is quite distinct from that of C.PvuII.

**DNA-bending by C.EcoO109I–Hisx6**

Sequence-specific binding of proteins to DNA is often accompanied by bending of the DNA. We have studied the ability of C.EcoO109I–Hisx6 to bend its recognition site. A synthetic DNA fragment that contained the C.EcoO109I
binding sequence was cloned into the SalI–XbaI site of pBend2, and the electrophoretic mobilities of the DNA fragments generated on restriction digestion were monitored after the binding to C.EcoO109I–Hisx6 (Fig. 9A). The results are shown in Figure 9B. It is clear that although free fragments do not show any intrinsic curving, C.EcoO109I induces bending in its recognition site. As was found with repressor-operator systems, such as Gal and Lac, and an activator-operator system, such as CRP (22), the degree of electrophoretic retardation of the C.EcoO109I recognition site was highest when the binding site was in the middle and lowest when the binding site was closer to either end of the 141-bp DNA fragment. We have estimated the apparent C.EcoO109I-induced bending angle by following an empirical relationship between the relative electrophoretic mobility retardation caused by bending and the bending angle (23). C.EcoO109I induced an angle of 54 ± 4° in the C.EcoO109I site. As described above, C.PvuII binds to the C box, but no C.PvuII-induced bending has been reported. We have shown here for the first time that a regulatory protein of R-M systems bends the target site.

DNA sequences on or near activation of transcription initiation are known to show activator-protein-induced bending. It was suggested that such contortions may be essential for the formation and activity of the DNA–protein transcription initiation complex. It is very likely that DNA bending facilitates additional protein–protein or protein–DNA contacts that involve the activation protein and RNA polymerase. In this study, we demonstrated that: (i) C.EcoO109I acts in trans to stimulate the expression of both the ecoO109IR and ecoO109IC genes; (ii) C.EcoO109I is a sequence-specific DNA-binding protein acting as a homodimer, which recognizes the sequence 5¢-CTAAG(N)5CTTAG-3¢, located upstream of ecoO109IC; (iii) C.EcoO109I bends its recognition site. The recognition sequence of C.EcoO109I was not found as far as 143 bp upstream of ecoO109IM, and C.EcoO109I did not bind upstream of ecoO109IM. These findings are compatible with the results of deletion analysis and the C.EcoO109I-induced ecoO109IM promoter activity. As there are 10 nt in each chain, at every turn of the helix, the recognition site for C.EcoO109I, CTAAG, faces the same side of the DNA. Therefore, it can be presumed that homodimeric C.EcoO109I interacts with its target DNA, located upstream of its translational start site, from one side of the DNA helix and induces bending, which facilitates the formation of the DNA–protein transcripational initiation complex at the ecoO109IC promoter. Further analysis of the three-dimensional structures of the C.EcoO109I and C.EcoO109I–DNA complexes will provide us with useful information on interactions such as those between RNA polymerase and C.EcoO109I, subunits of C.EcoO109I, and C.EcoO109I and DNA.
REFERENCES


