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DNA methylation at the *Cfr*BI site is involved in expression control in the *Cfr*BI restriction–modification system

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ABSTRACT

We have previously found that genes of the *Cfr*BI restriction–modification (R-M) system from *Citrobacter freundii* are oriented divergently and that their promoter regions overlap. The overlapping promoters suggest regulation of gene expression at the transcriptional level. In this study the transcription regulation of *Cfr*BI R-M genes was analyzed *in vivo* and *in vitro* in *Escherichia coli*. It was shown that in the presence of *Cfr*BI methyltransferase (M·*Cfr*BI), cell galactokinase activity decreases 10-fold when the galactokinase gene (*galK*) is under the control of the *cfrBIM* promoter and increases 20-fold when *galK* is under the control of the *cfrBIR* promoter. The *Cfr*BI site, proven to be unique for the entire *Cfr*BI R-M gene sequence, is located in the –35 *cfrBIM* promoter region and is in close vicinity of the –10 *cfrBIR* promoter region. A comparison of the *cfrBIM* and the *cfrBIR* promoter activities in the *in vitro* transcription system using methylated and unmethylated DNA fragments as templates demonstrated that the efficiency of *Cfr*BI R-M gene transcription is regulated by enzymatic modification at the N-4-position of cytosine bases of the *Cfr*BI site by M·*Cfr*BI. From the results of the *in vivo* and *in vitro* experiments we suggest a new model of gene expression regulation in type II R-M systems.

INTRODUCTION

Over 3000 restriction–modification (R-M) systems in almost all the major groups of prokaryotic organisms have been found to date (1). The structural organization of a number of R-M genes which have been cloned and sequenced is diverse (2). Some R-M genes are located on plasmids (3–6) which means regulation of R-M genes is essential, not only to prevent host DNA autorestriction but also for R-M systems entering a new host. Although the existence of R-M gene regulation is quite evident, the mechanism underlying such regulation remains almost completely unknown. It has been shown that the regulation of the *Pvu*II (7) and *Bam*HI (8,9) R-M systems is performed by small proteins, whose genes are located in the intergenic region of the genes for restriction endonuclease (ENase) and methyltransferase (MTase). Apart from the activator protein *Pvu*IIC (7) of the ENase gene, the gene *pvu*IIW was also described (10,11), and their function is the subject of intensive study. Some m5-MTases such as M·*Eco*RII (12,13), M·*Sso*II (14) and M·*Msp*I (15), beside MTase activity, display an additional repressor activity localized at the N-terminus. The N-terminal parts of these protein molecules contain the so-called 'helix–turn–helix' (HTH) motif typical of prokaryotic DNA binding proteins. In these cases the R-M system regulation mechanism is based on the specific MTase interaction with its own promoter, i.e. the expression of the respective MTase is autoregulated at the transcriptional level. Thus, the mechanism of control of R-M gene expression differs from system to system.

This paper is devoted to regulation of the *Cfr*BI R-M system. It presents evidence that the *Cfr*BI site located in the *cfrBIR–cfrBIM* intergenic region plays a key role in the regulation of expression of these genes.

MATERIALS AND METHODS

Construction of the pFD51 derivatives carrying various parts of the *cfrBIR* and *cfrBIM* promoter sequences

Plasmid pBGM5 (16) carrying the total nucleotide sequence of the *Cfr*BI R-M system (DDBJ/EMBL/GenBank accession no. X57945) was used as a template for PCR synthesis of DNA fragments containing the intergenic region. Plasmid variants of pFD51 (17) where the galactokinase gene (*galK*) was under the control of the *cfrBIM* promoter were referred to as pMet with an appropriate number. Similarly, plasmids with various parts of the *cfrBIR* promoter were named as pRes. The nucleotide sequences of the primers used to clone DNA fragments containing the intergenic region follow (the *Cfr*BI site is underlined and nucleotide substitutions are shown in lower case).

pMet3: 5′-CCCAAGCTTGATCTGTTACCATACAAC-3′,
5′-CGGGATCCCCATGGACATAGTAAAAATG-3′;
pMet4: 5′-CCCAAGCTTAACCTGCTATCTTAGC-3′,
5′-CGGGATCCCCATGGACATAGTAAAAA-3′;
pMet6: 5′-CCCAAGCTTCTTAGCATCTCATTTTT-3′,
5′-CGGGATCCCCATGGACATAG-3′;
pMet11: 5′-CCCAAGCTTGATCTGTTACCATACAAC-3′,
5′-CGGGATCCtCATGGACATAGTAAAAATG-3′;
pMet15: 5′-CCCAAGCTTGATCTGTTACCATACAAC-3′,
5′-CGGGATCCCCATGGtaATAGTAAAAATG-3′;

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pRes7: 5′-CGGGATCCGATCTGTTACCATACAAC-3′,
5′-CCCAAGCTTCCATGGACATAGTAAAAATG-3′;
pRes8: 5′-CGGGATCCAACCTGCTATCTTAG-3′,
5′-CCCAAGCTTCCATGGACATAGTAAAAATG-3′;
pRes9: 5′-CGGGATCCGATCTGTTACCATACAAC-3′,
5′-CCCAAGCTTCCATGGtaATAGTAAAAATG-3′;
pRes14: 5′-CGGGATCCAACCTGCTATCTTAG-3′,
5′-CCCAAGCTTtCATGGACATAGTAAAAATG-3′.

The nucleotides were substituted using PCR-dependent site-directed mutagenesis. The *Bam*HI and *Hin*dIII sites were introduced into the primer sequences. The conditions for PCR reactions (30 cycles) were: 94°C for 30 s, 55°C for 30 s and 72°C for 25 s. The amplified DNA fragments were subcloned into the pFD51 plasmid at the *Bam*HI and *Hin*dIII sites in both orientations. The pMet23 and pRes27 plasmids were derived from the pMet3 and pRes7 plasmids, respectively, which were hydrolyzed at the *Cfr*BI site and their protruding ends were filled in with Klenow fragment. The plasmid pXB4 carrying the native M·*Cfr*BI gene was constructed from plasmid pBGM5. A 1.25 kb *Hae*II-d fragment of pHSG415 (18) with the chloramphenicol acetyltransferase gene (*cat*) was inserted into the *Mlu*I site of pBGM5 and then a 0.3 kb *Bgl*II–*Xba*I fragment was deleted. The resulting recombinant DNAs were analyzed by restriction mapping and the cloned DNA sequences were tested as described (19). The isolation of plasmid DNA, restriction analysis and gene cloning were performed as described (20).

Determination of galactokinase activity

The *Escherichia coli* HB101 and HB101/pXB4 strains were transformed by plasmids of the pMet and pRes series. Expression of *galK* was estimated either visually from colony color on MacConkey plates containing 1% galactose or directly by determination of galactokinase activity as nmol of galactose phosphorylated per min per ml of cell suspension at $OD_{650} = 1$ (17).

Formation of the promoter–protein complex

To study promoter–protein binding, 0.2 µg of the purified M·*Cfr*BI (containing an N-terminal His_6 sequence as an affinity tag) was incubated with DNA fragments (1 µg) in 10 µl buffer (50 mM Tris–HCl pH 7.5, 7 mM β-mercaptoethanol, 150 mM NaCl). The reaction mixture was applied on 6% polyacrylamide gel after 20 min incubation at room temperature.

In vitro transcription

In vitro transcription experiments were carried out as described (21). The purified 50 ng DNA fragment was incubated with *E.coli* RNA polymerase (100 nM) in 20 mM Tris–HCl (pH 8.0) containing 100 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50 µg BSA and 5% glycerol in a 20 µl reaction vol for 20 min at 37°C. A 2 µl mixture of four ribonucleotide triphosphates (500 µM UTP plus 2 mM each of ATP, GTP and CTP) containing 0.5 µCi [α-^{32}P]UTP and 20 µg heparin was then added and incubation continued for another 10 min. The reaction was terminated with 200 µl cold stop solution (10 mM EDTA and 1 µg tRNA). The resulting RNA transcripts were phenol/chloroform extracted and ethanol precipitated. The pellets were redissolved in 10 µl formamide loading buffer, heated at 90°C for 5 min and the RNA fragments were analyzed by 7% PAGE under denaturating conditions using urea.

RESULTS AND DISCUSSION

Determination of the minimal DNA region with the functionally active *cfrBIM* gene promoter

The *cfrBIR* and *cfrBIM* genes were previously shown to be located on opposite DNA strands and separated by 76 bp (16). Analysis of the intergenic region yielded identification of nucleotide sequences for both genes which were typical of bacterial promoters (Table 1A). To test the theoretically proposed boundaries of the gene promoters, the PCR-constructed DNA fragments representing the varying in length intergenic region were subcloned into the specific plasmid pFD51. The transcription efficiency was determined from galactokinase activity in cells harboring recombinant plasmids. The 40 bp DNA fragment (pMet4) covering the consensus –35 and –10 *cfrBIM* promoter sequences provided a level of galactokinase synthesis almost as high as that produced by the plasmid pMet3 containing a 55 bp DNA fragment of the intergenic region (Table 1B and C). It should be mentioned that the 55 bp fragment includes the intergenic region but lacks the Shine–Dalgarno (SD) sequences of both genes. The plasmid pMet6 contains a DNA fragment with a partially deleted –10 promoter region of *cfrBIM*. The galactokinase activity in the pMet6-harboring cells was about 35 times lower than that in cells containing plasmids with the intact promoter region (Table 1B and C). These results show that the 40 bp DNA fragment includes the functionally active *cfrBIM* promoter, which is 2–2.5 times more efficient than the well studied *lac*UV5 promoter (P_{lacUV5}). It is noteworthy that the maximal galactokinase activity for the pRes-harboring cells (i.e. for cells transformed by pRes7 or pRes8) was only ~2% of the galactokinase activity determined in the cells harboring plasmids with the *cfrBIM* promoter (Table 1B and C). In this case the level of galactokinase activity was comparable to that observed in the pFD51-harboring cells (data not shown).

In vivo study of *Cfr*BI R-M gene regulation

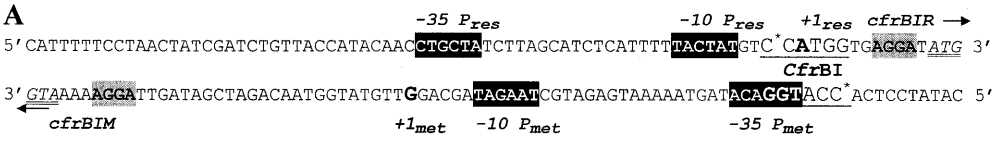
To study the MTase effect on transcription efficiency (shown previously for the *Eco*RII and *Sso*II R-M systems), the transcription experiments were carried out in the presence of M·*Cfr*BI whose gene was cloned into a pFD51-compatible plasmid providing for constitutive synthesis of M·*Cfr*BI. The introduction of such a plasmid (pXB4) into the cell was accompanied by an increase in galactokinase synthesis of ~20-fold for the pRes7- and pRes8-harboring cells and a >10-fold decrease for the cells harboring pMet3 and pMet4 (Table 1B and C). This suggests that M·*Cfr*BI is a regulatory protein that decreases the level of *cfrBIM* gene expression and increases the level of *cfrBIR* expression.

The galactokinase activity measurements for pRes8-harboring cells confirmed the theoretically proposed boundaries of the *cfrBIR* promoter (Table 1B and C). The results show that the maximal activities of the *cfrBIM* and *cfrBIR* promoters are in a 5:1 ratio. This ratio is in line with the coincidence of the *cfrBIM* and *cfrBIR* promoter sequences with the consensus sequences of bacterial promoters.

From the assumption that M·*Cfr*BI acts as an autorepressor, the deletion of the putative operator sequence would rule out the possibility of M·*Cfr*BI binding and, consequently, the repression of its own synthesis. Galactokinase activity in strains containing the plasmid pMet4, which lacks the major

Table 1. Effect of M·*Cfr*BI on the *cfrBIM* and *cfrBIR* gene promoter activities in the *in vivo* model system

A



B

		C: Gal phenotype		Level of Galactokinase %	
		- MTase	+ MTase	- MTase	+ MTase
pMET3	5′- CCATGGACATAGTAAAAATGAGATGCTAAGATAGCAGGTTGTATGGTAACAGATC -3′	RR	RW	263	22
pMET4	5′- ---------- -3′	RR	RW	208	23
pMET6	5′- ---------- -3′	W	W	7.4	8.3
pMET15	5′- -------TA---------- -3′	R	RW	68	24
pMET11	5′- T---------- -3′	RR	RR	347	368
pMET23	5′-CCATG---------- -3′	RR	RR	185	135
pRES7	5′- GATCTGTTACCATACAAGCTGCTATCTTAGCATCTCATTTTTACTATGTCCATGG -3′	W	R	2.3	53
pRES8	5′- ---------- -3′	W	R	1.8	57
pRES9	5′- ----------TA------ -3′	RW	R	19	46
pRES14	5′- ----------A -3′	W	W	2.7	3.4
pRES27	5′- ----------CATGG -3′	W	W	3.2	3.4

(**A**) Nucleotide sequence of the *Cfr*BI R-M intergenic region (16). Black boxes with light letters, the –35 and –10 promoter regions; indices *res* and *met*, elements belonging to *cfrBIM* and *cfrBIR* promoters, respectively; arrows, direction of transcription from these promoters; underlined text in larger typeface, the enzyme recognition site; *, methylated cytosine residues; double underlined text, ATG codons for each R-M gene; +1 (bold letters A and G), transcription start sites; grey shaded boxes, SD sequences.

(**B**) Plasmid constructs utilized to study *Cfr*BI R-M gene promoter activities. DNA fragments of various lengths containing the intergenic region were cloned in both orientations into plasmid pFD51 at the *Bam*HI and *Hin*dIII sites. The resulting plasmids with *galK* controlled by the *cfrBIM* promoter are referred to as pMet. Plasmids with various parts of the *cfrBIR* promoter are referred to as pRes. Only mutated bases in plasmids pMet11, pMet15, pMet23, pRes9, pRes14 and pRes 27 are shown.

(**C**) The *E.coli* strains HB101 (–MTase) and HB101/pXB4 (+MTase) were transformed by pMet and pRes plasmids. The colony color on the MacConkey agar medium containing 1% galactose (Gal phenotype) was visually differentiated as bright red (RR), red (R), pale red (RW) and white (W), respectively. The galactokinase activity was expressed as a percentage of the activity determined for cells of the *E.coli* strain HB101/pFD100 (P_{lacUV5}), which was 70 U (17). Each value represents the average of the determinations done in triplicate.

part of the putative operator sequence, differs only slightly from that found for pMet3 (Table 1B and C). Further, gel shift experiments on M·*Cfr*BI binding to methylated and unmethylated DNA fragments containing the intergenic region revealed no specific DNA–protein complexes for any DNA fragments (data not shown). This suggests that M·*Cfr*BI is incapable of specific binding to the regulatory region of its own gene. Thus, the above data present clear evidence for distinction of regulation of gene expression in the *Cfr*BI R-M system from that described for the *Eco*RII, *Sso*II and *Msp*I R-M systems where MTase acts as a repressor of its own synthesis.

Efficiency of transcription from the *cfrBIR* and *cfrBIM* gene promoters depends on cytosine methylation at the *Cfr*BI site

A peculiar feature of the *Cfr*BI R-M intergenic region is the existence of the enzyme recognition site which is unique for the entire gene sequence. The *Cfr*BI site is located in the –35 *cfrBIM* promoter region and is in close vicinity to that of the –10 *cfrBIR* (Table 1A). Specifically, 3 nt (TGG) of the *Cfr*BI site (5′-CCATGG-3′) belong to the –35 *cfrBIM* promoter region (5′-TGGACA-3′). Further, the *Cfr*BI site is separated from the –10 *cfrBIR* promoter region by only 2 nt. Two sets of experiments have been carried out to test the possible role of this site in *cfrBIM* and *cfrBIR* expression regulation.

The first set of experiments was designed to study promoter activity in the presence of M·*Cfr*BI under conditions which ruled out DNA modification at the *Cfr*BI site. The latter necessitated changes in the *Cfr*BI DNA sequence without affecting the functionally active promoter-essential regions. Thus, the *Cfr*BI site sequence (5′-CCATGG-3′) was replaced by 5′-TCATGG-3′. The C→T substitution affected neither the –35 *cfrBIM* promoter region nor that of the –10 *cfrBIR*. The plasmids

containing the mutated *Cfr*BI site were referred to as pMet11 and pRes14 (Table 1B). This substitution caused no marked changes in R-M gene promoter activities. However, these activities no longer depended on the presence of M·*Cfr*BI (Table 1C).

Plasmids pMet23 and pRes27 derived from plasmids pMet3 and pRes7 were constructed by filling in the *Cfr*BI site with Klenow fragment. Similar to pMet11 and pRes14 plasmids, the produced insertion mutation affected neither the –35 *cfrBIM* promoter region nor the –10 *cfrBIR* promoter regions but altered the *Cfr*BI site sequence, thereby preventing DNA methylation by M·*Cfr*BI. The 4 nt insertion caused only a slight decrease in *cfrBIM* promoter activity (pMet23) and a slight increase in *cfrBIR* promoter activity (pRes27) (Table 1B and C). Gene promoter activity measurements done in the presence of M·*Cfr*BI confirmed that the regulatory function is performed not by MTase itself, but by MTase-induced DNA modification at the *Cfr*BI site. Presumably, the absence of cytosine modification makes the stronger *cfrBIM* promoter preferable for the binding of RNA polymerase. This interaction could make initiation of transcription from the *cfrBIR* promoter more difficult. In contrast, cytosine modification at the *Cfr*BI site promotes RNA polymerase binding to the *cfrBIR* promoter. In this case, efficiency of transcription from the *cfrBIM* promoter drastically drops and is accompanied by an increase in the activity of the *cfrBIR* promoter.

It should be noted that the decrease in *cfrBIM* promoter activity can be caused either by DNA methylation at the *Cfr*BI site or by activity-inhibiting mutations. Thus, the second set of experiments was designed to study R-M gene promoter activities in the case where the *cfrBIM* promoter activity has been mutationally reduced while cytosine modification at the *Cfr*BI site is allowed. These experiments utilized plasmids pMet15 and pRes9 (Table 1B) with the modified –35 *cfrBIM* promoter region and the intact –10 *cfrBIR* promoter region. The activity caused by the *cfrBIM* mutant promoter (Table 1B and C) was about three times lower than that of the intact promoter. In contrast, the activity of the *cfrBIR* mutant promoter, as expected, increased ~8-fold. In these experiments the activities of both the promoters depended on the presence of a plasmid carrying the *cfrBIM* gene since the *Cfr*BI site remained intact. Thus, the co-transformation of pMet15 or pRes9 with plasmid pXB4 yielded activity of the *cfrBIM* promoter which was still low, while activity of the *cfrBIR* promoter was increased (Table 1C).

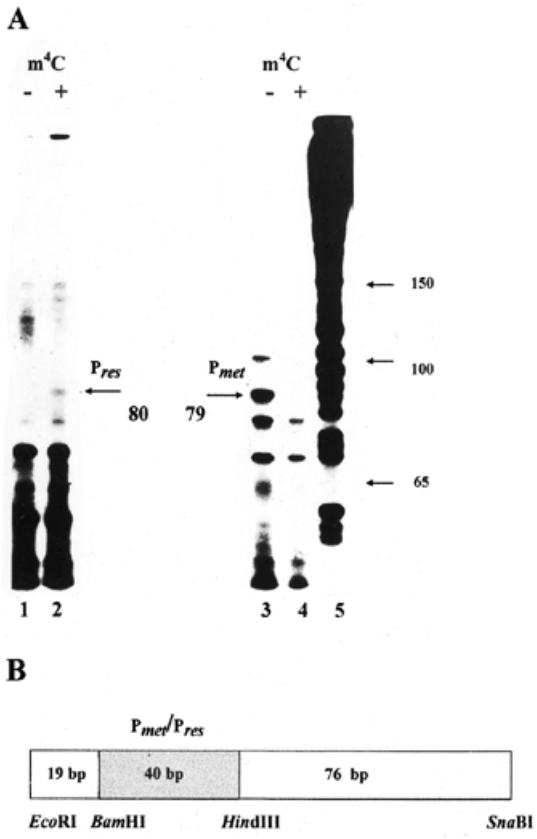


Figure 1. *In vitro* transcription from the *cfrBIM* and *cfrBIR* gene promoters. (**A**) The 7% PAGE autoradiogram of mRNAs produced by *in vitro* transcription from a 135 bp DNA fragment. *In vitro* transcription was performed as described (21) using *E.coli* RNA polymerase. The transcripts from the DNA fragment containing the *cfrBIR* gene promoter are presented in lanes 1 and 2 and those from the *cfrBIM* gene promoter in lanes 3 and 4. Transcription products in the absence of m^4C DNA modification at the *Cfr*BI site (lanes marked by a minus) and in the presence of MTase (lanes marked by a plus) were analyzed. The mRNA fragments resulting from transcription initiated by the *cfrBIR* and *cfrBIM* promoters were 80 nt (lane 2) and 79 nt (lane 3), respectively. Lane 5 represents DNA fragments of known length resulting from the sequencing reaction. (**B**) Schematic representation of the DNA fragment utilized for studies on *in vitro* transcription from the *Cfr*BI R-M gene promoters. The 135 bp *Eco*RI–*Sna*BI DNA fragment was derived from plasmids pMet4 and pRes8. The *Eco*RI–*Bam*HI (19 bp) and *Hin*dIII–*Sna*BI (76 bp) DNA fragments were from plasmid pFD51; the shaded *Bam*HI–*Hin*dIII (40 bp) DNA fragment containing either the *cfrBIM* or *cfrBIR* promoter was constructed from pMet4 or pRes8, respectively.

In vitro transcription from the *cfrBIR* and *cfrBIM* promoters

In vivo studies of the *cfrBIM* and *cfrBIR* promoter activities showed that DNA methylation at the *Cfr*BI site had a marked effect on the efficiency of transcription from the gene promoters. *In vitro* experiments on transcription from the *cfrBIM* and *cfrBIR* promoters were carried out for direct studies of the effect of the methylated cytosine on the efficiency of transcription from these promoters. The 135 bp DNA fragments used as templates for *E.coli* RNA polymerase were isolated from plasmids pRes8 and pMet4. To obtain base-modified fragments, the respective plasmids were isolated from strains that also contained the *cfrBIM*-carrying plasmid. The position of the studied promoter was asymmetric relative to the fragment ends and the transcripts of interest were expected to exceed 76 nt in length (Fig. 1B). The reaction products were separated on 7% PAGE under denaturating conditions and analyzed by densitometric autoradiogram scanning. The 79 nt transcript synthesized from the *cfrBIM* promoter was effectively produced only when the DNA fragment with unmodified bases was used as a template (Fig. 1A, lane 3). No transcript from the *cfrBIR* promoter was detected when the DNA fragments with the unmodified cytosine were used (Fig. 1A, lane 1). However, the use of the DNA fragment with the methylated *Cfr*BI site yielded a specific RNA fragment of 80 nt in length (Fig. 1A, lane 2). The transcript sizes allowed us to position the transcription start points for both genes (Table 1A). The intensity of the band corresponding to the transcript from the *cfrBIM*

promoter was about six times higher than that of the band corresponding to the transcript produced from the *cfrBIR* promoter. This relationship is in good agreement with that estimated from measurements of galactokinase activity. Thus, *in vitro* transcription experiments provided additional evidence for the direct effect of cytosine methylation at the *Cfr*BI site on the efficiency of transcription from R-M gene promoters.

A new model of regulation of R-M gene activity

These results clearly show that cytosine modification at the *Cfr*BI site plays a key role in regulation of *cfrBIR* and *cfrBIM* gene expression. We suggest that this modification alters the *E.coli* RNA polymerase affinity for R-M gene promoters and, consequently, the expression levels of these genes. In the absence of cytosine methylation at the *Cfr*BI site, transcription of the *cfrBIM* gene is more effective than that of the *cfrBIR* gene. Accumulation of M·*Cfr*BI results in modification of cell DNA including the recognition site located within the intergenic region. Methylation of this site causes a drastic reduction in *cfrBIM* promoter activity and, in contrast, *cfrBIR* promoter activity becomes much higher. The highly coordinated activity of *cfrBIM* and *cfrBIR* promoters regulated by DNA modification at the *Cfr*BI site reflects the essence of regulation of R-M system genes as genes of antagonistic proteins.

It should be noted that regulation of the *Cfr*BI R-M system has been studied in a heterologous background. However, *Escherichia* and *Citrobacter* are both representatives of the *Enterobacteriaceae* family and we believe that regulation in the original strain would be the same as in *E.coli*, but this has yet to be confirmed.

The function of MTase in the regulation of *cfrBIR* and *cfrBIM* gene expression is clearly different from that of MTases in known R-M systems (e.g. *Eco*RII, *Sso*II and *Msp*I) which accomplish their regulatory function as autorepressor proteins. M·*Cfr*BI belongs to the least abundant m^4C MTases, which also include M·*Pvu*II and M·*Bam*HI. An analysis of the *Cfr*BI R-M gene nucleotide sequences yielded no ORF coding for a regulatory C-protein which is characteristic of the *Pvu*II and *Bam*HI R-M systems (10). Thus, the mechanism of regulation described for the *Pvu*II and *Bam*HI R-M systems (7–9) differs from that found for the *Cfr*BI R-M system.

Numerous publications have reported involvement of DNA adenine methylation in the regulation of many important processes in bacterial cells (22,23). Thus, the dependence of transcription from a number of promoters on Dam MTase has been described (24–29). In the case of the *Cfr*BI R-M system studied here, *cfrBIM* and *cfrBIR* gene expression depends on m^4C DNA methylation at the *Cfr*BI site which is located in the gene promoter regions. It is noteworthy that in some R-M systems the recognition sites for the coding enzymes are also located in the regulatory regions of MTase and ENase genes. Specifically, two sites for *Pae*R7 are located in front of *paeR7M* (30). In the case of *Fok*I R-M system, two *Fok*I sites are located in front of *fokIR* and another pair of *Fok*I sites is upstream of the *fokIM* gene (31). However, the involvement of these sites in the regulation of gene expression for the above systems has not been reported. Presumably, the mechanism of gene regulation for the *Pae*R7 and *Fok*I R-M systems may be similar to the mechanism described here for the *Cfr*BI R-M system.

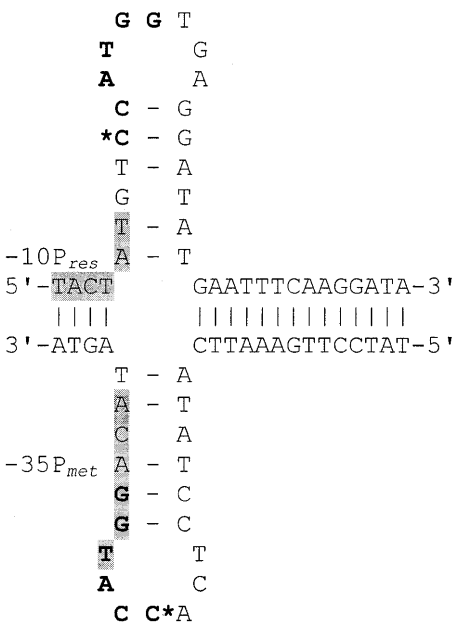


Figure 2. A putative cruciform structure of the *Cfr*BI-involving DNA sequence. The *Cfr*BI site is shown in bold. *, cytosine undergoing m^4-modification; shaded boxes, the –35 *cfrBIM* and –10 *cfrBIR* promoter regions. The PC/Gene (IntelliGenetics, Mountain View, CA) package of programs was used to analyze the nucleotide sequences.

The *Cfr*BI site is part of the inverted repeat sequence (Fig. 2). The formation of a cruciform structure suggested for such a sequence may be important for the binding of RNA polymerase to the *cfrBIR* promoter. In the absence of cytosine methylation at the *Cfr*BI site, this structure may act as a potential terminator of *cfrBIR* transcription. It was shown that the methylation of adenine within the inverted repeats results in a lowered local stability of the cruciform structure, because the methylation of adenine at N6 directly perturbs the amine participation in hydrogen bonding to thymine (32). The methylation of cytosine by MTase *Cfr*BI at N4 also affects the exocyclic amine forming hydrogen bonds with guanine and this should lead to lower stability of the CG base pair. So, the proposed cruciform structure may be considered as a possible additional regulator of gene expression control. Thus, the *Cfr*BI R-M system is a promising model for further studies of gene expression control.

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