The EMBO Journal vol.12 no.11 pp.4297–4303, 1993

Autogenous regulation of the *Eco*RII methylase gene at the transcriptional level: effect of 5-azacytidine

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Communicated by T.A.Trautner

mRNA of the *Eco*RII methylase (M.*Eco*RII), a type II modification enzyme, was induced when *Escherichia coli* carrying a cloned M.*Eco*RII gene was exposed to the bacteriocidal drug 5-azacytidine. Induction occurred only when transcription was initiated from its own promoter. When the 5′ promoter sequences were deleted or replaced with the *lac* promoter sequences, no induction occurred. The induction was independent of the template DNA level, but the presence of an intact M.*Eco*RII protein was a requirement. The drug is incorporated into DNA which then inhibits M.*Eco*RII by binding tightly to the enzyme. A deletion within the M.*Eco*RII coding region caused a marked increase in the basal level of mRNA transcribed from the M.*Eco*RII promoter, but no induction occurred upon 5-azacytidine treatment. The level could be reduced to normal by M.*Eco*RII *in trans*. *In vitro*, the enzyme bound to the sequences upstream of the transcription start sites and inhibited the initiation of transcription. These experiments indicate that expression of the M.*Eco*RII gene was autogenously regulated at the transcriptional level. Similar regulation is also noted in another DNA (cytosine-5) methylase, M.*Msp*I.

Key words: autoregulation/cytosine-5 methylase/DNA methylation/restriction–modification/transcription

Introduction

The *Eco*RII methylase (M.*Eco*RII) and *Eco*RII endonuclease both recognize the same DNA sequence, CC(A/T)GG, and are classified as a type II restriction–modification (R-M) system. In an R-M system, one function of the endonuclease is to defend against bacteriophage attack by hydrolyzing the foreign DNA, which lacks 'proper' methylation, whereas the role of the methylase is to protect the host DNA from digestion by the cognate endonuclease, while leaving foreign DNA unmethylated. The viability of the host organism depends on mechanism(s) regulating the expression of these two enzymes. The level and availability of the methylase protein must be limited so that the endonuclease can efficiently digest any foreign DNA before that DNA is methylated. On the other hand, when the R-M system is transferred to a new host, the host DNA would have to be methylated before the endonuclease acted. Therefore, an initial high level of expression of the methylase gene followed by its repression would be beneficial to the host. A wide range of R-M systems from various sources has been cloned and expressed in *E.coli*. This implies the existence of regulatory mechanisms for either or both of the genes within the system itself. Recently, several R-M systems have been shown to be regulated by a *trans*-acting protein encoded by a small open reading frame (ORF) present within the system (Tao *et al.*, 1991; Ives *et al.*, 1992; Tao and Blumenthal, 1992).

In this paper we describe a different type of regulation for the *Eco*RII R-M system. This system resides in the *E.coli* drug-resistance factor, N3 (Bannister and Glover, 1968; Yoshimori *et al.*, 1972), which can be transferred between bacteria. Cells carrying N3 were found to be sensitive to the drug 5-azacytidine (azaCyd). The sensitivity of the cells to the drug is due to the presence of methylase: the restriction enzyme is not necessary (Friedman, 1982). This drug is incorporated into DNA and inhibits M.*Eco*RII (Friedman, 1981, 1982). The enzyme forms a tight complex with azacytosine when the latter substitutes for the internal cytosine in the sequence CC(A/T)GG in DNA (Friedman, 1985, 1986) and presumably causes cell death. The complex between M.*Eco*RII and azacytosine-containing DNA (azaC-DNA) inhibits cellular processes *in vitro* such as DNA strand exchange (Huang and Friedman, 1991) and transcription (unpublished observation). Two other DNA (cytosine-5) methylases (C-Mtases), the Dcm enzyme and M.*Msp*I, also confer sensitivity to azaCyd when present on plasmids in *E.coli* (Bhagwat and Roberts, 1987; Lal *et al.*, 1988). These C-Mtases form complexes with azaC-DNA (Friedman, 1985, 1986). Recently, while investigating the effect of azaCyd on transcription in *E.coli* carrying a cloned M.*Eco*RII gene, we noticed that following treatment with the drug, the amount of M.*Eco*RII mRNA increased markedly when the gene was under the control of its own promoter. We have determined that control is exerted at the transcriptional level and that this is due to autogenous regulation mediated by M.*Eco*RII. Preliminary observations indicate that M.*Msp*I is also regulated similarly.

Results

Subcloning of M.EcoRII and M.MspI genes

For a better understanding of the regulation of M.*Eco*RII expression, we subcloned fragments bearing the structural gene for M.*Eco*RII with variable 5′ upstream sequences in both high and low copy number vectors. Recombinants of the pSS series contain fragments cloned in the multi-copy pUC vectors (Yanisch-Perron *et al.*, 1985), whereas the corresponding pAC constructs contain the same insert in the low-copy plasmid pACYC177 (Chang and Cohen, 1978). Constructions are described in Materials and methods and a physical map of the M.*Eco*RII gene is presented in Figure 1. In summary, plasmids pSS38/pAC38 and pSS12/pAC12 contain both the −35 and −10 regions of the native promoter. Plasmids pSSP/pACP contain only the −10 region while pSSQ/pACQ do not have any promoter-like sequences, but include the transcription start site(s). Although partially or completely lacking the native promoter,

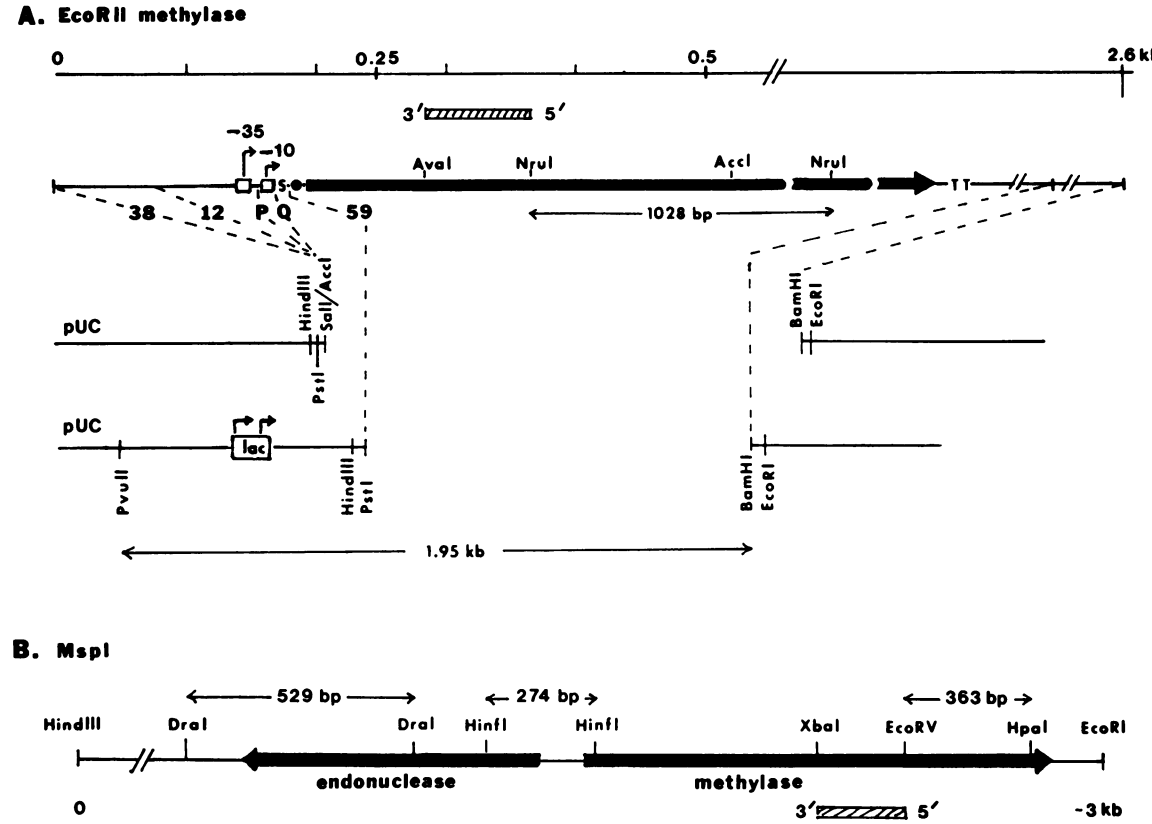

Fig. 1. Structure of M.*Eco*RII and M.*Msp*I subclones. (**A**) Map of the M.*Eco*RII gene. The M.*Eco*RII coding region is shown by the thick horizontal bar with an arrow. The promoters are shown by open boxes. S, transcription start site; ●, ribosome-binding site; TT, transcription stop site, are also shown. Selected restriction enzyme sites are indicated. The positions of the inserted sequences of the subclones pSS38, pSS12, pSSP, pSSQ and pSS59 are marked by dotted lines. Recombinants of the pAC series, except pAC59, contain the *Pst*I−*Bam*HI fragment from the corresponding pSS plasmid. pAC59 contains the 1.95 kb *Pvu*II−*Bam*HI fragment from pSS59. The 1028 bp *Nru*I fragment was deleted in pAC38Δ. The hatched box represents the single-stranded probe used for S1 protection. (**B**) Map of the 3.03 kb *Msp*I R-M system in plasmid p*Msp*I. The 529 bp *Dra*I fragment was deleted in plasmid pM*Msp*I-7, and the 363 bp *Eco*RV−*Hpa*I fragment was deleted in plasmid pM*Msp*IΔ. A 274 bp *Hin*fI fragment was used for enzyme binding. The hatched box represents the single-stranded probe used for S1 protection assays.

these two sets of recombinants confer M.*Eco*RII activity upon the host. In all of the pSS constructs mentioned above, the direction of transcription of the M.*Eco*RII gene is opposite to that initiated from the *lac* promoter. Transcription of the M.*Eco*RII gene in the multi-copy pSS59 (Som *et al.*, 1987) or in the low-copy pAC59 (this report) is initiated from the *lac* promoter but the methylase reading frame is not fused in-frame with *lacZ*.

Plasmids p*Msp*I and pM*Msp*I-7 confer M.*Msp*I activity upon the *E. coli* host. While p*Msp*I contains the entire *Msp*I R-M system, plasmid pM*Msp*I-7 does not code for a functional endonuclease (Figure 1).

Induction of M.EcoRII mRNA synthesis by azaCyd

The amounts of M.*Eco*RII mRNA synthesized by strains bearing different constructs, before and after azaCyd treatment, were compared by analyzing the degree of protection of a specific probe from S1 nuclease digestion after hybridization of an excess amount of that probe with a fixed amount of total cellular RNA. The results are presented in Figure 2. When an exponentially growing culture of GM271(pAC38) was exposed to azaCyd, induction of M.*Eco*RII mRNA occurred. The amount of mRNA was found to increase with time (Figure 2A). No such induction was noticed when the culture was treated with cytidine. Quantification of the protected DNA revealed that M.*Eco*RII mRNA synthesis increased >40-fold within 60 min of treatment with azaCyd. The induction occurred despite the inhibition of cell growth and cellular RNA synthesis (unpublished observation). Analysis of plasmid DNA isolated from control and drug-treated cells showed that the amount of plasmid DNA did not increase as a result of the drug action (data not given). The increased amount of mRNA is therefore not due to an elevated level of the template.

We investigated the role of 5′ sequences upstream of the M.*Eco*RII gene in azaCyd-induced RNA synthesis. The results are shown in Figure 2B. Cells containing pAC12 showed an induction similar to that found with cells carrying pAC38 but no such induction was noted with cells containing pACQ. Like pAC38, pAC12 contains both the −10 and −35 regions of the native promoter, whereas pACQ does not contain any promoter sequences. Cells bearing plasmid pAC59, in which the M.*Eco*RII gene was under the control of the *lac* promoter, had a low level of M.*Eco*RII mRNA which was not induced by azaCyd.

Figure 2B also shows the result obtained from studies done with multi-copy plasmids. Although the basal levels of M.*Eco*RII mRNA were high in cells containing multi-copy plasmids, the effect of azaCyd was found to be similar to that in cells with low-copy plasmids. Cells bearing pSS38 showed an effect similar to that found with pAC38. With pSSP, a construct containing a partially deleted M.*Eco*RII promoter, limited induction can be seen (lanes k and l). In GM271(pSS59), the level of M.*Eco*RII mRNA was found

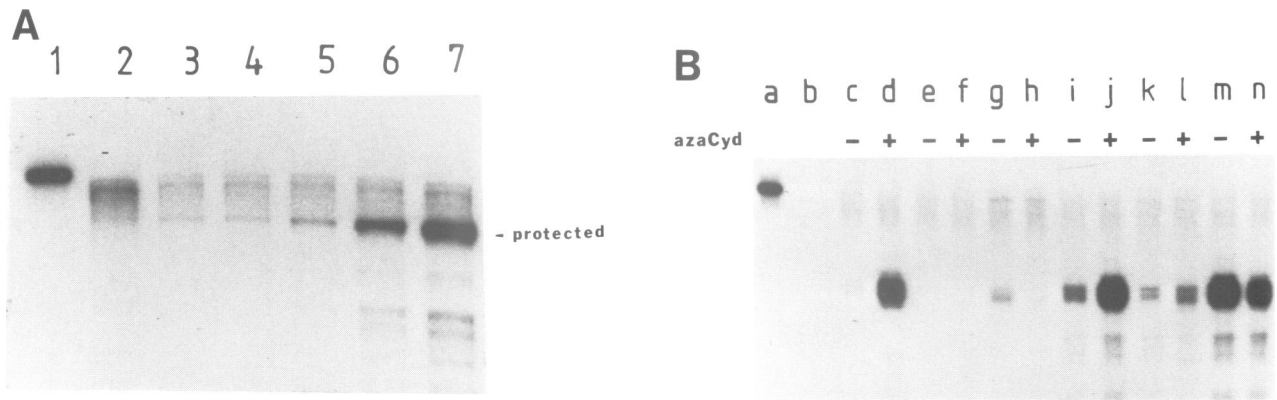

Fig. 2. Induction of transcription of the M.*Eco*RII mRNA by azaCyd as analyzed by S1 protection. (**A**) Time course of induction. *E.coli* GM271(pAC38) was treated with cytidine or azaCyd. Aliquots of the culture were removed at the indicated times and RNA was isolated. Twenty micrograms of total cellular RNA were hybridized with excess radiolabeled probe. After S1 nuclease digestion, fragments were resolved by electrophoresis on an 8% denaturing polyacrylamide gel. Lane 1, 500 c.p.m. untreated probe, 1% of the amount used in each hybridization; lane 2, 20 μg tRNA; lane 3, before treatment; lane 4, 60 min after cytidine treatment; lanes 5, 6 and 7, azaCyd treatment, 10, 30 and 60 min, respectively. The total reaction mix was loaded in lane 2. In each of lanes 3–7, one-fifth of the reaction mix was loaded. Protected fragments are indicated. (**B**) Effect of the upstream sequences and plasmid copy number on induction. GM271 bearing the indicated plasmids were grown to early log phase and treated with azaCyd. Twenty micrograms of RNA, isolated from cultures before (−) and after (+) the drug treatment, were tested for S1 protection with excess probe. Two-fifths of the product were analyzed in each of lanes b–n. Lane a, untreated probe, 1% of the amount used in each hybridization; b, tRNA; c and d, pAC12; e and f, pACQ; g and h, pAC59; i and j, pSS38; k and l, pSSP; m and n, pSS59.

to be very high because of initiation of transcription from the *lac* promoter, but no induction occurred after drug treatment. In fact, Cerenkov counting of the main bands in lanes m and n showed a 2.5-fold decrease in M.*Eco*RII mRNA isolated from azaCyd-treated cells. These experiments establish that induction of M.*Eco*RII mRNA synthesis upon azaCyd treatment depends on the presence of the endogenous promoter but is independent of plasmid copy number.

Effect of a deletion within the gene on transcription

The activity of M.*Eco*RII is destroyed *in vivo* by the drug, presumably because the enzyme binds to azaC-DNA. Inhibition of the enzyme by azaC-DNA has also been demonstrated *in vitro* (Friedman, 1981). We therefore determined if the catalytic activity of M.*Eco*RII was necessary for the azaCyd-induced synthesis of mRNA, initiated from the M.*Eco*RII promoter. We prepared a deletion derivative of pAC38, pAC38Δ (see Materials and methods). The DNA sequences upstream of the translational start codon of the methylase gene are identical in these two plasmids but in cells carrying the deletion derivative, only the first 56 N-terminal amino acids of M.*Eco*RII are synthesized from the mRNA. The amount of mRNA, initiated from the M.*Eco*RII promoter, was markedly higher in cells bearing pAC38Δ (Figure 3, lane 3) than in cells carrying pAC38 (lane 1). However, in the former, mRNA synthesis could not be induced by azaCyd treatment (lane 4). This high level of specific mRNA in GM271(pAC38Δ) was dramatically decreased when pSS38, a compatible multi-copy plasmid with a colE1 origin, was introduced into the cell (lane 6). This plasmid confers M.*Eco*RII activity on the strain. This suggests that M.*Eco*RII has a negative regulatory role on transcription initiated from its own promoter.

Binding of M.EcoRII to DNA containing its promoter sequence

The observation that the initiation of mRNA synthesis from the M.*Eco*RII promoter was markedly higher when the enzyme was absent, such as in GM271(pAC38Δ), or when

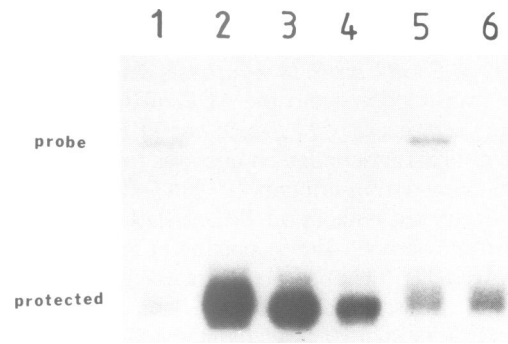

Fig. 3. Effect of a deletion of the M.*Eco*RII coding region on transcription as analyzed by S1 nuclease protection. A fragment of 1.03 kb was deleted from the M.*Eco*RII coding region of pAC38. The deletion recombinant pAC38Δ was used alone or together with pSS38 to transform *E.coli* GM271. RNA from cells carrying these plasmids was compared by S1 nuclease protection as described in the text and in the legend for Figure 2. Lanes 1 and 2, pAC38; lanes 3 and 4, pAC38Δ; lane 5, pSS38; lane 6, pSS38 plus pAC38Δ. In lanes 2 and 4, cells were treated with azaCyd.

the enzyme was inactivated by azaCyd treatment, as in the case of GM271(pAC38), led us to postulate an interaction between the enzyme and a sequence within or around the promoter. Fragments generated by *Acc*I digestion of plasmids pSSP, pSSQ, pSS12 and pSS38 were tested for binding with M.*Eco*RII (Figure 4). These fragments include sequences from the very beginning of the respective inserts up to the end of codon no. 107. Under the experimental conditions described, we found that binding occurred between M.*Eco*RII and DNA fragments from both pSS38 and pSS12. These fragments contain both the −10 and −35 region promoter sequences. No such binding was noticed with DNA fragments from pSSP and pSSQ. In these two constructs promoter sequences were deleted either partially (pSSP) or fully (pSSQ). The same DNA fragment from pSS12 did not show any binding with M.*Hpa*II, M.*Hha*I and M.*Msp*I.

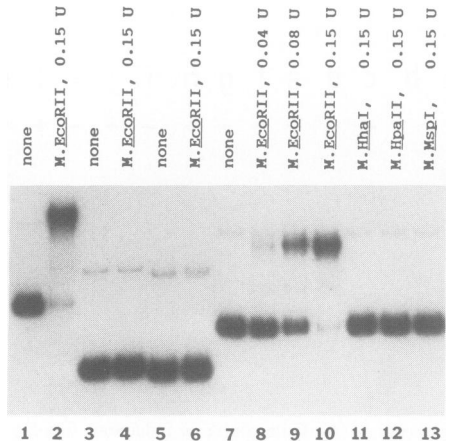

Fig. 4. DNA binding activity of M.*Eco*RII as detected by electrophoretic mobility shift. *Acc*I fragments from pSS38, pSS12, pSSP and pSSQ (Figure 1) were labeled with [α-^{32}P]dATP. Approximately 0.1 pmol of each of the fragments were incubated with the indicated amount of M.*Eco*RII or other indicated methylases as described in the text. The binding mixtures were analyzed on a 5% polyacrylamide gel. Origin of fragments: lanes 1 and 2, pSS38; lanes 3 and 4, pSSP; lanes 5 and 6, pSSQ; lanes 7–13, pSS12. The slowly moving faint band in each lane is probably due to a non-specific complex between the DNA and albumin, present in these reactions.

Effect of M.EcoRII on RNA synthesis in vitro

In vitro transcription from the M.*Eco*RII promoter was studied in the presence of M.*Eco*RII and M.*Hha*I (Figure 5). A single distinct transcription product was found when, prior to the initiation of transcription, 0.1 pmol of DNA template was pre-incubated without a C-Mtase (lane 2) or with 0.3 U of M.*Hha*I (lane 6). Pre-incubation of the same amount of DNA with 0.075 U of M.*Eco*RII markedly decreased the amount of transcript and 0.15 U of M.*Eco*RII (an enzyme:DNA ratio of 10:1) totally inhibited RNA synthesis. Therefore M.*Eco*RII, by binding to its own promoter or at an adjacent site, blocks the initiation of RNA synthesis.

Regulation of the M.MspI gene

To determine if similar regulation occurs in other type II R-M systems, we tested another C-Mtase, M.*Msp*I. Even though we subcloned the M.*Msp*I gene in a high-copy plasmid and although the DNA isolated from the transformed *E.coli* was fully protected from *Msp*I digestion, the level of M.*Msp*I mRNA, as detected by S1 nuclease protection assay, was found to be extremely low. Using the same amount of total RNA and labeled probes of the same specific activity, the signal obtained with cells bearing pM*Msp*I-7, a plasmid containing the M.*Msp*I gene, was very faint even after exposing the gel at −70°C for 4 days, as compared with that obtained with cells specifying M.*Eco*RII in a similar multi-copy plasmid such as pSS12. (Figure 6A, lanes 1 and 3 versus Figure 2B, lanes a and i). Probably the *E.coli* RNA polymerase does not efficiently transcribe from *Moraxella* promoters. However, after addition of azaCyd, induction of M.*Msp*I mRNA was detected in strains bearing pM*Msp*I-7 (lane 4, Figure 6A). Moreover, as was the case with pAC38Δ, a deletion made within the M.*Msp*I gene (construct pM*Msp*IΔ) resulted in elevated levels of mRNA (lane 5). As judged from ethidium bromide staining, no difference was found in the yield of plasmid DNA from equal amounts of cells between the strains bearing plasmids pM*Msp*I-7 and pM*Msp*IΔ (data not shown). Figure 6B shows that M.*Msp*I

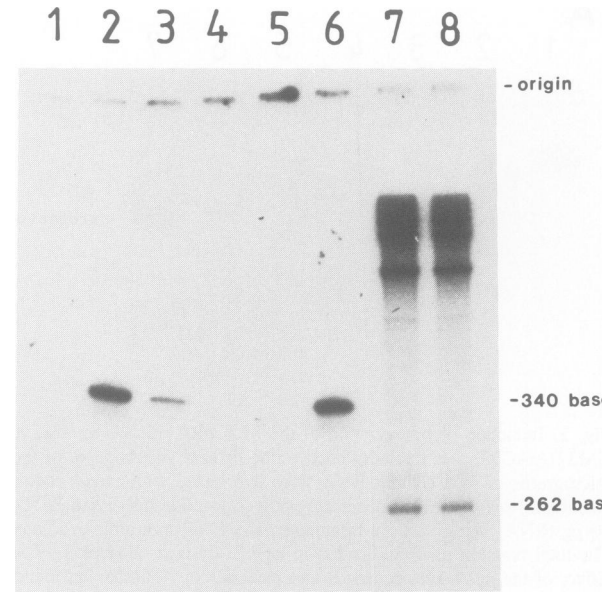

Fig. 5. Inhibition of *in vitro* transcription by M.*Eco*RII. Products of *in vitro* transcription from the M.*Eco*RII promoter were resolved on a 6% polyacrylamide–urea gel (lanes 1–6). A 443 bp *Acc*I fragment from pSS12, 0.1 pmol, was used as the template. The template was pre-incubated with the following proteins: lanes 1 and 2, none; lanes 3, 4 and 5, 0.075, 0.15 and 0.3 U (0.15 U = 1 pmol) respectively of M.*Eco*RII; lane 6, 0.3 U of M.*Hha*I. RNA synthesis was then initiated as described in the text, except in lane 1, where Mg^{2+} was omitted. In lanes 7 and 8, a 412 bp fragment containing the λ P_L promoter, 0.01 pmol, was used as template, pre-incubated in the absence (lane 7) or presence (lane 8) of 2 pmol M.*Eco*RII. The size of the transcripts are indicated.

efficiently binds with a 274 bp *Hin*fI fragment from pM*Msp*I, which includes the entire intergenic region between the *Msp*I R-M genes and extends 247 bp upstream of the M.*Msp*I translational start codon (Lin *et al.*, 1989). Although no sequence has been found which resembles a typical *E.coli* promoter sequence, in all probability this piece of DNA contains the promoter for M.*Msp*I. The same fragment failed to bind to three other C-Mtases, namely M.*Hpa*II, M.*Hha*I and M.*Eco*RII. Also, no binding was detected between M.*Msp*I and a 374 bp *Ava*II–*Hin*dIII fragment from M13mp18 containing the *lac* promoter. The binding of M.*Msp*I to its promoter sequence was therefore specific.

Discussion

It has been assumed that regulation of an R-M system is necessary for the survival of the host. The genes of type II R-M systems are located adjacent to one another (Wilson, 1991). Close linkage helps co-ordinate genetic transfer. Hence the presence of a regulatory mechanism within the system can be anticipated. Recently the *Pvu*II and *Bam*HI systems have been shown to be regulated by a *trans*-acting protein, the gene product of an ORF within the system. Similar ORFs have also been described in the *Sma*I and *Eco*RV systems (Tao *et al.*, 1991; Ives *et al.*, 1992; Tao and Blumenthal, 1992). These gene products are structurally similar. In each case the endonuclease gene is positively regulated. In the *Bam*HI system, the protein also acts as a repressor of the methylase gene. Whereas the polypeptide induces the endonuclease 1000-fold, it inhibits the methylase only 15-fold (Ives *et al.*, 1992).

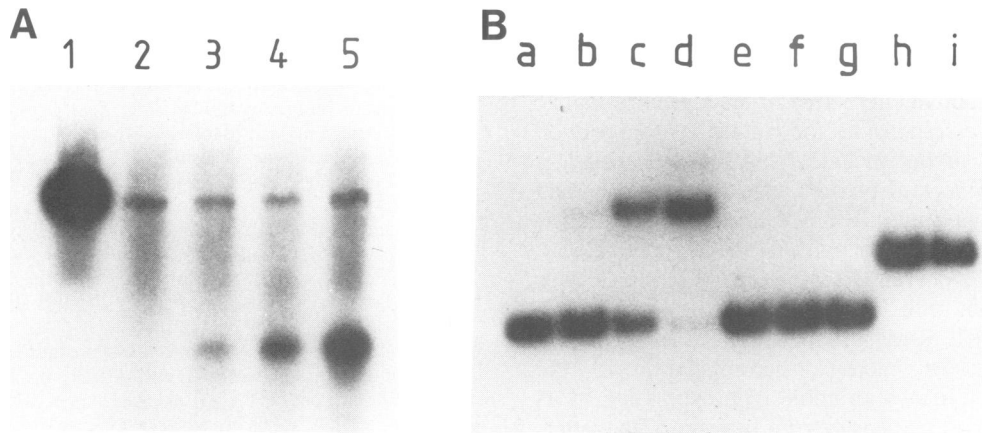

Fig. 6. Transcriptional regulation of M.*Msp*I. (**A**) S1 nuclease protection of RNA isolated from GM271 bearing plasmids containing the full-length or partially deleted M.*Msp*I gene. Experiments were carried out as described in the legend for Figure 2, except that a 5% denaturing gel was used for electrophoresis. Lane 1, probe, 200 c.p.m., 1% of the amount used in each hybridization; lane 2, tRNA; lane 3, pM*Msp*I-7; lane 4, pM*Msp*I-7, after azaCyd treatment; lane 5, pM*Msp*IΔ. In lanes 2–5 total reaction products were analyzed. (**B**) DNA binding activity of M.*Msp*I as analyzed by mobility shift assay. A 274 bp *Hin*fI fragment containing the intergenic region of the *Msp*I R-M system was incubated with the indicated amount of M.*Msp*I or other methylases. The binding mixtures were analyzed on a 5% polyacrylamide gel. Lane a, no addition; lanes b, c and d, 0.02, 0.05 and 0.1 U respectively of M.*Msp*I; lane e, 0.1 U of M.*Hpa*II; lane f, 0.1 U of M.*Hha*I; lane g, 0.1 U of M.*Eco*RII. Lanes h and i, reaction mixture containing a 374 bp DNA fragment with the *lac* promoter sequence in the absence (lane h) or presence (lane i) of 0.1 U of M.*Msp*I.

The genes of the *Eco*RII R-M system are transcribed towards one another from two non-overlapping sets of promoters separated by more than 2.7 kb (Som *et al.*, 1987; Kosykh *et al.*, 1989; Bhagwat *et al.*, 1990). Results presented in this paper demonstrate that M.*Eco*RII is autogenously regulated. We discovered autogenous regulation of M.*Eco*RII in attempting to understand the basis for the sensitivity of cells specifying M.*Eco*RII to the cytidine analog azaCyd. Recently we found that cells harboring a low-copy plasmid pAC59, in which the M.*Eco*RII structural gene was cloned under the control of the *lac* promoter, were not killed by azaCyd, whereas cells bearing pAC12, in which the gene was under the control of its own promoter, were (data not given). When M.*Eco*RII mRNA was assayed in these strains, the levels were found to be comparable. However, azaCyd treatment induced M.*Eco*RII mRNA synthesis greatly in the latter cells while inhibiting it in the former (Figure 2B). Therefore, induction of M.*Eco*RII mRNA is dependent on the structural gene being under control of its own promoter.

Proof that the induction was due to autogenous control and not a peculiarity of azaCyd treatment was obtained when part of the structural gene was deleted. Cells with plasmids containing the M.*Eco*RII promoter, but having a deletion in the coding region, had unusually high levels of mRNA transcribed from the promoter (Figure 3). Treatment of these cells with azaCyd did not increase M.*Eco*RII mRNA synthesis. More importantly, this transcription was inhibited when M.*Eco*RII was expressed *in trans*. These results were confirmed by *in vitro* transcription experiments. In summary, for azaCyd-induced expression of the M.*Eco*RII gene, the requirements are an intact promoter with both the −35 and −10 region sequences and an intact methylase protein, whereas for high expression without azaCyd treatment, an intact promoter and the absence of a functional methylase protein are needed. These findings lead to the conclusion that the enzyme negatively regulates its own expression by interacting with a sequence within or near its promoter. This sequence does not contain the target sequence for the enzyme. To demonstrate the interaction *in vitro*, both the −35 and −10 regions of the promoter were necessary. The interaction is specific. Other C-Mtases would not bind to, or inhibit transcription from the M.*Eco*RII promoter. The binding does not need *S*-adenosylmethionine (data not given). At this point we do not know whether any other factor is involved *in vivo*.

Evidence presented here also supports autoregulation of another C-Mtase, M.*Msp*I. Unlike *Eco*RII, the *Msp*I R-M system has divergently transcribed endonuclease and methylase genes separated by only 110 bp (Lin *et al.*, 1989). Like M.*Eco*RII, M.*Msp*I binds tightly to azaC-DNA (Friedman, 1985). Even though efficiency of transcription of the M.*Msp*I gene was extremely poor in *E.coli*, an increase in transcription was detected both as an effect of azaCyd or when a portion of the M.*Msp*I coding region was deleted. The changes are, however, not as striking as in the case of M.*Eco*RII.

The plasmid pAC38Δ, which carries a deletion within the M.*Eco*RII gene but has an intact promoter sequence, is derived from a low-copy plasmid, pAC38. We attempted to construct a similar deletion in a multi-copy plasmid, pSS38. The recombinant plasmid, pSS38Δ, proved to be extremely toxic to *E.coli* GM271. The transformed colonies were flat and small even after 24 h of incubation. Most of the colonies would not grow in liquid culture. Those that did, grew poorly, and plasmids isolated from such cultures gave extremely poor DNA yields. We have not studied the phenomenon further. One explanation for this behavior could be the inability of the cells to tolerate the level of uncontrolled transcription from the large amount of DNA template yielded by the multi-copy plasmid. At present we do not have evidence that any catalytically inactive mutant of M.*Eco*RII would fail to regulate the expression of the gene. Recently, Wyszynski *et al.* (1992) found that one of their plasmids, specifying a mutant M.*Eco*RII protein, was toxic to cells. Uncontrolled expression of the gene could be the cause of the toxicity they observed. On the other hand, the deletion in the M.*Msp*I gene, although in a multi-copy plasmid, is very stable in *E.coli*. This is probably because transcription from the M.*Msp*I promoter is so poor that even a 10-fold

increase would not affect cell viability.

Many autoregulatory proteins have been described (Maloy and Stewart, 1993). Regulation can be transcriptional or translational. A number of such proteins are regulatory factors, which are responsible for regulating transcription of other genes. Others include DNA or RNA binding proteins such as the λ *cI* protein, ribosomal proteins and threonyl-tRNA synthetase. The *E.coli ksgA* gene product, which has RNA methylase activity, is also autoregulated at the translational level (van Gemen *et al.*, 1989). C-Mtases are a new addition to the class of autoregulatory proteins.

Since the *Eco*RII R-M system is located in a plasmid transferable between bacteria, autoregulation of the methylase enzyme allows an initial high expression of its gene in the new host and ensures the survival of the latter. For chromosomal R-M systems, there is still the necessity to control expression of the methylase if foreign DNA is to be appropriately digested. We have studied the *Msp*I system and found the existence of autogenous control of the modification gene. At present we do not know whether other DNA methylases have similar properties. C-Mtases contain many structural domains that are very similar (Lauster *et al.*, 1989; Posfai *et al.*, 1989), one of which may be involved in promoter binding. We also do not know whether this is the only control mechanism for regulation of the *Eco*RII R-M system. Although the cellular balance of endonuclease and methylase can be adequately controlled by regulating the expression of one of them, in this case the methylase gene, regulation of the *Eco*RII endonuclease gene may also occur. The *E.coli* Dcm enzyme recognizes the same sequence and methylates the same cytosine residue as M.*Eco*RII. These two enzymes are structurally very similar (Som *et al.*, 1987; Hanck *et al.*, 1989). We are investigating whether the Dcm enzyme can regulate M.*Eco*RII and *vice versa*.

Materials and methods

Materials

Radiochemicals were purchased from Dupont–New England Nuclear. S1 nuclease and RNase-free DNase were from Gibco-BRL and Pharmacia, respectively. Sequenase was from United States Biochemicals; restriction enzymes were from BRL and New England Biolabs. C-Mtases, except for M.*Eco*RII, were from New England Biolabs. M.*Eco*RII was purified from an over-producing strain (Som and Friedman, 1990). All C-Mtases were assayed by a method described previously (Friedman, 1985). The unit used for C-Mtase activity in this study is defined as picomoles of methyl group transferred from tritiated *S*-adenosylmethionine to *E.coli* B DNA per minute under standard conditions.

Plasmids and strains

Multi-copy plasmid pSS38 (Som *et al.*, 1987), the largest of the constructs of the pSS series used in this study, contains sequences 194 bp upstream of the translational start codon of the M.*Eco*RII gene. Plasmids pSS12, pSSP and pSSQ were constructed from pSS38 by gradual removal of sequences upstream of the start codon. *Sal*I-linearized pSS38 was treated with Bal31, filled-in with dNTPs, and ligated with *Sal*I linkers. After digestion with *Sal*I and *Bam*HI, the fragments containing the M.*Eco*RII gene were cloned in the *Sal*I and *Bam*HI sites of pUC18. Plasmids pSS12, pSSP and pSSQ contain 118, 34 and 23 bp, respectively, upstream of the start codon. The corresponding low-copy pAC recombinants were constructed by cloning the *Pst*I–*Bam*HI fragments of the pSS plasmids into the *Pst*I and *Bam*HI sites of pACYC177. pAC59 was prepared by ligating a 1.95 kb *Bam*HI–*Pvu*II fragment from pSS59 (Som *et al.*, 1987), containing the M.*Eco*RII gene and the *lac* promoter region, to a 2.64 kb *Bam*HI–*Dra*I fragment of pACYC177. In pSS59/pAC59, the *lac* sequences are fused with the M.*Eco*RII sequences 9 bp upstream of the ATG start codon.

Plasmid pAC38Δ was constructed from pAC38 by substituting an *Xba*I linker for the internal 1028 bp *Nru*I fragment (Figure 1). This linker contains a termination codon.

Plasmid p*Msp*I was constructed by subcloning a 3.03 kb *Eco*RI–*Hin*dIII fragment containing the entire *Msp*I R-M system (Lin *et al.*, 1989) in pUC18. Removal of 529 bp from p*Msp*I by partial *Dra*I digestion and subsequent re-ligation created plasmid pM*Msp*I-7, which was devoid of a functional *Msp*I endonuclease gene. Plasmid pM*Msp*IΔ was constructed from pM*Msp*I-7 by deleting a 363 bp *Hpa*I–*Eco*RV fragment within the coding region for M.*Msp*I, and re-circularizing the plasmid by blunt-end ligation. Cells containing pM*Msp*IΔ have no methylase activity.

Escherichia coli GM271 (*leu*B-6 *dcm*-6 *his*G4 *thi*-1 *hsd*R2 *ara*-14 *lac*Y1 *gal*K2 *gal*T22 *xyl*-5 *mtl*-1 *rps*L136 *ton*A31 *tsx*-78 *sup*E44) was obtained from G.Marinus and used as the host for all plasmids.

Preparation of probes for S1 nuclease protection studies

To prepare the probe for M.*Eco*RII mRNA, a 365 bp *Sal*I–*Nru*I fragment from pSS38 was cloned in the *Sal*I and *Sma*I sites of M13mp19. The single-stranded viral DNA, which contained the message strand of the insert, was isolated from infected cells. The complementary strand was synthesized using the 17mer universal primer, dNTPs and Sequenase. The synthesized duplex was digested with *Ava*I and the 3′ end was filled in with dNTPs containing [α-^{32}P]dATP. The single-stranded probe, labeled at the 3′ end, was isolated by denaturing gel electrophoresis. The terminal 79 bases of the 3′ end of the 115 base probe are complementary to the M.*Eco*RII mRNA.

The single-stranded probe for M.*Msp*I mRNA was prepared in a similar way to that described above from a recombinant M13mp18 containing a cloned *Eco*RV–*Xba*I fragment of p*Msp*I (Figure 1). The 257 base probe contains, at the ^{32}P-labeled 3′ end, 216 bases complementary to the M.*Msp*I mRNA.

Cell growth and isolation of RNA

E.coli GM271 cells bearing different recombinant plasmids were grown in minimal medium supplemented with 0.2% casamino acid and the appropriate antibiotic. At A_{600} = 0.5, cytidine or azaCyd was added to a final concentration of 20 μg/ml. After the indicated time, the incubation was stopped by removing aliquots and immediately chilled to 0°C by mixing with crushed ice. The cells were collected by centrifugation and total RNA was isolated (Gilman and Chamberlin, 1983). The RNA was purified free of DNA by digestion with DNase, followed by extraction with phenol–chloroform and two precipitations with ethanol. The amount of total RNA was estimated by measuring the A_{260}. The integrity of each preparation was verified by gel electrophoresis.

S1 nuclease protection of RNA

Twenty micrograms of cellular RNA were mixed with ^{32}P-labeled probe and precipitated with ethanol. The amount of probe was the same for each RNA sample in one set of experiments and was at least in a 5-fold excess over the largest amount of the target mRNA present in any of the samples. The amount was determined by trial experiments. The precipitated nucleic acids were hybridized and digested with 100 U of S1 nuclease for 1 h at 30°C as described by Sambrook *et al.* (1989). The digestion mixture was extracted with phenol–chloroform and the protected hybrids were precipitated with ethanol. The pellet was dried, dissolved in loading buffer containing formamide, heated at 85°C for 3 min and analyzed on a sequencing gel. S1 nuclease-protected fragments were detected by autoradiography.

Gel retardation assays and in vitro transcription

The indicated amounts of DNA templates and C-Mtases were mixed together in a 10 μl reaction mix containing 40 mM Tris–Cl, pH 7.6, 100 mM KCl, 5 mM dithiothreitol, 50 μM *S*-adenosylmethionine, 50 μg/ml albumin, 5% glycerol and 1 μg tRNA. The mixture was incubated at 25°C for 1 h. Loading buffer was added and the products were electrophoresed through a 5% polyacrylamide gel in 0.5 × TBE buffer. For *in vitro* transcription, 0.1 pmol of the 443 bp *Acc*I fragment of pSS12 containing the M.*Eco*RII promoter sequence was pre-incubated, as described above, with or without M.*Eco*RII. After 1 h, all ribonucleotides, except CTP, were added to a concentration of 150 μM, [α-^{32}P]CTP was added to a concentration of 15 μM and the mixture was incubated with 0.5 U of RNA polymerase at 37°C for 10 min. Heparin was then added to a concentration of 100 μg/ml and 1 min later, transcription was initiated by adding $MgCl_2$ to the 15 μl reaction mix to a concentration of 10 mM. The reaction was stopped after 10 min by adding EDTA to a final concentration of 20 mM. After extraction with phenol–chloroform, the aqueous layer was removed, and the synthesized RNA was precipitated with ethanol and analyzed by electrophoresis on a denaturing polyacrylamide gel.

Acknowledgements

This work was supported by a grant from the State University of New York.

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Received on June 3, 1993; revised on July 20, 1993