Nucleic Acids Research, 1993, Vol. 21, No. 10 2309–2313

Gene structure and expression of the *Mbo*I restriction – modification system

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Received March 17, 1993; Revised and Accepted April 15, 1993 DDBJ accession no. D13968

ABSTRACT

The genes from *Moraxella bovis* encoding the *Mbo*I restriction – modification system were cloned and expressed in *Escherichia coli*. Three open reading frames were found in the sequence containing the genes. These genes, which we named *mboA*, *mboB*, and *mboC*, had the same orientation in the genome. Genes *mboA* and *mboC* encoded *Mbo*I methyltransferases (named M.*Mbo*A and M.*Mbo*C) with 294 and 273 amino acid residues, respectively. The *mboB* gene coded for *Mbo*I restriction endonuclease (R.*Mbo*I) with 280 amino acid residues. Recombinant *E.coli*-MBOI, which contained the whole *Mbo*I system, overproduced R.*Mbo*I. R.*Mbo*I activity from *E.coli*-MBOI was 480-fold that of *M.bovis*. The amino acid sequences deduced from these genes were compared with those of other restriction – modification systems. The protein sequences of the *Mbo*I system had 38 – 49% homology with those of the *Dpn*II system.

INTRODUCTION

Restriction endonucleases are needed for the molecular cloning of genes. Many restriction–modification systems have been discovered in a wide variety of microorganisms (1), where they protect the host from invasion by foreign DNA. More than 100 genes encoding restriction–modification systems have been cloned and expressed in *Escherichia coli* (2, 3). *Moraxella bovis*, which are facultative anaerobic and Gram-negative rods (4), have two restriction–modification systems, *Mbo*I and *Mbo*II, which recognize double-stranded DNA at the sequences 5′-GATC-3′ and 5′-GAAGA-3′, respectively (5). The genes encoding the *Mbo*II system have been cloned and sequenced (6).

The *Sau*3AI system (7), the *Dpn*II system (8, 9), *Dpn*I endonuclease (8), and Dam methylase (10) recognize the same DNA sequence as the *Mbo*I system. The structures of genes encoding their enzymes, except the genes for the *Mbo*I system, have been identified. Comparison of these enzymes may help to elucidate some general rules for DNA sequence recognition and cleavage.

*Mbo*I endonuclease (R.*Mbo*I) is one of the most widely used enzymes for molecular biology and was purified from *M.bovis* by Gelinas et al. (5). Cloning and expression of the R.*Mbo*I gene in *E.coli* will make it possible to obtain pure preparations of the enzyme in large amounts.

In this paper, we report the cloning of the genes coding for the *Mbo*I system and the expression of R.*Mbo*I in *E.coli*. The amino acid sequences predicted from the genes were compared with those of other restriction–modification systems.

MATERIALS AND METHODS

Enzymes and chemicals

Restriction endonucleases, modification enzymes, and kits for DNA sequencing, kilo-sequence deletion, 5′-end labeling, blunting, and ligation were products of Takara Shuzo Co., Ltd. (Kyoto, Japan), and were used according to the manufacturer's instructions.

Bacterial strains, plasmids, phages, and DNA preparation

M.bovis (ATCC 10900) was purchased from the American Type Culture Collection. *E.coli* strains GM33 [*sup85, dam3* (11)], HB101 [*F⁻, hsdS20(rB⁻,mB⁻), supE44, ara14, l⁻, galK2, lacY1, proA2, rpsL20, xyl-5, mtl-1, recA13* (12)], and JM109 [*recA1, endA1, gyrA96, thi, hsdR17(rK⁻,mK⁻), supE44, relA1, l⁻, D(lac-proAB), F′, traD36, proAB, lacIᵠZDM15* (13)] and plasmids pBR322 [Tc^r, Ap^r (14)], pACYC184 [Cm^r, Tc^r (15)], pUC18 [Ap^r (13)], pUC19 [Ap^r (13)], pKH1 [Ap^r (16)], and pNT203 [Tc^r (17)] were the host-vector systems used for cloning and expression. M13mp18 and mp19 (13) were used for DNA sequencing. *E.coli* competent cells were produced by the procedure of Hanahan (18). λ virulent phage (λgt·λc) was prepared from plate lysates. *E.coli* plasmid DNA was prepared on a small scale by the method of Birnboim and Doly (19). *M.bovis* chromosomal DNA was purified by the procedure of Ausubel et al. (20).

Construction of the *M.bovis* genomic library

An *Alu*I partial digest of chromosomal DNA was fractionated with agarose gel electrophoresis, giving fragments from 4 to 7 (kb) long. The fragments were ligated to the *Eco*RV site of plasmid pBR322 and used to transform *E.coli* GM33 (Dam⁻).

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Screening of the *Mbo*I methyltransferase gene

Total plasmids were prepared from the transformed *E.coli* GM33 cells and digested with an excess of R.*Mbo*I. *E.coli* GM33 was transformed again with the digested plasmids. The transformants obtained were plated onto L-plates (21) containing 100 μg/ml ampicillin. Plasmids were prepared from the surviving bacterial cells and analyzed for their ability to express *Mbo*I methyltransferase. A recombinant plasmid, pUM1, with a 4-kb insert was isolated and subcloned into pUC19 to yield plasmid pUM2. This plasmid was used for the construction of a series of deletion clones (pUM2-d1 to pUM2-d6; Fig. 1).

Analysis of N-terminal amino acid sequences

R.*Mbo*I was partially purified from a culture of *M.bovis* by the method of Gelinas et al. (5). The N-terminal amino acids were sequenced as described elsewhere (22, 23).

Screening for *Mbo*I system genes

A DNA probe (23-mer) was synthesized based on the N-terminal amino acid sequences of R.*Mbo*I for the screening of genes encoding the *Mbo*I system. After the probe was labeled with [γ-^{32}P]ATP and the kit for 5'-end labeling (Megalabel, Takara), it was used for genomic Southern hybridization as described elsewhere (23). A 5.1-kb *Bgl*II-*Nde*I fragment was isolated, blunt-ended, and cloned into the pUC18 vector to yield a plasmid, pMBO1. *E.coli* JM109 (Dam$^+$) cells carrying pMBO1 were assayed for their ability to produce R.*Mbo*I and M.*Mbo*I, and then used for the construction of a series of deletion clones (pMBO1-d1 to pMBO1-d7; Fig. 1).

Assay of enzyme activities

R.*Mbo*I activity *in vivo* was estimated from the plating efficiency of λ virulent phage. *In vitro*, activity was assayed by the incubation at 37°C of 25 μl of a reaction mixture containing 20 mM Tris−HCl (pH 8.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, 1 μg of N^6-methyladenine-free λ DNA, and the enzyme solution. One unit of enzyme activity was defined as the amount of enzyme needed for complete digestion of 1 μg of N^6-methyladenine-free λ DNA at 37°C for 1 hr. *Mbo*I methyltransferase activity was assayed *in vivo* by the resistance of plasmids to R.*Mbo*I digestion. This activity was assayed *in vitro* by the incubation of 10 μl of 50 mM Tris−HCl (pH 7.5) containing 5 mM 2-mercaptoethanol, 10 mM EDTA, 80 mM *S*-adenosylmethionine, 1 μg of N^6-methyladenine-free λ DNA, and the enzyme solution at various concentrations for 1 hr at 37°C. Then 40 μl of a reaction mixture containing R.*Mbo*I was added and the mixture was incubated at 37°C until digestion was complete. One unit of enzyme activity was defined as the amount of enzyme needed for complete protection from digestion by R.*Mbo*I for 1 hr of 1 μg of N^6-methyladenine-free λ DNA.

Deletion assays were done as follows. A series of deletion mutants was prepared with a kilo-sequence deletion kit and introduced into *E.coli*. The activities of the two enzymes from the clones were assayed *in vivo* and *in vitro* as described above.

Nucleotide sequencing

Nucleotide sequencing was done by the dideoxy chain termination method (24), except that 7-deaza-dGTP was used instead of dGTP. Restriction fragments were isolated from the *Mbo*I restriction−modification genes and subcloned into the appropriate sites of the vectors M13mp18 or mp19. Alternatively, deletion derivatives were generated from subclones containing the *Mbo*I system with a kilo-sequence deletion kit. The sequences were deduced from the results for both strands. The DNA sequences and the predicted amino acid sequences were analyzed by a computer-aided system for DNA analysis (DNASIS, Hitachi, Japan).

Construction of plasmids for overproduction of R.*Mbo*I

A *Pvu*II fragment of the plasmid pUM2-d4 was ligated with the *Eco*RV site of the pACYC184 vector. The plasmid obtained, pMBOC, included a *lac* promoter and the *mboC* gene region from pUM2-d4 (Fig. 2A). An *Eco*RI-*Xmn*I fragment from the pMBO1-d2 plasmid (Fig. 1) and the *Bgl*II-*Eco*RI linker (Fig. 2B) were ligated with pKH1 vector, which had been digested with *Eco*RI, blunt-ended with a blunting kit, and digested with *Bgl*II. The resulting plasmid, pMBOAB, contained the *mboA* and *mboB* genes. Both pMBOC and pMBOAB plasmids were introduced into *E.coli* HB101 together with the plasmid pNT203 for overproduction of R.*Mbo*I.

The *E.coli* recombinants obtained, named *E.coli*-MBOI, were cultured in 20 ml of LB broth (21) containing 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, and 15 μg/ml tetracycline at 30°C until the absorbance at 600 nm was 1.0−2.0, heated at 42°C for 15 min, and cultured at 37°C for 5 hr. The cells were harvested, suspended in 4 volumes of a buffer [20 mM potassium phosphate (pH 7.5) containing 10 mM 2-mercaptoethanol, 5% glycerol, and 0.15% Triton X-100], sonicated for 10 min, and centrifuged at 100,000×*g* for 30 min. The supernatant was assayed for R.*Mbo*I activity *in vitro* and checked for production of R.*Mbo*I by SDS-PAGE (Fig. 2C).

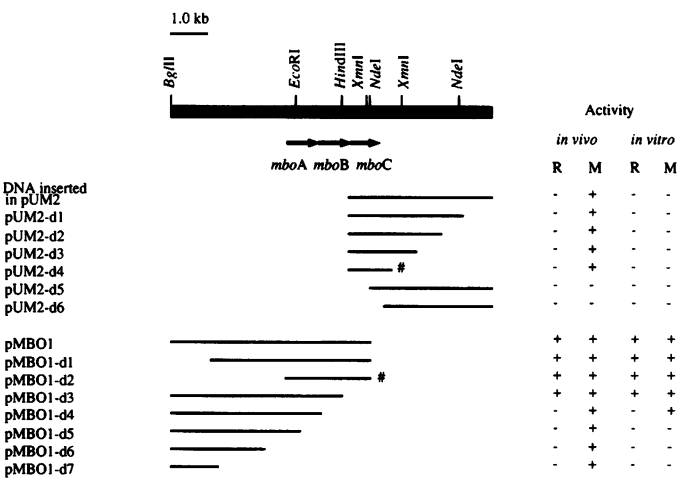

Figure 1. Restriction map and deletion analyses of pUM2 and pMBO1. Deletion derivatives, pUM2-d1, -d2, -d3, -d4, -d5, and -d6, of the fragment inserted in pUM2 and deletion derivatives, pMBO1-d1, -d2, -d3, -d4, -d5, -d6, and -d7, of the fragment inserted in pMBO1 were prepared with a kilo-sequence deletion kit. Enzyme activities were assayed *in vivo* and *in vitro* as described in 'Materials and Methods': +, detectable activity; −, no detectable activity; R, restriction activity; M, modification activity; #, the fragments sequenced.

SDS-PAGE

SDS-PAGE was done by the method of Laemmli (25) with a 12.5% polyacrylamide slab gel. Phosphorylase *b* (M_r 97,400), bovine serum albumin (66,200), ovalbumin (42,700), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400) were used as markers of molecular weight (Bio-Rad Labs).

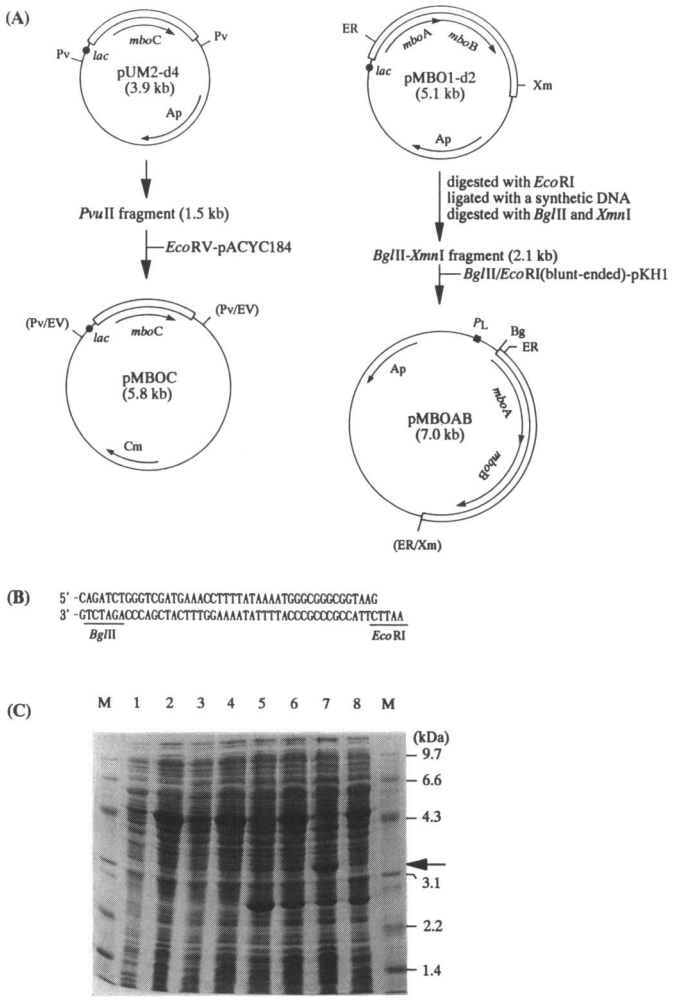

Figure 2. Construction of the plasmid pMBOAB and overproduction of R.*Mbo*I in *E.coli*-MBOI. (**A**), Plasmid pMBOC carried the *mbo*C gene and a *lac* promoter region from plasmid pUM2-d4. Plasmid pMBOAB contained the *mbo*A and *mbo*B genes from plasmid pMBO1-d2 and *P*L promoter from the vector pKH1. Bg, *Bgl*II; ER, *Eco*RI; EV, *Eco*RV; Pv, *Pvu*II; Xm, *Xmn*I; *lac*, *lac* promoter; *P*L, *P*L promoter; Ap, ampicillin resistance gene; Cm, chloramphenicol resistance gene. (**B**), The nucleotide sequence of a synthetic DNA fragment for construction of plasmid pMBOAB. (**C**), Overproduction and estimation of the molecular weight of R.*Mbo*I. Lanes M, molecular weight markers; lane 1, *E.coli* HB101 with heat-induction; lane 2, *E.coli* HB101 without induction; lane 3, *E.coli* HB101 containing plasmid pNT203 with heat-induction; lane 4, *E.coli* HB101 containing plasmid pNT203 without induction; lane 5, *E.coli* HB101 containing plasmids pNT203 and pMBOC with heat-induction; lane 6, *E.coli* HB101 containing pNT203 and pMBOC without induction; lane 7, *E.coli* HB101 containing pNT203, pMBOC, and pMBOAB with heat-induction; lane 8, *E.coli* HB101 containing pNT203, pMBOC, and pMBOAB without induction. The arrow indicates the position of the molecular weight of R.*Mbo*I.

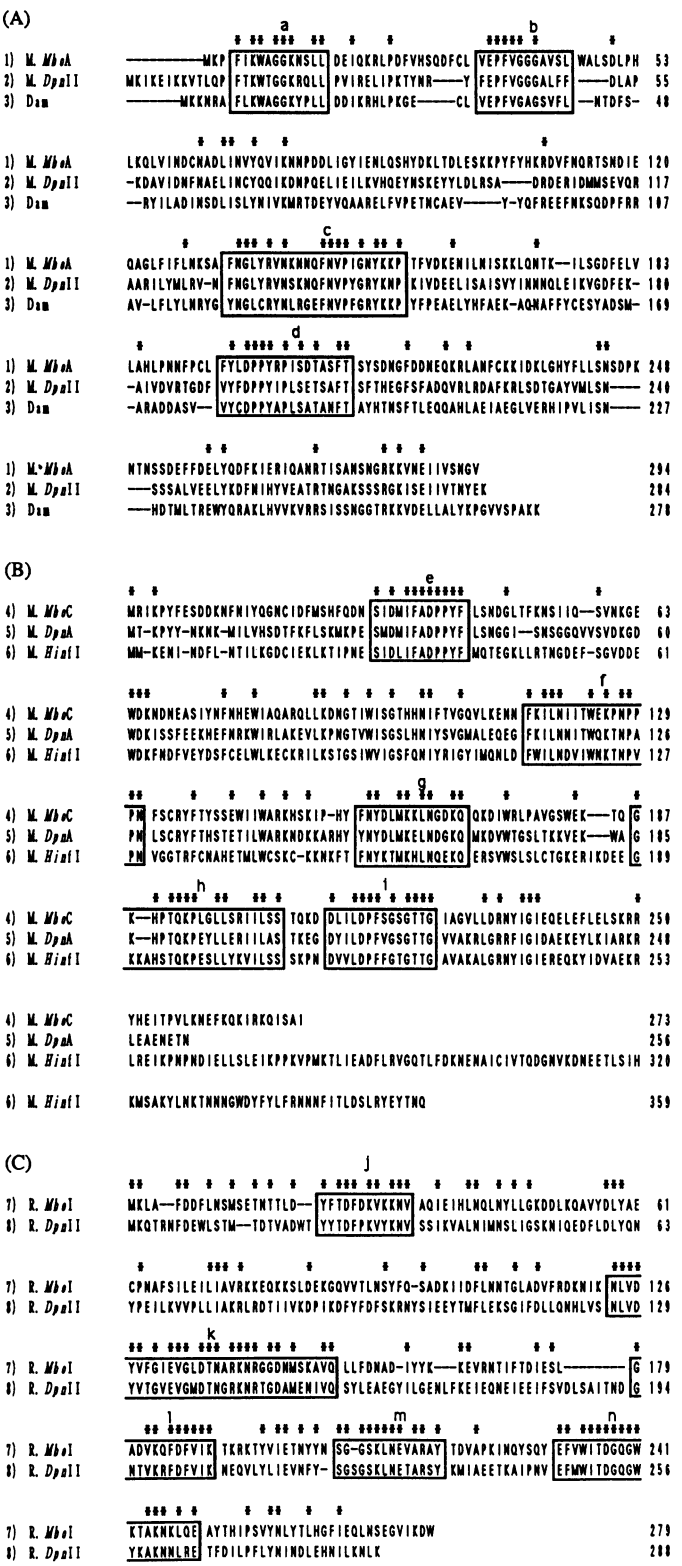

Figure 3. Alignments of M.*Mbo*A, M.*Dpn*II, and *E.coli* Dam methylases (**A**), M.*Mbo*C, *Dpn*A, and M.*Hin*fI methylases (**B**), and R.MboI and R.DpnII endonucleases (**C**). Identical amino acids of the methylases in A and B and of the endonucleases in C are indicated by asterisks. Bars show gaps in the aligned sequences. The highly conserved regions are boxed and labelled a to n.

RESULTS AND DISCUSSION

Isolation of the *Mbo*I methyltransferase gene

Selection of the gene encoding *Mbo*I methyltransferase was based on the resistance to digestion with R.*Mbo*I of self-modifying recombinant plasmids. One clone that expressed *Mbo*I methyltransferase was selected and subcloned into the pUC19 vector for the construction of a deletion series. The activities of *Mbo*I methyltransferase and endonuclease from the deletion clones were assayed *in vivo* and *in vitro* (Fig. 1). The region encoding *Mbo*I methyltransferase was found in the pUM2-d4 plasmid. Endonuclease activity was detected neither *in vivo* nor *in vitro*. Methyltransferase activity *in vitro* was not found; probably, it was not expressed at a high enough level for detection by our assay.

Analysis of N-terminal sequence of R.*Mbo*I

Thirty amino acids, Met- Lys- Leu- Ala- Phe- Asp- Asp- Phe- Leu- Asn- Ser- Met- Ser- Glu- Thr- Asn- Thr- Thr- Leu- Asp- Val- Phe- Thr- Asp- Phe- Asp- Lys- Val- Lys- Lys, were identified in the N-terminal sequence of purified R.*Mbo*I. A 23-mer DNA probe, 5'-ATGAAGITIGCITTTGATGATTT-3' ('I' indicates inosine residues), was synthesized based on this N-terminal sequence. The probe did not anneal with the pUM1 plasmid.

Isolation of *Mbo*I restriction−modification genes

Plasmid pMBO1 was obtained by Southern hybridization with the synthetic probe. Endonuclease and methyltransferase activities *in vivo* and *in vitro* were detected in the clones that contained the pMBO1 plasmid (Fig. 1). Deletion mutants containing pMBO1-d1, -d2, -d3, and -d4 had methyltransferase activity either *in vivo* or *in vitro*. The mutants containing pMBO1-d5, -d6, and -d7 had only *in vivo* methyltransferase activity. The methyltransferase activity *in vivo* was probably that of the Dam methylase of host *E.coli*. The level of expression of Dam methylase is low, which probably explains why the activity was not found *in vitro* under our assay conditions. Therefore, we expected that there was another methyltransferase gene in pMBO1 and analyzed pMBO1-d2, which probably contained the endonuclease and methyltransferase genes.

Gene structure of the *Mbo*I system

Nucleotides of the fragments inserted in the pUM2-d4 and pMBO1-d2 plasmids were sequenced as described in 'Materials and methods'. The fragments inserted in the pUM2-d4 and pMBO1-d2 plasmids overlapped with each other. There were three open reading frames (ORFs) in the same direction in the combined nucleotide sequence, 2826 base pairs (bp) long (Fig. 1). The first and third ORFs, 885 bp long and coding for positions 137 to 1021, and 822 bp long and coding for positions 1867 to 2688 (termed *mbo*A and *mbo*C, respectively) encoded methyltransferases (named M.*Mbo*A and M.*Mbo*C) of 294 and 273 amino acid residues (M_r 33,700 and 31,900), respectively. The second ORF, 843 bp long (1023 to 1865; the *mbo*B gene) encoded a restriction endonuclease (R.*Mbo*I) of 280 residues (M_r 32,200). The N-terminal amino acid sequence deduced from the *mbo*B gene was identical with that of purified R.*Mbo*I. There were putative Shine−Dalgarno (SD) sequences upstream of the three genes: GAAA at positions 126 to 129 for the *mbo*A gene, GGAG at 1013 to 1016 for the *mbo*B gene, and AGGA at 1855 to 1858 for the *mbo*C gene. A putative promoter sequence upstream of the *mbo*A gene resembled the *E.coli* consensus sequence; a −35 box, TTGTAA, at positions 61 to 66 and a −10 box, TATAAT, at positions 92 to 97. The GC content of the three genes was about 30%, which was lower than the median of *M.bovis* [41.0−44.5%, (4)]. The G+C content is low in the genes of the *Mbo*II system, also (6).

Overproduction of R.*Mbo*I

The *Mbo*I system, which had two methyltransferases, M.*Mbo*A and M.*Mbo*C, was similar to the *Dpn*II system, because both systems recognized the same sequence and both had two methyltransferases. If the two methylases of the *Mbo*I system had the same functions as those of the *Dpn*II system, one of the two proteins might help to regulate system activation. We constructed two plasmids, named pMBOC and pMBOAB, for expression of the R.*Mbo*I gene (Figs. 2A and B). The strain obtained by transformation, *E.coli*-MBOI, overproduced R.*Mbo*I. A polypeptide with the M_r of 31,000 was overproduced; this was probably R.*Mbo*I (Fig. 2C). A band at M_r26,000 (Fig. 2C, lanes 5 to 8) is likely to be a translational product of the chloramphenicol resistance gene. *In vitro* R.*Mbo*I activity in a crude extract from heat-induced *E.coli*-MBOI was 1,200,000 U/g cells, which was 480-fold that from *M.bovis*.

We also transformed *E.coli* with pMBOAB and pNT203, and this strain overproduced R.*Mbo*I, as well. However, the *mbo*B and *mbo*C genes could not be cloned in *E.coli* without the *mbo*A gene. These findings indicate that M.*Mbo*C might be a weaker and less specific methylase than M.*Mbo*A; the *Dpn*II system also has a pair of methylases different in this way (9).

Comparison of amino acid sequences

The deduced amino acid sequences of the *Mbo*I system were compared with the sequences of other restriction−modification systems published in the Gene/Protein database. The protein sequence of M.*Mbo*A was 44% homologous with that of M.*Dpn*II (9) and 34% homologous with that of *E.coli* Dam methylase (10). No homology was found between the sequences of M.*Mbo*A and M.*Mbo*C, which enzymes recognized and methylated the same DNA sequence. The sequence of M.*Mbo*C had 49% homology with that of *Dpn*A (8) and 35% homology with that of M.*Hin*fI (26, 27). The best alignments of the sequences of M.*Mbo*A, M.*Dpn*II, and *E.coli* Dam methylase (group I) and of M.*Mbo*C, *Dpn*A, and M.*Hin*fI (group II) are shown in Figs. 3A and B. There were four and five highly homologous regions in the sequences of group I and II methylases, respectively (boxes a to i). The b and i regions probably contained the binding site of *S*-adenosylmethionine (28, 29). The d and e regions containing the DPPY motif might be involved in the methylation of exocyclic amino acids (30). We cannot explain why regions other than b, i, d, and e were conserved.

The M.*Mbo*A and M.*Mbo*C protein sequences were homologous with the T4 Dam methylase (31) and M.*Eco*RV (32) sequences or with the M.*Mbo*II (6) and M.*Hpa*I (33) sequences, respectively. The only characteristic shared by these methylases was that they are N^6-adenine methylases.

The R.*Mbo*I protein sequence had homology (38%) with the R.*Dpn*II sequence only (9). Five highly homologous regions (Fig. 3C, boxes with j to n) were found between the two protein sequences. R.*Mbo*I recognizes and cleaves the same DNA sequence as R.*Dpn*II. Therefore, the homologous regions were probably involved in the specificities of these enzymes.

ACKNOWLEDGEMENTS

We thank Mr. T.Tanigawa of Bioproducts Development Center, Takara Shuzo Co., Ltd., for N-terminal amino acid sequence analysis.

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