Cloning of the *MspI* Modification Enzyme

THE SITE OF MODIFICATION AND ITS EFFECTS ON CLEAVAGE BY *MspI* AND *HpaII*+

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The gene for the *MspI* modification enzyme from *Moraxella* was cloned in *Escherichia coli* using the plasmid vector pBR322. Selection of transformants carrying the gene was based on the resistance of the modified plasmid encoding the enzyme to cleavage by *MspI*. Both chromosomal and plasmid DNA were modified in the selected clones. None of the clones obtained produced the cognate restriction enzyme which suggests that in this system the genes for the restriction enzyme and methylase are not closely linked. Crude cell extracts prepared from the recombinant strains, but not the host (*E. coli* HB101), contain an S-adenosylmethionine-independent methyltransferase specific for the *MspI* recognition site, CCGG. Production of the enzyme is 3-4 fold greater in the transformants than in the original *Moraxella* strain. 5-Methylcytosine was identified as the product of the reaction chromatographically. The outer cytosine of the recognition sequence, *CCGG*, was shown to be the site of methylation by DNA-sequencing methods. This modification blocks cleavage by both *MspI* and its isoschizomer *HpaII*. *HpaII*, but not *MspI*, is able to cleave the unmethylated strand of a hemimethylated substrate. The relevance of these results to the use of *MspI* and *HpaII* to analyze patterns of methylation in genomic DNA is discussed.

DNA methylation occurs as a postsynthetic modification in both prokaryotic and eukaryotic cells (1). In bacteria (2, 3), and also in lower eukaryotes (4), two sites of methylation are observed: at the 6-amino group of adenine, and on cytosine at the 5-position of the pyrimidine ring. Both of these sites are at accessible positions within the major groove of the standard B form double helix. In higher eukaryotes, 5-methylcytosine is the only modified base found within the DNA. In mammalian DNA, 3-10% of the total cytosine residues are converted to 5-methylcytosine, and about 90% of the modified cytosine residues occur within the dinucleotide sequence, CpG (5, 6).

The biological role of DNA methylation is best understood for the restriction-modification systems which have been found in a wide variety of bacterial species (3). In this case, methylation at a specific residue within the recognition sequence serves to protect the DNA from cleavage by the cognate restriction enzyme. In *Chlamydomonas*, a lower eukaryote, the coordination of restriction and modification activities appears to be responsible for the maternal inheritance of chloroplast DNA (7, 8). There is not as yet evidence of a restriction-modification system in higher eukaryotes, although nucleases from several mammalian species which have some cleavage specificity have been reported (9).

There is growing evidence linking DNA methylation to the control of transcription in eukaryotes. In the β-globin gene locus in the chicken (19), rabbit (11), and man (12) certain sites within and around the globin genes have been found to be unmethylated in erythroid cells but either partially or fully methylated in nonerythroid tissues where the genes are inactive. Similar observations have been made with the ovalbumin gene (13), the mouse metallothionein-I gene (14), and several viral genes (15-17). The basis for the association between reduced methylation of DNA and gene activity is not known. However, studies showing that demethylation induced by 5-azacytidine may lead to the activation of specific genes (14, 18) as well as the initiation of entire developmental programs (19-21) suggest a causal relationship. In responsive systems, methylation may block the binding of specific transcription factors, or alternatively, affect chromatin structure so as to interfere with recognition by the transcription complex. The recent observation that methylation of cytosine in the alternating purine-pyrimidine sequence poly(dG-dC) greatly facilitates the transition from B to Z DNA may be of importance in this regard (22).

Studies of DNA methylation in eukaryotes have depended on the use of restriction enzymes sensitive to cytosine methylation within their recognition sequence. The isoschizomers *MspI* and *HpaII* have been most commonly used. These enzymes both recognize the tetranucleotide sequence CCGG and cleave the DNA between the 2 cytosine residues. If, however, the internal cytosine is methylated, cleavage by *HpaII* is blocked (23) whereas restriction with *MspI* is unaffected (24, 25). Methylation at this site occurs within the commonly modified dinucleotide CpG, and is the major site of modification within the *HpaII* sequence. A second pattern of modification has been observed less frequently, characterized by resistance to cleavage by both *MspI* and *HpaII* (26). The DNA modification responsible has not yet been established, but presumably methylation has occurred at either the external cytosine or at both cytosine residues within the recognition site.

In the present study, we have cloned the gene for the *MspI* modification enzyme from *Moraxella* in *Escherichia coli*. Production of the enzyme is 3-4 fold greater in recombinant strains which carry the gene on the plasmid vector pBR322 than in the original *Moraxella* species. None of the clones isolated synthesize the cognate restriction enzyme, suggesting...
that in this system the genes for the restriction enzyme and methylase are not closely linked. The outer cytosine of the CCGG recognition sequence was shown to be the site of methylation by DNA sequencing methods. Contrary to the expectation from previous studies (23, 28–30) this modification blocks cleavage by both MspI and HpaII. HpaII, but not MspI, is able to cleave the unmethylated strand of a hemi-methylated substrate.

MATERIALS AND METHODS

Enzymes and Radiolabeled Reagents—HpaII, MspI, and other restriction enzymes used were purchased from New England Biolabs or Bethesda Research Laboratories. One unit of restriction enzyme is defined as the amount required to completely digest 1 mg of λ DNA in 1 h at 37 °C. T4 DNA ligase was obtained from Boehringer Mannheim, polynucleotide kinase from P-L Biochemicals, and bacterial alkaline phosphatase from Bethesda Research Laboratories. [γ-32P]ATP (3000 Ci/mmol) was purchased from Amersham and S-adenosyl[methyl-3H]methionine (55 Ci/mmol) was purchased from New England Nuclear.

Restriction Digests with MspI and HpaII—All reactions with MspI and HpaII were carried out in the same buffer: 10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 6 mM β-mercaptoethanol, 6 mM MgCl2, and 100 μg/ml of autoclaved gelatin. Unless otherwise noted, the temperature was 37 °C. Reactions with plasmid DNA substrates were analyzed by electrophoresis on 0.85% agarose gels in 0.4 mM Tris-acetate (pH 7.9), 1 mM EDTA, and 1 μg/ml of ethidium bromide. DNA bands were visualized under a UV lamp. Smaller restriction fragments were analyzed on 12% polyacrylamide gels in 0.5 mM Tris-borate (pH 8.3) and 1 mM EDTA. Individual 32P-labeled fragments were located by autoradiography.

Transformation and Methylase Selection—Plasmid libraries were constructed from EcoRI and HindIII restriction fragments derived from Moraxella DNA as described previously (31). pBR322 was used as the cloning vector and E. coli HB101 as the host. Transformants were selected on the basis of ampicillin resistance. The population of plasmids representing each library was isolated by the CaCl2/ethidium bromide procedure (32). The recombinant plasmids from each library (500 μg) were digested with 100 units of MspI for 17 h at 37 °C. No remaining intact plasmid DNA was detected by agarose gel electrophoresis. The enzyme was then inactivated by heating at 65 °C for 30 min. CaCl2 was added to a final concentration of 100 mM, and the entire reaction mixture was then added directly to 3 × 1010 competent HB101 cells prepared as described by Wensink et al. (33). The transformation protocol followed the sequence described by Bolivar and Backman (34). Approximately 6000 individual ampicillin-resistant transformants were obtained. Small scale DNA isolations from recombinant clones were carried out essentially as described by Klein et al. (35). All experiments using recombinant DNA were carried out in accordance with the guidelines of the National Institutes of Health.

Preparation and Assay of Cell-free Extracts—Crude cell extracts were prepared by the method of Jacobson and Moraxella as described by Gunthert et al. (36) except that cells were disrupted by sonication (1 min pulses with a Bronwill Biosonic sonicator at setting 90). Final samples were dialyzed exhaustively against 200 mM Tris-Cl (pH 8.0) with 5% glycerol. The protein concentration was adjusted to 50 mg/ml (37), and the samples stored at 4 °C.

The assay for the MspI methylase was based on the transfer of [3H]methyl groups from S-adenosyl[methyl-3H]methionine to plasmid DNA substrates. The typical reaction mixture contained 5 μl of protein extract, 12 μg of DNA and 25 mM S-adenosyl[methyl-3H]methionine (55 Ci/mmol) in a total volume of 100 μl. The buffer was 200 mM Tris-Cl (pH 8.0) and 8 mM EDTA. After incubation for 1 h at 37 °C, the reaction was stopped by the addition of SDS to a final concentration of 0.3%, and the sample heated at 100 °C for 5 min. Following addition of 500 μg of sonicated salmon sperm DNA, the reaction mixture was extracted three times with phenol saturated with 200 mM Tris-Cl (pH 8.0) and the DNA precipitated by the addition of 5 volumes of ethanol. The precipitated DNA was redissolved in 1 ml of water, transferred quantitatively to a scintillation vial containing 10 ml of ScintiVerse (Fisher) and counted.

Cell-free extracts were assayed for the MspI endonuclease using plasmid substrates which, when cut with MspI, give one or more distinct fragments larger than 1 kilobase. The reaction conditions were as described above. Since we could not detect the restriction enzyme in crude cell extracts prepared from the recombinant strains, the samples were chromatographed on phosphocellulose under identical conditions used to isolate the enzyme from Moraxella. After loading the extract on a phosphocellulose column equilibrated with 10 mM phosphate, 1 mM EDTA, and 0.2 M NaCl at pH 7.0, the column was developed with a 0.2 to 0.8 M NaCl gradient. MspI isolated from Moraxella elutes between 0.4 and 0.5 M NaCl.

Identification of Methylated Bases—pBR322 was methylated in vitro with S-adenosyl[methyl-3H]methionine using a crude cell extract from a recombinant strain carrying the MspI modification enzyme. To the modified DNA (1 μg) was added 10 μg of each of the bases: adenosine, guanine, cytosine, uracil, and thymine, and 25 μg of the nucleoside 5-methylcytidine. The mixture was heated in a sealed tube containing 100 μl of 88% formic acid at 175 °C for 30 min. The hydrolysate was dried under vacuum and redissolved in 10 ml of formic acid. A portion of this solution (2 ml) was spotted on a cellulose thin layer plate (20 × 20 cm) (Kodak 1235A). The chromatogram was developed using the solvent systems (a) methanol:concentrated HCl:H2O (70:20:10, v/v) and (b) 1-butanol:methanol:H2O:ammonium hydroxide (60:20:20:1, v/v) in the first and second dimensions, respectively. Spots were visualized under a UV lamp, scraped off the plate, suspended in 5 ml of ScintiVerse for 2 h and then counted for radioactivity.

DNA Sequencing—A 57-base pair HaeIII fragment from pBR322 (residues 991–1048) and the corresponding fragment from the modified plasmid pMER3 was isolated by electrophoresis on a 10% polyacrylamide gel. The purified fragments (2.5 μg) were labeled at their 5'-termini with [γ-32P]ATP and polynucleotide kinase as described by Maxam and Gilbert (39). The labeled complementary strands were separated by alkali denaturation and electrophoresis on an 8% polyacrylamide gel run at 200 V for 4 h at 4 °C. The sample, dissolved in 100 μl of 0.8 N NaOH, 1 mM EDTA, and 10% glycerol, containing 0.1% xylene cyanol and 0.1% bromphenol blue, was boiled for 3 min immediately before application. DNA-sequencing reactions were carried out as in Ref. 39. The end-labeled DNA fragments were resolved on an 8% polyacrylamide gel in the presence of 7 M urea. Spots were visualized under a UV lamp, excised, and extracted. The purified DNA bands were visualized under a UV lamp, excised, and extracted. The extracted DNA fragments were purified by electrophoresis on a 12% polyacrylamide gel.

RESULTS

Cloning of the MspI Modification Enzyme and Characterization of Recombinant Plasmids—If a recombinant plasmid carries the gene for the modification enzyme and it is expressed within the host, then all of the MspI sites within the plasmid should become modified, rendering it resistant to cleavage by the MspI restriction endonuclease. This provided the basis for the selection scheme. Plasmid DNA was isolated from genomic libraries constructed in E. coli (HB101) from EcoRI and HindIII restriction fragments of Moraxella DNA ligated into pBR322. The mixed population of plasmids from each library (500 μg) was digested to completion with an excess of MspI and then used to retransform E. coli. Each

2 The abbreviations used are: SDS, sodium dodecyl sulfate; °C, 5-
methylcytosine.
digest gave approximately 6000 individual recombinant clones. Small scale DNA isolations from selected colonies showed that in over half of the isolates neither plasmid or chromosomal DNA could be digested by MspI, indicating that the gene for the modification enzyme had been cloned and was expressed.

pMER3 and pMEH7 (Fig. 1) are the smallest recombinant plasmids isolated from the EcoRI and HindIII genomic libraries, respectively, which encode the modification enzyme. The cloned insert in pMER3 is a single EcoRI fragment 6.3 kb in length (lane 5 in Fig. 2). All of the larger methylase recombinants isolated from the EcoRI library included this fragment. The insert in pMEH7 is 5.1 kb in length and has an internal HindIII site which divides the fragment into 3.5- and 1.6-kb segments (lane 6). The smaller of these is included entirely within the EcoRI fragment cloned in pMER3 (lane 7). Fig. 3 shows a partial restriction map of the two inserts indicating the region of overlap.

To determine if the methylase recombinants also produce the restriction enzyme, cell-free extracts were prepared (see "Materials and Methods") from the recombinant strains having the plasmids pMER3, pMEH7, and pMER7. The latter results from a partial digest of the genomic DNA and contains a 1.7-kb sequence flanking the insert cloned in pMER3. We were unable to detect the restriction enzyme in these crude samples or after chromatography on phosphocellulose under conditions used to purify the enzyme from Moraxella. Because low levels of the enzyme may have been difficult to detect against the background of nonspecific nucleases present, we attempted to demonstrate the restriction enzyme by biological criteria: resistance to infection by bacteriophage λ and reduced transformation efficiency with the compatible plasmid pACYC184 (40). However, none of the three strains showed these phenotypes. Finally, we screened the entire population of methylase recombinants isolated from the two genomic libraries (greater than 6000 individual clones) by infection with λ, in mass liquid culture (31). No resistant clones were isolated. Under the conditions used for selection, if a restriction positive strain had been present in the population even at a frequency as low as 1 per 1000 it should have been detected (31). These results indicate that the restriction enzyme either is not closely linked to the methylase or requires some other regulatory factor for its expression. The latter possibility seems unlikely since, in the other class II restriction-modification systems studied, no other genetic loci are required for expression of the restriction enzyme or methylase (31, 41-44).

**Identification of the MspI Methylase in Recombinant Strains**—Cell-free extracts prepared from the recombinant strains and Moraxella were assayed for methylase activity by the incorporation of tritium label into pBR322 and related DNA substrates from S-adenosyl-[3H-methyl]methionine (Table I). The mec and dam methylases present in E. coli (2) do not interfere with the assay since their recognition sites within the plasmid are already modified. The level of activity found in the recombinant strains (A) is 3-4-fold greater than in the native Moraxella species (G). The enzyme is not present in E. coli transformed with pBR322 (E). Two further observations associate this activity with the modification enzyme. If pMER3, the plasmid from the methylase strain, is used as the substrate, no incorporation of radioactivity occurs (B and H). In this case, all of the MspI sites within the plasmid have already been blocked by modification within the host cell. If pBR322 is first cut with MspI to destroy all of the MspI sites within the plasmid, it no longer serves as a substrate for the methylase (C). In a control experiment, cutting pBR322 with HhaI, for which there are a similar number of sites in the plasmid, did not significantly affect the level of incorporation (D). These results show that the methyltransferase identified in the recombinant strain is specific for the MspI-recognition sequence.

To prove that the modification enzyme catalyzes the conversion of cytosine to 5-methylcytosine, pBR322 was labeled...
**TABLE I**

Identification of the MspI methylase in recombinant strains

Cell extracts prepared from the bacterial strains were incubated with different plasmid DNA substrates and S-adenosyl-[methyl-3H] methionine, and the incorporated radioactivity was assayed. Strain R13 carries the recombinant plasmid pMER3 encoding the MspI methylase. Strain 609 carries the cloning vector pBR322. MspI is the native *Moraxella* species which produces the MspI restriction and modification enzymes. The endogenous restriction enzyme is inactivated by EDTA (8 mM) in the reaction mixture. In the control there is no cell extract present.

| Strain | Substrate | S-Adenosyl-[methyl-3H]methionine |
|--------|-----------|----------------------------------|
| A      | R13 pBR322| $7.8 \times 10^4$               |
| B      | R13 pMER3 | $4.5 \times 10^5$               |
| C      | R13 pBR322 (cut with MspI)| $1.1 \times 10^5$               |
| D      | R13 pBR322 (cut with HhaI)| $6.3 \times 10^5$               |
| E      | 609 pBR322| $4.1 \times 10^5$               |
| F      | 609 pMER3 | $4.7 \times 10^5$               |
| G      | Msp pBR322| $2.6 \times 10^5$               |
| H      | Msp pMER3 | $1.9 \times 10^5$               |
| I      | Control   | $3.4 \times 10^5$               |

**TABLE II**

Methylated bases from pBR322 labeled with the cloned MspI modification enzyme

pBR322 DNA was modified in vitro with S-adenosyl-[methyl-3H] methionine using the crude cell extract prepared from the methylase positive recombinant strain R13 (see Table I). The modified DNA, with each of the unlabeled bases added, was hydrolyzed in 88% formic acid at 175 °C and chromatographed in 2 dimensions as described under “Materials and Methods.” Resolved spots for each of the bases, located under a UV lamp, were scraped off the plate and counted for radioactivity. The control is a spot taken from the chromatogram from a position distinct from the bases. "Cyt refers to 5-methylcytosine.

| Base | Counts/min |
|------|------------|
| Ade  | 51         |
| Gua  | 68         |
| Cyt  | 62         |
| Ura  | 54         |
| "Cyt | 982        |
| Thy  | 190        |
| Control | 65        |

in vitro with S-adenosyl-[H-methyl]methionine using the cell-free extract from the recombinant strain described in Table I. The modified DNA was hydrolyzed in 88% formic acid to the purine and pyrimidine bases and analyzed by two-dimensional thin-layer cellulose chromatography. As shown in Table II, the incorporated radioactivity is associated with 5-methylcytosine and, to a much smaller extent, thymine. The latter is due to the low level (approximately 10%) of deamination of 5-methylcytosine which occurs in the hydrolysis reaction (45).

The Site of Methylation—In order to determine the site of modification by the MspI methylase, a 57-base pair *Hae*III restriction fragment containing a single MspI site was isolated from pBR322 (residues 991-1048) and the corresponding fragment obtained from the modified plasmid pMER3. After labeling the 5' ends with 32P, the complementary strands of each fragment were separated by alkali denaturation and electrophoresis. Fig. 4 shows the DNA sequencing patterns for one of the strands isolated from each fragment. The resistance of 5-methylcytosine to the limited hydrazine cleavage reaction used in DNA sequencing results in a gap in the sequencing ladder at the position of the modified cytosine residue (46). In the modified strand (B) the band due to the external cytosine residue at the MspI-recognition site, *CCGG*, is completely absent (arrow). The intensity of the band due to the internal cytosine is comparable to the other cytosine residues within the sequence, indicating that this position is not modified. The same result was found with the complementary strand and with a second *Hae*III fragment isolated from pBR322 including two MspI sites (data not shown).

**Methylation of the Outer Cytosine Blocks *HpaI*—*HpaI* is an isoschizomer of MspI but, in contrast, the cognate modification enzyme methylates the internal cytosine residue at the recognition sequence, *CCGG* (23). As shown in Fig. 5 using the modified plasmid pMER3 as substrate, methylation of the external cytosine also blocks *HpaI*. Both in the supercoiled and linear forms, the modified plasmid was not cleaved under conditions in which pBR322 was digested to completion. The slow rate of nicking of the modified plasmid evident with the supercoiled substrate, lanes 4 and 5, is most likely due to
contaminating nonspecific endonuclease activity in the commercial enzyme. There was also no evidence of specific cleavage of the modified plasmid at elevated temperatures between 40 and 65 °C (not shown). Under these conditions, HpaII remains catalytically active and gives the characteristic cleavage pattern with pBR322.

The Activity of MspI and HpaII toward Hemimethylated Substrates—Three heteroduplex molecules were constructed to study the activity of MspI and HpaII toward hemimethylated substrates modified at the outer cytosine (see "Materials and Methods").

**Fig. 5.** Methylation of the outer cytosine within the recognition sequence blocks HpaII. 1, pBR322; 2, pBR322 digested with HpaII; 3, pMER3; 4, pMER3 digested with HpaII; 5, pMER3 and pBR322 digested with HpaII; 6, pMER3 linear form cut with PsI; 7, pMER3 linear digested with HpaII; 8, pMER3 linear and pBR322 digested with HpaII. In each reaction, 0.5 µg of DNA was digested with 4 units of HpaII in 25 µl for 30 min at 37 °C. These conditions represent approximately a 4-fold over-digestion of pBR322. To exclude the possibility of enzyme inhibition, pBR322 was included in samples 5 and 8. The same results were obtained with HpaII purchased from either Bethesda Research Laboratories or New England Biolabs. At higher concentrations of enzyme there was no evidence of cleavage of the linear plasmid. There was, however, progressively more nicking of the supercoiled substrate (lanes 4 and 5) probably due to contamination by nonspecific endonucleases.

**Fig. 6.** The reactivity of MspI and HpaII toward hemimethylated substrates. The preparation of the three heteroduplex derivatives studied is described in detail in the text. Substrate 1 is unmethylated; substrates 2 and 3 are hemimethylated, modified on the labeled and unlabeled strands, respectively. The DNA fragments were incubated with 4 units of the restriction enzyme for 1 h at 37 °C. The restriction digests were analyzed by electrophoresis on a 12% polyacrylamide gel and an autoradiogram obtained. Lane 1, fragment 1 uncut; lane 2, fragment 1 cut with HpaII; lane 3, fragment 2 cut with HpaII; lane 4, fragment 3 cut with HpaII; lane 5, fragment 1 cut with MspI; lane 6, fragment 2 cut with MspI; lane 7, fragment 3 cut with MspI.

**Fig. 7.** Analysis of the cleavage of the hemimethylated substrates by electrophoresis under denaturing conditions. The samples in Fig. 6 were denatured by boiling in 0.05 M NaOH and electrophoresed in the presence of 7 M urea in parallel with the four standard sequencing lanes shown at the left. The labeled strand A alone is in lane 0. The arrow marks the position of the band due to cleavage of the labeled strand within the recognition sequence. The presence of the band at this position in lane 7 excludes the possibility that the reaction observed with HpaII is due to contamination with the unmethylated B strand. Even with much greater exposure of the gel there was

The labeled strand (A) is the same as in the sequencing experiments shown in Fig. 4. Neither HpaII or MspI were found to cleave the hemimethylated substrates 2 and 3 under conditions in which the unmodified fragment 1 was completely digested (Fig. 6). Since the gel was run under native conditions only double stranded breaks would have been detected. Fig. 7 shows the analysis of the same samples by electrophoresis under denaturing conditions in parallel with the four standard sequencing lanes. In this case, the labeled bands are due only to the A strand. With the unmodified fragment, there is a band at the expected position (arrow) due to cleavage within the recognition site by both HpaII and MspI (lanes 2 and 5). There is also a weaker band at this position in lane 4, indicating that HpaII is able to cleave, although at a substantially slower rate, the unmethylated strand of the hemimethylated substrate 3. MspI did not cut this substrate (lane 7). This result excludes the possibility that the reaction observed with HpaII is due to contamination with the unmethylated B strand. Even with much greater exposure of the gel there was
no detectable cleavage of the methylated strand in fragment 2 with either HpaII or MspI (lanes 3 and 6).

**DISCUSSION**

The selection scheme which we have used to clone the MspI methylase appears to have been first suggested by Mann et al. (41) and has been used previously to clone the BspI methylase (47), and the deoxyadenosine methylase from E. coli (recognizing the sequence GATC). The facility of this approach is increased the greater the number of restriction sites for the enzyme within the cloning vector. In practice, this generally limits the method to methylases having a four-base pair recognition sequence. In the case of MspI, there are 26 restriction sites within pBR322, five of which lie within the ampicillin gene which was used as the selective marker. Efforts to clone the SmaI methylase (recognizing the sequence CCCGGG) using the plasmid vector pBR313, which has only one site for the restriction enzyme, have been unsuccessful, there being a very high background level of non-methylase recombinants. It is also important that the modification of the host DNA not be detrimental to the cell. Methylation at a specific sequence within the promoter of an essential gene, for example, may act as a lethal mutation.

None of the MspI methylase recombinants isolated produce the homologous restriction enzyme. Three specific clones were examined directly for enzyme activity, and the entire pool of methylase recombinants was screened on the basis of resistance to infection by λ. Since partial digests of the genomic DNA were included in the original construction of the plasmid libraries it is very unlikely that the restriction enzyme is located at the boundary of a neighboring restriction fragment. Unless a more complex regulatory mechanism is required for expression of the restriction enzyme, it should be possible to clone the MspI endonuclease utilizing a methylase positive strain as the host. It may simplify the cloning of the restriction enzyme if the methylase gene is first integrated within the host chromosome as a component of a λ lysogen.

The finding that methylation of the central cytosine residue within the recognition site does not prevent cleavage by MspI (24, 25) first suggested that the modification enzyme methylates the outer cytosine. Subsequently, indirect evidence has been presented that methylation of the outer cytosine does block cleavage by MspI (28-30). These same studies, however, concluded that this modification does not affect the reactivity toward HpaII. This interpretation appeared to be consistent with earlier studies of Mann and Smith (23) in which the effects of different patterns of methylation on cleavage by HpaII were investigated using a synthetic oligonucleotide substrate (see below). The observation that Moraxella DNA could not be cleaved with either MspI or HpaII (30, 49) thus required that either the modification enzyme methylates both cytosine residues or that a second methyletransferase exists specific for the internal cytosine. Similarly the identification of two CCGG sites at homologous positions 5' to the human Aγ and Gγ-globin genes not cut by either HpaII or MspI raised the possibility that both cytosine residues were modified (29).2

Having cloned the gene for the MspI methylase greatly facilitated our analysis of the specificity of the enzyme. As in other restriction-modification systems characterized previously, the modification enzyme is an S-adenosylmethionine-dependent methyltransferase and yields 5-methycytosine as the product of the reaction. Using DNA sequence methods, we have proven that only the external cytosine residue at the recognition site is modified (Fig. 4). Contrary to the expectation from the previous studies cited above, this modification also blocks HpaII (Fig. 5). The resistance of Moraxella DNA to cleavage by both MspI and HpaII, therefore, may be explained by methylation of the external cytosine alone. A second DNA methyltransferase may be present, but we could not detect this activity in crude extracts from Moraxella using the modified plasmid pMER3 as substrate (Table I, H).

In the work described by Mann and Smith (23) in which it was reported that methylation of the external cytosine does not block HpaII, only a hemimethylated substrate was studied and the electrophoretic analysis of the products was carried out under denaturing conditions. The results obtained could be explained either if double-stranded cleavage occurred or if only one of the strands within the recognition site were cut. To determine the possible activities of MspI and HpaII toward hemimethylated substrates, heteroduplex molecules were constructed from the complementary strands of corresponding fragments from pBR322 and the modified plasmid pMER3. As shown in Fig. 6, neither HpaII or MspI are able to effect double-strand cleavage at a hemimethylated site. However, analysis of the reaction products on a denaturing gel showed that HpaII is able to cut the unmethylated strand of a hemimethylated substrate (Fig. 7), although at a rate much slower than cleavage of the unmodified fragment. As expected, MspI does not share this activity. Immediately following replication, newly synthesized DNA is hemimethylated and must also be protected from cleavage by the homologous restriction enzyme.

In these studies, the heteroduplex derivatives were isolated following rennaming by polyacrylamide gel electrophoresis. In one case in which there was an excess of the labeled strand, it was clearly resolved from the duplex species. Nonetheless, in control experiments we also examined the reactivity of MspI and HpaII toward the single-stranded fragments used in the construction of the heteroduplex substrates. Under our usual assay conditions there was no detectable cleavage with either enzyme. Yoo and Agarwal (50) have shown that MspI can cleave single-stranded molecules. However, this reaction is dependent on high concentrations of both enzyme and substrate and is optimal at lower temperature. It appears that under these conditions a duplex forms at the recognition site between two single-stranded fragments, which are self-complementary in this region, facilitated by approximation at the active site of the enzyme.

Although 5-methycytosine occurs in higher eukaryotes predominantly within the dinucleotide sequence CpG, it is also found in varying frequencies at other positions. In particular, the two pyrimidine isopairs "CC and "C"C have been identified in mouse DNA (51). Either of these modifications would account for specific CCGG sequences within genomic DNA that are resistant to both MspI and HpaII (29). Since the spectrum of DNA methyltransferases present in eukaryotic cells has not been defined it will be important to distinguish between these two possibilities. If only the external cytosine is modified, a cloned (unmethylated) single-stranded DNA fragment hybridized to the genomic DNA should be cleaved within the recognition site by HpaII.

Exogenous DNA fragments cloned within a plasmid carrying the MspI methylase gene will become fully modified within the host cell. These derivatives should prove useful in deter-
mining the effects of different patterns of methylation on DNA structure and on protein-nucleic acid interactions.

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