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Purification of *Mbo* II methylase (GAAG^{m}A) from *Moraxella bovis:* site specific cleavage of DNA at nine and ten base pair sequences

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ABSTRACT

The restriction modification methylase M.Mbo II has been purified using a sensitive oligonucleotide linker assay. The enzyme methylates the Mbo II recognition sequence GAAGA at adenine to produce GAAG^{m}A. M.Mbo II can be used in conjunction with the methylation dependent restriction endonuclease Dpn I (G^{m}ATC) to produce cleavage at the 10 base sequence GAAGATCTTC. When M.Mbo II is used in combination with M.Cla I (ATCGATCGAT), cleavage by Dpn I occurs at the four ten base sequences GAAGATCTTC, GAAGATCGAT, ATCGATCTTC and ATCGATCGAT, which is equivalent to a nine base recognition site. The use of combinations of adenine methylases and Dpn I to generate highly selective DNA cleavages at a variety of sequences up to fourteen base pairs is discussed.

INTRODUCTION

Site specific cleavage of DNA at eight and ten base pair sequences has been reported previously (1). This technique employs the sequence specific modification methylases M.Taq I (TCG^{m}A) and M.Cla I (ATCG^{m}AT) and a restriction enzyme, Dpn I, which only cuts the methylated sequence G^{m}ATC (2,3). Methylation of DNA by M.Taq I or M.Cla I enables selective cleavage of DNA by Dpn I at TCGATCGA and ATCGATCGAT, respectively, in DNA which is otherwise unmethylated (1). We report here the isolation of Mbo II methylase which methylates the Mbo II recognition sequence (4) at GAAG^{m}A and which can be used in conjunction with Dpn I to produce cleavage at the 10 base sequence GAAGATCTTC. Furthermore, when M.Mbo II is used in conjunction with M.Cla I, cleavage by Dpn I occurs at the four ten base sequences GAAGATCTTC, GAAGATCGAT, ATCGATCTTC and ATCGATCGAT. Thus M.Mbo II/M.Cla I sites occur as frequently as a nine base recognition site.

METHODS

Strains and plasmids

Moraxella bovis (ATCC 10900) was supplied by ATCC. E.coli strain DS1312 Sm^R dam-3 Pl^s, a derivative of LE392, was supplied by Douglas Smith. pPB13 was kindly provided by T. Poteete. Plasmid pTR13 (5) was supplied by M. Silberklang and plasmid pHR-deltaB (6) was supplied by D.A. Clayton.

Enzymes and chemicals

The methylases M.Cla I and M.Taq I were purified by a modification of the published procedure (7,8) and all restriction endonucleases including Dpn I were supplied by New England Biolabs. Methylation reactions for all enzymes were performed in 50 mM Tris HCl (pH 7.5), 10 mM EDTA, 100ug/ml BSA and 80 uM SAM. ^3H SAM 84Ci/mmol was obtained from Amersham and New England Nuclear.

Purification of M.Mbo II

Moraxella Bovis (ATCC 10900) was grown to mid-log phase at 37°C in Brain Heart Infusion (Difco) media supplemented with 2ug/ml NAD and 10 ug/ml Hemin (11). 300g of cells were harvested by centrifugation at 10,000 rpm in a Sharpless rotor for one hour then dissolved in 1500 ml of SB (10mM KPO_4 pH6.5, 50mM KCl, 0.1 mM EDTA, 10mM 2-mercaptoethanol) and disrupted by sonication. The sonicated cells were centrifuged at 10,000 rpm for one hour and the supernatant loaded on a phosphocellulose P-11 column (Whatman) (5 cm x 10 cm) at 120 ml/hr, pre-equilibriated with SB. The column was run with a gradient of KCl from 0.05 to 0.75 M at pH 6.5. Three DNA methylase peaks and two restriction endonuclease peaks, Mbo I (GATC) and Mbo II (GAAGA) (4), were detected. Methylation was assayed at this step by incorporation of ^3H into lambda DNA or a ligated oligonucleotide $(GAAGATCTTC)_n$ (see below). M.Mbo II modification methylase co-eluted with Mbo II restriction endonuclease at about 0.4-0.5 M KCl. M.Mbo I eluted at about 0.3 M KCl and Mbo I eluted at about 0.2 M KCl. The two M.Mbo methylases were largely separated in this step. The fractions containing M.Mbo II were loaded onto a Hydroxyapatite column (2.5 cm x 5 cm) and eluted with a gradient of 10 to 400 mM PO_4 in 10mM 2-mercaptoethanol, 100mM KCl and 5% Glycerol. M.Mbo II eluted at approximately 250 mM PO_4. The active peaks were

dialysed against 2 liters of SB with 0.1 M KCl and loaded on Heparin Sepharose column (Pharmacia) (1 cm x 4 cm), which was eluted with a linear gradient from 0.1 to 0.8 M KCl in SB (pH 7.5) and 5% glycerol. M.Mbo II eluted at 0.4 M KCl. Active fractions were dialysed overnight in 20mM Tris-HCl, 10mM 2-mercaptoethanol 50 mM KCl pH 7.5 and loaded on a monoQ HPLC column (Pharmacia). The column was eluted with a 60 ml linear gradient from 50-950 mM KCl in the same buffer and 1 ml fractions were collected. M.Mbo II eluted as a sharp peak at exactly 0.2 M KCl in five separate purifications, whereas Mbo II restriction endonuclease eluted in the void volume.

Assay for methylases

Assay of column fractions employed 2 ul aliquots of enyme extract in a volume of 25 ul containing 2uCi of ^{3}H SAM (10-12 Ci/mMol), in 50mM Tris-Cl pH 7.5, 10mM EDTA, 100ug/ul BSA. Reactions contained 1 to 2 ug of lambda DNA or 100 ng of the oligonucleotide $(GAAGATCTTC)_n$, which had been previously ligated to produce TCA precipitable DNA. After one to two hours incubation at 37^{o}, counts incorporated into lambda DNA or oligomerized oligonucleotide were measured by 10% TCA precipitation onto Whatman 540 paper. After rinsing in isopropanol and subsequent drying, the samples were counted in Liquiflor scintillation fluid.

Plasmid preparation

Plasmid DNAs were prepared by standard procedures (9,10). End labelling was performed using AMV reverse transcriptase (Biosciences Inc.) and two appropriate alpha ^{32}P dNTP's (supplied by Amersham, 800Ci/mmol) using the vendors' recommended conditions.

RESULTS AND DISCUSSION

Purification of M.Mbo II

The methylase M.Mbo II which protects against the restriction endonuclease Mbo II (GAAGA) (4) was purified from Moraxella bovis using Phosphocellulose P-11, Hydroxylapatite and Heparin Sepharose column chromatography followed by monoQ HPLC chromatography. At each stage fractions containing methylase were detected by a method employing a polymerized oligonucleotide

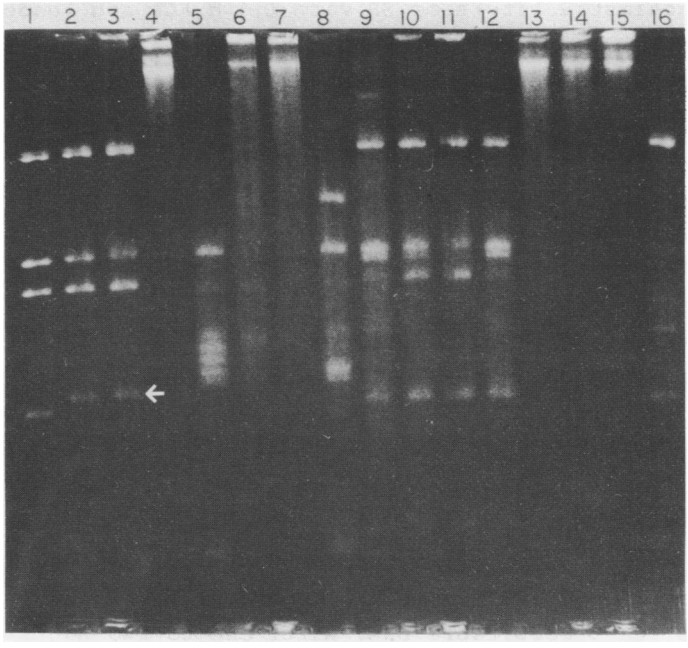

Figure 1:
1% agarose gel electrophoresis of pB13 dam- DNA. Each lane contains one ug of DNA. (1) Mbo I (2) 1ul M.Mbo II then Mbo I (3) 2ul M.Mbo II then Mbo I (4) 1ul dam then Mbo I (5) Mbo II (6) 1 ul M.Mbo II then Mbo II 7) 2 ul M.Mbo II then Mbo II (8) M.Mbo I then Mbo II (9) Sau 3A, (10) 1 ul M.Mbo II then Sau 3A (11) 2 ul M.Mbo II then Sau 3A (12) 1 ul dam then Sau 3A (13) Dpn I (14) 1 ul M.Mbo II then Dpn I (15) 2 ul M.Mbo II then Dpn I (16) 1 ul M.Mbo I then Dpn I

linker that contains the M.Mbo II recognition sequence. This oligonucleotide linker assay is very sensitive and specific for the desired methylase (see METHODS). Approximately 100 units of M.Mbo II were obtained per gram of Moraxella bovis cells. One unit of M.Mbo II protects one ug of lambda dam$^-$ DNA in one hour from a 10 fold excess of Mbo II restriction endonuclease. The methylase migrated as a single band on an SDS-polyacrylamide gel (data not shown).

Methylation specificity of M.Mbo II

To determine the methylation specificity of M.Mbo II advantage was taken of the fact that sequence-specific methylation can protect against heterologous restriction endonucleases at overlapping sites (7,12,13). For instance, it

was known that Dam sites (G^mATC) are protected from Mbo II digestion in the sequence $GAAG^mATC$ (12,13,14) and Figure 1, lanes 5 and 8, indicating that Mbo II is sensitive to adenine methylation. Conversely, M.Mbo II does not cross-protect against Sau 3A (GATC) at GAAGATC (Figure 1, lanes 9,10,11 and 12) or against Sac I at GAGCTCTTC (Nelson M. unpublished results). Since both Sau 3A and Sac I are sensitive to cytosine methylation but insensitive to adenine methylation (7,12,13,15) this evidence indicates that the purified M.Mbo II does not methylate at TmCTTC. However, Mbo I (GATC) sites are protected from digestion at the sequence GAAGATC (and GATCTTC) by methylation with our M.Mbo II preparation, but not at other GATC sequences (Figure 1, lanes 5 and 8). Since Mbo I is sensitive to adenine methylation but not cytosine methylation (7,12,15) this cross-protection experiment indicates that the M.Mbo II methylates at $GAAG^mA$. Furthermore, since only a subset of Mbo I sites are protected by our preparation it is clear that the purified M.Mbo II is largely free of Mbo I (G^mATC) methylase (Figure 1, lanes 1,2,3 and 4). The possibility that the preparation also methylates TCTTmC, G^mAAGA or GA^mAGA in addition to $GAAG^mA$ has not been excluded.

Since Moraxella Bovis contains a G^mATC specific methylase M.Mbo I (and at least two other DNA methylases (data not shown)) in addition to M.Mbo II ($GAAG^mA$) it was necessary to demonstrate that M.Mbo I did not contaminate the M.Mbo II preparation. M.Mbo I was assayed by overnight incubation of 20 units of the M.Mbo II preparation with one ug lambda dam^- DNA or pPB13 dam^- plasmid DNA which was then cut with a ten fold excess of Mbo I (GATC), Dpn I (G^mATC) or Mbo II (GAAGA) to determine the level of methylation at G^mATC and GAAGmA. Preparations were cut to completion by Mbo I except at GAAGATC sites but were not cut by Dpn I or Mbo II. M.Mbo I was not detectable in these assays nor was it detected when polymerized oligonucleotide $(CGGATCCG)_n$ was used as a substrate. It is estimated that there is less than 0.05 units of M.Mbo I per unit of M.Mbo II in our preparation.

Because the recognition sequence of Mbo II does not have dyad symmetry, the corresponding methylase(s) cannot protect both strands by methylating the same sequence in both strands. In contrast to all other type II modification methylases purified to

date, only M.Mbo II and M.Hph I (Feehery R and Nelson M, unpublished results) possess such asymmetry. This presents a problem since, during host replication, one daughter DNA molecule will be transiently unmethylated. This problem does not arise for palindromic methylases because both daughter molecules are hemimethylated after replication, which is sufficient to protect against the corresponding restriction endonuclease. In order to explain protection of host DNA against Mbo II during replication at least three possibilities can be proposed. First, two different M.Mbo II methylases may exist, one with 5' GAAG^{m}A 3' specificity and the other with 5' TCTTC 3' specificity. However, two different Mbo II specific methylases are unlikely because only one was found. Second, there may be two different specificities for Mbo II methylase, one of which methylates 5' GAAG^{m}A 3' and the other 5' TCTT^{m}C 3'. Note that 5'T^{m}CTTC 3' specific methylation has been excluded experimentaly, above, and we assume methylation does not occur at T. Finally, methylation may only occur on one strand in vivo. In the this case there would have to be a system for ensuring newly replicated DNA is not cut, either by sequestering the restriction endonuclease away from the DNA, or by allosteric regulation. A precedent for the final possibility occurs in the type III restriction systems (16,17), which only methylate one strand although the mode of protection for newly replicated DNA is not known yet.

Site specific cleavage by Dpn I after M.Mbo II methylation

As previously shown, Dpn I will only cut DNA which is methylated in both strands of the sequence G^{m}ATC (2,3). This sequence can be created in vitro using certain sequence-specific adenine methylases whose specificities overlap the Dpn I recognition sequence. For instance, M.Taq I (TCG^{m}A) methylation enables Dpn I to cleave at the eight base pair sequence TCGATCGA (1). Similarly, M.Mbo II has the specificity required to result, in principle, in new Dpn I cleavage sites at the inverted repeat

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5' G A A G^mA T C T T C 3'
3' C T T C TmA G A A G 5'
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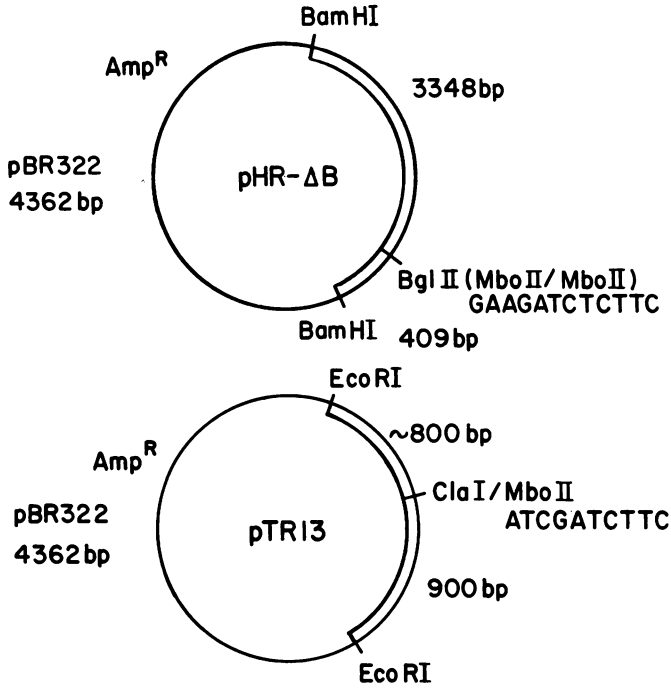

Figure 2:
Restriction maps of plasmids pHR-deltaB (6) and pTR13 (5)

Furthermore, as previously proposed (1), M.Mbo II can be used in conjunction with other methylases to produce cleavage by Dpn I at other rare sequences. For instance, the use of M.Mbo II and M.Cla I together should produce cleavage of DNA not only at the M.Mbo II/M.Mbo II inverted repeat GAAGATCTTC and the overlapping M.Cla I repeat ATCGATCGAT but also at hybrid sites containing both M.Mbo II and M.Cla I recognition sequences GAAGATCGAT and its compliment ATCGATCTTC. These four ten base recognition sequences give the M.Mbo II/M.Cla I/Dpn I site a frequency equivalent to a nine base recognition sequence. Such a sequence should occur on average once in 256,000 base pairs of random DNA sequence.

By searching the GENBANK (Bolt Beranek and Newman, Boston) database of published DNA sequences the sequences GAAGATCTTC and GAAGATCGAT were found to occur by chance in a number of available DNAs. Two pBR322 derivatives containing the sequence GAAGATCTTC (pHR-deltaB, a mouse mitochondrial DNA subclone) and ATCGATCTTC

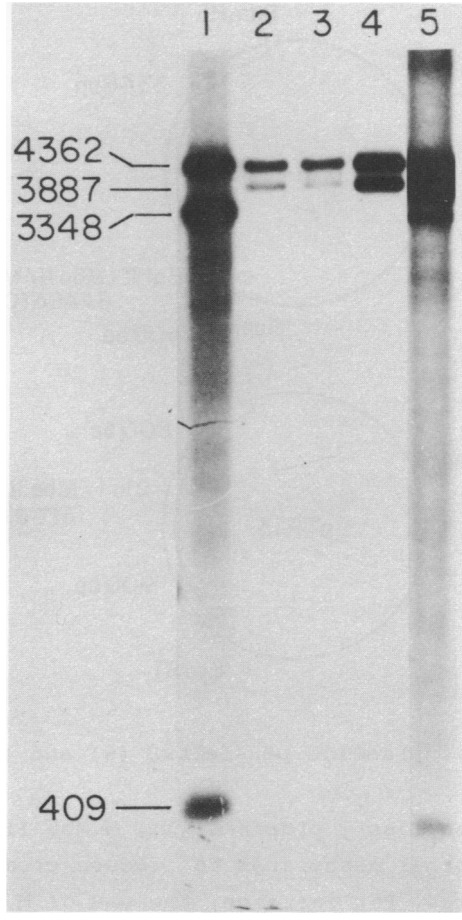

Figure 3:
End labelled digests of pHR-dB run on 1% agarose TBE. Lane (1) Bam HI, Bgl II (2) Bam HI (3) M.Mbo II then Bam HI + Bgl II (4) M.Mbo II then Bam HI (5) M.Mbo II then Bam HI + Dpn I.

(pTR13, a Drosophila tRNA gene) were transformed in to dam⁻ E.coli DS1312 and selected on ampicillin. Plasmid DNA from these cells was isolated and methylated in vitro (see methods). Restriction maps of these plasmids are given in Figure 2. Figure 3 shows sequence specific cleavage of the sequence GAAGATCTTC in pHR-AB by Dpn I after M.Mbo II methylation. Figure 4 shows Dpn I cleavage of GAAGATCGAT in pTR13 after methylation with both M.Mbo II and M.Cla I. Figure 4, lane 1 shows that M.Cla I methylation,

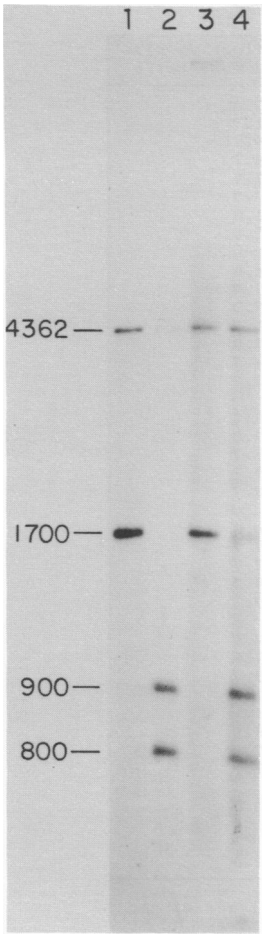

Figure 4:
End labelled digests of pTR13 run on 1% agarose TBE. Lane (1) M.Cla I then Eco RI + Dpn I (2) Eco RI + Cla I, (3) M.Mbo II + M.Cla I then Eco RI (4) M.Mbo II + M.Cla I then Eco RI + Dpn I.

alone, is insufficient for Dpn I cleavage at the sequence GAAGATCGAT.

In conclusion, a preparation of M.Mbo II (GAAG^{m}A) free of detectable M.Mbo I (G^{m}ATC) has been used to demonstrate sequence specific cleavage by Dpn I restriction endonuclease at the sequences GAAGATCTTC and, in conjunction with M.Cla I, at the sequence GAAGATCGAT. However, we have found that M.Mbo II/M.Mbo II sequence GAAGATCTTC is relatively slowly methylated compared to the single methylation site GAAGA (data not shown). A similar phenomenon occurs with the M.Taq I/M.Taq I sequence TCGATCGA (1). On the other hand, the hybrid M.Mbo II/M.Cla I sequence

Table 1 Methylase/Dpn I recognition sequences

Methylase(s)	Recognition Sequence#	Effective size@ in base pairs
M.Bsp M II/M.Bsp M II	TCCGGATCCGGA	12.0
M.Bsp M II/M.Eco B	TCCGGATCAN$_5$TGCT	11.7
M.Bsp M II/M.Cla I	TCCGGATCGAT	9.7
M.Bsp M II/M.Mbo II	TCCGGATCTTC	9.7
M.Bsp M II/M.Nru I	TCCGGATCGCGA	11.0
M.Bsp M II/M.Taq I	TCCGGATCGA	7.9
M.Bsp M II/M.Taq II	TCCGGATCGGTC	11.0
M.Bsp M II/M.Xba I	TCCGGATCTAGA	11.0
M.Eco B/M.Eco B	AGCAN$_5$TGATCAN$_5$TGCT	14.0
M.Eco B/M.Cla I	AGCAN$_5$TGATCGAT	9.9
M.Eco B/M.Mbo II	AGCAN$_5$TGATCTTC	9.9
M.Eco B/M.Nru I	AGCAN$_5$TGATCGCGA	11.7
M.Eco B/M.Taq I	AGCAN$_5$TGATCGA	7.9
M.Eco B/M.Taq II	AGCAN$_5$TGATCGGTC	11.7
M.Eco B/M.Xba I	AGCAN$_5$TGATCTAGA	11.7
*M.Cla I/M.Cla I	ATCGATCGAT	10.0
*M.Cla I/M.Mbo II	ATCGATCTTC	9.0
M.Cla I/M.Nru I	ATCGATCGCGA	9.7
M.Cla I/M.Taq II	ATCGATCGGTC	9.7
M.Cla I/M.Xba I	ATCGATCTAGA	9.7
*M.Mbo II/M.Mbo II	GAAGATCTTC	10.0
M.Mbo II/M.Nru I	GAAGATCGCGA	9.7
*M.Mbo II/M.Taq I	GAAGATCGA	7.7
M.Mbo II/M.Taq II	GAAGATCGGTC	9.7
M.Mbo II/M.Xba I	GAAGATCTAGA	9.7
M.Nru I/M.Nru I	TCGCGATCGCGA	12.0
M.Nru I/M.Taq I	TCGCGATCGA	7.9
M.Nru I/M.Taq II	TCGCGATCGGTC	9.7
M.Nru I/M.Xba I	TCGCGATCTAGA	11.0
*M.Taq I/M.Taq I	TCGATCGA	8.0
M.Taq I/M.Taq II	TCGATCGGTC	7.9
M.Taq I/M.Xba I	TCGATCTAGA	7.9
M.Taq II/M.Taq II	GACCGATCGGTC	12.0
M.Taq II/M.Xba I	GACCGATCTAGA	11.0
M.Xba I/M.Xba I	TCTAGATCTAGA	12.0

Available and potential cleavage systems using Dpn I and either one or two site specific methylases are presented. More complicated schemes employing more than two methylases can be envisioned but are not included here.

* These cutting systems have been demonstrated (this paper and (1)). The others are theoretical, based on known restriction recognition sequences. M.Hph I (GGTGA) has been excluded because it is now known to be a cytosine methylase (Feehery R. and Nelson M., unpublished results). Schemes using M.Eco B have not been tried but are likely to work as M.Eco B has the correct specificity (TGCTN$_8$TG^{m}A) (20). Both Nru I and Xba I do not cleave DNA methylated at adenine in their recognition sequences (13), a prerequisite if the corresponding methylase is to have adenine specificity. The Bsp M II recognition sequence is from R. Morgan and I. Schildkraut (unpublished results). The methylation sensitivity of this enzyme is not known. Isoschizomers of Dpn I include Cfu I, Nmu EI, Nmu DI and Nsu DI (Camp R., Hurlin P. and Schildkraut I., unpublished results). Isoschizomers of Mbo II include Nsu I. Isoschizomers of Nru I include Ama I and Sbo 13 (21).

Where there is more than one recognition sequence for Dpn I only one of the combinations is shown.

@ The effective size is the reciprocal of the fourth root of the sum of the cutting frequency per base pair. For example, for M.Cla I/M.Mbo II this is calculated from the frequency of GAAGATCTTC ($1/4^{10}$) + GAAGATCGAT ($1/4^{10}$) + ATCGATCTTC ($1/4^{10}$) + ATCGATCGAT ($1/4^{10}$) = ($1/4^9$). Effective size = 9 base pairs.

GAAGATCGAT is methylated at the normal rate by either M.Mbo II or M.Cla I.

A number of highly selective DNA cutting tools using sequence specific adenine methylation and Dpn I cleavage have been demonstrated. Others may be predicted based on the known and potential modification specificities of modification methylases. All the known and some of the potential cleavage systems derived from known restriction systems are listed in Table 1. These cleavage systems potentially generate fragments averaging from 50,000 to 125,000,000 base pairs. When combined with the ability to separate the large fragments generated by pulsed gel electrophoresis (19) these cutting strategies should be useful in generating physical maps of very large DNA molecules and in isolating large fragments from chromosomes.

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* For all sequences only one strand is shown, oriented 5' to 3'. ^{m}A represents 6-methyladenine.

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