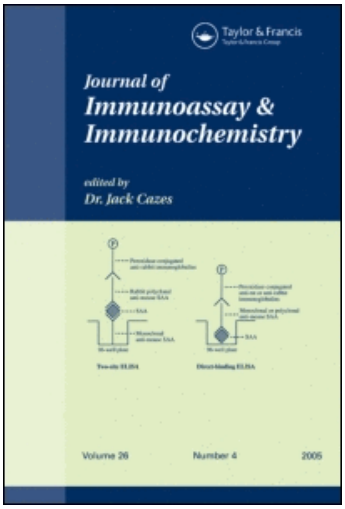

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Genotyping of DNA Using Sequence-Specific Methyltransferases Followed by Immunochemical Detection

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ABSTRACT

Modern molecular genetics relies on the ability to map the positions of genes on chromosomes, relative to known DNA markers. The first such DNA markers described were Restriction Fragment Length Polymorphisms, but any restriction endonuclease used for RFLP mapping is just one member of a restriction-modification pair. For each restriction endonuclease, there is a companion methyltransferase (MTase) that has the same DNA sequence specificity. Therefore, in principle, it should be possible to use MTases rather than

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restriction enzymes to detect polymorphic sites in DNA. We have used sequence-specific DNA MTases to detect genetic polymorphisms in closely related viral pathogens. If at least one MTase recognition site is present in PCR-amplified DNA, then methyl groups are incorporated; if no MTase site is present, then methyl groups are not incorporated. When several different sequence-specific DNA MTase reactions are carried out, the pattern of methyl incorporation defines a DNA MTase genotype. DNA MTase Genotyping (DMG) can be used to rapidly diagnose heritable or infectious diseases, to immunochemically detect DNA at defined 2 to 8 base pair sites, or to characterize the amplicons by constructing ordered maps.

Key Words: Methyltransferase; Polymorphisms; 6-methyl-A; 5-methyl-C antibodies.

INTRODUCTION

Gene mapping and DNA fingerprinting are normally carried out using restriction endonucleases.[1] Over 2000 different sequence-specific endonucleases have been characterized, of which about 200 are commercially available (http://rebase.neb.com). These restriction endonucleases have been widely used in RFLP mapping[2,3] and to detect mutations responsible for heritable human diseases.[4] However, methyltransferase (MTase) enzymes have been used sparingly in gene mapping experiments.[5] DNA MTases have been used neither to map genes nor to detect mutant alleles.

It is well-known that each restriction endonuclease has a companion sequence-specific DNA methyltransferase[6] and several hundred different DNA MTase specificities are known.[7,8] DNA methyltransferases (MTases) catalyze the transfer of methyl groups from *S*-adenosylmethionine (SAM) to specific sites in double-stranded DNA, yielding methylated DNA and *S*-adenosylhomocysteine (SAH).

$$\text{DNA} + \text{SAM} \xrightarrow{\text{DNA MTase}} \text{methyl-DNA} + \text{SAH}$$

In principle, there is no reason why MTases cannot be used to identify polymorphic sites in DNA. After all, any 4 to 8 b.p. DNA sequence which is cut by a restriction endonuclease is also recognized by its isospecific DNA MTase partner. Moreover, some sites not recognized by endonucleases can be methylated by sequence-specific MTases, especially those which have short (2 to 3 b.p.) specificities. For example, 75% of point mutations responsible for heritable human diseases occur at

hypermutable CG sites.[9] Several DNA MTases, but no known endonucleases, recognize the CG dinucleotide sequence.[10–12]

In this paper, we describe how sequence-specific DNA MTases can be used to characterize DNA of different sources and to generate ordered maps which allow to characterize amplicons. A new method for genetic fingerprinting of DNA, termed 'DNA MTase Genotyping' (DMG), is described. Four different DMG formats are employed:

(a) A radiometric ^{3}H-methyl incorporation assay;
(b) A non-radioactive immunochemical detection assay for in vivo methylated DNA;
(c) A combination of sequence-specific DNA methylation, restriction digestion, and radiometric detection of ^{3}H-methyl incorporation; and
(d) An immunochemical streptavidin-anchored PCR format in which biotinylated oligonucleotide primers and combinations of DNA MTases and endonucleases are employed to characterize amplicons by constructing ordered maps.

EXPERIMENTAL

Radiometric DNA MTase Genotyping of 191 b.p. pBluescript DNA

If radioactive ^{3}H-methyl-SAM is used as a substrate, then the number of methyl groups incorporated into DNA can be measured by trichloroacetic acid (TCA) precipitation of ^{3}H-methyl-DNA, followed by liquid scintillation counting.[13] T3 and T7 DNA sequencing primers (Stratagene, La Jolla, CA) were used to PCR amplify 10 ng of the polylinker region of pBluescript (SK+) plasmid. DNA was amplified with primers T3 and T7 using 30 cycles of (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C) in a 100 μL reaction volume containing 2.5 units of Taq Polymerase (Promega, Madison, WI) in (20 mM Tris–HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.25 μM of oligonucleotide primers). About 1 μg of PCR-amplified plasmid DNA was either undigested or else digested by EcoRI, BamHI, or PstI endonuclease (10 units, 2 h at 37°C). These DNAs were then phenol extracted, alcohol precipitated in 1.5 mL Eppendorf tubes, and resuspended in 30 μL of (50 mM Tris–HCl pH 7.5, 5 mM EDTA, 100 μg/mL BSA, ~2 μM ^{3}H-methyl-SAM (New England Nuclear, ~85 Ci/mmole). Five units of M.EcoRI or M.BamHI MTases

(New England Biolabs, Beverly, MA) were added and DNA methylation was carried out for 1 h at 37°C. The amount of ^{3}H-methyl-DNA was measured by TCA precipitation onto Whatman No. 1 filter paper, followed by liquid scintillation counting.[13]

Immunochemical Detection of ^{6m}A and ^{5m}C In Vitro Methylated DNA

DNA fragments which are methylated in vitro using sequence-specific MTases can be immuno-chemically detected after Southern blotting using rabbit anti-^{6m}A or rabbit anti-^{5m}C antisera. DNA was first enzymatically methylated at specific sites and then digested using restriction enzymes having different sequence specificities. After gel electrophoresis and Southern blotting to nylon filters, only those DNA fragments containing methyl groups whose positions were defined by in vitro methylation were immunochemically detected.

Immunochemical Detection of G^{6m}ATC-Methylated 536 b.p. Human β-Globin Amplicon

A 536 b.p. amplicon was obtained by PCR amplification from human placental DNA using primers BG536F: 5′GGTTGGCCAATCTACTCCCAGG-3′ and BG536R: 5′GCTCACTCAGTGTGGCAAAG-3′.[4] One microgram of human placental DNA (Sigma Chemical Co., St. Louis, MO) was submitted to 30 cycles of PCR amplification (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C) in a 100 μL reaction volume containing 2.5 units of Taq Polymerase (Promega, Madison, WI) in (20 mM Tris–HCl pH 8.4, 1.5 mM $MgCl_2$, 0.25 μM of primers). Ten microliters of the PCR product was treated with 5 U of M.dam MTase (New England Biolabs, G^{6m}ATC) in (50 mM Tris–HCl pH 7.5, 50 mM Potassium glutamate, 1 mM EDTA, 50 μg/mL BSA, 100 μM SAM). When methylated amplicons were submitted to endonuclease digestion, 5 U of the respective restriction endonuclease was incubated in 1X KGB buffer[14] for 30 min. The in vitro modified amplicons and digestion products were electrophoresed in 1.5% agarose in TBE buffer at 40 V for 2 h. The DNA was stained with ethidium bromide and photographed under UV light. Subsequently, the DNA was transferred to a nylon membrane (Hybond^{TM}, Amersham-Pharmacia Biotech, UK) by Southern blotting.[15] DNA was fixed to a nylon membrane for 75 s using 1,200 J/cm^2 UV irradiation (Stratalinker, Stratagene, La Jolla, CA) followed by photooxidation for 15 min using a

500 W lamp[16] in (0.1 M Tris–HCl pH 9.0, 60% formamide, 25 μg/mL methylene blue). The membrane-bound DNA was blocked in (100 mM Tris–HCl pH 8.0, 200 mM NaCl, 1 mM EDTA (TBS) 10% nonfat milk) overnight at 4°C. The membrane was then incubated for 1 h at room temperature in 10 mL of a 1/5000 dilution of a rabbit anti-^{6m}A antiserum (Megabase Research Products, Lincoln, NE) in the above blocking solution. Filters were washed 3 times with TBS-T (0.5% Tween 20) and then 3 times with TBS. A 1/5000 dilution of an alkaline-phosphatase conjugated goat anti-rabbit IgG (Vector Labs, Burlingame, CA) was used as a secondary detection antibody. After washing as described above, Fast Red substrate for alkaline-phosphatase (Pierce Chemical Company, Rockford, IL) was added. ^{6m}A-methylated DNA was detected as red bands on a white nylon filter background (Fig. 1).

Immunochemical Detection of In Vitro ^{5m}CG-Methylated λ Bacteriophage dcm-DNA

Five micrograms of λ bacteriophage *dam-dcm*-DNA (Fermentas, Hanover, MD) was specifically methylated at ^{5m}CG sites using 5 U of M.SssI MTase (New England Biolabs, Beverly, MA) in (50 mM Tris–HCl pH 7.5, 1 mM EDTA, 50 μg/mL BSA, 100 μM SAM) for 2 h at 37°C. The ^{5m}C-modified DNA was then digested for 1 h with 5 U of BamHI restriction endonuclease in 1X KGB buffer; or else left undigested. Five microgram of Chlorella virus NY-2A DNA (44.9% ^{5m}C; ref 17) was digested with 5 U of BamHI endonuclease as a positive control. DNA samples were electrophoresed in a 1.5% agarose gel at 20 V overnight. The gel was Southern blotted to a nylon membrane, UV-fixed to the membrane, and photooxidized as described above. Subsequent ^{5m}C immunochemical detection was carried out as described for the ^{6m}A-DNA, but a 1/1000 dilution of rabbit anti-^{5m}C antiserum (Megabase Research Products, Lincoln, NE) was used instead of the anti-^{6m}A antiserum; and BCIP/NBT substrate for alkaline phosphatase (KPL Laboratories, Gaithesburg, MD) was employed. Methylated DNA was detected as purple bands on a white nylon filter background (Fig. 2).

Generation of Ordered Maps from Short PCR Products Using Radiometric ^{3}H-Methyl Detection

Herpes Simplex Virus (HSV-1 or HSV-2) DNA (Sigma, St. Louis, MO) was amplified using a 5′ biotinylated forward primer

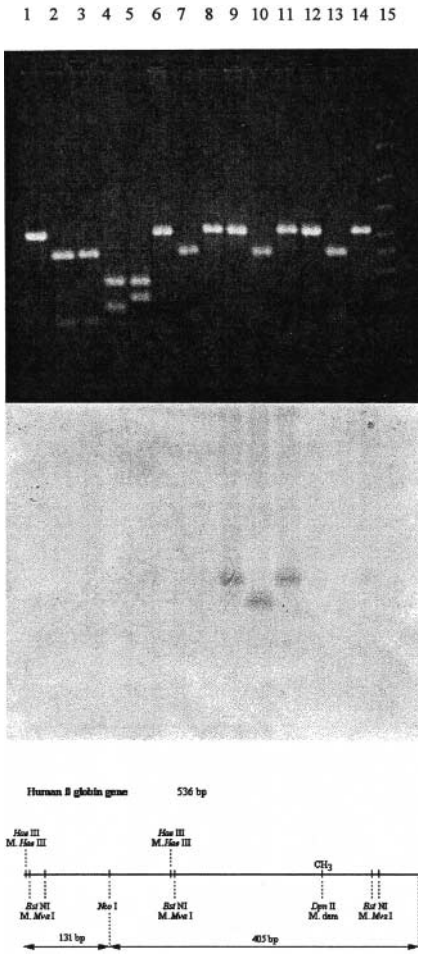

Figure 1. Immunochemical detection of in vitro $G^{6m}ATC$ methylated PCR amplicon from human β-globin gene after NcoI, DpnII, BstNI or HaeIII digestion and southern blotting to a nylon membrane. A. PCR products (536 b.p.) were digested with 5 U of endonucleases: NcoI (C↓CATGG, lanes 2, 7, 10, and 13), DpnII (↓GATC, lanes 3 and 11), BstNI (CC↓WGG, lanes 4 and 14), HaeIII (GG↓CC, lanes 5 and 8). Control no MTase (lanes 1–5). M·HaeIII MTase ($GG^{5m}CC$, lanes 6–8), M·dam MTase ($G^{6m}ATC$, lanes 9–11) M·MvaI MTase ($C^{4m}CWGG$, lanes 12–14). Unmethylated and uncut PCR product (lane 1), methylated (M·HaeIII) and uncut product (lane 6), methylated (M·dam) and uncut product (lane 9), methylated (M·MvaI) and uncut product (lane 12), molecular weight:50–2000 b.p. BioRad (lane 15). B. The ^{6m}A-methylated DNA was detected using an anti-^{6m}A rabbit antiserum as described in Experimental.

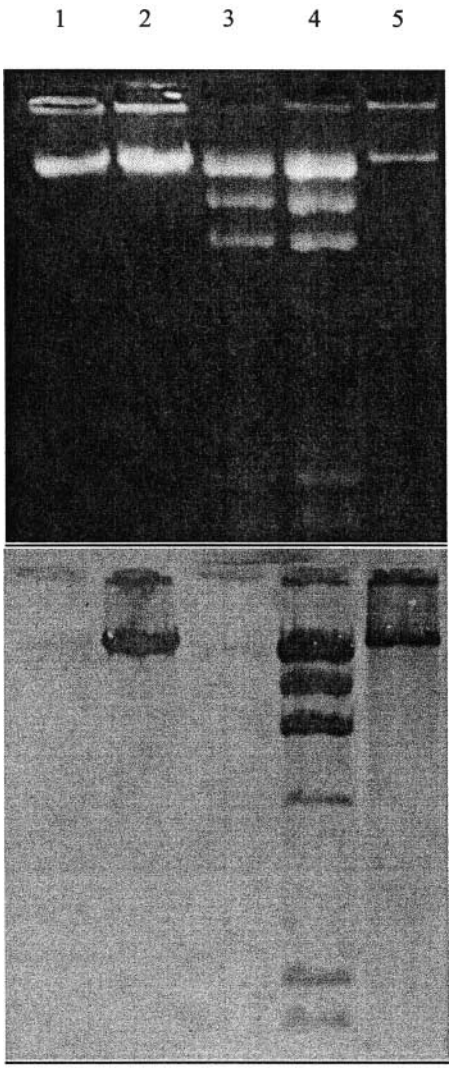

Figure 2. Immunochemical detection of ^{5m}C in DNA fragments immobilized to a nylon membrane after southern blotting using anti-^{5m}C antibodies. Negative control: unmethylated bacteriophage λ dcm-DNA (lanes 1 and 3). Positive control: in vivo ^{5m}C-methylated Chorella virus NY-2A DNA (44.9% ^{5m}C) lane 5. Positive control: in vitro ^{5m}CG-methylated (M·SssI) phage λ dcm-DNA (lanes 2 and 4). DNAs were digested with 10 U of HindIII endonuclease (A↓AGCTT, lanes 3 and 4). B. The ^{5m}C-methylated DNA was detected using anti-^{5m}C rabbit antiserum as described in Experimental.

(5′CCAAGCTGACGGCCATTACAA GGTCC-3′) and a reverse primer (5′AAGTGGCTCTGGCCTATGTCCCACACGC-3′). A 100 μL PCR reaction was carried out using 30 cycles of (5 s at 94°C, 5 s at 68°C, and 5 s at 72°C) with 2.5 units of Taq Polymerase (Promega, Madison, WI) in (20 mM Tris pH 8.4, 1.5 mM $MgCl_2$, 0.25 μM of oligonucleotide primers) in a PTC-150 MJ Research Thermocycler. Using this device, the PCR reaction was completed in 17 min. The resulting amplicons (97 b.p. long) were specifically ^{3}H-methylated (~2 μM ^{3}H-methyl-SAM (New England Nuclear, ~85Ci/mmoles) using 5 U of different sequence-specific methyltransferases in (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 μg/mL BSA). Subsequently, methylated and unmethylated HSV amplicons were digested for 30 min with 5 U of restriction endonucleases in 1X KGB buffer. In vitro modified and/or digested amplicons were added to streptavidin-coated Flash Plates™ (New England Nuclear, Boston, MA) in a volume of 200 μL and ^{3}H-methyl cpm were measured in a Packard Top Count™ scintillation counter (Packard Instruments, Meriden, CT). The advantage of the Scintillation Proximity Assay (SPA) detection method is that precursor ^{3}H-methyl-SAM need not be removed from the ^{3}H-methyl-DNA reaction product; i.e., SPA is a homogenous assay. The 5′ biotinylated DNA is simply enzymatically methylated in vitro, followed by capture on a streptavidin-coated scintillator plastic. Bound ^{3}H-methyl-DNA results in emission of light.

Generation of Ordered Maps from Short PCR Products Using a Streptavidin Capture ELISA

The generation of ordered maps by ELISA involves two major steps: the capture of biotinylated PCR products using streptavidin coated microtiter wells and the detection of ^{6m}A using an anti-^{6m}A monoclonal antibodies.

Immulon 4 HB strip assemblies (Dynex Technologies Inc., Chantilly, VA) were coated with Streptavidin (S4762, Sigma Chemicals, St. Louis, MO) by incubating each well with 50 μL of 10 μg/mL streptavidin in water. ELISA strips were left uncovered in a dry incubator at 37°C overnight to allow complete evaporation. Dried strips were stored in a sealed bag at 4°C for up to 3 months. Before use in ELISA, the wells were hydrated by washing with PBS (10 mM Sodium Phosphate, pH 7.5, 150 mM NaCl). After PCR, sequence specific methylation and digestion with restriction enzyme was performed.

Ten microliters of samples were added to wells along with 90 μL of PBS containing 0.5% blocking reagent (Boehringer Mannheim, Germany) and incubated at room temperature (RT) for 30 min. The wells were emptied and 100 μL of a mouse monoclonal anti-^{6m}A antibody (F. J. Hoffman, S. L. Mundo, and O. J. Lopez, Characterization of a monoclonal antibody specific to 6-methyl-Adenine, manuscript in preparation) diluted 1/5000 in blocking buffer was added to wells. After 20 min incubation at RT the wells were washed three times with PBS-T (1% Tween-20) and received 100 μL of goat-anti mouse IgG conjugated to horseradish peroxidase. After incubating for 20 min at RT, the wells were washed four times with PBS-T and once with PBS. The wells were developed with 100 μL of TMB substrate (Kirkegaard and Perry Laboratories, Gaithersberg, MD). The color reaction was stopped after 15 min by the addition of 100 μL of 0.18 M sulfuric acid per well. Absorbance was read on an Opsys MR ELISA plate reader (Dynex) at 450 nm. Controls included wells with buffer alone and PCR products, with and without biotin.

RESULTS

Radiometric DNA MTase Genotyping of pBluescript DNA

Radiometric ^{3}H-methyl DNA incorporation experiments were carried out to test the feasibility of DNA MTase genotyping, using pBluescript plasmid DNA as a test substrate in a simple model system. As seen in Table 1, the specificity of DNA MTase genotyping is the same as that of RFLP mapping: sites pre-cut by EcoRI or BamHI restriction endonucleases cannot be methylated by M.EcoRI or M.BamHI MTases, respectively. In addition, EcoRI digestion blocks subsequent M.BamHI methylation; there is a decrease from 69,719 to 1,306 ^{3}H-methyl cpm. In pBluescript DNA, EcoRI and M.BamHI sites are only 18 b.p. apart. Apparently, prior digestion using EcoRI produces DNA which is not a substrate for methylation by M.BamHI.

The sensitivity of the radiometric (^{3}H-methyl-DNA incorporation) assay is very high. Using M.EcoRI (GA^{6m}ATTC) or M.BamHI (GGAT^{4m}CC) MTases, sequence-specific incorporation of 70,000 to 85,000 cpm of ^{3}H-methyl groups into 1 μg of PCR-amplified 191 b.p. DNA was observed against a background of ~600 to 800 cpm, which corresponds to a signal-to-noise ratio of >1000 to 1. Less than 50 ng of PCR-amplified DNA could be detected.

Table 1. Demonstration of radiometric DNA MTase genotyping using pBluescript plasmid.

	^{3}H-methyl-c.p.m. in DNA digested by			
DNA MTase	—	EcoRI	BamHI	PstI
None	574	750	661	836
M·EcoRI (GA^{6m}ATTC)	85,043	770	32,830	27,674
M·BamHI (GGAT^{4m}CC)	69,719	1,306	999	817

BamHI PstI EcoRI

pBluescript 683 – ACTAGT**GGATCC**CCCCGGG**CTGCAGGAATTC**GATAT-717

The 191 b.p. polylinker region of plasmid pBluescript was PCR amplified and methylated using either M·EcoRI or M·BamHI MTase enzymes, either without prior endonuclease treatment or after digestion by EcoRI or BamHI restriction endonuclease. ^{3}H-methyl-DNA c.p.m. were measured by TCA precipitation followed by liquid scintillation counting.[13] EcoRI (GAATTC) and BamHI (GGATTC) sites in pBluescript are separated by 18 b.p. (DNA sequence of top strand of pBluescript is shown). The results are expressed as TCA precipitable ^{3}H-methyl counts per minute.

Immunochemical DNA MTase Genotyping of Human β-Globin Amplicon

In order to develop an alternative to radiometric ^{3}H-methyl detection, experiments were carried out to adapt DNA MTase genotyping to an immunochemical format. The specificity of a rabbit anti-^{6m}A and anti-^{5m}C polyclonal antiserum was tested using methylated or control unmethylated DNAs which had been immobilized on nylon membranes (data not shown). These preliminary experiments demonstrated that ^{6m}A- or ^{5m}C-methylated DNA could be immunoenzymatically detected, using rabbit anti-^{6m}A or anti-^{5m}C immune serum and alkaline-phosphatase conjugated goat anti-rabbit IgG.

Figure 1 shows DNA MTase genotyping of a 536 b.p. amplicon obtained from the human β-globin gene using immunochemical (anti-^{6m}A antibody) detection. Beta-globin DNA fragments containing a single M.dam (G^{6m}ATC) recognition site were bound to the membrane, UV fixed to the membrane and immunochemically detected using anti-^{6m}A antibody and goat anti-rabbit alkaline-phosphatase conjugate. DpnII cleavage (↓GATC) was specifically blocked by M.dam MTase (G^{6m}ATC), BstNI cleavage (CC↓WGG) was blocked by M.MvaI MTase (C^{4m}CWGG), and HaeIII cleavage (GG↓CC) was blocked by

M.HaeIII MTase (GG^{5m}CC), confirming that the DNA fragment was methylated in a sequence-specific fashion.

The undigested (536 b.p.) amplicon and large (405 b.p.) NcoI (C↓CATGG) restriction fragments containing the M.dam (G^{6m}ATC) site were selectively immunostained in this ''Southwestern'' blot format. Detection was specific for ^{6m}A-modified DNA, since DNA fragments, which were either unmethylated, ^{4m}C-methylated using M.MvaI (C^{4m}CWGG), or ^{5m}C-methylated using M.HaeIII (GG^{5m}CC) were not detected using the anti-^{6m}A antiserum.

As shown in Fig. 2, anti-^{5m}C antibodies can also be used to immunochemically detect DNA which has been methylated in vitro using sequence-specific MTases. A ''Southwestern'' blot of bacteriophage λ *dam-dcm*-DNA methylated in vitro with M.SssI MTase (^{5m}CG), and developed with the rabbit anti-^{5m}C antiserum, followed by immunostaining using alkaline phosphatase-anti-rabbit IgG conjugate, demonstrates that DNA fragments with ^{5m}C at specific internal ^{5m}CG sites could also be immunochemically detected. Thus, it was possible to detect both ^{6m}A and ^{5m}C in DNA immunoenzymatically.

Generation of Physical Maps of PCR-Amplified DNA

The relative positions of restriction (*r*) and modification (*m*) sites in PCR-amplified DNA can be determined in reference to a fixed 5′ biotinylated end, which is captured on a streptavidin-coated matrix. Physical maps may be constructed by amplification of DNA using a 5′ biotinylated oligonucleotide primer, followed by sequence-specific DNA methylation and/or cleavage. The 5′ biotinylated amplicon is bound to a plastic plate coated with streptavidin prior to radiometric detection. If the bound amplicon contains a ^{3}H-methyl group, then a positive signal is achieved. If the bound amplicon has no MTase sites, then no ^{3}H-methyl groups are detected. Furthermore, if one or more restriction endonuclease recognition sites is located between the 5′ biotin group and the methylation site, then the signal is eliminated. In this fashion, ordered maps of *r–m* sites relative to the 5′ end can be determined without the need for gel electrophoresis.

Table 2 shows results from an experiment in which amplicons from two strains of Herpes Simplex Viruses (HSV-1 and HSV-2) were methylated with ^{3}H-methyl-SAM and several different sequence-specific MTases. The relative location of the recognition sites for DNA restriction and modification was determined by anchoring amplicons at one end via a 5′ biotin group, which was captured on a streptavidin-coated matrix.

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Table 2. Ordered mapping of HSV-1 and HSV-2 using radiometric DMG.

			HSV-1		HSV-2	
Biotinylated primer	MTase	RE	c.p.m.	bc	c.p.m.	bc
Reverse	—	—	66	0	36	0
Reverse	M·TaqI	—	57	0	1457	1
Reverse	M·MvaI	—	1916	1	29	0
Forward	M·TaqI	RsaI	58	0	1516	1
Reverse	M·TaqI	RsaI	48	0	55	0
Reverse	M·TaqI	MspI	59	0	67	0
Forward	M·MvaI	RsaI	1923	1	30	0
Reverse	M·MvaI	RsaI	234	0	142	0
Reverse	M·MvaI	MspI	145	0	258	0

M.MvaI RsaI

HSV-1 CCAAGCTGACGGACATTTACAAGGTCCCCTGGACGGGTACGGCCGCATG

HSV-2 CCAAGCTGACGGAGATTTACAAGGTCCGCTCGACGGGTACGCGCGCATG

M.TaqI RsaI

MspI

HSV-1 AACGGCCGGGGCGTGTTTCGCGTGTGGGACATAGGCCAGAGCCACTTCCAGAA

HSV – 2 AACGGCCGGGGTGTGTTCCGCGTGTGGGACATCGGCCAGAGCCACTTGGTCGC

MspI

One microgram of a 97 b.p. amplicon obtained by PCR amplification of HSV-1 and HSV-2 DNAs using a biotinylated forward or reverse primer was methylated using M·TaqI (TCGA) or M·MvaI ($C^{4m}CWGG$) in presence of ^{3}H-methyl-SAM. Subsequently, the DNA was digested with RsaI (GT↓AC) or MspI (C↓CGG) restriction endonucleases. The 5′biotinylated ^{3}H-methyl DNA was added to streptavidin-coated Flash-Plates® and counted in a Packard TopCount™ scintillation counter. The results are the average of three measurements. *Key*: Forward and reverse primer are underlined. RE: restriction endonuclease. MTase and RE recognition sites are bolded. bc: binary code (key: $0 < 260$ c.p.m.; $1 > 260$ c.p.m.).

The presence or absence of ^{3}H-methyl groups could be rapidly determined using Scintillation Proximity Assay[18] on streptavidin-coated plastic plates (Flash-Plate®; New England Nuclear, Boston, MA).

Combinations of DNA methylases and restriction endonucleases were used to distinguish these two Herpesvirus strains, as well as to build ordered maps of *r–m* sites in PCR-amplified HSV-1 and HSV-2 DNAs. For example, there is an M·TaqI (TCG^{6m}A) site in the HSV-II DNA amplicon which is not present in the homologous HSV-1 DNA amplicon. Thus, M·TaqI MTase methylates HSV-2, but not HSV-1 DNA amplicons. Furthermore, since we are using a 5′ biotinylated reverse primer and the M·TaqI site in HSV-2 is located 5′ to a unique RsaI (GT↓AC) cleavage site, digestion by RsaI endonuclease eliminates the ^{3}H-methyl signal from HSV-2 amplicons. On the other hand, when the forward primer was 5′ biotinylated, the signal was not eliminated by RsaI digestion. The relative positions of [5′ biotin anchor—RsaI—M·TaqI] sites was thus determined without the need for gel electrophoresis.

Generation of Ordered Maps from Short PCR Products Using a Streptavidin Capture ELISA

The radiometric ordered mapping can be adapted to an ELISA format. DNA is amplified by PCR with one primer biotinylated. The PCR product is then methylated. The methylated amplicon is digested with different restriction enzymes. Then, the amplicon is added to an avidin-coated ELISA plate. The methylated DNA is detected using anti-^{6m}A antibodies. Table 3 shows that the HSV-2 amplicon can be detected using a monoclonal antibody against ^{6m}A. One hundred ng of DNA specifically ^{6m}A-methylated at one site can be detected. Furthermore, this signal can be eliminated by digesting with restriction enzymes RsaI and MspI, whose recognition sites are located between the recognition site for the methyltransferase and the 5′ biotinylated primer used for PCR amplification. Thus, characterization of amplicons by ordered mapping can be achieved in an ELISA format in less than 2 h.

DISCUSSION

DNA MTase genotyping (DMG) is a new method for DNA fingerprinting that offers certain technical advantages over existing methods. In particular, DMG has the virtues of speed, sensitivity, and high

Table 3. Characterization of HSV-2 amplicon by ordered mapping by ELISA-DMG.

Biotinylated primer	M.TaqI	Restriction endonuclease	OD at 450 nm
Reverse	−	—	0.03 ± 0.01
Reverse	+	—	1.32 ± 0.12
Reverse	+	MspI	0.12 ± 0.04
Reverse	+	RsaI	0.23 ± 0.08
Forward	+	—	1.11 ± 0.11
Forward	+	MspI	1.23 ± 0.11
Forward	+	RsaI	1.45 ± 0.08

The HSV-2 amplicon was methylated in vitro using 5 units of M.TaqI ($G^{6m}ATC$) methylase for 30 min at 37°C. After methylation, 20 μL of the amplicon was digested with 10 units of MspI (CCGG) or RsaI (GTAC) at 37°C for 30 min. After sequence specific-methylation and endonuclease digestion, 10 μL of methylated, unmethylated or digested PCR products were immunostained for ^{6m}A in an ELISA format using a anti-6 Methyl Adenine monoclonal antibody. The results are the average of three measurements.

specificity. The availability of anti-6 methyl adenine and anti-5 methyl cytosine antibodies allows for the coupling of the PCR-DMG to immunochemistry detection. MTases allow sequence-specific internal labeling of DNA at 2 to 8 b.p. sites and can be used alone or in combination with restriction endonucleases to genotype DNA. Unlike standard electrophoretic restriction endonuclease or RFLP mapping, ordered maps of restriction-modification (*r–m*) can be constructed without the need for gel electrophoresis. Therefore, DNA MTase genotyping can be easily automated. The entire DNA MTase genotyping procedure can be carried out in less than 1 h in the radiometric format (Table 2) when PCR-amplified DNA is anchored to a solid support using 5′-biotinylated primers and streptavidin-coated polystyrene plates.[19] In the ELISA format, the results are obtained in less than 2 h (Table 3). In principle, ordered maps of PCR-amplified DNA can be constructed in an automated ELISA format with the appropriate liquid handling devices.

Due to its high (>1000:1) signal-to-noise performance, DNA MTase genotyping is a very accurate genetic fingerprinting method when either immunochemical or radiometric detection is employed. The sensitivity of DMG is sufficient to distinguish closely related viral pathogens (Table 2) and at least 100 ng of specifically methylated DNA at a unique site by ELISA.

The specificity of a DNA MTase is, at a first aproximation, the same as that of its companion restriction endonuclease.[8] Therefore, any mutation or polymorphism which can be detected as an RFLP marker can also be detected as a well-defined DNA MTase polymorphisms (Table 2).

In contrast to other enzymatic DNA labeling protocols (i.e., 5′ or 3′ end-labeling or uniform labeling using DNA polymerases), when sequence-specific MTases are employed, DNA is internally labelled at defined 2 to 8 b.p. sites. For example, if DNA is first methylated in vitro using M·EcoRI MTase followed by digestion using BamHI endonuclease, then only those BamHI restriction fragments containing methylated GA^{6m}ATTC sites are detected by Southern blotting and immunochemical detection using anti-^{6m}A antibody.

More generally, it is possible to use sequence-specific DNA MTases and antibodies to methylated nucleosides to specifically detect DNA at a variety of defined 2 to 8 b.p. sites. Rabbit antibodies to ^{6m}A and ^{5m}C have been used to detect DNA which has been methylated at in vitro methylated G^{6m}ATC and ^{5m}CG sites (Figs. 1 and 2). In principle, it is possible to immunochemically detect almost any 2 to 8 DNA MTase recognition site for which a ^{6m}A- or ^{5m}C-specific DNA MTase is known.

The immunochemical methods for detecting modified nucleosides can also be applied to in vivo modified DNAs (Fig. 2, lane 5). For example preliminary results have shown detectable levels of ^{6m}A in *Zea mays* B73 cultivar DNA using anti-^{6m}A antibody (unpublished results).

In practice, we have found that anti-^{6m}A antisera has a high titer (>1:10,000) and can detect a single methyl group pair in 2,000 b.p. PCR-amplified DNA (Fig. 1 and unpublished results). Results with anti-^{5m}C antisera are encouraging (Fig. 2). However, the titer of rabbit anti-^{5m}C antisera is ten fold lower than that of anti-^{6m}A (around 1:1000) and the detection limit is aproximately one ^{5m}C methyl group pair in 500 b.p. of PCR-amplified DNA. These results are consistent with data of Kong et al.[20] who also prepared anti-^{4m}C antibodies. Immunochemical detection of ^{6m}A in DNA is technically much easier and more sensitive than ^{5m}C or ^{4m}C immunodetection. The use of a monoclonal antibody anti-^{6m}A allows to increase sensitivity, 100 ng of 97 b.p. long amplicon methylated at a single site can be detected (Table 3). Furthermore, the amplicon can be thoroughly characterized by ordered mapping. The whole procedure takes less than 2 h (17 min of PCR plus 90 min of ELISA).

DNA MTases can be used alone or in combination with restriction endonucleases to genotype DNA. The data in Table 2 can be arranged in a 3×3 array: (no MTase, M·TaqI, M·MvaI) × (no restriction enzyme, RsaI, MspI). In general, for m different MTases and r restriction enzymes an $(m+1) \times (r+1)$ array is used, including null controls, to generate

ordered maps of *r–m* sites. Such gel free *r–m* mapping in ordered arrays lends itself to automation. Since there are over 200 different *r–m* enzymes, there are at least $200^2 = 40{,}000$ two-way combinations of *r–m* enzyme pairs which might be used to diagnose diseases or map genes. In general, for *m* different MTases and *r* restriction endonucleases, there are ($r \times$ m) enzyme combinations and $[(r+1) \times (m+1)]$ different reactions which can be employed for DNA diagnostic applications (Table 2, Table 3). In principle, very large $(r+1) \times (m+1)$ arrays can be carried out in a 8×12 ELISA or miniature "DNA chip" format.

In certain cases (Table 1) the close proximity of a DNA MTase recognition sequence to a nearby restriction site may produce anomalous results. For example, prior cleavage of pBluescript DNA by EcoRI nearly completely abolishes subsequent methylation at the nearby M.BamHI site (18 b.p. away). However, prior cleavage by BamHI does not substantially inhibit methylation by M. EcoRI. This result suggest that the M.BamHI MTase footprint on its substrate is >18 b.p. long, whereas the DNA footprint of M.EcoRI is <18 b.p. long.

The presence or absence of a DNA MTase recognition site can be determined without the need for gel electrophoresis; and relatively short (70–100 b.p.) DNA amplicons can be genotyped. Rapid (< 2 h) ordered maps of PCR-amplified DNA can be constructed in an automated ELISA format with the appropriate liquid handling devices.

In summary, DNA MTase genotyping (DMG) is a rapid, sensitive method for detecting polymorphisms, distinguishing closely related pathogens, detecting DNA at specific sites, and generating ordered maps without the need for gel electrophoresis. DNA which has been methylated in vitro using sequence-specific MTases can be detected using either radiometric (^{3}H-methyl) or immunochemical methods (using anti-^{6m}A or anti-^{5m}C antibodies). Immunochemical detection of methylated DNA using anti-^{4m}C[20] and anti ^{5m}C[21] antibodies has also been described. Since a large number of DNA MTases are available as side fractions from commercial restriction endonuclease purifications, DMG is a practical, versatile new DNA finger-printing method, which may be useful in a variety of gene mapping and diagnostic applications.

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