

# Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands

(DNA methylation/tumor suppressor genes/*p16/p15*)

JAMES G. HERMAN\*<sup>†</sup>, JEREMY R. GRAFF\*, SANNA MYÖHÄNEN\*, BARRY D. NELKIN\*, AND STEPHEN B. BAYLIN\*<sup>‡</sup>

\*Oncology Center and <sup>‡</sup>Department of Medicine, The Johns Hopkins Medical Institutions, 424 North Bond Street, Baltimore, MD 21231

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**ABSTRACT** Precise mapping of DNA methylation patterns in CpG islands has become essential for understanding diverse biological processes such as the regulation of imprinted genes, X chromosome inactivation, and tumor suppressor gene silencing in human cancer. We describe a new method, MSP (methylation-specific PCR), which can rapidly assess the methylation status of virtually any group of CpG sites within a CpG island, independent of the use of methylation-sensitive restriction enzymes. This assay entails initial modification of DNA by sodium bisulfite, converting all unmethylated, but not methylated, cytosines to uracil, and subsequent amplification with primers specific for methylated versus unmethylated DNA. MSP requires only small quantities of DNA, is sensitive to 0.1% methylated alleles of a given CpG island locus, and can be performed on DNA extracted from paraffin-embedded samples. MSP eliminates the false positive results inherent to previous PCR-based approaches which relied on differential restriction enzyme cleavage to distinguish methylated from unmethylated DNA. In this study, we demonstrate the use of MSP to identify promoter region hypermethylation changes associated with transcriptional inactivation in four important tumor suppressor genes (*p16*, *p15*, *E-cadherin*, and von Hippel-Lindau) in human cancer.

In higher order eukaryotes, DNA is methylated only at cytosines located 5' to guanine in the CpG dinucleotide (1). This modification has important regulatory effects on gene expression, especially when involving CpG-rich areas known as CpG islands, located in the promoter regions of many genes (2, 3). While almost all gene-associated islands are protected from methylation on autosomal chromosomes (3), extensive methylation of CpG islands has been associated with transcriptional inactivation of selected imprinted genes (4, 5) and genes on the inactive X-chromosome of females (6, 7). Aberrant methylation of normally unmethylated CpG islands has been documented as a relatively frequent event in immortalized and transformed cells (8) and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers (9–12). In this last situation, promoter region hypermethylation stands as an alternative to coding region mutations in eliminating tumor suppressor gene function (9, 10). Therefore, mapping of methylation patterns in CpG islands has become an important tool for understanding both normal and pathologic gene expression events.

Mapping of methylated regions in DNA has relied primarily on Southern hybridization approaches, based on the inability of methylation-sensitive restriction enzymes to cleave sequences that contain one or more methylated CpG sites. This method provides an assessment of the overall methylation status of CpG islands, including some quantitative analysis (13), but requires large amounts of high molecular weight

DNA (generally 5  $\mu$ g or more), can detect methylation only if present in greater than a few percent of the alleles and can only provide information about those CpG sites found within sequences recognized by methylation-sensitive restriction enzymes. A more sensitive method of methylation detection combines the use of methylation-sensitive enzymes and PCR (14). After digestion of DNA with the enzyme, PCR will amplify from primers flanking the restriction site only if DNA cleavage has been prevented by methylation (15, 16). Like Southern-based approaches, this method can only monitor CpG methylation in methylation-sensitive restriction sites. Moreover, the restriction of unmethylated DNA must be complete, since any uncleaved DNA will be amplified by PCR yielding a false positive result for methylation. This approach has been useful in studying samples where a high percentage of alleles of interest are methylated, such as the study of imprinted genes (5, 15, 16) and X chromosome-inactivated genes (14). However, difficulties in distinguishing between incomplete restriction and low numbers of methylated alleles make this approach unreliable for detection of tumor suppressor gene hypermethylation in small samples or in samples where methylated alleles represent a small fraction of the population.

The chemical modification of cytosine to uracil by bisulfite treatment has provided another method for the study of DNA methylation that avoids the use of restriction enzymes (17). In this reaction, all cytosines are converted to uracil, but those that are methylated (5-methylcytosine) are resistant to this modification and remain as cytosine (18). This altered DNA can then be amplified and sequenced, providing detailed information within the amplified region of the methylation status of all CpG sites (17). However, this method is technically rather difficult and labor-intensive, and, without cloning of the amplified products, the technique is less sensitive than Southern analysis, requiring  $\approx 25\%$  of the alleles to be methylated for detection (19).

We now report a novel PCR method, methylation-specific PCR (MSP), which is sensitive and specific for methylation of virtually any block of CpG sites in a CpG island. We designed primers to distinguish methylated from unmethylated DNA in bisulfite-modified DNA, taking advantage of the sequence differences resulting from bisulfite modification. Unmodified DNA or DNA incompletely reacted with bisulfite can also be distinguished, since marked sequence differences exist between these DNAs. The frequency of CpG sites in CpG islands renders this technique uniquely useful and extremely sensitive for such regions. Herein, we detail the MSP procedure and show its use for detecting the aberrant methylation of four tumor suppressor genes in human neoplasia.

## MATERIALS AND METHODS

**DNA and Cell Lines.** Genomic DNA was obtained from cell lines, primary tumors, and normal tissue as described (10–12).

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Abbreviations: MSP, methylation-specific PCR; *VHL*, von Hippel-Lindau.

<sup>†</sup>To whom reprint requests should be addressed.

The renal carcinoma cell line was kindly provided by Michael Lerman (National Cancer Institute, Frederick, MD).

**Bisulfite Modification.** DNA (1  $\mu$ g) in a volume of 50  $\mu$ l was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. For samples with nanogram quantities of human DNA, 1  $\mu$ g of salmon sperm DNA (Sigma) was added as carrier before modification. Thirty microliters of 10 mM hydroquinone (Sigma) and 520  $\mu$ l of 3 M sodium bisulfite (Sigma) at pH 5, both freshly prepared, were added and mixed, and samples were incubated under mineral oil at 50°C for 16 hr. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer (Promega) and eluted into 50  $\mu$ l of water. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at -20°C.

**Genomic Sequencing.** Genomic sequencing of bisulfite-modified DNA (17) was accomplished using the solid-phase DNA sequencing approach (19). Bisulfite modified DNA (100 ng) was amplified with *p16* gene-specific primers 5'-TTTTAGAGGATTTGAGGGATAGG (sense) and 5'-CTACCTAATTCGAATTCCTACA (anti-sense). PCR conditions were as follows: 96°C for 3 min and 80°C for 3 min, after which 1 unit of *Taq* polymerase (BRL) was added; then 35 cycles of 96°C for 20 sec, 56°C for 20 sec, 72°C for 90 sec; and finally 5 min at 72°C. The PCR mixture contained 1 $\times$  buffer (BRL) with 1.5 mM MgCl<sub>2</sub>, 20 pmol of each primer, and 0.2 mM dNTPs. To obtain products for sequencing, a second round of PCR was performed with 5 pmol of nested primers. In this reaction, the sense primer, 5'-GTTTCCCAGTCACGACAGTATTAGGAGGAAGAAAGAGGAG, contains M13-40 sequence (underlined) introduced as a site to initiate sequencing, and the anti-sense primer 5'-TCCAATTCCTACAAACTTC is biotinylated to facilitate purification of the product before sequencing. PCR was performed as above, for 32 cycles with 2.5 mM MgCl<sub>2</sub>. All primers for genomic sequencing were designed to avoid any CpGs in the sequence. Biotinylated PCR products were purified using streptavidin-coated magnetic beads (Dyna, Oslo), and sequencing reactions were performed with Sequenase and M13-40 sequencing primer under conditions specified by the manufacturer (United States Biochemical).

**PCR Amplification.** Primer pairs described in Table 1 (20–24) were purchased from Life Technologies. The PCR mixture contained 1 $\times$  PCR buffer (16.6 mM ammonium sulfate/67 mM Tris, pH 8.8/6.7 mM MgCl<sub>2</sub>/10 mM 2-mer-

captoethanol), dNTPs (each at 1.25 mM), primers (300 ng each per reaction), and bisulfite-modified DNA ( $\approx$ 50 ng) or unmodified DNA (50–100 ng) in a final volume of 50  $\mu$ l. PCR specific for unmodified DNA also included 5% dimethyl sulfoxide. Reactions were hot-started at 95°C for 5 min before the addition of 1.25 units of *Taq* polymerase (BRL). Amplification was carried out in a Hybaid OmniGene temperature cyclor for 35 cycles (30 sec at 95°C, 30 sec at the annealing temperature listed in Table 1, and 30 sec at 72°C), followed by a final 4-min extension at 72°C. Controls without DNA were performed for each set of PCRs. Each PCR (10  $\mu$ l) was directly loaded onto nondenaturing 6–8% polyacrylamide gels, stained with ethidium bromide, and directly visualized under UV illumination.

**Restriction Analysis.** Of the 50  $\mu$ l of PCR mixture, 10  $\mu$ l was digested with 10 units of *Bst*UI (New England Biolabs) for 4 hr, according to conditions specified by the manufacturer. Restriction digests were ethanol precipitated before gel analysis.

## RESULTS

**Validating the Design Strategy for MSP: Genomic Sequencing of *p16*.** An initial study was required to validate whether our strategy for MSP would prove feasible for assessing the methylation status of CpG islands. We needed to determine whether the density of methylation, in key regions to be tested, was great enough to facilitate our primer design. We chose to test this for the *p16* tumor suppressor gene in which we (10, 25) and others (26, 27) have documented that hypermethylation of a 5' CpG island is associated with complete loss of gene expression in many cancer types. However, other than for CpG sites located in recognition sequences for methylation-sensitive enzymes, the density of methylation and its correlation to transcriptional silencing has not been established. We thus employed the genomic sequencing technique to explore this relationship. As has been found for other CpG islands examined in this manner (19, 28, 29), the CpG island of *p16* had no methylation at any CpG site in those cell lines and normal tissues previously found to be unmethylated by Southern analysis (Fig. 1; refs. 10 and 25). However, it was extensively methylated in cancer cell lines shown to be methylated by Southern analysis (Fig. 1). In fact, all cytosines within CpG dinucleotides in this region were completely methylated in the cancers lacking *p16* transcription. This marked difference in sequence following bisulfite treatment suggested that our

Table 1. PCR primers used for MSP

Primer set	Sense primer,* 5' $\rightarrow$ 3'	Antisense primer,* 5' $\rightarrow$ 3'	Size, bp	Anneal temp., °C	Genomic position†
<i>p16</i> -W‡	CAGAGGGTGGGGCGGACCGC	CGGGCCGCGGCGGTGG	140	65	+171
<i>p16</i> -M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACC CGACCGTAA	150	65	+167
<i>p16</i> -U	TTATTAGAGGGTGGGGTGGATTGTT	CAACCCAAACCACAACATAA	151	60	+167
<i>p16</i> -M2	TTATTAGAGGGTGGGGCGGATCGC	CCACCTAAATCGACCTCCGACCG	234	65	+167
<i>p16</i> -U2	TTATTAGAGGGTGGGGTGGATTGTT	CCACCTAAATCAACCTCCAAACC	234	60	+167
<i>p15</i> -W	CGCACCCCTGCGGCCAGA	AGTGGCCGAGCGGCCGG	137	65	+46
<i>p15</i> -M	CGTTCGTATTGCGGTT	CGTACAATAACCGAACCCGA	148	60	+40
<i>p15</i> -U	TGTGATGTTGTTGTTGTTGTTGTTGTT	CCATACAATAACCAACAA	154	60	+34
<i>VHL</i> -M	TGGAGGATTTTTTTGCGTACGC	GAACCGAACCGCCGAA	158	60	-116
<i>VHL</i> -U	GTTGGAGGATTTTTTTGTTGTTGTT	CCCAAACC AACCCACAAA	165	60	-118
<i>Ecad</i> -M	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	116	57	-205
<i>Ecad</i> -U	TAATTTTAGGTTAGAGGGTTATTGTT	CACAACCAATCAACAACA	97	53	-210

\*Sequence differences between modified primers and unmodified DNA are in boldface type and differences between methylated/modified and unmethylated/modified are underlined.

†Primers were placed near the transcriptional start site. Genomic position is the location of the 5' nucleotide of the sense primer in relation to the major transcriptional start site defined in the following references and Genbank accession numbers: *p16* (most 3' site), X94154 (20); *p15*, S75756 (22); *VHL*, U19763 (23); and *E-cadherin*, L34545 (24).

‡W represents unmodified or wild-type primers. M, methylated-specific primers; and U, unmethylated-specific primers.

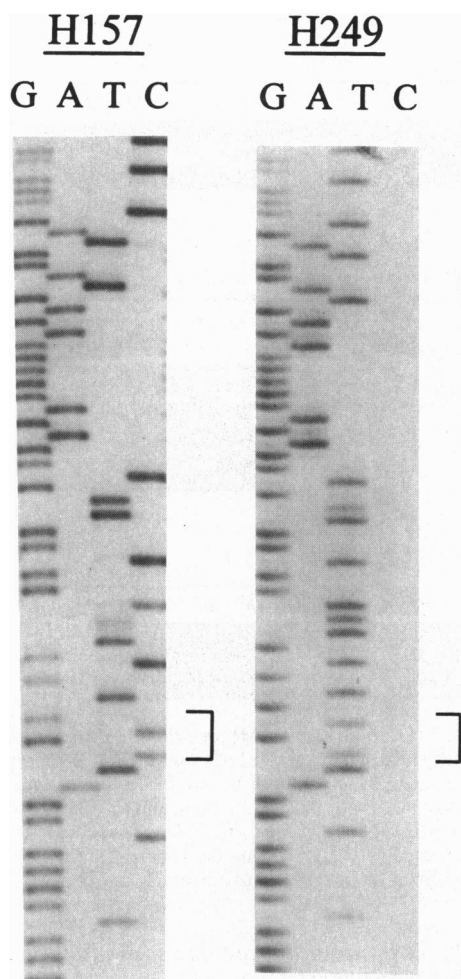


FIG. 1. Genomic sequencing of *p16*. The sequence shown has the most 5' region at the bottom of the gel, beginning at +175 in relation to a major transcriptional start site (20). All cytosines in the unmethylated cell line H249 have been converted to thymidine, while all Cs in CpG dinucleotides in the methylated cell H157 remain as C, indicating methylation. Designated by the bracket (]) is a *Bst*UI site, which is at -59 in relation to the translational start site in GenBank sequence U12818 (21), but which is incorrectly identified as CGAG in sequence X94154 (20). This CGCG site represents the 3' location of the sense primer used for *p16* MSP.

strategy for specific amplification of either methylated or unmethylated alleles was feasible.

**Primer Design for MSP.** Primers were designed to discriminate between methylated and unmethylated alleles following bisulfite treatment and to discriminate between DNA modified by bisulfite and that which had not been modified. To accomplish this, primer sequences were chosen for regions containing frequent cytosines (to distinguish unmodified from modified DNA), and CpG pairs near the 3' end of the primers (to provide maximal discrimination in the PCR between methylated and unmethylated DNA). Since the two strands of DNA are no longer complementary after bisulfite treatment, primers can be designed for either modified strand. For convenience, we have designed primers for the sense strand. The fragment of DNA to be amplified was intentionally small, to allow the assessment of methylation patterns in a limited region and to facilitate the application of this technique to samples, such as paraffin blocks, where amplification of larger fragments is not possible. In Table 1, primer sequences are shown for all genes tested, emphasizing the differences in sequence between the three types of DNA that are exploited for the specificity of MSP. The multiple mismatches in these

primers, which are specific for these different types of DNA, suggest that each primer set should provide amplification only from the intended template.

**MSP Analysis of *p16*.** We first tested the primers designed for *p16* on DNA from cancer cell lines and normal tissues for which the methylation status had previously been defined by Southern analysis (10, 25). In all cases, the primer set used confirmed the methylation status determined by Southern analysis. For example, lung cancer cell lines U1752 and H157, as well as other cell lines with methylated *p16* alleles, amplified only with the methylated primers (Fig. 2A). DNA from normal tissues (lymphocytes, lung, kidney, breast, and colon) and the lung cancer cell lines H209 and H249, having only unmethylated *p16* alleles, amplified only with unmethylated primers (examples in Fig. 2A). PCR with these primers could be performed with or without 5% dimethyl sulfoxide. DNA not treated with bisulfite (unmodified) failed to amplify with either set of methylated or unmethylated specific primers, but readily amplified with primers specific for the sequence before modification (Fig. 2A). DNA from the cell line H157 after bisulfite treatment also produced a weak amplification with unmodified primers, suggesting an incomplete bisulfite reaction. We have occasionally observed this in other samples. However, this unmodified DNA, unlike partially restricted DNA in previous PCR assays relying on methylation-sensitive restriction enzymes, is not recognized by the primers specific for modified DNA. It therefore does not provide a false positive result or interfere with the ability to distinguish methylated from unmethylated alleles.

We next sought to define the sensitivity of MSP for detection of methylated *p16* alleles. DNA from cell lines with methylated *p16* alleles was mixed with DNA with unmethylated *p16* alleles before bisulfite treatment. We could consistently detect 0.1% of methylated DNA ( $\approx 50$  pg) present in an otherwise unmethylated sample (Fig. 2B). We have also determined the sensitivity limit for the amount of input DNA. As little as 1 ng of human DNA, mixed with salmon sperm DNA as a carrier, was detectable by MSP (data not shown).

Fresh human tumor samples often contain normal and tumor tissue, making the detection of changes specific for the tumor difficult. However, the sensitivity of MSP suggests it would be useful for primary tumors as well, allowing for detection of aberrantly methylated alleles even if they contribute relatively little to the overall DNA in a sample. In each case, while normal tissues (lymphocytes, lung, kidney, and colon) were unmethylated at the *p16* locus, tumors found to be methylated at the *p16* CpG island by Southern analysis also contained methylated DNA detected by MSP, in addition to some unmethylated alleles (examples in Fig. 2B). Analysis of DNA from paraffin-embedded tumors revealed methylated and unmethylated alleles (example in Fig. 2B), as shown for the same primary lung cancer in Fig. 2B. To confirm that these results were not unique to this primer set, we used a second downstream primer for *p16* that would amplify a slightly larger fragment (Table 1). This second set of primers reproduced the results described above (Fig. 2C), confirming the methylation status defined by Southern blot analysis.

To verify further the specificity of the primers for the methylated alleles and to check specific cytosines for methylation within the region amplified, we took advantage of the differences in sequence at a methylation-sensitive restriction site between methylated/modified DNA and unmethylated/unmodified DNA. Specifically, the *Bst*UI recognition site, CGCG, will remain CGCG if both Cs are methylated after bisulfite treatment and amplification but will become TGTG if unmethylated. Digestion of the amplified products with *Bst*UI will then distinguish these two products, as restriction of *p16* amplified products illustrates. Only unmodified products and methylated/modified products, both of which retain the

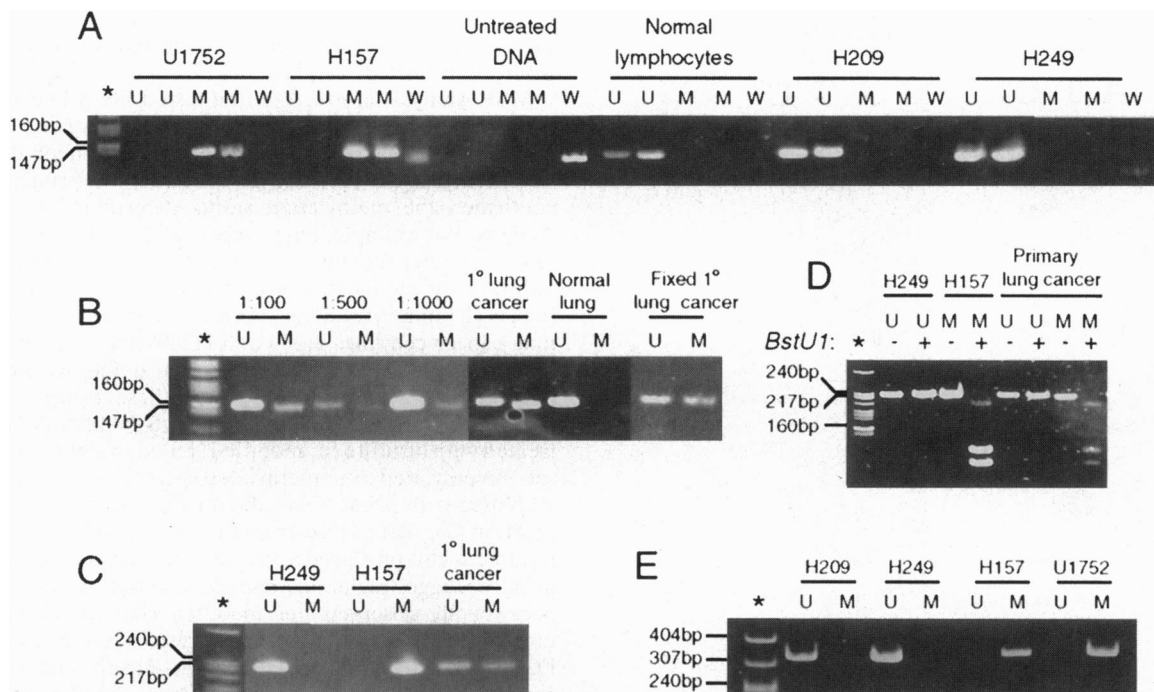


FIG. 2. MSP of *p16*. Primer sets used for amplification are designated as unmethylated (U), methylated (M), or unmodified/wild-type (W). \*, Molecular weight marker pBR322-*MspI* digest. (A) Amplification of bisulfite-treated DNA from cancer cell lines and normal lymphocytes, and untreated DNA (from cell line H249). (B) Mixing of various amounts of H157 DNA with 1  $\mu$ g of H249 DNA before bisulfite treatment to assess the detection sensitivity of MSP for methylated alleles. Modified DNA from a primary lung cancer sample, normal lung, and the paraffin-embedded (fixed) tissue block of this primary lung cancer are also shown. (C) Amplification with the p16-U2 (U) primers, and p16-M2 (M) described in Table 1. (D) The amplified *p16* products of C were restricted with *Bst*UI (+) or were not restricted (-). (E) Testing for regional methylation of CpG islands with MSP, using sense primers p16-U2 (U) and p16-M2 (M), which are methylation-specific, and an antisense primer that is not methylation-specific.

CGCG site, are cleaved by *Bst*UI. Products amplified with unmethylated/modified primers failed to be cleaved (Fig. 2D).

The primer sets discussed above were designed to discriminate heavily methylated CpG islands from unmethylated alleles. To do this, both the upper (sense) and lower (antisense) primers contained CpG sites that could produce methylation-dependent sequence differences after bisulfite treatment. MSP might be employed to examine more regional aspects of CpG island methylation. To examine this, we tested whether methylation-dependent differences in the sequence of just one primer would still allow the discrimination between unmethylated and methylated *p16* alleles. The antisense primer used for genomic sequencing, 5'-CTACCTAATTCCAATTCCTACA, was used as the antisense primer, which contains no CpG sites, and was paired with either a methylated or unmethylated sense primer (Table 1). Amplification of the predicted 313-bp PCR product only occurred with the unmethylated sense primer in H209 and H249 (unmethylated by Southern) and only with the methylated sense primer in H157 and U1752 (methylated by Southern), indicating that methylation of CpG sites within a defined region can be recognized by specific primers and distinguish between methylated and unmethylated alleles (Fig. 2E).

**The Use of MSP for the Analysis of Other Genes.** We extended our study to include three other genes transcriptionally silenced in human cancers by aberrant hypermethylation of 5' CpG islands. The cyclin-dependent kinase inhibitor *p15* is aberrantly methylated in many leukemic cell lines and primary leukemias (11). For *p15*, MSP again verified the methylation status determined by Southern analysis. Thus, normal lymphocytes and cancer cell lines SW48 and U1752, containing only unmethylated alleles of *p15* alleles by Southern analysis (11), amplified only with the unmethylated set of primers, while the lung cancer cell line H1618 and leukemia cell line KG1A amplified only with the methylated set of primers (Fig. 3A), consistent with previous Southern analysis

results (11). DNA from the cell line Raji produced a strong PCR product with methylated primers and a weaker band with unmethylated primers. This was the same result for methylation obtained previously by Southern analysis (11). Noncultured leukemia samples, like the primary tumors studied for *p16*, had amplification with the methylated primer set as well as the unmethylated set. This heterogeneity also matched Southern analysis (11). Again, as for *p16*, differential modification of *Bst*UI restriction sites in the amplified product of *p15* was used to verify the specific amplification by MSP (Fig. 3B). Amplified products using methylated primer sets from cell lines H1618 and Raji or unmodified primer sets, were completely cleaved by *Bst*UI, while amplified products from the unmethylated primer set did not cleave. The smaller sizes of products observed in the unmodified product reflect the 11-bp difference in size of the original PCR product. Primary acute myelogenous leukemia samples also demonstrated cleavage only in the methylated product but had less complete cleavage. This suggests a heterogeneity in methylation, where methylation is extensive in the region underlying the methylation-specific primers, allowing amplification by MSP but is not inclusive of all CpG sites between the primers for each allele.

Aberrant CpG island promoter region methylation is associated with inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene in  $\approx 20\%$  of clear cell renal carcinomas (9). This event, like mutations for *VHL* (30), is restricted to clear cell renal cancers (9). Primers designed for the *VHL* sequence were used to study DNA from the renal cell cancer cell line RFX393, which is methylated at *VHL* by Southern analysis (data not shown), and DNA from the lung cancer cell line U1752, which is unmethylated at this locus (9). In each case, the methylation status of *VHL* determined by MSP confirmed that found by Southern analysis (Fig. 3C), and *Bst*UI restriction site analysis validated the PCR product specificity (Fig. 3D).

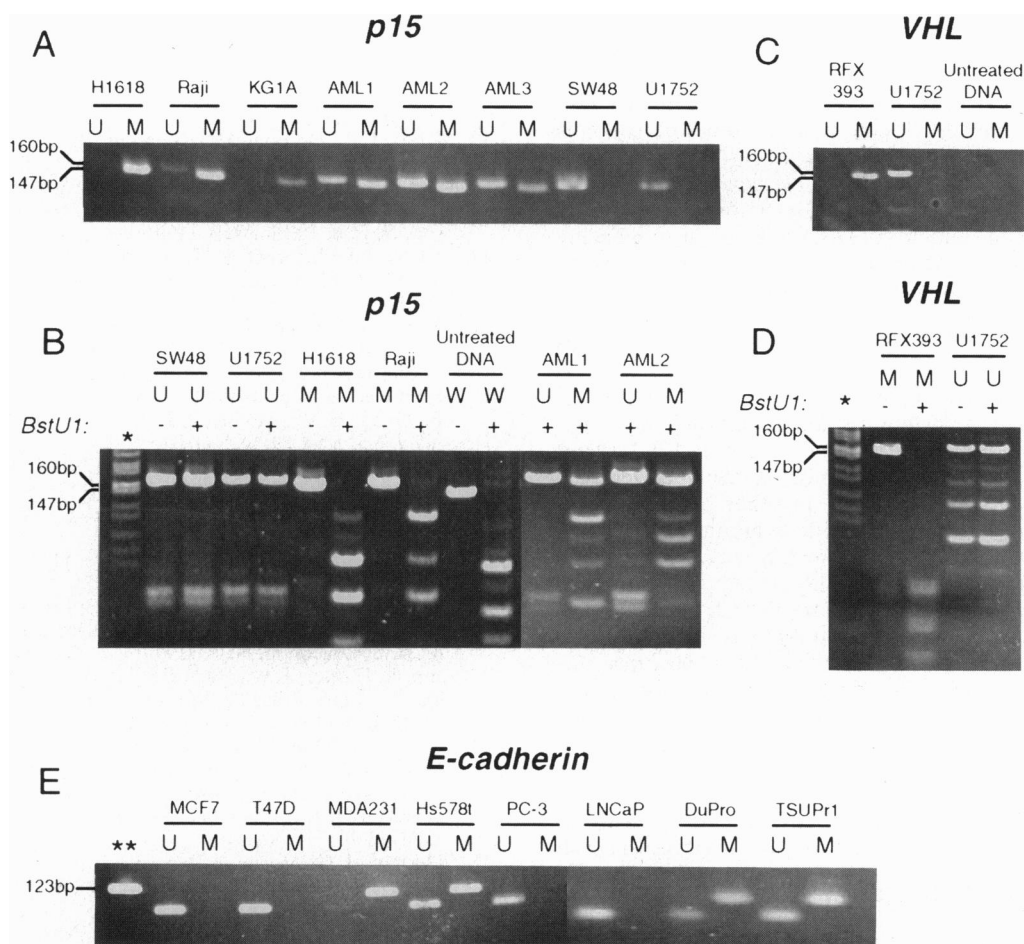


FIG. 3. MSP analysis of several genes. Primer sets used for amplification are designated as unmethylated (U), methylated (M), or unmodified/wild-type (W). \*, Molecular weight marker pBR322-*Msp*I digest; and \*\*, 123-bp molecular weight marker. All DNA samples were bisulfite-treated except those designated untreated. (A) MSP for *p15*. (B) The *p15* products were restricted with *Bst*UI (+) or were not restricted (-). (C) MSP for *VHL*. (D) The *VHL* products were restricted with *Bst*UI (+) or were not restricted (-). The smaller molecular weight fragments seen in the U lanes represent primer dimers, which are present in lanes without template DNA and can be faintly seen in C. (E) MSP for *E-cadherin*.

The expression of the invasion/metastasis suppressor gene, *E-cadherin*, is often silenced by aberrant methylation of the 5' promoter in breast, prostate, and many other carcinomas (12, 31). We designed primers for the *E-cadherin* promoter region to test the use of MSP for this gene. In each case, MSP analysis paralleled Southern blot analysis for the methylation status of the gene (12). DNA from breast cancer cell lines MDA-MB-231, HS578t, and prostate cancer cell lines DuPro, and TSUPr1, all heavily methylated by Southern blot, displayed prominent methylation. DNA from MCF7, T47D, PC-3, and LNCaP, all unmethylated by Southern, showed no evidence for methylation in the sensitive MSP assay (Fig. 3E). MSP analysis revealed the presence of unmethylated alleles in Hs578t, TSUPr1, and DuPro, consistent with a low percentage of unmethylated alleles in these cell lines previously detected by Southern analysis (12). *Bst*UI restriction analysis again confirmed the specificity of the PCR amplification (data not shown).

### DISCUSSION

We have described a novel PCR approach, MSP, for rapid analysis of the methylation status of CpG islands. As illustrated, this technique provides significant advantages over previous PCR-based techniques and other methods used for assaying methylation. MSP is markedly more sensitive than Southern analysis, facilitating detection of low numbers of methylated alleles and the study of DNA from small samples. MSP allows the study of paraffin-embedded materials, which

could not previously be analyzed by Southern analysis. MSP also allows examination of all CpG sites, not just those within sequences recognized by methylation-sensitive restriction enzymes. This markedly increases the number of such sites that can be assessed and will allow rapid, fine mapping of methylation patterns throughout CpG-rich regions. This latter point was demonstrated for *p16*, where the discrimination between methylated and unmethylated alleles could be attributed to differences in methylation in an 8-bp region. MSP also eliminates the frequent false positive results due to partial digestion of methylation-sensitive enzymes inherent in previous PCR methods for detecting methylation. Furthermore, with MSP, simultaneous detection of unmethylated and methylated products in a single sample confirms the integrity of DNA as a template for PCR and allows a semiquantitative assessment of allele types that approximates the quantitation determined by Southern analysis. Finally, the ability to validate the amplified product by differential restriction patterns is an additional advantage.

The only technique that can provide more direct analysis than MSP for most CpG sites within a defined region is genomic sequencing. However, MSP can provide similar information and has the following advantages. First, MSP is much simpler and requires less time than genomic sequencing, with a typical PCR and gel analysis taking 4–6 hr. In contrast, for genomic sequencing, amplification, cloning, and subsequent sequencing may take days. Second, MSP avoids the use of expensive sequencing reagents and the use of radioactivity.

Both of these factors make MSP better suited for the analysis of large numbers of samples. Third, the use of PCR as the step to distinguish methylated from unmethylated DNA in MSP allows for a significant increase in the sensitivity of methylation detection. For example, if cloning is not used before genomic sequencing of the DNA, <10% methylated DNA in a background of unmethylated DNA cannot be seen (19). The use of PCR and cloning does allow sensitive detection of methylation patterns in very small amounts of DNA by genomic sequencing (17, 32). However, in practice, this would require sequencing analysis of 10 clones to detect 10% methylation, 100 clones to detect 1% methylation, and, to reach the level of sensitivity we have demonstrated with MSP (1:1000), one would have to sequence 1000 individual clones.

In summary, MSP is a simple, sensitive, and specific method for determining the methylation status of virtually any CpG-rich region. In addition to detecting aberrant CpG island methylation of tumor suppressor genes, MSP will be useful for monitoring CpG islands important in other biological processes. For example, MSP should facilitate monitoring patterns of methylation in imprinted genes at key stages of embryogenesis. Assays used to define clonality of cell populations, as assessed by detecting methylation patterns of X chromosome-inactivated genes in female cells, should be readily adaptable to the MSP approach. Finally, MSP should prove extraordinarily valuable clinically for the detection of methylation patterns in small DNA samples associated with disease states such as the fragile X syndrome, altered gene imprint states, and cancer.

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