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Secondary Structure at a Hot Spot for DNA Methylation in DNA from Human Breast Cancers

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Abstract. *The VNTR at c-Ha-ras resides in a hotspot for DNA methylation on chromosome 11 in human tumors, where it is flanked by two MspI restriction sites. We have investigated the nature of the MspI site polymorphism at the c-Ha-ras VNTR observed in variety of tumors including breast cancer. We find that the MspI site 5' to the VNTR is present in a Non-B DNA structure with single-strand character that renders it accessible to bisulfite modification under native conditions, while the MspI site 3' to the VNTR appears to reside in a normal B-form structure that is inaccessible to bisulfite. The non-B DNA structure accounts for the observed polymorphism since MspI cannot cleave single-stranded DNA and control experiments show that the MspI sites were neither mutated nor abnormally methylated. Southern blotting showed that structural polymorphism was present in tumor DNA and tumor adjacent normal tissue DNA but absent from lymphocyte DNA from the same patients. We conclude that the non-B DNA structural polymorphism detected in human tumors near the c-Ha-ras VNTR is a self-perpetuating epigenetic mark that manifests itself spontaneously during breast carcinogenesis in a methylation hot spot.*

Non-B DNA structures are a plausible description of mutational hotspots (1-3), sites of triplet repeat expansion (2, 4, 5) and clustered sites of somatic recombination (6). However, many sites of unusual structure appear to be both stable and favored conformations that are present in living cells. These occur as structures associated with chromosomal centromeres (7), telomeres (8) and the control regions of genes (9-14). In general such sequences can be heavily methylated (15), highly polymorphic in the human population (16, 17), known breakpoint clusters in cancers (15), or unusual sequences associated with chromosome fragile sites (18).

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One such carefully studied site is the VNTR at c-Ha-ras. This sequence of tandem 28 bp repeats resides in a known hotspot (11p15) for DNA methylation in tumors (19), and is highly polymorphic in the human population (20). Although unusual structures associated with the c-Ha-ras VNTR have not been reported, the presence of multiple length variants in the population suggests that the sequence is prone to the formation of unusual DNA structures associated with unequal recombination or slippage.

Since non-B DNA structures in isolated DNA appear to be exceptionally sensitive to modification by bisulfite, we have asked whether or not sites known to reside in the hotspot for DNA methylation on chromosome 11p15 that is associated with human solid tumors were sensitive to bisulfite modification. Our results show that a sequence adjacent to the highly polymorphic VNTR 3' to the c-Ha-ras coding sequence is selectively sensitive to bisulfite modification in breast tumor DNA and in DNA from adjacent histologically normal margins, suggesting that the unusual structure is propagated clonally. The presence of an unusual structure in tumor and tumor adjacent tissue specimens was confirmed by restriction analysis using an enzyme that is unable to cleave single-stranded DNA. Inspection of patients' blood lymphocyte DNA with the same methods showed that lymphocyte DNA from these same patients remained in the B-DNA conformation.

Materials and Methods

Isolation of DNA from patient samples. Adjacent specimens of malignant and normal breast tissues were obtained under an IRB approved protocol at resection and stored at –70˚C prior to use. The tissue was pulverized under liquid nitrogen and then digested with proteinase K (Applied Biosystems, Foster City, CA, USA) overnight at 50˚C. High-molecular-weight DNA was purified with an automated DNA extractor (Applied Biosystems). Purified DNA was treated with 0.05 μg of RNase (Sigma Chemical Co., St. Louis, MO, USA) per ml for one hour at 37˚C and collected by ethanol precipitation.

Amplification and visualization, of bisulfite-treated PCR products. Genomic DNAs isolated as described above from Human Breast Tumor, Tumor Adjacent, and Blood Lymphocyte specimens (1 μg

each) were bisulfite treated using the EZ DNA Methylation Kit (Zymo, Orange, CA, USA) as described by the manufacturer except that, the sodium hydroxide denaturation step was omitted when non-denatured DNA was treated with bisulfite. 1 μg of DNA was added to a final volume of 45 μL, for both conditions. For denatured samples, 5 μL of M-Dil Buffer (NaOH) was added and was incubated at 37˚C for 15 min. The non-denatured samples were likewise incubated for 15 min at 37˚C. Then 100 μL of CT-reagent (bisulfite) was added to the denatured samples. 5 μL of M-dil buffer was added to 100 μL of CT-Reagent, mixed and then 105 μL added to the 45 μL non-denatured samples. A set of denatured and non-denatured DNAs was then incubated 55˚C overnight protected from light, and likewise a similar set was incubated at 37˚C overnight and protected from exposure to light. The DNA was subsequently desulfonated and purified per the manufacturer's instructions. The DNA was eluted and adjusted with 10 mM Tris 1 mM EDTA, pH 7.5 to 40 ng/μg assuming 100% recovery. The resulting four samples were then amplified with 3' *Msp*I or 5' *Msp*I primers directed at the upper and lower strands of each region (Figure 1). The amplification reaction consisted of 0.25 μl Qiagen Hotstart Taq (Qiagen, Valencia, CA, USA), 2.5 μl 10x Qiagen buffer, 320 mM dNTPs, 2.0 mM added $MgCl_2$, 1.0 μl Q-Solution, 900 nM each for forward and reverse primers, 5.0 μl DNA. The conditions for 5' MspI were 95˚C 10 min, then 35cycles of 95˚C 30s, 50˚C 30s, 72˚C 60s, then extension for 7 min at 72˚C. The conditions for 3' *Msp*I were 95˚C 10 min, then 35 cycles of 95˚C 30s, 60˚C 30s, 72˚C 60s, then extension for 7 min at 72˚C. The PCR reactions were then run on a 2% agarose gel run at 100 volts for 1.5hrs, with the xylene cyanol dye marker at 6 cm. The Gels were then stained with ethidium bromide and visualized with a UV transilluminator. The sequences of the amplification primers were as follows: Lower Reverse 5' *Msp*I 5'-CTACTAAAAACCCACAACGC-3'; Lower Forward 5' *Msp*I 5'-TTTTGGTTAGTTAGCGGTAT-3'; Upper Reverse 5' *Msp*I 5'-CCAACCAACGACATACCCTA-3'; Upper Forward 5' *Msp*I 5'-TTTTGTTGGGGGTTTATAGCGT-3'; Upper Forward 3' *Msp*I 5'ATTAGTTTTTTTATCGATAGATTTTTC-3'; Upper Reverse 3' *Msp*I 5'-TCACCTACATCTAACGCCCCCAACA-3'; Lower Forward 3' *Msp*I 5'-ACCAACTTCCCCATCGATAAACTTCCC-3'; Lower Reverse 3' *Msp*I 5'-TTATTTGTATTTGACGTTTTTAGTA-3'. Untreated DNA PCR reactions were identical to those used for bisulfite treated DNA, except that for the 5' *Msp*I site, where the following PCR conditions were used: 95°C 10 min, then 35 cycles of 95°C 30 s, 60°C 30 s, 72°C 30 s, then extension for 7 min at 72°C, and for the 3' *Msp*I site 95°C 10 min, then 35 cycles of 95°C 30 s, 56°C 30 s, 72°C 30 s, then extension for 7 min at 72°C.

Cloning and sequencing of the bisulfite-treated PCR products. The bands corresponding to the 155 bp product from the upper strand and the 153 bp product from the lower strand of the 5' *Msp*I target and the 120 bp products from the upper and lower strands of the 3' *Msp*I target were gel-extracted using the QIAquick Gel Extraction Kit (Qiagen). The products of the untreated 3' *Msp*I (154 bp) and 5' *Msp*I (120 bp) were also gel-extracted in the same fashion. Once isolated, the PCR product was cloned from 4 μl of extracted DNA using the pCR 2.1-TOPO Cloning Kit (Invitrogen, Carlsbad, CA, USA), as described by the manufacturer. The resulting colonies were inoculated into liquid cultures and plasmid DNA was isolated from each. Plasmids with inserts of the appropriate size were sequenced at the City of Hope DNA Sequencing Lab as previously described (21).

Restriction enzyme digestion. DNA was suspended in a solution of 6 mM KC1, 10 mM Tris-HC1 (pH 7.4), 10 mM $MgC1_2$, 1 mM dithiothreitol, and 100 μg of bovine serum albumin per ml. Restriction endonucleases *Hpa*II, *Msp*I, and *Mbo*I (New England Biolabs, Beverly, MA) were used to digest the DNA. The DNA was pre-incubated for 15 minutes at 37˚C. Separate digests containing *Hpa*II (5 U/μg of DNA), *Msp*I (10 U/μg of DNA), or *Mbo*I (5 U/μg of DNA) were incubated for two hours at 37˚C. After one hour of digestion, additional *Msp*I (5 U/μg) was added to the *Msp*I digest. To ensure completeness of the digestion, co-digestion control experiments with ΦX174 DNA were performed for each DNA digest as previously described (22). Simian virus 40 DNA was used as a co-digestion control for *MboI* restriction digests since ΦX174 contains 6-methyladenine at the 5' GATC recognition site and cannot be cleaved by *Mbo*I. *Mbo*I is not sensitive to the presence of 5-methylcytosine in DNA.

DNA was precipitated with ethanol and suspended in water, and its concentration was determined from its absorbance at 260 nm. DNA fragments were separated by electrophoresis on 1.2% agarose gels (Sigma Chemical Co.) in a solution of 50 mM Tris-acetate, 10 mM EDTA, and 30 mM sodium acetate (pH 8.7) overnight at 30 V.

Southern blotting. Southern transfers (23) were prepared using the alkaline transfer procedure and nylon membranes (Zeta-probe, Bio-Rad Laboratories, Richmond, CA, USA). The 987 bp *Msp*I fragment containing the VNTR region of plasmid pT24-C3 was isolated from *Msp*I digests of plasmid DNA by the freeze-squeeze technique (24). The isolated DNA was subsequently 1abeled with ^{32}P to a specific activity greater than 10^9 cpm/μg by using the random priming procedure (25) and was then used to probe Southern blots. Hybridization was performed (23) and the membranes were used for autoradiography.

Results

We chose to inspect the regions flanking the VNTR since they have been shown to be refractory to restriction digestion by *Msp*I in tumor DNA (26). In order to confirm that the reported polymorphism affects the DNA from the patients studied here, we performed a series of Southern blotting experiments on eleven different patient specimens.

MspI restriction site polymorphism. Digestion controls like those depicted in Figure 2 were performed on each DNA specimen prepared for Southern blotting. These controls show that a limit digest of the human DNA had been achieved in each case. The number of bands in *Msp*I digests confirms the unusual degree of polymorphism in sequences homologous to the c-Ha-ras VNTR region. Samples from individual patients showed either two or four prominent *Msp*I bands (Figure 3). Additional (higher molecular weight) bands were often observed as minor bands with normal DNAs. Length polymorphism at these sites is well known, and allelic variants are well characterized (27). However, it seems unlikely that a normal individual would inherit four alleles for the locus. Thus, some of this polymorphism must be generated somatically.

Somatic insertion-deletion events at the VNTR could

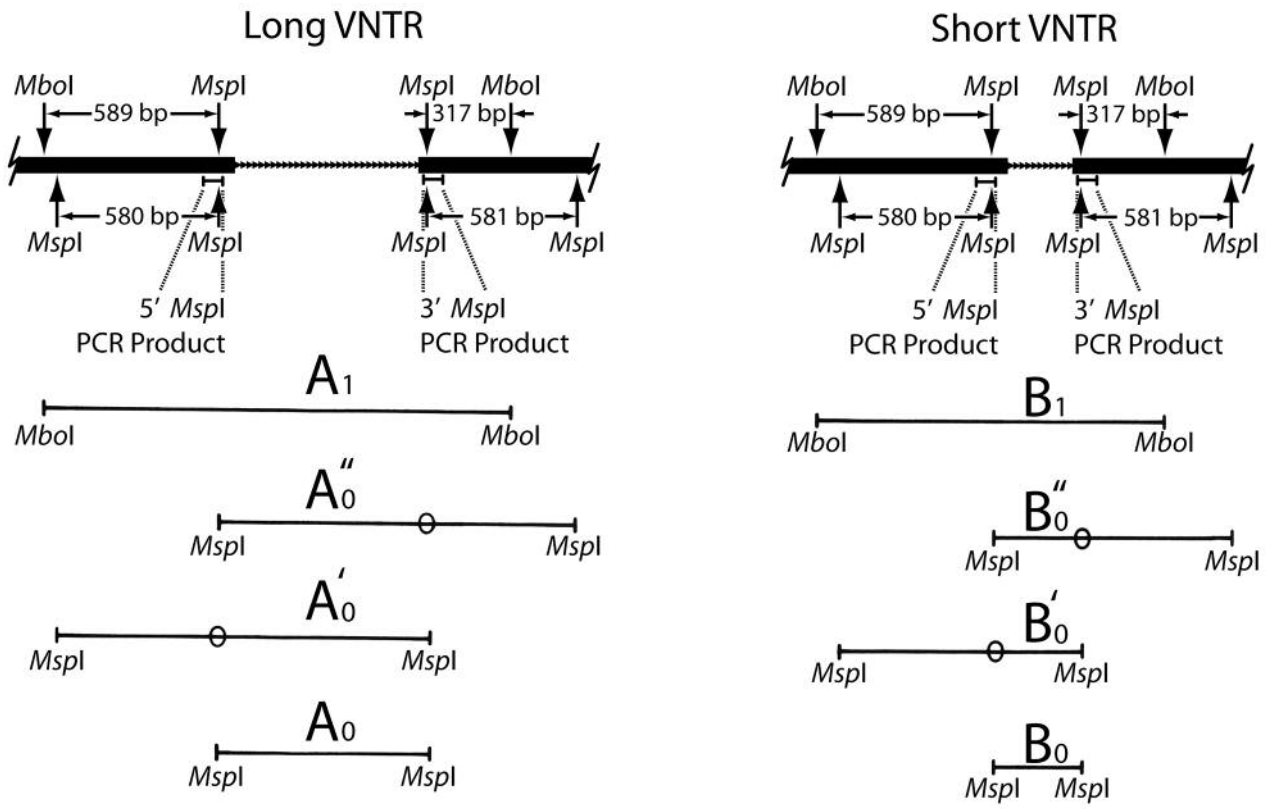


Figure 1. *Restriction patterning in the immediate vicinity of the c-Ha-ras VNTR. Relationships between MspI and MboI fragments containing the VNTR are shown schematically for fragments produced from a region containing a long VNTR (A series) and a short VNTR (B series). The repetitive element in the VNTR is depicted by short tandem arrowheads, while single copy DNA is shown as a thick dark line. The positions of the PCR target sites covering the 3' MspI and 5' MspI sites are also indicated.*

generate the observed polymorphism by creating length variants. Alterations at the *Msp*I sites directly flanking the VNTR could generate the polymorphism by creating restriction site variants. To distinguish between these possibilities, DNA samples were digested with *Mbo*I and Southern blots were hybridized with the VNTR probe. *Mbo*I does not cleave within the smallest VNTR fragment produced by *Msp*I (Figure 1). Thus, the number of *Msp*I fragments produced from a given specimen must equal the number of fragments in MboI digests if the patterns observed with MspI reflect VNTR length. MboI cleavage experiments showed that this is not the case.

Patients exhibiting two prominent bands in *Msp*I digests (Figure 3A) exhibited a single prominent band in DNA digested with *Mbo*I (Figure 3B). DNA from patients exhibiting four prominent MspI fragments generated only two fragments with *Mbo*I (Figure 3C and Figure 3D).

These findings suggest that the polymorphism is associated with unusual properties at *Msp*I sites flanking the VNTR that render some of these sites refractory to *Msp*I cleavage. Since cleavage kinetics for restriction enzymes in general and for *Msp*I in particular are known to be altered by the sequence context near the recognition site (28) we tested the reproducibility of our Southern blotting results with selected patient samples. Those tests showed that patterns of cleavage and band intensity could be reproduced in experiments performed completely independently more than eighteen months apart. Moreover, sequential digestion with *Msp*I followed by *Hpa*II did not alter the patterns. These findings indicate that the patterns are produced from true limit digests of DNA.

Sequence information is available for the region in the vicinity of a representative VNTR. By using that data, the fragmentation pattern can be analyzed from the restriction map as shown in Figure 1. Fragments carrying a long VNTR are designated with the letter A. Fragments carrying a short VNTR are designated with the letter B. The fragments produced by cleavage of both of the sequenced *Msp*I sites directly flanking the VNTR are designated A_0 or B_0. Fragments produced by cleavage of only one of these two sites, followed by cleavage of the next available *Msp*I site, add 580 or 581 bp of DNA to the primary fragment. The two

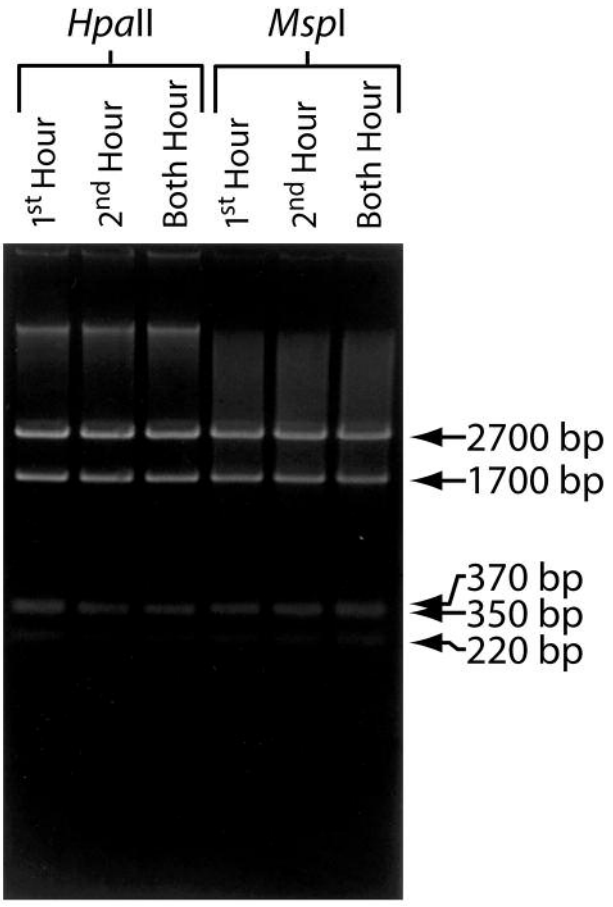


Figure 2. *Controls for complete digestion by restriction endonucleases. Digestion controls were performed as described in reference (48). A representative gel shows that ΦX174 marker DNA added to a sample of the restriction digestion mixture was completely cleared during the first hour of digestion and that enough enzyme activity remained in the digestion mixture to cleave a second aliquot of the phage DNA in the second hour. Thus, the human DNA present during both hours was exposed to at least a two-fold excess of restriction enzyme. Patterns like that shown were obtained for each patient DNA sample analyzed. Tumor DNA was used in this example. Labels on lanes (1st hr., 2nd hr., Both hrs.) indicate the length of exposure for the phage DNA during digestion. The positions of the ΦX174 DNA bands are indicated to the right. Human DNA forms a background smear in each lane. Cleavage of human DNA by MspI is more extensive than that by HpaII because of the methylation at the 5'CCGG 3' site recognized by these enzymes.*

possible secondary fragments differ by only 1 bp, and were not separated from one another on these gels. These are designated A_0' or A_0'' for the long variant and B_0'or B_0'' for the short variant in Figure 1. Fragments produced by *Mbo*I are designated A_1 or B_1.

The analysis is illustrated by data in Figures 1 and 3. In the first patient, the *Msp*I digest shows two bands at about 2720 and 3320 bp (Figure 3A). This might be interpreted as heterozygosity for length alleles at this locus; however, the intensity of the 3320 bp band as determined from gel scans is inappropriately lower than that of the 2720 bp band. Heterozygosity is ruled out for this patient by the *Mbo*I digestion pattern (Figure 3B), which shows a single length variant of 3650 bp. Since the distance between the *Msp*I site and the *Mbo*I sites that flank the VNTR is 589 bp on the left and 317 bp on the right (Figure 1), the length of the *Msp*I fragment containing the VNTR (*i.e.*, fragment A_0) should be 906 bp shorter than the 3650 bp *Mbo*I fragment containing the VNTR. Since the expected value of 2734 bp agrees well with the observed value of 2720 bp for the shorter of the two *Msp*I fragments, we conclude that the short fragment is fragment A_0 with reference to the restriction map. Since the long *Msp*I band is about 580 bp longer than the shorter band, we conclude that this band represents the A_0'or A_0'' fragments produced by lack of cleavage of one of the *Msp*I fragments immediately flanking the VNTR.

The second patient (Figure 3C) was clearly heterozygous for allelic length variants, as judged from the *Mbo*I digests. This enzyme produced fragments of about 3175 bp (A_1) and 2050 bp (B_1). Moreover, these appear to be true length alleles, since scans of the hybridization signal in the autoradiograph showed that the two fragments were present at about equivalent ratios.

Four bands were detected in *Msp*I digests of DNA from the same patient. The fragment sizes were again completely consistent with the map if one assumes that a small fraction of the *Msp*I sites immediately adjacent to the VNTR are refractory to *Msp*I digestion. The largest fragments (designated A_0' or A_0'') were about 2900 bp long and differed from the next largest fragment A_0 (2325 bp) by about 580 bp. B_0' or B_0'' fragments were about 1650 bp long and differed from the shortest fragment, B_0 (1050 bp) by about 580 bp. This is again consistent with the restriction map, and is confirmed by comparing the two primary *Msp*I bands (designated A_0 and B_0 in Figure 1) with the corresponding *Mbo*I fragments (A_1 and B_1). In each case the observed difference was very close to the expected 906 bp.

*Msp*I cleavage might be blocked by point mutation at either of these two sites. Thus, we asked whether or not the sites were mutated by sequencing representative clones of the PCR amplified regions surrounding the affected sites (Figure 1). Mutations were not observed in any of the 20 clones sequenced from each site, all of which exhibited the normal 5'CCGG 3' sequence that is susceptible to *Msp*I cleavage (Figure 4).

Since single-stranded DNA is known to block *Msp*I cleavage, it seemed likely that one or both of the restriction sites near the VNTR might possess single-stranded character that would render it accessible to bisulfite modification under native conditions. Bisulfite attacks cytosines and 5-methylcytosines that are accessible to the reagent and water (*i.e.* present in single-strands or exposed in unusual DNA

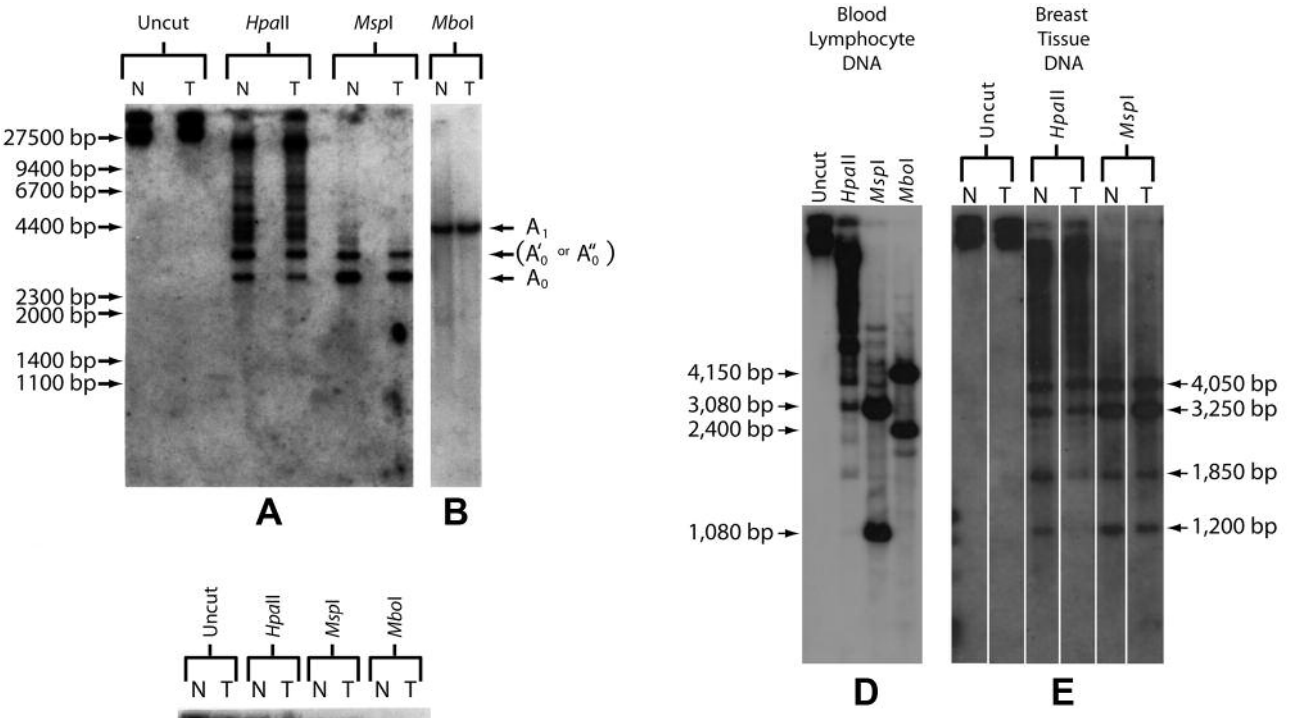


Figure 3. *Methylation and polymorphism in a homozygous patient. A. Southern blot comparing HpaII, MspI, and MboI cleavage patterns in normal (N) and tumor (T) DNA from a single patient. HpaII digests indicated only a slightly elevated level of DNA methylation in tumor tissue. The presence of two prominent bands in the MspI pattern suggests that the patient carries two length alleles or an MspI restriction site polymorphism. B. The patient is shown to be homozygous for length alleles by the presence of only a single band in MboI digests. The Southern blots were probed with the 987bp MspI fragment containing 812bp VNTR from plasmid pT24-C3. Marker fragments are from HindIII digests of phage λ DNA and HaeIII digests of ΦX174 DNA. Letters at the right refer to fragment assignments indicated in the map in Figure 1. C. A comparison of HpaII digests indicates a moderate elevation of DNA methylation in tumor tissue in this patient. The four prominent bands present in the MspI patterns indicate unusual restriction site polymorphism at MspI sites. The patient is shown to be heterozygous for length alleles by the presence of two bands (with appropriate intensity ratios) in MboI digests. D. Southern blot of uncut and HpaII, MspI, and MboI digested DNA from blood lymphocytes of a representative patient. A comparison of HpaII digests indicates a moderate elevation of DNA methylation in tumor tissue. The four prominent bands present in the MspI patterns indicate unusual restriction site polymorphism at MspI sites. The patient is shown to be heterozygous for length alleles by the presence of two bands (with appropriate intensity ratios) in MboI digests. E. Southern blot of uncut, HpaII and MspI digested DNA from histologically normal margin (N) and adjacent breast tumor (T) of the same patient studied in Figure 2D.*

structures). Cytosines are rapidly deaminated while 5-methylcytosines are deaminated much more slowly (29). In general cytosines can be converted to uracils under conditions where 5-methylcytosines remain intact. This results in strands that are no longer complementary so that PCR amplification can focus on either strand (Figure 5) when primers complementary to one or the other of the converted strands are used (30).

PCR systems (Figure 1) specific for each of the two converted strands (Upper and Lower) were used to amplify regions containing the *Msp*I site 5' to the VNTR (5' *Msp*I) and the *Msp*I site 3' to the VNTR (3' *Msp*I). Only 5' *Msp*I was amplified under native conditions at 37˚C indicating that it was in a structure accessible to bisulfite under these conditions (Figure 6). In order to assess the degree of accessibility at 5' *Msp*I under native conditions, representative amplicons corresponding to both the upper

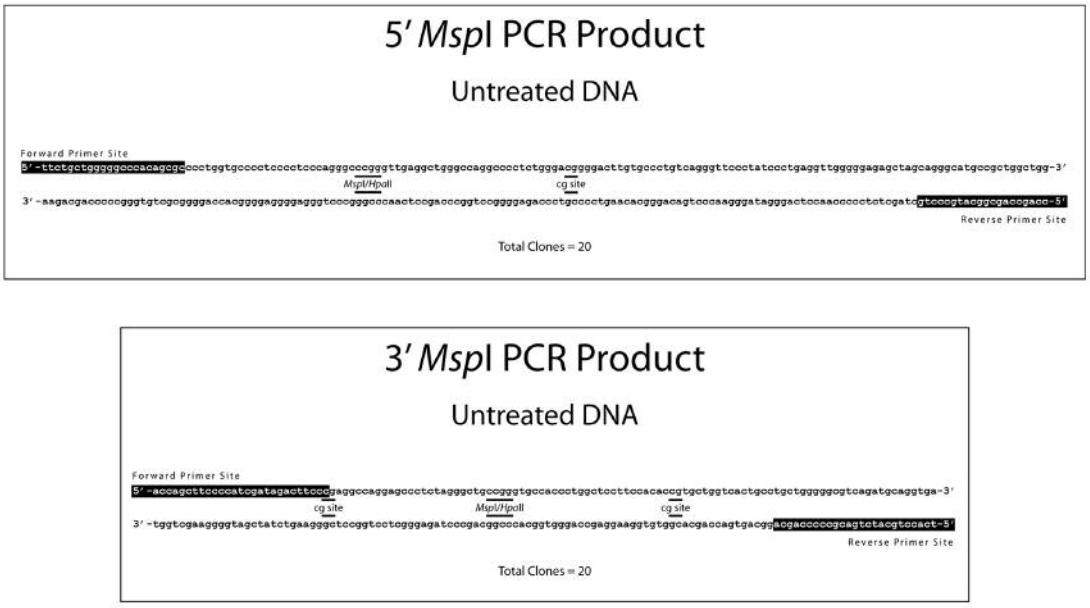


Figure 4. *Untreated DNA was amplified with a PCR system designed to amplify the unconverted DNA sequence. Twenty clonal isolates from the 3' MspI site and twenty clonal isolates from the 5' MspI were sequenced. None of the isolates showed an alteration in sequence at the CCGG MspI site.*

and lower strands of 5' *Msp*I were cloned and sequenced from the amplification product obtained after bisulfite treatment under native conditions (non-denatured DNA treated with bisulfite at 37˚C). The results are depicted in Figure 7A. Nearly all cytosines except those at the two CG sites in the amplicon were converted to uracils in the region. When amplification was carried out after bisulfite treatment under the denaturing conditions used in determining CG methylation state (30) sequencing of the cloned representatives of the amplification products from the upper and lower strands showed that the cytosines in the two CG sites in the region were symmetrically methylated (Figure 7B). Thus the methylated state of the two CG sites in the region is associated with bisulfite accessibility in the region.

We next asked whether or not tumor tissue differed from lymphocyte DNA from the same patient. When PCR amplification was carried out after bisulfite treatment under native conditions using DNA isolated from lymphocyte or from tumor DNA isolated from the same patient, tumor DNA was found to be about 2.5 fold more accessible to bisulfite than lymphocyte DNA. This finding was confirmed by Southern blotting (Figure 3D and 3E). In these experiments, limit digests of both blood and tumor DNA were obtained (see representative digestion control in Figure 2). Southern blotting demonstrated partial digestion of the tumor and tumor adjacent DNA contrasted with complete digestion of DNA from the blood lymphocyte DNA.

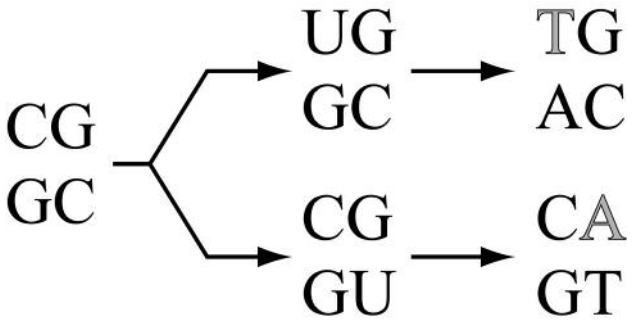


Figure 5. *Bisulfite Conversion. Since the two strands created by bisulfite conversion are not complementary, if the primers lie within the converted region, then the clonal isolates of bisulfite deaminated DNA can take either of two forms (either C→T or G→A) within a given sequence context. For convenience, these are designated upper-strand isolates or lower-strand isolates in Figure 6 and Figure 7.*

Discussion

Polymorphism of the *Msp*I sites near the VNTR region at c-Ha-*ras* has been observed a variety of tumors (19, 26, 31). In general polymorphism at the site could result from point mutation, non-CG methylation, or an unusual DNA structure affecting the expected site of *Msp*I attack. The Southern blotting results reported here (Figure 3) extend the results reported by others (19, 26, 31) by demonstrating that the polymorphism affects one or both

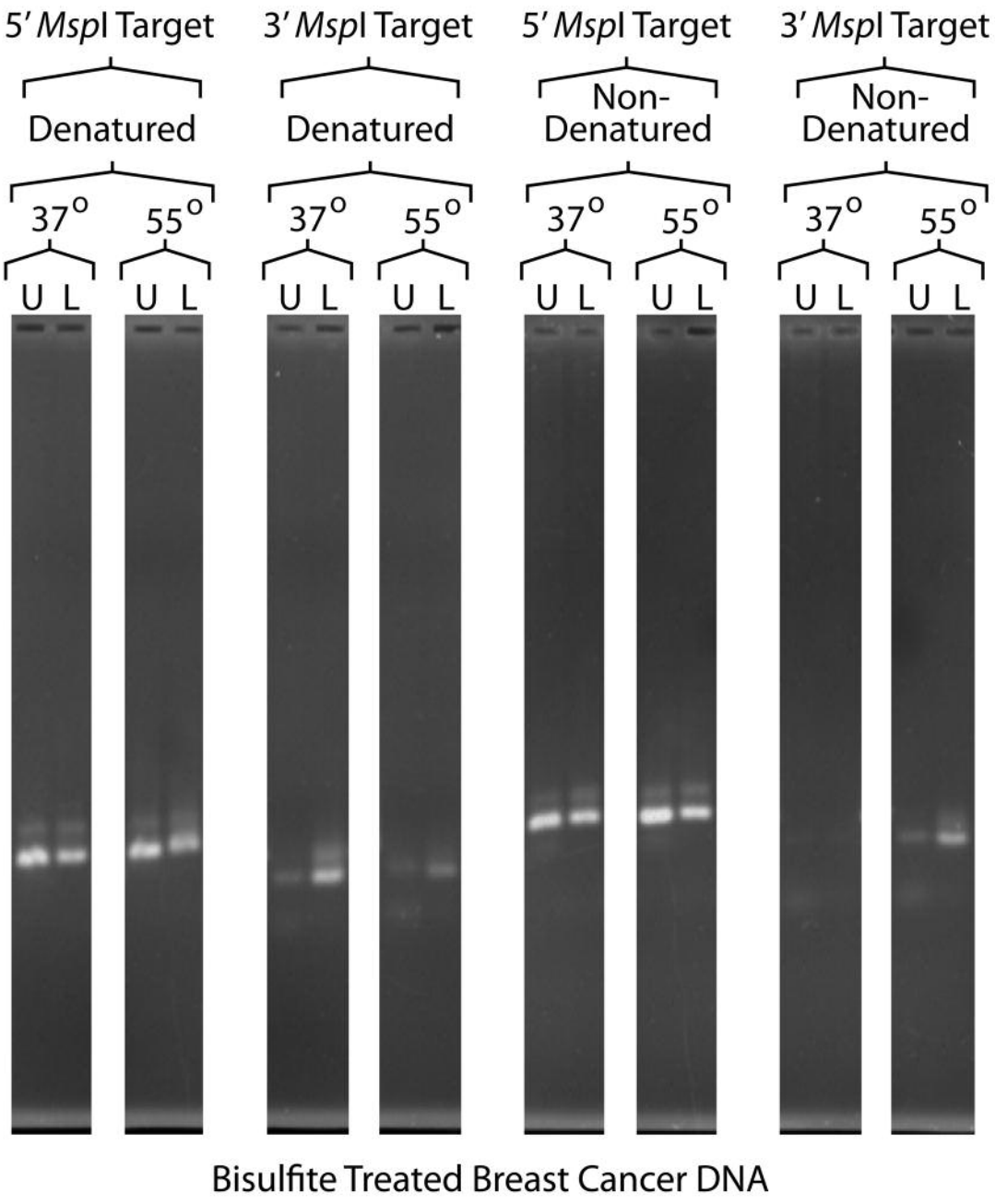
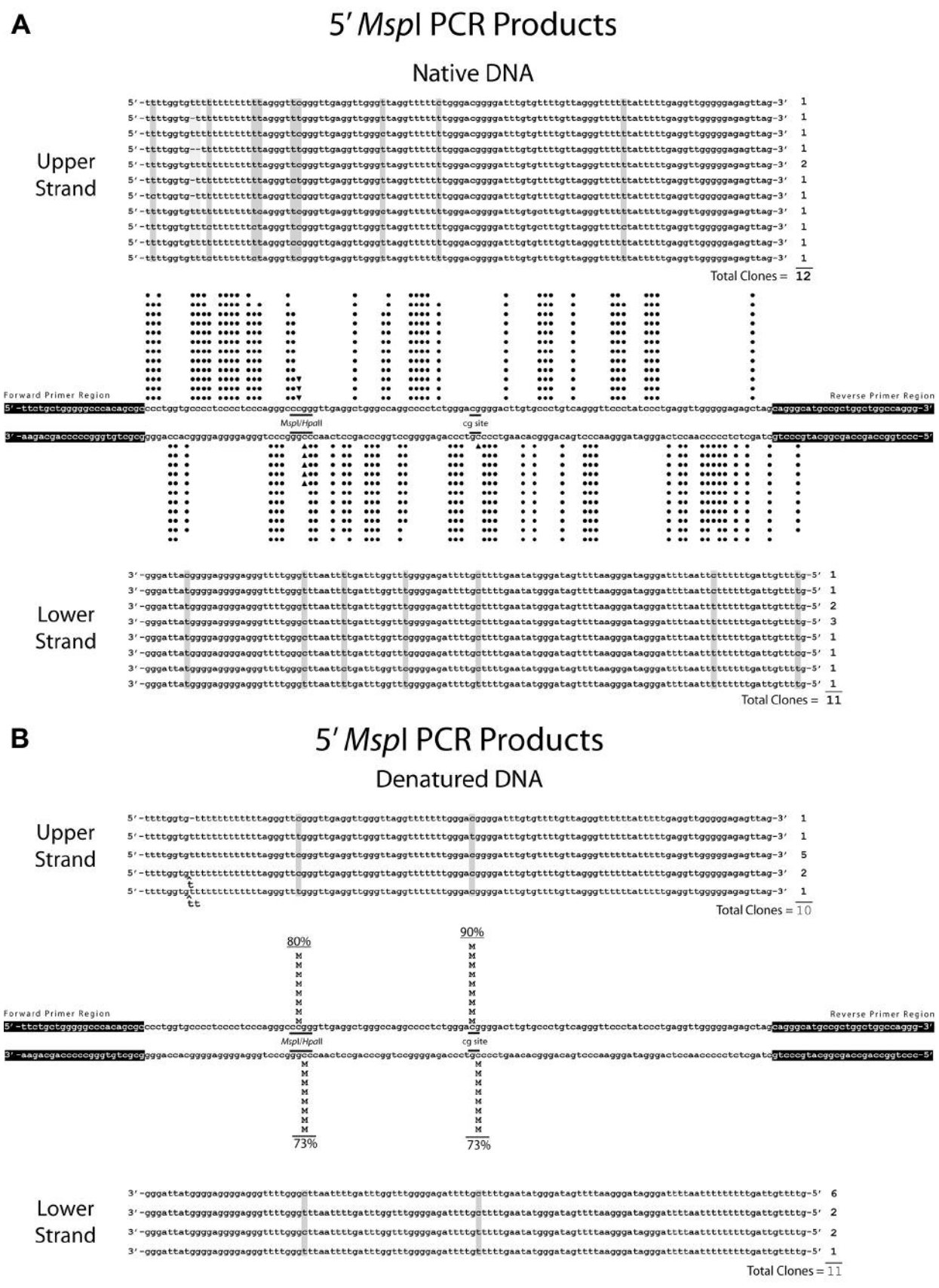


Figure 6. *PCR amplification systems targeting the two regions spanning the MspI sites labeled 3' MspI and 5' MspI were designed so as to amplify the bisulfite treated (i.e. converted) upper and lower strands at each site. Only the PCR systems targeting the 5' MspI region were amplified from native DNA treated with bisulfite at either 37°C or 55°C.*

of the two *Msp*I sites immediately adjacent to the VNTR since partial digestion products were about 580 bp longer than the primary bands detected in the blot (Figure 1). With reference to Figure 1 homozygous individuals were found to possess two fragment lengths in limit digests that differed by about 580 bp in length (Figure 3A and Figure 3B), while heterozygous individuals (Figure 3C, Figure 3D and Figure 3E) were found to possess four fragment lengths among which two sets were found to differ by about 580 bp each. Partial digestion could be ruled out by the digestion controls performed on each digest (Figure 2). Sequence polymorphism could also be ruled out by


A
5′ MspI PCR Products
Native DNA
Upper Strand
Total Clones = 12
Forward Primer Region
Reverse Primer Region
MspI/HpaII
cg site
Lower Strand
Total Clones = 11
B
5′ MspI PCR Products
Denatured DNA
Upper Strand
Total Clones = 10
80%
90%
73%
73%
Lower Strand
Total Clones = 11

inspecting cloned representatives of the two sites (Figure 4). Non-CG methylation was ruled out by sequencing the methylation-sensitive PCR products of each strand (Figure 7B). Thus we conclude that the data demonstrating bisulfite modification of native DNA at 5' *Msp*I (Figure 7A) coupled with the Southern blotting results outlined above indicate the presence of an unusual DNA structure that spans this 5' *Msp*I site.

A gap in the duplex in the region is ruled out because both strands were amplifiable and both were accessible to bisulfite (Figure 7A). This suggests a possible cruciform structure at the site or any one of the canonical unusual structures that can be adopted by cruciform DNA (*e.g.* quadruplex or triplex). However the data do not allow us to determine the exact nature of the unusual structure present at this site. Although the non-B DNA structure survives DNA isolation as has been shown for the structure observed by Raghavan *et al.* (6) at the Bcl_2 locus, it remains possible that the structure present *in vivo* is altered somewhat during DNA isolation. Moreover, given the close proximity to the VNTR it is possible that secondary structure extending into VNTR itself contributes to the unusual structure at 5' *Msp*I.

Since the structure is present at high levels in both tumor and tumor-adjacent (histologically normal) DNA but present in much reduced levels in lymphocytes it constitutes either a tissue specific or tumorigenesis specific epigenetic mark. Preliminary evidence (Clark unpublished) in prostate cancer specimens suggests that it is specific to tumorigenesis. The presence of the structure in non-tumor tissue in the breast suggests a field cancerization of the cells involved in clonal propagation of the structure. A clonal loss of function associated with the inability of the cells to suppress the formation of the unusual structure after replication would provide a simple explanation for its propagation. Preliminary experiments with nuclease S1 sensitivity suggest that the breast tumor DNA is rapidly digested indicating that additional sites of non-B DNA structure are present in the breast cancer DNA (Smith unpublished).

Finally, we note that this unusual structure is methylated at both CG sites inspected by the methylation sensitive PCR (Figure 7B). Given recent reports demonstrating that Dnmt1 is responsible for both *de novo* methylation (42) and the maintenance of methylation patterns (43) in human cancer cells, it seems likely that the unusual structure promotes *de novo* methylation by Dnmt1 (39, 44, 45). Thus the hypermethylation of these sites is consistent with a simple loss-of-function hypothesis in which a component of the cellular system that suppresses unusual structure formation is lost permitting the spontaneous formation of unusual structures that are known to be activated substrates for human Dnmt1 (2, 34, 46, 47). This is consistent with reports that the 3' *Msp*I fragment resides in a known hot spot for DNA methylation (19). Moreover, it suggests that the known tendency of DNA binding proteins important in epigenetic signaling in both mice and humans [*e.g.* DNA (Cytosine-5) Methyltransferase I [Dnmt1] (32-39) and Methylated Cytosine Binding Protein 2 [MeCP2] (40, 41)], to interact strongly with unusual structures present in DNA is biologically meaningful.

Figure 7. *A. Bisulfite accessibility of cytosines in native DNA and 37°C. PCR amplicons obtained from the bisulfite treated native DNA were cloned and sequenced. Twelve clonal isolates of the upper strand and eleven clonal isolates from the lower strand were sequenced. Cytosines marked with an M are methylated (See B). Most cytosines in the region were converted to uracil indicating that the region is accessible to bisulfite under native conditions. Cytosines that were modified to uracil in a give clone are indicated by a ●. Cytosines in the heavily methylated CG sites were also sometimes modified by the reagent. Those sites are indicated by a ▲. B. Bisulfite accessibility of cytosines in denatured DNA at 55°C. PCR amplicons obtained from the bisulfite treated denatured DNA were cloned and sequenced. Ten clonal isolates of the upper strand and eleven clonal isolates from the lower strand were sequenced. Under these conditions all unmethylated cytosines are converted to uracils. Symmetrical methylation of the two CG sites in the region is apparent. Sites that were not converted to uracil in a given clone are indicated with an M.*

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