Single-Site Methylation within the p53 Promoter Region Reduces Gene Expression in a Reporter Gene Construct: Possible in Vivo Relevance during Tumorigenesis

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ABSTRACT

It is not known whether transcriptional suppression by de novo methylation occurs within the promoter region of the p53 gene during multistage tumorigenesis. To address this question, in vivo alterations in the CpG methylation within the rat p53 promoter region were evaluated in control, preneoplastic, and tumor tissue during tumor progression using the folate/methyl-deficient model of hepatocarcinogenesis. Alterations in CpG methylation were found to be site-specific and to vary depending on the stage of carcinogenesis. To further explore the effect of site-specific methylation on p53 promoter activity, reporter gene constructs were prepared containing specifically methylated sites within the p53 promoter region, and the transcriptional activity in cultured mammalian cells was determined in a transient transfection assay. Relative to the unmethylated construct as a positive control, single-site methylation at nucleotide (nt) -450, which occurs 216 nt upstream from the 85-nt minimal promoter region, suppressed promoter activity by 85%. In contrast, single-site methylation at nt −179, which occurs within the minimal essential promoter region, suppressed activity by only 20%. The p53 promoter constructs containing the singly methylated CpG site at nt −450 were then reevaluated for progressive changes in methylation status 48 h after transfection, during maximum suppression of promoter activity. Restriction analysis with methylation-sensitive enzymes revealed that de novo methylation had occurred after transfection at previously unmethylated sites. These findings suggest that nt −450 may constitute a critical site for initiation of de novo methylation and progressive spreading of methylation associated with transcriptional inactivation of the p53 gene. Furthermore, the results suggest a possible alternative mechanism for the silencing of the p53 gene in tumors that do not have p53 mutations.

INTRODUCTION

Progressive dysregulation and disruption of the heritable patterns of DNA methylation have been a consistent observation during multistage carcinogenesis. During tumor progression, the DNA becomes paradoxically hypomethylated, despite the presence of regional hypermethylation and an increase in DNA methyltransferase activity (1, 2). In several human tumors, ectopic de novo methylation within promoter regions containing CpG islands has been linked to transcriptional silencing of tumor suppressor genes (3–5). CpG islands occur within the promoter region of ~60% of human genes, and despite increased CpG content relative to the rest of the genome, CpG islands tend to remain remarkably unmethylated in normal cells (6). The inappropriate promoter region methylation during tumor progression has been shown to be functionally equivalent to coding region mutation as a mechanism to inactivate tumor suppressor genes (3, 7). Whereas mutations in the p53 gene are a common late event in many human cancers, recent evidence suggests that for primary hepatocellular carcinoma, p53 mutations are quite rare (with the exception of aflatoxin adducts at codon 249) and do not appear to contribute to liver tumor progression (8). Using PCR single-strand conformational polymorphism and sequencing analysis, we have similarly concluded that p53 mutations are a rare event in the methyl-deficient model of hepatocarcinogenesis (9).

Although transcriptional silencing of tumor suppressor genes has been associated with extensive de novo methylation throughout their 5′ promoter regions, the pattern and extent of promoter region hypermethylation appear to vary in different genes and tumor types (1, 3–5). In certain genes, promoter region CpG methylation is sufficient to reduce gene expression by directly blocking transcription factor binding (10–12) or by directing the binding of repressor proteins (13, 14). In contrast, aberrant silencing of the human O6-methylguanine methyltransferase gene does not involve direct methylation of transcription factor binding sites but depends on chromatin condensation indirectly influenced by distant sites of methylation (15). In other studies, promoter region methylation has been shown to alter normal nucleosomal positioning such that transcription factor accessibility is restricted (16–18). Recently, it has been demonstrated that one molecular mediator of transcriptional repression is the methyl-binding protein, MeCP2, that binds to methylated CpG sites in a complex with histone deacetylase to induce local histone deacetylation and a repressive chromatin configuration that is inaccessible to the transcription complex (19, 20).

Experimental evidence to date suggests that the regulation of p53 expression is multifactorial and involves both translational and post-translational mechanisms to control the intracellular levels of p53 protein (21–25). A posttranslational mechanism appears to contribute to increased p53 protein levels after acute DNA damage (21); however, with chronic genotoxic stress, transcriptional mechanisms appear to be operative (26). Although de novo methylation and transcriptional repression have been clearly established in other tumor suppressor genes, promoter region methylation in the p53 gene during tumorigenesis has not been described. The 5′ region of the p53 gene is unique among tumor suppressor genes and housekeeping genes in that it does not contain a CpG island, nor does it contain consensus TATA or CAAT motifs or multiple Sp1 sites (27). The p53 promoter region has been sequenced, and basal promoter activity has been localized to an 85-bp region that is essential for full promoter activity (27, 28). Putative binding sites for NF-1, NFκB, and the basic helix-loop-helix family of transcription factors occur within this minimal 85-bp region that extends into the noncoding exon 1 (28–30). Because of the high degree of sequence homology among the p53 promoter regions of the human, rat, and mouse genes, it is likely that the mechanisms of transcriptional regulation are conserved between these species (27). A second promoter is located in the intron between the noncoding exon 1 and the expressed exon 2 in human DNA; however, the RNA transcribed from this promoter is unrelated to p53 expression (31). Recent evidence has demonstrated that a reduction in wild-type p53 gene dosage without mutation is sufficient to promote tumorigenesis (32) and is consistent with the possibility that p53 promoter region methylation and reduced p53 gene expression contribute to the selection and expansion of preneoplastic cells. In the present study, the folate/methyl-deficient model of multistage...
hepatocarcinogenesis was used to evaluate in vivo changes in p53 promoter region methylation in preneoplastic and tumor tissue. Previous studies of this model demonstrated a paradoxical increase in methyltransferase activity associated with genome-wide hypomethylation, regional hypermethylation, and dysregulation of gene expression (33–36). Using multiple methylation-sensitive restriction enzymes and a PCR-based assay for methylation status, alterations in CpG methylation within the p53 promoter were found to occur at specific sites and with variable methylation, depending on the stage of carcinogenesis. To further explore the impact of site-specific methylation on p53 promoter activity, reporter gene constructs were created with differentially methylated sites within the p53 promoter region, and the relative transcriptional activity was compared in transfected mammalian cells. The evidence presented here suggests that, at least in the p53 gene, methylation at specific sites may be critical for the initiation of transcriptional repression.

MATERIALS AND METHODS

Rats and Diets. Weaning male F344 rats were housed two per cage in a temperature-controlled (24°C) room with a 12:12 h light/dark cycle and given ad libitum access to water and NIH-31 pelleted diet. On reaching 50 g of body weight (approximately 4 weeks of age), rats were randomly allocated to receive either a semipurified diet lacking in folic acid and choline and low in methionine (0.18%; Dyets, Inc., Bethlehem, PA) or the same diet supplemented with 0.4% methionine, 0.3% choline, and 2 mg/kg folic acid as a control. Four to six rats per diet group were killed by exsanguination under light ether anesthesia at 36 weeks (preneoplastic stage) and 54 weeks (hepatic carcinoma) after diet initiation. Tumors were whitish, solid masses, 1–1.5 cm in diameter, with no gross necrotic areas; parenchymal tissue was collected from the liver of the rats and immediately frozen in liquid nitrogen and stored at −80°C. Genomic DNA was purified by digestion with proteinase K, phenol-chloroform extraction, and ethanol precipitation as described previously (37).

PCR-based Assay for Site-specific p53 Promoter Region Methylation. To determine the methylation status of the 5′ region of the p53 gene, genomic DNA was treated with methylation-sensitive restriction enzymes followed by PCR amplification of a 418-bp fragment containing the basal promoter region of the p53 gene (27). A diagram of the promoter region of the p53 gene is shown in Fig. 1. The 85-bp region essential for basal transcriptional activity occurs between nt −216 and nt −131 and extends into the first noncoding exon [numbering according to Bienz-Tadmor et al. (27)]. The 15 CpG dinucleotides within the promoter region are represented by ○ and do not constitute a CpG island. The locations of the HindIII (Hha methylase site), BsrUI (FnuDII methylase site), and the AccI methyl-sensitive restriction sites are indicated. Note that the HindIII site and the BsrUI site occur only once within the amplified sequence, whereas there are four AccI sites. A 6.2-kb intron separates the noncoding exon 1 from exon 2, which contains the translation start site. Methylated cytosines at the restriction sites prevent enzyme cleavage and can be detected by PCR amplification product recovery that is equivalent to untreated control samples. Conversely, restriction enzyme cleavage at unmethylated sites induces DNA strand breaks and abolishes PCR amplification. Ten μg of DNA from control, preneoplastic, and tumor tissues were digested to completion for 12 h with 10 units/μg of the methylation-sensitive enzymes AccI, HindIII, or BsrUI, according to the manufacturer’s recommendations (New England Biolabs, Beverly, MA). The PCR primer set was designed to amplify the 418-bp region between −514 and −92 of the rat p53 gene, which includes the 85-bp minimal promoter. For PCR amplification, the sense primer was 5′-GTTTCAAAAGCCAAAAAG-3′, and the antisense primer was 5′-GGACCAAGTTGAAGGCTAATAG-3′. Each PCR reaction contained 1 μg of DNA (with or without enzyme pretreatment), 1.5 μl MgCl2, 200 μM of each deoxynucleotide triphosphate, 100 pm of each primer, and 2.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA) in 50 μl of PCR buffer. The cycling conditions consisted of an initial denaturation at 95°C for 7 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 54°C for 60 s, and extension at 72°C for 90 s. The semiquantitative aspects of the procedure were verified by a linear increase in PCR product recovery with increasing cycle number and DNA template concentration. After amplification, 10 μl of each PCR product were applied to a 2% NuSieve GTG-agarose gel in 1× Tris-borate EDTA, electrophoresed, and visualized with ethidium bromide staining.

Ms-SNuPE Assay. To more precisely quantify the in vivo methylation status of the CpG site at nt −450 in control, preneoplastic, and tumor tissues, the Ms-SNuPE assay was used, as described previously in detail (38). Briefly, 5 μg of genomic DNA were initially treated with sodium bisulfite. PCR primers for the top strand of the bisulfite-modified p53 promoter region including nt −450 were: (a) sense, 5′-AAAAAGTAAAGAGTAGT-3′; and (b) antisense, 5′-AACACTAGATTAAACAAA-3′. The resulting PCR products were electrophoresed on a 2% agarose gel, and DNA fragments were excised from the gel and isolated using GenElute spin columns. The Ms-SNuPE reactions were performed in a 25-μl reaction mixture containing 50 ng of purified PCR template, 1× PCR buffer, 1 μM single-nucleotide primer extension primer (5′-AGTTTGATAGGAAAGTAGT-3′), 1 μl of either [32P]TTP or [32P]dCTP, and 1 unit of AmpliTaq polymerase. After single-nucleotide extension at −450, equal amounts of radiolabeled DNA were loaded onto a 10% polyacrylamide gel containing 7 M urea. The extent of [32P]TTP and [32P]dCTP incorporation into the DNA fragments was quantified using phosphorimager analysis. The results were quantified as the average of C/(C + T) signal ratios (n = 3/group) and presented as the percentage methylation.

Plasmid Construction and in Vitro Methylation. A 418-bp fragment between −514 and −92 in the p53 promoter region was amplified using the following primers containing artificial PstI and SalI restriction sites: (a) sense, 5′-CTCGAGTTTACAAAAAGCTA-3′; and (b) antisense, 5′-GTC-GACCGAAGTTGAAGGCTAAC-3′. The resulting 430-bp PCR product was subsequently cloned into the pcR 2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The pcR 2.1 TOPO plasmid containing the p53 promoter insert (hereafter referred to as TOPO*p53) was linearized with SalI and methylated in vitro using SsI methylase, which nonspecifically methylates all CpG dinucleotides, or methylated at specific CpG sites using either HpaII or FnuDII methylases. The efficiency of in vitro methylation was confirmed by resistance to cleavage by the methylation-sensitive restriction enzymes BstUI (FnuDII methylase site), HinfI (HhaI methylase site), and AvrI. The linearized unmethylated and unmethylated TOPO*p53 vectors were then digested with PstI to excise the p53 promoter fragments. After fractionation on a 1% agarose gel, the DNA bands corresponding to 430 bp were cut from the gel, isolated using GenElute spin columns (Sigma, St. Louis, MO), and ethanol-precipitated. To determine whether global or site-specific CpG methylation of the p53 5′-promoter region affected gene expression in a reporter gene construct, 0.5 μg of the methylated and unmethylated control fragments were then ligated into 5 μg of pCAT Basic vector (Promega, Madison, WI) between the PstI and SalI restriction sites (hereafter referred to as pCAT*p53). The pCAT Basic plasmid contains the CAT reporter gene but lacks eukaryotic promoter and enhancer sequences. The ligation reaction was performed at 14°C for 20 h according to the manufacturer’s instructions. The efficiency of ligation and equivalent of incorporated DNA into the methylated and unmethylated constructs were confirmed by agarose gel electrophoresis and densitometry. The pCAT Control plasmid (Promega) containing the SV40 promoter and enhancer sequences results in strong CAT expression and was used as a positive control for transfection efficiency and an internal standard in the CAT assay. The positive control for p53 promoter activity was a totally unmethylated insert. The negative control was the pCAT Basic vector with no promoter insert. The effect of total methylation (SsI methylase) or targeted site-specific methylation on the transcriptional activity of the inserted p53 promoter fragment was expressed as the percentage change in reporter gene CAT activity relative to the unmethylated control.

Cell Culture, Transient Transfection, and CAT Assay. CHO K1 cells were grown in Ham’s F-12 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum at 37°C in a humidified 5% CO2 incubator. Plasmid transfection was performed using LipofectAMINE PLUS (Life Technologies, Inc.) according to the manufacturer’s instructions. Briefly, 0.5 × 106 cells were incubated with 1 μg of pCAT*p53 and LipofectAMINE reagent without serum for 3 h. To normalize for transfection efficiency, pCAT*653 constructs were cotransfected with 1 μg of pSV-β-galactosidase.
Fig. 1. Diagram of the promoter region of the p53 gene. An 85-bp region essential for basal transcriptional activity is present between nt −216 and nt −131 and extends into the first noncoding exon (numbering according to the work of Bienz-Tadmor et al. (27)). There are 15 CpG dinucleotides within the promoter region (C) that do not constitute a CpG island. The methylation-sensitive restriction sites analyzed are indicated and include the HinP1I at nt −450 (HhaI methylase site), the BstUI at nt −179 (FnuDII methylase site), and the four AciI sites at nt −410, −304, −364, and −180. The location and sequence of the 7-bp inverted repeat with cytosine mispairs at nt −450 and −410 is shown. A 6.2-kb intron separates the unexpressed exon 1 from the expressed exon 2. Dashed arrows, location of PCR primers.

RESULTS

To obtain preliminary evidence regarding the methylation status of the p53 promoter region during tumor progression in vivo, hepatic DNA from control, preneoplastic, and tumor tissue was treated with the methylation-sensitive enzymes AciI, HinP1I, and BstUI, which cleave their respective restriction sites only if the cytosines on both strands are unmethylated. The extent of PCR product amplification without (−) or with (+) enzyme pretreatment was visualized on ethidium bromide-stained agarose gels (Fig. 2). In Fig. 2A, the low level of qPCR product with enzyme pretreatment in control and preneoplastic tissue indicated that at least one of the four AciI sites within the p53 promoter was partially methylated; however, in the tumor tissue, all four sites appear to have become methylated (not cleaved). In Fig. 2B, the HinP1I site at nt −450 was predominantly unmethylated in control and preneoplastic liver but was partially methylated (partially cleaved) in tumor DNA. Although not clearly visible on the agarose gel, 32P labeling during the PCR amplification indicated a 2-fold increase in PCR product recovery in the tumor DNA (data not shown) that was subsequently confirmed with using the Ms-SNuPE assay. These results indicate that de novo methylation had occurred at the AciI and HinP1I sites in tumor DNA. In contrast, the BstUI site at nt −179 (Fig. 2C) was methylated in control tissue but had undergone extensive loss of methyl groups in preneoplastic and tumor DNA. The significant loss of cytosine methyl groups at the BstUI site in tumors would suggest that decreased p53 mRNA levels, as reported previously in these tumors (36), are not dependent on total methylation of the p53 promoter region. Interestingly, the sites of de novo methylation (AciI and HinP1I) occur at positions upstream of the minimal p53 promoter region, whereas the site of demethylation (BstUI) occurs inside the 85-bp minimal promoter. Fig. 3 shows a more quantitative estimate of the extent of CpG methylation at the HinP1I site (nt −450) in DNA extracted from control, preneoplastic, and tumor tissues. Relative to control and preneoplastic tissues, CpG methylation at nt −450 increased approximately 35% in tumor DNA (P < 0.001), consistent with significant de novo methylation at this region.
site during tumorigenesis in vivo. Taken together, these changes are consistent with disruption of the normal patterns of methylation during the carcinogenic process in vivo and further suggest that alterations in methylation may be site specific within the p53 promoter.

In Fig. 4, the relative CAT activity in a transient transfection assay stimulated by differentially methylated p53 promoter inserts in the pCAT Basic vector is presented as constructs 1–5. The promoter activity of the unmethylated insert served as a positive control and is presented as 100% (construct 1). The pCAT Basic vector without a promoter insert (construct 5) served as a negative control with CAT activity of <3% of the unmethylated control. In the totally methylated p53 promoter insert (construct 2), the CAT activity was reduced 70% relative to the unmethylated control, confirming that extensive promoter region methylation is associated with transcriptional repression. Most interesting, however, was the 85% suppression of CAT activity induced by HhaI methylase methylation of a single CpG site occurring at nt −450, which occurs 216 bp upstream of the minimal 85-bp promoter (construct 3). To determine whether site-specific methylation of a single CpG site targeted within the 85-bp minimal promoter region would also suppress gene expression, plasmid constructs were treated with FnuDII methylase. As shown in construct 4, site-specific methylation at nt −179, within the minimal promoter region, suppressed CAT activity by only 20%. These observations suggest that methylation at nt −450 of the p53 promoter region may represent a critical site for progressive inactivation of the p53 gene.

Theoretically, methylation of the single CpG site at nt −450 could serve as a focus for the subsequent spreading of methylation to other CpG sites within the p53 promoter region (39). To determine whether downstream de novo methylation occurred after transfection into the mammalian cells or whether the singly methylated site was retained in isolation during suppression of gene expression, plasmid DNA was isolated from the mammalian cells after 48 h and treated with methylation-sensitive enzymes. Subsequent PCR of the p53 promoter (using plasmid-specific primers) is presented in Fig. 5. Fig. 5A, Lane 2, shows that pretreatment with the methylation-sensitive enzyme HpaII (nt −450) did not reduce qPCR amplification relative to the untreated control (Lane 1), indicating that this site had remained methylated after transfection. The methylation status of the four AciI sites and the BsrUI site (nt −179) after transfection is presented in Lanes 3 and 4, respectively. These sites, originally unmethylated in the p53 promoter insert, were now resistant to methyl-sensitive restriction, indicating that de novo methylation had occurred at these sites, on at least one strand, within 48 h after transfection. To our knowledge, these results are the first to demonstrate spreading of methylation in a mammalian somatic gene sequence associated with reduced promoter activity. In Fig. 5B, the methylation status of the unmethylated control construct 48 h after transfection is presented. The HinP1II and AciI sites remained unmethylated throughout the transient transfection assay and were associated with maximal promoter activity. Interestingly, the BsrUI site became partially methylated after transfection, as indicated by the increase in the PCR product; however, methylation at this site was not associated with suppression of promoter activity in the reporter gene construct as shown in Fig. 4. In contrast to de novo methylation at the BsrUI site in the transfected plasmid p53 promoter, this site lost methyl groups in preneoplastic liver and tumor tissue in the in vivo rat model (Fig. 2C). Methylation instability is common during preneoplasia, and the methylation pattern in tumor cells may reflect the tumor selection process.

DISCUSSION

A major gap in the understanding of methylation dysregulation and neoplastic transformation is a lack of knowledge about the mechanisms underlying sequential changes in methylation patterns during the preneoplastic period in vivo. Current knowledge is based primarily on comparisons of methylation profiles between normal cells and tumor cells, and it is not clear whether methylation instability in tumor cells stems from an isolated determining event or from progressive...
alterations in heritable methylation patterns. The methyl-deficient model provides a unique opportunity to follow progressive changes in DNA methylation as they occur in vivo and to correlate these changes with alterations in p53 expression during tumor progression. The results reported here indicate that methylation dysregulation in the p53 promoter region begins during preneoplasia and is accompanied by additional alterations after the transition to tumor. Although the p53 promoter does not contain a CpG island, these results indicate that de novo methylation and demethylation do occur at specific sites during tumor development and may relate to alterations in p53 mRNA levels previously reported in this model (36). To further probe the functional significance of the site-specific de novo methylation at nt −450 in the tumor DNA, the 5′ promoter region of the p53 gene was PCR amplified, methylated in vitro with HhaI methylase (specific for the CpG site occurring at nt −450), and inserted into plasmid constructs to determine the effect on promoter activity. An approximately 85% inhibition of CAT gene expression was obtained with single-site methylation at nt −450 in the original plasmid construct. The fact that this same site was found to be significantly de novo methylated in tumor DNA suggests that methylation at nt −450 could be an important initiating signal or binding site for transcriptional repression of the p53 gene in vivo.

Transcriptional repression mediated by single-site methylation in promoter regions is not unprecedented in the literature. The presence of a single methylated CpG dinucleotide within the promoter region of the Herpes simplex virus tk gene is sufficient to allow transcriptional inactivation of the tk gene (40). Conversely, demethylation of a single critical CpG site in the EBV latency C promoter is sufficient for transcriptional activation (41). In human cells, a candidate tumor suppressor gene, S100A2, is unmethylated in normal cells but becomes hypermethylated and down-regulated during breast cancer progression (42). Within the promoter region of S100A2, in vitro methylation at a single site at nt −287 in the upstream promoter region suppressed promoter activity >70% in a gene reporter system. The exact same site was shown to be de novo methylated in DNA from breast cancer biopsies. These data support potential functional significance for site-specific methylation within the S100A2 promoter in breast tumor development. In another report, Gonzalgo et al. (43) show that in vitro methylation of a single HpaII site within the promoter region of the p16 gene in human bladder cell lines was sufficient to reduce p16 promoter activity by 48%, and methylation of three HpaII sites reduced activity by 67% in a CAT reporter system. Using similar in vitro promoter methylation and plasmid transfection experiments, Robertson et al. (44) subsequently demonstrated that methylation at a single HpaII site within the human ARF gene promoter was more effective in repressing promoter activity (80–85%) than methylation at four HhaI sites in the same region (60–70%). Taken together, these independent observations indicate that, at least in certain genes, methylation at specific CpG sites rather than total promoter region methylation density is sufficient to initiate the progressive events leading to transcriptional repression. Consistent with this notion, MeCP2 has been shown to bind to as few as one to three methylated cytosines (45).

Despite a plethora of descriptive studies indicating that de novo methylation in promoter regions is associated with transcriptional silencing of tumor suppressor genes in tumor DNA, the mechanistic basis and the initiating signal for ectopic de novo methyltransferase activity during cancer development remain obscure. A gene for a de novo DNA methyltransferase that is expressed in developing mouse embryos has been identified; however, it has not yet been shown to be inappropriately activated and reexpressed during carcinogenesis (46). Although the mechanism for de novo methylation in the p53 promoter of rat liver tumors is far from clear, the spontaneous formation of single-stranded conformers in repetitive single-stranded DNA that create high affinity binding sites and targets for the DNA methyltransferase presents an interesting possibility (47, 48).

Supporting the preferential binding of DNA methyltransferase to abnormal DNA conformations, Smith et al. (49) demonstrated that cytosines (5′ to a normally paired G) opposite mispairs, abasic sites, or gaps within synthetic oligonucleotides were preferential targets for the human DNA methyltransferase. De novo methylation has also been shown to occur preferentially at sites of mispaired cytosines within the spontaneous hairpin loops formed by CGG triplet repeats in the 5′-untranslated region of the human fragile-X FMR-1 gene (50, 51). The authors speculate that the presence of a mismatch at the target CpG site creates abnormal bp stacking interactions that mimic the transition state analogue for the methyltransferase and that the enzyme “stalls” after methyl transfer, forming a stable complex with the conformationally unusual DNA. The preferential binding at cytosine mispairs may also reflect the lower energy requirement for enzymatic extrahelical base rotation at these sites. In other studies, using purified bacterial DNA methyltransferases, replacement of the target cytosine in synthetic oligonucleotides by a mispair, abasic site, uracil, or a gap similarly created binding sites with higher affinity for the methyltransferase than the cognate hemimethylated CpG sites (52, 53).

Based on these considerations, we searched the p53 promoter region for inverted repeat sequences capable of forming stem-loop structures containing a mispair opposite the target cytosine. A 7-bp inverted repeat was identified upstream of the basal promoter that is capable of forming such a stem-loop structure (see Fig. 1). The target mispaired cytosines occur at nt −450 and nt −410, the same CpG sites that were found to be de novo methylated in DNA from the methyl-deficient liver tumors in vivo. Interestingly, 5-bp inverted repeats also exist in the same upstream region of both the mouse and human p53 promoter and similarly create cytosine mispairs at nt −442 and nt −441, respectively. Mouse numbering is based on the work of Bienz-Tadmor et al. (27); human numbering is based on the
work of Tuck and Crawford (28). Supporting a conserved mechanism, a “negative regulatory element” has been reported in the mouse p53 promoter that overlaps the sequence containing the inverted repeat at nt \(-442\) (27). The existence and conservation of these inverted repeats in the same proximal promoter region in three different species strengthen the possible functional significance of mis-paired cytosines within alternative stem-loop structures as binding sites and targets for DNA methyltransferase.

In previous studies, methylation within inverted repeat sequences has been shown to negatively affect transcriptional activity. Based on site-specific endonuclease cleavage within inverted repeats of ColE1 plasmid DNA, Lilley (54) proposed that 9–13-bp inverted repeats could adopt foldback hairpin structures, stabilized by negative superhelicity, that could dynamically alter local DNA conformation. He further postulated that the secondary structure imposed by self-association of inverted repeats could create unique recognition sites for protein-DNA interactions. In mouse P815 mastocytoma cells, methylation of inverted repeats was correlated with transcriptional repression, and it was suggested that the 2-fold rotational symmetry of inverted repeats would make them ideal substrates for regulatory proteins involved in gene expression (55). Bestor (56) brought mechanistic significance to these observations by showing that negative superhelicity in a plasmid DNA fragment, encompassing a cluster of inverted repeat sequences, dramatically increased the sequence specificity of the mammalian DNA methyltransferase. In subsequent studies, several authors have suggested that de novo methylation in vivo may be directed by DNA structural polymorphisms within repetitive elements (57–59).

Because intramolecular foldback structures such as hairpins or stem-loops in single-stranded synthetic oligonucleotides have been shown to direct methylation to a distant site, it has been suggested that the formation of such alternative structures in vivo could provide a mechanism for spreading of methylation in specific regions (60, 61). Using synthetic oligonucleotides methylated in vitro, a mispaired cytosine at the base of a 3-bp-long stem-loop was shown to be a recognition site for de novo methylation (61). These observations may provide mechanistic insights into the apparent spreading of de novo methylation within the p53 promoter after transfection of a singly methylated construct (Fig. 3, construct 3).

In the methyl-deficient model of hepatocarcinogenesis, global DNA hypomethylation has been associated with a decrease in the methyl donor S-adenosylmethionine (62), an increase in DNA methyltransferase activity (63, 64), and an increase in hepatocyte proliferation within 1–2 weeks of dietary intervention (35, 65, 66). These and other observations prompted the hypothesis that global hypomethylation was due to passive demethylation during DNA synthesis due to the lack of sufficient methyl donor (35, 63, 66, 67). Based on the results of the present study and those of Smith et al. (48, 49), it may be further hypothesized that high affinity binding of the DNA methyltransferase to unusual DNA conformations may contribute to the paradoxical global hypomethylation as well as regional hypermethylation during multistage carcinogenesis. Although rare in the normal cell, abasic sites, gaps, and strand breaks are often present as chronic unpaired DNA lesions in the premalignant cell. In the folate/methyl-deficient model of carcinogenesis, the early appearance and chronic presence of this type of lesion has been shown to parallel DNA hypomethylation, uracil misincorporation, and inefficient DNA repair activity (36, 64, 65, 68). Sites of DNA damage that mimic the transition state of DNA methyltransferase reaction (base mispairs, abasic sites, gaps, and strand breaks replacing the target C) could bind and preoccupy available enzyme such that maintenance of DNA methyltransferase activity becomes compromised. Conversely, enhanced enzyme binding to the same kind of lesions opposite a target C would increase the probability for de novo methylation. Thus a deficiency in DNA repair capacity would be expected to increase both the frequency of ectopic methyltransferase binding and DNA hypomethylation as well as the frequency of methylation at some specific CpG sites. Further research will be required to determine whether sites of unpaired DNA damage and/or mispairs in foldback secondary structures in DNA are mechanistically related to inappropriate de novo methylation and subsequent selection for neoeplastic expansion in the rare premalignant cell.

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