Breaks in Genomic DNA and within the p53 Gene Are Associated with Hypomethylation in Livers of Folate/methyl-deficient Rats

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ABSTRACT

Male weanling Fischer 344 rats were fed either a semipurified diet deficient in the methyl donors methionine, choline, and folic acid or a supplemented control diet for a period of 9 weeks. At intervals of 2, 5, and 7 days, 3 weeks, and 9 weeks after initiation of the respective diets, the relative level of DNA strand breaks and the degree of cytosine methylation were quantified in high molecular weight DNA and also within the p53 gene in liver samples from these rats. Genome-wide strand break accumulation was associated with progressive genomic hypomethylation and increased DNA methyltransferase activity. With the use of quantitative PCR as a gene-specific DNA strand break assay, unique DNA strand breaks were detected in exon 5 but not in exons 6–8 of the p53 gene, and were accompanied by significant p53 gene hypomethylation. DNA hypomethylation has been shown to alter the conformation and stability of the chromatin structure, rendering affected regions more accessible to DNA-damaging agents. To determine whether methylation status alters the sensitivity of DNA to strand breakage, DNA in isolated nuclei was methylated in vitro and exposed to endogenous calcium/magnesium-dependent endonuclease activated under defined conditions. The incidence of enzyme-induced DNA strand breaks was decreased significantly with increased DNA methylation. In nuclei isolated from livers of methyl-deficient rats, the hypomethylated DNA was found to be more sensitive to enzyme- and oxidant-induced DNA strand break induction. Taken together, these results provide evidence that DNA strand breaks are induced in high molecular weight DNA and also within the p53 gene in liver tissue from methyl-deficient rats. The increased incidence of these strand breaks in DNA from methyl-deficient rats may be related to alterations in chromatin accessibility associated with DNA hypomethylation.

INTRODUCTION

Chronic dietary insufficiency of the lipotropic nutrients choline and methionine has been reproducibly shown to be hepatocarcinogenic in the rat and in certain mouse strains with or without chemical initiation (1–3). Several hypotheses have emerged over the years in attempt to understand the biochemical and molecular basis for this nutritional model of multistage hepatocarcinogenesis. While none of these is mutually exclusive, the major hypotheses that have been proposed implicate: (a) DNA hypomethylation leading to inappropriate expression of growth regulatory oncogenes (4–6); (b) oxidative free radical DNA damage secondary to early lipid accumulation and peroxidation (1, 7, 8); (c) aberrant membrane phospholipid metabolism, receptor expression, and protein kinase C-mediated signal transduction (3, 9, 10); (d) abnormal deoxynucleotide metabolism promoting DNA base misincorporation transition mutations (11, 12); and (e) oncogene and tumor suppressor gene aberrations (4, 13, 14). The one common factor in all these hypotheses is the absolute requirement for regenerative proliferation to promote the heritable genetic and/or epigenetic changes that lead to neoplastic transformation. In fact, several of these hypotheses are difficult to separate from, and may be partially explained by, events that are secondary to the regenerative proliferation stimulated by the cytotoxic effects of the diet.

The global DNA hypomethylation associated with methyl deficiency has been causally linked to a diet-induced depletion of SAM,3 the substrate for the methyltransferase (6). Hepatic levels of S-adenosylmethionine in rats are reduced by 25% within the first week of consuming a methyl-deficient diet (15) and continue to decrease progressively in subsequent weeks (16). Maintenance methyltransferase activity is stimulated in the livers of methyl-deficient rats in order to maintain the methylation status of newly synthesized DNA strands during diet-induced regenerative proliferation. However, in the presence of reduced SAM availability, progressive DNA hypomethylation is promoted (5).

Genome-wide alterations in DNA methylation, as well as regional variations in gene-specific methylation patterns, have been observed in a variety of preneoplastic and transformed cell models (17). However, a mechanistic relationship between DNA methylation and malignant transformation has proven difficult to define since both global hypomethylation and regional hypermethylation may occur simultaneously during various stages of tumor progression (18, 19). The implicit hypothesis in many studies of DNA methylation and carcinogenesis is that hypomethylation of growth-promoting proto-oncogenes and/or hypermethylation of tumor suppressor genes will alter transcription factor binding and expression of these genes to promote a selective growth advantage for the initiated cell (6). An alternative consideration is that DNA hypomethylation may promote malignant transformation by inducing regional alterations in DNA conformation and chromatin structure. Local conformational changes can promote genomic instability by increasing the accessibility of specific sequences to DNA-damaging agents (20, 21). Loss of methylated cytosines alters the conformation and stability of the chromatin structure presumably by decreasing binding sites for methyl-specific proteins. In the absence of methyl-directed protein binding, affected DNA sequences are rendered more accessible to oxidant and/or enzyme-induced DNA strand breakage (22–24). Thus, dysregulation of DNA methylation patterns and associated changes in DNA-protein binding may promote neoplasia not only by altering the transcription of cancer-related genes but also by altering local DNA structure and sequence accessibility to DNA-damaging agents.

In light of these considerations, we examined the kinetics of DNA strand breakage as related to methylation status of the DNA in livers from folate/methyl-deficient rats at intervals from 2 days to 9 weeks after diet initiation. Increased expression of the p53 gene is a DNA damage response that is initiated by the presence of DNA strand breaks (25). The p53 gene product also appears to function as a competence factor that is normally up-regulated during cellular proliferation (25, 26). Both genome-wide and p53 gene-specific hypomethylation and DNA strand break accumulation were evaluated in the present study. The results are consistent with the hypothesis that hypomethylation induces regional alterations in chromatin/DNA

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3 The abbreviations used are: SAM, S-adenosylmethionine; qPCR, quantitative PCR.
accessibility such that certain sequences become more vulnerable to oxidative and/or enzyme-induced strand breakage.

MATERIALS AND METHODS

Animals and Diets. Male weanling F344 rats were obtained from the National Center for Toxicological Research breeding facility, housed 2/cage in a temperature-controlled (24°C) room with a 12-h light/dark cycle, and given ad libitum access to water and NIH-31 pelleted diet (Purina Mills, Richmond, IN). Four-week-old rats (body weight, 50 g) were allocated randomly to either the low methionine, choline-deficient diet (Dyets, Inc., Bethlehem, PA) additionally lacking in folic acid, or a control diet supplemented with 0.4% methionine, 0.3% choline, and 2 mg/kg folic acid. Folic acid was omitted from the low methionine/choline-devoid diet in order to enhance the severity of methyl group deficiency in this semipurified diet preparation (11, 27). The diets were stored at 4°C and given ad libitum with biweekly replacement. Body weights and food consumption were recorded weekly. Four to six rats per group were killed by exsanguination under light ether anesthesia at intervals of 2 days, 5 days, 1 week, 3 weeks, and 9 weeks after diet initiation. There were no overt signs of deficiency until week 9 when body weight gain was decreased by 30% in the folate/methyl-deficient rats. The livers were excised, frozen immediately in liquid nitrogen, and stored at −80°C for subsequent isolation of nuclei or DNA for analyses.

Genome-wide DNA Strand Break Accumulation. A modification of the random oligonucleotide-primer synthesis assay was used to detect the presence of low frequency DNA strand breaks in high molecular weight DNA (28). Briefly, 3'-OH DNA fragments present in the high molecular weight DNA extract are separated initially into single strands by a denaturation step. After reassociation, these DNA fragments serve as primer and the excess of high molecular weight DNA serves as template in a reaction with DNA polymerase. The [32P]dCTP incorporation initiated by Klenow fragment of DNA polymerase I under strictly defined conditions (time, temperature, and precursor concentration) is proportional to the number of 3'-OH breaks (fragment primers) present. For the procedure, DNA was isolated according to Ausubel et al. (29), dialyzed against an excess of TE buffer [10 mM Tris-HCl (pH 7.4) - 1 mM EDTA], denatured by exposure at 100°C for 5 min, and then immediately cooled on ice. The mixture contained 0.25 μM heat-denatured DNA sample, 0.5 μM [32P]dCTP (3000 Ci/mmol; DuPont New England Nuclear, Boston, MA), 0.05 mM concentrations of each dGTP, dATP, and dTTP, 0.6 μM dCTP, 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 7.5 mM DTT, and 0.5 unit Klenow polymerase (New England Biolabs, Beverly, MA) in a total volume of 25 μl. After incubation for 30 min at 16°C, the reaction was stopped by the addition of an equal volume of 12.5 mM EDTA. The samples (in triplicate) were subsequently applied onto Whatman DE-81 ion exchange filters and washed in 0.5 M sodium phosphate buffer (pH 6.8) to remove unincorporated precursors. The radioactivity remaining on the filters was measured in a 1900TR Packard scintillation counter (Packard Instruments, Meriden, CT). The data were expressed as pmol [32P]dCTP incorporation/μg DNA.

qPCR. Quantitative PCR is a highly sensitive technique that can be used to detect DNA strand breaks within a defined gene. It is based on the ability of a DNA lesion to halt the progression of the Taq polymerase during PCR amplification. Under optimized conditions, the decrease in the absolute amount of radiolabeled PCR product amplified over a given time will be proportional to the level of DNA lesions present within the gene (30). High molecular weight DNA was extracted from nuclei with the use of the Ausubel protocol for RNAase digestion (29). The DNA was initially digested with 4 units/μg DNA Sall restriction (New England Biolabs) for 12 h at 37°C to increase accessibility of the DNA to primer annealing. Intron and flanking 20-mer primer sequences for each of the exons 5–8 of the rat p53 gene have been published previously (31) and were synthesized by Genosys Biotechnologies, Inc. (Woodlands, TX). The use of intron primers obviates amplification of p53 pseudogenes present in the rat (31). Thin walled reaction tubes were used for the 50-μl PCR reaction mix which contained for each exon: 0.5 μg DNA template, PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 1.5 mM MgCl₂, 100 pmol of each primer, 200 μM concentrations of each dNTP, plus an AmpliTaq PCR Gem (Perkin Elmer Cetus, Norwalk, CT) for hotstart PCR. The samples were denatured at 94°C for 10 min in a Perkin Elmer thermocycler (Model 9600) before adding 2.5 units of AmpliTaq DNA polymerase and 10 μCi of [32P]dCTP (3000 Ci/mmol). The amplification protocol consisted of 27–30 cycles of denaturation at 94°C for 50 s, annealing at 58°C for 50 s, and extension at 72°C for 70 s. The quantitative aspects of the procedure were verified by the linear increase in product recovery with increasing cycle number and DNA template concentration. A 20-μl aliquot of PCR product was analyzed by electrophoresis in 3% NuSieve agarose gel in Tris-borate EDTA buffer containing ethidium bromide to verify the quality of the amplification. The radiolabeled single-band product of each amplified exon was cut from the gel, transferred to scintillation vials in 2 ml H₂O, and melted by microwave heating; the 32P incorporation was quantified in a Model 1900TR Packard scintillation counter.

Quantitative HpaII-PCR Assay for p53 Gene Methylation. The methylation status of the p53 gene was assessed with a modification of the qPCR procedure described by Singer-Sam et al. (32). With the use of primers flanking the HpaII cleavage sites within the p53 gene, quantitative recovery of PCR product will vary directly with the extent of HpaII-induced strand breaks at unmethylated CCGG sites. Briefly, 5 μg DNA was incubated with 10 units HpaII restriction endonuclease (New England Biolabs) in 1× HpaII buffer [10 mM Tris-propane-HCl, 10 mM MgCl₂, 1 mM DTT (pH 7.0)] at 37°C for 4 h in a total volume of 60 μl. The HpaII activity was inactivated by heating samples for 20 min in a 65°C heat block. A 3-μl aliquot of the HpaII reaction mixture was added to the PCR reaction mix, followed by amplification and quantitative recovery of PCR product as described above.

Genome-wide DNA Methylation. The relative methylation status of DNA extracted from livers of deficient and control rats was determined with the use of the method described by Balaghi and Wagner (33). The conditions of the assay are such that the radiolabeled methyl group acceptance in vitro is related inversely to the number of methylated CpG sites in the extracted DNA. Briefly, the 30-μl reaction mixture contained 0.5 μg DNA isolated from frozen livers of deficient and control rats, 3 μM S-adenosyl-l-[methyl-3H]methionine containing 2 μCi (DuPont New England Nuclear), 3 units SssI CpG methylase (New England Biolabs) in 1× SssI buffer [10 mM Tris-HCl (pH 7.9)-120 mM NaCl-10 mM EDTA-1 mM DTT]. Duplicate tubes were incubated at 30°C for 1 h. The in vitro methylated DNA was isolated from a 15-μl aliquot of the reaction mixture by filtration on a Whatman DE-81 ion exchange paper. The DNA was washed three times with 20 ml of 0.5 M sodium phosphate buffer (pH 7.0), followed by 2 ml of 70% ethanol and 2 ml absolute ethanol. The air-dried filters were placed in scintillation vials, and the incorporated radioactivity was quantified in a 1900TR Packard scintillation counter.

DNA Methytransferase Activity in Nuclear Extracts. Nuclei were isolated from frozen liver samples obtained from deficient and control rats and protein extracts prepared as described by Basnakian et al. (34). The protein concentration in the nuclear extracts was measured by the method of Sedmak and Grossberg (35). The DNA methytransferase reaction conditions were such that both the methyl donor and the DNA template were in excess so that extent of methyl group incorporation was dependent on the level of methytransferase activity in the nuclear extract. Extracts were incubated for 4 h at 37°C in a 130-μl reaction mixture containing 10 mM Tris-HCl buffer (pH 7.8), 5 mM MgCl₂, 10 μg of calf thymus DNA as substrate (Sigma Chemical Co., St. Louis, MO), and 2 μl of S-adenosyl-l-[methyl-3H]methionine as the methyl donor. The samples were applied to Whatman DE ion exchange filters, and radioactivity was quantified by liquid scintillation counting. The results are expressed as [3H]methyl incorporation/100 μg protein in nuclear extract.

Endonuclease Digestion of DNA in Nuclei Before qPCR. The endonucleolytic calcium/magnesium-dependent endonuclease in isolated liver nuclei was activated in vitro as a means to induce controlled (enzyme-mediated) strand breaks in DNA. Nuclei were isolated as described by Basnakian et al. (34). For endonuclease digestion of DNA, the nuclei were resuspended in a buffer consisting of 50 mM Tris-HCl (pH 7.9), 0.25 mM sucrose, 5 mM 2-mercaptoethanol, 2 mM CaCl₂, and 5 mM MgCl₂. The samples were incubated for predetermined intervals at 37°C. The reaction was stopped by placing samples in an ice bath and immediately extracting the DNA as described by Ausubel et al. (29). The DNA samples were stored at −20°C until qPCR amplification of the p53 gene.

In Vitro DNA Methylation in Nuclei. In order to determine whether an increase in methylation density of the DNA would protect against endonuclease-mediated DNA strand breaks in vitro, the DNA in liver nuclei isolated from control rats was methylated in a reaction tube containing 10 μg DNA in nuclei, 10 mM Tris-HCl (pH 7.9), 0.25 mM sucrose, 10 mM CaCl₂, 5 mM NaCl,
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The relative changes in DNA strand break accumulation in livers of rats fed the folate/methyl-deficient diet or the supplemented control diet over a 9-week period is presented. In the assay used, the level of DNA strand breaks present is proportional to the incorporation of \(^{32}P\)dCTP into DNA under defined conditions. The data is expressed as pmols dCTP incorporation/\(\mu\)g DNA. At each time point assayed, DNA strand break accumulation was significantly higher in livers from the deficient rats relative to control (n = 4-6/group; P < 0.001, day 2 and day 63; P < 0.01, days 5, 7, and 21). Points, mean; bars, SEM.

In Vitro Lipid Peroxidation in Nuclei. Nuclear membrane lipid peroxidation was induced in nuclei isolated from livers of rats fed the folate/methyl-deficient diet for 3 and 9 weeks. To 100 \(\mu\)g DNA in nuclei, 200 \(\mu\)l Tris-HCl (pH 7.5), 50 \(\mu\)g FeSO\(_4\).7H\(_2\)O, and 100 \(\mu\)g ascorbic acid were added to generate free radicals as described by Milne et al. (36). Lipid peroxidation was confirmed in parallel samples by a significant increase in malondialdehyde production (data not shown). After a 60-min incubation at 37\(^\circ\)C, DNA was extracted as described above and applied to a 1% agarose gel. The extent of DNA degradation after gel electrophoresis was visualized by ethidium bromide staining and compared between diet groups.

Statistical Analysis. Comparisons for statistically significant differences between means were made with the use of the Student's t test and Sigmastat (Jandel Scientific, San Rafael, CA) software.

RESULTS

Genome-wide DNA Strand Break Accumulation. In Fig. 1, the extent of \(^{32}P\)dCTP incorporation is a direct reflection of the relative level of 3'-OH-DNA fragments present in DNA extracted from livers of folate/methyl-deficient and control rats. Within 48 h after diet initiation, the level of DNA strand breaks in livers of the deficient rats was twice that observed in the control rats. This acute increase in DNA damage was followed by an apparent repair of strand breakage as evidenced by the reduction in strand break levels from day 5 to 3 weeks. This acute DNA “damage control” response was maintained for 3 weeks on the deficient diet but appeared to fail after 9 weeks when DNA strand break levels were observed to increase approximately 4-fold over that in control livers. Despite the variation in magnitude, genomic DNA strand break accumulation was significantly higher in livers from deficient rats at all time points (P < 0.01). It should be noted, however, that a difference in DNA fragmentation between diet groups was not visible with agarose gel electrophoresis of genomic DNA (Fig. 2B). Thus, the strand breaks detected were of relatively low frequency in genomic DNA although significantly different between groups.

Quantitative PCR of Exons 5–8 of the p53 Gene from Livers of Folate/Methyl-deficient and Control Rats. As shown in Fig. 2A, the recovery of \(^{32}P\)-labeled qPCR product from exon 5 of the p53 gene was assessed subsequently by qPCR, and the comparison was made between control DNA and the in vitro methylated DNA.

In Vitro Lipid Peroxidation in Nuclei. Nuclear membrane lipid peroxidation was induced in nuclei isolated from livers of rats fed the control or deficient diet for 3 and 9 weeks. To 100 \(\mu\)g DNA in nuclei, 200 \(\mu\)l Tris-HCl (pH 7.5), 50 \(\mu\)g FeSO\(_4\).7H\(_2\)O, and 100 \(\mu\)g ascorbic acid were added to generate free radicals as described by Milne et al. (36). Lipid peroxidation was confirmed in parallel samples by a significant increase in malondialdehyde production (data not shown). After a 60-min incubation at 37\(^\circ\)C, DNA was extracted as described above and applied to a 1% agarose gel. The extent of DNA degradation after gel electrophoresis was visualized by ethidium bromide staining and compared between diet groups.

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Fig. 3. A, the calcium/magnesium-dependent endonuclease in nuclei isolated from control livers was activated for 40 and 80 min in order to generate endonuclease-mediated breaks in the DNA. After DNA extraction, exons 5–8 of the p53 gene were amplified by PCR. The quantitative recovery of PCR product from nuclei exposed to activated endonuclease for 40 and 80 min is expressed as mean percentage change from simultaneously amplified DNA from control nuclei kept on ice for 80 min during endonuclease activation. The decrease in qPCR product recovery in exon 5 from the deficient rat liver is significant (P < 0.01). B, electrophoretic mobility in agarose gel of DNA extracted from control and deficient livers after 40–80 min of endonuclease exposure. Columns, mean; bars, SEM.

Quantitative PCR of Exons 5–8 after DNA Digestion in Nuclei by Calcium/Magnesium-dependent Endonuclease. To determine whether the diet-induced hypersensitivity of the exon 5 sequence to DNA strand breakage could be reproduced in vitro, DNA in nuclei isolated from control rats was exposed to endogenous calcium/magnesium-dependent nuclear endonuclease digestion before qPCR amplification of exon 5. The results presented in Fig. 3A demonstrate a remarkable similarity in the pattern of strand break sensitivity in exons 5–8 of the hepatic p53 gene when compared to that induced by dietary folate/methyl deficiency (Fig. 2A). The amplification of p53 exons 6–8 from deficient livers was not different from control; however, exon 5 appeared to contain a sequence or sequences uniquely sensitive to enzyme-induced DNA cleavage as evidenced by reduced recovery of radiolabeled PCR product after quantitative PCR. In contrast to the high molecular weight DNA template amplified from methyl-deficient livers (Fig. 2B), the DNA template amplified by qPCR after in vitro endonuclease digestion was considerably degraded as indicated in Fig. 3B. Thus, considerable fragmentation of DNA was required to reproduce this phenomenon in hepatic DNA from control rats. In contrast, the diet-induced DNA strand breaks in exon 5 of the p53 gene from deficient rats were detectable in high molecular weight DNA (Fig. 1) and may reflect an enhanced sensitivity (or accessibility) of this sequence to strand breakage under conditions of methyl deficiency.

Kinetics of Genome-wide Hypomethylation and DNA Methyltransferase Activity. In Fig. 4, the progressive alterations in global DNA methylation in livers of folate/methyl-deficient rats are superimposed on the simultaneous increase in DNA methyltransferase activity over the same interval in the same tissue. These parameters were also measured in DNA from the control-fed rats and did not vary significantly over this interval (data not shown). An acute decrease in the DNA methylation density occurred within the first 2 days of exposure to the deficient diet (P < 0.001). Between 2 and 7 days on the deficient diet, the methyltransferase activity increased sharply without change in the methylation density. However, between 1 and 3 weeks on the deficient diet, DNA methylation density again decreased significantly (P < 0.01), despite a further 2-fold increase in methylase activity. It is interesting that between 3 and 9 weeks, the methyltransferase activity appeared to reach a maximum or plateau that coincided with the maximum decline in methylation status. A comparison of Figs. 1 and 4 reveals that the major decrease in methylation status occurring during the interval between 3 and 9 weeks paralleled the increase in genome-wide DNA strand breaks during the same interval in the same tissue.

Kinetics of p53 Gene Hypomethylation and Strand Break Accumulation. In Fig. 5, a comparison of p53 gene-specific DNA strand break accumulation (measured as the decrease in recovery of
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Fig. 5. Methylation status of CCGG sites within exons 5–8 of the *p53* gene was measured as the recovery of radiolabeled qPCR product after *HpaII*-biased DNA pretreatment. The data are expressed as percentage change in qPCR product recovery from *HpaII*-biased DNA from deficient livers relative to *HpaII*-biased DNA from control livers (n = 4). The methylation status of the *p53* gene from the control liver did not change with time on the control diet (data not shown). Progressive changes in methylation density with time on the deficient diet (A) and the accumulation of DNA strand breaks within exons 5–8 of the *p53* gene (B) (DNA strand break accumulation is proportional to the decrease in qPCR amplification over exons 5–8 as described in "Materials and Methods"). wk, week. Points, mean; bars, SEM.

The relative induction of enzyme-induced DNA strand breaks in the *p53* gene was then quantified by qPCR. A comparison of Fig. 6, A and B, indicates that *in vitro* methylation could partially protect the DNA from endonuclease-mediated DNA strand breaks.

Sensitivity of Genomic DNA to Enzyme and Oxidant-induced DNA Strand Breaks *in Vitro*. Nuclei isolated from deficient and control livers at 3 and 9 weeks after diet initiation were incubated under conditions designed to activate the endogenous calcium/magnesium-dependent endonuclease (see "Materials and Methods"). The electrophoretic mobility of ethidium bromide-stained DNA in agarose gel after enzyme activation is presented in Fig. 7A. It is apparent that

qPCR product amplified over exons 5–8 is compared to the level of *p53* gene methylation (measured by qPCR amplification of exons 5–8 after *HpaII*-biased strand breaks at unmethylated CpG sites). After 1 week on the folate/methyl-deficient diet, an increase in *p53* gene hypomethylation was paralleled by an increase in *p53* gene-specific strand breaks, similar to genome-wide observations (Fig. 4). However, in contrast to the genome-wide effects, the acute response within the *p53* sequence on day 2 was significant hypermethylation of the gene, suggesting nonrandom gene-specific *de novo* methylation of the *p53* gene (P < 0.01). Thereafter, *p53* gene hypomethylation paralleled an increase in gene-specific strand breaks.

*In Vitro* Methylation of DNA in Nuclei Protects against Enzyme-mediated Strand Breaks. In an attempt to determine whether the sensitivity to DNA strand breakage within the *p53* gene could be modified by the methylation status of the DNA, the endogenous methylinase in isolated nuclei was activated in the presence of [methyl-[^3]H]SAM. An increase in DNA methylation status was verified by a significant increase in the uptake of radiolabeled methyl groups into DNA of incubated nuclei compared to control nuclei maintained on ice (data not shown). The endogenous calcium/magnesium-dependent endonuclease was activated subsequently in both the untreated control nuclei (Fig. 6A) and the *in vitro* methylated nuclei (Fig. 6B) for 40 min as described in "Materials and Methods."

Fig. 6. A, mean percentage change in qPCR product recovery after endonuclease digestion (n = 4). A, the decrease in qPCR product recovery after 40-min exposure of control liver nuclei to the calcium/magnesium-dependent endonuclease reflects the induction of enzyme-induced DNA strand breaks. B, the DNA in nuclei has been methylated *in vitro* before qPCR amplification. The relative increase in qPCR product recovery after *in vitro* methylation is consistent with a protective effect of DNA methylation on enzyme-induced DNA strand breaks. Columns, mean; bars, SEM.

In Vitro* Methylation of DNA in Nuclei Protects against Enzyme-mediated Strand Breaks. In an attempt to determine whether the sensitivity to DNA strand breakage within the *p53* gene could be modified by the methylation status of the DNA, the endogenous methylinase in isolated nuclei was activated in the presence of [methyl-[^3]H]SAM. An increase in DNA methylation status was verified by a significant increase in the uptake of radiolabeled methyl groups into DNA of incubated nuclei compared to control nuclei maintained on ice (data not shown). The endogenous calcium/magnesium-dependent endonuclease was activated subsequently in both the untreated control nuclei (Fig. 6A) and the *in vitro* methylated nuclei (Fig. 6B) for 40 min as described in "Materials and Methods."

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the level of enzyme-induced internucleosomal cleavage was higher in the livers of deficient rats relative to control. These results are consistent with the hypothesis that the accessibility of internucleosomal sites to endonuclease cleavage was greater in the hypomethylated DNA from the deficient rats. In a second experiment, nuclear membrane lipid peroxidation in isolated nuclei was stimulated in vitro as described in “Materials and Methods.” The DNA in nuclei from deficient and control livers was subsequently extracted, and the electrophoretic mobility of DNA in agarose gel is presented in Fig. 7B. These results suggest that the DNA from the deficient livers was more sensitive to an equal level of oxidant stress relative to DNA from control livers.

**DISCUSSION**

Agents and conditions that induce breaks in the DNA template stimulate a rapid and transient elevation of p53 protein levels (25, 37). The p53-dependent DNA damage-response pathway leads to G1 arrest and strand break repair or to apoptotic cell death depending on the cell type and extent of DNA damage (38, 39). Unrepaired single or double strand breaks persisting in the genome can interfere with gene expression (40), induce neoplastic transformation in cultured cells (41, 42), or trigger apoptotic cell death (43, 44). As such, unrepaired DNA strand breaks may constitute a considerable threat to genomic integrity and cell viability, especially under conditions of increased cell proliferation. In the present study, we demonstrate that dietary methyl deficiency, a well established model of multistage hepatocarcinogenesis, induces a biphasic pattern of genome-wide DNA strand breaks in liver DNA over a 9-week period. Within 48 h after initiating the methyl-deficient diet, an acute increase in DNA strand breaks occurs, which is followed by a second peak after 9 weeks of chronic diet consumption. This biphasic response (Fig. 1) appears to reflect an attempt at DNA damage control during the early weeks of methyl deficiency that cannot be sustained with chronic exposure to the biochemical stress of the diet.

Consistent with a p53-dependent damage control response to dietary methyl deficiency, Christman et al. (5) recently reported an increase in p53 mRNA within 2 weeks after feeding an amino acid-defined methyl-deficient diet. However, no increase in p53 protein levels by Western blot analysis was detectable in liver from deficient rats (data not shown) and most likely reflects the short half-life of this DNA damage-inducible protein. In earlier studies, Rushmore et al. (45) reported an increase in “alkali-labile lesions” in hepatic DNA from rats exposed to a methyl-deficient diet for only 3 days. The etiology of the DNA strand breaks is not clear. The several possibilities are not mutually exclusive and include (a) early lipid peroxidation and free radical-induced breaks; (b) apoptosis-related breaks; (c) DNA repair-related breaks; and (d) hypomethylation and altered conformation/accessibility of DNA to damaging agents.

Quantitative PCR amplification of the p53 gene of genomic liver DNA yielded the unexpected observation that an apparent “hotspot” for DNA strand breakage exists in exon 5 of the p53 gene from the methyl-deficient rats that is not present in exons 6, 7, or 8, nor in p53 exons amplified from control DNA (Fig. 2). Quantitative PCR can be utilized as a highly sensitive gene-specific DNA strand break assay based on the ability of a strand break to interrupt the progression of the Taq polymerase during the linear phase of PCR amplification of a given sequence (30). As a result, preexisting strand breaks in DNA or restriction enzyme-induced DNA strand breaks can be detected in a gene-specific manner as a decrease in the absolute amount of 32P-labeled amplification product recovered relative to control (46). In Fig. 3, activation of the endogenous calcium/magnesium-dependent endonuclease in liver nuclei in vitro was found to induce the identical pattern of breaks (p53 exon 5 but not 6–8) in DNA from control-fed rats. A possible explanation for this observation is that the conformation of chromatin was altered with diet-induced DNA hypomethylation such that accessibility (sensitivity) to endonuclease cleavage was enhanced at this site. Consistent with this interpretation, Keshet et al. (21) have demonstrated that cytosine hypomethylation alters chromatin configuration, resulting in an increased sensitivity to DNase I cleavage. Other studies have shown that methylated DNA sequences assume a nuclease-resistant conformation in mammalian nuclei (47, 48). More recently, cytosine methylation has been shown to modify topoisomerase I and II sequence-specific cleavage of the c-myc gene in DNA fragments (22). The simplest explanation for these observations is that methylation or methyl-specific protein binding directly protects the DNA at methylated cytosines or indirectly protects it by promoting a condensed (inaccessible) chromatin configuration (23, 48).

In the present study, progressive genomic DNA hypomethylation was induced with increased time on the methyl-deficient diet and was associated with a progressive increase in DNA methyltransferase activity over a 9-week period (Fig. 4). A similar reciprocal relationship between methyltransferase activity and genomic methylation density has been observed previously in cultured tumor cells in vitro (49) and during progressive colorectal cancer in humans (50). However, the fact that normal cell proliferation is similarly associated with increased methyltransferase activity and hypomethylation complicates a comparison with tumorigenesis (18). An inverse relationship between enzyme activity and product occurs under conditions of limited substrate availability, and a diet-induced depletion of SAM with methyl deficiency is well documented (16, 27). Recent evidence in a highly sensitive prokaryotic model indicated that the DNA methyltransferase can facilitate the deamination of cytosine to uracil under conditions of reduced SAM, raising the possibility that enzyme-induced deamination (C to U to T) may promote C to T transition mutations with methyl deficiency (46). In preliminary studies, we have recently demonstrated that sequence-specific demethylation occurs at certain CpG sites within the p53 gene, whereas other CpG sites remain persistently methylated in preneoplastic liver of methyl-deficient rats (51). Similarly, codon 248 in the human liver p53 gene has been shown to be highly resistant to demethylation despite genomewide hypomethylation (52). These persistent methylated cytosines may represent hotspots for spontaneous deamination mutagenesis as proposed by Jones et al. (53). Of interest, the presence of p53 transition mutations has been identified in liver tumors from rats chronically fed a methyl-deficient diet (14). Whether the hotspots for strand break induction in the p53 gene observed in the present study correspond to sites of mismatch repair of spontaneous and/or enzyme-induced cytosine deamination events in p53 is currently under investigation in our laboratory.

The kinetics of p53 gene hypomethylation with time on the methyl-deficient diet was evaluated in a separate experiment with the use of HpaII pretreatment, followed by qPCR detection of enzyme-induced strand breaks. An unexpected and transient hypermethylation of the p53 gene was observed after 2 days on the deficient diet (Fig. 5). Clearly, this response was nonrandom since global DNA methylation density was reduced significantly at this time (Fig. 4). Theoretically, an increase in cytosine methylation at HpaII sites would require sequence-specific de novo methyltransferase activity (assuming that HpaII cleaves only at fully nonmethylated sites). Abnormal regional DNA hypermethylation associated with increased methyltransferase expression has been demonstrated by Issa et al. (50) in models of tumor progression and also during transformation of NIH 3T3 cells (17). In other studies, Smith (23) has shown that sites of DNA damage are selectively methylated by de novo methyltransferase activity and has postulated that an important function of DNA methylation is to...
stabilize chromatin regions containing DNA damage. In Fig. 5, A and B, the accumulation of DNA strand breaks within the p53 gene as measured by qPCR is compared to the alterations in p53 methylation status in the deficient rat livers over the 9-week feeding period. It is interesting to note that the increase in p53 methylation on day 2 coincided with a transient increase in gene-specific DNA strand breaks. The hypermethylation response was followed by a reduction in the incidence of strand breaks on days 5 and 7 of diet exposure. These data are consistent with an initial methyl-protective response to DNA damage, as proposed by Smith (23), that could not be sustained under chronic conditions of reduced SAM availability. After 3 and 9 weeks of methyl deficiency, a progressive increase in p53 strand breaks was associated with progressive p53 hypomethylation.

In an experiment designed to more directly address the possibility that DNA methylation provides a protective mechanism against DNA strand break induction, the methyltransferase was activated in isolated nuclei, and the incorporation of [3H]methyl groups was monitored to verify an increase in DNA methylation density. The relative ability of the calcium/magnesium-dependent endonuclease to cleave hypermethylated and control DNA was subsequently monitored by qPCR strand breaks analysis of the p53 gene. The results shown in Fig. 6 demonstrate that an increase in DNA methylation conferred partial resistance to enzyme-induced strand breaks in the p53 gene consistent with a protective role for DNA methylation.

In final experiments, the relative sensitivity to endonuclease cleavage and to oxidant stress in vitro was evaluated in liver nuclei isolated from rats fed the control or deficient diet for 3–9 weeks. In both cases (Fig. 7, A and B), the DNA extracted from nuclei of the deficient rats at 3 and 9 weeks was visibly more fragmented than was the DNA from the control rats, despite equal exposure to the DNA-damaging conditions. Although enzyme- and oxidant-induced DNA strand breaks represent very different mechanisms of DNA fragmentation, increased sensitivity to both types of DNA damage was explained by an increase in accessibility (vulnerability) of hypomethylated DNA.

In summary, the results of the present study provide evidence that DNA strand breaks are induced in hepatic DNA of methyl-deficient rats as early as 2 days after diet initiation and persist for at least 9 weeks. Both genome-wide and p53 gene-specific strand breaks are associated with progressive genome-wide and p53 gene hypomethylation, which may contribute to altered gene expression, genomic instability, and tumor progression in this model of nutritional carcinogenesis.

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Breaks in Genomic DNA and within the p53 Gene Are Associated with Hypomethylation in Livers of Folate/methyl-deficient Rats

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