Progressive Alterations in Global and GC-Rich DNA Methylation during Tumorigenesis

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DNA methylation plays a key role in the regulation of gene expression, and failure to maintain normal patterns of methylation often contributes to carcinogenesis. We have characterized progressive methylation changes during the promotion stage of carcinogenesis using a SENCAR mouse skin initiation/promotion tumorigenesis model. Mice were initiated with a dermal application of 75 μg dimethylbenz[a]anthracene (DMBA) and promoted with 9, 18, 27, and 36 mg cigarette smoke condensate (CSC) thrice weekly for time periods up to 29 weeks, when a large increase in tumor number was produced by the highest three doses. Global and GC-specific methylation were assessed using SssI methylase and arbitrarily primed PCR, respectively. Changes in GC-specific methylation were dose- and time-dependent. CSC doses required to detect these changes were 27 mg at 6 weeks and 18 mg at 9 weeks. This effect appears to be reversible; changes in GC-specific methylation were less marked after 9 weeks promotion with 27 mg CSC followed by 6 weeks of recovery in comparison to 9 and 15 weeks promotion with 27 mg CSC and no recovery period. Both tumor and non-tumor tissue promoted with 27 mg CSC for 29 weeks exhibited changes in GC-specific methylation that were more pronounced in tumors. Tumor tissue was globally hypomethylated, whereas non-tumor tissue did not exhibit changes in global methylation. In conclusion, as expected for a mechanism underlying tumor promotion, CSC alters methylation in a threshold-exhibiting, reversible, progressive fashion during promotion. Progressive alterations in global and GC-rich methylation appear to be mechanistically important during tumor promotion.

Key Words: DNA methylation; tumorigenesis; promotion; SENCAR mouse; skin tumorigenesis; epigenetics.

Carcinogenesis is a multistage, multistep process with three experimentally defined stages: initiation, promotion, and progression (Pitot and Dragan, 1994). Initiation involves a heritable alteration to the genome of a normal cell that provides a selective growth advantage over surrounding cells in response to promoting agents. Initiation is thought to be irreversible and due to mutation; however, epigenetic factors may also play a role (Goodman and Watson, 2002). During promotion, initiated cells clonally expand, and increasingly aberrant subclones develop. Promoting agents might facilitate this expansion by increasing the proliferation rate and/or decreasing the rate of apoptosis in these cells (Schulte-Hermann et al., 1990). The promotion stage is reversible, and the dose-response relationship for promoters exhibits a threshold (Goodman, 2001; Pitot and Dragan, 1994). In progression, continued subclone expansion no longer requires a promoting agent, and more extensive alterations to the genome such as chromosomal damage and aneuploidy are observed (Pitot and Dragan, 1994).

Changes required for the basic cancer phenotype include evasion of apoptosis, self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion, and metastasis, limitless replicative potential, and sustained angiogenesis (Hanahan and Weinberg, 2000). While these events might occur via mutation, epigenetic events can play a fundamental role in carcinogenesis (Watson and Goodman, 2002b). Epigenetic regulation of gene expression occurs through heritable transcriptional modulation superimposed on the primary DNA sequence. Thus, epigenetic mechanisms such as DNA methylation, i.e., the 5-methylcytosine content of DNA, have the capacity to change transcriptional levels without changing the sequence (Holliday, 1994). Genes commonly found to have altered transcriptional levels in cancer, such as the often underexpressed tumor suppressor p53 and the often overexpressed oncogene ras, can be altered by mutation or epigenetic mechanisms (Hosaka et al., 2002). Importantly, a mutated oncogene needs to be expressed in order to contribute to carcinogenesis (Hahn et al., 1999), and the expression level might be governed by epigenetic mechanisms.

Methylation facilitates a remodeling of chromatin to an inactive state. Increased methylation in GC-rich promoter regions of genes is generally associated with decreased transcription and vice versa (Ballestar and Esteller, 2002). Much of the promoter-specific methylation occurs at CpG islands, 200 bp or longer stretches of DNA with a 50% or greater GC content and a higher than expected CpG content (Gardiner-Gardner and...
In several types of cancers, increased methylation in the promoter regions of tumor suppressors such as p16, E-cadherin, and O6-methylguanine DNA methyltransferase (MGMT) is associated with, and believed to be the cause for, decreased expression of these genes (Esteller et al., 2001). Both hyper- and hypomethylation may contribute to carcinogenesis via silencing of tumor suppressor genes, upregulation of oncogenes, and/or decreased genome stability (Counts and Goodman, 1995; Goodman and Watson, 2002). Tumors characteristically exhibit increases in methylation at GC-rich regions with a decreased overall or global methylation (Gama-Sosa et al., 1983) that can facilitate oncogene expression. Changes in methylation precede tumor formation, indicating that these alterations might contribute to tumorigenesis (Robertson and Jones, 2000). There has been limited research on mouse skin methylation, but a few reports indicate methylation differences between normal and tumor skin tissue (Ramsden et al., 1985; Winter et al., 1990).

We have examined both global and GC-specific methylation using a SENCAR mouse skin initiation/promotion model of tumorigenesis. The SENCAR mouse stock was generated in the 1960s and 1970s from selective breeding of mice sensitive to epidermal papilloma formation in response to 7,12-dimethylbenz[a]anthracene (DMBA) initiation and the croton oil (containing TPA) promoting agent (Stern and Conti, 1996). These mice are extremely sensitive to carcinogenesis, and generally respond more rapidly and uniformly to the induction of skin tumors than other available strains or stocks. Importantly, the initiation and promotion stages are clearly demarcated, thus facilitating the study of biochemical and molecular mechanisms involved in a particular stage of carcinogenesis (Slaga et al., 1996).

SENCAR mice were initiated with a dermal application of DMBA, followed by administration of various doses of cigarette smoke condensate (CSC), a presumptive tumor promoter, for different lengths of time. We are testing the hypothesis that specific types of methylation alterations play a role during the promotion stage of carcinogenesis. Four specific aims were addressed: (1) to assess methylation status during tumorigenesis in this classic two-stage model system, (2) to ascertain whether particular methylation changes correlate to tumor formation in a sequential fashion, (3) to determine whether changes in methylation exhibit a dose-response relationship with regard to promoter treatment, and (4) to assess the potential for reversibility of altered methylation in precancerous tissue.

**MATERIALS AND METHODS**

**Animals.** Weanling female SENCAR mice were obtained from the National Cancer Institute, at the Frederick Cancer Research and Development Center (Frederick, Maryland). Mice (5–7 weeks of age at receipt) were allowed 2 weeks to acclimate to the testing environment, and then randomly assigned to treatment groups according to body weight (Fig. 1). To ensure groups of similar mean body weight, all groups were compared by ANOVA and least significant difference criteria and were demonstrated not to be significantly different at a 5%, two-tailed assumption. Animals were housed and cared for in accordance with the Institute of Laboratory Animal Resources (ILAR, 1996), Commission of Life Sciences, National Research Council document entitled, *Guide for the Care and Use of Laboratory Animals*. Experimental animals were initiated with either 75 mg DMBA or acetone (vehicle control). Initiation was followed by thrice-weekly CSC promotion at concentrations of

![FIG. 1. Experimental design indicating control and CSC-promoted groups of SENCAR mice used in this study. Initiation was performed with a single dermal application of 75 μg DMBA. Promotion with CSC (cigarette smoke condensate) was performed with thrice weekly dermal applications of the various doses indicated. The duration of treatment and sacrifice time are presented. There were at least seven animals in each group. + indicates initiation; * indicates acetone administration.](https://academic.oup.com/toxsci/article-abstract/75/2/289/1655863)
0, 9, 18, or 27 mg CSC per application (in acetone) for 6 or 9 weeks or a concentration of 27 mg for 15 or 29 weeks. Another group of 5- to 7-week-old SENCAR mice were initiated with 75 μg DMBA or acetone and promoted with 36 mg CSC for 29 weeks in the same laboratory during this same time period for a concurrent study. Though we did not use tissues from this last group, we do present tumor incidence data from these animals (see Fig. 7 later in this article). Following completion of the exposure regimen, animals were euthanized with 70% CO₂, and skin collected from the chemical application site was snap-frozen at –80°C until use. DNA was isolated by a phenol/chloroform procedure as described in Strauss (1990).

**Preparation of CSC.** Cigarettes supplied by R. J. Reynolds were conditioned to the laboratory environment (64.4–78.8°F and 30–70% relative humidity) and smoked using modified AMESA smoke generators operated under Federal Trade Commission standard conditions. Mainstream smoke collected from the vacuum port of the smoke machines was delivered to a condensate collection system that consisted of glass-felled impingers maintained at temperatures approximating –10°C, –50°C, and –70°C. Condensate was extracted from the glass beads using high-purity acetone, then subjected to rotary evaporation to reduce acetone and water content; this procedure was designed to yield a condensate sample with a total water content approximating 8%. To reduce variation in condensate composition, several daily condensate collections were combined to create a “pooled condensate” sample. CSC dosing solutions were prepared as needed by serial dilution of the pooled condensate using a solution of 8% water in high purity acetone to create dosing solutions of 45, 90, and 135 mg “tar”/mL. Both the pooled condensate and the prepared CSC dosing solutions were stored in amber glass bottles at –20°C or 4°C.

**Global DNA methylation analysis using the SSSI methylase assay.** SsI methylase utilizes S-adenosyl methionine as a methyl group donor to methylate the 5′ position of cytosine at unmethylated CpG sites in DNA. Thus, the level of global DNA methylation can be determined by the amount of tritiated methyl groups from [3H-CH₃] S-adenosyl-L-methionine incorporated into DNA, since there is an inverse relationship between incorporation of radioactivity and the original degree of methylation. DNA (1 mg) was incubated with 2 μCi [3H-CH₃] S-adenosyl-L-methionine (New England Nuclear, Boston, MA) and 3 units of SsI methylase (New England Biolabs, Beverly, MA) for 1 h at 30°C. Results are presented as counts per min per microgram (cpm/μg) DNA. Five replicates were performed per sample. Graphical presentation was performed using Excel®. Statistical analysis was performed with Excel using 2-tailed t-tests to compare the average cpm/μg DNA measurements between treatment groups and controls. A p value of <0.05 was considered statistically significant.

**Methylation Analysis of GC-Rich Regions**

**Restriction digests.** For each DNA sample, three restriction digests were performed as follows: Rsal alone, Rsal and MspI, and Rsal and HpaII. Rsal is a methylation-insensitive enzyme used to cut the DNA into smaller fragments. Both MspI and HpaII are methylation-sensitive enzymes that cut between cytosine residues at 5′-CCGG-3′ sites. MspI will not cut if the external cytosine is methylated, while HpaII will not cut if the internal cytosine is methylated; both will cut if the site is unmethylated (Mann and Smith, 1977). All enzymes used were from Roche (Indianapolis, IN). Restriction digests were performed with 1 μg of DNA and 5.0 units of Rsal in Roche buffer L. After a 1-h incubation (with shaking) in a water bath at 37°C, two 2.5-unit aliquots of MspI or HpaII were added, 2 h apart. The total incubation time was 18 h. The enzymes were inactivated by a 10-min incubation at 65°C, and the digests were stored at 4°C until use.

**Arbitrarily primed (AP)-32P PCR.** PCR was performed on restriction digests using a single primer (5′-AACCTTACCTAAACGCGG-3′) that arbitrarily binds within GC-rich regions of DNA (Gonzalgo et al., 1997). Reactions were composed of 5 ml of the restriction digest (containing 1 mg digested DNA), 0.4 μM each primer, 1.25 units of Taq polymerase (Gibco BRL, Rockville, MD), 1.5 mM MgCl₂, 60 mM Tris, 15 mM ammonium sulfate, 1.65 mM α-32P-dATP (New England Nuclear, Boston, MA), and glass-distilled water to volume. Samples were heated for 5 min at 94°C before addition of dNTPs in order to minimize the possibility of primer-dimer formation. Cycling conditions included a single denature cycle for 2 min at 94°C, followed by 5 cycles under the following conditions: 30 s at 94°C, 1 min at 40°C, 1.5 min at 72°C; then 30 cycles of 94°C for 30 s, 55°C for 15 s, and 72°C for 1 min, a time delay cycle for 5 min at 72°C, and a soak cycle at 4°C. PCR products (5 μl of each) were separated on a 6% polyacrylamide sequencing gel at 45 watts for 2 ½ h. The gel was soaked for 10 min in a fixing solution with 5% acetic acid and 5% methanol, rinsed for 10 min in glass-distilled water, dried, and placed into a cassette with a storage phosphoimage screen to visualize labeled PCR products. Compared to larger DNA fragments on the upper halves of gels, smaller fragments on the lower halves of gels sometimes required a longer exposure to clearly discern bands. Thus, a short exposure of 3 days followed by a longer exposure of 8 days was often performed on a gel. Phosphoimages were analyzed using Quantity One® Bio-Rad software.

**Quantification of band intensity.** Regions of the phosphoimages in which bands appear more or less prominently compared to controls were boxed and numbered. Bands within these regions were outlined and measured for pixel number and intensity using NIH image. The total pixel intensity units for each band were obtained by multiplying the pixels in the band by the mean intensity units within the outlined region. Reference rows of bands (R) with reasonable lane-to-lane consistency were chosen to represent lane-to-lane background and/or loading differences. In order to compensate for any differences in lane background levels, the ratio of band intensity for each numbered region to the band intensity within the corresponding lane’s reference (R) region was determined. Ratio fold change differences between CSC-promoted and control animals were calculated by dividing the ratios of the CSC-promoted animals by the ratios of the control animals in corresponding regions.

**RESULTS**

Global methylation was assessed for non-tumor tissue from treatment groups 1, 4, 9, and 11, and tumor and non-tumor tissue from treatment group 12 (Figs. 1 and 2). Only the tumor samples from treatment group 12 exhibited a statistically significant difference in the level of global methylation compared to untreated controls (group 1). The global methylation levels of non-tumor tissue from animals given the identical 27 mg CSC for 29 weeks treatment did not exhibit a statistically significant difference from controls. Similarly, tissues from animals promoted with 27 mg for 9 or 15 weeks (groups 9 and 11, respectively) and sacrificed immediately afterwards did not exhibit a level of global methylation that was significantly different from either untreated (at 29 weeks) or initiated-only animals (groups 1 and 4, respectively).

Analysis of GC-rich methylation of tumor and non-tumor tissue (29 weeks promotion) from treatment group 12 is shown in Figure 3. Increased methylation at the external C of 5′-CCGG-3′ sites is expected to result in more prominent bands in Rsal/MspI-treated samples, while increased methylation at the internal C is expected to result in more prominent bands in Rsal/HpaII-treated samples. Compared to untreated controls, there were seven regions in the tumor tissue for which bands in the Rsal/MspI were seen more prominently, indicative of methylation at the external cytosine of the 5′-CCGG-3′ site. In four of these seven regions, bands in the Rsal/MspI lanes of the non-tumor tissue from CSC-promoted animals were also more...
prominent than what was seen in the untreated controls (Fig. 3a). A quantitative representation of band intensity for each of the seven regions is provided in Figure 3b. Within a particular region, an increase in the average ratio of CSC-promoted samples compared to corresponding controls (untreated at 29 weeks, or acetone-promoted at earlier time points) represents a more prominent band in the CSC-promoted samples, indicative of CSC-induced hypermethylation. Conversely, a decrease in the average ratio of the CSC-promoted samples compared to controls represents a less prominent band in the CSC-promoted samples, indicative of CSC-induced hypomethylation. In this case, the higher ratios shown for the CSC-promoted tumor and non-tumor groups compared to untreated controls indicates hypermethylation at the external cytosine of the 5'-CCGG-3' site. Additional regions of GC-rich DNA were methylated at the external cytosine in tumor compared to non-tumor tissue.

The fact that we observe a very different degree of inhibition of both $M_spl$ and $H_paiI$, which share a common 5'-CCGG-3' recognition site, rules out the likelihood that adduct formation, rather than methylation changes, at the recognition site underlie the changes in band intensity observed. Specifically, we have observed many examples of sites where $M_spl$ digestion was inhibited and relatively few examples of sites where $H_paiI$ digestion was inhibited. In some cases, $M_spl$ digestion was inhibited within the same region where $H_paiI$ was not and vice versa.

In order to determine whether the effect of promoter treatment requires initiation to induce increases in methylation, the GC-rich methylation patterns of non-tumor tissue collected at 9 weeks from animals initiated with DMBA and promoted with 27 mg CSC (group 9) were compared with non-tumor tissue from animals initiated with acetone and similarly promoted with CSC (group 13). Regardless of whether the skin was initiated or not, increases in methylation were detected at the external cytosine site, indicating that prior application of an initiator was not necessary for CSC to effect methylation at the 9 week time point (data not shown). Furthermore, there were no differences seen in the GC-rich methylation patterns between DMBA-initiated, acetone-promoted (9 or 15 weeks), and untreated (29 weeks) animals (data not shown).

Next, we examined GC-rich methylation in non-tumor tissue from animals treated with various doses following 6 (Fig. 4) and 9 (Fig. 5) weeks promotion to determine the concentration of CSC necessary to induce detectable changes at each time point. The lowest dose found to cause changes in GC-rich methylation at 6 weeks was 27 mg (Fig. 4b). At this dose, there were 2 regions in both $R_sal/M_spl$ and $R_sal/H_paiI$ lanes at which bands were more prominent in CSC-promoted animals compared to acetone-promoted controls. The corresponding increases in pixel intensity ratios of the promoted animals (Fig. 4c) are indicative of increased methylation at both the internal and external cytosine sites. There were also two regions at which bands were seen less prominently in the $R_sal/M_spl$ lanes of the CSC-promoted animals compared to controls. The corresponding decreases in pixel intensity ratios (Fig. 4c) are indicative of a decrease in methylation at the external cytosine site. No changes in GC-rich methylation were detected with 18 mg CSC promotion at 6 weeks (Fig. 4a). Thus, the threshold dose needed to elicit detectable changes in GC-rich methylation at 6 weeks is between 18 and 27 mg CSC.
DMBA-initiated non-tumor skin promoted with 18 mg CSC for 9 weeks induces increases in band intensity in three regions in the RsaI/MspI lanes compared to acetone-promoted controls (Fig. 5b). The corresponding increases in pixel intensity ratios of the CSC-promoted animals (Fig. 5c) are indicative of increases GC-rich methylation at the external cytosine. Treatment of initiated tissues with 9 mg CSC does not elicit observable changes in methylation (Fig. 5a), indicating that the threshold dose needed for detectable increases in GC-rich methylation at 9 weeks is between 9 and 18 mg CSC.

In order to determine if CSC-induced changes in GC-rich methylation were reversible, we compared the effects of a 9-week treatment of 27 mg (group 9, Fig. 6a), a 9-week treatment of 27 mg followed by 6 weeks of no treatment (group 10, Fig. 6b), and a 27-mg treatment for 15 weeks (group 11, Fig. 6c) in non-tumor tissue. In all of these groups, an in-
increased amount of methylation at the external cytosine was observed. More prominent increases in external cytosine methylation were observed for treatment groups 9 and 11 compared to those seen for treatment group 10, consistent with the finding that the CSC-induced changes in GC-rich methylation are reversible. Table 1 presents quantification of the phospho-images depicted in Figure 6, showing that the most prominent increases in pixel intensity ratios in promoted animals compared to corresponding controls are seen in animals treated with 27 mg for 9 and 15 weeks (Figs. 6a and 6c, respectively; note in particular regions 1 and 4 in Table 1) in comparison to animals which were promoted with 27 mg CSC for 9 weeks and sacrificed after a 6-week recovery period (Fig. 6b; note region 3 in Table 1). These data support the conclusion that the increases in methylation induced by CSC are reversible.

Finally, when we examined tumor incidence after 29 weeks of CSC promotion (Fig. 7), we found that there was a very low incidence of tumors in uninitiated animals treated with 36 mg of CSC, demonstrating that initiation is required for a marked increase in tumor formation in response to CSC promotion.
under the experimental conditions examined. Also, the most significant increase in tumor number was seen between 9 and 18 mg CSC (Fig. 7), an observation that parallels the threshold dose required to discern increases in GC-rich methylation at 9 weeks (Fig. 5).

DISCUSSION

We have characterized changes in global and GC-specific methylation that occur as a result of promotion with various doses of CSC for different time periods in a two-stage initiation/promotion SENCAR mouse skin tumorigenesis model. Our goal was to determine the overall effect of the promoter on genome-wide patterns of methylation in order to discern particular aspects of methylation that are dysregulated during tumorigenesis. A frequent finding in tumor tissue is that global levels of methylation are decreased, while there appear to be selective increases and/or decreases in the GC-rich promoter regions of genes (Baylin et al., 1998). This is consistent with our observation that DMBA-initiated, CSC-promoted tumor tissue is globally hypomethylated with increases in GC-rich methylation. Additionally, our study reveals a progressive in-
crease in GC-rich methylation, in a time- and dose-dependent, threshold-exhibiting manner that precedes the appearance of tumors. Global hypomethylation appears to be a relatively late event that is observed in tumor tissue and not in surrounding non-tumor tissue. Therefore, distinct mechanisms might underlie alterations in global and GC-rich patterns of methylation.

Maintaining normal patterns of methylation is dependent on multiple, interdependent factors including maintenance and de novo methylation, demethylation not linked to DNA replication, the availability of methyl group sources (S-adenosyl methionine is the proximate methyl donor), cellular proliferation, and cellular differentiation (Goodman and Watson, 2002). Alteration of one or more of these factors may lead to hyper-and/or hypomethylation, both of which have been shown to contribute to carcinogenesis. Maintenance methylation following DNA replication is accomplished by Dnmt1, which acts preferentially at hemimethylated sites in DNA, while de novo methylation is primarily accomplished by Dnmt3a and b (Okano et al., 1998). Changes in the cellular proliferation rate challenge a cell’s methylation machinery to adjust the maintenance methylation rate accordingly, and cellular differentiation is controlled by changes in DNA methylation. In addition, methylation can be directed to specific regions of DNA. For example, the leukemia-promoting promyeloid leukemia reti-

FIG. 6. Reversibility of GC-rich methylation changes. The methylation status of GC-rich regions of initiated non-tumor mouse skin DNA promoted with 27 mg of CSC for 9 weeks (a), 27 mg of CSC for 9 weeks followed by 6 weeks of recovery (b), and 27 mg of CSC for 15 weeks (c) is presented. “C” indicates control animals initiated with DMBA and promoted with acetone, sacrificed at 9 weeks in (a) and 15 weeks for (b) and (c); “P” indicates DMBA-initiated, CSC-promoted animals. Numbers underneath the brackets at the top of the gel indicate individual animals. For each sample, RsaI, RsaI/MspI, and RsaI/HpaII digests were performed. Numbered solid boxes indicate rows of bands seen more prominently in CSC-promoted animals compared to controls. Dashed boxes indicate reference (R) rows of bands that are reasonably constant and highlighted to illustrate that lane-to-lane loading was relatively consistent. In (a) and (c), the area of the gel above the black line is from a 3-day exposure, while the area of the gel below the black line is from an 8-day exposure of the same gel. In (b), the entire image shown is from a gel exposed for 3 days. Results shown for the two animals in the control group and the four animals in each promoted group are representative of six animals per group.
Corresponding Reference Bands for Each Group in Figure 6a

- The bands in the corresponding reference regions (Figs. 6a–c). The fold change values were calculated by dividing the pixel intensity ratio in the designated regions as compared to that of the control samples (C) within the same region.

### TABLE 1

<table>
<thead>
<tr>
<th>Region</th>
<th>C</th>
<th>P</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.94</td>
<td>14.48</td>
<td>2.09</td>
</tr>
<tr>
<td>2</td>
<td>0.55</td>
<td>1.12</td>
<td>2.04</td>
</tr>
<tr>
<td>3</td>
<td>3.98</td>
<td>6.02</td>
<td>1.51</td>
</tr>
<tr>
<td>4</td>
<td>4.07</td>
<td>10.72</td>
<td>2.63</td>
</tr>
</tbody>
</table>

*Regions 1 and 2 are shown in Figure 6a, region 3 is shown in Figure 6b, and region 4 is shown in 6c.*

*P: promoted with 27 mg CSC for 9 weeks (regions 1 and 2), 27 mg CSC for 9 weeks with a 6-week recovery period (region 3), and 27 mg CSC for 15 weeks (region 4).

HpaII unique feature of our study is that through the use of methylation of both the internal and external cytosines of the 5′-CCGG-3′ site was assessed. We report that the majority of persistent methylation changes found within the GC-rich regions occur at the external C of 5′-CCGG-3′ sites, indicating that CSC promotion may have a targeted effect on this particular type of methylation. While the bulk of methylation research focuses on methylation within the symmetrical CpG dinucleotides, CpNpG methylation has been detected in mammalian cells (Clark et al., 1997; Stirzaker et al., 1997). The specific basis for CpNpG methylation in mammalian systems is not known. However, in Arabidopsis, CpNpG-specific methylation occurs through an interaction of the DNA methyltransferase with histone 3, which first must be methylated by a specific methyltransferase (Jackson et al., 2002).

Altered methylation of the GC-rich promoter regions of genes is a common event in carcinogenesis and is detectable prior to the appearance of a clinically evident tumor (Lehmann et al., 2002). For instance, methylation of the promoter regions of p16 and MGMT tumor suppressor genes has been detected in the sputum DNA of all patients with squamous cell carcinoma of the lung up to 3 years before clinical diagnosis (Palmisano et al., 2000). Furthermore, methylation of tumor suppressor genes p16, MINT1 (methylation in tumor 1), MINT2, MINT31, MGMT, or hMLH1 is frequently observed in colorectal cancer (Chan et al., 2002). Increases in promoter methylation of at least one of these genes was the only molecular abnormality identified in 16% of aberrant crypt foci, which are postulated to be the earliest precursor lesions in colorectal carcinogenesis (Chan et al., 2002). The arbitrarily primed PCR procedure used in our study has been shown to amplify GC-rich, CpG-containing promoter regions of a variety of genes (Gonzalgo et al., 1997; Kohno et al., 1998).

Both hyper- and hypomethylation of promoter regions might contribute to carcinogenesis by facilitating the transcriptional silencing of suppressor genes and enhanced expression of oncogenes, respectively (Laird, 1997). Furthermore, hypomethylation of non-promoter regions may lead to a decreased stability of the genome due to an increase in the expression of transposons that are typically silenced by methylation (Roberts and Jones, 2000). Therefore, alterations in DNA methylation may play a variety of roles in carcinogenesis (Counts and Goodman, 1995).

The SENCAR mouse skin model allows for demarcation of the initiation and promotion stages of carcinogenesis (Slaga et al., 1996). Additionally, the rate of tumor formation in animals treated with initiator only has been shown to be virtually the same as that in untreated animals (Ewing et al., 1988). Consistent with this observation, our studies demonstrate a clear dose-response relationship for tumor formation following promotion with CSC, while treating uninitiated animals with a high dose of promoter resulted in minimal tumor incidence, indicating that CSC does not appear to possess a significant initiating potential (Fig. 7). Therefore, CSC appears to be acting primarily as a tumor promoter, and the SENCAR model...
is ideal for permitting examination of both qualitative and quantitative effects on methylation during the promotion stage. We have demonstrated that the promoter effects on GC-rich methylation exhibit a threshold. Moreover, threshold doses required for detectable GC-rich methylation decreased with increased time of promotion, indicating that the effects of the promoter were both time- and dose-dependent, and that the altered methylation observed fits well with the classic criteria for a mechanism involved in tumor promotion (Pitot and Dragan, 1991, 1994). The promoting effects of CSC on methylation are similar to those elicited by the classic rodent liver tumor promoter phenobarbital (PB), which also causes global hypomethylation (Counts et al., 1996) and hypermethylation of GC-rich regions at both the external and internal cytosine sites at 5′-CCGG-3′ sequences (Watson and Goodman, 2002a). In addition, the effects of both PB and CSC are reversible, a hallmark characteristic of a tumor promoter (Pitot and Dragan, 1991, 1994).

Furthermore, we have found that the threshold dose required to induce detectable changes in GC-rich methylation (18 mg. Fig. 5) at 9 weeks is the same threshold dose required to elicit a dramatic increase in tumor incidence at 29 weeks (Fig. 7). This suggests that methylation changes at early times might be predictive of future tumorigenesis. Indications that methylation changes might serve as biomarkers of carcinogenesis have become increasingly prevalent. For instance, it has been reported that aberrant methylation of p16 is an early event in lung cancer and a potential biomarker for early diagnosis (Belinsky et al., 1998). Here, the GC-rich alterations detected prior to global decreases in methylation might be indicative of methylation-mediated silencing of particular tumor suppressor genes, followed by facilitation of expression of oncogenes and transposable elements. This model supports a causal role for altered methylation in skin tumorigenesis. CSC acts as a classic promoter, inducing methylation changes in a progressive, threshold-exhibiting, and reversible manner, as expected for a mechanism underlying tumor promotion. It is important to stress the fact that methylation change(s) per se, particularly at early times following chemical treatment, do not indicate that tumor formation is inevitable, since these changes are potentially reversible.

Carcinogenesis involves a progressive clonal selection/expansion of cells that are increasingly abnormal, both genetically and phenotypically. The specific sequence by which key heritable alterations to the genome occur may be an important determinant of carcinogenesis. However, it appears likely that the individual crucial alterations to critical genes stem from a stochastic process, and one can expect this to be enhanced under conditions where control of DNA methylation is decreased. Indeed, whether a particular modification predominates, e.g., hypo- versus hypermethylation and/or alterations in global and/or GC-rich regions, at a certain stage of tumor development can depend upon the species, target organ, and chemicals involved (Counts and Goodman, 1995). The current characterization of stepwise, progressive, promoter-induced alterations in methylation in the SENCAR two-stage mouse skin tumorigenesis model provides further support for the multiple roles that aberrant methylation may play in this process. Multiple changes in methylation are observed during CSC tumor promotion; increased methylation of GC-rich regions precedes global decreased methylation. Hence, progressive alterations in global and GC-rich methylation appear to be mechanistically important in tumor promotion.

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