Altered DNA methylation, an epigenetic mechanism, likely contributes to tumorigenesis, with an inverse relationship existing between methylation in a promoter region and transcription. Using the SENCAR two-stage mouse skin tumorigenesis model, altered methylation was characterized in precancerous tissue and in tumor tissue. Mouse skin was initiated with 7,12-dimethylbenz[a]anthracene and promoted three times a week with 3, 9, 18, or 27 mg cigarette smoke condensate (CSC) for 4, 8, or 29 weeks; tumors were collected at 29 weeks. In addition, reversibility of changes in methylation was assessed following cessation of the promoting stimulus. DNA was isolated, and GC-rich methylation was assessed quantitatively via methylation-sensitive restriction digestion, arbitrarily primed PCR, and electrophoretic separation of PCR products. Analysis focused on regions of altered methylation (RAMs), which persisted from 4 to 8 weeks and from 8 weeks to tumor tissue. Persistent RAMs (i.e., seen in precancerous tissue and carried forward to tumors) are likely to play a key role in tumorigenesis. Twenty-two CpG sites in the upstream region of the Ha-ras promoter were unmethylated in control skin, 27 mg CSC, and tumor tissue. At two CpG sites closer to the transcriptional start site the incidence of hypomethylation increased with the dose of CSC. Hypomethylation was detected in all tumor samples. Expression of Ha-ras increased with 18 and 27 mg CSC promotion and more so in tumor tissue. These data support our hypothesis that tumor promotion involves instability of the epigenome, providing an environment where changes in the methylation status of specific regions of the genome accumulate progressively and contribute to the clonal expansion of initiated cells that leads to tumor formation.

Key Words: DNA methylation; epigenetic; Ha-ras; promotion; SENCAR; skin tumorigenesis.
Epigenetics is defined broadly as processes that establish heritable states of gene expression without altering the DNA sequence. Specifically, altered patterns of DNA methylation (i.e., 5-methylcytosine content of DNA) have been shown to occur during the promotion stage of skin tumorigenesis. Previous studies in our laboratory showed that cigarette smoke condensate (CSC), an effective promoting agent in the two-stage model, induced reversible dose- and time-dependent changes in methylation in GC-rich regions of DNA as well as global decreases in methylation in tumor tissue (Watson et al., 2003).

GC-rich regions of DNA are frequently associated with the promoter regions of genes, and the methylation status of promoter regions can be linked to the regulation of gene expression. Specifically, increased methylation in a promoter region may decrease gene expression, while a decrease in promoter methylation possesses the potential for upregulating gene expression (Jones and Laird, 1999). The silencing of tumor suppressor genes via an increase in promoter methylation has been demonstrated for genes such as O'6-methylguanine-DNA methyltransferase, cyclin-dependent kinase inhibitor 2B, and RASSF1A (Jones and Baylin, 2002; Watson et al., 2004). In addition, HoxA5, which upregulates p53 expression, was shown to be hypermethylated following promotion with 27 and 36 mg CSC for 9 weeks, which was associated with a decrease in expression (Watson et al., 2004). Aberrant patterns of methylation, specifically hypomethylation, may also facilitate the activation of oncogenes. Ha-ras, a classic oncogene, is reproducibly activated in mouse epidermal tumors (Balmain and Pragnell, 1983; Balmain et al., 1984). This has mainly been attributed to early mutation induced by initiating agents such as DMBA and dibenzo[a,l]pyrene (DB[a,l]P). However, one study suggests that mutations in codon 61 of Ha-ras induced by DB[a,l]P simply result in a transient proliferation of cells. Over time only a small subpopulation of these cells persist, whereas the majority are lost (Khan et al., 2005). It is possible that continued upregulation of Ha-ras may be governed by changes in DNA methylation. In support of this assertion, decreased methylation at one Xhol site within the vicinity of the Ha-ras gene exhibited hypomethylation as compared to normal epidermis in some papillomas and carcinomas (Ramsden et al., 1985). Based on these intriguing but limited data, further investigation of Ha-ras promoter methylation is necessary.

We hypothesize that progressive, nonrandom changes in DNA methylation contribute to tumorigenesis. SENCAR mice were initiated with DMBA and promoted with increasing doses of CSC for 4 or 8 weeks. The GC-rich methylation patterns were analyzed for dose and time relationships as well as reversibility in precancerous skin tissue via a sensitive and quantitative arbitrarily primed PCR (AP-PCR) and capillary electrophoretic approach. Regions of altered DNA methylation that persist from precancerous to tumor tissue are identified, and changes in the methylation status of the promoter region of Ha-ras are associated with changes in gene expression.

**MATERIALS AND METHODS**

**Animals**

Female SENCAR mice (ages 7–10 weeks) were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). Animals were quarantined and allowed to acclimate for a minimum of 10 days prior to being randomly assigned to treatment groups based on 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 risk level to ensure groups of similar mean body weight. Animals were housed and cared for at the R. J. Reynolds Tobacco Co. facilities and in accordance with the Institute of Laboratory Animals Resources, Commission of Life Sciences, National Research Council document entitled, *Guide for the Care and Use of Laboratory Animals*. Mice, six to seven animals per group, were initiated with a single, 200 μl topical application of 75 μg DMBA, followed by three weekly applications of 3, 9, 18, or 27 mg CSC or 8% water in acetone (vehicle control) per 200-μl application for 4, 8, or 29 weeks and sacrificed immediately afterward. The recovery groups were treated with 3, 9, 18, or 27 mg CSC for 8 weeks and allowed an 8-week recovery period prior to sacrifice. Two additional recovery groups for mice treated with 27 mg for 4 weeks were allowed a 4- or 8-week recovery period prior to sacrifice. Each dose was applied to the midback of the animal and evenly spread across the application site, which extended from the nape of the neck to the base of the tail and laterally from the shoulders to the hips. Hair was removed from the application sites with electric clippers prior to dosing. All applications were conducted according to a standardized protocol (Meckley et al., 2004). Mice were euthanized with 70% CO₂, and skin tissue was collected from the chemical application site. Skin masses that arose during the 29-week post-initiation period were excised and identified histologically for tumor type, and papilloma tissue was employed for these experiments. All collected samples were snap-frozen at –80°C and kept until analyzed.

**DNA and RNA Isolation**

In order to isolate DNA, frozen skin tissue or tumor tissue was pulverized using a mortar and pestle and then allowed to thaw following the addition of TRI Reagent (Sigma St. Louis, MO). A dounce homogenizer was used to thoroughly homogenize the sample. RNA was isolated according to the manufacturer’s protocol. DNA isolation was carried out according to an alternative protocol obtained from the manufacturer. DNA was extracted with back extraction buffer in lieu of ethanol precipitation.

**Preparation of CSC**

CSC was prepared as described previously (Watson et al., 2003), and doses of 3, 9, 18, and 27 mg were applied to the animals three times a week.

**AP-PCR and Capillary Electrophoresis**

A comparison of data obtained from DNA isolated from control and treated tissues permits the simultaneous detection of treatment-related increased methylation (more methylation in a region that was methylated in control), decreased methylation (less methylation in a region that was methylated in control), and new methylation (methylation in regions that were not methylated in control). Therefore, the procedure we have developed provides an in-depth picture of treatment-related altered methylation in multiple regions of the genome (Bachman et al., 2006).

**Restriction Digests**

DNA samples, of which duplicates are prepared, are subjected to double digests with restriction enzymes: (1) a methylation-insensitive enzyme and (2) a methylation-sensitive enzyme. Rsal is the methylation-insensitive enzyme used initially to cut the DNA into fragments which facilitates complete digestion by the second enzyme, a methylation-sensitive restriction enzyme. The methylation-sensitive enzymes used in this study were *MspI* and *HpaII*. 

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Restriction digestion with Rsal/MspI and Rsal/HpaII was performed as described previously (Bachman et al., 2006).

AP-PCR and Capillary Electrophoretic Separation of PCR Products

AP-PCR was performed on restriction digests using a single arbitrary primer, 5′ AAC CCT CAC CCT AAC CCC GG 3′ (modified from Gonzalgo et al., 1997), fluorescently labeled at the 5′ end with HEX (purchased from Integrated DNA Technologies Coralville, IA). This primer was designed to bind well to GC-rich regions, and the 5′ CCGG 3′ sequence at its 3′ end increases the probability of primer annealing to the MspI and HpaII restriction site, allowing for the detection of methylation at the site of primer annealing and between sites of primer annealing. The procedures for AP-PCR and capillary electrophoresis were performed as described previously (Bachman et al., 2006).

Data Analysis

The results represented as size of PCR products, in base pairs, and their corresponding peak areas were analyzed using the Excel program. A consensus, average, peak area for each PCR product reporting in control and treated groups was prepared, and the consensus control and treated peaks at a specific PCR product were compared. This permitted us to detect treatment-related (1) hypomethylations which include both 100% decreases and decreases which are statistically significant when compared to control, (2) hypermethylation which are increases that are statistically significant when compared to the control, and (3) new methylations which are indicated by the formation of a PCR product following treatment which was not formed under control conditions. Significance was determined via a Student t-test, p < 0.05. The detailed procedure for data analysis has been described previously (Bachman et al., 2006).

Reversibility of Altered Methylation: Calculations

A detailed description of the approach by which reversibility of altered methylation was calculated is provided as Supplementary Information.

Assumptions of the AP-PCR Data Analysis

Analysis of the data includes the following assumptions: (1) each separate PCR product of a defined size represents a distinct region of the genome; (2) a region can include one or more recognition sequences for the specific methylation-sensitive restriction enzyme employed located between the annealing sites of the upstream and downstream primers, thus, the amount of each PCR product formed can be viewed as representing an “average” of the methylation status of the particular recognition sequences located between the upstream and downstream primers; and (3) changes in the amount of each PCR product represents the altered methylation status of a particular GC-rich region of DNA.

Common and Unique Regions of Altered Methylation

PCR products of identical size that occur in two treatment groups (e.g., 27 mg CSC promotion and tumor tissue) were considered to be common regions of altered methylation (RAMs). The methylation changes associated with RAMs in common between two treatment groups were considered equivalent and persistent if the changes in methylation are in the same direction and the extents of change are statistically no different as determined by two-way ANOVA, p = 0.05. RAMs in common between two treatment groups were considered unique RAMs if (1) the changes in methylation were opposite in direction (i.e., a hypomethylation is elicited by one treatment and a new methylation is elicited in the same region by the comparison treatment) or (2) the changes in methylation were in the same direction but the extents of change were statistically different as determined by two-way ANOVA, p = 0.05. RAMs were deemed to be unique if they were only observed in a particular treatment group.

Evaluating Total RAMS

Although HpaII and MspI both restrict CCGG sites, HpaII identifies altered methylation at the internal cytosine while MspI identifies altered methylation at the external cytosine. Therefore, the Rsal/MspI and Rsal/HpaII digests were considered separate experiments to determine regions of the genome which exhibit altered methylation in response to treatment. When we look at the total number of RAMs identified by HpaII and MspI, there is the unavoidable possibility that a slight amount of double counting might occur. This would be the case if methylation was altered at both the internal and external cytosine within CCGG sites of a particular genomic region. Therefore, we would be considering differences in methylation within a given region even if there were some “double counting” of RAMS.

Percent Dissimilarity Calculations

The dissimilarity between the extents of altered methylation due to treatment in comparison to control was calculated. The total number of PCR products reporting in control was added to the total number of unique PCR products (i.e., those PCR product sizes that were not formed under control conditions) reporting from treatment to get the total number of combined PCR products. This represents the total number of regions (PCR products) analyzed between the two groups.

The total number of regions (PCR products) exhibiting a statistically significant change (hypomethylations, hypermethylations, and new methylations) divided by the total number of combined PCR products times 100 equals the percent dissimilarity from control.

Gene-Specific Methylation Analysis: Bisulfite Sequencing of 5′ Promoter Region of Ha-ras

Bisulfite conversion and PCR amplification. Bisulfite conversion of DNA effectively deaminates all unmethylated cytosines to uracil, leaving methylated cytosines unaffected. Two micrograms of DNA was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA). PCR is performed with bisulfite-converted DNA which allows for the replacement of uracil with thymine and 5-methylcytosine with cytosine. Consequently, only cytosines that were originally methylated remain in the DNA sequence. PCR was carried out using primers specific for bisulfite-converted DNA and containing no CpG sites. The two Ha-ras primers, 5′ GTG GGG TTA GAG TGT TTA AGA TTT G 3′ and 5′ CTC TTA CTC TAA AAG ACA TTT CCA C 3′ were used to amplify the −950 to −1232 nt (283 bp) region of the Ha-ras promoter relative to the transcriptional start site. Primers were designed based on sequence information obtained from Brown et al. (1988) and Neades et al. (1991). Each PCR contained 0.5 μg bisulfite-converted DNA, 1 × Failsafe Buffer G (Epicentre Technologies, Madison, WI), 0.3 μM each primer, 1.5 units Taq polymerase (Invitrogen Carlsbad, CA), and glass distilled water (GDW) to a final volume of 25 μl. Cycling conditions were 95°C for 3 min, 38 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 1 min, followed by a 1 time delay cycle of 72°C for 5 min and a 4°C soak. Amplification of the target region was verified by gel electrophoresis on a 3% agarose gel. Duplicate Ha-ras PCR products were combined and purified using the Qiagen (Valencia, CA) Qiaquick PCR Purification Kit. Samples were quantified fluorometrically.

Sequencing. Automated sequencing of purified Ha-ras PCR products was carried out at the Genomics Technology Support Facility at Michigan State University using an ABI PRISM3100 Genetic Analyzer. Two separate sequencing reactions were performed for each gene. Sequencing reactions are composed of a 20-ng PCR product, 30 pmol of either the forward primer or reverse primer, and GDW to 12 μl.

Gene-Specific Methylation Analysis: Methylation-Sensitive Restriction Digestion of Ha-ras

Restriction digests. DNA was restricted with Smal endonuclease (Invitrogen), a methylation-sensitive enzyme which recognizes the CCCGGG sequence and restricts DNA only if the cytosine immediately 5′ to guanine is not methylated. Methylated DNA will not be restricted by Smal; therefore, the target region will be amplified with subsequent PCR. Unmethylated DNA will be restricted by Smal preventing amplification of the target region. Each reaction was composed of 1 μg DNA, 3 units Smal enzyme, 1 × React4 Buffer (Invitrogen), and GDW to a final volume of 10 μl. Negative control digests
i.e., Smal was omitted) for each animal were also prepared. Reactions included, 1 μg DNA, 1× React4 Buffer, and GDW to a final volume of 10 μl. All samples were incubated at 37°C for 16 h.

**PCR amplification.** PCR was carried out on digested (i.e., incubated with Smal enzyme [+ Smal]) or undigested (i.e., incubated without Smal enzyme [− Smal]) DNA. Forward (5’ CAG GGT GGA GGC TCT GTA GT 3’) and reverse (5’ GAG AGG AGC AAG GAA GCA CC 3’) primers (Okoji et al., 2002) amplify the ~325 to +200 region spanning the transcriptional start site of the Ha-ras gene which contains two Smal restriction sites. Reactions consisted of 1× Failsafe Buffer H (Epicentre Technologies), 2 μM each primer, 0.43 μg digested or undigested DNA, and GDW to a final volume of 25 μl. Cycling conditions were 80°C for 5 min, 94°C for 2 min, 24 cycles of 96°C for 1 min, 65°C for 1 min, and 72°C for 2 min, followed by 1 time delay cycle of 72°C for 5 min. PCR products were electrophoresed (3% agarose gel) and visualized by ethidium bromide staining. A Polaroid picture of each gel was taken; the Polaroid picture was scanned, and the image was analyzed using the NIH image program. The number of PCR cycles (24) was chosen following a pilot study in which 21, 23, and 25 cycles were tested. The chosen number of cycles was estimated to maximize the difference in PCR product band intensities between the undigested control DNA and the digested DNA. Because a heterogeneous mixture of cells expressing varying degrees of methylation were sampled, increases and decreases in band intensity compared to control is proportional to increases and decreases in methylation of Smal restriction sites.

**Quantification of band intensity.** The relative intensity of a PCR product band corresponds to the relative starting concentration of methylated DNA. Each target PCR product band was outlined and measured for pixel number and intensity by using the NIH image program. The number of pixels defined the size of the outlined region. The same sized region was used to measure both the PCR product band and the lane background. Total pixel intensity (TPI) units were calculated by multiplying the number of pixels by the mean intensity units within the outlined region. This was done separately for both the background and PCR product band. The TPI units of the background were subtracted from the TPI units of the PCR product band to give a normalized TPI for the outlined region within a lane.

**Calculation of ratios and 95% confidence interval.** TPI units were calculated for PCR product bands resulting from amplification of either digested (+ Smal) or undigested (− Smal) DNA. For each animal, a ratio was calculated to determine the amount of PCR product formed from digested DNA versus undigested DNA. For this, a ratio of the TPI units from + Smal DNA to TPI units from − Smal DNA was calculated. The mean (n = 5) ratio with standard deviation and 95% confidence interval (CI) (±0.05) was calculated for the control animals only. Treated animal ratios were compared individually to the control 95% CI. If a ratio from a treated animal fell above the upper limit of the CI, the internal cytosome within the Smal sites was considered to be hypermethylated. If a ratio from a treated animal fell below the lower limit of the CI, the internal cytosome within the Smal sites was considered to be hypomethylated.

**Expression of Ha-ras.**

**Reverse transcription of RNA.** Triplicate RNA samples were treated with DNaseI (Invitrogen) to purify the RNA from contaminating DNA remaining after isolation. Each reaction contained 2 μg RNA, 1× DNaseI reaction buffer, 2 units DNaseI, and DEPC (Diethylpyrocarbonate)-treated GDW to a final volume of 20 μl. Samples were incubated at room temperature for 15 min followed by addition of MgCl₂ to a final concentration of 2.7 mM. RNA was heated to 65°C for 10 min to inactivate the DNaseI enzyme. The TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to reverse transcribe the DNaseI-treated RNA. Each reverse transcription reaction contained 1× Reverse Transcription Reaction Buffer, 5.5 mM MgCl₂, 200 μM of each dNTP, 2.5 μM random hexamer, 20 units RNase Inhibitor, 62.5 units Multiscribe Reverse Transcriptase, and DEPC-treated GDW to a final volume of 50 μl. The reactions are incubated at 25°C for 10 min, 42°C for 1 h, and 95°C for 5 min. All samples were stored at 4°C until needed.

**Real-time PCR.** A custom TaqMan assay including primers (For 5’ TGG TGG GCA ACA AGT GTG A 3’ and Rev 5’ GGC CTG CCG AGA CTC A 3’) and probe (5’ FAM CTG GCT CGC ACT GT 3’) specific for exon 3 of the Ha-ras gene was purchased from Applied Biosystems. This custom assay employs a nonfluorescent quencher dye. The assay was designed based on sequence information obtained from Brown et al. (1988) and Neades et al. (1991). In addition, an Applied Biosystems custom TaqMan assay including primers (For 5’ CTA CTA CCG ATT GGA TGG TTT AGT GA 3’ and Rev 5’ GTC AAG TTC GAC CGT CTT CTC A 3’) and probe (FAM 5’ CCG TGG GCC GAC CC 3’) was used for the control gene, 18s rRNA (accession no. X00686). Triplicate reactions for both the gene of interest (Ha-ras) and the control gene (18S) were prepared per sample. Standards were also prepared for 18S and Ha-ras and ranged from 5 × 10⁵ copies/μl to 5 × 10⁷ copies/μl. Each Ha-ras reaction contained 1× Custom Assay Mix (Applied Biosystems), 1× TaqMan Universal PCR MasterMix, 11 μl cDNA, and GDW to a final volume of 25 μl. Each 18S reaction contained 1× Custom Assay Mix (Applied Biosystems), 1× TaqMan Universal PCR MasterMix, 11 μl cDNA, and GDW to a final volume of 25 μl. Reactions for each standard contained 1× Custom Assay Mix (Applied Biosystems), 1× TaqMan Universal PCR MasterMix, 2 μl standard, and GDW to a final volume of 25 μl. Cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The absolute standard curve method for quantifying fold change over control was employed.

**RESULTS**

Analysis of GC-rich regions of DNA allowed for a genome-wide snapshot of altered methylation including hypomethylations, hypermethylations, and new methylations in response to treatment. Dose-dependent changes in methylation were discerned following promotion of mouse skin with 3, 9, 18, or 27 mg CSC for 8 weeks. In addition, tumor tissue was evaluated for aberrant patterns of methylations. During promotion, a treatment-related increase in the total number of RAMs was observed, whereby the RAMs induced by 3 and 9 mg CSC were approximately half of the total RAMs induced by 18 and 27 mg CSC (Table 1). Regions of increased methylation, including hypermethylations and new methylations, were predominant and clearly increased with dose (Fig. 1). In contrast, regions of hypomethylation did not exhibit a dose-response relationship. Tumor tissue was somewhat distinct from precancerous tissue in that the incidence of hypermethylations and new methylations decreased while total regions of hypomethylation increased and comprised over half of the total alterations detected (Fig. 1). A comparative evaluation of the total changes in altered methylation between treatment groups allowed for the identification of developing patterns and trends which developed with increasing doses of CSC. However, in order to clearly and simply demonstrate the extent to which RAMs that developed during promotion and in tumor tissue differed from controls, a percent dissimilarity was calculated for each separate dose and for tumor tissue. Methylation patterns arising from 3 and 9 mg CSC promotion were 49 and 48% dissimilar to methylation...
patterns of controls (Table 2). With 18 and 27 mg CSC promotion, dissimilarity increased to 70 and 66% (Table 2). This illustrates that patterns of methylation become more abnormal as the dose of CSC increases from 9 to 18 mg CSC.

As expected, the status of methylation in tumor tissue was the most dissimilar (79%) to control (Table 2). Dose-dependent changes in methylation were consistent with tumor multiplicity reported for each dose of CSC. At 29 weeks, just under eight tumors per mouse were reported in response to 27 mg CSC promotion (Fig. 2). Approximately five tumors per mouse were induced by 18 mg CSC promotion and two tumors per mouse were seen with 9 mg CSC promotion; 3 mg tumor promotion did not increase tumor incidence above that of the control (Fig. 2). Therefore, tumor multiplicity could reflect, in part, the extent of altered patterns of methylation and the increasing dissimilarity of patterns as the dose of CSC is increased.

With promoter stimulation, an accumulation of changes in methylation could occur over time. Time-dependent changes in methylation were tracked following promotion of mouse skin with 27 mg CSC for 4 and 8 weeks. Total RAMs increased from 21 at 4 weeks to 40 at 8 weeks where most changes were attributable to hypermethylations and new methylations (Table 3). This supports the notion that the number of changes in methylation increases and accumulates with time.

A unique feature of the arbitrarily primed PCR, capillary electrophoresis approach employed is that it allows multiple methylation changes within particular regions of the genome to be identified and it permits comparisons to be made with regard to dose and time (Bachman et al., 2006). The actual, basic underlying data for the Figures 3–5 are presented as Supplemental Information. Changes in methylation induced by 4-week, 27-mg CSC promotion were compared to those induced by 8-week, 27-mg CSC promotion. Common RAMs and unique

### Table 1

**Summary of GC-Rich RAMs: Comparison of DMBA-Initiated, CSC-Promoted (8 week) Tissue and Tumor Tissue to Control (DMBA/Acetone)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Digest</th>
<th>Regions of hypomethylation$^a$</th>
<th>Regions of hypermethylation$^b$</th>
<th>Regions of new methylation$^c$</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mg CSC</td>
<td>HpaII</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>MspI</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Total$^d$</td>
<td></td>
<td>14</td>
<td>0</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>9 mg CSC</td>
<td>HpaII</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>MspI</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Total$^d$</td>
<td></td>
<td>2</td>
<td>8</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>18 mg CSC</td>
<td>HpaII</td>
<td>6</td>
<td>10</td>
<td>19</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>MspI</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Total$^d$</td>
<td></td>
<td>7</td>
<td>10</td>
<td>21</td>
<td>38</td>
</tr>
<tr>
<td>27 mg CSC</td>
<td>HpaII</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>MspI</td>
<td>0</td>
<td>10</td>
<td>27</td>
<td>37</td>
</tr>
<tr>
<td>Total$^d$</td>
<td></td>
<td>2</td>
<td>10</td>
<td>28</td>
<td>40</td>
</tr>
<tr>
<td>Tumor tissue</td>
<td>HpaII</td>
<td>19</td>
<td>0</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>MspI</td>
<td>3</td>
<td>1</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>Total$^d$</td>
<td></td>
<td>22</td>
<td>1</td>
<td>14</td>
<td>37</td>
</tr>
</tbody>
</table>

$^a$Hypomethylated RAMs include both statistically significant (Student $t$-test, $p < 0.05$) decreases and 100% decreases.

$^b$Hypermethylated RAMs are only those increases which are statistically significant (Student $t$-test, $p < 0.05$).

$^c$New methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not formed under control conditions.

$^d$Total RAMs including hypomethylations, hypermethylations, and new methylations for the RsaI/MspI and RsaI/HpaII digests combined are reported for each treatment.

FIG. 1. Increases (hypermethylations and new methylations) in GC-rich methylation are dose dependent. RAMs are categorized as increased methylation and decreased methylation. Increases in methylation exhibit a clear dose-response relationship. Tumor tissue is shown for comparison. A table tallying the RAMs for each dose and tumor tissue is shown as an inset in the chart. RAMs exhibiting increased methylation include all hypermethylations and new methylations. Hypermethylations are increases that are significantly different from control values (Student $t$-test, $p < 0.05$). RAMs exhibiting decreased methylation include all partial hypomethylations and complete hypomethylations (100% decreases from control). Partial hypomethylations are decreases that are significantly different from control values (Student $t$-test, $p < 0.05$).
RAMs were identified (Fig. 3A). Common RAMs exhibiting equivalent changes in methylation persisted from 4 to 8 weeks. The persistent, “carry forward,” RAMs included 1 hypomethylation, 3 hypermethylations, and 10 new methylations (Fig. 3B). A further comparison was made between 8-week, 27-mg CSC–induced methylation changes and those identified in tumor tissue (29 weeks). We were able to categorize the nine persisting changes into two groups: (1) those that persisted from 8 to 29 weeks and (2) those that originated with 4 weeks promotion and persisted through the 8-week time point to the tumor tissue (Fig. 3B). Of the nine persisting changes one of the two hypomethylations and four of the six new methylations originated from the 4-week, 27-mg CSC promotion (Fig. 3B). This stepwise accumulation of RAMs over time as precancerous skin is promoted with 27 mg CSC and progresses to tumor tissue likely represents critical changes in methylation.

RAMs exhibited by the tumor tissue were a compilation analysis of nine tumors; three tumors each arose from the 9-, 18-, and 27-mg CSC promotion. Common and equivalent RAMs (persisting changes) were identified by separately comparing 8-week, 9-, 18-, and 27-mg CSC–induced changes to tumor tissue. Persisting changes in methylation originating from 9, 18, and/or 27 mg CSC are depicted in Figure 4B along with the unique changes exhibited by the tumor tissue. As expected, the highest number of critical changes in methylation which carried through to tumor was seen with 27 mg CSC. Of the 13 total persistent changes in tumor, four (one hypomethylation, one hypermethylation, and two new methylations) were solely attributable to the 27-mg CSC promotion and three hypomethylations were solely attributable to the 18-mg CSC promotion. Notably, one hypomethylation and one new methylation induced by all three promoting doses persisted to

### TABLE 2
Measure of the Percent Dissimilarity of DMBA-Initiated, 8-week, 3-, 9-, 18-, and 27-mg CSC–Promoted Tissue or Tumor Tissue to DMBA/Acetone Control

<table>
<thead>
<tr>
<th>Total number</th>
<th>Total RAMs</th>
<th>Percent dissimilarity to DMBA/acetone control</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mg CSC</td>
<td>39</td>
<td>20/39 51%</td>
</tr>
<tr>
<td>9 mg CSC</td>
<td>44</td>
<td>21/44 48%</td>
</tr>
<tr>
<td>18 mg CSC</td>
<td>54</td>
<td>38/54 70%</td>
</tr>
<tr>
<td>27 mg CSC</td>
<td>61</td>
<td>40/61 66%</td>
</tr>
<tr>
<td>Tumor</td>
<td>47</td>
<td>37/47 79%</td>
</tr>
</tbody>
</table>

*aThe total number of regions includes every PCR product size reporting between control and treated groups. Each PCR product represents a region of the genome.

*bTotal RAM represents the number of regions exhibiting complete hypomethylation (100%), partial hypomethylation, hypermethylation, or new methylation.

### FIG. 2.
Tumor multiplicity increases with dose of CSC. Tumor multiplicity for animals initiated with DMBA and promoted thrice weekly with 3, 9, 18, or 27 mg CSC for 29 weeks is shown. Total number of tumors per mouse in each dosing group is expressed over time.

### TABLE 3
Summary of GC-Rich RAMs: Comparison of DMBA-Initiated, CSC 4- and 8-week Promotion to Control

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Digest</th>
<th>Regions of hypomethylation</th>
<th>Regions of hypermethylation</th>
<th>Regions of new methylation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-week promotion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 mg CSC</td>
<td>HpaII</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>MspI</td>
<td>0</td>
<td>3</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1</td>
<td>5</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>8-week promotion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 mg CSC</td>
<td>HpaII</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>MspI</td>
<td>0</td>
<td>10</td>
<td>27</td>
<td>37</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>2</td>
<td>10</td>
<td>28</td>
<td>40</td>
</tr>
</tbody>
</table>

*aHypomethylated RAMs include both statistically significant (Student t-test, p < 0.05) decreases and 100% decreases.

*bHypomethylated RAMs are only those increases which are statistically significant (Student t-test, p < 0.05).

*cNew methylations indicate the formation of a PCR product following treatment due to a gain of methylation either at the site of primer annealing or between sites of primer annealing which was not formed under control conditions.

*dTotal RAMs including hypomethylations, hypermethylations, and new methylations for the RsaI/MspI and RsaI/HpaII digests combined are reported for each treatment.
the tumors. Each dose of CSC elicited changes in methylation that persisted and could contribute to the altered pattern of methylation observed in tumor tissue (Figs. 4A and 4B).

Reversibility, as an operational definition, is characteristic of tumor promotion as continued exposure to the promoting agent is necessary for progressive clonal expansion of initiated cells. In light of this, changes in methylation were assessed following a 4- or 8-week recovery period. Specifically, RAMs previously induced by 4- or 8-week, 27-mg CSC promotion were reanalyzed following the recovery period. Reversal of the methylation changes induced by 4-week, 27-mg CSC was more complete as the duration of recovery was lengthened. Following 4 weeks recovery, two hypermethylations and seven new methylations had reversed (Fig. 5A). With 8 weeks recovery, an additional five RAMs had reversed (Figs. 5A and 5B). In comparison, 8 weeks promotion with 27 mg CSC followed by 8 weeks recovery resulted in the reversal of 1 of 2 hypomethylations, 10 of 10 hypermethylations, and 24 of 28 new methylations (Fig. 5B). Importantly, all changes, with the exception of one new methylation, which persisted from the 4-week promotion, were recoverable, which clearly demonstrates that changes in methylation which accumulate in response to the promoting stimuli are largely reversible.

GC-rich regions of the genome are frequently found in the promoter regions of genes, and changes in methylation can be associated with changes in gene expression. Therefore, in light of the number of CSC- and tumor-induced RAMs within GC-rich regions, a gene-specific approach was used to assess CSC-induced changes in methylation within the promoter region of Ha-ras. Upregulation of Ha-ras, an oncogene, via

**FIG. 3.** Progressive changes in methylation: changes which persist from 4 to 8 weeks and from 8 weeks to tumor. The flowchart illustrates the steps necessary to determine progressive changes in methylation (A). RAMs induced by 4-week, 27-mg CSC promotion were compared to those resulting from 8-week, 27-mg CSC promotion. Common RAMs in which the magnitudes of change were equivalent (two-way ANOVA, \( p < 0.05 \)) were considered persistent changes. RAMs unique to 8-week, 27-mg CSC included common RAMs in which the magnitude of change was different or the RAMs were only observed with 8-week, 27-mg CSC. Similarly, RAMs induced by 8-week, 27-mg CSC promotion were compared to those in tumor. Common RAMs in which the magnitudes of change were equivalent (two-way ANOVA, \( p < 0.05 \)) were considered persistent changes. RAMs unique to tumor included common RAMs in which the magnitudes of change were statistically different or the RAMs were only observed in tumor tissue. RAMs in which the magnitude of change was different included common regions in which the changes in methylation were opposite in direction and common regions in which the change was in the same direction, but the magnitudes of change were statistically different (two-way ANOVA, \( p < 0.05 \)). Persistent and unique RAMs induced by 4-week, 27-mg CSC (\( \text{O} \)), 8-week, 27-mg CSC (\( \text{O} \)), and tumor (hexagons) are represented (B). Hypomethylations (HYPM), hypermethylations (HYPERM), and new methylations (NEWM) are segregated. Total unique changes (minus any persistent changes) are tallied and reported for each category of methylation change.
FIG. 4. Progressive changes in methylation: changes induced by 8-week, 9-, 18-, 27-mg CSC and persist to tumor. The flowchart illustrates the steps necessary to determine progressive changes in methylation (A). RAMs induced by 8-week, 9-, 18-, and 27-mg CSC promotion were compared to those identified in tumor tissue. Common RAMs in which the magnitudes of change were equivalent (two-way ANOVA, \( p < 0.05 \)) were considered persistent changes. RAMs unique to tumor included common RAMs in which the magnitude of change was different or the RAMs were only observed in tumor tissue. RAMs in which the magnitude of change was different included common regions in which the changes in methylation were opposite in direction and common regions in which the change was in the same direction, but the magnitudes of change were statistically different (two-way ANOVA, \( p < 0.05 \)). Persistent and unique RAMs induced by 8-week, 9-, 18-, or 27-mg CSC (\( \bigcirc \)) and tumor (\( \bigcirc \)) are represented (B). A total of six RAMs each persisted from 9 and 18 mg CSC to tumor while nine RAMs persisted from 27 mg CSC to tumor. Hypomethylations (HYPOM), hypermethylation (HYPERM), and new methylations (NEWM) are segregated for each treatment. Total unique changes (minus any persistent changes) are tallied and reported for each category of methylation change. * Represents three identical RAMs which persisted from both 9 and 27 mg CSC promotion. ** Represents one identical RAM which persisted from both 9 and 18 mg CSC. *** Represents identical RAMs which persisted from 9, 18, and 27 mg CSC.
hypomethylation of its promoter region could lead to increased expression, which might contribute to tumorigenesis. We first analyzed a 283-bp region of the Ha-ras promoter containing 22 CpG dinucleotides which was 950 nt upstream of the transcriptional start site (Fig. 6A). Virtually all 22 CpG sites were unmethylated in control skin tissue; 27 mg CSC promoted precancerous skin tissue and tumor tissue (Fig. 6B). Analysis of changes in methylation occurring closer to the transcriptional start site was similarly conducted. This was achieved via methylation-sensitive restriction digestion with Smal endonuclease. Figure 7A outlines the target 525-bp region which spanned the transcriptional start site of the Ha-ras gene and contained two Smal CCCGGG recognition sites. The percentage of animals exhibiting hypomethylation at these two sites progressed from 43 to 57 to 85% as the 8-week promoting dose of CSC was increased from 3 to 9 to 18 mg CSC (Fig. 7B). Promotion with 27 mg CSC resulted in hypomethylation being observed in 67% of the total animals (Fig. 7B). Hypomethylation of the region of interest in the Ha-ras promoter was discerned in all nine of the tumor tissues (Fig. 7B).

Due to the fact that the 5’ promoter region of Ha-ras was determined to be unmethylated and two CpG sites within Smal recognition sequences were hypomethylated in a generalized dose-dependent manner, changes in gene expression were assessed. Promoting doses of 18 and 27 mg CSC resulted in statistically significant (10- and 6-fold) increases in gene expression over control (Fig. 8), which can be associated with the higher incidence of hypomethylation at the CpG sites.
analyzed. Although 3 and 9 mg CSC also seemed to increase gene expression, the fold changes did not reach statistical significance and this may indicate that a threshold incidence of hypomethylation is required for the upregulation of Ha-ras.

Strikingly, in the tumor tissue the expression of Ha-ras increased by 104-fold (Fig. 8). Hypomethylation of CpG sites close to the transcriptional start site coupled with the absence of methylation in a distant 5' promoter region could contribute to the dramatic upregulation of Ha-ras in tumor tissue.

FIG. 6. Methylation status of the promoter region of Ha-ras. A diagram of the Ha-ras promoter indicating the location of PCR primers and CpG sites (gray lollipops) in relation to the transcriptional start site is presented (A). Bisulfite-sequencing analysis within the region spanning 283 bp revealed that all 22 CpG sites were unmethylated (open circles) in control, 27-mg CSC–promoted skin, and tumor tissues except for three samples that exhibited methylation (closed circles) at one or two CpG sites (B).

FIG. 7. SmaI restriction digest analysis of the Ha-ras promoter. A schematic illustrates the targeted region spanning 325 bp around the transcriptional start site of Ha-ras which contains two SmaI recognition sequences CCCGGG (A). SmaI will not cut its recognition sequence if the internal cytosine is methylated. The amount of PCR product generated is representative of the level of methylation at the internal cytosine. In general, the incidence of hypomethylation of the two CpG sites increased with the dose of CSC at the 8-week time point. Hypomethylation was detected in 100% of the tumor tissues (29-week time point) analyzed (B).

FIG. 8. Expression of Ha-ras. Changes in the gene expression of Ha-ras as detected by real-time PCR are expressed as fold change over control. A statistically significant increase (Student t-test, p < 0.05) over control was observed in response to 8-week, 18- and 27-mg CSC promotion. Tumor tissue exhibited a very large and highly significant (p < 0.001) induction of Ha-ras expression.
DISCUSSION

The AP-PCR and capillary electrophoresis method described here is a novel approach to evaluating the methylation status of GC-rich regions of DNA, and it allows for the simultaneous identification of three possible types of methylation changes: hypomethylations, hypermethylation, and new methylations. As demonstrated, dose- and time-dependent relationships, as well as aspects of methylation reversibility are clearly defined. The scope of this method, when employing the methylation-sensitive isoschizomers HpaII and MspI, relies on changes in methylation within their CCGG recognition site. Approximately 35.25% of these CCGG sequences lie in transposable elements, while 64.19% are located in gene-coding and promoter regions of the mouse genome (Frazarri and Greally, 2004). By targeting CCGG sites, we are sampling ~7.45% of all CpG dinucleotides estimated for the mouse genome of which 4.37% are found within gene-coding and promoter regions (Frazarri and Greally, 2004). Importantly, the methylation status of CpCpG sites are concurrently evaluated which expands the capacity and power of our technique to detect overall altered methylation.

Comparatively, common alternative genome-wide methylation status assays (i.e., restriction landmark genomic scanning [RLGS] and amplification of intermethylated sites) which rely on methylation-sensitive restriction with enzymes such as NotI and SmaI, further limit the proportion of CpG dinucleotides that can be sampled (Costello et al., 2002; Frigola et al., 2002). RLGS targets CCGGCCGC sites via NotI and drastically reduces the proportion of CpG dinucleotides evaluated in CpG islands in the mouse genome to 0.03% (Frazarri and Greally, 2004). Approximately 14% of all HpaII and MspI recognition sequences are located within CpG islands. Therefore, our method is less restricted, allowing for a more comprehensive approach to addressing treatment (i.e., CSC)-related disruption of DNA methylation during the promotion stage of skin tumorigenesis.

Promoting agents have been shown to act via a threshold exhibiting dose-response with regard to changes in methylation (Watson et al., 2003). Consistent with this dose-response characteristic, CSC was shown to induce a clear dose-dependent increase in hypermethylation and new methylations. However, a fundamental shift in the nature of the observed changes in methylation over time was evident. Hypermethylation and new methylations were the predominant alteration detected in precancerous tissue at early and intermediate time points while tumor tissue (29 weeks) was predominantly hypomethylated. This key feature was reported previously in that global hypomethylation was infrequently detected in precancerous skin (Watson et al., 2003). In a separate study, global loss of 5-methylcytosine content was reported to progress in two steps. The first occurred during the early stages of benign tumor growth and further loss of 5-methylcytosine content was seen during the transition from an epithelial phenotype to a highly metastatic dedifferentiated/spindle morphology (Fraga et al., 2004). Given that distinct methylation profiles are consistently identified for precancerous and tumor tissues, defining the intermediary steps is critical. In light of the fact that carcinogenesis involves a progressive clonal expansion of cells bearing heritable alterations of their genomes, the key question, addressed in this report, is “Do the changes in methylation at early stages of promotion persist and accumulate over time to facilitate tumorigenesis?”

The promotion stage of tumorigenesis involves the stepwise accumulation of heritable changes which are critical for the selection and clonal expansion of initiated cells (Dragan et al., 1993). A number of changes in DNA methylation persisted from 4 to 8 weeks with the highest promoting dose of CSC and from 8 weeks to the tumors. Importantly, a few changes originating with the 4-week promotion were identified at 8 weeks and, also, persisted to the tumors. Those altered regions of methylation which persisted during promotion are likely to be critical epigenetic changes that contributed to the clonal expansion of subsets of initiated cells. As this effect was observed with the doses that caused tumors (i.e., 9, 18, and 27 mg/CSC), a relationship appears to exist between the tumor incidence elicited with each dose of CSC and the number and type of persisting changes in methylation. This supports the view that altered DNA methylation is a causative factor in tumor formation. The notion that altered DNA methylation is a cause and not an effect was previously tested in mice with decreased Dnmt1 expression and substantial genome-wide DNA hypomethylation in all tissues. These mice developed aggressive T-cell lymphomas which were linked to the development of chromosomal instability and the inappropriate activation of oncogenes (Gaudet et al., 2003). Although, at this time, cause and effect cannot be established unequivocally, these are fundamental observations illustrating that changes in DNA methylation occur during the promotion stage of tumorigenesis and precede the formation of tumors.

Reversibility is a key aspect of the promotion stage of tumorigenesis (Dragan et al., 1993), and the potential exists for reversing altered methylation of DNA; therefore, altered methylation may be a mechanism underlying promotion (Goodman and Watson, 2002). The balance of methylation-demethylation reactions is regulated by numerous enzymes including maintenance methylases, de novo methylases, and demethylases. Therefore, changes in DNA methylation can “reverse” by a number of mechanisms (Goodman and Watson, 2002). Active demethylation can involve removing the methyl group from cytosine which could effectively restore a hypermethylated state to control levels (Ramchandani et al., 1999). Erroneous maintenance methylation following DNA replication could lead to both loss of methylation in hypermethylated regions and gain of methylation in hypomethylated regions. Finally, proliferation of “normal” cells and apoptosis of cells exhibiting abnormal methylation profiles could both contribute to a “reversal” of altered
methylations. Within 8 weeks following cessation of the 4- and 8-week promoting stimuli, regions exhibiting altered methylation were seen to “reverse.” With the exception of one new methylation, all persistent changes were recoverable. This is a clear demonstration that progressive, critical changes in methylation are reversible, consistent with the notion that reversibility is a hallmark of tumor promotion.

The altered methylation observed in GC-rich regions could lead to aberrant gene expression. Increased methylation in the promoter regions of p16, and MGMT and E-cadherin, among others was observed during mouse skin promotion (Fraga et al., 2004; Watson et al., 2004). As demonstrated by Estellar et al. (2001), unique profiles of promoter hypermethylation can be created and used to characterize the disruption of critical pathways in tumorigenesis. Hypermethylation-associated gene silencing is extremely important when considering the mechanisms involved in tumor formation. However, both hypermethylation and hypomethylation are occurring simultaneously in GC-rich regions, illustrating the point that a variety of alterations in methylation may play a role in carcinogenesis (Counts and Goodman, 1995).

Activation of Ha-ras is a common feature of papillomas (Balmain and Pragnell, 1983; Balmain et al., 1984). A novel aspect of the current research is the finding that tumors arising from CSC-promoted skin exhibited an increased incidence of hypomethylation of the promoter region of Ha-ras. Decreased methylation, in addition to significant increased expression of the oncogene, suggests that altered methylation during promotion, in addition to the possibility of mutation in response to DMBA initiation, might contribute directly to the activation of Ha-ras which likely plays a role in tumorigenesis. At first glance, the 100-fold increase in expression of Ha-ras that was observed in the papillomas might appear extreme. However, in a related study, expression of Ha-ras in normal skin was virtually undetectable by Western blot analysis (Rodriguez-Puebla et al., 1999). Therefore, given that basal levels of Ha-ras expression in normal skin are extremely low, the 100-fold increase observed in tumor tissue is not unrealistic, and it may play a fundamental role in facilitating tumorigenesis.

The aberrant activation of Ha-ras has been implicated in facilitating numerous aspects of a malignant phenotype (i.e., proliferation, invasion, and metastasis) (Giehl, 2005). Over-expression of Ha-ras may lead to a cascade of protein kinases, resulting in the phosphorylation of Jun, which can then upregulate the activity of DNA methyltransferase, thereby increasing the methylation capacity of the cell and resulting in aberrant methylation patterns (MacLeod et al., 1995). A consequence of this upregulated methylation capacity includes the possibility of silencing tumor suppressor genes through promoter hypermethylation. It was demonstrated that an oncogene, v-src, induced overexpression of Dnmt1 which led to downregulation of a candidate tumor suppressor gene, tsg, through promoter hypermethylation (Sung et al., 2004). Therefore, upregulation of ras in response to promoter hypomethylation could facilitate further aberrant patterns of methylation, which can facilitate tumorigenesis including hypermethylation of tumor suppressor genes, through indirect activation of DNA methyltransferases.

Genetic instability is a basic feature of carcinogenesis. Mutations in genes that normally function to maintain genetic stability may cause the formation of a mutator phenotype (Loeb, 2001). DNA hypomethylation may lead to both elevated mutation rates (Chen et al., 1998) and chromosomal instability (Edean et al., 2003) in addition to a CpG island methylator phenotype (Abe et al., 2005). We have discerned widespread altered DNA methylation on all three levels, i.e., hypomethylation, hypermethylations, plus new regions of methylation which occur in a progressive fashion during tumorigenesis. Thus, it is now appropriate to talk about instability of the epigenome as a fundamental component of the genetic instability that provides an environment which fosters the aberrant gene expression involved in the transformation of a normal cell into a frank carcinoma.

SUPPLEMENTAL DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/. Supplementary data contain a detailed description of the approach taken to calculate the reversibility of RAMs identified by the AP-PCR and capillary electrophoretic procedure. Additionally, a series of figures which provide the actual, basic underlying data for Figures 3–5 are presented as Supplemental Information. These figures provide the data for RAMs for which the methylation change was (1) statistically significant (hypomethylations, hypermethylations), (2) a 100% loss of methylation, and (3) new methylations depicted for the 8-week promotion with 3, 9, and 18 mg CSC, 4- and 8-week promotion with 27 mg CSC, and tumor tissue. This information also provides the basis for the data that are reported in Tables 1 and 3.

ACKNOWLEDGMENTS

A.N.B. was a predoctoral fellow supported by NIH-NIEHS Training Grant No. T32-ES-07255. Research support, in the form of a gift, from the R. J. Reynolds Tobacco Co. is acknowledged gratefully.

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