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Transcriptional Gene Silencing Promotes DNA Hypermethylation through a Sequential Change in Chromatin Modifications in Cancer Cells

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ABSTRACT

It is well established that DNA hypermethylation of tumor suppressor and tumor-related genes can occur in cancer cells and that each cancer subtype has specific gene sets that are commonly susceptible to methylation and silencing. Glutathione *S*-transferase (*GSTP1*) is one example of a gene that is hypermethylated and inactivated in the majority of prostate cancers. We previously reported that hypermethylation of the *GSTP1* CpG island promoter in prostate cancer cells is initiated by a combination of transcriptional gene silencing (by removal of the Sp1 sites) and seeds of methylation that, instead of being constantly removed because of demethylation associated with transcription, acts as a catalyst for the spread of methylation across the CpG island. In this study, we now demonstrate that the seeds of DNA methylation also play an important role in initiating chromatin modification. Our results address a number of central questions about the temporal relationship between gene expression, DNA hypermethylation, and chromatin modification in cancer cells. We find that for the *GSTP1* gene, (*a*) histone acetylation is independent of gene expression, (*b*) histone deacetylation is triggered by seeds of DNA methylation, (*c*) the spread of DNA hypermethylation across the island is linked to MBD2 and not MeCP2 binding, and (*d*) histone methylation occurs after histone deacetylation and is associated with extensive DNA methylation of the CpG island. These findings have important implications for understanding the biochemical events underlying the mechanisms responsible for abnormal hypermethylation of CpG island-associated genes in cancer cells.

INTRODUCTION

Methylation of DNA occurs at cytosine residues at CpG dinucleotides by the addition of a methyl group to the carbon-5 position of cytosine through the action of the DNA methyltransferase enzymes (1). Approximately 70% of all CpG dinucleotides in the mammalian genome are methylated and these sites commonly occur in repetitive DNA elements (2). In contrast, most unmethylated CpG sites are located within the 29,000 CpG islands found spanning the promoter and first exon regions of housekeeping genes and tumor suppressor genes. The precise mechanism by which these regions remain methylation free is unclear, but active transcription appears to be an essential component (3, 4). Methylation of CpG islands, however, is associated with gene silencing such as in X chromosome inactivation and genomic imprinting (5).

In cancer cells, DNA hypermethylation of tumor suppressor genes is thought to play a critical role in carcinogenesis (6). Methylation-induced suppression is thought to occur either by directly interfering with the binding of transcription factors or through the action of methylated DNA binding (MBD) proteins and chromatin modification. The specific MBD proteins, which include MeCP2, MBD1, MBD2, MBD3, and MBD4, all have a conserved methyl-CpG binding domain and specific methylated DNA binding properties (7). MeCP2 was the first described member of the MBD family and can bind to single methylated CpG dinucleotides regardless of sequence context (8). MeCP2 is reported to repress transcription by binding to methylated DNA and recruiting Sin3 through its transcriptional repression domain, which interacts with histone deacetylases (HDACs; Ref. 9). MBD2 can also bind to a single methylated CpG site in solution binding assays and localizes to major satellite DNA (10). MBD2 was reported to be the methyl binding component of the MeCP1 complex (11), another transcriptional repression complex, which interacts with the Sin3 (12) or NuRD complex (13), and is also associated with HDAC1 and HDAC2 and MBD3. In contrast to MeCP2, the MeCP1 complex requires a densely methylated DNA fragment (11 or more methyl CpG sites) for productive binding.

The structure of chromatin and the activity of the associated genes are clearly mediated by multiple modifications of its components. Histone proteins are subject to many different chemical modifications, including phosphorylation, acetylation, and methylation, and these modifications affect access of regulatory factors and complexes to chromatin and influence gene expression. Acetylation of lysine residues on histones H3 and H4 leads to formation of open chromatin structure (14), whereas deacetylation is associated with repressed chromatin. Methylation at lysine 4 of histone H3 (H3-K4) is associated with promoters of active genes, whereas methylation at H3-K9 is associated with promoters of inactive genes (15, 16).

To understand the process that triggers hypermethylation of CpG island-associated genes in cancer cells, it is important to understand the temporal relationship between CpG methylation, the binding of MBD proteins, and the acetylation and methylation of associated histones. In a previous study, we reported that a combination of both gene silencing and random seeds of CpG methylation is necessary to trigger hypermethylation of the unmethylated CpG island spanning the promoter and first exon of the glutathione *S*-transferase (*GSTP1*) gene in prostate cancer cells (3). The CpG island promoter region of the *GSTP1* gene becomes methylated in the majority of prostate tumors. In normal tissues, the gene is expressed and unmethylated; however, in prostate cancer, the gene is inactivated, and both alleles are extensively methylated (17). In this study, we have followed the sequential changes in histone H3 acetylation and methylation and binding of MeCP2 and MBD2 during the process of DNA hypermethylation of the *GSTP1* gene in prostate cancer cells. Our findings have important implications for understanding the relationship between chromatin modification and abnormal hypermethylation of CpG islands in cancer cells.

MATERIALS AND METHODS

Cells Culture. LNCaP prostate cancer cells that had been stably transfected with *GSTP1* plasmid constructs were cultured in T-medium with 10% heat-inactivated FCS, 1% penicillin/streptomycin (Invitrogen), and 900 μg/ml Geneticin (G418; Life Technologies, Inc.), as described previously (3). The cells were grown at 37°C in 5% CO_2 atmosphere and split 1:3 every 4–5 days.

GSTP1 Plasmid Constructions. The constructs used in this study have been described in detail in Song *et al.* (3). Briefly, The *Shuttle vector* contains the entire *GSTP1* CpG island extending from 1.2 kb upstream of the transcription start site to intron 4 cloned into pBluescript SK^+. A modification of the *Nar*1 site resulted in an extra CpG dinucleotide at CpG –23, which was used as an informative marker to distinguish the endogenous *GSTPI* gene from

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transfected *GSTP1* construct. The *Sp1 deletion vector* was constructed by deletion of the minimal promoter region [*Nar*1(1150)/*Stu*I(1216) fragment] from the GSTP1 shuttle vector. The *seeded shuttle vector* and the *seeded Sp1 deletion vector* were prepared by methylation of the shuttle and Sp1 deletion vectors with *Hpa*II methylase (New England Biolabs) according to the manufacturer's protocol. Complete methylation of the *Hpa*II sites was confirmed by resistance to restriction by *Hpa*II enzyme and by bisulfite sequencing.

Genomic Bisulfite Sequencing. DNA was extracted from the LNCaP cells using the Puregene extraction kit (Gentra Systems). The bisulfite reaction was carried out on 2 μg of restricted DNA for 16 h at 55°C under conditions as described previously (18). After neutralization, the bisulfite-treated DNA was ethanol precipitated, dried, resuspended in 50 μl of H_2O, and stored at −20°C. Approximately 2 μl of DNA were used for each of the nested PCR amplifications (3). The *GSTP1* primers used for the amplification were GST9 and GST10 for first round and GST11 and GST12 for second round, as described by Millar *et al.* (17). At least three independent PCR reactions were performed to ensure a representative methylation profile. PCR fragments were directly purified using the Wizard PCR DNA purification system and cloned into the pGEM-T-Easy Vector (Promega) using the Rapid Ligation Buffer System (Promega). Approximately 12 individual clones were sequenced from the pooled PCR reactions using the Dye Terminator cycle sequencing kit with AmpliTaq DNA polymerase FS (Applied Biosystems) and the automated 373A NA Sequencer (Applied Biosystems). The methylation status for each CpG site was determined, and the average was calculated from the sequencing data of at least 12 individual clones.

Quantitative Real-Time Reverse Transcription-PCR. RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was reverse transcribed from 2 μg of total RNA using SuperScript III RNase H$^-$ Reverse Transcriptase (Invitrogen-Life Technologies, Inc.), according to the manufacturer's instructions. The reaction was primed with 200 ng of random hexamers (Roche). The reverse transcription reaction was diluted 1:20 with sterile H_2O before addition to the reverse transcription-PCR. Expression from the *GSTP1*-transfected plasmid was quantitated using a fluorogenic real-time detection method using the ABI Prism 7000 Sequence Detection System. Five μl of the reverse transcription reaction were used in the quantitative real-time PCR reaction using 2× SYBR Green 1 Master Mix (P/N 4309155) with 50 ng of each primer. The forward primer (5′-TCAAAGCCTCCTGCCTATACG-3′) was designed to cover the exon 3/exon 4 junction of *GSTP1*, and the reverse primer (5′-GCGAGCTCTAGCATTTAGGTGA-3′) was designed to the bovine growth hormone 3′-polyadenylation signal, which had been cloned into the *GSTP1* shuttle vector (3). To control for the amount and integrity of the RNA, the Human 18S rRNA kit (P/N 4308329; Applied Biosystems), containing the rRNA forward and reverse primers and rRNA VIC probe, was used. Five μl of the reverse transcription were used in a 20-μl reaction in TaqMan Universal PCR Master Mix (P/N 4304437) with 1 μl of the 20× Human 18S rRNA mix. The reactions were performed in triplicate, and the SD was calculated using the Comparative method (ABI PRISM 7700 Sequence Detection system User Bulletin no. 2, P/N 4303859). The cycle number corresponding to where the measured fluorescence crosses a threshold is directly proportional to the amount of starting material. The mean expression levels are represented as the ratio between *GSTP1* and 18S rRNA expression.

Chromatin Immunoprecipitation (ChIP) Assays. ChIP assays were carried out according to the manufacturer (Upstate Biotechnology). Briefly, $\sim 1 \times 10^6$ LNCaP cells, in a 10-cm dish, were fixed by adding formaldehyde at a final concentration of 1% and incubating for 10 min at 37°C. The cells were washed twice with ice-cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A), harvested, and treated with SDS lysis buffer for 10 min on ice. The resulting lysates were sonicated to shear the DNA to fragment lengths of 200–500 bp. The complexes were immunoprecipitated with antibodies specific for acetylated-histone H3 (no. 06-599), MeCP2 (no. 07-013), dimethyl-histone H3(lys9) (no. 07-212), and MBD2 (no. 07-198) from Upstate Biotechnology. Ten μl of antibody were used for each immunoprecipitation according to the manufacturer. No antibody controls were also included for each ChIP assay, and no precipitation was observed. The antibody/protein complexes were collected by salmon sperm DNA/protein A agarose slurry and washed several times following the manufacturer's instructions. The immune complexes were eluted with 1% SDS and 0.1 M $NaHCO_3$, and the cross-links were reversed by incubation at 65°C for 4 h in the presence of 200 mM NaCl. The samples were treated with proteinase K for 1 h, and the DNA was purified by phenol/chloroform extraction, ethanol precipitation, and resuspended in 30 μl of H_2O.

Quantitative PCR Analysis. The amount of *GSTP1* DNA that was immunoprecipitated with each antibody was measured by Real-Time PCR using the ABI Prism 7900HT Sequence Detection System. Amplification primers: forward primer, GST 82U (1034–1048) 5′-GCTGCGCGGCGACTC-3′ or GST85U 5′-TTCCCCGGCCAGCTG-3′ and reverse primer, GST 83U (1091–1003) 5′-GGCGGCCGCTGCA-3′. A TaqMan probe [labeled with FAM (1056–1074) 5′-6FAM-TCCAGGGCGCGCCCCTCT-TAMRA-3′] was designed so that it only detected transfected *GSTP1* sequences and did not detect endogenous *GSTP1*. A TaqMan probe [labeled with VIC (1054–1073) 5′-VIC-ACTCCAGGGCGCCCCTCTGC-TAMRA-3′) was designed to detect endogenous *GSTP1* sequences. PCR reactions were set up according to the SDS compendium (version 2.1) for the 7900HT Applied Biosystems Sequence Detector. Twenty-μl reactions were set up using the TaqMan Universal PCR Master Mix (2×) in a 396-well plate. Four μl of either immunoprecipitated DNA, no-antibody control, or input chromatin were used in each PCR, and the PCRs were set up in triplicate. Universal thermal cycling conditions were used: 50°C for 2 min, then 95°C for 10 min, followed by 95°C for 15 s and 60°C for 1 min repeated for 40 cycles. At the completion of the run, the threshold bar was set in the exponential phase of the logarithmic graph of the amplification plot and the C_T values recorded. The cycle number that the measured fluorescence crosses a threshold is directly proportional to the amount of starting material. Outlying samples were excluded from the analysis. SD was calculated using the Comparative method (ABI PRISM 7700 Sequence Detection System User Bulletin no. 2, P/N 4303859). For each sample, an average C_T value was obtained for immunoprecipitated material and for the input chromatin. The difference in C_T values (δC_T) reflects the difference in the amount of material that was immunoprecipitated relative to the amount of input chromatin, and this was then expressed relative to the C_T value for LNCaP cells (ABI PRISM 7700 Sequence Detection system User Bulletin no. 2, P/N 4303859).

RESULTS

Silencing and Seeding Triggers Hypermethylation of *GSTP1* in LNCaP Cells. We previously reported that *de novo* methylation of the CpG island, spanning the promoter and first exon of a transfected copy of the *GSTP1* gene, can be triggered in LNCaP prostate cancer cells, if the gene is inactive (deletion of Sp1 sites) and has a low level of methylation (3). In this study, to address what the relationship is between the process that triggers DNA hypermethylation and modification of the associated chromatin, we have used a subset of the *GSTP1* constructs for transfection into LNCaP cells, summarized in Fig. 1*A*. These include two pairs of vectors, one pair that express *GSTP1* (shuttle) and one pair where the Sp1 sites had been deleted (Sp1 deletion). We used quantitative real-time reverse transcription-PCR to confirm that the *GSTP1* shuttle vector was transcriptionally active and the Sp1 deletion vector was transcriptionally inactive (Fig. 1*B*). For each pair of constructs, one partner was transfected in the unmethylated state, and one was transfected with seeds of methylation after *Hpa*II methylation. After transfection and selection by G418 resistance, colonies were selected and pooled. DNA was isolated after a number of serial passages and analyzed for methylation of the transfected *GSTP1* CpG island by bisulfite genomic sequencing. Fig. 2 summarizes the methylation status of the endogenous and transfected *GSTP1* gene constructs. In contrast to the endogenous *GSTP1* CpG island that is densely methylated in LNCaP cells (Fig. 2*A*), the two expressing constructs (shuttle and seeded shuttle) remained unmethylated or became substantially demethylated (Fig. 2, *B* and *C*). This is in accordance with our previous findings where we also found that active transcription from the *GSTP1* shuttle vector promoted demethylation of the *Hpa*II seeds of methylation (3). Hypermethylation of the CpG island was only triggered in the construct that was transcriptionally inactive (by removal of the Sp1 sites) and seeded with a low level of methylation at *Hpa*II sites. In this construct, *de novo* methylation of the CpG sites flanking the *Hpa*II sites occurred, and in addition, the density of methylation was shown to increase across the island with additional doublings (Fig. 2, *E* and *F*).

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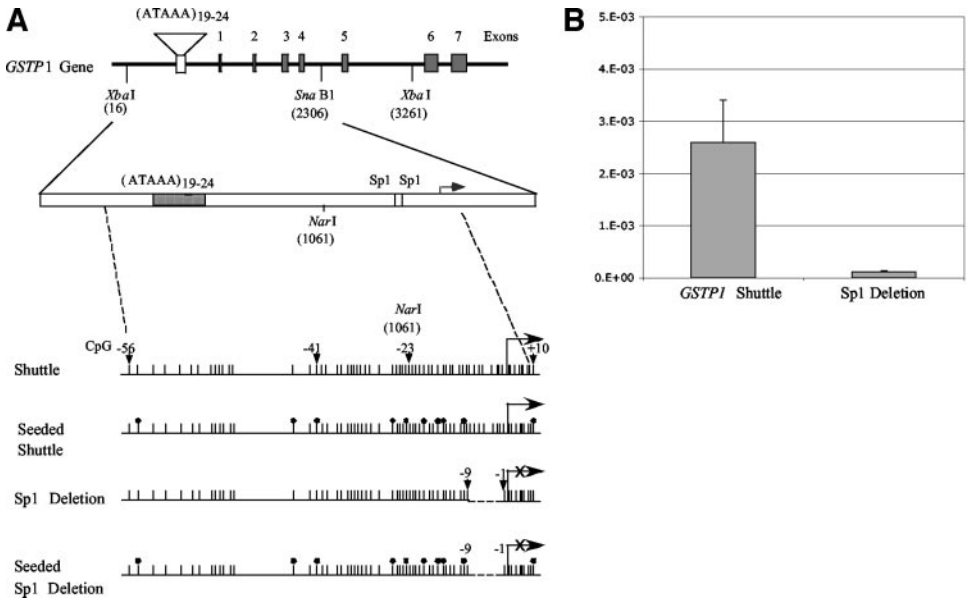

Fig. 1. Map of the *GSTP1* constructs transfected into LNCAP cells. *A*, the *GSTP1* constructs used for stable transfection studies were constructed from a 2.3-kb *Xba*I/*Sna*BI fragment containing the *GSTPI* gene from sites 16 to 2306 (GenBank accession no. M24485) and included 1.2 kb upstream from the start of transcription, the $(ATAAA)_{19-24}$ repeat CpG island boundary element and exons 1–4 (3). The constructs shown are shuttle vector: the *Nar*I site (1061) was modified to include an extra CpG dinucleotide at CpG −23, and this was used as an informative marker to distinguish the endogenous *GSTP1* from the transfected *GSTP1* gene. Seeded shuttle: the shuttle vector was methylated at *Hpa*II sites as indicated by ●. Sp1 deletion vector: this construct was prepared from the shuttle vector by deleting CpG sites (−2 to −8), as indicated by the [sdash] [sdash] [sdash]. This region included the 2 Sp1 sites in the *GSTP1* promoter. Seeded Sp1 deletion: the Sp1 deletion vector was methylated at *Hpa*II sites, as indicated by ●. The *vertical lines* indicate the relative positions of the CpG sites of the CpG island. An *arrow* indicates active *GSTP1* transcription, and a *cross* indicates inactive transcription. *B*, quantitative real-time RT-PCR of *GSTP1* expression. Expression levels for the transfected *GSTP1* constructs were estimated using fluorescent quantitative RT-PCR in triplicate, using a reverse primer specific to the polyA sequence in the transfected *GSTP1* constructs and a forward primer that covers the exon 3/exon 4 junction of *GSTP1*. The human18S rRNA kit (P/N 4308329) was used to control for the amount and integrity of the RNA. The mean expression levels are represented as the ratio between *GSTP1* and 18S expression.

Analysis of the Transfected *versus* Endogenous *GSTP1* Promoter. To address how the seeds of methylation aid in triggering the subsequent hypermethylation of the island, we analyzed the chromatin modification across the *GSTP1* CpG island in the various constructs after transfection. We used a ChIP assay using antibodies against acetylated and methylated histone H3 and measured the amount of *GSTP1*-specific DNA that was released from the immunoprecipitates by quantitative real-time PCR. To differentiate the chromatin state of the transfected *GSTP1* sequences from the endogenous *GSTP1* gene, we designed Taqman probes specifically to either the transfected *GSTP1* sequence or the endogenous *GSTP1* sequence (Fig. 3*A*). All of the transfected *GSTP1* constructs have an extra CG at base 1061. A FAM-labeled Taqman probe was designed to detect this extra CG, and a VIC-labeled Taqman probe was designed that was specific to the wild-type *GSTP1* gene for the same region using the same reverse primer and a different forward primer (GST85U). The FAM probe was totally specific for transfected *GSTP1* DNA, and it did not detect the endogenous gene sequence (Fig. 3*B*). The VIC probe detected the endogenous gene in preference to the transfected sequence by 5 cycles (Fig. 3*B*); however, because of the reduced specificity, we only used this probe to measure endogenous *GSTP1* chromatin modification in untransfected LNCaP cells. The specificity of the FAM probe ensured we would only detect transfected *GSTP1* sequence in the chromatin immunoprecipitation assays.

Histone H3 Acetylation Is Independent of *GSTP1* Expression. To determine whether seeding methylation triggered subsequent DNA hypermethylation by altering the acetylation state of the associated histones, we studied the H3 histone acetylation of the *GSTP1* promoter region. Using ChIP and a polyclonal antibody generated to acetylated H3, we performed ChIP analysis on LNCaP cells and LNCaP cells transfected with different *GSTP1* constructs (Fig. 1). After immunoprecipitation, the DNA was released, and the *GSTP1* promoter region, associated with the transfected DNA, was analyzed by real-time PCR and distinguished from the endogenous gene by using the FAM-labeled Taqman probe. Fig. 4*A* summarizes the extent of *GSTP1* promoter region associated with acetylated H3 histones in the various *GSTP1* constructs relative to the endogenous *GSTP1* promoter from LNCaP cells. In the LNCaP cells, the endogenous *GSTP1* promoter is deacetylated, *i.e.*, it does not bind acetylated histone H3 antibodies, and this correlates with the promoter being extensively methylated and the gene silent. In contrast, the transfected shuttle or *Hpa*II-seeded shuttle vector, which are both hypomethylated and actively transcribed, are associated with acetylated histone H3. Interestingly, the chromatin associated with the Sp1 deletion construct is also acetylated at histone H3, although in this construct, *GSTP1* expression is inactive because of the promoter deletion. Deacetylation of histone H3 was only triggered in the chromatin of the *Hpa*II-seeded *GSTP1* construct that had become hypermethylated with increasing doublings (25d and 36d). Therefore, inactive transcription alone was insufficient to change the acetylation state of the histones: extensive DNA methylation triggered by seeds of methylation was required before histone deacetylation occurred.

DNA Methylation Precedes H3-K9 Histone Methylation. To determine whether there was a difference in the H3 histone methylation state of the various *GSTP1* constructs, we performed ChIP analysis using a polyclonal antibody generated to dimethylated H3-K9 on LNCaP cells and LNCaP cells transfected with different *GSTP1* constructs. Fig. 4*B* summarizes the extent of H3-K9 methylation associated with the various *GSTP1* constructs relative to the endogenous *GSTP1* promoter from LNCaP cells. In the LNCaP cells, the endogenous *GSTP1* promoter histones are methylated, *i.e.*, the promoter region binds methylated histone H3-K9 antibodies, and this correlates with the promoter being extensively methylated and the gene silent. Conversely, the shuttle and *Hpa*II-seeded shuttle, which are unmethylated and actively transcribed, are not associated with H3-K9 histone methylation. The Sp1 deletion, which is unmethylated but is transcriptionally silent, is also not associated with methylated H3-K9 histones. Interestingly, the Sp1 deletion *Hpa*II-seeded con-

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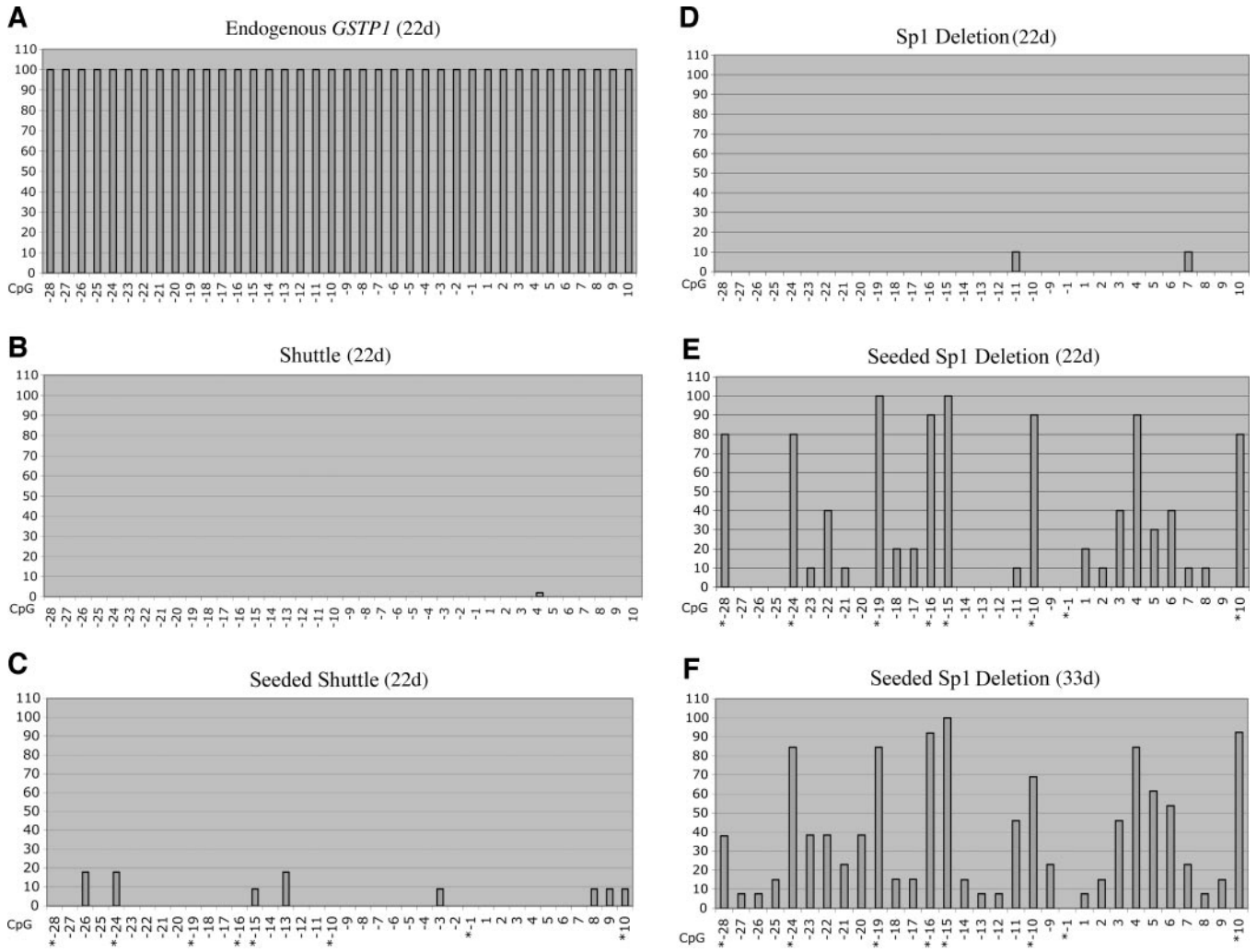

Fig. 2. Extent of methylation across the CpG island of the *GSTP1* constructs transfected into LNCaP cells. The degree of methylation across the CpG island of the *GSTP1* gene was determined using bisulfite sequencing. At least two to three independent PCR amplifications were performed from bisulfite-treated DNA and six clones from each sequenced and the methylation from at least 18 clones was averaged, as described in Song *et al.* (3). *A*, endogenous *GSTP1*; *B*, *GSTP1* from transfected shuttle vector (22 doublings); *C*, *GSTP1* from *Hpa*II-seeded shuttle construct (22 doublings); *D*, *GSTP1* from Sp1 deletion construct (22 doublings); *E*, *GSTP1* from *Hpa*II-seeded Sp1 deletion construct (22 doublings); *F*, *GSTP1* from *Hpa*II-seeded Sp1 deletion construct (33 doublings). The percentage methylation status of each CpG dinucleotide from CpG −28 to CpG +10 is indicated. The *Hpa*II sites that were methylated before transfection are indicated by an *asterisk*.

struct was also not significantly associated with methylated H3-K9 histones, although the promoter region is hypermethylated. Increasing the number of doublings (25d to 36d) increased the level of DNA methylation (Fig. 3*F*) but did not increase the level of histone methylation (Fig. 4*B*). Therefore, we conclude that DNA methylation of the *GSTP1* promoter precedes histone methylation.

MeCP2 Is Not Associated with Triggering *GSTP1* Hypermethylation. To determine whether hypermethylation and deacetylation of the *GSTP1* was associated with binding of MeCP2 proteins, we performed ChIP analysis using a polyclonal antibody generated to MeCP2 on LNCaP cells and LNCaP cells transfected with different *GSTP1* constructs. Fig. 4*C* summarizes the extent of MeCP2 binding associated with the *GSTP1* constructs relative to the endogenous *GSTP1* promoter from LNCaP cells. MeCP2 was found to bind to the endogenous *GSTP1* promoter from LNCaP cells, which is extensively methylated and not expressed. In contrast, there was no significant level of MeCP2 binding to the actively transcribing unmethylated shuttle and *Hpa*II-seeded shuttle, nor to the unmethylated but silent SpI deletion construct. In addition, there was little MeCP2 binding across the *GSTP1* promoter from the *Hpa*II-seeded Sp1 deletion construct. However, there was a slight increase in MeCP2 binding after additional doublings (25d to 36d; Fig. 4*C*), and this was associated with an increase in hypermethylation density across the *GSTP1* promoter region (Fig. 2, *E* and *F*). However, the level of MeCP2 binding relative to binding on the endogenous promoter is minimal and therefore does not appear to be associated with triggering hypermethylation of the *GSTP1* promoter.

MBD2 Binding Is Associated with *GSTP1* Hypermethylation. To determine whether MDB2 is associated with initiating hypermethylation, we performed ChIP analysis using a polyclonal antibody generated to MBD2 on LNCaP cells and LNCaP cells transfected with different *GSTP1* constructs. Fig. 4*D* summarizes the extent of MBD2 binding associated with the various *GSTP1* constructs relative to the endogenous *GSTP1* promoter from LNCaP cells. Similar to the MeCP2 results, MBD2 was found to bind to the endogenous *GSTPI* promoter from LNCaP cells that is extensively methylated and not expressed. Also similar to the MeCP2 ChIP results, there was no significant binding of MBD2 in the actively transcribing unmethylated shuttle or the *Hpa*II-seeded shuttle nor in the unmethylated but silent SpI deletion construct. However, in contrast to MeCP2 lack of binding, MBD2 protein was found to be associated with the silent *Hpa*II-seeded vector at a similar level to the endogenous *GSTP1* gene. Therefore, although MBD2 and MeCP2 are both found to bind to the

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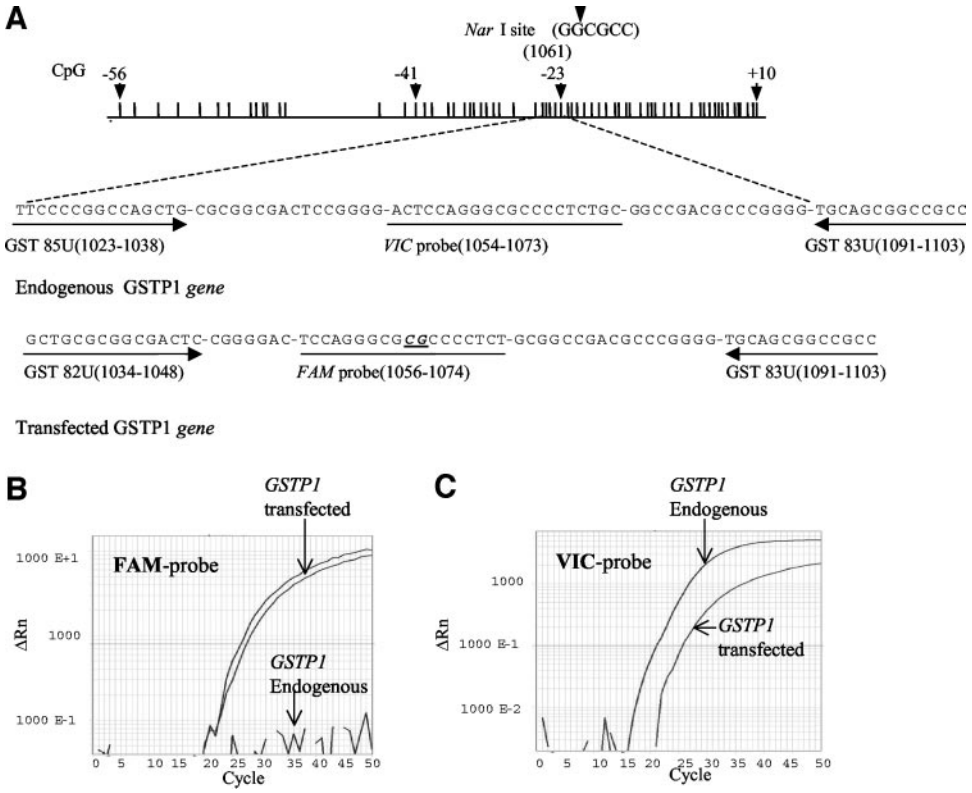

Fig. 3. Specific detection of endogenous and transfected *GSTP1* sequences. TaqMan primers and probes were designed to specifically distinguish the endogenous *GSTP1* sequence from the transfected *GSTP1* DNA sequences. *A*, the forward primer GST 82U and GST 85U and the reverse primer GST 83U sequences are shown and *underlined*. *GSTP1* sequence coordinates are indicated (GenBank accession no. M24485). The TaqMan probe to endogenous *GSTP1* is labeled with VIC and *underlined*. The TaqMan probe designed to transfected *GSTP1* was labeled with FAM and *underlined*. The extra CpG dinucleotide distinguishing it from endogenous sequence is in *bold*. *B*, specificity of the FAM TaqMan probe complementary to the transfected *GSTP1* sequence, comparing signals of the endogenous *versus* transfected *GSTP1* sequence using plasmid DNA. *C*, specificity of the VIC TaqMan probe complementary to the endogenous *GSTP1* sequence, comparing signals of the endogenous *GSTP1* sequence and transfected *GSTP1* sequence.

endogenous *GSTP1* gene, MBD2 binding appears to bind initially to this region and is associated with the spread of *de novo* methylation of the *GSTP1* CpG island.

DISCUSSION

The mechanism responsible for initiating hypermethylation of CpG islands promoter regions in cancer cells is poorly understood. CpG islands in normal cells are hypomethylated, and we previously hypothesized that when the gene is actively transcribed, hypomethylation is maintained by a constant flux between a low level of *de novo* methylation and demethylation (3). However, if the gene is inactivated in the cell, either because of a lack of transcription factors or transiently, *de novo* methylation is favored and appears to spread from the seeds of methylation. Gene inactivity is also associated with changes in chromatin modification. In this study, we wished to investigate the temporal relationship between hypermethylation and chromatin modification. This is difficult to assess in genes that are already hypermethylated because the chromatin modification has already occurred in the cancer cell. Therefore, we decided to explore the change in histone modification of an introduced *GSTP1* gene in LNCaP prostate cancer cells to establish what effect transcriptional gene silencing and seeds of methylation have on the chromatin structure during the process of hypermethylation of the *GSTP1* CpG island.

The CpG island spanning the promoter of the endogenous *GSTP1* gene is extensively methylated, and the gene is inactive in the majority of prostate cancer cells, including the prostate cancer cell line LNCaP (19). Using ChIP analysis, we found that the H3-K9 histones associated with this hypermethylated region were deacetylated and methylated. In addition, there was significant binding of the methyl binding proteins MeCP2 and MBD2. Previous studies on the endogenous *GSTP1* in breast cancer cells (20) and in hepatocellular carcinoma cells (21) have also showed substantial binding of MBD2 to the methylated promoter, but in contrast to our findings, they did not observe MeCP2 binding. This difference in our results could simply reflect the different antibodies to MeCP2 used in our studies or could reflect a difference in protein levels in prostate cancer cells. However, the chromatin modification and binding of methyl binding proteins all correlate with the hypermethylated state of the gene and inactive transcription. In contrast to the endogenous gene, we found that the H3-K9 histones associated with the transfected *GSTP1* gene were acetylated and unmethylated, and there was no binding of the MeCP2 or MBD2 correlating with the transfected *GSTP1* CpG island remaining unmethylated and the gene active.

To determine whether inactivation of the *GSTP1* gene alone could trigger a change in chromatin structure, we examined the modification of histones spanning the *GSTP1* CpG island of a construct where the Sp1 sites had been deleted and thus resulted in gene silencing. Interestingly, in this construct, although the promoter was inactivated, the associated H3 histones remained acetylated and unmethylated at the lys9 residue. Therefore, silencing alone is insufficient to alter the chromatin modification of the *GSTP1* CpG island. Deacetylation of histone H3-K9 only occurred when the CpG island was seeded with methylation in combination with gene inactivation. Therefore, a low level of methylation across the CpG island is necessary to induce deacetylation of the histones. Seeding methylation only occurs when the gene is inactive because when the gene is active demethylation of the island is constantly promoted. Our results indicate that a low level of CpG methylation can induce histone deacetylation but does not affect histone H3-K9 methylation. Interestingly, the endogenous *GSTP1* gene from LNCaP cells, which was extensively methylated across the CpG island, was associated with methylated H3-K9 histones, whereas the transfected DNA that was less methylated (average 50% in comparison to 99%) was still associated with H3 histones unmethylated at the lysine 9 residue. Therefore, the degree of DNA methylation across the CpG island appears to dictate the methylation state of the associated histones.

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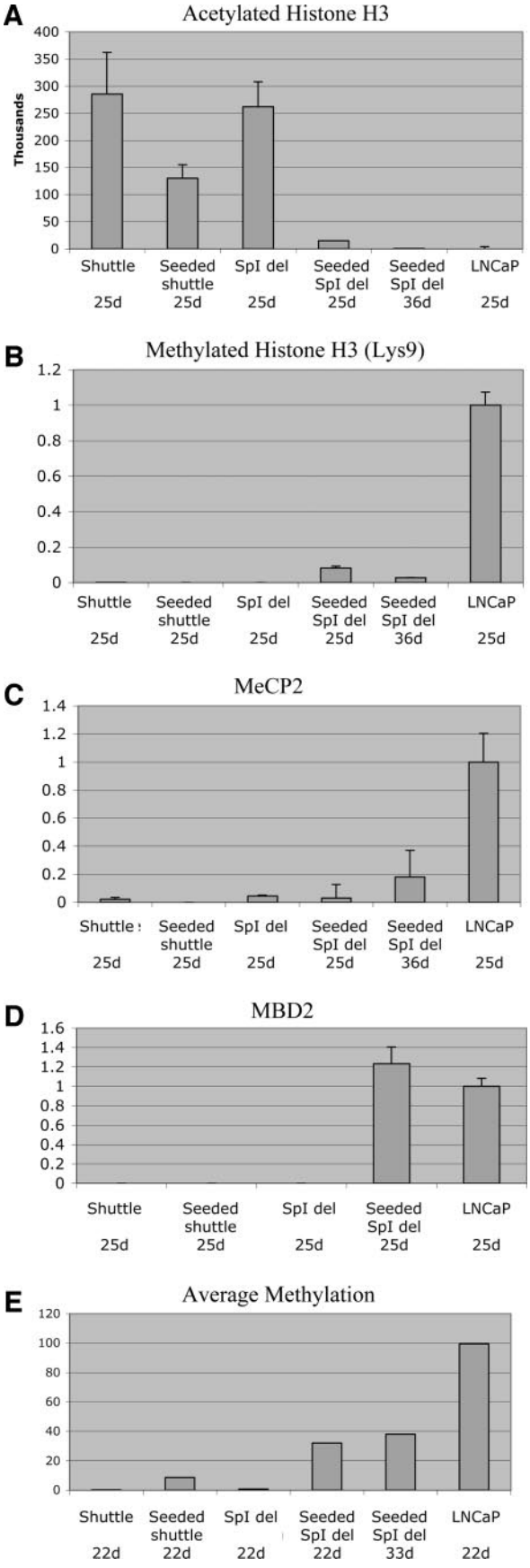

Fig. 4. Chromatin immunoprecipitation (ChIP) of the *GSTP1* CpG island promoter region. Chromatin from LNCaP and transfected cell lines was immunoprecipitated with various antibodies (*A*); antiacetylated histone H3 (*B*); anti-dimethyl-Histone H3 (lysine 9; *C*); anti-MeCP2 (*D*); anti-MBD2. The amount of immunoprecipitated DNA was quantified by real-time PCR, and the chromatin modification and binding of MDB proteins to the endogenous and transfected *GSTP1* sequences were distinguished using sequence specific probes as shown in Fig. 3. The amount of immunoprecipitated *GSTP1* DNA is calculated as a ratio of immunoprecipitated DNA to the total amount of input DNA in the PCR reaction. All results in the graph are expressed relative to LNCaP. *E*, average methylation across the *GSTP1* CpG island from CpG site −28 to +10 was calculated as a percentage of the number of methylated sites over the number of sites sequenced using bisulfite sequencing.

To determine whether the seeds of methylation stimulated the methylated DNA binding proteins to associate with the *GSTP1* CpG island, we assayed for binding of MeCP2 and MBD2 to the transfected constructs. Interestingly, we found that seeding methylation initiated MBD2 binding in preference to MeCP2 binding. We propose that the seeds of methylation initiate MBD2 binding and that MBD2, as part of the MeCP1 complex, recruits HDACs (11, 22), resulting in subsequent histone deacetylation of the *GSTP1* CpG island. MBD2 has also been shown to bind DNMT1 (23), and DNMT1 and DNMT3a bind a variety of HDACs (24–26); therefore, a combination of MBD2 binding and HDAC and DNA methyltransferase recruitment would contribute to the additional spread and maintenance of DNA methylation across the CpG island. We found that MeCP2 binding only occurred with multiple passages after transfection and was associated with an increase in the average DNA methylation level of the *GSTP1* CpG island, whereas MBD2 binding was independent of the degree of methylation. Interestingly, MeCP2 binding has been shown to facilitate H3-K9 methylation (27), suggesting that it is the later binding of MeCP2 that initiates subsequent histone H3-K9 methylation as seen in the *GSTP1* endogenous gene.

In our system, we have initiated gene silencing by genetic deletion of Sp1 binding sites and studied the subsequent effect on DNA hypermethylation and histone remodeling. In contrast, other studies have examined the relationship between aberrant DNA hypermethylation and key histone code components by reversing DNA methylation using 5-aza-deoxycytidine treatment and histone deacetylation using HDACs inhibitors to follow the change in histone modification after gene activation and concluded that DNA methylation acts as the dominant switch for directing the modification of the histone proteins and chromatin state (28, 29). Our results support this observation in that DNA methylation precedes any change in the histone state of the *GSTP1* gene. In contrast, Bachman *et al.* (30) have reported that histone modification can occur independently of DNA methylation. In this study, the authors showed the wild-type $p16^{INKAa}$ gene could be activated with demethylation in HCT116 cells that were lacking DNMT1 and 3B expression. With continued cell passaging, the $p16^{INKAa}$ gene became inactivated, and the associated histones were methylated, but the DNA remained unmethylated and DNA methylation only occurred subsequently after additional passage. However, a low level of seeding DNA methylation may not have been detected because methylation-specific PCR and pooled genomic sequencing were used to study methylation levels. Histone methylation has also been shown to direct DNA methylation and gene silencing in *Neurospora* and *Arabidopsis* (31, 32): a reverse scenario to our system where we have shown that gene silencing can equally direct DNA methylation and subsequent histone methylation. It is possible that two alternate mechanisms can occur to remodel the chromatin and the mechanism used is governed by the transcriptional state of the gene.

The combination of our results allows us to suggest a model to explain the sequential process leading to DNA hypermethylation of the *GSTP1* CpG island in prostate cancer cells (Fig. 5). In our model, if *GSTP1* is inactivated in a prostate cell as the initial event, then the low level of random *de novo* methylation is no longer counterbalanced by demethylation, resulting in an accumulation of methylation that spreads slowly

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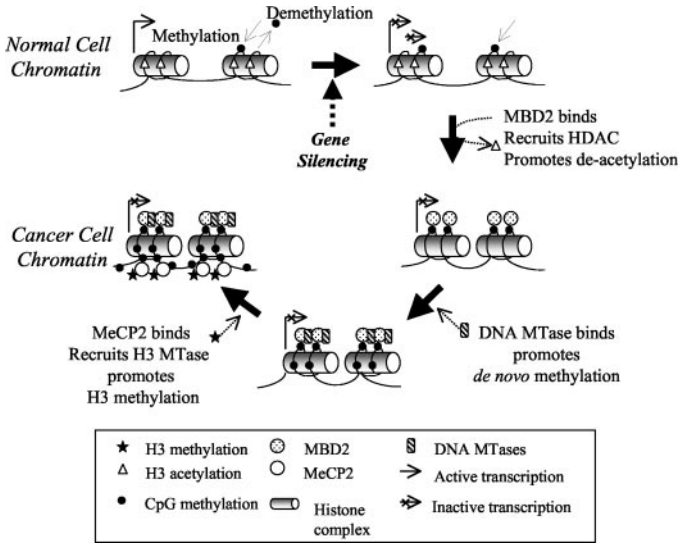

Fig. 5. Model depicting the sequential changes in the chromatin modification with hypermethylation of the *GSTP1* gene in prostate cancer cells. Our model suggests that if *GSTP1* is initially silenced in prostate cancer cells by removal of Sp1 sites, hypermethylation of the CpG island promoter can occur because of sequential changes in the chromatin structure. Gene silencing prevents demethylation and promotes a low level of *de novo* methylation. Deacetylation of H3-K9 histones and binding of MBD2 protein occurs with the increasing seeds of methylation that accumulate with additional cell passage. DNA MTase binds and promotes additional *de novo* methylation. Histone H3-K9 methylation occurs subsequently and is associated with extensive DNA methylation of the *GSTP1* CpG island.

across the island from these methylation seeds. The seeds of methylation promote binding of MBD2, which in turn promotes binding of histone deacetylases and DNA methyltransferases, resulting in histone deacetylation and additional *de novo* methylation. MeCP2 binds after extensive DNA methylation of the *GSTP1* CpG island and recruits histone methyltransferase resulting in H3-K9 methylation consolidation of an inactive chromatin structure. In this model, gene inactivation of tumor suppressor genes occurs before chromatin remodeling. We propose that gene silencing in cancer cells is stochastic and can occur randomly at a low frequency in any normal cell, and it is the silencing of the gene that promotes DNA hypermethylation and subsequent chromatin remodeling over successive cell generations.

Previously, we proposed that the genes susceptible to methylation in different cancer subtypes reflect the set of genes that are more likely to undergo random inactivation in the normal cell equivalent or those genes that once silent give the cell a selective growth advantage (3, 4, 33). We now suggest that if the genes are inadvertently silenced, their continued inactivation is ensured by a combination of DNA hypermethylation and chromatin remodeling. We find that deacetylation of the *GSTP1* CpG island is independent of transcriptional gene silencing but is dependent on a low level of seeding methylation that accumulates with lack of gene transcription. Deacetylation of the promoter is associated with binding of MBD2 and the localized spread of DNA methylation from seeds within the CpG island. Histone methylation occurs subsequently and is associated with MeCP2 binding and extensive DNA methylation. These findings have important implications in understanding the temporal relationship between transcriptional gene silencing and the triggers associated with DNA hypermethylation and chromatin remodeling in cancer cells.

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