Demethylase Activity Is Directed by Histone Acetylation*

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Mammalian genomes are compartmentalized into dense inactive chromatin that is hypermethylated and active open chromatin that is hypomethylated. It is generally accepted that this bimodal pattern of methylation is established during development and is then faithfully inherited through subsequent cell divisions by a maintenance DNA methyltransferase (DNMT1). The pattern of methylation is believed to direct local histone acetylation states. In contrast to this well accepted consensus, we show here using a transient transfection model that an active demethylase is involved in shaping patterns of methylation in somatic cells. Demethylase activity is directed by the state of histone acetylation, and therefore, the resulting methylation pattern is determined by local histone acetylation states contrary to the accepted model. Our data support a new model suggesting that the pattern of methylation is maintained by a dynamic balance of methylation and demethylation activities and the local state of histone acetylation. This provides a simple mechanism for explaining why active genes are not methylated.

A hallmark of mammalian genomes is the compartmentalization of the genome into dense inactive chromatin that is hypermethylated and active, open chromatin that is hypomethylated (1). However, the mechanism responsible for establishing this tight relationship remains unclear. The accepted model is that a sequence of methylation and demethylation events fashion the methylation pattern during development, but it is then faithfully inherited by a semiconservative DNA methyltransferase, DNMT1 (2). Methylation of newly synthesized DNA is exclusively determined by the state of methylation of the parental strand. The pattern of methylation is therefore believed to be fixed in somatic cells. Based on the assumption that DNA conserves its pattern of methylation in somatic cells, numerous experiments used transiently transfected methylated DNA to study the effects of DNA methylation on gene expression. In many of these studies the state of methylation of these ectopically methylated genes following transfection was not determined assuming that the pattern of methylation of the transfected gene did not change. Methylated DNA is associated with methyl CpG binding proteins, such as MeCP2, which reside in a complex with histone deacetylase activity (3). The current model is therefore that the pattern of methylation dictates the state of histone acetylation and chromatin configuration (4).

This attractive model explains the compartmentalization of the genome and its inheritance in somatic cells, but it can not explain how genes are demethylated upon their activation. An alternative and opposite interpretation of the tight correlation between histone acetylation is that active chromatin causes demethylation of associated sequences. Such a model can explain why active genes are not methylated and how their unmethylated state is maintained through cell division.

This hypothesis that active chromatin can cause demethylation is supported by previous data. Treatment of mammalian cells with general histone deacetylase inhibitors can cause global demethylation of human Epstein-Barr virus producer cell lines’ genomes (5). Similarly in Neurospora the deacetylase inhibitor TSA causes selective demethylation (6). Recent data support the claim that inhibition of histone deacetylation can cause selective demethylation of some genes such as the IgfII receptor (7) but not certain tumor suppressor genes (8). Whereas this data shows that activation of genes by histone deacetylase inhibitors can lead toward loss of methylation, the mechanism is unclear. This loss of methylation might either be caused by inhibition of the maintenance DNA methyltransferase during replication, by site-specific proteins, by triggering site-specific repair activity, or by active site-specific or general demethylation.

In this report we use a transient transfection approach to directly measure demethylation activity in human cells (HEK 293) and show that the state of methylation of DNA is not fixed in somatic cells but is dynamically modulated to correlate with the state of gene activity. This is accomplished by active demethylase activity that is directed by histone acetylation. These data provide a simple mechanism for explaining how active genes are demethylated and maintained in their unmethylated state.

MATERIALS AND METHODS

Cell Culture and CAT Assays—HEK 293 cells were plated at a density of 8 × 10^4/well in a six-well tissue culture dish and transiently transfected with 80 ng of plasmid DNA using the calcium phosphate precipitation method as described previously (9). Transfections were repeated a minimum of three times using different cultures of HEK 293 cells. CAT assays were performed in triplicate as described previously (9).

Cell Culture and Flow Cytometry—HEK 293 cells were maintained as a monolayer in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% calf serum (Colorado Serum Co). To serum starve the cells, confluent HEK 293 cells were cultured in a medium containing 0.5% fetal calf serum for 72-h post-transfection. To determine the percentage of cells at different stages of the cell cycle, cells were stained with propidium iodide and the DNA content was measured by flow cytometry.

In Vitro Methylation of Substrates—pMetCAT+, SV40CAT, pCMV-
GFP, and GFP plasmids were methylated in vitro by incubating 10 μg of plasmid DNA with 20 units of SssI CpG DNA methyltransferase (10) (New England BioLabs Inc.) in a buffer recommended by the manufacturer containing 160 μM S-adenosylmethionine, at 37 °C for 2 h. After repeating this procedure three times, full protection from HpaII digestion was observed.

**Bisulfite Mapping**—Bisulfite mapping was performed as described previously with minor modifications (11). 5 μg of bisulfite-treated DNA samples was subjected to PCR amplification using the first set of primers described below. PCR products were used as templates for subsequent PCR reactions utilizing nested primers. The PCR products of the second reaction were then subcloned using the Invitrogen TA cloning kit (we followed the manufacturer's protocol), and the clones were sequenced using the T7 sequencing kit (Amersham Pharmacia Biotech) (we followed the manufacturer’s protocol, procedure C). The primers used for the bacterial DNA chloramphenicol acetyltransferase (CAT) genomic region (GenBank accession number U65077) were:

- **Primers for CAT**:<br> 1. 5’-tggattatgtgattaaatc-3’<br> 2. 5’-taaagaaaaaaatattttta-3’<br> 3. 5’-taaaaataaaaaatataaatc-3’<br> 4. 5’-ggggttgtttatggaattg-3’<br> 5. 5’-ctaaaaaaaatataaatc-3’<br> 6. 5’-ctaaaaaaaatataaatc-3’<br>

The primers used for the enhanced green fluorescence protein (pEGFP-1) (CLONTECH) (GenBank accession number U55761) were:

- **Primers for GFP**:<br> 1. 5’-gttattatggtgagtaaggg-3’<br> 2. 5’-ggttgtttatggaattg-3’<br> 3. 5’-tataactattataatatc-3’<br>

Both the outside and nested primers used for amplifying bisulfited DNA are indicated as *hatched arrows*. The result of this analysis is presented. Each line indicates one clone, filled circles are methylated, and empty circles are unmethylated.

**Chromatin Immunoprecipitation**—CHIP assays (12) were performed by following the Upstate Biotechnology Chromatin Immunoprecipitation (CHIP) assay kit protocol (catalog no. 17-295). HEK 293 cells were transfected with 80 ng of *in vitro* methylated *pMetCAT*+, *SV40CAT*, and *pCMV-GFP* plasmids, using the calcium phosphate method (see above). A final concentration of 0.3 μM TSA was added or not added to fresh medium 24 h after transfection. Formaldehyde was added to the culture media at a final concentration of 1%, 96 h post-transfection, and incubated at 37 °C for 10 min, and chromatin was immunoprecipitated using an anti-acetylated histone H3 antibody (Upstate Biotechnology) as recommended by the manufacturer. One-tenth of the lysate was kept to quantitate the amount of DNA present in different samples before immunoprecipitation. DNA purified from both the immunoprecipitated and pre-immune (pre) samples was diluted from 1:10,000 serially and was subjected to PCR amplification using the following primers for the CAT and GFP genes:

- **Primers for CAT**<br> 1. 5’-catcgatataacccggtt-3’<br> 2. 5’-aaccctcattggaattagc-3’<br> 3. 5’-caaggcgaggagctgtg-3’<br> 4. 5’-eggcatatagataacctggt-3’

- **Primers for GFP**<br> 1. 5’-catcgatataacccggtt-3’<br> 2. 5’-aaccctcattggaattagc-3’

**MNase Assay**—Micrococcal nuclease (MNase) (Amersham Pharmacia Biotech) digestion (0–60 units) were performed on purified nuclei (13) for 20 min at 20 °C in 50 mM Tris-HCl, pH 8.0, 0.05 mM CaCl₂, and 20% glycerol. The MNase reaction was terminated and DNA was extracted using the DNeasy Tissue Kit (Qiagen).

**Western Blot Analysis**—Total cell extracts were prepared using standard protocols and resolved on a SDS-polyacrylamide gel electrophoresis (7.5% for dMTase and 12.5% for GFP protein). After transferring to nitrocellulose membrane and blocking the nonspecific binding with 5% milk, GFP protein was detected using rabbit polyclonal IgG (Santa Cruz, sc-8541) at 1:5000 dilution, followed by peroxidase-conjugated anti-rabbit IgG (Sigma) at 1:5000. Transfected dMTase protein was detected using Anti-Xpress mouse monoclonal IgG (Invitrogen, R910-25) at 1:5000 dilution, followed by peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch) at 1:20,000 dilution and enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

**RESULTS**

**Ectopically Methylated Sequence of DNA Is Actively Demethylated in Mammalian Cells When It Resides Downstream from a Strong Promoter**—If the methylation pattern of genes in somatic cells is static as predicted by the semiconservative model of methylation, an ectopically methylated gene will remain methylated in somatic cells. If the methylation pattern and its correlation with gene activity are a dynamic balance of methylation and demethylation, then the cell should recognize ectopically methylated DNA and adjust its pattern of methylation in accordance with its state of activity. We therefore first tested the hypothesis that somatic cells can recognize an ectopically methylated gene and demethylate it when it resides downstream from a strong promoter but not when it is found downstream from an inactive or weak promoter. We chose to compare the state of methylation of an identical reporter gene sequence to exclude the possibility that any observed differences in demethylation are a result of a sequence-specific property of the demethylated region. The *pMetCAT* fusion construct expressing the bacterial (chloramphenicol acetyl transferase) CAT reporter gene under the direction of 2 kb of the DNA *dnmt1* regulatory region was previously shown to be highly active in P19 cells (9) but is inactive in HEK 293 cells (Fig. 1A). In comparison, the CAT reporter gene, which is controlled by the SV40 promoter, is highly active in HEK 293 cells (Fig. 1A). Both fusion constructs were fully methylated in *vitro* with SssI methylase and transfected into HEK 293 cells using the calcium phosphate protocol (9). After 48 h, DNA from these cells was isolated and the methylation state of CpGs residing in a 265-bp region within the CAT reporter gene was determined by bisulfite mapping. 45% of CpGs became demethylated within the CAT region under the control of the SV40 promoter, whereas almost no demethylation (3.5%) was detected within the CAT region downstream from the *dnmt1* regulatory region (Fig. 1B). This experiment demonstrates that identical sequences can be differentially demethylated in HEK 293 cells depending on their state of expression.

We next determined whether this demethylation is unique to the CAT reporter gene and whether it occurs exclusively when a reporter gene is found downstream from the SV40 promoter. Therefore, we performed the same assay using an *in vitro* methylated GFP (green fluorescence protein) reporter gene,
which is positioned under the control of a different strong viral promoter from CMV (Fig. 2A). Sodium bisulfite mapping of a region within the GFP gene, downstream from the CMV promoter, revealed that the GFP reporter became demethylated albeit to a lesser extent than SV40CAT (Fig. 2A, −TSA). This experiment confirms that HEK 293 cells bear a demethylation activity that demethylates ectopically methylated sequences residing downstream from strong promoters. Most or all the CpGs residing in the entire region of 250 bp of GFP are demethylated in some plasmid molecules suggesting that demethylation is regional and not site-specific.

**Demethylation Is Directed by a Local State of Acetylation**—Multiple mechanisms are responsible for modifying and activating chromatin, including binding of activators, histone acetylation and deacetylation, ATP-dependent remodeling and formation of a pre-initiation complex (14). To determine whether regional demethylation requires histone acetylation, we tested whether the histone deacetylase inhibitor, trichostatin A (TSA) increases the demethylation of ectopically methylated reporter genes, (Fig. 2, A and B). Bisulfite mapping of the GFP reporter in transfectants treated with 0.3 μM TSA for 72 h, showed almost complete demethylation compared with limited demethylation in the absence of TSA (Fig. 2A). A representative Southern blot of in vitro methylated GFP plasmid transfected into HEK cells treated or untreated with TSA is shown in Fig. 2C. The histogram in Fig. 2D represents the quantification of demethylation of the GFP reporter gene as determined by Southern blot analyses. The degree of demethylation was determined by quantifying the relative abundance of the fully digested HpaII fragment (529 bp) per lane, by image densitometry. The numbers and standard errors represent the average of demethylation from three independent experiments. A 4-fold increase in demethylation of transfected plasmid occurs in the presence of TSA. These results imply that deacetylated histones protect ectopically methylated DNA from active demethylation in HEK 293 cells.

TSA also induces a change in the state of expression of the GFP reporter plasmid, as expected. Fig. 2E shows HEK cells as examined under fluorescent light with regular light overlaid with the same field. Within the same size field, an average of 18% of cells expressed GFP when treated with TSA, versus only 0.13% of the absence of TSA.

**Histone Acetylation Can Trigger Demethylation Irrespective of the Presence of a Specific Promoter or Regulatory cis Elements**—Regional demethylation downstream from strong promoters may be a consequence of the interaction of specific factors with distinct cis elements in the promoter region, or the formation of a pre-initiation complex that might be stimulated by TSA inhibition of histone deacetylase (14). To determine whether histone acetylation can trigger demethylation irrespective of the presence of a specific promoter, we tested whether a promoterless GFP construct becomes demethylated in HEK cells. As illustrated in Fig. 2F, TSA stimulates demethylation of a GFP reporter sequence residing in a promoterless plasmid, as indicated by the almost complete digestion of the GFP sequence with the endonuclease HpaII (which cleaves the sequence CCGG only when it is not methylated). The relative abundance of fully demethylated HpaII fragments from four independent experiments was quantified using image densitometry and graphed in Fig. 2G. A 5-fold increase in demethylation of transfected plasmid occurs in the presence of TSA. This data supports the hypothesis that histone acetylation and not necessarily the presence of a unique regulatory region directs the demethylation of a sequence of DNA in HEK cells.

TSA Increases Demethylation in a Dose- and Time-dependent Manner—pCMV-GFP-transfected cells were treated with increasing concentrations of TSA (Fig. 3A) and subjected to Southern blot analysis to investigate the dose response between demethylation and inhibition of histone acetylation. HpaII sensitivity goes from undetectable with 0 μM TSA, to complete with 0.3 μM TSA, demonstrating that demethylation increases with increasing TSA concentration. The extent of demethylation of GFP in the presence of TSA increases with increasing time (Fig. 3B). HpaII sensitivity goes from undetectable at 48 h post-transfection, to complete after 96 h. This time course also illustrates stability of the transfected plasmid throughout the transfection experiment under these conditions and suggests that demethylation of exogenously introduced methylated plasmid occurs slowly, beginning only 72 h post-transfection.

**The Effect of TSA on Demethylation Is Independent of Changes in Cell Cycle**—Because the experimental paradigm used to detect demethylation requires the incubation of cells with TSA for 72 h, we determined whether cell cycle arrest is not a factor in changing the methylation pattern. In vitro methylated GFP-transfected HEK cells were replenishehd with either Dulbecco’s modified Eagle’s medium containing either 10% fetal calf serum in the presence or absence of TSA, or 0.5% fetal calf serum (fcs), and harvested 72 h post-transfection. Harvested cells were stained with propidium iodide and subjected to fluorescence-activated cell sorting analysis to determine the cell cycle profile (Fig. 4A). Both cell populations that were either serum-starved (0.5% fetal calf serum) or treated with TSA displayed the same shift in cell cycle, from G1 toward G2 (Fig. 4A). The state of methylation of transfected GFP plasmid under the different conditions was analyzed by sodium bisulfite mapping (Fig. 4B). Contrary to cells treated with TSA, which displayed 100% demethylation, no significant demethylation occurred in transfected GFP plasmid in serum-starved cells. Because the alterations in the cell cycle profiles of serum-starved and TSA-treated cells were similar, this experiment is consistent with the conclusion that cell cycle changes caused by TSA per se are not responsible for the observed demethylation.

**Demethylated Sequences Exhibit an Increased Association with Acetylated Histones in the Presence of TSA**—To demonstrate that transiently transfected DNA is associated with either acetylated or deacetylated histones and that TSA can alter the state of acetylation of histones bound to the GFP and CAT reporter sequences, we performed a chromatin immunoprecipitation assay (CHIP) using anti acetyl-histone H3 antibody. The results presented in Fig. 5 show that there is an increased association of the CAT gene sequence with acetylated histones when they reside downstream from the strong SV40 promoter/enhancer (Fig. 5A, −TSA, +AB) relative to those residing downstream from the pMet regulatory region (Fig. 5E, −TSA, +AB). TSA increases the abundance of both CAT and GFP sequences associated with acetylated histones (Fig. 5, A and C, +TSA, +AB). Interestingly, TSA treatment does not increase acetylation of histones associated with pMetCAT+ (Fig. 5E, +TSA, +AB), which is consistent with previous studies that demonstrate that not all sequences are acetylated following TSA treatment (8). Perhaps, the context of factors interacting with some regions renders them inaccessible to HATs. Consistent with our hypothesis, the pMetCAT+ plasmid is not demethylated even after TSA treatment (data not shown).

To provide a direct assessment of whether DNA associated with acetylated chromatin is demethylated, SV40CAT plasmid DNA isolated from anti-acetyl H3 histone immunoprecipitated samples, treated or not treated with TSA, was subjected to bisulfite mapping (Fig. 5B). All clones analyzed displayed al-
Histone deacetylase inhibitor TSA enhances demethylation. A, a physical map of the GFP region that was analyzed by bisulfite mapping is shown with the position of the CpGs indicated and numbered according to accession number U55781. In vitro methylated pCMV-GFP plasmid was transfected into HEK293 cells and treated with and without a final concentration of 0.3 μM TSA, 24 h post-transfection, and harvested after 96 h. DNA prepared from the transfectants was treated with bisulfite. The GFP region indicated in the map by dashed angled lines was amplified, subcloned, and sequenced. Hatched arrows indicate the location of both the outside and nested primers used to amplify bisulfited DNA. Each line within the boxes represents an independent clone. A filled circle represents a methylated CG dinucleotide, and an empty circle represents a demethylated CG dinucleotide. The top box displays clones from control transfectants (−TSA), and the bottom box displays clones from cells treated with a final concentration of 0.3 μM TSA (+TSA). B, representative sequencing gels of clones originating from cells treated and untreated with TSA. Arrows indicate the location of CG dinucleotides within the GFP gene. C and F, a fully methylated promoter or promoterless GFP plasmid, respectively, was transfected into HEK293 cells. The transfectants were either treated or not treated with a final concentration of 0.3 μM TSA 24 h post-transfection and harvested after 96 h. 10 μg of isolated DNA was digested with 50 units of EcoRI followed by either digestion with 20 units of MspI or HpaII restriction enzymes, fractionated on a 1.7% agarose gel, and was then subjected to Southern blot transfer and hybridization with a 32P-labeled GFP fragment (see map of probe in panel A, indicated by a dashed line flanked by restriction sites Cfr10I and AvaII). D and G, histograms represent the quantification of demethylation of the GFP reporter gene in the promoter (D) and promoterless (G) constructs as determined by Southern blot analyses. The degree of demethylation was determined by quantifying the relative abundance of the fully digested HpaII fragment (529 bp) per lane, by image densitometry. The numbers and standard errors represent the average of demethylation from three independent experiments for each the promoter and promoterless experiments. E, fluorescence microscopy of representative fields from HEK cells transfected with GFP treated with or without TSA. The average percentage of expressing cells per field was calculated from between 5 and 10 fields examined from three independent experiments.
most complete demethylation, in contrast to non-immunoprecipitated DNA (Fig. 5B). The same degree of demethylation was also apparent for pCMV-GFP plasmid DNA isolated from immunoprecipitated samples; however, in this case DNA was amplified only from cells treated with TSA (Fig. 5D). DNA could not be immunoprecipitated when attempting to IP from GFP-transfected cells, which were not treated with TSA, nor from cells transfected with pMetCAT plasmid in the presence or absence of TSA. This is consistent with previous studies demonstrating that methylated DNA is associated with deacetylated histones as a result of recruitment of histone deacetylases by methylated DNA binding proteins such as MeCP2 (15). The SV40 promoter seems to be less affected by methylation as evident by the fact that it is partially immunoprecipitated by antibodies directed against acetylated histones. The fact that DNA that is associated with acetylated histones is demethylated even in the absence of TSA support the hypothesis that demethylation following TSA treatment requires acetylation of the histones associated with the specific gene and is not a consequence of a general increase in demethylation activity. This result can explain why TSA treatment per se does not result in demethylation of all endogenous genes (8).

Transfected GFP Plasmid Assembles into a Nucleosome Structure—Experiments with transiently transfected DNA have resulted in the abundance of information on the regulation of gene expression in eukaryotes as well as the effects of DNA methylation and histone acetylation on gene expression. However, the issue of whether transiently transfected DNA is assembled into nucleosomes has been controversial (16). Using plasmids transfected by calcium phosphate precipitation, Reeves et al. (17) showed that nucleosomal arrays are indicated (1 = mononucleosome, 2 = dinucleosome, etc.). The fractionated DNA was subjected to Southern blot analysis and hybridization with a 32P-labeled GFP fragment (Fig. 6, second panel). Mononucleosomes and dinucleosomes can be detected but at lower doses of MNase than bulk DNA, consistent with previous findings showing that digestion of transcriptionally active genes is more rapid than bulk DNA (19). Our data is consistent with the previously proposed model that nucleosomes are deposited onto non-replicating DNA in transfected cells; however, it is possible that the overall structure may not be as organized as replicated cellular chromatin. Nevertheless, the transiently transfected DNA allows one to dissect some of the basic principles of the interaction between demethylase and DNA assembled into nucleosomes.

Histone Acetylation Induces Replication-independent Active Demethylation of DNA—Recent data have demonstrated that binding of a transcriptional activator to its cognate site results in site-specific demethylation by a passive replication-dependent mechanism (20). Such a mechanism was proposed to explain the demethylation of active genes during development.
Histone Acetylation and Demethylation

FIG. 5. Bisulfite mapping and CHIP analysis of the association of transfected plasmids with acetylated histones. A, C, and E, HEK 293 cells transfected with in vitro methylated plasmids (SV40CAT, pCMV-GFP, and pMetCAT+, respectively) were either treated or not treated with a final concentration of 0.3 μM TSA. The cells were formaldehyde cross-linked after 96 h and subjected to a chromatin immunoprecipitation assay using an antibody against acetylated histone 3 (H3). PCR was performed for each experiment on serial dilutions of DNA not treated with TSA (or input) and not treated with TSA (anti H3 IP - TSA; B only) are presented.

To test whether the demethylation detected after transient transfection and treatment with TSA is occurring by a similar mechanism, we digested DNA prepared from TSA-treated HEK cells that were transiently transfected with in vitro methylated pCMV-GFP, with the restriction enzymes DpnI and XbaI. DpnI cleaves the sequence GATC only when the adenine is methylated on both strands (21). Because mammalian cells do not bear a methylase that methylates the adenine in GATC, replication of the plasmid in HEK cells will render it resistant to DpnI digestion. XbaI, on the other hand, does not cleave the sequence TCTAGAtc (which is present in pCMV-GFP) when the adenine is methylated (22). Replication of the plasmid in mammalian cells will render it sensitive to XbaI digestion. However, the data presented in Fig. 7 show that, although the transfected pCMV-GFP is fully cleaved with HpaII (Fig. 7A) indicating that it is fully demethylated following TSA treatment, it is completely digested with DpnI and resistant to XbaI (Fig. 7B). Control experiments shown in Fig. 7C demonstrate that the plasmids are completely methylated prior to their transfection into HEK cells, because they are resistant to HpaII cleavage. These data demonstrate that the plasmid did not replicate in HEK cells. Therefore, these data support the hypothesis that increasing histone acetylation induces replication-independent active demethylation of DNA.

Ectopic Expression of Mbd2b/Demethylase Stimulates Demethylation of Ectopically Methylated DNA—We recently cloned and demonstrated that the methylated DNA binding protein MBD2b bears a demethylase (dMTase) activity (23). The availability of a cloned demethylase cDNA allowed us to determine whether the rate-limiting step for demethylation is exclusively the state of acetylation of histones or whether increasing the level of demethylase in the cell could increase demethylation. Methylated GFP was cotransfected with or without the Mbd2b/demethylase expression vector His-dMTase (23) into HEK 293 cells. We mapped the state of methylation of the transfected GFP gene by bisulfite mapping shown in Fig. 8A. The extent of demethylation within the highly CG rich area of the GFP gene is 27% compared with 66% demethylation when dMTase is cotransfected with GFP. Fig. 8B shows a representative Southern blot of transfected GFP in the absence or presence of His-dMTase plasmid. The histograms in Fig. 8C represent the quantification of demethylation of the GFP reporter gene as determined by Southern blot analyses. The degree of demethylation was determined by quantifying the relative abundance of the fully digested HpaII fragment (529 bp) per lane, by image densitometry. The numbers and standard errors represent the average of demethylation from three independent experiments. These experiments demonstrate that demethylation of a given sequence is dependent on the abundance of demethylase in addition to the state of histone acetylation. Our data differ from another report that has shown that Mbd2b/demethylase acts as a transcriptional repressor of a cotransfected methylated granulocyte/macrophage-specific lysozyme gene enhancer and that it did not cause demethylation (24). A simple explanation to resolve this inconsistency is that the interaction of MBD2b/demethylase with methylated genes depends on the promoter context as well as the accessibility of acetylated histones. Our unpublished data suggest that ectopic expression of His-dMTase does not demethylate pMetCAT+, which is not associated with acetylated histones even after TSA treatment (Fig. 5).

Because we have previously shown that His-dMTase demethylates DNA indiscriminately in vitro (23), we also determined whether demethylase could demethylate DNA in the absence of a distinct regulatory sequence in the living cell. As seen in Fig. 8, B and D, forced expression of His-dMTase enhances the demethylation of pCMV-GFP and the promoterless GFP (Fig. 8D) as indicated by the increase in the relative fraction of the plasmid that is digested with HpaII. The relative
Histone Acetylation and Demethylation

Abundance of fully demethylated HpaII fragments from four independent experiments was quantified using image densitometry and shown graphically in Fig. 8E. A Western blot analysis using the Anti-Xpress antibody (which recognizes the histidine-tagged protein) demonstrates expression of His-dMTase in transfected cells but not in untransfected HEKS (Fig. 8F), thereby confirming the expression of exogenously added dMTase in transfected cells. This experiment shows that increasing the abundance of demethylase results in demethylation of a gene in different contexts and that demethylase does not require specific regulatory sequences, nevertheless some promoters can inhibit its activity as discussed above.

Ectopic Expression of Mbd2b/Demethylase Increases Expression of Ectopically Methylated GFP—To determine whether the increased demethylation is reflected in increased expression of the transfected pCMV-GFP plasmid and to demonstrate that His-dMTase does not suppress the activity of the pCMV-GFP promoter, as has been shown for other promoters (25), cell extracts from HEK cells transfected with GFP plasmid and His-dMTase or GFP alone, were subjected to Western blot analysis using an antibody directed toward GFP protein. Cell extracts were loaded in triplicate, and equal loading was determined by Amido black staining (Fig. 9A). HEK cells cotransfected with both GFP and His-dMTase consistently expressed more protein than GFP transfectants alone. In Fig. 9B, HEK cells expressing GFP, which were either cotransfected with GFP plasmid and His-dMTase or GFP alone, were counted 96 h post-transfection by use of a hemacytometer with a fluorescence microscope. The graph shows the results obtained from two independent experiments. Whereas ectopic expression of His-dMTase increases the percentage of cells expressing GFP, it is clear that induction of expression by ectopic dMTase is significantly less than by TSA treatment (compare with Fig. 2E). These data suggest that demethylation is not sufficient to remove all the repression triggered initially by methylation.

DISCUSSION

The DNA methylation pattern is a fundamental constituent of the mechanisms regulating gene expression in vertebrates. It is therefore clear why it is important to understand how DNA methylation patterns are formed and how they cast their intimate interrelationship with chromatin structure. The data presented in this report support a simple but attractive model that explains the remarkable correlation between active genes and hypomethylation. Our data suggest that the state of methylation is determined by chromatin structure. The state of methylation of a sequence is determined by a dynamic balance of the abundance of demethylase activity in the cell and the state of acetylation of histones.

We show that an identical sequence of DNA, which is ectopically methylated, is actively demethylated in HEK 293 cells when it is associated with acetylated histones. Histone acetylation is induced either by the SV40 enhancer that possibly recruits histone acetyl transferases to regulatory regions (Figs. 1 and 5A) or by a pharmacological inhibitor of histone deacetylation (Figs. 2, 5A, and 5C). DNA that is bound to acetylated histones also becomes demethylated (Fig. 5, B and D), thus providing direct proof that DNA associated with acetylated histones is actively demethylated. The fact that SV40 DNA associated with acetylated histones is completely demethylated, even in cells that were not treated with TSA, supports the hypothesis that active demethylation is a consequence of the association with acetylated histones and not other side-effects of TSA treatment. Augmenting demethylase activity by forced expression of a cloned demethylase also stimulates demethylation (Fig. 8) and gene expression (Fig. 9). Taken together, the data presented in this paper fit a model proposing that the limiting step in demethylation is the interaction between the demethylase and DNA. The probability of such an interaction is enhanced by either histone acetylation or an increase in the abundance of demethylase. It is tempting to speculate that demethylase is inhibited from accessing the DNA by deacetylated histone tails as has been shown to be the case with multiple transcription factors (26).

Bisulfite mapping, which is followed by PCR amplification,
allows one to look at the demethylation events in a single DNA molecule. A striking observation that emerges from this analysis is that, with a few exceptions, active demethylation of ectopically methylated plasmids is regional and not site-specific. Complete clusters of CGs are demethylated in some plasmid molecules and none in others (Figs. 2 and 8). This pattern of demethylation is inconsistent with distributive demethylation but is consistent with a processive mechanism as has previously been suggested based on an in vitro analysis (27).

Regional and processive demethylation of CpG clusters is also consistent with the picture that emerges from the mapping of the state of methylation of CpG-rich sequences in vivo. For example, all the CpG sequences in the CpG-rich first exon of the tumor suppressor p16 (28, 29) and the tumor suppressor p21 (30) are not methylated in most tissues, but the first exon is progressively completely methylated in some tumors that do not express p16 (28).

If histone acetylation is involved in regional demethylation of
CpG islands outside of a promoter region as suggested by our data, then histone acetylation should not be restricted only to the promoter region per se. We show that the reporter genes we analyzed are associated with acetylated histones. This observation is in accordance with previous data showing that histone acetylation is not restricted to nucleosomes within the promoter region but appears downstream of the transcription start sites (31), which can explain the demethylation of sequences residing a few hundred bases downstream from a promoter (Figs. 1 and 2). Recent data suggest that extended domains of histone acetylation can occur at large distances from matrix attachment regions (32).

Significant attention has been directed in the past toward site-specific demethylation, particularly demethylation associated with differentiation and activation of specific genes. Razin and Riggs (2) proposed that demethylation occurs in a site-specific and passive manner by binding of factors to specific sites during discrete time points in development that mask it from methylation during replication. This model has recently gained experimental support (33). However, more recent data have shown that passive demethylation is not simply dependent on the presence of DNA binding transcriptional activators (34). Yet still, other experiments have demonstrated site- and cell-specific active demethylation in transient transfection assays (35), suggesting the presence of site- and cell-specific demethylation machinery (36). The data presented here are not necessarily in contradiction with these previous observations. It is possible that different mechanisms are responsible for the site-specific demethylation that accompanies the activation of genes during development and the regional demethylation demonstrated here.

An alternative reasonable hypothesis is that mechanisms similar or identical to the demethylation activity described in this report are also responsible for site-specific demethylation. Although regional demethylation might be triggered by a regional non-cell-selective histone acetylation, site-specific and cell type-specific demethylation might be directed by a specific recruitment of histone acetyl transferases by certain activator proteins. This model is consistent with a requirement for enhancer or cis-acting sequences for site- and cell-specific demethylation (35). For example, demethylation that is initiated at the small pre-B cell stage of a single kappa light-chain allele requires the presence in cis of both the intronic and 3' kappa enhancers (37). It is tempting to speculate that the activators that interact with these cis elements trigger demethylation by recruiting HATs and inducing histone acetylation.

The specific physiological function of the demethylase activity detected by our transient transfection assay is unclear. One of the outstanding questions is: What is the physiological role of demethylase in somatic cells? Is it responsible for repair of ectopic methylation or does it play a dynamic role in maintaining the DNA methylation pattern and its correlation with chromatin structure? It is also possible that the demethylase activity studied here is an embryonic function that is aberrantly activated in some transformed cells. Nevertheless, the assay described in this study reveals some of the basic principles of the demethylation reaction in living cells. These principles might explain a wide range of observations of demethylation events in vivo as well as shed light on one of the fundamental properties of mammalian genomes, the correlation of gene expression, chromatin structure, and the DNA methylation pattern.

The presence of demethylase that demethylates broad regions of ectopically methylated DNA in a somatic cell line forces us to revisit our understanding of the DNA methylation pattern in somatic cells and the relationship between DNA methylation and histone acetylation. The DNA methylation pattern at a given point like other biological signals might be a steady-state equilibrium of methylation and demethylation, whereas the direction of the reaction is determined by the local state of histone acetylation. Recent data focused on how DNA methylation changes histone acetylation; our data suggest that it is possible that histone acetylation determines DNA methylation.

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REFERENCES
Histone Acetylation and Demethylation