Heritable Gene Repression through the Action of a Directed DNA Methyltransferase at a Chromosomal Locus*

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The ability to exogenously impose targeted epigenetic changes in the genome represents an attractive route for the simulation of genomic de novo epigenetic events characteristic of some diseases and for the study of their downstream effects and also provides a potential therapeutic approach for the heritable repression of selected genes. Here we demonstrate for the first time the ability of zinc finger peptides to deliver DNA cytosine methylation in vivo to a genomic integrated target promoter when expressed as fusions with a mutant prokaryotic DNA cytosine methyltransferase enzyme, thus mimicking cellular genomic de novo methylation events and allowing a direct analysis of the mechanics of de novo DNA methylation-mediated gene silencing at a genomic locus. We show that targeted methylation leads to gene silencing via the initiation of a repressive chromatin signature at the targeted genomic locus. This repression is maintained after the loss of targeted methyltransferase enzyme from the cell, confirming epigenetic maintenance purely through the action of cellular enzymes. The inherited DNA methylation pattern is restricted only to targeted sites, suggesting that the establishment of repressive chromatin structure does not drive further de novo DNA methylation in this system. As well as demonstrating the potential of these enzymes as tools for the exogenous, heritable control of cellular gene expression, this work also provides the most definitive confirmation to date for a transcriptionally repressive role for de novo DNA methylation in the cell and lends some weight to the hypothesis that the aberrant methylation associated with certain diseases may well be a cause rather than a consequence of transcriptional gene repression.

Hypermethylation at CpG sequences is commonly observed in the promoters of a number of genes in cancer and is often associated with transcriptional down-regulation (reviewed in Ref. 1); however, whether such patterns of DNA methylation are the end result, or the initiating cause, of gene shutdown is a longstanding question (2). The observed increases in levels of de novo methylation over time associated with some diseases, such as chronic myeloid leukemia and the progression from myelodysplastic syndrome to acute myeloid leukemia (3, 4), might suggest a potentially more passive role for DNA methylation in these arenas, especially as a number of the genes seen to be aberrantly methylated in leukemia and cancer are sometimes similarly methylated in aging healthy cells. However, the ability to study the characteristics and consequences of such de novo methylation has been limited by the inability to simulate this event in vivo.

The only direct route currently available for the authentic delivery of de novo DNA methylation is the use of gene-targeted DNA cytosine methyltransferase (Mtase) enzymes. These enzymes comprise fusions of gene-specific DNA-binding proteins, e.g. rationally designed zinc finger proteins, with DNA cytosine methyltransferases. The ability to design zinc finger proteins to recognize virtually any DNA sequence using well established zinc finger/DNA recognition codes or to select zinc fingers specific for a particular DNA sequence via phage display strategies has enabled the evolution of a number of novel proteins with cellular function. These include gene-specific transcriptional activators and repressors as well as targeted restriction enzymes capable of gene modification in vivo (5, 6). Following the initial demonstration of in vitro functionality of targeted cytosine methyltransferase enzymes (7), they have since been shown to be capable of delivering targeted methylation in bacteria, yeast, and the mammalian mitochondrial compartment (8–10) as well as inducing gene repression of plasmid-based reporter genes and viral vectors in transient assays (11). One potential problem with these enzymes has been the level of associated nontargeted methylation, which is sometimes unacceptably high due to the avidity of the wild-type Mtase components used and is often overlooked. Recently we showed that, employing four zinc finger arrays capable of binding to chromatin target sites (12, 13) in combination with reduced affinity/activity prokaryotic DNA cytosine methyltransferase mutants, methylation could be targeted to predetermined sequences with virtually no nonspecific methylation occurring (14). The HpaII methyltransferase F38H mutant used in these latter studies (henceforth referred to as FH) dem-

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1 The abbreviations used are: Mtase, methyltransferase; FH, F38H mutant; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay.

2 The abbreviations used are: Mtase, methyltransferase; FH, F38H mutant; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay.
onstrated a significantly overall reduced methyltransferase activity relative to the wild-type enzyme because of a mutation in the保守的FXGXF motif involved in Mtase cofactor binding and target base interaction, which allowed the zinc finger protein component to dominate in DNA-protein interactions. The use of non-mammalian Mtases as fusion components, additionally, was thought to be more likely to reduce any potential interactions arising with regulatory cellular factors, such as has been shown to occur with the endogenous DNMT3A and DNMT3B Mtases (15, 16) and which might result in the mistargeting of Mtase activity in vivo.

To examine the applicability of targeted methylation in modulating gene expression in the context of mammalian genomic DNA generally and as a prelude to targeting more functionally complex endogenous promoters, we have transiently expressed four zinc finger/HpaII (FH)-based Mtases in cells harboring an integrated reporter gene in which expression is driven by a minimal promoter flanked with a high density of HpaII sites (5′-CCG-3′) immediately adjacent to zinc finger recognition sites. The short- and longer-term consequences of transient expression of targeted DNA methyltransferases on the appearance of de novo DNA methylation patterns, gene expression levels, modulation of chromatin marks, and epigenetic inheritance were subsequently examined.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture and CAT Analysis**—NIH3T3 cells were stably transfected with a CAT reporter gene construct based on pBLCAT (39) as described previously (14). Clonal cell lines with demonstrated CAT activity were transiently transfected using the Nucleofector system (Amaza GmbH) at an average of ≥80% efficiency, confirmed by green fluorescent protein/fluorescence-activated cell sorter analysis. Internal control β-galactosidase activity was assessed 48 h post-transfection to confirm equivalent transfection efficiencies within each experiment. CAT assays involved monitoring the level of acetyl transfer activity relative to the wild-type enzyme because of a mutation in vivo.

**Methylation Analysis**—Isolated genomic DNA was subjected to bisulfitite modification using the EpiTect bisulfite kit (Qiagen Ltd.) according to the manufacturer’s instructions. DNA corresponding to the target site and flanking regions was amplified using nested PCR with two different sets of primers for comparative purposes to conclusively demonstrate that PCR amplification was not biased toward methylated sequences. Primers were designed to bind 200–300 bp flanking the target site and thymidine kinase promoter regions, embedded in the pBLCAT vector. The modified vector sequence is available on request.

**Western Blot Analysis**—Immunoprecipitation was performed essentially as described previously (14). FLAG antibody (F1804) was used to detect the plasmid harboring a targetable site bound to HpaII Mtase restriction of plasmid DNA, or restriction of plasmids purified from bacteria that were cotransformed with expression and target site vectors.

**Chromatin Immunoprecipitation Assays**—Chromatin immunoprecipitation assays were performed essentially as described previously (40). Antibodies against histone H3K4me3 (ab8580) and H3K9me2 (ab1220) were from Abcam (Cambridge, UK). Primers used for TaqMan analysis of immunoprecipitated chromatin fragments were F1 (5′-gacggagcctgccaac-3′), R1 (5′-ctgccgatgcgggctct-3′), and TAM (5′-caacagagcagatgcca-3′).

**RESULTS**

**De Novo Methylation of a Genomic Target Site**—We constructed a clonal cell line containing a single integrated CAT reporter gene driven by a minimal thymidine kinase promoter, modified to contain 4aZf recognition sites (5′-GACGCA-GAACCC-3′) flanked by a high density of target HpaII sites and a low density of HhaI (5′-GCCG-3′) and CG sites. This distribution of sequences allowed us to discriminate between purely targeted methylation by the targeted HpaII enzyme (4aZf-FH) and the action of endogenous Mtases, which could potentially methylate these additional embedded HhaI and CpG sites and at flanking CpG sites. The 4aZf protein is a derivative of the well-characterized anti-p190Bcr-Abl three-zinc finger protein (17) modified by a single zinc finger extension (12, 13). Zinc finger peptides were expressed as N-terminal fusions with the HpaII (FH) mutant Mtase and were linked by a flexible linker
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(Gly<sub>4</sub>Ser)<sub>3</sub>. For a schematic of the proposed Mtase action and a more detailed description of the target site, see Fig. 1, A and B. In <i>vitro</i> DNA binding and methylation analysis confirmed specific targeted methylation within a complex DNA population (supplemental Fig. S1, A and B) (14) and that tethering of the Mtase component at the zinc finger recognition site allows site-specific binding and targeted methylation to occur up to 40–45 bp away from the zinc finger site (supplemental Fig. S1D).

Cells were transiently transfected at a high efficiency with 4aZf-FH or control constructs including ΔZf-FH, 4aZf alone, and 4bZf-FH (for a vector schematic, see Fig. 1C). The 4bZf-FH enzyme served as a control for any background methylation mediated via nonspecific zinc finger/methyltransferase-DNA interactions because the target region lacks 4bZf recognition sites (5′-GACGCGGCGCTC-3′). Isoschizomer restriction analysis (comparing restriction patterns generated by methylation-sensitive versus insensitive restriction enzymes with the same sequence specificity) of plasmids coding for the 4aZf-FH and 4bZf-FH enzymes revealed similar levels of enzyme activity in bacteria, although these high copy number plasmids were seen to possess a low level of nonspecific methylation (supplemental Fig. S1C). Low copy number plasmids expressing these enzymes remained largely unmethylated. This effect of high cellular protein concentrations "overriding" the intrinsic specificity of zinc finger proteins has been reported previously by ourselves and others (13, 18) and demonstrates the need for moderate levels of such targeted Mtase enzymes in cellular systems. The ΔZf-FH (zinc finger deletion) construct represented an additional control for monitoring any untargeted methylation due solely to mutant methyltransferase activity at the target region. Transfection with wild-type HpaII or 4aZf-HpaII Mtase constructs ultimately proved toxic to the cell over the time scale of the experiments and was not studied further, confirming the requirement for mutational approaches in the development of these targeted Mtase enzymes.

Western blot analysis confirmed equivalent expression levels for constructs used (Fig. 2A). EMSA analysis using whole cell extracts also confirmed that cellularly expressed 4aZf-FH protein bound significantly only to DNA containing 4aZf with multiple flanking HpaII recognition sites, but not to probe containing just multiple HpaII sites (Fig. 2B). Similarly, the 4bZf-FH protein bound only to probe containing the 12-bp 4bZf and multiple HpaII sites, albeit less strongly than 4aZf-FH to its target site. EMSA for the weakly binding 4bZf-FH protein at relatively higher protein levels is also shown to more clearly confirm specific binding to its target probe (Fig. 2B, inset). More detailed analysis of the binding and methylation of these proteins is given elsewhere, both describing the ability of the 4aZf-FH enzyme to target methylation specifically in the context of complex genomes in <i>vitro</i> assays and demonstrating similar general enzymatic activities between the 4aZf-FH and 4bZf-FH enzymes (supplemental Fig. S1) (14).

To determine the extent of any targeted DNA methylation, genomic DNA was isolated 16 days post-transfection with various constructs, and methylation status was assessed initially by BsiEI restriction of post-bisulfite-modified DNA (COBRA assay) (19), sites for which are preserved through methylation of consecutive HpaII sites within the target sequence (Fig. 3A). Significant target site DNA methylation was associated with 4aZf-FH-transfected cells only, indicated by the observed cleavage of the PCR product (Fig. 3B, arrow, but not for 4bZf-FH- and ΔZf-FH-transfected cells, confirming target-specific methylation by the 4aZf-FH enzyme. Importantly and as a prerequisite to the further studies described in this work, the target site itself was not subject to <i>de novo</i> methylation by the endogenous Mtases of the cell. However, this may be a consequence of selecting clonal cell lines with a demonstrable reporter gene activity and hence a reduced likelihood of being the subject of cellular <i>de novo</i> methylation because methylated genes are more likely to be inactive.

Clonal bisulfite sequencing analysis of the DNA methylation status of the target and flanking regions from independent
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The effect of directed de novo methylation on gene expression was measured after 6, 12, and 24 days, together with target region methylation status over the same time period as
assessed by combined bisulfite restriction analysis assay. Expression of the 4aZf-FH enzyme resulted in a nearly 70% drop in CAT activity compared with controls (Fig. 5A) and correlated with the observed high levels of methylation at the target site (Fig. 5B). Significantly, this repression could be partially alleviated by treatment of cells with the DNA-demethylating agent 5-azacytidine or the histone deacetylase inhibitor MS275 and further alleviated by cotreatment with these drugs to a level above that of normal gene expression (see supplemental Fig. S1).

A weak alleviation of repression over time was observed for 4aZf-FH-transfected cells, which was mirrored by a similar slight fall in methylation levels observed at the target site (Fig. 5B). This effect may be due to a gradual loss of maintenance methylation or an outgrowth of untransfected cells over multiple passages. Expression of the 4bZf-FH enzyme resulted in an initial drop in CAT activity of about 20% at day 6 but returned to control levels by day 12. This observation of low level methylation by the 4bZf-FH protein was attributed to low density and transient nonspecific methylation, which may have been below the threshold for maintenance and perhaps working in concert with the effects of transient promoter occupancy by the enzyme. The suggestion that the 4bZf zinc finger component is not able to fully suppress nonspecific methylation by the Mtase component is in line with the relatively weak binding of the 4bZf-FH enzyme to its target site (Fig. 2B).

Zinc finger protein binding alone (4aZf) did not result in gene repression. Expression of weakly active 4aZf-HhaI mutant enzyme also failed to result in any significant gene repression (Fig. 5A) or methylation at any of the HhaI sites present within the target region above background (data not shown), in line with previous results for this enzyme (14), and confirms the lack of general zinc finger fusion protein binding-induced effects in the absence of a fully functional Mtase component.

To address issues relating to the possible contributions of reporter gene integration sites to de novo methylation-mediated gene repression, we performed CAT assays on pooled clonal populations harboring the integrated reporter vector (~25 clones) that had been transfected with targeted Mtase and control vectors. Targeted Mtase expression induced a similar drop in gene expression levels to that of individual clones that were examined, suggesting no locus-specific bias toward gene repression (Fig. 5A). Given that intuitively, sites of integration are more likely to occur in euchromatic, i.e. normally transcriptionally active, regions of the genome, the demonstration of targeted methylation-mediated gene silencing at multiple regions of this type would suggest that this route for exogenous control of gene repression is robust.

De Novo Methylation Initiates the Acquisition of a Repressive Histone Mark—Given the strong correlation historically observed between the methylation status of DNA and heterochromatin formation, we examined the chromatin composition associated with the target site/promoter region as a result of transient 4aZf-FH expression using chromatin immunoprecipitation analysis. Chromatin immunoprecipitation analysis of the promoter region showed that 4aZf-FH-targeted methylation was associated with a nearly 8-fold enrichment of histone H3K9me2 methylation (Fig. 6A), a histone modification known to be associated with transcriptional repression. This clearly pointed toward the initiation of a repressive histone mark for
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This region of DNA in response to de novo methylation. Similarly, analysis of histone H3K4me3, a marker for transcriptionally active chromatin (21), showed a relative 2.5-fold reduction in response to 4aZf-FH expression compared with controls. There was no observable change in histone H3K9me2 or H3K4me3 in c-fos and afm gene promoters, which were examined as additional controls for the potential effects of any non-targeted background methylation on chromatin composition throughout the genome (Fig. 6B).

**DISCUSSION**

It is widely accepted, mostly through the correlative observation that methylated genes in vivo are inactive, that DNA methylation is intricately associated with transcriptional repression. Historically, experiments involving transient transfection of plasmids methylated in vitro have gone a significant way to confirming the generally repressive effect of DNA methylation on gene transcription. However, there has always been some concern as to whether the methylation-mediated effects observed for cell-based methylases translate fully to genomic chromatin, given the differences in the nature of the structure (topology) and replication control between these two DNA species. For example, origins of replication associated with some episomal vectors have been shown to possess unique chromatin structures (22). A sought-after goal has therefore been the ability to impose DNA methylation in vivo at a defined region of the genome so that the direct effect of such methylation on gene expression and chromatin structure could be evaluated in a genuine chromosomal context. One potential route that has recently suggested itself for the targeted delivery of cytosine methylation to genomic DNA, although specifically limited to promoter regions, is that of RNA interference-mediated epigenetic silencing. Increasingly, it has been shown that small interfering RNAs and other nucleic acids complementary to promoter regions can give rise to DNA methylation at that region through a mechanism that in *Schizosaccharomyces pombe* is thought to involve histone H3 lysine 9 methylation (23). However, it is now unclear whether this is a universal response because experiments have failed to show this consistently (24–30). It is also unclear whether such induced methylation precedes or derives from the formation of a repressive chromatin state since in some instances such small interfering RNA-induced epigenetic gene silencing has been shown clearly not to involve DNA methylation (27). Therefore, the applicability of this approach in delivering de novo DNA methylation to predetermined regions is unclear.

Recently, the laboratory of Jeltsch and co-workers (11) demonstrated that targeted methylation could be directed to plasmid-borne reporter gene promoters in transient cotransfection assays using the Gal4 DNA-binding domain or zinc finger peptides fused to DNA Mtases and that targeted methylation led to reporter gene transcriptional repression in this extra-chromosomal context. The longer-term characteristics associated with targeted methylation of these sequences generally, such as the duration of repression and maintenance of methylation pattern and the acquisition of altered chromatin marks over time, were not examined, presumably due to the transient lifetime and extra-chromosomal nature of the reporter plasmids used in that study, and thus a direct comparison to the time-dependent analysis of methylation at the genomic locus presented here cannot be made at this point. However, the demonstrated maintenance of methylation and repression seen in our experiments after only a brief exposure to the targeted methyltransferase enzyme suggests distinct advantages over RNA interference-based methods, in which the sustained presence or expression of RNA effector molecules is required for long-term gene repression.

We observed no methylation at targeted regions that lacked HpaII sites but contained methylatable CpG sites, and expression of the essentially inactive targeted HhaI enzyme failed to repress gene expression to any detectable level. Taken together these results suggest a lack of any interaction between cellular proteins and the prokaryotic Mtases used in this study, which would possibly give rise to gene shutdown and/or methylation at CpG sequences. Interestingly, targeting the inactivated catalytic domains of DNMT3A or DNMT3B to the promoters of transient reporter plasmids has been shown to result in an ∼10–15% drop in reporter gene expression levels compared with a 60–65% reduction for targeted active enzymes in the same experiment (11). Whether this was due to the steric effect of binding of an inactive Mtase at the target promoter or the recruitment of endogenous factors to the target promoter through Mtase interactions is unknown.

One of the first attempts to study the function of DNA methylation in the cell in a genomic context, and the closest comparable study to the work reported here, i.e. analysis of de novo methylation at a genomic locus, focused on the introduction of in vitro variable “patch”-methylated reporter cassettes into the genome using Cre/loxP methodology (31). Although this elegant approach generally confirmed the correlation between observed DNA methylation and transcription repression, one concern is that it is not possible to use this approach to fully divorce the observed transcriptional repression from cellular events associated with the recombination process or from
effects due to further epigenetic modification of methylated episomal DNA prior to its integration. That said, however, an analysis of relative histone H3K4me3 levels at a methylated versus unmethylated region within the body of a green fluorescent protein reporter gene, introduced via Cre/loxP methodology, revealed an approximate 2.5-fold reduction at methylated regions (32), which agrees with the values reported for our own experiments at the targeted promoter region. The additional ability shown in our studies to target DNA methylation directly to “natural chromatin,” negating the potential complications associated with upstream genetic events, has enabled a clear demonstration of the downstream histone responses to de novo DNA methylation.

Neither the 4aZf nor 4aZf-HhaI enzyme induced any significant transient or long-term gene repression in our experiments, confirming that the repressive effects observed for the 4aZf-FH enzyme are indeed attributable to the targeted Mtase action of this enzyme and not purely to initial steric occlusion at the promoter region or cellular piggybacking events mediated through the Mtase component, as mentioned previously. Steric effects have been thought previously to make promoters more susceptible to endogenous (de novo) Mtase activity due to reduced transcriptional activity, for example, the observed DNA hypermethylation of the GSTP1 promoter as a result of transcriptional gene silencing due to deletion of Sp1 sites within the promoter region (33).

In the experiments described here, the target promoter is initially clearly maintained in the genome in a “naïve” unmethylated state and with a chromatin signature characteristic of actively transcribed genes. The promoter then acquires and maintains a specific methylation pattern through the action of the targeted Mtase enzyme. The target region subsequently acquires a repressive histone signature, which is also maintained long after the loss of the initial methylating stimulus. Such observations, in addition to quite clearly pointing to a direct role for de novo methylation-mediated gene silencing in this system, additionally imply that the de novo DNA methylation patterns that emerge in cells associated with cancer and disease progression have the potential to initiate gene repression. Whether or not this is the case, especially considered in the context of such methylation occurring aberrantly at normally functional endogenous promoters, is a question that can perhaps now be addressed by the further development of targeted methyltransferases specific for those loci. However, we acknowledge that the stably integrated target DNA used in our experiments may not behave generally in the same manner as cellular genes or fully mimic the methylation-mediated responses of an endogenous promoter for which the presence of distal and proximal regulatory elements and pre-set chromatin states and boundaries may modulate the response to exogenously applied de novo methylation.

The mechanistic connection among established DNA methylation, transcriptional repression, and histone modification is well documented (34, 35). Components of each epigenetic pathway have now been shown to cross-interact to a significant extent. Methyl-CpG-binding proteins, for example, have been shown to associate with histone deacetylases, histone methyltransferases, and methylated DNA, providing a clear link between DNA methylation and heterochromatin formation (36, 37). This link is further reinforced by the demonstrated interaction between the endogenous DNA Mtases, such as DNMT3A and the SETDB1 histone methyltransferase (38), and between other chromatin-modifying factors (reviewed in Refs. 15 and 16). The methylation pattern seen throughout the course of our experiments is maintained predominantly at targeted HpaII sites, but not at embedded or adjacent CpG (non-HpaII) sites. This suggests a purely maintenance activity by the endogenous cellular enzymes, with no evidence of any Mtase action by the endogenous de novo Mtases DNMT3A and DNMT3B, which might be sequestered at heterochromatic regions through protein-protein interactions (38).

This work describes the first demonstration of the functional consequences of targeted de novo methylation at a genomic locus, directly confirming the role of de novo genomic DNA methylation in the control of gene expression. The ability to target cytosine methylation directly to specific genomic DNA sequences represents an important step toward studying spatial and temporal issues associated with genuine de novo methylation and the exogenous control of the heritable repression of cellular genes.

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