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MINI-REVIEW

Application of DNA methyltransferases in targeted DNA methylation

Albert Jeltsch · Renata Z. Jurkowska · Tomasz P. Jurkowski · Kirsten Liebert · Philipp Rathert · Martina Schlickenrieder

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Abstract DNA methylation is an essential epigenetic modification. In bacteria, it is involved in gene regulation, DNA repair, and control of cell cycle. In eukaryotes, it acts in concert with other epigenetic modifications to regulate gene expression and chromatin structure. In addition to these biological roles, DNA methyltransferases have several interesting applications in biotechnology, which are the main focus of this review, namely, (1) in vivo footprinting: as several bacterial DNA methyltransferases cannot methylate DNA bound to histone proteins, the pattern of DNA methylation after expression of DNA methyltransferases in the cell allows determining nucleosome positioning; (2) mapping the binding specificity of DNA binding proteins: after fusion of a DNA methyltransferase to a DNA-binding protein and expression of the fusion protein in a cell, the DNA methylation pattern reflects the DNA-binding specificity of the DNA-binding protein; and (3) targeted gene silencing: after fusion of a DNA methyltransferase to a suitable DNA-binding domain, DNA methylation can be directed to promoter regions of target genes. Thereby, gene expression can be switched off specifically, efficiently, and stably, which has a number of potential medical applications.

Keywords DNA methyltransferase · Epigenetics · Enzyme design · DamID

A. Jeltsch (✉) · R. Z. Jurkowska · T. P. Jurkowski · K. Liebert · P. Rathert · M. Schlickenrieder
Biochemistry Laboratory,
School of Engineering and Science,
Jacobs University Bremen,
Campus Ring 1,
28759 Bremen, Germany
e-mail: a.jeltsch@iu-bremen.de

Introduction to bacterial and mammalian DNA methyltransferases

A variety of molecules including proteins, RNA, DNA, and small molecules like neurotransmitters are methylated in living cells (Cheng and Blumenthal 1999). The methyl group is transferred from the cofactor *S*-adenosyl-L-methionine by a class of enzymes called methyltransferases (MTases) (reviews, Cheng 1995; Hermann et al. 2004; Jeltsch 2002; Wion and Casadesus 2006). In bacteria, DNA methylation occurs at the N6 atom of adenines as well as the N4 and C5 atoms of cytosines; in metazoans only the latter type of modification is observed. The methyl groups add additional information to the DNA sequence. This so-called epigenetic information often plays central regulatory roles in the control of the cellular physiology.

Technically, DNA methyltransferases provide unique opportunity to mark DNA inside a cell in a site-specific manner. This property can be employed as a tool to study the binding of proteins to DNA and to regulate the expression of target genes. In this short review, we will first summarize the properties and biological roles of DNA MTases in prokaryotes and eukaryotes and then describe new developments in the biotechnological application of these enzymes.

Properties and biological role of bacterial DNA methyltransferases

Bacterial DNA methyltransferases are generally small in size (300–500 amino acids). They usually methylate the DNA specifically within or next to short recognition sequences, which are often palindromic. The structures of several bacterial DNA MTases have been solved, indicating that all the enzymes share a common fold (reviews, Cheng

1995; Cheng and Roberts 2001). Structural and biochemical studies demonstrated that within the subclasses of DNA methyltransferases, the catalytic mechanisms are conserved as well (review, Jeltsch 2002). In bacteria, DNA methylation has a multitude of different biological functions, which can be summarized as follows:

1. Coordination of gene expression and cell cycle: After DNA replication, the transient phase of hemimethylated DNA can be used for specific regulation of the gene expression. In *Escherichia coli* and related bacteria, DNA methylation takes place at the adenine residue within the GATC sites by the action of the DNA adenine methylation (Dam) enzyme. As this site is palindromic, the methyl mark resides on both strands of the DNA. After DNA replication, the DNA is hemimethylated and usually gets rapidly converted into the fully methylated state. However, the origin of replication of the *E. coli* chromosome, which is enriched in GATC sites, stays hemimethylated for 15–20 min because the binding of the SeqA protein prevents the remethylation. As hemimethylated origins are not active, this prevents reinitiation of DNA replication before the cell cycle is completed (review, Lobner-Olesen et al. 2005).
   In the *Caulobacter crescentus*, the CcrM system methylates the DNA at GANTC sites. In these bacteria, the replicative conversion into hemimethylated GANTC sites activates the promoter region of the *ctrA* gene, which is an important cell cycle regulator (review, Reisenauer and Shapiro 2002).
2. DNA repair: The brief period during which methylation sites are hemimethylated after DNA replication allows to distinguish the parental and daughter strand of DNA synthesis. In *E. coli*, this property enables a directed repair of the replication errors by the MutHLS mismatch repair system. MutS will recognize mismatches, recruit MutL and MutH, which then move to a GATC site. MutH cleaves the unmethylated strand, and the daughter strand is removed. The repair of the mismatch is finalized by the synthesis of the new DNA strand (review, Joseph et al. 2006).
3. Gene expression and phase variation: Methylation of MTase target sites can influence the binding of bacterial transcription factors to the DNA and thereby regulate gene expression. In the case of the *E. coli* Dam system, there are some promoters that contain sets of Dam sites, whose methylation is variable. Mutual exclusive methylation of Dam sites at the promoter of P-pili, for example, regulates the expression of these genes and influences pathogenicity of *E. coli*. Involvement of DNA methylation in bacterial phase variation and pathogenicity has been demonstrated in other cases as well (review, Hernday et al. 2002, 2004; Heusipp et al. 2007).
4. Control of DNA uptake: The majority of bacterial DNA MTases are parts of the restriction–modification systems (review, Pingoud et al. 2005; Pingoud and Jeltsch 2001). In such systems, the MTase acts in concert with a restriction enzyme of a matching specificity. The restriction enzyme cuts incoming DNA, whereas genomic DNA is protected against cleavage by methylation at specific sites. This mechanism is employed to protect the bacteria against phage infection, as well as the uptake of DNA from the environment. These systems thereby constitute a barrier to horizontal gene transfer that might have an important role in bacterial speciation (Jeltsch 2003).

Properties and biological role of eukaryotic DNA methyltransferases

DNA MTases are found in the majority of lower and higher eukaryotes, for example, in fungi, plants, and mammals. In this brief review, we will restrict the discussion to the mammalian enzymes. In this group, DNA methylation occurs at CG sites, which are methylated in both strands of the DNA in a cell-type-specific pattern. Three families of mammalian DNA MTases have been found, called Dnmt1, Dnmt2, and Dnmt3 (review, Hermann et al. 2004). Dnmt2 has only a low DNA MTase activity and has recently been shown to methylate a cytosine residue in the anticodon loop of transfer RNA-Asp (review, Jeltsch et al. 2006).

Dnmt1 shows a high preference for hemimethylated CG sites and is responsible for copying the pattern of DNA methylation after each round of DNA replication. This enzyme comprises a large N-terminal part, which is implicated in cellular targeting, protein/protein interaction, and regulation of the catalytic domain. The C-terminal catalytic domain resembles bacterial DNA Mtases, but so far has not been demonstrated to have activity on its own (Fatemi et al. 2001).

The Dnmt3 family comprises two active DNA methyltransferases Dnmt3A and 3B as well as Dnmt3L, which is inactive but functions as an activator of Dnmt3A and 3B. Like Dnmt1, the Dnmt3A and 3B enzymes consist of a large N-terminal domain, which is involved in protein/protein interaction and targeting, and a C-terminal domain responsible for catalysis. However, in contrast to Dnmt1, the isolated catalytic domains of Dnmt3A and 3B are enzymatically active (Gowher and Jeltsch 2002; Reither et al. 2003). The Dnmt3A and 3B enzymes do not distinguish between unmethylated and hemimethylated DNA and do not show large sequence specificity, apart from a preference for CG sites and certain flanking sequences (Gowher and

Jeltsch 2001; Handa and Jeltsch 2005). They are essential for setting the initial pattern of DNA methylation, a process that certainly requires the recruitment of the enzymes to the target DNA by interaction with other proteins. This can be illustrated, for example, by the fact that Dnmt3B—but not Dnmt3A—is responsible for methylation of pericentromeric repeats (Okano et al. 1999; Xu et al. 1999), an observation that cannot be explained only by the catalytic properties and target sequence preferences of Dnmt3A and 3B.

In mammals, the central effect of DNA methylation at CG sites in promoter regions of genes is gene repression (review, Klose and Bird 2006). In concert with other epigenetic marks, like histone tail modification and expression of noncoding RNA, DNA methylation constitutes the cellular memory that saves the transcriptional profile of the cell and passes it to daughter cells (review, Fuks 2005). In this context, DNA methylation is involved in the regulation of gene expression during development and in response to environmental signals (review, Jaenisch and Bird 2003). In addition, genetic imprinting (review, Reik and Walter 2001) and the inactivation of one X-chromosome in females is mediated by DNA methylation (review, Heard and Disteche 2006). As most CG sites in the genome are methylated and the chromatin condensed, DNA methylation contributes to a general inactive state of mammalian DNA. Thereby, it protects the genome against the mobilization of repetitive DNA elements and hinders recombination. Consequently, loss of DNA methylation can lead to genomic instability (Gaudet et al. 2003).

Aberrant DNA methylation contributes to the development of many diseases, as it leads to loss of transcriptional control (reviews, Egger et al. 2004; Feinberg and Tycko 2004; Rodenhiser and Mann 2006). In many cancers, for example, methylation of tumor suppressor genes leads to downregulation of their expression. Downregulation of the expression of tumor suppressor genes is observed with almost equal prevalence as inactivation of the gene product by somatic mutations. Furthermore, a general reduction in DNA methylation causes genomic instability, which is a hallmark of cancer cells (review, Ducasse and Brown 2006; Jones and Baylin 2002).

Biotechnological applications of DNA methyltransferases

DNA MTases have been used for different purposes in biotechnology, which are summarized in Fig. 1 and will be explained in detail in the next paragraphs of this minireview.

Application of DNA methyltransferases for in vivo and in vitro footprinting

One biotechnological application of DNA MTases is to map the positioning of histones on chromatin in vivo and in vitro (Fatemi et al. 2005; Kladde and Simpson 1996; Kladde et al. 1996). For this approach, the M.SssI MTase from *Spiroplasma* sp. has been used that methylates CG sites, like the mammalian MTases, but cannot access DNA bound to histone proteins (Gowher et al. 2005; Kladde et al. 1999; Okuwaki and Verreault 2004). Therefore, after exposure to M.SssI, the methylation pattern of the DNA preserves the nucleosomal footprints allowing to follow the dynamics of nucleosome positioning. The advantages of M. SssI for such applications include its very short recognition

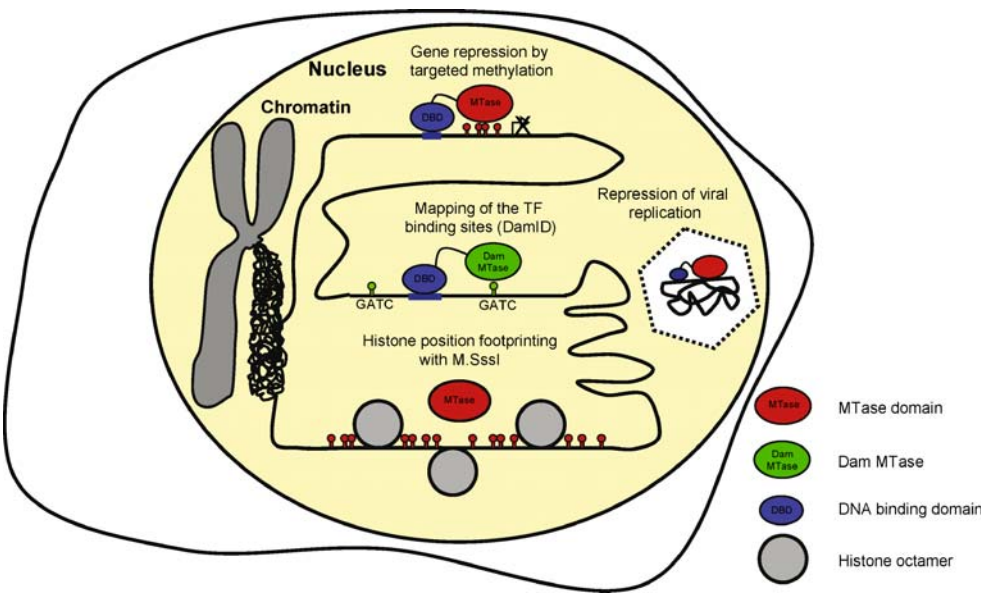

Fig. 1 Schematic drawing of biotechnological applications for DNA MTases in the cell. *Green lollipops* represent adenine–N6 methylation at GATC sites, *red lollipops* denote for cytosine–C5 methylation at CG sites

sequence and good activity, which results in a high density of the methylation. Furthermore, the methylation status of each cytosine residue on DNA substrates up to several hundreds base pairs can be rapidly detected by bisulfite conversion of the DNA followed by subcloning and sequencing (Clark et al. 1994; Frommer et al. 1992).

Application of DNA methyltransferases in targeted DNA methylation

Due to their abilities of site-specific covalent modification of DNA and the potential of gene regulation, applications of DNA MTases concentrate on the idea of targeted DNA methylation. This concept implies that a DNA MTase is directed to defined genomic sites by fusion to a targeting module. In principle, targeting modules could include small molecules, polynucleotides, and protein domains. While, currently, no small molecules are available that would direct proteins to specific DNA sequences, successful employment of polynucleotide or protein domains as targeting device has been demonstrated in different studies. It should be noticed that in all these applications, the specificity of the targeting process and the efficiency of methylation is essential and has to be established experimentally.

Targeting of DNA methyltransferases via triple helix formation

This approach relies on the potential of polypurine and polypyrimidine single-stranded DNA to bind to polypurine double-stranded DNA in a sequence-specific manner and form a triple-stranded DNA (review, Frank-Kamenetskii and Mirkin 1995). Successful application of this strategy has been recently demonstrated using a restriction endonuclease as model system (Eisenschmidt et al. 2005). The authors chemically fused an oligonucleotide to the restriction enzyme *Pvu*II, purified the enzyme–DNA conjugate and demonstrated very specific targeting of the conjugate to a single recognition site of the restriction enzyme positioned next to the triple helix-forming site in vitro. One principal advantage of this approach is that, by following the rules of triple helix formation, targeting oligonucleotides can be designed from scratch for any polypurine DNA sequence. Further development and application of chemically modified base analogs and modified DNA backbone frameworks, like peptide-nucleic acids, might help to stabilize the triple strands and target non-polypurine sites as well (Fox 2000; Seidman and Glazer 2003).

For a successful application of this method in vivo, two major obstacles have to be overcome, namely, the slow rate of the triple helices formation (Paes and Fox 1997; Seidman et al. 2005) and the delivery of the enzyme–DNA conjugates into the cell. Although protein transfection reagents are available for that purpose, their efficiency often depends on the nature of the cargo protein, and it is difficult to transfect a large fraction of cells. Furthermore, it is not known if the methyltransferase would stay active after delivery. However, a major advantage of the approach is that additional chemical modifications could be made on the enzyme to influence its properties. For example, it has been recently shown that it is possible to reversibly inactivate a DNA MTase by chemical modification (so-called caging) and reactivate it with good yield by laser irradiation (Rathert et al. 2007). In principle, this provides an opportunity to deliver a caged MTase and activate it only in the target cell or tissue by a laser beam.

Targeting of DNA methyltransferase by fusion to a DNA-binding domain

The approach of targeted methylation by fusing an MTase to a DNA-binding domain (DBD), that directs the enzyme to its target on the DNA, currently has the most applications and has been developed the furthest (Fig. 2). One inherent advantage of this procedure is that the MTase–DBD fusion proteins are constructed at the genetic level, and the fusion genes are transfected into cells such that the chimeric MTase is produced in the cell. The application of DNA MTase–DBD fusion proteins was pioneered by Xu and Bestor (1997) who first achieved targeted methylation in vitro using a fusion protein consisting of the DBD of Zif268 and the bacterial CG-specific DNA methyltransferase M.SssI.

However, the use of protein domains for DNA recognition bears disadvantages as well. While, in principle, each DNA sequence could be targeted, a potential problem is that protein domains that specifically bind to the target site might not be available. The advances in the engineering of artificial zinc-finger proteins might help to overcome that bottleneck because successful design strategies have been developed that allow the construction of zinc-finger

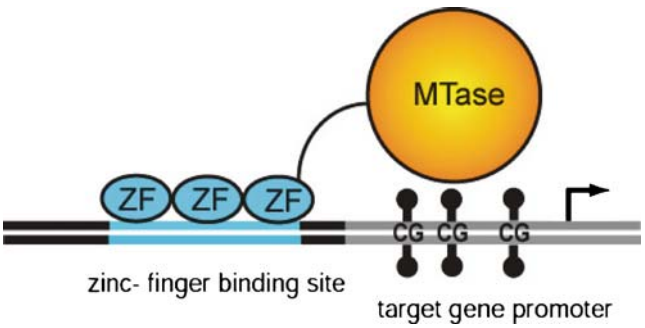

Fig. 2 Schematic drawing of the architecture of chimeric DNA MTases comprising a targeting zinc finger (*ZF*) and a methyltransferase (*MTase*) domain. The *lollipops* denote for CG sites that got methylated by the targeted MTase

modules for arbitrary DNA targets (reviews, Nomura and Sugiura 2007; Uil et al. 2003). These advances (which are truly exceptional because there are no general rules available to design specificity of DNA-interacting proteins) explain the prevalence of using zinc-fingers for such applications.

Possible applications of DBD–MTase fusion constructs go into two directions. First, the MTase can be used to label the DNA and thereby keep record of the DNA-binding properties of the DBD fusion partner. This approach has been used to study the specificity of transcription factors binding to DNA and the accessibility of chromatin to DNA-binding proteins. Alternatively, a DBD of a known specificity could be fused to the MTase domain to methylate the DNA at predetermined sites and thereby silence the expression of target genes.

Study of the DNA interaction of DNA-binding proteins by fusion to DNA methyltransferases

Different DNA MTases have been fused to DNA-interacting proteins (Carvin et al. 2003a, b; Greil et al. 2006; Jessen et al. 2004). After expression of the fusion proteins in the cell, the DBD binds to the DNA and the neighboring methylation sites get modified. Therefore, the methylation pattern of the DNA will reflect the specificity of the DBD and the accessibility of the chromatin for binding. Among the many inherent advantages of this approach, the most important are that the experiment is performed under physiological conditions inside the living cell and that the methylation status can be easily determined after DNA isolation. Therefore, methylation probing is an attractive alternative to other in vivo footprinting approaches, like UV cross-linking followed by ligation-mediated polymerase chain reaction (PCR) analysis (Pfeifer and Tommasi 2000) or protection of DNA against modification by dimethyl sulfate (DMS) and analysis by DNA sequencing (Gregory et al. 1998).

Among the different MTases employed for methylation probing, the *E. coli* Dam enzyme has been proven to be the most successful one (review, Greil et al. 2006). The advantages of this approach (called DamID; Fig. 3) are that the Dam enzyme methylates adenine residues, which is

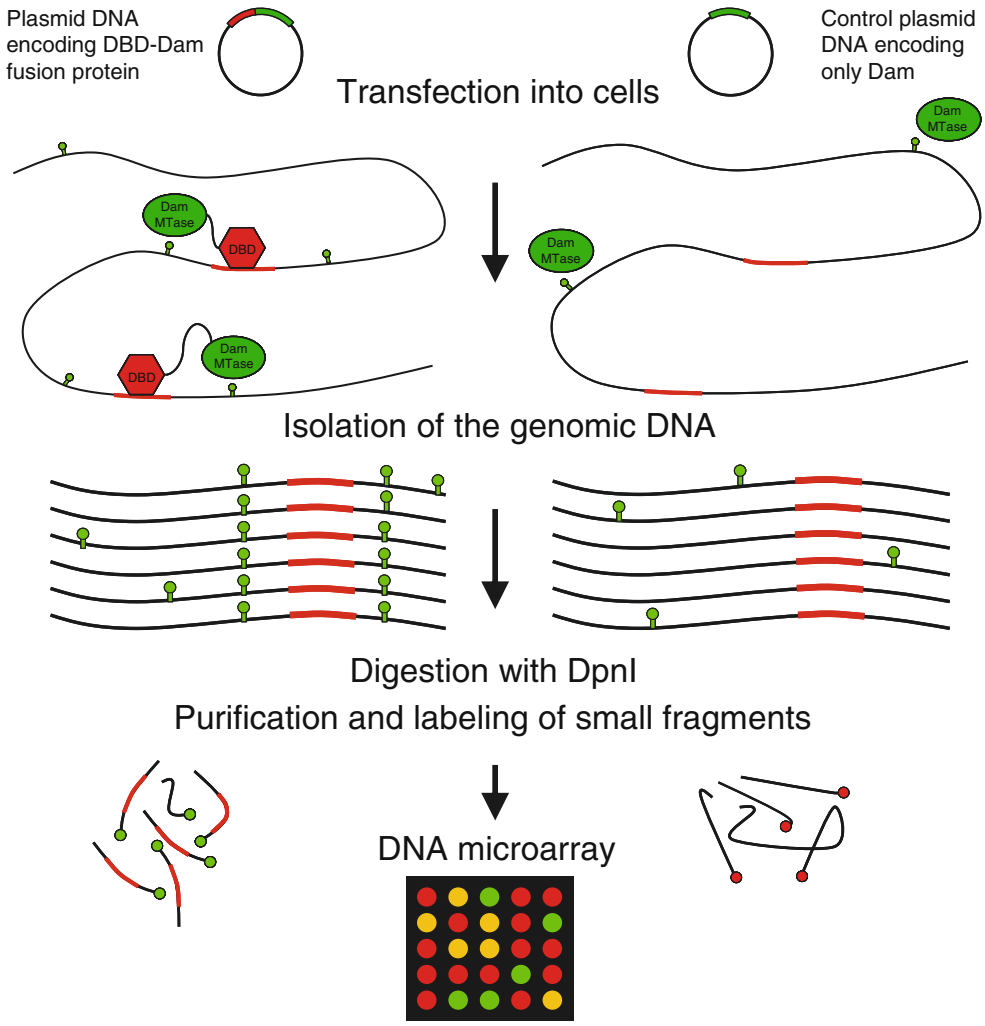

Fig. 3 Schematic drawing of the principle of DamID. *Left* A fusion protein comprising a DNA-binding domain (*DBD*) and the Dam DNA MTase is expressed in a cell. Dam recognition sites close to binding sites of the DBD (*red line*) become methylated (indicated by *green lollipops*). As a control the untargeted Dam MTase is expressed (*right*). DNA is isolated from both samples, cleaved with *Dpn*I restriction enzyme, which cleaves at methylated dam sites. DNA cleavage fragments are isolated and analyzed, for example, by differential labeling and hybridization to DNA microarrays

a modification that does not naturally occur in metazoans and does not cause a detectable phenotype in flies (Wines et al. 1996). Dam methylation at GATC sites can be easily detected using several restriction enzymes that are inhibited by the methylation. Moreover, Dam methylation is unique among all bacterial methylation events because the methylated GATC sequence is recognized by the *Dpn*I restriction enzyme, which does not cleave unmethylated DNA. Therefore, both negative and positive display of Dam methylation is possible by restriction digestion because enzymes like *Dpn*II or *Mbo*I cleave unmethylated sites but leave the methylated sites intact, whereas *Dpn*I only cleaves the methylated site. DamID has been employed to map in vivo binding sites for various transcription factors including HP1, cyclin D3, Myc, Max, and Mad/Mnt (de Wit et al. 2005; Orian et al. 2003; Song et al. 2004; van Steensel et al. 2001).

Targeted gene silencing using chimeric DNA methyltransferases

The second application of DNA MTase–DBD fusion proteins aims to incorporate methyl groups into the DNA to regulate the expression of endogenous genes. Potential applications of this approach include the repression of oncogenes in cancer cells, the silencing of viral proteins in cells after viral infection, and the downregulation of genes, whose aberrant expression contributes to the development of disease (review, Egger et al. 2004). Examples include diseases accompanied by accumulation and aggregation of endogenous proteins, like the amyloid precursor protein (APP) and tau proteins in Alzheimer's disease (review, Weiner and Frenkel 2006), mutated Huntingtin in Chorea Huntington (review, Borrell-Pages et al. 2006), α-synuclein in Parkinson's disease (review, Cookson 2005), or prion proteins in bovine spongiform encephalopathy (BSE)-related diseases (review, Caughey and Baron 2006). In all these examples, it is well conceivable that the reduction in the expression of these proteins could be an approach for treatment of the disease.

Several papers from different groups demonstrated recently the principal applicability of this technology. In yeast cells, Carvin et al. demonstrated successful targeted methylation after fusing the transcriptional factor PHO4 to the M.CviPI methyltransferase from Chlorella virus NYs-1 as well as fusing the DBDs of zinc-finger proteins based on Zif268 with M.*Cvi*PI and M.SssI (Carvin et al. 2003a, b). However, no effects on gene expression were reported, which might be due to the absence of a functional DNA methylation system in yeast. In addition, nontargeted methylation was observed, which has been attributed to the high catalytic activities of the MTase domains. The level of nontargeted methylation could be reduced by introducing mutations into the targeted MTase that lower its catalytic activity (Smith and Ford 2007).

Li et al. (2007) showed in mammalian cells that chimeric MTases comprising the Gal4 DNA-binding domain fused to the catalytic domain of the mouse Dnmt3A and 3B enzymes led to dense methylation of the DNA at the targeted Gal4 site and neighboring regions, which resulted in a strong repression of the targeted gene promoters (Li et al. 2007). In an independent study, recruitment of Dnmt3a–Gal4 fusion proteins to artificial promoters containing upstream activation sequences (UAS; which are targets for Gal4) has been reported as well (Li et al. 2006). With a similar approach, DNA methylation could also be targeted to mitochondrial genes (Minczuk et al. 2006).

After fusing the catalytic domain of Dnmt3A to an artificial zinc-finger DBD, which targets the promoter region of the herpes simplex virus 1 IE175k protein, targeted methylation could be used to repress the propagation of HSV-1 virus in cell culture (Li et al. 2007). This promising result hints toward potential medical applications of this technology. The high specificity of targeting observed in this study might be due to the fact that the Dnmt3A catalytic domain was used, which does not show high endogenous methylation activity. The high level of targeted methylation observed by Li et al. (2007) could be explained by the ability of the mammalian DNA MTase domain to interact with the endogenous silencing apparatus and recruit additional silencing factors to the target site. Employing mammalian DNA MTase domains has therefore two important advantages for directed DNA methylation: First, mammalian enzymes have the potential to cooperate with other endogenous silencing factors (including other DNA MTases and enzymes that chemically modify histone tails); second, their inherent activity is low, which reduces the risk of nontargeted methylation.

Due to several major advantages, targeted DNA methylation has a greater potential for the regulation of the endogenous genes than alternative strategies like RNAi. First, chimeric DNA MTases can be constructed at genetic level, and the DNA methylation is induced after transient expression of the enzymes. After its establishment, the newly generated DNA methylation pattern is copied by the cellular maintenance methylation machinery, such that permanent expression of the chimeric MTase is not required. Thereby, many of the risks associated with stable delivery of genes in mammalian cells are avoided. Second, DNA methylation cooperates with other epigenetic processes, including histone tail modifications, and by inducing chromatin condensation, it leads to a very strong and stable repression of gene expression.

The process of targeted gene silencing by chimeric DNA MTases resembles endogenous regulation, which often includes the piggyback transporting of DNA MTases to target genes by transcription factors and other chromatin interacting proteins. Different studies have demonstrated an interaction of Dnmt3a with p53 and RP58 transcription

factors (Fuks et al. 2001; Robertson et al. 2000; Wang et al. 2005), the retinoic acid receptor (Di Croce et al. 2002), EZH2 and SetDB1 (both histone methyltransferases; Li et al. 2006; Vire et al. 2006), Karopsi's sarcoma-associated herpes virus protein LANA (Shamay et al. 2006), human papilloma virus protein E7 (Vire et al. 2006), as well as Mbd3 and Brg1 (Datta et al. 2005). Therefore, the application of chimeric MTases for external gene regulation is one example of successful imitation of nature in biotechnology. Future work will show if targeted gene silencing by chimeric DNA MTases will hold its promises.

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