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Molecular Enzymology of Mammalian DNA Methyltransferases

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Abstract DNA methylation is an essential modification of DNA in mammals that is involved in gene regulation, development, genome defence and disease. In mammals 3 families of DNA methyltransferases (MTases) comprising (so far) 4 members have been identified: Dnmt1, Dnmt2, Dnmt3A and Dnmt3B. In addition, Dnmt3L has been identified as a stimulator of the Dnmt3A and Dnmt3B enzymes. In this review the enzymology of the mammalian DNA MTases is described, starting with a description of the catalytic mechanism that involves covalent catalysis and base flipping. Subsequently, important mechanistic features of the mammalian enzyme are discussed including the specificity of Dnmt1 for hemimethylated target sites, the target sequence specificity of Dnmt3A, Dnmt3B and Dnmt2 and the flanking sequence preferences of Dnmt3A and Dnmt3B. In addition, the processivity of the methylation reaction by

Dnmt1, Dnmt3A and Dnmt3B is reviewed. Finally, the control of the catalytic activity of mammalian MTases is described that includes the regulation of the activity of Dnmt1 by its N-terminal domain and the interaction of Dnmt3A and Dnmt3B with Dnmt3L. The allosteric activation of Dnmt1 for methylation at unmodified sites is described. Wherever possible, correlations between the biochemical properties of the enzymes and their physiological functions in the cell are indicated.

1 Introduction

The first mammalian DNA methyltransferase (MTase) activity was discovered byRazin's group in the early 1980s (Gruenbaum et al. 1982). The enzyme responsible for this activity is called Dnmt1 today [the name derives from *DN*A *m*ethyl*t*ransferase; the systematic nomenclature of DNA MTases is described in Roberts et al. (2003)]. The murine Dnmt1 enzyme was the first mammalian DNA MTase to be cloned and expressed recombinantly (Bestor et al. 1988; Pradhan et al. 1997). During the last decade, three more members of the mammalian Dnmt enzyme family have been discovered and cloned (Fig. 1; reviews: Chen and Li 2004; Hermann et al. 2004a). All these enzymes contain a domain of approximately 400–500 amino acid residues, which is characterised by the presence of 10 conserved amino acid motifs, shared between prokaryotic and eukaryotic DNA-(cytosine-C5)-MTases (reviews: Cheng 1995; Jeltsch 2002). The catalytic centre and coenzyme binding site of MTases reside within this domain. In addition, the Dnmt1 and the Dnmt3 en-

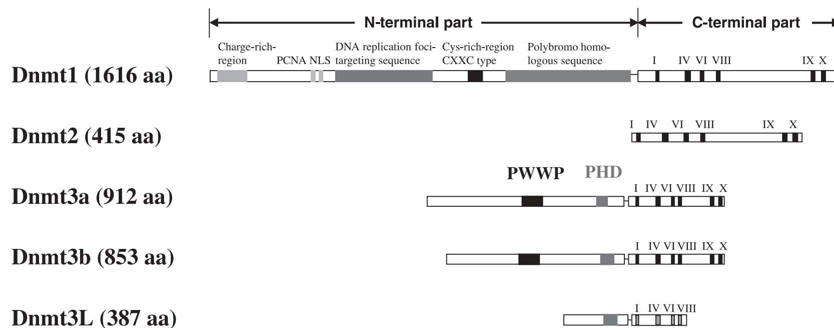

Fig. 1 Domain organisation of the mammalian Dnmts. The mammalian methyltransferases are divided into an N-terminal part and a C-terminal part. The C-terminal part shows strong amino acid sequence homology to prokaryotic DNA-(cytosine-C5)-MTase and contains 10 conserved catalytic amino acid motifs (indicated by *Roman numerals*) characteristic for this enzyme family

zymes harbour large N-terminal regulatory parts (reviews: Chen and Li 2004; Hermann et al. 2004a). The N-terminal regulatory domain of Dnmt1 contains different motifs and subdomains which interact with many other proteins (Chuang et al. 1997; Fuks et al. 2003; Liu and Fisher 2004; Margot et al. 2003; Pradhan and Kim 2002; Robertson et al. 2000; Rountree et al. 2000). One example of these interacting proteins is the proliferating cell nuclear antigen (PCNA) known as processivity factor for the DNA polymerases ε/δ (Chuang et al. 1997; Maga and Hubscher 2003). It seems that the N-terminus is forming a platform for binding of proteins involved in chromatin condensation, gene regulation and DNA replication. In addition, Dnmt1 has a role in mismatch repair of mammalian cells (Kim et al. 2004; Wang and James Shen 2004).

Dnmt1 has a strong preference for methylation of hemimethylated CG sites (Fatemi et al. 2001; Gruenbaum et al. 1982; Hermann et al. 2004b), which implicates it as having a function in maintenance of the methylation pattern of the DNA after replication. Dnmt1 knock-out mice die during embryogenesis; embryos show almost complete loss of DNA methylation (Li et al. 1992). Interestingly, the catalytic domain of Dnmt1 is inactive in the absence of the N-terminal part (Fatemi et al. 2001), which implies an important regulatory function of the N-terminal domain on the enzyme.

Dnmt2 is the smallest enzyme among the eukaryotic MTases and it comprises only the catalytic domain (Fig. 1). It has a very slow turnover rate (Hermann et al. 2003; Kunert et al. 2003; Liu et al. 2003; Tang et al. 2003). The protein is conserved in many eukaryotic species (also some that only have low or even undetectable levels of DNA methylation like *Drosophila melanogaster* or *Schizosaccharomyces pombe*). The biological function of Dnmt2 is not known, although it has been associated to longevity in *D. melanogaster* (Lin et al. 2004).

The mammalian Dnmt3 enzyme family consists of three different proteins, Dnmt3A, Dnmt3B and Dnmt3L (Fig. 1). The regulatory N-terminal domain of Dnmt3A and Dnmt3B is not essential for catalysis (Gowher and Jeltsch 2002; Reither et al. 2003). Both enzymes contain an ATRX-like Cys-rich domain (also called PHD domain) and a PWWP domain, which are involved in interactions with other proteins and targeting to heterochromatin (Aapola et al. 2002; Bachman et al. 2001; Chen and Li 2004; Fuks et al. 2003; Ge et al. 2004). Despite significant amino acid sequence and biochemical similarities, Dnmt3A and Dnmt3B have distinct biological roles. Dnmt3B is responsible for methylation of pericentromeric satellite regions (Hansen et al. 1999; Okano et al. 1999; Xu et al. 1999). Dnmt3B$^{-/-}$ knock-out mice die during the late embryonic stage and the embryos lack methylation in pericentromeric repeat regions (Okano et al. 1999). Loss of Dnmt3B activity in human leads to ICF (immunodeficiency, centromere instability, facial anomalies) syndrome, a genetic disorder that

is accompanied by low methylation in the pericentromeric satellite regions of chromosomes 1, 9 and 16 (Ehrlich 2003). Dnmt3A knock-out mice show developmental abnormalities and die a few weeks after birth (Okano et al. 1999). This enzyme has been associated with the methylation of single copy genes and retrotransposons (Bourc'his and Bestor 2004; Bourc'his et al. 2001; Hata et al. 2002) and it is required for the establishment of the genomic imprint during germ cell development (Kaneda et al. 2004). The N-terminal part of Dnmt3L is shorter than those of Dnmt3A and Dnmt3B and only contains the PHD domain. The C-terminal part of this protein is truncated and all its "catalytic" motifs are crippled, indicating it cannot be an active DNA MTase. Dnmt3L acts as a stimulator of the catalytic activity of Dnmt3A and Dnmt3B activity (Chedin et al. 2002; Gowher et al. 2005; Suetake et al. 2004).

In the following sections, the enzymology of the mammalian DNA MTases will be reviewed. Starting with a description of the catalytic mechanism, some important mechanistic features like the degree of specificity for the target base and preference for flanking sequences, the processivity of DNA methylation and the mechanism of control of enzyme activity will be discussed. It is written under the presumption that a detailed knowledge of the enzymes' properties is an essential prerequisite for the understanding of their cellular roles.

2
Catalytic Mechanism of DNA-(Cytosine-C5)-MTases

All DNA MTases use the coenzyme *S*-adenosyl-L-methionine (AdoMet) as the source for the methyl group being transferred to the DNA bases. The methyl group of AdoMet is bound to a sulphonium centre, which activates it towards nucleophilic attack. The AdoMet binding site is remarkably conserved in all DNA (and also non-DNA) MTases. It is created by residues from the motifs I–III and X, which form conserved contacts to almost every hydrogen bond donor and acceptor of the AdoMet and, in addition, several hydrophobic interactions to the cofactor. The roles of many of these residues have been confirmed by mutagenesis experiments in prokaryotic MTases (review: Jeltsch 2002).

2.1
Reaction Mechanism of DNA-(Cytosine-C5)-MTases

The reaction mechanism of cytosine-C5 methylation was uncovered for the prokaryotic DNA-(cytosine-C5)-MTase M.HhaI (Fig. 2; Wu and Santi 1985; Wu and Santi 1987). A key feature of the catalytic process is a nucleophilic

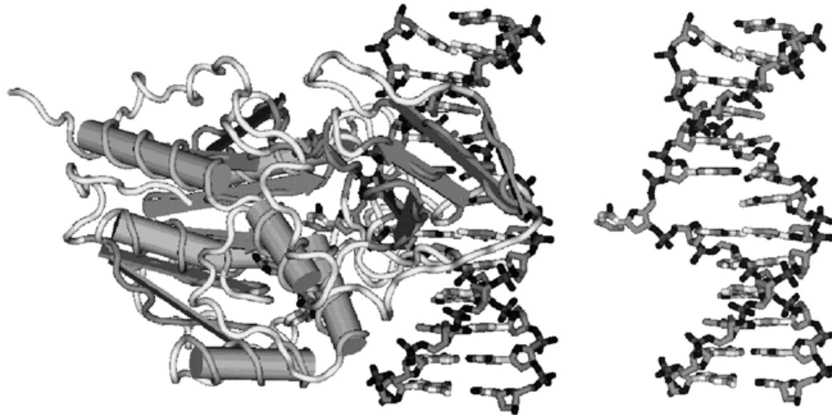

Fig. 2 Structure of the prokaryotic M.HhaI DNA MTase. The *left part* shows the protein in schematic view, in the *right part* only the DNA is shown to illustrate the rotation of the target base out of the DNA helix

attack of the enzyme on the carbon-6 of the target cytosine. This attack is performed by the thiol group of the cysteine residue that is part of the conserved PCQ motif in the active site of cytosine-C5-MTases (motif IV). This reaction is catalysed by the protonation of the cytosine N3 position carried out by the glutamic acid of the amino acid motif ENV (motif VI). The formation of the covalent bond activates the cytosine C5 atom towards nucleophilic attack on the methyl group leading to the addition of the methyl group to carbon-5. The reaction cycle is closed by the elimination of the 5-position proton and the thiol moiety, which resolves the covalent intermediate and re-establishes aromaticity (review: Jeltsch 2002).

This description of the catalytic mechanism of DNA-(cytosine C5)-MTases by a combination of covalent catalysis and acid base catalysis is supported by a large body of experimental evidence: The covalent reaction intermediate between methylated DNA and the active site cysteine has been observed in all structures of DNA-(cytosine-C5)-MTase in complex with DNA known so far (Klimasauskas et al. 1994; Reinisch et al. 1995). In addition, the covalent intermediate has been detected biochemically with several DNA MTases including Dnmt1 and Dnmt3A (Chen et al. 1991; Hanck et al. 1993; Osterman et al. 1988; Reither et al. 2003; Santi et al. 1984; Wyszynski et al. 1993; Yoder et al. 1997) and covalent complex formation has been shown to involve the cysteine residue in the PCQ motif (Chen et al. 1991; Everett et al. 1990; Hanck et al. 1993; Reither et al. 2003). In addition, the importance of the cysteine residue in motif IV for catalysis by prokaryotic MTases has been demonstrated by

site-directed mutagenesis (Hurd et al. 1999; Wyszynski et al. 1992, 1993). The formation of a stable covalent intermediate comprising the enzyme and the target base is the basis of the efficient inhibition of DNA MTases by cytidine analogues incorporated into DNA, which currently is being investigated with respect to its therapeutic potential (review: Gowher and Jeltsch 2004).

Surprisingly, in the case of the Dnmt3A catalytic domain, the glutamic acid residue in motif VI has been shown to be very important for activity, but the removal of the active site cysteine residue did not result in a complete loss of catalytic activity (Reither et al. 2003). This finding suggests that, in addition to covalent catalysis, other mechanisms of enzyme catalysis are operative in DNA MTases (at least in the case of Dnmt3A) such as positioning of the target base and the cofactor with respect to each other and stabilisation of the transition state of methyl group transfer. In this context, it is interesting to note that Dnmt3A purified from *Escherichia coli* but also from insect cells shows only relatively low turnover rates (Aoki et al. 2001; Gowher and Jeltsch 2001; Okano et al. 1998). This indicates that the active site of Dnmt3A is not in an ideal conformation and the cysteine residue is not ideally positioned to perform a nucleophilic attack on the C6 position. It might be possible that a covalent modification of the enzyme or an interaction with another protein could induce a conformational change of the catalytic site that activates the enzyme and switches the catalytic mechanism to the covalent catalysis scheme (Reither et al. 2003). The mammalian Dnmt1 enzyme might be a precedent for this kind of activation, because although the full-length enzyme is highly active, its catalytic domain is not active in an isolated form, which implies that an interaction of the catalytic domain with the rest of the protein is essential for the catalytic domain to adopt a catalytically competent conformation.

2.2
Base Flipping

The first X-ray structure of a DNA-(cytosine-C5)-MTase in complex with DNA was determined with M.HhaI (Klimasauskas et al. 1994; Fig. 3). It demonstrated that DNA MTases completely rotate their target base out of the DNA helix prior to its methylation, a process called base flipping. After base flipping the target cytosine is no longer buried in the double helix of the DNA but is turned about its flanking sugar-phosphate bonds such that it projects out into the catalytic pocket of the enzyme. The base pairing hydrogen bonds are broken and the stacking interactions with the adjacent base pairs are lost during this process. Base flipping has been observed in all MTase-DNA complex structures known so far (Goedecke et al. 2001; Klimasauskas et al. 1994; Reinisch et al. 1995) and also in many other enzymes interacting with

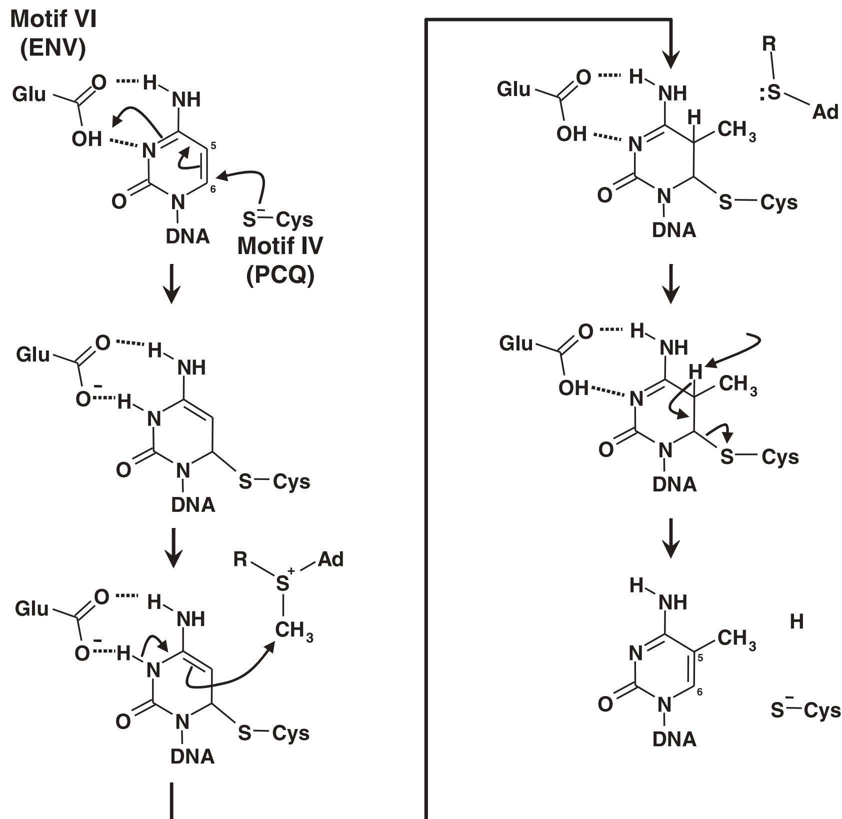

Fig. 3 Chemistry of the DNA methylation reaction

DNA, for example many DNA repair enzymes (reviews: Cheng and Roberts 2001; Roberts and Cheng 1998). It brings the target base into close contact to the enzyme, allowing for the intricate chemical reactions to occur and for accurate recognition of the flipped base, an important requirement for the function of DNA repair glycosylases. Also, it is a prerequisite for the catalytic mechanism as described above, because it makes the C5, C6 and N3 positions of the cytosine accessible to the enzyme.

The structure of M.HhaI is typical for all enzymes of the DNA-(cytosine-C5)-MTase family (reviews: Cheng 1995; Cheng and Roberts 2001). It comprises two domains. The larger, catalytic domain is conserved among all enzymes of this type. It consists of a central, parallel, 6-stranded β-sheet flanked by α-helices. The domain can be divided into two subdomains, one forming

the binding pocket for the flipped target base, the other for the AdoMet cofactor. The structures of both subdomains are similar, and the catalytic domain most likely arose by gene duplication (Malone et al. 1995). The smaller domain is involved in the recognition of the target sequence and structurally diverse (review: Jeltsch 2002). The only structure of a mammalian MTase catalytic domain solved so far is that of Dnmt2 (Dong et al. 2001). The protein is folded very similarly to M.HhaI; unfortunately, the reason(s) why the Dnmt2 enzyme has only a very low catalytic activity cannot be deduced from its structure.

3 Target Sequence Specificity of Mammalian DNA MTases

All mammalian DNA MTases modify DNA at CG sites. However, the degree of specificity for the target sequence and the preference for different methylation states of the target site varies considerably among the different enzymes.

3.1 Specificity of Dnmt1 for Hemimethylated DNA

In 1982, Razin's group isolated DNA MTase activity from mammalian cells that displayed a very high preference for hemimethylated CG sites (Gruenbaum et al. 1982). Later this enzyme was identified as Dnmt1, but, using oligonucleotide substrates, different factors for the preference of hemimethylated DNA over unmethylated were found which range from 2- to 50-fold (Fatemi et al. 2001; Flynn et al. 1996; Pradhan et al. 1999; Tollefsbol and Hutchison 1995, 1997). These differences could be due to deviations in the experimental setup (allosteric activation, see Sect. 5.1), different substrates, different sources of the proteins and different degrees of purity. For example, Bestor reported in the early 1990s that treatment of Dnmt1 with proteases leads to the loss of preference for hemimethylated target sites (Bestor 1992). Similarly, we observed that the preference for hemimethylated DNA decreased from its original level of about 50-fold during prolonged storage of the enzyme (Fatemi et al. 2001). In the context of longer hemimethylated DNA, a 24-fold preference for a hemimethylated target site has been detected (Hermann et al. 2004b). In vivo, this property is very important, as it enables the enzyme to copy the existing methylation pattern of the DNA after DNA replication and, therefore, to work as a maintenance MTase. The high specificity of Dnmt1 for hemimethylated target sites is a fascinating example of molecular recognition, because the presence of a single methyl group switches on the enzyme's activity at hemimethylated CG sites. The detailed mechanism of this process is not yet known.

3.2
CG and Non-CG Methylation by Dnmt3A and Dnmt3B

Both Dnmt3A and Dnmt3B do not differentiate between unmethylated and hemimethylated substrates, and both are involved in de novo DNA methylation in vivo (Gowher and Jeltsch 2001; Okano et al. 1998, 1999). Interestingly, Dnmt3A and Dnmt3B also methylate cytosine residues in a non-CG context in vitro (Aoki et al. 2001; Gowher and Jeltsch 2001; Hsieh 1999; Ramsahoye et al. 2000). Depending on the substrate and assay system, the activity at non-CG sites varies between 0.5% and 10% of the activity observed at CG sites. In general, CA sites were found the second-best substrate for Dnmt3A and Dnmt3B. Methylation of non-CG sites by Dnmt3A has been detected also in mouse DNA (Dodge et al. 2002). However, since Dnmt1 cannot maintain this asymmetric methylation, the biological function of this activity is not known. One could speculate that non-CG methylation is important to ensure a rapid onset of a strong repression of gene expression during early embryogenesis. After some time, when additional epigenetic mechanisms like histone modification and chromatin condensation have become effective, the non-CG methylation might no longer be required.

3.3
Flanking Sequence Preference of Mammalian DNA MTases

Another facet in the DNA interaction of mammalian DNA MTases is their flanking sequence preferences. Since it contains only two bases, the recognition sequence of these enzymes is much shorter than typical DNA interaction sites of proteins of that size, which are in the range of 8 to 14 base pairs. Therefore, it is likely that interactions between the protein and the DNA also occur outside of the central CG site, which could lead to preferences of methylation of CG sites within a certain sequence context. Such differences are usually called "flanking sequence preference" and they are conceptually distinct from the "sequence specificity", because a change in the flanks will only modify the rate of methylation, while a change in the central target site will abolish methylation. The flanking sequence preferences of Dnmt3A and Dnmt3B have been studied in detail. Dnmt3A exhibits strong strand preference for CG sites flanked by pyrimidines and a loose consensus sequence of YNCGY (Lin et al. 2002). Later, the consensuses sequence could be refined and extended also to Dnmt3B, showing that both enzymes prefer methylation of CG sites in a RCGY context and disfavour YCGR sites (Handa and Jeltsch 2005). Interestingly, the rates of methylation of substrates differing in 4 base pairs on each site of the central CG site varied by more than more than 500-fold. Comparing these numbers with the actual preference for CG over CA

in a given sequence context, which is approximately 10- to 100-fold, one has to conclude that the concept of flanking sequence and central site is not fully applicable to Dnmt3A and Dnmt3B, because changes in the flanking sequence influence the reaction rate to a similar degree as a change of the central CG to CA. The flanking sequence preferences of Dnmt1 for the methylation at unmethylated CG sites have been studied as well, demonstrating the enzyme shows a clear preference for methylation within a CCGG context (R. Goyal and A. Jeltsch, in preparation).

Interestingly, a statistical analysis of human DNA methylation patterns revealed that there is a clear correlation between the average methylation level of CG sites and their flanking sequence that closely fits to the flanking sequence preferences of Dnmt3A and Dnmt3B (Handa and Jeltsch 2005). This finding demonstrates that the intrinsic preferences of Dnmt3A and Dnmt3B for certain target sites shaped the human epigenome. However, the biological implications of the sequence preferences of the Dnmt3A and Dnmt3B de novo MTases might extend even to immunology. DNA containing unmethylated CG dinucleotide sequences is immunogenic in mammals (Krieg 2002; Rui et al. 2003). In several reports it has been shown that DNA with CG flanked by purine at the 5′ end and pyrimidine at the 3′ end has a higher immunogenic response when compared to other sequences (Klinman et al. 1996; Krieg 2002). This consensus sequence is identical to the high preference consensus sequence for Dnmt3A and Dnmt3B. Therefore, those flanking sequences that render high immunogenicity to unmethylated CG dinucleotide sites belong to the most preferred consensus sequence for de novo DNA MTases and hence have the lowest probability to be unmethylated in the human DNA. Thereby, the risk of an autoimmune response generated from self-DNA is minimised. This observation indicates co-evolution of de novo DNA MTases and the immune system in context with CG dinucleotides and the flanking sequences (Handa and Jeltsch 2005).

3.4 Specificity of Dnmt2

The substrate specificity of the Dnmt2 enzyme is still not fully understood. The human enzyme has a preference for CG sites (Hermann et al. 2003) whereas *D. melanogaster* Dnmt2 was found to prefer CT and CA sites (Kunert et al. 2003). It is not clear whether or not these differences are due to the amino acid differences between both enzymes, which are only moderate. However, all these studies are hampered by the low methylation activity of the enzymes, leading to an insufficient statistical sampling. Therefore, additional experiments will be required to resolve this issue.

4
Processivity of DNA Methylation by Mammalian DNA MTases

Since DNA MTases are enzymes that work on a long polymeric substrate containing several potential target sites, the processivity of the methylation reaction is an important issue for this class of enzymes. Here, processivity is defined as the preference of the enzyme to transfer more than one methyl group to one DNA molecule without release of the DNA.

4.1
Processivity of Dnmt1

Evidence for a processive reaction mechanism of Dnmt1 dates back to 1983 when Bestor and Ingram demonstrated that Dnmt1 methylates longer substrates faster than shorter ones (Bestor and Ingram 1983). Recently, long hemimethylated substrates were used to study the processivity of Dnmt1 in more detail using a physiological substrate. This study demonstrated that Dnmt1 modifies DNA in a highly processive reaction, and during the processive movement on the DNA it accurately copies the exiting methylation pattern (Hermann et al. 2004b). Such processive methylation of DNA implies that Dnmt1 moves along the DNA after each turnover. The mechanism of this movement is not yet clear; it might involve a sliding and a hopping process. It also is not known if Dnmt1 moves on the DNA with a directional preference.

It is tempting to speculate that the ability of Dnmt1 to methylate DNA in a processive reaction and to interact with PCNA are co-adaptations that enable the enzyme to bind to the replication fork in vivo and methylate nascent DNA immediately after DNA replication. However, its catalytic activity might not suffice to cope with the high density of CG sites in heterochromatin. Therefore, Dnmt1 might impede the progression of the replication fork if it remained tightly attached to the replication fork during replication of heterochromatic DNA. To avoid this potential complication, one could suppose that Dnmt1 is released from the replication fork during the heterochromatin replication phase, and that the methylation of heterochromatic DNA is restored after replication has taken place. This model is supported by the finding that the time gap between replication and methylation is larger for the heterochromatic than for the euchromatic DNA (Gruenbaum et al. 1983; Leonhardt et al. 1992; Liang et al. 2002). Furthermore, it has been demonstrated that Dnmt3A and Dnmt3B also play a role in the preservation of methylation levels at heterochromatic DNA (Chen et al. 2003; Liang et al. 2002; Rhee et al. 2002).

4.2 Processivity of Dnmt3A and Dnmt3B

Similar experiments with Dnmt3A and Dnmt3B yielded the interesting result that Dnmt3A modified DNA in a distributive reaction, but Dnmt3B was processive (Gowher and Jeltsch 2001, 2002). This was an unexpected observation because the catalytic domains of Dnmt3A and Dnmt3B are about 84% identical in amino acid sequence. However, among the 44 amino acid residues that are not identical between human and murine Dnmt3A and Dnmt3B catalytic domains, 15 include charged residues. The exchanges observed among these residues are highly biased such that, in the end, Dnmt3B carries 6 more positive charges than Dnmt3A. Therefore, Dnmt3B has a much more positively charged DNA binding cleft than Dnmt3A, which could explain why Dnmt3B methylates DNA in a processive reaction whereas Dnmt3A is distributive (Fig. 4; Gowher and Jeltsch 2002).

The difference in the kinetic mechanisms of the catalytic domains of Dnmt3A and Dnmt3B could be related to the distinct biological functions of these enzymes in the cell, because satellite 2 repeats (one of the major targets of Dnmt3B) are exceptionally rich in CG sites when compared with the rest of the genome (Gowher and Jeltsch 2001). Dnmt3B is well suited to modify

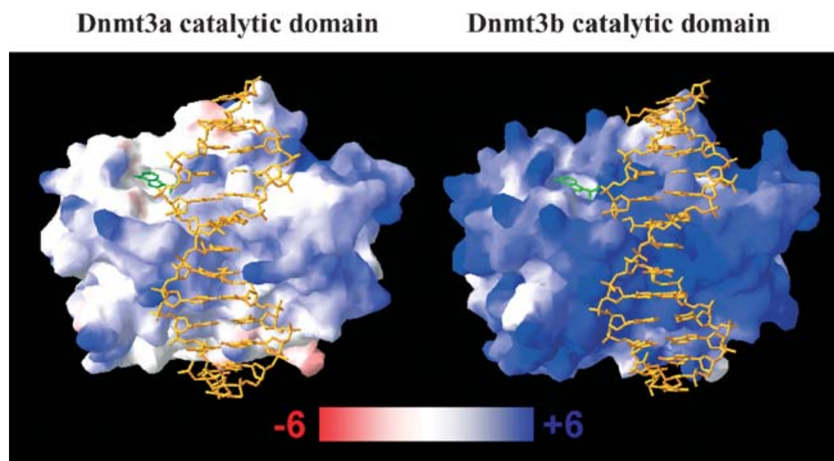

Fig. 4 Models of the catalytic domains of Dnmt3A and Dnmt3B. The models were prepared using M.*Hha*I as template as described in (Gowher and Jeltsch 2002). The surface of the proteins was coloured according to the electrostatic potential calculated using Swiss PDB viewer version 3.7.b2. To illustrate the location of the DNA binding cleft in the enzymes, the DNA as seen in the M.*Hha*I-DNA complex is shown in *orange*, the AdoMet is shown in *green*

these regions, because after targeting to the DNA it can methylate several cytosine residues in a processive reaction. The distributive reaction mechanism of Dnmt3A might explain why it cannot replace Dnmt3B at satellite repeats in vivo, although the Dnmt3A enzyme can methylate these regions.

So if the processive mechanism has such obvious advantages, why did Nature invent distributive enzymes like Dnmt3A? One advantage of a distributive enzyme could be that its activity is under better control, because it has to be directed to the DNA for each single methylation event. Therefore, a distributive enzyme depends on a mechanism targeting it to the sites of action much more so than a processive enzyme, where one targeting event will lead to the transfer of several methyl groups to the DNA. In line with these considerations, Dnmt3A has been associated with the methylation of single-copy genes and retrotransposons (Bourc'his and Bestor 2001, 2004; Hata et al. 2002) and it is critical to the establishment of the genomic imprint during germ cell development (Kaneda et al. 2004). Therefore, Dnmt3A is involved in the methylation of defined target sites, whereas Dnmt3B (at least as far as the methylation of heterochromatic repeats is concerned) catalyses the complete methylation of large DNA domains. One could envisage that Dnmt3A contacts a targeting factor and thereby keeps indirect contact (via the targeting protein) to the DNA. This mechanism would allow for efficient methylation of the DNA at sites that are determined by the specificity of the targeting complex.

5
Control of DNA MTase Activity in Mammalian Systems

The mechanism by which mammalian DNA MTases create a specific DNA methylation pattern that carries additional information is one of the most fascinating questions regarding the function of these enzymes. Although the exact mechanism of pattern generation is not certain, it clearly depends on the control of the enzyme's activity by different instances that include control of gene transcription, covalent modification and interaction with regulatory proteins. The transcriptional control of mammalian Dnmts has been reviewed recently (Pradhan and Esteve 2003b) and is beyond the scope of this review, which focuses on enzymology. Dnmt1 isolated from mammalian cell lines has been shown to carry some phosphoryl groups (Glickman et al. 1997). However, the functional relevance of this modification is not yet known, and it is not clear if post-translational modifications occur with Dnmt3A, Dnmt3B or Dnmt2 as well. In the following paragraphs the interactions of MTases with regulatory proteins will be discussed.

5.1 Allosteric Activation of Dnmt1

Surprisingly, the isolated catalytic domain of Dnmt1 is not catalytically active, although it contains all the amino acid motifs characteristic for cytosine-C5 MTases (Fatemi et al. 2001; Margot et al. 2000; Zimmermann et al. 1997). These results demonstrate that the N-terminal part of Dnmt1 has an important role in controlling the activity of the protein, such that Dnmt1's N-terminal part could be considered a "regulatory protein". A similar observation was already made by Bestor (1992) by demonstrating that a proteolytic cleavage of Dnmt1 just between the catalytic domain and the N-terminal domain leads to a strongly increased activity of Dnmt1 towards unmethylated target sites (Bestor 1992). In this study, the C- and N-terminal parts of Dnmt1 most likely remained in contact, but the proteolytic cleavage induced a conformational change that activated the enzyme.

Interestingly, Dnmt1 bears at least two separate DNA binding sites, at least one in the N-terminal part and one in the C-terminal part (Araujo et al. 2001; Fatemi et al. 2001; Flynn and Reich 1998). The enzyme can interact with its target DNA and, in addition, with a second DNA molecule that functions as an allosteric regulator. Binding to methylated DNA activates Dnmt1 for methylation of unmodified target sites (Bacolla et al. 1999; Fatemi et al. 2002; Fatemi et al. 2001). Steady-state kinetic experiments demonstrate that the N-terminal part of Dnmt1 has a repressive function on the catalytic domain, which is relieved after binding of methylated DNA to the N-terminus (Bacolla et al. 2001). Experimental evidence suggests that binding of methylated DNA occurs within the Zinc-domain, which forms a direct protein/protein contact to the catalytic domain of the enzyme (Fatemi et al. 2001) or to a short motif in between the PCNA interaction site and the nuclear localisation signal (NLS) (Pradhan and Esteve 2003a). Given these results, at least three different states of Dnmt1 can be distinguished: The isolated catalytic domain is inactive towards hemimethylated and unmethylated DNA. With unmethylated DNA the full-length enzyme shows low activity. In the presence of methylated DNA, the activity of Dnmt1 is much higher, suggesting that the N-terminal part has two effects: (1) It stimulates the C-terminal part for general activity and (2) either unmethylated DNA binding to the N-terminal part inhibits the enzyme or binding of methylated DNA stimulates the enzyme, leading to an increased methylation of unmodified sites.

This allosteric activation is a surprising effect, as it means that, in the presence of methylated DNA, Dnmt1 loses specificity for hemimethylated DNA and also starts working as a de novo MTase. Therefore, activated Dnmt1 is less accurate in copying an existing methylation pattern, which at first

sight appears as a mis-adaptation for a maintenance MTase. After allosteric stimulation, Dnmt1 has a similar activity on unmethylated and hemimethylated DNA, suggesting that this enzyme could also have a role in de novo methylation of DNA. Activated Dnmt1 could support Dnmt3A and Dnmt3B in de novo methylation, a conclusion that is in agreement with in vivo data demonstrating Dnmt1 is required for de novo methylation (Liang et al. 2002) and overexpression of Dnmt1 can cause de novo methylation of DNA (Biniszkiewicz et al. 2002). This assumption is also supported by the finding that Dnmt1 and Dnmt3A interact with each other (Datta et al. 2003; Kim et al. 2002).

The allosteric activation mechanism of Dnmt1 makes DNA methylation behave in an all-or-none fashion, because some methylation will always attract more methylation. In addition, epigenetic signalling comprises several positive feedback loops: Initial DNA methylation could induce histone 3 lysine 9 methylation or histone deacetylation (Cameron et al. 1999; Fahrner et al. 2002; Sarraf and Stancheva 2004; Tariq et al. 2003). These responses in turn could trigger additional DNA methylation (Bachman et al. 2003; Jackson et al. 2002; Lehnertz et al. 2003; Tamaru and Selker 2001). Furthermore, methylation of DNA could attract MeCP2 that itself would target Dnmt1 to the DNA (Kimura and Shiota 2003). Therefore, in a steady-state situation only completely unmethylated and fully methylated regions of the DNA coexist, which are separated by chromatin boundary elements. This all-or-none behaviour might increase the efficiency of epigenetic circuits in switching on and off gene expression. These mechanisms also explain the observation that methylation tends to spread from heavily methylated regions of the DNA into neighbouring unmethylated regions, which is often observed in cancer cells.

5.2
Stimulation of Dnmt3A and Dnmt3B by Dnmt3L

De novo methylation by Dnmt3A and Dnmt3B is regulated by at least one additional protein, namely Dnmt3L, which shows clear homology to the Dnmt3A and 3B enzymes (Aapola et al. 2000). However, Dnmt3L carries mutations within all conserved DNA-(cytosine-C5)-MTase motifs. This observation suggests that Dnmt3L adopts the typical MTase fold, but it does not have catalytic activity. In co-transfection experiments, Dnmt3L has been shown to stimulate DNA methylation by Dnmt3A in human cell lines (Chedin et al. 2002). In vitro studies demonstrated an approximately 15-fold activation of Dnmt3A and Dnmt3B by Dnmt3L (Gowher et al. 2005). Biochemical studies demonstrate Dnmt3L directly interacts with Dnmt3A and Dnmt3B via its C-terminal domain (Gowher et al. 2005; Hata et al. 2002; Suetake et al.

2004) and induces a conformational change of Dnmt3A that facilitates DNA and AdoMet binding. However, the interaction of Dnmt3A and Dnmt3L is transient, and Dnmt3L dissociates from Dnmt3A-DNA complexes. Therefore, Dnmt3L acts as a substrate and coenzyme exchange factor on Dnmt3A and Dnmt3B (Gowher et al. 2005).

Dnmt3L is expressed during gametogenesis and embryonic stages (Bourc'his and Bestor 2004; Bourc'his et al. 2001), showing a similar expression pattern as the Dnmt3A and Dnmt3B enzymes. Dnmt3L knock-out mice display a normal phenotype (Bourc'his and Bestor 2004; Bourc'his et al. 2001; Hata et al. 2002). Homozygous female mice are fertile, but when crossed with wild-type males their pups die at embryonic day 10.5. Analysis of the DNA methylation pattern showed that the female imprint was not properly established in oocytes of Dnmt3L knock-out females (Bourc'his et al. 2001; Hata et al. 2002). Homozygous male knock-out animals are sterile because of defects in spermatogenesis. Methylation analysis showed major loss of methylation in spermatogonial stem cells, leading to male infertility (Bourc'his and Bestor 2004; Hata et al. 2002). These strong phenotypes of Dnmt3L knock-out mice illustrate the importance of the stimulatory effect of Dnmt3L on Dnmt3A and Dnmt3B in vivo. It is to be expected that more regulators (inhibitors and stimulators) of Dnmts will be discovered in the future.

6
Future Perspectives

The cellular memory of developmental decisions is crucial in the development and maintenance of multicellular organisms. Failure in the propagation of the cellular memory of differentiated states is a major reason for cancer and other diseases. Cellular memory is mediated by epigenetic switches including DNA methylation in mammals. DNA MTases, the enzymes that set up the pattern of DNA modification and thereby impose additional information on the DNA, are central actors in epigenetic information transfer. However, many mechanistic features of these fascinating enzymes are incompletely characterised so far. Future biochemical experiments will address issues like substrate specificity, reaction mechanism, control of enzyme activity, targeting of methylation to certain DNA regions and interaction of MTases with other proteins involved in epigenetic processes. A more detailed understanding of the behaviour of DNA MTases shall enable us to get a better grasp of epigenetic regulation as a whole.

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