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Review

Biochemistry and biology of mammalian DNA methyltransferases

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Abstract. DNA methylation is a stable but not irreversible epigenetic signal that silences gene expression. It has a variety of important functions in mammals, including control of gene expression, cellular differentiation and development, preservation of chromosomal integrity, parental imprinting and X-chromosome inactivation. In addition, it has been implicated in brain function and the development of the immune system. Somatic alterations in genomic methylation patterns contribute to the etiology of human cancers and ageing. It is tightly interwoven with the modification of histone tails and other epigenetic signals. Here we review our current understanding of the molecular enzymology of the mammalian DNA methyltransferases Dnmt1, Dnmt3a, Dnmt3b and Dnmt2 and the roles of the enzymes in the above-mentioned biological processes.

Key words. DNA methylation; DNA methyltransferase; Dnmt1; Dnmt3a; Dnmt3b; epigenetics; regulation of gene expression.

Introduction

DNA methylation was discovered in calf thymus DNA by Hotchkiss in 1948 [1]. It occurs at the N6 position of adenine residues and the N4 and C5 positions of cytosine residues, only the last type being observed in higher eukaryotes, including mammals. In any case the methyl groups are positioned in the major groove of the DNA, where they do not interfere with the Watson/Crick base-pairing capacities of the nucleotides (fig. 1A). In 1964, Gold and Hurwitz [2] identified the first DNA methyltransferase (MTase) in *Escherichia coli*. DNA methylation since then has been discovered in nearly all the organisms investigated, ranging from bacteria to man. The first mammalian DNA MTase activity was discovered by the group of Razin [3]. The enzyme responsible for this activity today is called Dnmt1 (the Dnmt abbreviation is derived from DNA methyltransferase; the systematic nomenclature of DNA methyltransferases is described in [4]). Its murine homolog was the first mammalian DNA methyltransferase to be cloned and expressed recombinantly [5, 6]. During the last decade, three more members of the mammalian Dnmt enzyme family have been discovered and cloned [7–9], and biochemical, genetic and molecular biology approaches have been systematically applied to further our understanding of this important class of enzymes.

DNA methylation at the C5 position of cytosines is the only covalent modification of DNA known in metazoans. 5-methylcytosine, sometimes considered as the fifth base of mammalian DNA, occurs at levels of about 3–8% of the cytosine residues. It is found predominantly in the short canonical sequence 5′-CG-3′, and rarely at non-CG sites. It is an important carrier of epigenetic information extending the information content of the DNA [10]. The

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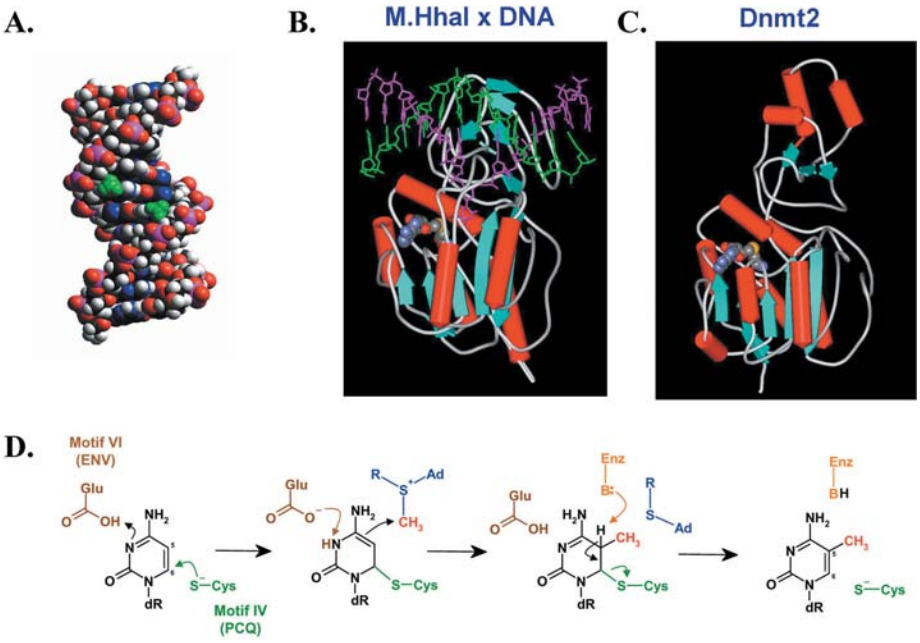

Figure 1. (*A*) Structure of B-DNA methylated at one central CG site. The DNA is coloured according to the atom types, the methyl groups attached to the cytosines in the central CG site are coloured green. (*B* and *C*) Structures of the bacterial M.HhaI MTase in complex with DNA and of the mammalian Dnmt2 enzyme. The proteins are shown in a schematic view (α-helices are displayed as red tubes, β-strands as cyan arrows) in a similar orientation. Both structures contain the product of the coenzyme of the methylation reaction *S*-adenosyl-L-homocysteine shown in a space-filled view. The lower part of both structures comprising the conserved catalytic subdomain is similar. In contrast, the structures of the smaller domains pointing upwards differ from each other. In figure 1B, the DNA structure shows the target base (in yellow) being rotated out of the DNA helix. (*D*) Chemistry of the methylation reaction of DNA-(cytosine-C5)-MTases. The methylation reaction catalysed by DNA MTases involves the formation of a dihydrocytosine intermediate that is covalently bound to the enzyme and releases *S*-adenosyl-L-homocysteine as product. In the second step of the reaction, the covalent bond is broken and the methylated cytosine released.

fact that methylation at the C5 position of cytosines occurs at all is intriguing, since it has the crucial disadvantage the deamination of methylated cytosines results in an $^{m}C \rightarrow T$ transition mutation. In contrast, deamination of unmethylated cytosine leads to uracil in the DNA, which can be recognized and repaired efficiently by the uracil-deglycosylase pathway. As a result, CG sites are a major mutational hotspot that account for about 30% of point mutations in the germline [11] and for acquired somatic mutations [review: 12], and they have been depleted from vertebrate genomes over evolutionary time. Small regions of the genome in which CG sequences are not underrepresented are known as CpG islands. These CpG islands are frequently found near the 5′ ends of genes and are mostly unmethylated, which prevented them from depletion [13, review: 14].

DNA methylation has a variety of important functions in mammals, such as gene repression [review: 15, 16], control of cellular differentiation and development [reviews: 17, 18], preservation of chromosomal integrity [19], parental imprinting [review: 20] and X-chromosome inactivation [review: 21, 22]. In addition, DNA methylation silences endogenous retroviruses and suppresses homologous recombination [review: 23]. It has been implicated in brain function [24, 25] and the development of the immune system [reviews: 26, 27]. Somatic alterations in genomic methylation patterns contribute to the genesis of human cancers [review: 28, 29] and ageing [review: 30]. As a consequence, DNA methylation is essential for normal embryonic development in mammals, as illustrated by the finding that DNA methyltransferase knockout mice die early in development [31, 32].

In this review, a brief overview of the fundamental chemistry of DNA cytosine-C5 methylation will be given, followed by details on the biochemical and biological aspects of this process. Mammalian DNA methyltransferases will be discussed, including their mechanism, regulation, expression and their roles in the methylation processes ongoing in every cell.

Chemistry of DNA cytosine-C5 methylation

DNA methylation is introduced into DNA after replication by specific enzymes, called DNA methyltransferases (MTases). All DNA MTases use *S*-adenosyl-L-methionine (AdoMet) as the source of the methyl group being transferred to the DNA bases. The methyl group of AdoMet is bound to a sulphonium atom, which thermodynamically destabilizes the molecule and makes the relatively inert methylthiol of the methionine moiety very reactive towards nucleophilic attack by nitrogen,

oxygen and sulphur atoms or activated C atoms (carbanions).

The reaction mechanism of cytosine-C5 methylation was uncovered for the prokaryotic DNA-(cytosine-C5)-MTase M.HhaI [33, 34]. Prokaryotic and eukaryotic cytosine-C5 MTases share a number of conserved amino acid motifs that have structural roles and are involved in catalysis [review: 35]. A key feature of the catalytic process is a nucleophilic attack of the enzyme on the carbon-6 of the target cytosine. This attack is performed by the thiol group of the cysteine residue in a PCQ motif conserved in the active site of cytosine-C5-MTases (motif IV). The formation of the covalent bond activates the C5 atom towards electrophilic attack and leads to the addition of the methyl group to carbon-5 of the cytosine followed by elimination of the 5-position proton and resolution of the covalent intermediate (fig. 1D). The glutamic acid of the amino acid motif ENV (motif VI) is important to stabilize the DNA-protein complex [review: 10]. This description of the catalytic mechanism is supported by a large body of experimental evidence: the existence of a covalent reaction intermediate between methylated DNA and the active site cysteine has been demonstrated for many DNA MTases [23, 36–40] and has been observed in all structures of DNA-(cytosine-C5)-MTase in complex with DNA known so far [41, 42]. Covalent complex formation has been shown to be dependent on the cysteine residue in the PCQ motif [38, 40, 43]. In addition, the importance of the cysteine residue for catalysis has been demonstrated by site-directed mutagenesis [39, 44, 45].

The first X-ray structure of a DNA-(cytosine-C5)-MTase in complex with DNA also was determined with M.HhaI [41] (fig. 1B). It demonstrated that DNA MTases pull their target base out of the DNA helix prior to methylation, a process called base flipping. After base flipping, the target cytosine is no longer buried in the double helix but is rotated about its flanking sugar-phosphate bonds so 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 [41, 42, 46] and also in many other enzymes interacting with DNA, for example many DNA repair enzymes [reviews: 47, 48]. It brings the target base into close contact to the enzyme, allowing for intricate chemical reactions to occur and for accurate recognition of the flipped base, an important prerequisite for the function of DNA repair glycosylases. The M.HhaI enzyme has been demonstrated to have a structure typical for all enzymes of its family [reviews: 48, 49]. It comprises two domains. The larger, catalytic domain is conserved among all DNA-(cytosine-C5)-MTases. It consists of a central parallel six-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. The smaller domain is involved in the recognition of the target sequence and structurally diverse [review: 10].

DNA methylation in mammals

In prokaryotes, methylation takes place at almost all recognition sites of the MTase. In contrast, mammalian genomes are only methylated at certain CG sites, resulting in a pattern of methylation. This pattern carries epigenetic information. It is significantly conserved between individuals of the same species within corresponding cell types, but it is different in different cell types of the same organism. One can distinguish two types of DNA methylation: Whole regions and domains of DNA (comprising hundreds to several thousands of base pairs) can be extensively methylated (like repetitive DNA). This type of information is designated 'level of DNA methylation' in this review. In addition, each part of the DNA can carry a specific methylation state at each CG site, creating a very complex type of information, called 'pattern of DNA methylation' here. Both types of methylation must be distinguished with respect to their biological consequences (i.e. the readout of the information) and the ways to establish and inherit them.

The analysis of the methylation information is still in its infancy. The Human Epigenome Project (HEP) (http://www.epigenome.org) is a collaboration of public and private institutions trying to identify the methylation pattern of human DNA and catalog variable methylation positions [50]. Initial HEP pilot studies concentrated on the methylation pattern of chromosome 6 within the region of the major histocompatibility complex. This region is highly diverse towards its methylation pattern, and the region is associated with several diseases (fig. 2).

DNA methylation states are clonally inherited and therefore have to be maintained in dividing cells. The pattern of methylated and unmethylated CG sites is transformed into a pattern of hemimethylated and unmethylated sites by DNA replication. Therefore, DNA methylation is required in conjunction with every DNA replication cycle. After replication, the methylation pattern is read and copied onto the daughter strand by a maintenance MTase that has a high specificity for methylation at hemimethylated sites.

DNA methylation as epigenetic signal

The methylation of single or few CG sites can influence the binding of individual transcription factors to the DNA or recruit methyl-C binding proteins that function as tran-

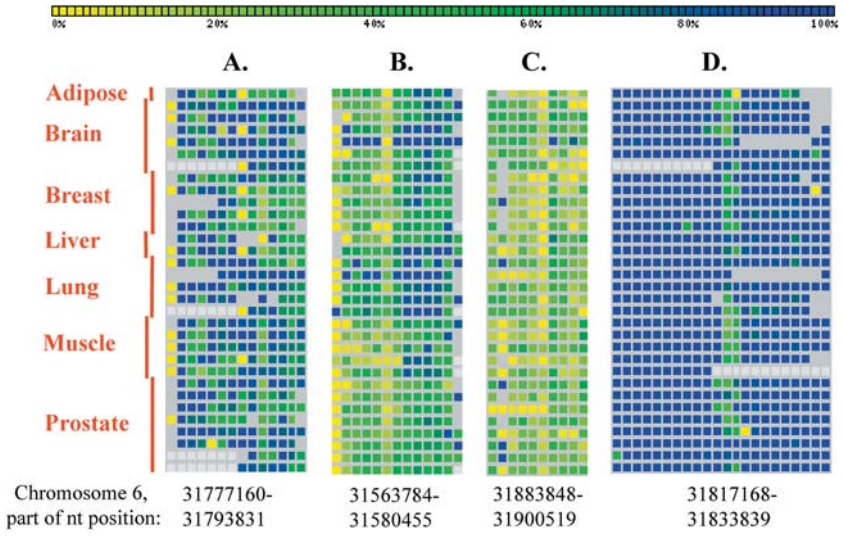

Figure 2. Examples of differentially methylated regions within the region of the major histocompatibility complex. Each square indicates a CG sequence, intervening bases are omitted. The methylation level of each CG site is color-coded, blue showing the highest methylation percentage (all data are taken from www.sanger.ac.uk/PostGenomics/epigenome/). (*A*) a highly diverse methylation pattern, different CG sites are methylated to a different extent; (*B*) a more tissue specific pattern of methylation; (*C*) an example of an almost unmethylated CG island; (*D*) a region with all CGs being almost fully methylated.

scriptional co-repressors and thereby regulate the expression of individual genes [review: 16]. Thereby, the methylation level is connected to the broad organization of the chromatin, with undermethylated DNA usually being part of euchromatin, whereas heavily methylated DNA is part of heterochromatin.

DNA methylation is functionally connected to the modification pattern of histone tails, which can be acetylated, methylated, ubiquitinated or phosphorylated [review: 51]. Whereas unmethylated DNA often is acetylated, methylated DNA often is deacetylated and displays methylation of histone 3 lysine 9 (H3K9 methylation) (fig. 3). DNA methylation and histone tail deacetylation act in a synergistic manner and stimulate each other, conferring a repressive state to the chromatin [52]. In addition, H3K9 methylation triggers de novo methylation of DNA in plants and fungi [53–55]. Conversely, erasure of CG methylation in *Arabidopsis* and human alters the H3K9 methylation [56, 57]. Therefore, DNA methylation, H3K9 methylation and histone deacetylation constitute a self-enforcing epigenetic circuit which sets up a metastable epigenetic switch to control gene expression, changing between an active state (euchromatin) and an inactive state (heterochromatin) of the DNA. Most important, epigenetic gene regulation functions in a heritable but not irreversible manner. In addition, euchromatin and heterochromatin differ by the mechanism and time of replication, with heterochromatic and euchromatic DNA being replicated late and early, respectively [review: 58]. Since euchromatin is active in transcription, whereas heterochromatin is not, this distinction is very important for the regulation of gene expression in metazoan (even in species that do not display DNA methylation). Formation of heterochromatin represents a very efficient and stable tool of gene silencing on a large scale.

Dynamics of DNA methylation

In mammals, genome-wide reprogramming of DNA methylation is known to occur in the germ cells and preimplantation embryos, initiated by an almost complete demethylation of the DNA in the preimplantation embryos [reviews: 12, 59]. This process is important to erase existing epigenetic information and reset the system for a new developmental cycle. Remodelling of sperm chromatin starts immediately after fertilization through replacement of protamines by (acetylated) histones accompanied by genome-wide demethylation which is completed before DNA replication commences [60]. Although this is the best evidence so far for active demethylation in vivo, the mechanisms by which it occurs are still being discussed. The maternally inherited genome is progressively demethylated over several rounds of cell division by a passive mechanism, which results from a lack of maintenance methylation during the early cycles of DNA replication [61]. During this time, methylation declines in housekeeping genes and repeated sequences throughout the genome. Remarkably, imprinted genes and retroviral sequences [62] are largely exempt from this process and maintain their methylation marks. A wave of de novo methylation remethylates the DNA around the time of implantation to different extents in embryonic and extraembryonic lineages [63]. This restoration of methylation is dependant on de novo

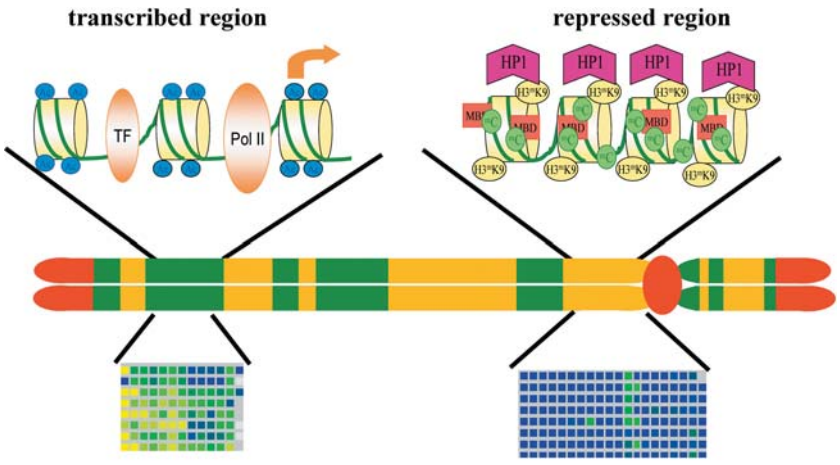

Figure 3. Schematic drawing of the characteristic properties of chromatin in the open and repressed states. A chromosome is schematically divided into green: euchromatic regions (green), constitutive heterochromatic regions (red) and facultative heterochromatic regions (orange). In the upper part of the figure different features of the chromatin states are indicated. In transcribed regions, transcription factors can bind and histone proteins are acetylated; in non-transcribed regions, DNA is methylated, histones are methylated (H3K9 methylation), MBD (methyl-CpG-binding) proteins and HP1 (heterochromatin-binding protein 1) can bind. In the lower part, typical CG methylation patterns are shown (compare fig. 2).

methylation activity. DNA methylation is maintained thereafter in somatic lineages.

In comparison to methylation of the genomes of somatic cells, mature sperm and egg in mammals are highly methylated [59]. During maturation of germ cells, a similar reprogramming cycle occurs: early in the development of both male and female primordial germ cells genome-wide demethylation occurs. Remethylation of the male and female germline takes place later at different stages of gametogenesis. This reprogramming in germ cells is needed for the resetting of imprints and plays a role in the removal of acquired epigenetic modifications.

Mammalian DNA methyltransferases

As a consequence of the dynamic state of DNA methylation, two different methylation processes occur: De novo methylation establishes the methylation state; maintenance methylation copies it onto daughter DNA strands after DNA replication. In mammals, several MTases have been discovered and characterized in the past decade. The first mammalian MTase discovered was Dnmt1, which is highly conserved among eukaryotes [review: 64]. Later, Dnmt2 and the Dnmt3 family of DNA MTases were found. All the known mammalian MTases have a common structure of the catalytic domain which resembles the prokaryotic enzymes and is characterized by the 10 conserved amino acid motifs implicated in the catalytic function. In addition, the Dnmt1 and Dnmt3 enzymes contain a large N-terminal regulatory domain (fig. 4). In the following sections the mammalian DNA MTases will be discussed in more detail. Afterwards, we will return to a discussion about the biological roles of the individual enzymes in the context of the processes described in the last section.

The Dnmt1 enzyme

As already mentioned, Dnmt1 comprises a large N-terminal domain with regulatory function and a smaller C-terminal catalytic domain (fig. 5). The regulatory domain harbours different motifs, like a charge-rich domain that interacts with the Dmap1 transcriptional repressor [65] and contains different start codons; a nuclear localization signal, a PCNA (proliferating cell nuclear antigen) interacting domain [66], a replication foci targeting region [67] and a cysteine-rich Zn^{2+} binding domain of the CXXC type also found in the HRX protein [68, 69]. The zinc domain comprises eight conserved cysteine residues in two CXXCXXC clusters and two isolated cysteines. Since a single CXXCXXC…C domain binds one Zn^{2+} ion [70], the zinc finger in Dnmt1 most likely comprises two of these structures binding two zinc ions. It has been implicated in DNA binding by Dnmt1 [71]. Whereas Dnmt1 is localized at replication foci during S-phase [review: 72], it is actively excluded from the nucleus in fertilized eggs and stored in the cytoplasm [61], leading to so-called passive demethylation of the female genome that occurs because DNA replication is not accompanied by DNA methylation during the first DNA replication cycles of fertilized eggs. One part of the N-terminal domain shows homology to the Polybromo-1 protein from chicken [review: 73]; this domain contains two BAH (Bromo adjacent homology) domains that may be in-

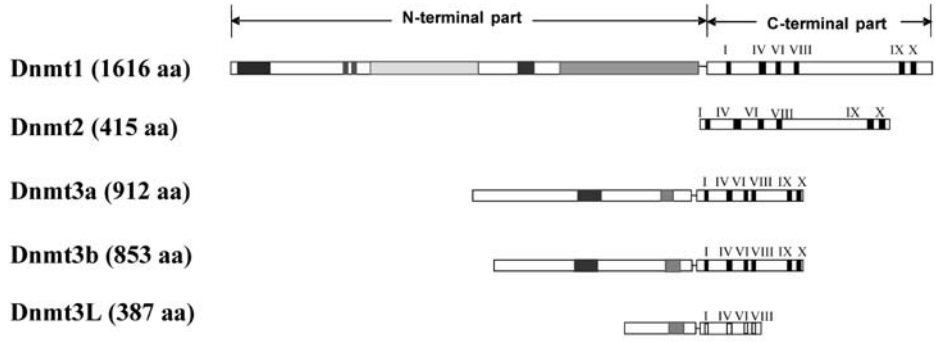

Figure 4. Domain organization of the mammalian Dnmts. The mammalian methyltransferases are divided into an N-terminal regulatory part and a C-terminal catalytic part. The C-terminal part shows strong amino acid sequence homology to prokaryotic DNA-(cytosine-C5)-MTase and contains all the conserved catalytic amino acid motifs (abbreviated by Roman numerals) defined for the prokaryotic enzymes. The boxes shown in the MTase sequence indicate the various domains, structural and sequence motifs identified in these proteins. For details see figs. 5 and 6.

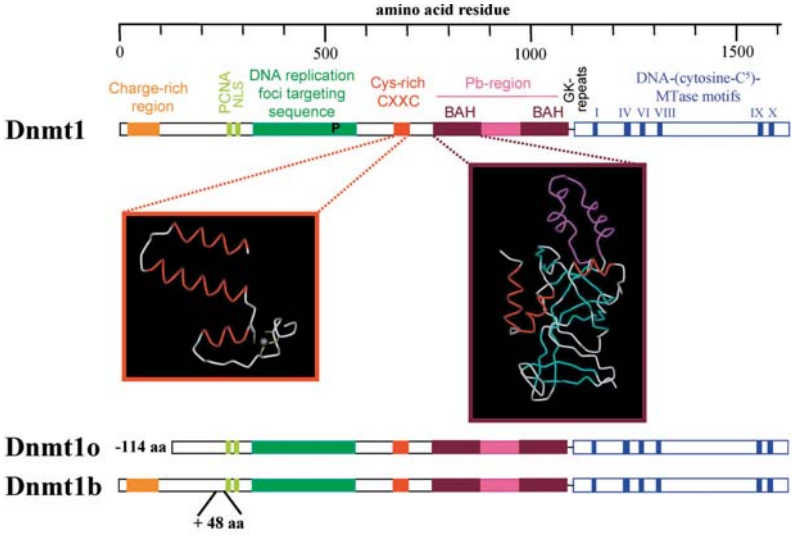

Figure 5. Schematic drawing of the architecture of Dnmt1 (human: 1616 aa) and its splicing isoforms. *Charge-rich region*: contains several translation start points, and the interaction site with DMAP1. *PCNA*: PCNA-interaction site; *NLS*: nuclear localization signal; *P*: major phosphorylation site at Ser 514, *Cys-rich-region*: cysteine-rich zinc binding motif (ATRX type zinc finger); *Pb-region*: polybromo-1 protein homologous region containing two BAH domains; *GK-repeats*: glycine-lysine-repeats. In the central part of the figure the structures of the BAH domain in the N-terminal region of *S. cerevisiae* Orc1p (PDB code: 1m4z) and of a CXXC type zinc finger in the RecQ helicase catalytic core (1OOY) are shown. Two helices and a connecting loop that are not part of the conserved BAH structure are coloured violet. Note that the Cys-rich domain of Dnmt1 contains two complete CXXC motifs and therefore most likely is equivalent to two of the RecQ zinc fingers. The proteins are represented by their Cα-ribbon, α-helical parts are coloured red, β-strands cyan.

volved in protein-protein interaction. The Polybromo-1 protein mediates interactions of different chromatin components. The Dnmt1 polybromo domain is supposed to play a role in the transport of Dnmt1 to the replication foci as well [74]. The BAH domain is found in a number of chromatin-associated proteins. Its structure has been solved for the BAH domain in the N-terminal region of *Saccharomyces cerevisiae* Orc1p protein. The BAH domain has an elongated shape comprising mainly antiparallel β-strands and a small helical domain [75]. In summary, the N-terminal part of Dnmt1 seems to be involved in the intracellular delivery and regulation of catalytic activity of Dnmt1. The essential function of Dnmt1 in the mammalian cell is demonstrated by the observation that mice deficient for Dnmt1 die in midgestation with significantly reduced levels of DNA methylation [31].

Dnmt1 does not function in the cell in an isolated manner, and many interacting proteins have been reported to bind to the N-terminal part of Dnmt1 by yeast two-hybrid and/or biochemical interaction assays. These interactions demonstrate that Dnmt1 is embedded in the cell cycle control, e.g. it shows interaction with the processivity factor of the replication fork, PCNA, and p21WAF1, an inhibitor of cyclin-dependent kinases (CDKs) [66]. Also,

p21WAF1 can interact directly with PCNA, and as such inhibit DNA replication. Inhibition of Dnmt1 has been shown to interfere with DNA replication [76], and the expression of Dnmt1 is tightly coordinated with DNA replication [77]. The N-terminal part of Dnmt1 also interacts with the retinoblastoma (Rb) protein, another cell cycle regulator [78, 79]. It was shown that Dnmt1 interacts with the transcriptional repressor DMAP1 [65] and its inhibitor RGS6 [80]. Dnmt1 can bind Rb to repress transcription of E2F1-responsive promoters as well as bind to the transcription factor E2F1 directly [78]. Since its promoter is regulated by E2F1-Rb as well, this feedback loop could constitute a self-regulatory mechanism to maintain a balanced expression of Dnmt1 [81]. In addition, Dnmt1 directly interacts with histone modifying enzymes such as histone methyltransferase SUV39H1 [82], and histone deacytelase HDAC1 and HDAC2 [65, 78]. Additionally, Dnmt1 can interact with methyl CpG binding proteins such as MBD2, MBD3 [83] and MeCP2 [84] and with the heterochromatin binding protein HP1 [82], which binds to H3K9 methylated chromatin via its chromodomains [85]. All these interactions result in repression of transcription and stabilization of the repressed state of the DNA. Finally, Dnmt1 is reported to interact directly with the de novo methyltransferases Dnmt3a and Dnmt3b [86]. Taken together, these observations draw a complicated network of connections between Dnmt1 and several cellular proteins involved in gene regulation and epigenetic signalling that could mediate methylation-dependent but also some non-methylation-dependent functions of Dnmt1 at different cell stages.

The C-terminal domain of Dnmt1 contains all the conserved motifs characteristic for cytosine-C5-MTases, and harbours the active centre of the enzyme. The only protein reported to interact with Dnmt1 by the C-terminal domain is the chaperone p23 [87]. The C- and N-termini are connected via a lysine-glycine repeat hinge region. Dnmt1 has also been shown to undergo posttranslational modifications such as phosphorylation with unknown biological implications [88].

Dnmt1 has different translational start points [6], and exists in different splice variants [89] (fig. 5). The predominant splicing isoform in somatic cells in human comprises 1616 amino acid residues. A shorter germ-cell-specific form of Dnmt1 called Dnmt1o [90] is found in growing oocytes and during preimplantation development. Dnmt1o lacks the N-terminal 114 amino acid residues and displays an increased stability in vivo against degradation [91]. The intrinsic stability of the Dnmt1o protein allows creating stable ooplasmic stores of Dnmt1o that are available in the nuclei of the eight-cell-stage embryo and maintain methylation patterns on alleles of imprinted genes during the fourth embryonic S-phase [91, 92]. Which form of Dnmt1 is responsible for maintaining the pattern earlier is not clear, but residual Dnmt1 activity could be enough for this task.

Another splice form of Dnmt1 is Dnmt1b, which incorporates in-frame an additional 48 nt between exons 4 and 5 [93, 94]. Since the amount of Dnmt1b protein in somatic cells is only 2–5% the level of the known Dnmt1, and its enzymatic properties are similar, the biological functions of Dnmt1b are not clear at present.

In contrast to the normal tissues, where the CpG islands remain mostly methylation free, it has been observed that inactivation of genes in cancer cell lines is frequently associated with methylation of CpG islands. This altered pattern of methylation is often associated with elevated levels of DNA MTases. It has been shown that an increased DNA MTase activity is associated with the establishment of transformed states in human cells [95], and many reports demonstrate upregulation of Dnmt1 in vivo in several different tumour types [96–99], although this seems not to be a universal phenomenon [100].

Dnmt1 methylates DNA highly specifically at CG sites, with methylation at non-CG sites being almost undetectable in vitro [71]. Dnmt1 has been shown in vitro to prefer hemimethylated over unmethylated DNA 15- to 40-fold [71, 101-103], which is the basis for its function in copying the existing methylation pattern after DNA replication. The structural and mechanistic basis for the specificity of the enzyme for CG sites as well as its preference for hemimethylated DNA is unknown. Dnmt1 methylates hemimethylated DNA in a highly processive reaction [A. Hermann, unpublished results].

Despite strong homology to prokaryotic MTase and the presence of all conserved MTase motifs, the catalytic domain of Dnmt1 alone is not sufficient for the enzymatic activity [71]. Enzyme activity was only observed in the presence of a substantial part of the N-terminal region [104, 105]. Most likely through intramolecular interaction of these domains, a conformational change of the catalytic domain of Dnmt1 into an active conformation is induced. The hypothesis that the protein might have various states is further strengthened by the observation that the pre-existing methylation allosterically activates the catalytic centre of Dnmt1 [71, 106–109]. In the activated form, the preference of the enzyme for hemimethylated DNA drops because of increased activity towards unmethylated DNA. The mechanism of this allosteric activation is unknown; the zinc domain [71] and residues 284–287 [109] have been implicated in the process. The presence of this highly elaborated activation mechanism suggests a role of Dnmt1 in de novo methylation of DNA.

The Dnmt3 family

The Dnmt3 enzymes were identified in mouse and human expressed sequence tag (EST) databases by their homology to the bacterial $5^{m}C$ MTases [7]. The Dnmt3 family consists of Dnmt3a and Dnmt3b, which are highly related to one another but encoded by separate genes. The general architecture of both Dnmt3 enzymes resembles

Dnmt1 with a C-terminal catalytic domain that bears all the MTase motifs (fig. 4). *Baculovirus*- and *E. coli*-expressed Dnmt3a and Dnmt3b proteins were found to methylate CG dinucleotides without preference for hemimethylated DNA [7, 110], thus assigning them a de novo methylation function. The de novo methylation activity of Dnmt3a and 3b was also detected in vivo, using a stable episomal system that employs plasmids as targets for DNA methylation in human cells overexpressing the Dnmt3a or Dnmt3b proteins [111] and after expression of Dnmt3a in transgenic *Drosophila melanogaster* [112]. The targeted disruption of Dnmt3a and 3b in the mouse embryonic stem cells showed that both proteins are essential for mouse development [32]. The Dnmt3a and 3b proteins partially substitute for each other, as indicated by the finding that a Dnmt3a/3b double knockout has a more severe phenotype than each individual deletion [32]. However, both proteins also exhibit specialized roles. Mutations in human Dnmt3b are linked with a syndrome called ICF (immunodeficiency, centromeric instability, facial abnormalities) [32, 113, 114], a rare recessive autosomal disorder characterized by hypomethylation at pericentromeric satellite regions [115]. Since Dnmt3a is not affected in these patients, it cannot replace Dnmt3b in this function.

In vitro studies on the specificity of Dnmt3a and 3b have shown that apart from methylation at CG sites, cytosines in non-CG sites are also significantly methylated [110, 116, 117]. The biological function of this non-CG methylation is not yet known. It is not maintained by Dnmt1 because of its high specificity for CG methylation. Dnmt3a shows preference to methylate sites that are flanked by pyrimidines rather than purines [118]. The catalytic domains of both Dnm3a and 3b have been cloned and were shown to possess catalytic activity [119, 120]. Further, Dnmt3a and 3b catalytic domains were shown to differ mechanistically, because Dnmt3a is distributive but 3b processive in its activity [119]. This difference can be speculated to relate to the function of the enzymes. Dnmt3b is known to methylate the pericentromeric repeats carrying high CG content; a processive reaction mechanism supports methylation of this DNA region in a given, short time window. Dnmt3a cannot replace Dnmt3b in this function, possibly because of its distribution mechanism, which is less efficient in methylating highly CG rich DNA. However, DNA methylation by Dnmt3a can be controlled more accurately because it requires targeting for each methylation event. Evidence suggests that this enzyme is involved in the establishment of methylation patterns at single copy genes [121].

The N-terminal parts of Dnmt3a and 3b contain an ATRX-like Cys-rich domain and a PWWP domain (figs 6, 7). The ATRX domain is also called PHD domain (plant homeodomain). It is an ~50-residue motif found mainly in proteins involved in eukaryotic transcription regulation characterized by a conserved Cys_4-$HisCys_3$ zinc binding motif. The domain folds into an interleaved zinc finger which binds two Zn^{2+} ions. The structure reveals a conserved zinc binding core, together with two variable loops that are likely candidates for interactions with other domains and ligands [122]. The PHD domain of Dnmt3a is sufficient to repress transcription, independent of the methyltransferase activity [123, 124]. The structure of the PWWP domain has been solved for Dnmt3b [125]. The N-terminal half of the PWWP domain resembles a barrel-like five-stranded structure,

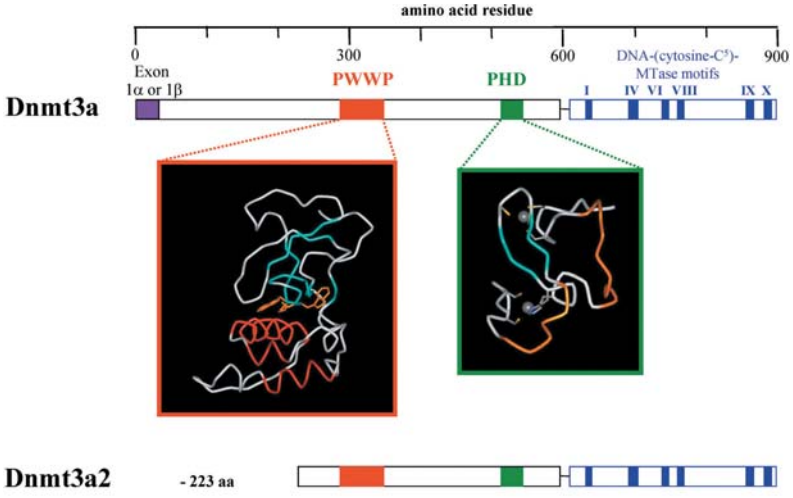

Figure 6. Schematic drawing of the architecture of the Dnmt3a (human 912 aa) protein and its Dnmt3a2 isoform. The central part of the figures shows the structures of PWWP domain of Dnmt3b2 (pdb code: 1KHC) and structure of the PHD zinc-finger domain of the Wstf transcription factor (pdb code: 1F62). In the PWWP domain the conserved proline and tryptophane residues are displayed in orange. The PHD domain contains two loops that are likely to mediate interactions to other proteins (shown in orange). The proteins are represented by their Cα-ribbon, α-helical parts are coloured red, β-strands cyan.

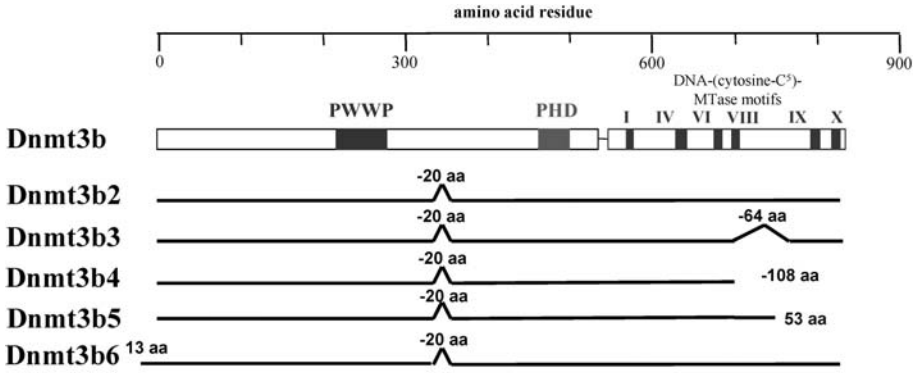

Figure 7. Schematic drawing of the architecture of the human Dnmt3b (853 aa) protein and some of its splicing isoforms. The structures of the PWWP and PHD motifs are shown in figure 6. The different isoforms are discussed in the text.

whereas the C-terminal half contains a five-helix bundle. The two halves are packed against each other to form a single structural module that exhibits a prominent positive electrostatic potential. The PWWP domain of Dnmt3b alone binds DNA in vitro, probably through its basic surface, and Dnmt3b binds DNA stronger than a mutant without the domain. In addition, the PWWP domain seems to target the de novo methyltransferases to chromatin [126].

Like Dnmt1, Dnmt3a and Dnmt3b were shown to exist in different splicing isoforms. There are two known forms of Dnmt3a, which differ in the first 223 amino acid residues [127] (fig. 6). The short form Dnmt3a2 is transcribed from an internal start site under the control of an intronic promoter. It lacks the N-terminal 223 amino acid residues of the full-length protein. Dnmt3a is the major form in adult tissues, where it co-localizes with heterochromatin. In contrast, Dnmt3a2 is the major form during embryogenesis and localized to euchromatin, suggesting a targeting function of the N-terminal part of the long Dnmt3a that is not present in Dnmt3a2. Additionally, there exists alternative use of exon 1 in Dnmt3a transcripts [128]. It seems that exon 1β is used in embryonic stem cells, whereas exon 1α is used somatic cells. Several different *dnmt3b* transcripts, resulting from alternative splicing of exons 10, 21 and/or 22, have been reported which are expressed at different levels during development [7, 113, 129, 130] (fig. 7). Dnmt3b1 and Dnmt3b2 are enzymatically active in DNA methyltransferase assays, whereas Dnmt3b3, which lacks parts of motif IX, appears to be inactive [7, 131]. Dnmt3b4 and Dnmt3b5 encode truncated proteins that lack motifs IX and X and are presumably inactive as well [113, 130]. They probably play roles that are independent of methylation or as positive and negative regulators of DNA methylation. Although the enzymes are inactive, their targeting to pericentromeric regions is not influenced [132].

Dnmt3a and Dnmt3b have been shown to undergo post-translational modifications such as sumoylation [133, 134] and to interact with a number of proteins. The Dnmt3 enzymes have also been shown to interact with a number of proteins. Dnmt3a interacts with the H3K9 methyltransferase Suv39 [82] and Dnmt3a, and Dnmt3b interacts with Dnmt1 [86]. In addition, both Dnmt3 enzymes interact with HDACs [124]. Dnmt3a acts as a co-repressor for RP58 in a manner that does not require its de novo methyltransferase activity [124] and is mediated by recruitment of HDACs to the DNA.

Dnmt3b is overexpressed in various tumors, while the expression level of Dnmt3a was only modestly increased in identical tumors [32, 130], showing that Dnmt3b plays an important role in tumorigenesis. This finding agrees with the mechanistic difference between both enzymes, which leads to a higher intrinsic methylation activity for Dnmt3b than for 3a.

The third member of the Dnmt3 family, Dnmt3L, shows clear homology to the Dnmt3a and 3b enzymes [135] (fig. 4). Its N-terminal part comprises just the PHD domain; the C-terminal part extends to the conserved motif VIII. Strikingly, key catalytic residues are deleted or mutated in almost all of the conserved motifs, suggesting that Dnmt3L folds like a DNA MTase but cannot have any catalytic activity. In co-transfection experiments, Dnmt3L has been shown to stimulate DNA methylation by Dnmt3a in human 293/EBNA cells [136]. Stimulation of Dnmt3b was not detectable in these experiments. Co-purification studies revealed an interaction of Dnmt3L with Dnmt3a and Dnmt3b [121], as well as interaction with histone deacetylase [137]. Dnmt3L is expressed during gametogenesis and embryonic stages [121, 138], showing an expression pattern similar to the Dnmt3a and 3b enzymes.

Dnmt2

The *dnmt2* gene is conserved among eukaryotes both in the organisms that show methylation and those that lack detectable methylation, such as *Schizosaccharomyces pombe*. Like bacterial MTases, Dnmt2 lacks the large

N-terminal regulatory domain (fig. 4). The structure of Dnmt2 has been solved in complex with *S*-adenosyl-L-homocysteine, demonstrating a canonical DNA MTase structure with high similarity to prokaryotic DNA-(cytosine-C5)-MTases [139] (fig. 1C). In contrast, the structure of the smaller domain differs from that of known MTases. However, these domains also diverge in bacterial MTases, presumably because they are involved in the specific binding of the target DNA. Dnmt2 was identified in 1998, but initially no DNA MTase activity was detected [8, 9, 140].

Dnmt2 messenger RNA (mRNA) is ubiquitously expressed at low levels in most human and mouse adult tissues and in mouse embryonic stem cells [140]. A higher expression level can be detected in cancer cell lines [8, 9, 140]. The murine embryonic stem cells homozygous for a Dnmt2 null mutation are viable and display normal levels of methylation at endogenous sequences [140]. A weak but reproducible methyltransferase activity was recently demonstrated for the recombinant Dnmt2 protein in vitro [141–143] and in vivo [144]. The specificity of the enzyme is not yet known; evidence suggests preferences for CG [141] or CT and CA sites [143]. The weakness of the activity observed in vitro could be due to the absence of an important posttranslational modification and/or interacting proteins, or even to a high specificity of this protein for special DNA targets. The biological function of this enzyme is not yet known.

Regulation of DNA methylation in mammals

Given its fundamental importance, DNA methylation is tightly regulated in mammals. In particular, unregulated de novo methylation activity which would destroy the methylation pattern of the DNA is not present during any stage of the cellular and developmental cycle. Part of this regulation is due to the strictly controlled expression of MTases. True de novo MTases are expressed at very low levels in differentiated tissues. In addition, despite the sequence similarity and the occurrence of all characteristic MTase motifs, the catalytic domains of mammalian DNA MTases display one very obvious difference to the prokaryotic enzymes: they all have only very low catalytic activity in the absence of stimulatory proteins. The catalytic domain of Dnmt1 is inactive without the N-terminal part (which can be considered a stimulating protein in this respect), and Dnmt2 displays very weak methylation activity. Potential activators of Dnmt2 remain to be identified. The catalytic domains of Dnmt3 enzymes are active in isolated form, but their activity is low, and mutational studies have suggested that it does not make use of the full repertoire of catalytic residues [120] because a Dnmt3a variant in which the catalytic cysteine residue was replaced by alanine displayed only an about fivefold decrease in activity, whereas similar exchanges in prokaryotic MTases usually inactivate the enzyme.

Therefore, the catalytic domains of Dnmt3a and 3b might be activated by a conformational change that positions all the catalytic residues for efficient DNA methylation. If such a conformational change of the active site of the Dnmt3a would allow for a full contribution of the cysteine to catalysis, the enzyme could be activated by several orders of magnitude. By this mechanism, stimulatory proteins could control the activity of Dnmt3a and 3b. With Dnmt3L, one stimulator of the Dnmt3 MTases has already been identified; others may remain to be discovered.

The dependence of the MTases on interaction with stimulatory proteins could strongly increase the efficiency of delivering the MTase activity to target regions. If, for example, one assumes that an MTase is directed with 90% efficiency to a DNA target, the methylation accuracy will also be in that range. This means about 1 in 10 methyl groups introduced in the DNA will be at a wrong place. However, if the enzyme is only active in the presence of a stimulator that is also targeted with an efficiency of 90%, the overall accuracy will be increased to 99%, meaning that only 1 in 100 methyl groups will be at a wrong place. By a similar mechanism, the efficiency of transcriptional control of the activity of DNA MTases present in the cell could be amplified if the MTase requires the presence of a stimulator protein for high catalytic activity.

Biological functions and cooperative roles of mammalian Dnmts

With the discovery of Dnmt3 family of MTases, it apparently seemed that these proteins would exclusively perform the function of de novo DNA methylation, and Dnmt1 would maintain the methylation information at every cell division. But these definitions proved to be too simplistic. On the one hand, Dnmt1 is known to possess *de novo* methylation activity [review: 64], and it is stimulated by methylated CGs in cis which can lead to de novo spreading of methylation [71, 107, 108, 145]. De novo methylation takes place in somatic cells where the expression of Dnmt3a and Dnmt3b is low, also suggesting a role of Dnmt1 in this process. On the other hand, Dnmt3a and 3b have been shown to be required for maintaining methylation at specific sequences [32, 146, 147]. In addition, it has been shown that methylation levels can be maintained in cell lines in the absence of Dnmt1 [148, 149]. These observations lead to the questions: Why is Dnmt1 required for de novo methylation, and How can the Dnmt3 enzymes provide help in maintenance methylation?

To answer these questions, one has to consider that the genome can be roughly divided into two parts: heterochromatic DNA with a very high methylation level and euchromatic DNA, which carries a detailed pattern of

methylation (fig. 3). Therefore, one has not only to distinguish between de novo and maintenance methylation but also to take into account that both these processes occur on heterochromatic and euchromatic DNA.

Maintenance of methylation patterns

There is solid evidence that methylation, like replication of the genome, occurs in at least two phases: euchromatin is replicated early and heterochromatin is replicated late [review: 58]. In fact, gene knockouts in ES cells show that Dnmt1 can maintain the methylation pattern in eukaryotic regions without help from de novo methyltransferases, whereas they are required for maintenance of methylation at heterochromatic regions [146]. As described above, euchromatin carries a detailed pattern of DNA methylation. Given its preference for hemimethylated CG sites, Dnmt1 is the only enzyme in the cell that is able to copy this pattern. The interaction of Dnmt1 with PCNA and its localization at replication foci suggests that Dnmt1 might be a part of the replication fork during the replication of euchromatic DNA. Due to its high processivity, the enzyme could methylate the DNA immediately after replication, which would explain the short time gap between DNA replication and methylation [67, 77, 150]. Since the density of DNA methylation is relatively low within these regions, Dnmt1 is not allosterically activated at this time.

Since Dnmt3a and Dnmt3b cannot differentiate between unmethylated and hemimethylated CG sites, they obviously cannot copy a specific pattern of methylation. Therefore, it is not conceivable that Dnmt3 enzymes could contribute to the maintenance of methylation patterns. In addition, it is questionable whether changes in the histone modification state could contribute to encoding a pattern of DNA methylation that is defined at the level of individual base pairs. The reason for this is that the modification state of the histone tails defined for a whole nucleosome comprising 146 bps of DNA, such that one would expect the histone tail modification should influence the epigenetic state of the DNA in units of 146 (or more) bp. It would be difficult to rationalize how the modification state of histone tails could specifically determine the methylation state of individual CG sites.

Preservation of methylation levels

In contrast to euchromatin, the heterochromatic part of the genome does not carry a detailed pattern of methylation but it is methylated by default. Therefore, preservation of the level of methylation in these regions does not depend on the ability to read a pattern of CG methylation, but just requires presence of an active enzyme. In addition to being heavily methylated, heterochromatic DNA contains other epigenetic marks as well, such as H3K9 methylation or deacetylated histones. Moreover it is replicated late. Given these differences, methylation of heterochromatic DNA could be induced by the histone modification pattern of the DNA (such as H3K9 methylation) or by a default methylation mechanism of late replicating DNA. Dnmt3a and Dnmt3b could contribute to the methylation activity in the cell during the late replication phase and in part replace Dnmt1. In addition, it is possible that the preference of Dnmt1 for hemimethylated DNA contributes to the process. Evidence suggests that all three mechanisms are followed, because both Dnmt3 and Dnmt1 are required at this stage, and it has been documented that H3K9 methylation can induce methylation of heterochromatic DNA [55].

The connection of DNA methylation to other epigenetic processes could be mediated by the interaction of Dnmt3 enzymes with HDACs [124]. In addition interaction with HP1, which itself binds K9 methylated histone 3 tails, has been reported for Dnmt1 and Dnmt3a [82]. Cooperation of Dnmt1, Dnmt3a and Dnmt3b could be mediated by the mutual interaction of the proteins [86, 151].

This complicated network of interacting proteins for the methylation of heterochromatic DNA could be required because it is essential to establish the methylation of these regions as soon as possible after DNA replication owing to the importance of methylation in silencing transposable elements located in the heterochromatic part of the genome. It is conceivable that the activity of Dnmt1 alone is not sufficient to guarantee the high methylation level of these sequences, which sometimes contain high levels of CG sites, since repetitive heterochromatic DNA is often CG rich. For example, the pericentromeric satellite 2 repeats known to be methylated by Dnmt3b [32, 152] have a observed/expected CG density of >0.6 [153, 154], whereas it is around 0.25 in euchromatin. Dnmt1 might be released from the replication fork at the phase of heterochromatin replication, such that methylation has to be restored in heterochromatic regions 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 [67, 146, 150]. This model could also explain the role of allosteric activation of Dnmt1, which could activate the enzyme to ensure the complete methylation of the highly methylated parts of the genome. By similar means methylation at heterochromatic DNA could be established during embryogenesis. In this sense 'maintainance' methylation (defined here as the methylation of a hemimethylated CG site) and 'de novo' methylation (defined here as the methylation of an unmethylated CG site) of heterochromatic DNA is mechanistically very similar.

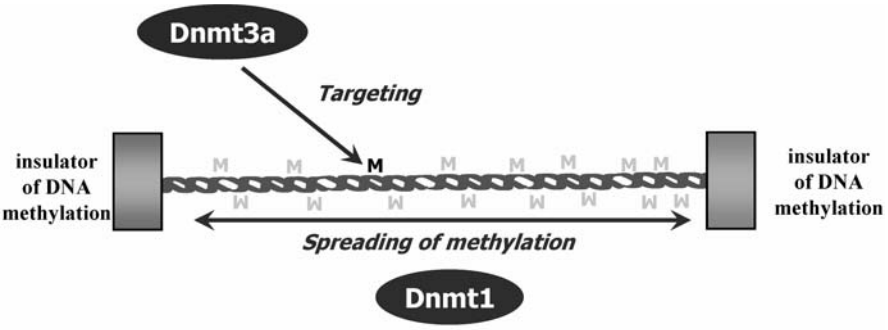

Figure 8. Model of the functional cooperation of Dnmt1 and Dnmt3 or Dnmt3b in de novo methylation of DNA (modified after: [10]). After delivery of one (or a few) methyl groups to the DNA by a *de novo* MTase, Dnmt1 becomes activated and spreads the methylation over the whole domain of the DNA.

Generation of methylation patterns by de novo methylation

The last question is to explain how complex patterns of methylation are set up during de novo methylation. Although the process is not fully understood, evidence suggests that during embryogenesis a global de novo methylation of the genome occurs, with certain parts of the DNA being protected from methylation by tightly bound transcription factors or other proteins [155]. Thereby, the methylation pattern of the DNA established at that time reflects the pattern of gene transcription in embryonic cells. During embryogenesis Dnmt3a and 3B are heavily expressed and could perform the function of global remethylation of the DNA. Dnmt1 could provide help in de novo methylation by means of its cis spreading activity. This model explains why many housekeeping genes are accompanied by CG islands which could be considered a footprint of transcription factors tightly bound to the promoters of these essential genes. In a similar way, imprints are established by active protection of DNA regions from de novo methylation. In agreement to this model, Dnmt3a and Dnmt3L are essential for establishment of the imprinted regions in oocytes [62, 121]. The CTCF protein that is involved in reading the imprint also has a role in this step, because CTCF binding maintains the hypomethylated state of differentially methylated domain in the H19/Igf2 locus during oogenesis [156].

Another mechanism to generate complex methylation patterns could be targeting of the DNA MTases to certain parts of the genome or targeted demethylation of DNA regions. Since the process of DNA demethylation is rather enigmatic, here we focus on the targeted methylation approach. Targeted methylation of DNA could be initiated by de novo MTases directed to the target region. Once some CG sites are modified on a DNA domain, Dnmt1 will be activated and tend to spread the methylation over the whole domain (fig. 8). In this model, the de novo enzymes are just required to mark a DNA domain for methylation by Dnmt1. Targeting of MTases could be achieved by interaction of MTases with transcriptional repressors that could lead to preferential binding of MTases to repressed parts of the chromatin. Thereby transient repression could induce DNA methylation, which would render the repressive state more stable. In this model, DNA methylation could memorize the transcriptional state of a gene [157]. Methylation could have a similar function in the brain, which is a fascinating hypothesis that still waits systematic experimental testing. The accuracy of the process could be elevated by MTase activator proteins that are also targeted, which might explain the role of Dnmt3L in the methylation of single copy genes.

Outlook

DNA methylation in mammals is far from being understood. The targeting and regulation of the DNA MTases in the cell that results in the generation of defined patterns of DNA methylation is mysterious. The functions of the several splicing isoforms of mammalian MTases are unclear, and we are just beginning to explore the effects of posttranslational modification of the Dnmts. In addition to Dnmt3L, more stimulators and potentially inhibitors of Dnmts are to be discovered. Finally, the enzymology of DNA demethylation is still in its infancy.

Finding answers to these questions is even more pressing because DNA methylation is a very important process implicated in normal functions such as development, brain function and preservation of genomic integrity and diseases like cancer. In addition, epigenetic differences might contribute to the etiology of many multifactorial diseases. The direct impact of problems with methylation reprogramming is seen in cloning. Somatic cloning has been inefficient in all species, with high abortion and fetal mortality rates. These developmental defects have been attributed to incomplete reprogramming of the somatic nuclei by the cloning process [12, 158]. Finally, directed methylation and demethylation of DNA could be a valuable tool to manipulate gene expression for therapeutic purposes.

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