Biological aspects of cytosine methylation in eukaryotic cells

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Abstract. The existence in eukaryotes of a fifth base, 5-methylcytosine, and of tissue-specific methylation patterns have been known for many years, but except for a general association with inactive genes and chromatin the exact function of this DNA modification has remained elusive. The different hypotheses regarding the role of DNA methylation in regulation of gene expression, chromatin structure, development, and diseases, including cancer are summarized, and the experimental evidence for them is discussed. Structural and functional properties of the eukaryotic DNA cytosine methyltransferase are also reviewed.

Key words. 5-methylcytosine; DNA methylation; CpG islands; chromatin structure; protein-DNA interactions; X-chromosome inactivation; demethylation; genomic imprinting; DNA cytosine-5 methyltransferase.

List of abbreviations. Ac, activator; CRE, cAMP-responsive element; CREB protein, cAMP-responsive element binding protein; EC cells, embryocarcinoma cells; HIV, human immunodeficiency virus; IGF-II, insulin-like growth factor II; LTR, long terminal repeat; 5-mC, 5-methyl cytosine; MDBP, methylated DNA-binding protein; MeCP, methyl-CpG binding protein; MEL cells, murine erythroleukemia cells; NHP-1, non-histone protein-1; SDR, sequence-specificity determining region; TF, transcription factor.

Introduction

Only a subset of the about 50,000–100,000 genes of a vertebrate genome determine the phenotype of any cell of the organism. The expression of developmental stage or tissue-specific groups of genes is the result of a complex regulatory network acting on the different levels such as chromatin structure, transcription initiation, RNA splicing, RNA stability, translation initiation, and protein stability. It has been shown for many genes that hypomethylation of the promoter region is necessary for transcription[36,40,87,157], and methylation of the nucleotide cytosine in the dinucleotide 5′-CpG-3′ (fig. 1) has therefore been connected with the first two levels of regulation. CpG dinucleotides[76] (and CpXpG trinucleotides in higher plants[77]) are the preferential sites of this cytosine methylation. The maintenance after DNA replication of an initially established methylation pattern by methylation of the hemimethylated CpG dinucleotides provides a heritable signal, for instance, the activity or inactivity of a transcription factor binding site (fig. 1 B). In this case, a change in methylation could result in a change in transcription factor binding and transcriptional activity.

Although the biological significance of DNA methylation is still not entirely clear, it must be an important one, because otherwise the highly unstable base 5-methylcytosine (5-mC) would have vanished due to natural selection. 5-mC is readily converted to thymine by oxidative deamination[46] (fig. 1 A). A sophisticated DNA repair apparatus has evolved in mammals which catalyzes the selective repair of the T-G mismatches (resulting from 5-mC deamination) back to C-G pairs[208,209]. Nevertheless, these C to T transitions cause many gene mutations in humans with often deleterious consequences, such as hereditary diseases and cancer[73,165]. Probably due to the deamination of 5-mC to thymine, CpG dinucleotides are underrepresented in the DNA of higher eukaryotes, while the resulting dinucleotides 5′-TpG-3′ and 5′-CpA-3′ are overrepresented, when compared to the expected frequency calculated from the frequency of individual nucleotides[24].

A number of experiments have been reported in support of two prevailing hypotheses concerning the function of DNA methylation: The first assumes that DNA methylation is mainly important to perpetuate the structure of inactive chromatin, and that changes of DNA methylation are the result of developmental processes initiated by other signals[25]. The second hypothesis proposes that changes of DNA methylation are initial and decisive steps in developmental processes, including ontogenesis[41], senescence[86] and carcinogenesis[59]. While the first hypothesis concentrates on the static aspects of genome organization, the second hypothesis emphasizes the importance of the changes in genome methylation. In particular, it is not clear if demethylation of promoter DNA is the cause or the effect of gene activation, and if methylation of regulatory DNA sequences is a primary regulatory signal, or if it is 'invited' by preceding inactivation. This review focuses on the argument that both aspects of DNA methylation have evolved in parallel and are essentially two sides of the same coin.

The percentage of methylated cytosines varies widely between eukaryotes (table 1). The 'fifth base' 5-mC (fig. 1 A) is absent from the genomes of several eukaryotes with small genomes, like *Drosophila melanogaster*[197], and *Saccharomyces cerevisiae*[154]. While the degree of cytosine methylation in a genome is determined by nearest neighbor analysis or high performance liquid chromatography, the pattern of CpG methylation has commonly been analyzed by the cleavage of DNA using the pair of restriction endonucleases HpaII and MspI.

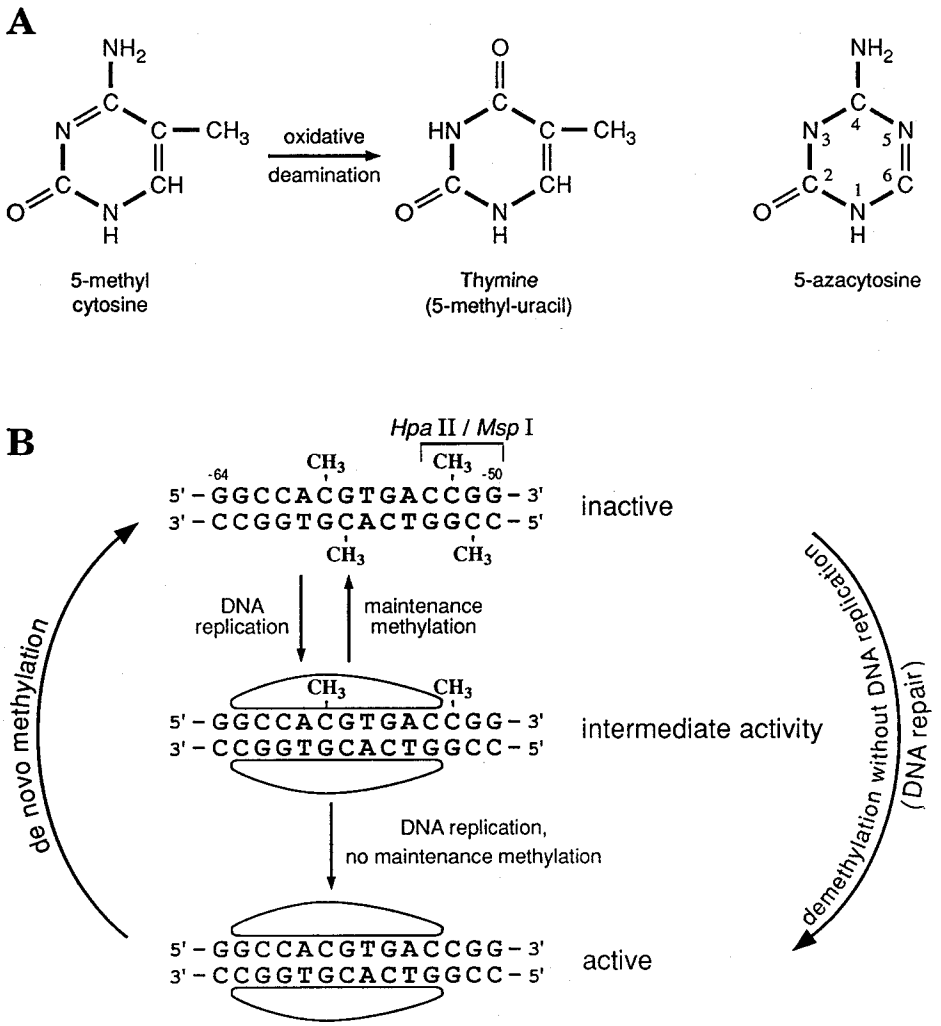

Figure 1. (*a*) Structure of 5-methylcytosine (5-mC), thymine, which arises by deamination of 5-mC, and of the DNA methylase inhibitor 5-azacytosine. (*b*) Reactions of methylated DNA using the binding site of the major late transcription factor/upstream regulatory factor (MLTF/USF) from the major late promoter of adenovirus as an exemplary sequence[205]. It also contains an HpaII/MspI recognition site 5'-CCGG-3', which is used to test the methylation status of a subset of 5'-CpG-3' dinucleotides (see text).

Table 1. Genome size and degree of methylation. Genome size data are from[116, 134, 161].

Species name	Genome size (haploid, bp)	*mdC*100* mdC+dC	Reference
Saccharomyces cerevisiae	$2.20*10^7$	< 0.05	154
Neurospora crassa (stationary)	$1.50*10^7$	1.5	167
Aspergillus nidulans	$4.00*10^7$	< 0.05	5
Drosophila melanogaster	$1.10*10^8$	< 0.05	197
Paracentrotus sp.	$6.40*10^8$	6.6	43
Xenopus laevis	$2.20*10^{10}$	6.9	47
Gallus domesticus (liver)	$2.30*10^9$	3.7	3
Mus musculus (liver)	$2.30*10^9$	4.6	62
Rabbit (liver)	$2.50*10^9$	4.2	199
Homo sapiens (liver)	$2.80*10^9$	4.2	56
Triticum aestivum	$1.65*10^{11}$	25.5	140
Hordeum vulgare	$1.16*10^{11}$	22.6	140

MspI, an isoschizomer of HpaII, cleaves DNA irrespective of a methyl group present at the internal C residue of its recognition site CCGG, whereas HpaII cannot cleave methylated or hemimethylated DNA[201] (fig. 1 B). A number of isoschizomeric restriction enzymes with different sensitivity to 5-mC have since been used for the study of DNA methylation[117]. Since 1984, the technique of genomic sequencing has made possible the determination of the methylation status of every cytosine in the investigated sequence without cloning of the DNA[44]. Cloning, i.e. propagation in *E. coli* would eliminate the methylation specific for eukaryotic DNA.

Because there seems to be a rough correlation between genome size and genome methylation (table 1), it has been argued that the major role of DNA methylation in eukaryotes is the inactivation of non-transcribed DNA and, thereby, the compartmentalization of a large genome. This would reduce the amount of DNA sequence that can interact in a non-specific manner with DNA-binding regulatory proteins[25]. In invertebrates such as fungi[166], sea urchins and insects[26, 27], only repetitive (satellite) sequences seem to be methylated. In many fungi, DNA methylation is a mechanism for the

specific inactivation of repetitive sequences [177]. In vertebrate DNA, too, a high percentage of all 5-mC is situated in satellite DNA, which interestingly enough is not CpG-depleted [63, 135]. Most importantly, DNA methylation is also found in non-repetitive vertebrate DNA. From an evolutionary perspective, cytosine methylation has evolved from a component restricted to repetitive DNA into a component of non-repetitive DNA in vertebrates and vascular plants, and during this evolutionary process 5-mC has probably become associated with the different biological processes discussed below.

CpG islands

Many genes contain DNA segments of 0.5 to 3 kilobases with a high G + C content and a high frequency of CpG dinucleotides relative to the frequency in the whole genome, which are referred to as CpG islands [25]. Typically CpG islands contain the promoter/mRNA leader region of genes, especially of ubiquitously expressed genes (housekeeping genes) or genes which are potentially active in many tissues, e.g. the protooncogenes c-fos, c-int-1, and c-myc. CpG islands are unmethylated [25, 28, 65], and this observation has been confirmed by genomic sequencing of CpG islands [128, 136, 148, 149]. Unmethylated CpG islands have been found also in the genomes of higher plants [6, 136]. Because these sequences never became methylated and therefore never were subjected to the deamination of 5-mC, they escaped CpG depletion. As shown for a human α-globin pseudogene [29], a CpG island of a pseudogene becomes methylated and rapidly loses most CpG sites. The CpG depletion of a maize glyceraldehyde-3-phosphate dehydrogenase pseudogene CpG island was found to occur over a period of about one million years [155].

Most CpG islands are contained in GC-rich chromosome bands of several hundred thousand basepairs in length, which have been named isochores and which are characteristic for genomes of vertebrates and higher plants [17]. Only in these taxa have CpG islands been identified. It has been speculated that GC-poor isochores are the result of CpG-depletion in the methylated domains of invertebrate genomes, while the unmethylated domains containing most of the single-copy DNA gave rise to the GC-rich isochores, which constitute only 3–5% of the genome [17]. CpG islands were probably selected for, when cytosine methylation was no longer confined to repetitive DNA, because the permanent activity of genes would have been impaired by methylation of the regulatory regions [25]. Interestingly, the ubiquitous transcription factor Sp1, which binds to almost all CpG-rich promoters, has up to now only been identified in vertebrate cells [129]. This suggests a parallel evolution of CpG islands and a transcription factor involved in constitutive transcriptional activity. As binding of Sp1 is not affected by methylation of its binding site, an Sp1 binding site would be able to potentiate transcription regardless of its methylation status [80, 85]. A number of other regulatory factors specific for CpG-rich promoters have been characterized [101, 102, 124]. It would be very interesting to know if these proteins are also able to bind to their target sites regardless of the methylation status of the DNA, and if they are also restricted to organisms containing CpG islands.

While CpG islands are kept essentially unmethylated in all cells, there is a prominent exception: On the inactive X-chromosome of mammalian (eutherian) females most of the promoters are kept in an inactive state by means of CpG methylation [72]. The recent analysis of the human X-linked housekeeping gene phosphoglycerate kinase I by genomic sequencing showed that every cytosine of all CpG dinucleotides is methylated on inactive X-chromosomes, while no 5-mC was found in the same sequences on active X-chromosomes [148, 149]. The methylation of CpG islands on inactive X-chromosomes has important medical implications with regard to the fragile X-chromosome syndrome. This frequent (one in 1500 newborn males) heritable form of mental retardation is associated with a specific break on the X-chromosome, and it was recently found that a CpG island very near to the breakage site is methylated in most, if not all, affected people [14, 81, 200]. This methylation most probably results from an insufficient reactivation of the inactive, mutated chromosome in the transmitting mother, but the causative mutation and its function are unknown.

Methylation of CpG islands has also been observed in cultured cells, where it is associated with transcriptional silencing of the corresponding gene. This results in a loss-of-function phenotype [8, 98]. These loss-of-function mutations are referred to as epimutations and can be reverted by treatment with inhibitors of the cellular maintenance methylase such as 5-azacytidine (fig. 1 A), leading to demethylation of the inactivated CpG islands [86]. Because the same CpG islands are never methylated in the DNA from animals or primary cells, cultured cell lines are not always a reliable model system for the investigation of DNA methylation.

The principal question concerning CpG islands is how the islands are kept unmethylated. CpG islands of genes introduced into transgenic mice (transgenes) remain also unmethylated [108] unless the transgene is present in high copy numbers, in which case the mechanism(s) responsible for the unmethylated state of CpG islands seem to be disturbed [126]. The binding of abundant transcription factors, e.g. of Sp1 factor, could prevent the access of methylase and thus DNA methylation [25, 85]. This hypothesis suggests that non-transcribed DNA is not bound by transcription factors and 'invites' methylation [25]. Alternatively, CpG-rich DNA may be a poor substrate for DNA methylase, for which there is indeed evidence [38]. However, CpG-rich satellite DNA becomes fully methylated [120]. Recent experiments provide evidence for a third explanation, namely an active demethylation of methylated CpG-islands in embryonic

cells[61, 193]. This hypothesis suggests that components of the cell recognize CpG islands as a different entity. The undermethylation of CpG-rich satellite sequences in germline cells points in a similar direction, namely that CpG-rich sequences are subjected to developmental controlled methylation-demethylation cycles[171, 218]. This would also explain how CpG-rich satellite DNA has escaped CpG depletion. To what extent the different mechanisms suggested contribute to the unmethylated status of CpG islands is unknown.

Chromatin structure and DNA methylation

There could be more than one mechanism by which CpG methylation causes gene inactivation: promoter inactivation can be the result of cooperativity of several 5-mC nucleotides[33, 133], or of the methylation of one CpG dinucleotide[92] (table 2). The first hypothesis, which suggests that methylated DNA is accompanied by a change in chromatin structure, is supported by several experiments:

1) Methylated reporter genes were transcriptionally active when injected into *Xenopus* oocytes, but transcription decreased and stopped after several hours. When in vitro reconstituted chromatin was injected into *Xenopus* oocytes, it was always inactive[35]. Similar results have been found for hemimethylated DNA[51].

2) Methylated DNA was shown to have a DNaseI-insensitive chromatin structure characteristic for 'closed', inactive chromatin[7, 106]. In addition, methylated DNA (e.g., the inactive X-chromosome and repetitive DNA) is replicated late during the S phase of the cell cycle, while active, non-methylated chromatin or DNA experimentally demethylated by treatment of cells with 5-azacytidine is replicated early in the S phase[94, 176].

3) At least 80% of the methylated cytosines are found in chromatin containing histone H1, which is a component of non-transcribed chromatin[9]. Conversely, chromatin containing unmethylated DNA is devoid of histone H1 and contains hyperacetylated histones, which are also characteristic features of transcribed chromatin[194].

4) In experiments with the γ-globin gene, in vitro methylation of the promotor region, rather than the coding sequence, abolished gene activity after transfection of the methylated construct into cells[36]. But none of the six CpG sites in the 302 bp surrounding the transcription

Table 2. Cytosine methylation and protein-DNA interactions.

Organism	Protein (function) (promoter/enhancer)	Binding site	Reference
DNA binding reduced by cytosine methylation			
Mammals			
	E2F (TF)	TTTCGCGC (adenovirus E2A promoter)	109
	Activating protein 2 (TF)	CCCGCCGGC (preproenkephalin promoter)	45, 82
	cAMP responsive element binding protein (TF)	(T/G)(T/A)CGTCA (Somatostatin promoter)	92
	Aromatic hydrocarbon receptor (TF)	TNGCGTG (cytochrome P-450Ia1 promoter)	178
	Major late transcription factor/upstream stimulatory *f*actor (TF)	GGCCACGTGACC (major late promoter of adenovirus)	205
	Enhancer binding protein-80 (TF)	GCGCATATGCCGA (promoter of intracisternal particle A)	58
	Tyrosine amino transferase factor (TF?)	TGCACATGCGCAGAG	12
	c-Myc (TF)	GGCCACGTGACC	153
Tobacco			
	CG-1 (TF)	CACGTG (chalcone synthase promoter)	186
Maize			
	Transposase (?) encoded by the maize transposon *Activator* (binding to one hemimethylated form is enhanced)	AAACGG	110
	Transposase (?) encoded by the maize transposon Enhancer	CCGACATCTTA	69
DNA binding enhanced by cytosine methylation (only mammalian proteins analyzed)			
	Methylated DNA-binding protein (MDBP)	RTm^{5}YRYYAm^{5}YRGm^{5}YRAY (R, A or T; m^{5}Y, 5-mC or T; Y, C or T)	89, 218
	Methyl-CpG binding protein (MeCP)	methylated, CpG-rich DNA	125
	Methylated DNA-binding protein 2 (MDBP-2)	TTCACCTTmCGCTATGAGG-GGGATCATACTGG	142
DNA binding not affected by cytosine methylation			
	Sp1 (TF)	GGCGGG	80, 85

start site is solely responsible for repression of transcription by 5-mC, rather it seems to be a cooperativity effect between these six methylated cytosines. Only removal of all six cytosines contained in CpG dinucleotides by site-directed mutagenesis restored transcription [133].

5) DNA demethylated by 5-azacytidine treatment is more efficiently repaired after mutagenesis, probably because DNA repair enzymes have better access to demethylated and therefore more open chromatin [84].

A biochemical basis for the observations summarized under (i)–(v) is most likely provided by proteins binding specifically to methylated DNA (*m*ethylated *D*NA *b*inding *p*roteins, MDBPs [89], or *m*ethylated *C*pG binding *p*roteins, MeCPs [125]), which have been characterized by several groups [89, 125, 142, 219]. It has been suggested that by binding to 5-mC MeCPs play a crucial role for the structure of 'closed', transcriptionally inactive, DNase-resistant chromatin [7, 125]. A family of genes coding for proteins with a role in heterochromatin structure has been described in *Drosophila* and other insects, vertebrates and plants [183]. Whether the different MDBPs/MeCPs characterized so far are functionally and/or structurally similar to the proteins involved in heterochromatin formation, remains to be seen. One of the MDBPs shows some sequence-specificity [219], MeCP-1 binds only to DNA containing a very high density of methylated CpGs [125], and for MDBP-2 a specific, methylated binding sequence has been identified [142]. The proposed role of the MDBPs has been tested by adding purified MDBP-2 to in vitro transcription reactions, which resulted in an inhibition of in vitro transcription [142]. Evidence for the involvement of MeCP-1 in promoter inactivation was provided by a similar experiment: the reactivation of methylated promoters by addition of methylated DNA to the in vitro transcription reactions. This has been interpreted as competitive binding of a limited amount of MeCP-1 in the transcription extracts [33].

The evidence for a cause and effect relationship between an 'open', nuclease-sensitive chromatin structure and gene expression is as inconclusive as in the case of DNA methylation [74]. It has been shown that both mutation of a transcription factor [71] and mutation of a factor binding site [99] result in a 'closed', nuclease-insensitive chromatin structure of the non-transcribed gene. That shows that chromatin can be 'opened' by binding of a transcription factor to DNA, and consequently chromatin could remain in an inactive conformation when transcription factor binding is blocked by a methylated cytosine, as discussed in the following paragraph.

DNA methylation and protein-DNA interaction

The inverse correlation between methylation of promoter DNA and gene expression has been generalized by many investigations, including for example phosphoenolpyruvate carboxykinase [15, 93] and albumin [138]. In model experiments, promoters containing several methylated cytosines were transfected into cells and the transcriptional activity measured. A promoter with *c*AMP *r*esponsive sequence *e*lements (CREs) was inactivated in vitro as well as in vivo after methylation of the site TGACGTCA in vivo, and DNA-binding by the *c*AMP *r*esponsive *e*lement *b*inding protein (CREB) was also abolished [92]. Transcription factors unable to bind to their methylated target sequences in vitro have also been defined in other systems [12]. These ubiquitous factors also did not bind to their methylated binding site in vivo, which resulted in a tissue-specific protein binding pattern as a result of the tissue-specific methylation pattern [12]. A compilation of DNA-binding proteins tested for their ability to bind to their methylated target site is found in table 2. While most of the proteins in table 2 are transcription factors, DNA-binding proteins encoded by the maize transposable elements Enhancer and Activator (Ac), which have a putative role in excision of the transposons from the chromosome, are also affected by the methylation status of their target sequences [69, 110]. For a number of plant transposons it has been shown that the inability to transpose is associated with a methylation of the transposon's sequence [206]. It is not known, however, if methylation of transposable elements is the primary block to potentially damaging mobility of transposons, or if methylation occurs after inhibition of transposon mobility by other mechanisms.

An important role has been suggested for intermediary hemimethylated DNA, which is the product of the replication of methylated DNA (fig. 1 B). It has been speculated that factors unable to bind their methylated target site, but recognizing hemimethylated DNA, could induce demethylation by steric inhibition of DNA methylase binding (fig. 1 B). Binding to hemimethylated DNA has been shown for the mammalian transcription factor MLTF/USF, so this mechanism of demethylation is theoretically possible [205]. Especially interesting are the DNA-binding properties of a protein encoded by the maize transposon Activator (Ac, table 2) [110]: It binds better to its unmethylated than to its methylated target sequence, and one of the two hemimethylated sequences resulting from DNA replication is recognized to an even greater extent by the putative transposase. This result might be the molecular explanation for the phenomenon known from genetic studies that Ac transposition occurs only during DNA replication.

It is not possible yet to conclude from the data summarized in table 2 that DNA binding of these factors is precisely regulated by methylation of their binding site(s). To prove this, the methylation at a transcription factor binding site, which is known to be inhibited by this specific methylation event, must be determined by genomic sequencing. Only then will it become possible to judge if demethylation precedes transcription factor binding, or if a protein can somehow bind in vivo to its

methylated target site, with subsequent demethylation of the site.

Mechanisms of promoter activation and inactivation

It has been argued in the preceding paragraphs that 5-mC functions as a long-term inactivation mechanism on several levels of genomic organization. How can this locking mechanism be relieved during cell determination and differentiation? In figure 1 B, two possible mechanisms are schematically shown: passive demethylation due to incomplete maintenance methylation during DNA replication, and active demethylation. There have been several reports of promoter demethylation without cell division (table 3). Under these conditions a passive demethylation as consequence of several rounds of DNA replication is not possible, and an active demethylation event, probably induced by transcription factor binding, seems to happen (fig. 1 B). Many studies have therefore attempted to clarify the temporal relation of gene transcription and promoter demethylation. It is known that apparently fully methylated genes can be transcribed, e.g. the vitellogenin genes of *Xenopus laevis*[67]. A trivial explanation would be that the methylation status of only a fraction of all CpG dinucleotides can be tested with methylation-sensitive restriction enzymes and crucial CpG sites are demethylated in these genes. An adenoviral promoter specifically inactivated by three methylated CpG dinucleotides around the transcription start site can still be transcribed even when still methylated: either by overexpression of the transcription activating protein E1A[207], or by the action of an upstream positioned enhancer[107]. An involvement of DNA methylation in *h*uman *d*eficiency *v*irus (HIV) latency has also been reported: methylation of two CpG dinucleotides[13] or all CpG dinucleotides[175] in the transcription regulating *l*ong *t*erminal *r*epeat (LTR) of HIV results in reduction of transcription of viral genes. Similar to the results with adenovirus[207], overexpression of the viral transactivator Tat results in transcription from the LTR methylated at only two CpG dinucleotides[13]. But activation of the methylated HIV LTR is not possible when all CpG dinucleotides are methylated[175]. It is still not known if the CpG depletion observed in the genomes of lentiviruses (to which HIV belongs) results from deamination of methylated latent viral genomes or from selection against CpG-rich sequences which would be susceptible to complete inactivation by cytosine methylation[180]. Transcription of fully methylated genes by RNA polymerase I and RNA polymerase III has also been described, and in both cases it was shown that all CpG dinucleotides were methylated[18,120]. But inhibition of RNA polymerase III transcription by promoter methylation has been found for an adenovirus type 2 gene and for a chicken tRNA gene[18,100]. The demethylation of the chicken liver-specific vitellogenin promoter, which takes place without DNA replication, has been studied in detail by genomic sequencing[168,169]. The kinetics of demethylation of several CpG dinucleotides either parallels the increase in transcription or lags somewhat behind. Strand-specific demethylation leading to hemimethylated DNA was also found. A protein has been characterized which binds during estrogen induction together with the estrogen receptor to the estrogen-responsive element of the chicken vitellogenin II gene (*n*on-*h*istone *p*rotein 1, NHP1). It appears that NHP1 makes single-strand cuts in the DNA surrounding the 5-mC and it was speculated that this might be an early step in an induced DNA repair process removing 5-mC upon DNA binding of the estrogen receptor[91]. Direct evidence for a DNA repair-like mechanism of active demethylation has been found during induced differentiation of *m*urine *e*rythro*l*eukemia cells (MEL cells): it was shown that non-replicating, demethylated DNA from differentiating MEL cells had incorporated deoxy[5-^{3}H]cytidine, but not deoxy-[5-^{3}H]adenosine, which was interpreted as specific replacement of 5-mC by cytosine[159]. In other experiments genes methylated in vitro become demethylated in the regulatory region after stable transfection into cells[179,216]. It has also been shown that this demethylation of tissue-specific promoters methylated before transfection depends on tissue-specific factors: a methylated α-actin promoter is only transcribed and demethylated in myogenic cells, but not in fibroblasts, which do not express the α-actin gene[216]. Using transient transfection in the same system, it was found that demethylation proceeds in two steps with a hemimethylated DNA as an intermediate[141]. This is a confirmation of the evidence by genomic sequencing for a hemimethylated demethylation intermediate. There is also evidence that the demethylation of the α-actin is independent of its transcription, and that demethylation is induced by signals in the DNA sequence, similar to the events during demethylation of CpG islands in embryonic cells[61]. It can be concluded that transcription from methylated promoters can be induced by defined developmental and physiological situations and that this transcription is associated with a replication-independent demethylation. The latter can also happen in the absence of transcription.

Several experiments have addressed the temporal order of gene inactivation and promoter methylation in different experimental systems, namely globin gene expression, virus inactivation, and X-chromosome inactivation in early embryos[57,66,114]. For all three cases, transcrip-

Table 3. Demethylation in the absence of DNA replication.

Species name	Gene	Tissue	Reference
Chicken	Vitellogenin	Liver	211
Chicken	δ-crystallin	Lens	188
Chicken	Myosin light chain LC1f and LC3f	Skeletal muscle	111
Epstein-Barr-Virus (human)	Early region	Lymphoma cells	192

tional inactivity was found to precede DNA methylation. Therefore, increased methylation of a DNA segment can be interpreted as a general inhibitory mechanism which permanently turns off already inactivated genes[41,131].

Changes in DNA methylation during differentiation and development

A distinct change in the degree of methylation of the genome has been observed during early mammalian embryogenesis. While many (but not all) genes are highly methylated in sperm DNA[75,157], genes are often less methylated in oocytes[54,130,171]. During development, the DNA of the extraembryonal membranes (yolk sac and placenta) becomes dramatically demethylated, while the DNA of fetal tissues is subjected to a de novo methylation process[130,158,170,171] (table 4). This de novo methylation(s), which result(s) in tissue-specific differences in the degree of DNA methylation (table 4), could have a function in the fixation of the activity status of a specific set of genes in a defined developmental stage, and thereby making this specific activity pattern stable and heritable for many cell generations[41,96,131]. Changes in genome methylation were also shown during in vitro development of MEL cells, a model system for blood cell development, and of embryocarcinoma (EC) cells, representing early embryonic development[21,158,217]. The induced hypomethylation during MEL cell development is an early and transient event preceding differentiation[160]. This result is in agreement with the hypothesis that a demethylation event allows activation of a new, tissue-specific set of genes and the imprinting of this new pattern of gene activity by de novo methylation. Differences in the degree of methylation between different tissues and developmental stages of mammals are summarized in table 4. Changes of methylation in several genes during in vitro development of EC cells and erythroleukemia cells have also been found[158,217]; for instance, onset of transcription of the collagen IV gene during EC cell differentiation is associated with developmentally regulated demethylation of its promoter[34]. The demethylation of tissue-specific immunoglobulin κ genes is probably also developmentally regulated, because the gene(s) remain always methylated in pre-B cells, even when transcribed, and become demethylated only in B cells[105]. Based on these results, it has been speculated that changes in methylation occurring in a developmental regulated manner or after induction by environmental stimuli predestinate genome domains or genes for activation and further demethylation. This hypothesis is backed by the observation that the non-transcribed repetitive sequences are also subjected to the developmental changes in methylation[62,63,170,171]. Model calculations show that changes in genome methylation would result from changes in the fidelity of the maintenance methylase and in the ratio between maintenance methylase and de novo methylase activity[150]. Whether the observed changes of DNA methylation are the result of fluctuations in DNA methylase activity, and whether active demethylation contributes to these changes, is unknown.

Changes in genome methylation during development of higher plants have also been proposed as important regulatory steps of developmental stages[152]. Because the genetic activity of transposable elements is correlated with their methylation status, transposons have been used as indicators of changes in genome methylation during plant development. From genetic studies it is known that transposons are activated at well-defined time points during development, and recently it has been shown that the decrease in activity of a transposon with increasing age of plant tissue is correlated with increasing methylation of its DNA[122]. It was also shown that inactivated maize transposons could be activated by plant cell culture, probably by going through a developmental phase with low DNA methylation[143]. However, the question whether or not inactivity preceded methylation remains unanswered in this model system, too.

Demethylation of the whole genome or large parts of it can also be induced by environmental stress. Methylated, inactive genes can become demethylated and activated by UV- or carcinogen-induced DNA damage and following DNA-repair, probably because the DNA repair processes are not matched by a sufficiently large maintenance methylase activity. Examples of this DNA-repair induced demethylation are the activation of metallothionein genes by UV[113], and the carcinogen-induced activation of the hamster thymidine kinase gene[11]. A fascinating analogy to this hypothetical gene activation by stress-induced demethylation is found in the 'genome stress hypothesis' put forward by Barbara McClin-

Table 4. (*a*) Changes in DNA methylation during development of the mouse. (*b*) Tissue-specific levels of methylation in man.

Tissue	Degree of methylation % 5-mC $\frac{mdC*100}{mdC+dC}$	Reference
a) Mouse		
Sperm	4.0	62
Heart	4.1	62
Spleen	4.3	62
Brain	4.7	62
Splenic macrophages	5.3	62
b) Man		
Sperm	4.0	56
Placenta	3.6	56
Spleen	4.5	56
Brain	4.7	56
Thymus	4.8	56
c) Human tumor cells		
Primary tumors:	3.2–4.9	64
Ovarian carcinoma	3.3–4.6	64
Breast adenocarcinoma	3.6–4.7	64
Colon carcinoma	3.7–4.4	64
Tumor metastases:	1.8–4.7	64
Melanoma in lymph nodes	3.2, 3.6	64
Melanoma in liver	3.4	64

tock[118]. McClintock found that mutagenic agents (X-rays, etc.) activated genome rearrangements in maize, mainly by activation of transposable elements. She proposed that a selective advantage provided by transposons is their ability to function as agents of genome rearrangements, thus creating new genotypes fitting into a rapidly changing environment. Transposons are also activated by virus infection[50]. As discussed above, a transposon's genetic activity is correlated with its methylation status. Therefore, stress-induced demethylation is probably the molecular mechanism underlying the genomic stress hypothesis of Barbara McClintock. This hypothesis has been tested by gamma-irradiation of maize kernels containing an inactive Mutator transposon, which became demethylated and active after DNA damage[202]. For mammalian model systems, however, induction of transcription factors by damaged DNA has been found, which are responsible for transcription of the UV-induced genes without preceding DNA-repair[187,198].

Demethylation of large parts of the genome can be experimentally induced by treatment of the cells with the DNA methylase inhibitor 5-azacytidine[97] (fig. 1 A). A number of genes can be activated by this treatment, including the promoters contained in methylated CpG islands on the inactive female X-chromosome[150]. The demethylating action of 5-azacytidine has been used to test the hypothesis that DNA demethylation is an important event during cell differentiation. Indeed, a number of cell lines can be induced to differentiate after demethylation with 5-azacytidine, the best studied example being the fibroblast cell line 10T1/2, which differentiates into a number of differentiated cell types upon 5-azacytidine treatment. However, the regulatory gene MyoD1, which has an important function during this differentiation process, is transcribed from a promoter contained within a CpG island, and it was recently shown by genomic sequencing that the promoter is methylated only in the cell line 10T1/2[98]. Therefore, DNA methylation probably does not have a physiological function in the developmental regulation of MyoD1. In general, reactivation by demethylating agents works probably only for methylated promoters contained within CpG islands. It does not seem possible to activate tissue-specific promoters by demethylation in tissues in which they are normally not expressed[88,138].

Genome hypomethylation has been found in a number of carcinomas and might occur during an early phase of cell transformation[60,64,70] (table 4). Hypomethylation is not the result of reduced DNA methylase activity[104]. It has been speculated that hypomethylation leads to a less heterochromatic chromosome structure, which in turn might result in an unequal distribution of chromosomes during mitosis[59]. Chromosome loss resulting in a loss of tumor suppressor genes is an important step during tumorigenesis. From the data in table 4 it is, however, clear that DNA methylation changes in cancer cells are distributed over a large scale, and hypermethylation also seems to take place in cancer cells[48]. Therefore, the role of changes in DNA methylation during carcinogenesis is not clear.

An important role of changes in DNA methylation during aging has been proposed, especially a loss of methylation with increasing age leading to uncontrolled activation of silenced genes[86,123]. A decrease in DNA methylation has been observed in aging primary cells, but not in immortal transformed cells[212]. An important model system to test this hypothesis has been the maintenance of inactivity of one female X-chromosome by DNA methylation. The activity of two X-linked heterozygous loci was measured as a function of increasing age, ornithine carbamoyltransferase in mice[204] and hypoxanthine phosphoribosyl-transferase in humans[127]. While in the first study an increase in the activity of the inactivated allele with increasing age was found, in the second study no age-related changes were identified. In addition, no age-related changes in X-chromosome methylation were found in the human glucose 6-phosphate dehydrogenase gene and two other genes with unknown function (GDX and MIC2) located on the X-chromosome[139]. If changes in DNA methylation are related to age, it is necessary to assume that this mechanism does not apply to germ-line cells.

Genomic imprinting

Embryological experiments aimed at the construction of mouse zygotes containing either two maternal or two paternal pronuclei have provided evidence for the idea that the presence of both parental genomes is essential for normal mammalian development to occur[132,184,189]. Chromosome translocations allowed the analysis of genomes, in which only a part of a chromosome was inherited from one parent[39]. The preferential inactivation of the paternal X-chromosome in extraembryonal tissue (as opposed to the random inactivation of paternal or maternal X-chromosome in embryonic female tissue) is also a case of genomic imprinting[72].

A number of transgenes have been found to be differentially expressed depending from which parent they have been inherited. In addition, the transgenes were methylated to a different degree when inherited from the father or from the mother[164]. For example, a c-myc gene which was transcriptionally regulated by a Rous sarcoma virus regulatory region was unmethylated when inherited from the father, and methylated when inherited from the mother. Only when inherited from the father was the transgene transcribed in a tissue-specific manner. When a methylated, inactive transgene inherited from a mother was transmitted to the following generation by a male, it was demethylated and transcribed in this second generation[190]. It has also been shown that differentially imprinted transgenes are expressed in a mosaic fashion, i.e. that some cells of the transgenic mouse express the transgene and other cells do not. The inactivity of the transgene and its corresponding methylation depended on the

genotype of the parent from which it was inherited[4, 119, 173, 174]. In humans, inherited differences in the methylation of repetitive sequences have been found[181]. The conclusion drawn from these results is that the methylation pattern of the imprinted transgenes is determined in a direct or indirect fashion by modifier genes, which are present in the population in several alleles of different activity. These modifier alleles act on the DNA of the gamete (on the oocyte during maternal imprinting) or of the embryo during a well-defined 'time window', which is 'open' only for maternal influence in the case of maternal imprinting. It has been suggested that heterochromatin-forming proteins fulfill the function of these modifier genes[183]. The imprinted status of the affected genes is then fixed by one of the waves of de novo methylation that occurs during development[131]. The elucidation of the function and the molecular mechanism of genomic imprinting awaits the identification of genes which are differentially expressed and/or methylated depending on whether they have been inherited from the father or the mother. Transgenes subjected to genomic imprinting have been used as tags for imprinted chromosome regions, but interestingly the genomic DNA surrounding an imprinted transgene is not imprinted[174]. In the case of the human oncogene c-Haras[42], a restriction length polymorphism between two alleles allowed the mapping of differences in methylation by HpaII/MspI, but the origin of the alleles from either the paternal or the maternal genome was not established, nor was a functional significance of the methylation differences found. By a similar approach, allele-specific methylation has been found in the human insulin gene[68]. Recently, the genes coding for the insulin-like growth factor II (IGF-II)[49] and for the IGF-II receptor[10] have been shown to be imprinted. The IGF-II gene is only expressed when inherited from the father and not transcribed when inherited from the mother. Conversely, the IGF-II receptor gene is only expressed from the maternal chromosome but not from the paternal chromosome. These two genes will be important tools for the elucidation of the function of methylation in differentially imprinted states.

Genomic imprinting is also of considerable medical importance[79, 162]. Some inherited diseases like Huntington's Chorea and spinocerebellar ataxia become manifest earlier in life when the mutated allele is inherited from the father. Genetically determined mental retardation in the Prader-Willi syndrome and Angelman syndrome are associated with deletions in the same region of chromosome 15[121]. The Prader-Willi syndrome always results from a mutation of the paternally inherited chromosome, while the Angelman syndrome is associated with deletions on the maternally inherited chromosome. The familial disposition for several tumors including retinoblastoma and Wilms tumor is caused by the transmission of mutated tumor suppressor genes, and a cancer originates when the second, wild-type allele is also mutated or lost by mitotic nondisjunction. It has been shown for retinoblastoma that the germline mutation occurs predominantly on the allele inherited from the father[55, 219]. This phenomenon has been described also for the von Recklinghausen type neurofibrimatosis[95]. It has been speculated that differences in methylation resulting from genomic imprinting could result in different mutation rates by deamination of 5-mC. Alternatively, genomic imprinting can result in different expression of the alleles, as shown for the IGF-II and IGF-II receptor genes, and a tumor develops only after mutation of the non-methylated, expressed allele[163].

Structure, regulation and expression of the mammalian cytosine methylase

Thus far, DNA cytosine-5-methyltransferase (DNA methylases, EC 2.1.1.37) isolated from mammalian tissue or cell culture cells have been characterized as large proteins of 190 kDa in size[20, 83, 144, 146, 156]. Smaller proteins with DNA methylase activity have been identified as degradation products of the 190 kDa protein, and it is not clear if partial proteolysis of the DNA methylase is a regulatory process occurring in vivo, or if it is an artefact of the purification procedure[1, 144, 147]. The purified eukaryotic DNA methylase shows a substrate preference for hemimethylated versus unmethylated DNA (which is expected from the necessity to maintain methylated CpG sites over many cell generations), and also for fully or partially single-stranded unmethylated DNA over unmethylated duplex DNA. RNA is also a substrate for the enzyme[30, 83]. Electron microscopy studies with purified methylase showed that it binds most efficiently to single-stranded CpG dinucleotides[145], and these studies also allowed a visualization of the hemi-elliptical globular structure of the enzyme[185]. With the help of an indirect immunoassay the number of DNA methylase molecules per cell nucleus has been estimated as 20,000–40,000[53]. A plant DNA methylase of about 160 kDa molecular weight has also been isolated and characterized[215].

It was also possible to obtain a partial protein sequence from purified methylase, which was used to isolate overlapping cDNA clones encoding a protein of 1573 amino acids. The cloning of a cDNA for murine methylase was an important step towards elucidation of the structure-function relationship of the enzyme[23]. Sequence comparisons between the murine and thirteen prokaryotic DNA cytosine-5 methyltransferases have identified five blocks of homology separated by non-homologous sequences[151] (fig. 2). The homologous block IV contains the putative reaction center of the cytosine methylases containing a conserved proline-cysteine dipeptide. The variable sequences between blocks VIII and IX are responsible for recognition of target sequences in bacteriophage cytosine-methylases[203, 210], and a similar role has been suggested for these regions in bacterial and mam-

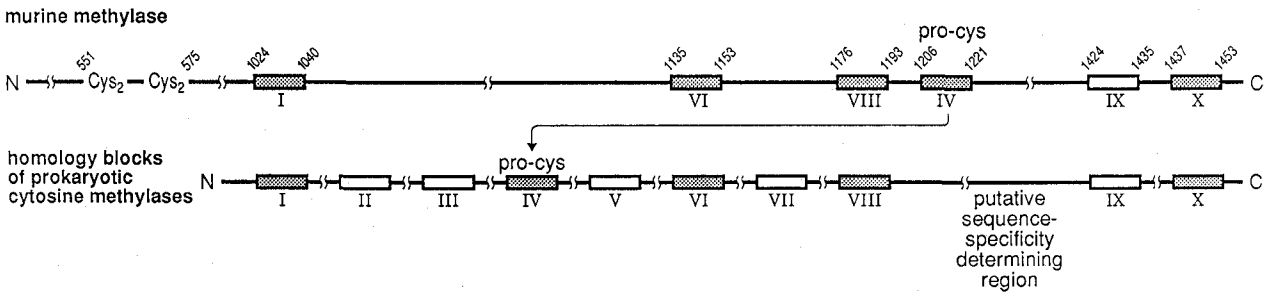

Figure 2. Structural features of murine DNA cytosine-5 methyltransferase (DNA methylase) and blocks of homology with prokaryotic DNA cytosine-5 methyltransferases, modified from [23, 151]. The shaded boxes are highly conserved between bacterial cytosine methylases, the open boxes show less homology [151]. The conserved proline-cysteine dipeptide is suggested as catalytic center of all cytosine methylases [151, 214]; a putative 'zincfinger' DNA binding region [16] and the sequence-specificity determining regions of the bacterial cytosine methylases [203, 210] are also indicated.

malian methylase [23, 151]. The methyl donor of all DNA methylases identified to date is S-adenosyl-methionine. A reaction mechanism has been suggested analogous to the mechanism of other enzymes catalyzing the transfer of 1-carbon units to the C5 of pyrimidine nucleotides, e.g. thymidylate synthase [214]. This mechanism suggests formation of an intermediate enzyme-substrate complex, in which the pyrimidine is covalently bound to the cysteine of the conserved proline-cysteine dipeptide. It has been suggested that 5-azacytidine inhibits the mammalian DNA methylase by a suicide mechanism – covalent binding to the cysteine in the catalytic center [172].

It is not clear if methylation of cytosines is confined exclusively to CpG dinucleotides. A DNA methylase from the rat methylated CpA and CpT in vitro at a 50-fold lower initial rate than CpGs [90]. Methylation of dinucleotides other than CpG has also been observed in vivo, e.g. in human spleen [213] and has also been found by genomic sequencing [196]. These sites would not be maintained in vivo after separation of the 5-mCpA from the TpG antistrand during the course of DNA replication. Other genomic sequencing experiments provided no evidence for methylation of cytosines outside of CpG dinucleotides [12, 148–150, 168, 169]. Of importance is the time span during which freshly replicated DNA remains hemimethylated. In prokaryotes, it has been shown that the level of methylation of the genome is determined by the level of methylase [191], and the 'window' of hemimethylation after DNA replication is important for the regulation of the bacterial cell cycle [32, 37, 112]. It has been supposed that hemimethylation is also important in eukaryotes. This assumption is supported by the finding that specific CpG dinucleotides remain hemimethylated for a considerable time during development [168], even for several cell generations [196]. The time window during which the replicated DNA remains hemimethylated is dependent on the rate of maintenance methylation, which is dependent on the available methylase activity. The kinetics of the maintenance methylation reaction have been determined by introduction of (α-^{32}P)dGTP into permeabilized cells and subsequent nearest-neighbor analysis of CpG sites. The maintenance methylation of hemimethylated CpG sites has been shown with these experiments to occur within 1 min after DNA replication [78]. DNA MTase activity copurifies with a so-called DNA replitase complex, which might be associated with the nuclear matrix [137]. These results suggest a temporal and spatial association between DNA replication and DNA methylation, which would increase the DNA methylase concentration at the replication fork. This proposed association is in agreement with the processivity of the DNA methylase, which methylates stretches of hemimethylated DNA [52, 182]. This tight association of maintenance methylation and DNA replication makes the formation of hemimethylated DNA during DNA replication unlikely. However, the much slower maintenance methylation during DNA repair processes [103] suggests that the generation of hemimethylated DNA is possible in the absence of DNA replication [141, 168]. At least one of the methylation-inhibited transcription factors (MLTF/USF) is able to bind to its hemimethylated recognition sequence [205]. Whether this has a biological function, e.g. for gene activation after DNA replication (fig. 1 B), remains to be seen.

As mentioned above, DNA methylases of different molecular weights, which most probably arise by proteolysis of the 190 kDa protein, have been isolated. It has been observed that the full size 190 kDa DNA methylase is the predominant form in rapidly dividing cells, while the shorter DNA methylase is the main DNA methylase in non-dividing cells [2, 20, 22]. It has also been found that DNA methylase partially proteolysed in vivo or in vitro has an enhanced de novo DNA methylase activity and a relaxed sequence specificity, and it has been suggested that this is due to the loss of a regulatory domain [1, 2]. The methylation of cytosine outside CpG dinucleotides discussed above could also result from this proteolysis. It is not known whether this change in the catalytic properties of the DNA methylase by proteolysis is a process occurring in vivo, and whether it is of significance for the developmental regulation of DNA methylation. The ratio of maintenance DNA methylase activity to de novo

DNA methylase activity, which has been determined to lie between 10 and 100[20, 30, 77, 146, 156], and which is of importance for changes in genome methylation, could also be affected by limited proteolysis. Cellular factors affecting DNA methylase activity and specificity have also been found[115, 195], and de novo methylation is probably also affected by the degree of DNA supercoiling[19].

We have assembled a full size cDNA of the murine DNA methylase from overlapping partial cDNAs into a bacterial/mammalian shuttle vector under the control of the strong enhancer/promoter of cytomegalovirus[31]. When transfected into monkey kidney cells, a several fold increase of methylase activity was observed (Czank, A., Häuselmann, R., Hergersberg, M., and Schaffner, W., unpublished observations). The possibility of controlled expression of a cloned mammalian methylase cDNA will allow the testing of different hypotheses concerning developmental regulation of DNA methylation and regulation of the DNA methylase.

Conclusions

It has been shown in different biological systems that DNA methylation stabilizes a gene's inactivity by affecting chromatin structure and protein-DNA interactions. It is now also known that gene-specific activation and demethylation is associated with a DNA repair-like process. DNA methylation serves as a heritable signal of chromatin inactivation, which imprints a tissue- and developmental-stage specific pattern of gene activity for following cell generations. To what extent cytosine methylation has evolved into a primary biological signal in gene regulation during development, will be seen by concentrating on differences in the methylation of DNA sequences during development in vivo.

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