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Minireview

Eukaryotic DNA methylation: facts and problems

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Patterns of DNA methylation in complex genomes like those of mammalian cells have been viewed as indicators of different levels of genetic activities. It is as yet unknown how these complicated patterns are generated and maintained during cell replication. There is evidence from many different biological systems that the sequence-specific methylation of promoters in higher eukaryotes is one of the important factors in controlling gene activity at a long-term level. In general, the fifth nucleotide 5-methyldeoxycytidine can be considered as a modulator of protein-DNA interactions. The degree and direction of this modulation has to be assessed experimentally in each individual instance. The establishment of de novo patterns of DNA methylation is characterized by the gradual non-random spreading of DNA methylation by an essentially unknown mechanism. In this review, some of the general concepts of DNA methylation in mammalian systems are presented, and research currently performed in the authors' laboratory has been summarized.

Eukaryotic DNA methylation; DNA methyltransferase; Pattern of DNA methylation; Promoter methylation and long-term inactivation; Spreading of DNA methylation; DNA methylation and DNA-protein interaction

1. INTRODUCTION

In the five letter genetic text, the embellishment by a fifth nucleotide, 5-methyldeoxycytidine (5-mC), generates specific patterns in eukaryotic DNA. While we begin to decipher the ways in which the presence of 5-mC in a sequence motif affects or fails to influence detectably the interaction with a specific protein, the biological significance of apparently highly specific and rather stable patterns of DNA methylation is still unknown. The genetic signal 5-mC can be considered as a modulator of DNA-protein interactions. The direction of this interaction cannot be predicted but has to be determined experimentally. Frequently, the interaction of a specific protein with a sequence motif is abrogated by 5-mC, e.g. in motifs in the E2A promoter of adenovirus DNA [1,2]. In other motifs, e.g. SP1, 5-mC does not interfere detectably with the binding of this transcription factor [3,4]. On the other hand, there are mammalian proteins which bind specifically to methylated DNA sequences [5,6]. Lastly, several restriction endonucleases from prokaryotes, e.g. *DpnI* (*Diplococcus pneumoniae*), *Nmu*DI, *Nmu*EI (*Neisseria mucosa*) [7] require for activity the presence of a methylated nucleotide, in these cases N^6-methyldeoxadenosine (N^6-mA) in the recognition sequence 5′-GCAT-3′. It is conceivable that the presence of a methylated nucleotide in a DNA sequence alters locally the structure of the DNA double helix. Definite information is not available on this problem.

In eukaryotic systems, the most extensively studied and best-documented effect of DNA methylation on a specific biological function is the inhibition or inactivation of promoters by sequence-specific methylation (for reviews, see [8–11]). Via promoter inactivation, DNA methylation obviously could interfere with many highly complex biological phenomena. Effects on differentiation, oncogenesis, parental imprinting [12], the inactivation of the X-chromosome [13], and others have been invoked in this context.

Obviously, many additional mechanisms, e.g. DNA replication, recombination, repair, and DNA structure need to be investigated for possible modulating effects of sequence-specific DNA methylations. So far, we have only a limited understanding of the influence of a few isolated 5-mC residues in eukaryotic (mammalian) DNA on biochemical reactions involving DNA-protein interactions. In mammalian DNA complex patterns of DNA methylation have been described (e.g. [14,15]), but the problem of 'pattern recognition' remains to be studied. Such patterns are likely to affect a host of different DNA-protein interactions, and in that way might

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alter structure and functions of entire domains of DNA.

2. BASIC FINDINGS ON DNA METHYLATION

In the DNA of higher eukaryotes, in particular of mammals, 5-mC appears to be the only modified nucleotide. The occurrence of N^6-mA in mammalian DNA is unlikely, but cannot be rigorously excluded. It is thought that the modified nucleotide 5-mC abounds in the nucleotide combinations 5′-CG-3′ or perhaps 5′-CXG-3′, but the other dinucleotide combinations, 5′-CA-3′, 5′-CC-3′, or 5′-CT-3′, have also been identified as sites for 5-mC. There is evidence that in human DNA the presumably atypical dinucleotide combinations may contain an unexpectedly high proportion of 5-mC [16]. It has been recognized that 5′-CG-3′ dinucleotides are frequently clustered in the 5′-region of eukaryotic genes [17,18]. In fact, so-called CG-islands have been described in the 5′-regions of many polymerase II-transcribed genes [19,20]. It is thought that the methylation of certain 5′-CG-3′ dinucleotides in the 5′-region of genes contributes to the long-term silencing of these genes [8–11].

A given pattern of DNA methylation is preserved, inherited as it were, by the enzymatic mechanism of maintenance methylation. Postreplicationally, certain cytidine (C) residues are methylated by the enzyme DNA methyltransferase which uses S-adenosylmethionine as the methyl donor. For the sequences 5′-CG-3′ or 5′-CXG-3′ the parental strand, which remains methylated after DNA replication, can serve as the memory strand for the DNA methyltransferase to impose symmetrically methyl groups on the newly synthesized, hence not yet methylated, DNA complement. More complicated controlling elements seem to be operative in the process of maintenance methylation, because, at least in continuous cell lines in culture, hemimethylated DNA sequences have been observed over several cell generations [21–23]. Apparently, maintenance methylation can be delayed after DNA replication.

The gene for at least one mammalian DNA methyltransferase has been cloned from mouse cells, and its nucleotide sequence has been determined [24]. The nucleotide sequence reveals a DNA-binding domain in the DNA methyltransferase gene and a second domain responsible for the methyl transfer. This latter domain has a high degree of homology [24] to a consensus sequence for many of the prokaryotic DNA methyltransferases [25]. The cloned gene for the mammalian DNA methyltransferase is of low abundance and there are no apparent homologies to other parts of the mammalian genome. This finding together with the high degree of similarity to the consensus sequence for prokaryotic DNA methyltransferases has prompted the idea that there might be only one such enzyme in mammalian cells. This proposal deserves critical evaluation. It is also conceivable that additional factors are essential for the regulation and/or specificity of the mammalian DNA methyltransferase.

Formally, one can distinguish a second type of DNA methylation, that of de novo methylation. An example studied in this laboratory may serve to illustrate this point. The DNA in the adenovirus particle is not detectably methylated, nor is free intranuclear DNA replicating or persisting in infected cells [26,27]. The viral DNA can become integrated into the genome of mammalian cells by covalent phosphodiester bonds [28–30]. The integrated viral DNA eventually becomes de novo methylated in highly specific patterns [29], such that the early viral genes, which are expressed in adenovirus-transformed cells, are undermethylated. The late, silenced viral genes are, however, strongly methylated [14,15]. This inverse correlation between DNA methylation and levels of gene expression provided at the time one of the first examples for an involvement of DNA methylation in the regulation of gene expression. Many cell generations in culture are required for these specific patterns of methylation to become established after the integration of the viral genome ([31], G. Orend, I. Kuhlman and W. Doerfler, manuscript in preparation). It is one of the unresolved problems to understand how de novo patterns of methylation arise and what enzymes and cofactors are involved in generating these patterns. In a practical sense, this phenomenon is of importance in that very frequently genes that are added to an existing genome, e.g. in transfection and transgenic animal experiments, become rapidly inactivated, presumably as a consequence of highly specific de novo methylation.

There is evidence that segments of DNA can become demethylated in the absence of concomitant DNA replication [23,32]. The enzymatic mechanism for this direct demethylation has not yet been elucidated. Apparently, this demethylation can be transient and restricted to only one DNA complement. Demethylation seems to precede the reactivation of the corresponding promoter and gene sequences. It is as yet unknown, whether the demethylation of one strand suffices in all cases for gene reactivation to occur. There is evidence for one of the adenovirus promoters that hemimethylation of the promoter in either complement leads to promoter inactivation ([2], U. Freisem-Rabien, and W. Doerfler, manuscript in preparation), but there may be differences from promoter to promoter depending, e.g. on whether hemimethylation alone can interfere with the binding of an essential transcription factor.

3. TECHNIQUES USED FOR THE ANALYSIS OF DNA METHYLATION

The first investigations on DNA methylation [33]

relied on the chemical characterization of the modified nucleotide. In later years, 5-mC has been identified and quantitated in total hydrolysates of DNA by a combination of chromatographic and electrophoretic procedures (e.g. [26,34, 35]). The recognition of genetically relevant patterns of DNA methylation in specific segments of a genome has become possible after the detection of methylation-sensitive restriction endonucleases (review in [7]). An important role in this respect was played by the discovery of the isoschizomeric restriction endonuclease pair *Hpa*II (*Haemophilus parainfluenzae*) and *Msp*I (*Moraxella* species) [36]. *Hpa*II cannot cleave the sequence 5′-CCGG-3′ when the 3′-located C residue is methylated or hemimethylated, but cuts the unmethylated sequence. *Msp*I is blocked only when the 5′-located C is modified. Thus, methylated or unmethylated 5′-CCGG-3′ sequences can be distinguished by differential cleavage of the same DNA with these two enzymes. Of course, a sequence like 5′-CCGG-3′ comprises only 10–15% of all the 5′-CG-3′ dinucleotide combinations in a sequence that might become methylated. Therefore, the results of a restriction analysis of DNA methylation are necessarily limited. The method of genomic sequencing [37] allows the determination of all the 5-mC residues in a nucleotide sequence. This method is based on the failure of hydrazine to modify 5-mC, and thus piperidine cannot cleave at the position of 5-mC, whereas the C reactions can proceed normally. Hence, the presence of 5-mC causes an interruption, a missing rung, in a sequencing ladder. The existence of a presumptive 5-mC residue in a sequence must be verified by the demonstration of a G residue in the complementary position of the opposite strand to exclude the possibility of a mutation.

4. SEQUENCE-SPECIFIC METHYLATION CAN CAUSE PROMOTER INACTIVATION

This topic has been extensively reviewed in the past (e.g. [8–11,38]; contributions in [39]). A summary of the adduced experimental evidence will be presented. In this laboratory, we have used the promoter of the E1A gene of adenovirus type 12 (Ad12) [40] and the late promoter of the E2A gene of adenovirus type 2 (Ad2) [41–43] to demonstrate the inactivation of promoters by sequence-specific promoter methylations. The late E2A promoter was methylated in vitro in positions −215, +6, and +24 by the *Hpa*II DNA methyltransferase (5′-CCGG-3′). Promoter inactivation was assessed in transient microinjection (oocytes of *Xenopus laevis*) or transfection experiments (mammalian cells) [42,43]. The same promoter as a fusion construct with an indicator gene was integrated into the genome of mammalian cells, and the inactivation by methylation was documented also in this system [44]. Lastly, an in vitro transcription system was used to demonstrate the inhibition of the late E2A promoter by 5′-CCGG-3′ methylation at the three aforementioned sequences [45]. The promoter sequences, the methylation of which led to promotor inactivation, were originally identified by studying inverse correlations between promoter methylation and gene inactivation in Ad12-or Ad2-transformed cell lines [14,15,45a]. These data taken together have led to the conclusion that sequence-specific promoter methylation can cause promoter inactivation. The decisive sites in any promoter have apparently to be elucidated experimentally, since their locations in the promoter and upstream or downstream sequences cannot be predicted. For the late E2A promoter, it has been demonstrated that the methylation of the +6 and +24 cytidine residues abrogates the binding of specific protein(s) to these downstream sequences [2], even when these sequences are only hemimethylated. In other parts of that promoter, methylated nucleotides interfere with the binding of proteins in certain regions, but not in others [22].

5. REVERSAL OF THE INACTIVATION

Promoter inactivation by sequence-specific methylation is not unconditional, but can be reversed by transactivation [43,46,47] or by a strong viral enhancer [48]. At least partial reactivation has been effected by the 289 amino acid protein encoded in the E1A region of the adenovirus genome. The methylated E2A promoter can be located both in the cellular genome or extrachromosomally in a plasmid construct and can be reactivated by the viral transactivator gene either in the chromosomal or extrachromosomal localization [47,49]. Reactivation is not accompanied by changes in the status of methylation in the reactivated E2A promoter, at least not in both DNA strands. E1A transactivation is thought to operate via protein-protein interactions [50]. This mechanism has, however, not yet been proven for the reactivation of a methylated and reactivated promoter. Since foreign genes artificially added to an existing genome frequently become inactivated by methylation, it would be sensible, for practical purposes, to introduce these foreign genes jointly with a transactivator gene or with a strong enhancer in order to counteract the inactivation due to methylation.

6. THE SPREADING OF DNA METHYLATION AND THE ESTABLISHMENT OF DE NOVO PATTERNS OF DNA METHYLATION

Foreign DNA inserted into an established mammalian genome becomes methylated in specific patterns. We have studied this phenomenon with Ad12 DNA integrated into the genome of hamster tumor cells. The previously unmethylated viral DNA is methylated in specific patterns in the course of several,

sometimes many, cell generations [31]. In colinearly inserted viral DNA, methylation is often initiated in the central part of the integrated foreign genome, and the terminal sequences abutting the cellular DNA are methylated much later or not at all (G. Orend, I. Kuhlmann, and W. Doerfler, manuscript in preparation). On the other hand, viral DNA replicating or persisting in the nucleus of mammalian cells in the non-integrated form is not detectably methylated. Moreover, human cellular DNA sequences, which are highly methylated in the cellular genome, remain unmethylated when the same cellular sequences are integrated as part of the genome of a viral-cellular symmetric recombinant (SYREC) and replicate in the nucleus of the same human cells in the non-integrated form [51]. Hence, chromosomal localization and organization may be important prerequisites for specific patterns of DNA methylation to arise. Nucleotide sequence alone does not seem to be the determining factor.

In foreign DNA that has been methylated in vitro in a few C residues and has subsequently become integrated into mammalian DNA, a gradual spreading of DNA methylation has been observed until eventually an entire region of the viral genome is completely methylated in all 5′-CG-3′ sequences [21,22]. This spreading effect may involve even non-5′-CG-3′ dinucleotides. The spreading of DNA methylation appears to be an essential element in the establishment of de novo patterns of DNA methylation. The spreading seems to proceed in a non-contiguous manner in that DNA segments bound to protein are initially excluded from the immediate spreading process and are methylated only at a later stage. As a consequence, the binding of many of the proteins is obliterated in the completely methylated region. These conclusions have been derived from genomic sequencing and footprinting experiments [21,22]. The spreading of DNA methylation may explain the programmed inactivation of genes in development and on one of the X-chromosomes [13].

The problem has been discussed whether DNA methylation was cause or consequence of promoter inactivation. In a sense, both considerations can apply. The introduction of one or a few 5-mC groups into decisive sequences in a regulatory region of a gene causes promoter inactivation. This initially very limited level of promoter methylation will probably escape detection by restriction analyses. The spreading of DNA methylation in this segment is initiated by the few 5-mC residues [21,22] and will successively lead to the complete methylation of the promoter and the neighboring sequences. At this extent of DNA methylation, it will be demonstrable by cleavage with appropriate restriction endonucleases. Now, it appears that DNA methylation may be the consequence of promoter inactivation. This interpretation is only partly correct, because it failed to consider the few 5-mC groups that initially caused promoter inactivation but were not detected by the limited technology.

7. METHYLATION PATTERNS IN HUMAN DNA

As a working hypothesis, it is proposed that the patterns of methylation in an entire genome reflect the states of activity in that genome. Presumably, states of activity and patterns in the distribution of methylated nucleotides differ depending on cell type or tissue. It is, therefore, the goal of our research to determine these patterns in selected areas of the human genome. Since cell lines represent a highly selected population of cells with cell line-specific patterns of gene activity and DNA methylation, it is mandatory to determine these patterns directly in primary human cells. Human lymphocytes and granulocytes are easily available as sources for human DNA. Two techniques have been chosen for the analysis. On the one hand, the 5′-upstream and promoter regions of the human genes for tumor necrosis factors α and β have been analyzed by the genomic sequencing technique to localize all 5-mC residues in these sequences. A surprisingly high concordance in the distribution of 5-mC groups among different individuals, even of different ethnic origins, has been observed [52]. In other experiments, larger segments of the human genome have been screened for their methylation patterns by the more conventional *Hpa*II-*Msp*I restriction analysis. As hybridization probes, randomly selected cosmid clones of human DNA with a low content of repetitive DNA sequences have been utilized. Even with that less sensitive analytical approach, that allows a survey of larger segments of the genome, very similar, if not identical, patterns have been revealed in different individuals (A. Behn-Krappa, I. Hoelker and W. Doerfler, manuscript in preparation). On the other hand, when human cell lines of lymphatic, leukemic or lymphoma origin have been investigated for methylation patterns, a remarkable heterogeneity has been found (S. Achten, A. Behn-Krappa, D. Heuss, B. Schmitz, M. Jücker, H. Tesch, V. Diehl and W. Doerfler, manuscript in preparation). It is apparent that cell lines will not constitute the cell type of choice for investigations on methylation patterns in the human genome.

In a cell, information about patterns of DNA methylation in many segments of the genome might allow one to interpret the state of genetic activity. Of course, we are far from even a vague comprehension of genetic activity levels in different human cell types. Would the determination of patterns of methylation provide one tool for obtaining an improved insight into the genetic activities, and probably not only of transcriptional activities, in different segments of the human genome?

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