Chapter 5

Basics on the Fifth Nucleotide in DNA, 5-Methyldeoxycytidine: A Regulatory Genetic Signal

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In mammalian as well as in plant DNA, and in the DNA of many other organisms, there occurs a fifth nucleotide, 5-methyldeoxycytidine (5-mC), in addition to the traditionally recognized four nucleotides A, C, G, and T. Although the presence of 5-mC in DNA has been known for a long time, only during the last 30 years has there been progress in elucidating its functional significance. This brief chapter will give an introduction to the field and address biological processes in which 5-mC has been shown to assume a major role.

1. On the early history of 5-mC

The fifth nucleotide, 5-methyldeoxycyidine (5-mC), was first described in DNA from the tubercle bacillus (Johnson & Coghill, 1925) and in calf thymus DNA (Hotchkiss, 1948). Subsequently, 5-mC had a biochemical future as 5-hydroxymethyl-C (5-hm-C) in the DNA of the T-even bacteriophages. The biological function of this C modification was never elucidated. Daisy Dussoix and Werner Arber (Arber & Dussoix, 1962; Dussoix & Arber, 1962) discovered the phenomena of restriction and modification in bacteria. It was recognized later that DNA modifications, such as 5-mC and/or N^6-methyladenosine (N^6-mA), had important biological consequences. A major endeavour followed in many laboratories that worked on the biochemistry of DNA modifications in bacteria and their phages (review by Arber & Linn, 1969). Around 1970, Hamilton Smith and his colleagues discovered the restriction endonucleases (Kelly & Smith, 1970) whose application to the analyses of DNA was pioneered by Daniel Nathan's laboratory (Danna & Nathans, 1971). It was soon appreciated that enzymes, whose activity was compromised by the presence of a 5-mC or an N^6-mA in the recognition sequence, could be of great value in assessing the methylation status of a DNA sequence (Waalwijk & Flavell, 1978; McClelland & Nelson, 1988).

In 1975, two papers (Holliday & Pugh, 1975; Riggs, 1975) alerted the scientific community to the importance of methylated DNA sequences in eukaryotic biology. At more or less the same time, my laboratory at the Institute of Genetics in Cologne independently analyzed DNA in the human adenovirus and in adenovirus-induced tumour cells for the presence of 5-mC residues (Günthert et al., 1976) and discovered that integrated adenovirus, perhaps any foreign, DNA had become *de novo* methylated (Sutter et al., 1978). DNA methyltransferases in human lymphocytes were studied early on by Drahovsky and colleagues (1976). Vanyushin's laboratory in Moscow analyzed the DNA of many organisms for the presence of 5-mC and N^6-mA (Vanyushin et al., 1968).

Church and Gilbert (1984) were the first to develop a genomic sequencing technique, based on the chemical modification of DNA by hydrazine, and thus provided a means to survey all possible C-residues for the occurrence of 5-mC in a sequence. The bisulfite sequencing technique introduced by Marianne Frommer and colleagues (Frommer et al., 1992; Clark et al., 1994) allowed for a positive display of methylated sequences. This method and some of its modifications have now become the 'gold standard' in analytical work on DNA methylation. The

method is precise and yields reproducible results but is laborious and expensive. At present, however, there is no better method available.

Constantinides, Jones and Gevers (1977) reported that the treatment of chicken embryo fibroblasts with 5-azacytidine, a derivative of cytidine which was known to inhibit DNA methyltransferases (reviewed by Jones, 1985), activated the developmental programme in these fibroblasts leading to the appearance of twitching myocardiocytes, adipocytes, chondrocytes, and others in the culture dish. Their interpretation, at the time, that alterations in DNA methylation patterns activated whole sets of genes involved in realizing a developmental program, has stood the test of time. There is now a huge body of literature on changes in DNA methylation during embryonal and foetal development (for an early contribution to this topic, see Razin et al., 1984). The observation of inverse correlations between the extent of DNA methylation and the activity of integrated adenovirus genes in adenovirus type 12-transformed hamster cells (Sutter & Doerfler, 1980 a; 1980 b) elicited a surge of similar investigations on a large number of eukaryotic genes. Today, it is generally accepted that specific promoter methylations in conjunction with histone modifications (acetylation and methylation, among others) play a crucial role in the long-term silencing of eukaryotic genes (Doerfler, 1983). There is no rule, however, without exceptions – Willis and Granoff (1980) have shown that the genes of the iridovirus frog virus 3 (FV3) are fully active, notwithstanding the complete 5€ -CG-3€ methylation of the virion DNA and of the intracellular forms of this interesting viral genome.

Since many foreign genomes in many biological systems and hosts frequently became *de novo* methylated, several authors have speculated on whether this phenomenon reflects the function of an ancient cellular defence mechanism (Doerfler, 1991; Yoder et al., 1997) against the uptake and expression of foreign genes, much as the bacterial cell has developed the restriction modification systems to counter the function of invading viral genomes. In eukaryotes, integrated foreign viral, in particular but not exclusively, retrotransposon genomes, which make up a huge proportion of the mammalian and other genomes, are frequently hypermethylated (Bestor, 1998). This finding is in keeping with the cellular defence hypothesis of *de novo* methylation mechanisms. In my laboratory at the Institute of Genetics in Cologne (Schubbert et al., 1997) and also by others (Forsman et al., 2003), these considerations have prompted investigations on the stability of food-ingested DNA in mammals as a possible source of foreign DNA taken up with high frequency by mammalian organisms.

In research on the function of 5-mC, many questions remain to be investigated: How have the patterns of DNA methylation, i.e. the distribution of 5-mC residues in any genome, evolved over time? How different are these patterns from cell type to cell type and under what conditions are they preserved, even interindividually maintained in a given species? In what way do these patterns codetermine the structure of chromatin by providing a first-line target for proteins binding preferentially to methylated sequences (Huang et al., 1984; Meehan et al., 1986) or by being repulsive to specific protein-DNA interactions?

Chromatin structure and specific patterns of DNA methylation, which differ distinctly from genome region to genome region, are somehow related. There is growing experimental evidence that the presence of 5-mC residues affects the presence of a large number of proteins in chromatin. However, we do not understand the actual complexity of these interactions or the role that histone modifications can play in conjunction with DNA methylation in the control of promoter activity. Imaginative speculations abound in the literature but there is little novel experimental evidence. I suspect we will have to unravel the exact structural and functional biochemistry of chromatin before real progress on these crucial questions will become possible. A recent review (Craig, 2005) phrased the chromatin enigma thus: ‘there are many different

architectural plans … leading to a seemingly never-ending variety of heterochromatic loci, with each built according to a general rule'.

With the realization and under the premise that promoter methylation could contribute to the long-term silencing of eukaryotic genes, researchers have approached the fascinating problem of genetic imprinting. Several groups provided evidence that genetically imprinted regions of the genome can exhibit different methylation patterns on the two chromosomal alleles (Sapienza, 1995; Chaillet et al., 1995). For one of the microdeletion syndromes involving human chromosome 15q11-13, the Prader-Labhart-Willi syndrome, a molecular test was devised on the basis of methylation differences between the maternally and the paternally inherited chromosome (Dittrich et al., 1992).

Problems of DNA methylation, of the stability and flexibility of the patterns of DNA methylation are also tightly linked to many unresolved questions on reproductive and/or therapeutic cloning. In an effort to correlate gene expression with survival and foetal overgrowth, imprinted gene expression has been investigated in mice cloned by nuclear transfer or in embryonic stem (ES) cell donor populations from which they were derived. The epigenetic state of the ES cell genome appears to be extremely unstable. Variation in imprinted gene expression has been observed in most cloned mice. Many of the animals survived to adulthood despite widespread gene dysregulation, indicating that mammalian development may be rather tolerant to epigenetic aberrations of the genome. These data imply that even apparently normal cloned animals may have subtle abnormalities in gene expression (Humpherys et al., 2001). In cloned animals, lethality occurs only beyond a threshold of faulty gene reprogramming of multiple loci (Rideout et al., 2001). However, malformations are frequent among cloned animals which appear also to have a limited lifetime.

Similarly, the idea of replacing defective genes with their wild type versions or of blocking neoplastic growth by introducing cogently chosen genes and stimulating the defences against tumours and metastases has captured the fascination of many scientists working towards realistic regimens in gene therapy. However, many unsolved problems have remained with viral gene transfer vectors: (i) Stable DNA transfer into mammalian cells was frequently inefficient; (ii) The site of foreign DNA insertion into the recipient genomes could not be controlled; (iii) The integrates at random sites were often turned off unpredictably due to cellular chromatin modifications and/or the *de novo* methylation of the foreign DNA.

Of course, there have been prominent voices cautioning against the premature application of insufficiently scrutinized concepts and techniques (cited in Stone, 1995). Adenovirus vectors proved highly toxic in topical applications to the bronchial system of cystic fibrosis patients (Crystal et al., 1994). In a tragic accident, the administration of a very high dose of a recombinant adenovirus, which carried the gene for ornithine-transcarbamylase, led to the death of 18 year old Jesse Gelsinger (Raper et al., 2003). Retroviral vectors as apparent experts in random integration were thought to assure continuous foreign gene transcription in the target cells. By using a retroviral vector system, ten infant boys suffering from X-linked severe combined immunodeficiency (X-SCID) had presumably been cured. However, the scientific community was alarmed soon thereafter by reports that two of these infants developed a rare T-cell leukemia-like condition (Hacein-Bey-Abina et al., 2003). Presumably, the integration of the foreign DNA construct had activated a protooncogene in the manipulated cells – perhaps a plausible explanation and in line with long-favoured models in tumour biology.

In this latter context, I submit to consider a different concept. The possibility exists that the insertion of foreign DNA into established mammalian genomes, with a preference at actively

transcribed loci, can alter the chromatin configuration even at sites remote from those immediately targeted by foreign DNA insertion (Doerfler, 1995; 2000). In cells transgenic for adenovirus or bacteriophage lambda DNA, extensive changes in cellular DNA methylation (Heller et al., 1995; Remus et al., 1999) and cellular gene transcription patterns (Müller et al., 2001) have been documented. Foreign DNA insertion at one site may, hence, affect the genetic activity of a combination of loci which might be disseminated over the entire genome. The chromosomal sites of the cellular genes thus afflicted might depend on the location of the initial integration event. Oncogenic transformation of the cell, according to this model, would ensue because of alterations in specific combinations of genes and loci and in extensive changes in the transcriptional programme of many different genes.

If valid, this concept could shed doubts on apparently useful procedures in molecular medicine – the generation of transgenic organisms, current gene therapy regimens, perhaps even on the interpretation of some knock-out experiments. The functional complexities of the human, or any other, genome cannot yet be fathomed by the knowledge of nucleotide sequences and the current textbook wisdom of molecular biology. At this stage of our 'advanced ignorance' in biology, much more basic research will be the order of this and, I suspect, many future days, in order to be able to heed the primary obligation in medicine – *primum nil nocere*.

2. Onward to new projects

Today, the concept of an important genetic function for 5-mC in DNA has been generally accepted. Moreover, many fields in molecular genetics have included studies on the fifth nucleotide in their repertoire of current research: regulation of gene expression, structure of chromatin, genetic imprinting, developmental biology, even in *Drosophila melanogaster* (Lyko et a., 2000), an organism whose DNA has been previously thought to be devoid of 5-mC, cloning of organisms, human medical genetics, cancer biology, defence strategies against foreign DNA, and others. Progress in research on many of these topics has been rapid, and the publication of a number of concise reports within the framework of Current Topics in Microbiology and Immunology is undoubtedly timely (Doerfler & Böhm, 2006 a; 2006 b). When screened for 'DNA methylation' in early June of 2007, PubMed[1] responded with a total of 12,357 entries dating back to 1965; a search for 'DNA methylation and gene expression' produced 5,322 citations.

A conventional review article on DNA methylation or on one of its main subtopics, therefore, would have to cope with serious limitations, omissions and oversimplifications. With more than 30 years of experience in active research in the field, I wish to briefly outline questions, problems and possible approaches for further research. Seasoned investigators in the field undoubtedly will have their own predilections. For the numerous newcomers to studies on DNA methylation, my listing might provide an introduction, or more likely might arouse opposition, which will be just as useful as an aid to initiate original research.

1. Chromatin structure

Patterns of DNA methylation in the genome and the topology of chromatin structure and composition are tightly linked. Studies on the biochemical modifications of histones – amino acid sequence-specific acetylations and methylations (Allfrey et al., 1964; and many references since) have revealed the tip of the iceberg. A much more profound understanding of the biochemistry of all the components of chromatin and their possible interactions with unmethylated or methylated DNA sequences will have to be elaborated. I would rate such studies as the number one priority

[1] PubMed is an online reference service of the National Library of Medicine and the National Institutes of Health.

and primary precondition for further progress in the understanding of the biological significance of DNA methylation.

2. Promoter studies

We still do not understand the details of how specific distributions of 5-mC residues in promoter or other upstream and/or downstream regulatory sequences affect promoter activity. It is likely, though still unproven, that there is a specific pattern for each promoter, perhaps encompassing only a few 5€ -CG-3€ dinucleotides, which leads to promoter inactivation. It would be feasible to modify one of the well-studied promoters in single, or in combinations, of 5€ -CG-3€ sequences and follow the consequences for promoter activity with an indicator gene. Moreover, for each methylated 5€ -CG-3€ sequence, the promotion or inhibition of the binding of specific proteins, transcription factors and others will have to be determined. It is still unpredictable whether there is a unifying system applying to classes of promoters or whether each promoter is unique in requiring specific combinations of 5€ -5m-CG-3€ residues for activity or the state of inactivity. Of course, in this context, the question can be answered as to whether the activity of a promoter can be ratcheted down by methylating an increasing number of 5€ -CG-3€ dinucleotides step by step in increments of one.

3. Correlations between DNA methylation and histone modification in eukaryotic promoters

In what functional and enzymatic ways are these two types of modifications interrelated? Can one be functional without the other; is one the precondition for the other one to occur? Ever since the search began for the class of molecules which encodes the genetic information, the 'battle has raged', as it were, between proteins and DNA to exert the decisive impact. A similar, though less fundamental, debate on the essential mechanisms operative in long-term gene inactivation is occupying the minds of researchers today. In most instances, the 5-mC signal is relevant mainly in long-term gene silencing. For frequent fluctuations between the different activity states of a promoter, the DNA methylation signal would be a poor candidate for a regulatory mechanism, because promoter methylation is not easily reversible.

4. On the mechanism of *de novo* methylation of integrated foreign or altered endogenous DNA

One of the more frequent encounters for molecular biologists with DNA methylation derives from the analysis of foreign DNA which has been chromosomally integrated into an established eukaryotic genome. Foreign DNA can become fixed in the host genome not only after infection with viruses, but also in the wake of implementing this integration strategy in the generation of transgenic organisms. In knock-in and knock-out experiments, in regimens of gene therapy and others, investigations on this apparently fundamental cellular defence mechanism against the activity of foreign genes – *de novo* methylation – has both theoretical and practical appeal. During the embryonic development of mammals, methylation patterns present at very early stages are erased and new patterns are re-established *de novo* in later stages. Hence, we lack essential information on a very important biochemical mechanism. There are only few systematic studies on the factors that influence the generation of *de novo* methylation patterns. Size and nucleotide sequence of the foreign DNA as well as the site of foreign DNA insertion could have an impact, but in what way remains uncertain. Other aspects of *de novo* methylation relate to the availability, specificity and topology of the DNA methyl-transferases in the chromatin structure.

5. Levels of DNA methylation in repetitive DNA sequences

Studies on repetitive DNA sequences and their functions are one of the very difficult areas in molecular biology, mainly for the want of new ideas to contribute to their study. Perhaps, the elucidation of the patterns of 5-mC distribution in these sequences could shed light on possibly novel approaches of how to proceed further. Repetitive DNA sequences, particularly

retrotransposon-derived DNA or endogenous retroviral sequences, are in general heavily methylated. Exact studies on the methylation and activity of specific segments in the repetitive DNA are available only to a limited extent. The difficulty for a systematic analysis certainly lies in the high copy number and the hard to prove or disprove possibility that individual members of a family of repetitive sequences might exhibit different patterns.

6. Foreign DNA insertions can lead to alterations of DNA methylation *in trans*

Studies on this phenomenon have occupied our laboratory for several years, and we are still investigating whether these alterations might be a general consequence of foreign DNA insertions or occur only under distinct conditions. We, therefore, propose to pursue the following strategies:

(i) Random insertion of a defined cellular DNA segment with a unique or a repetitive sequence at different chromosomal sites and follow-up of changes in DNA methylation in different locations of the cellular genome. In this context, methylation patterns in unique genes and in retrotransposons or other repetitive sequences will be determined.

(ii) In individual transgenic cell clones transgene location should be correlated with methylation and transcription patterns in the selected DNA segments. Could the chromosomal insertion site of the transgene be in contact with the regions with altered DNA methylation at the level of interphase chromosomes?

(iii) Studies on histone modifications in or close to the selected DNA segments in which alterations of DNA methylation have been observed.

(iv) Influence of the number of transgene molecules, i.e. the size of the transgenic DNA insert, at one site on the extent and patterns of changes in DNA methylation in the investigated *trans*-located sequences.

(v) I consider this topic of fundamental importance because its pursuance could shed light on unforeseen and unforeseeable problems arising during the generation of transgenic (gene manipulated) organisms, the cloning of organisms and in gene therapeutic strategies, possibly also in knock-in and knock-out experiments that are so frequently the basis of medically relevant conclusions. While the technical advantages and potential economic spin-off in the mentioned fields have been heralded in an exaggerated way, basic research dealing with the consequences of foreign DNA insertion has been deplorably under-represented.

7. Stability of transgene and extent of transgene methylation

Hypermethylated transgenes appear to be more stably integrated than hypomethylated ones (Hochstein et al., 2007). A refined approach to this problem could be to fix genomically differently pre-methylated transgenes and follow their stability in individual cell clones.

8. Enzymes involved in *de novo* methylation of integrated foreign DNA

It is still uncertain which DNA methyltransferases or which combinations of these enzymes are involved in the *de novo* methylation of integrated foreign DNA. Enzyme concentration by itself might not be the rate-limiting step. Rather, chromatin structure and the topical availability of DNA methyltranferases could be the important factors that need to be investigated.

9. The role of specific small RNAs in triggering DNA methylation

There is a lack of studies on this problem in mammalian systems.

10. Complex biological problems connected to DNA methylation

A great deal of very interesting research on DNA methylation derives from the work on epigenetic phenomena, on genetic imprinting, and more generally, from the fields of embryonal

development, medical genetics and tumour biology. From the presently available evidence, DNA methylation or changes in the original genomic patterns of DNA methylation are most likely implicated in any one of these phenomena.

Concluding remarks

The structural and functional importance of the 'correct' patterns of DNA methylation in all parts of a mammalian genome is, unfortunately, not well understood. The stability, inheritability, and developmental flexibility of these patterns all point to a major role that these patterns play in determining structure and function of the genome. Up to the present time, studies on the repetitive sequences, which comprise > 90% of the DNA sequences in the human or other genomes, have been neglected. We only have a vague idea about the patterns of DNA methylation in these abundant sequences, except that the repeat sequences are often hypermethylated, and that their patterns are particularly sensitive to alterations upon the insertion of foreign DNA into an established genome. Upon foreign DNA insertion into an established genome, during the early stages of development, or when the regular pathways of embryonal and/or foetal development are bypassed, e.g. in therapeutic or reproductive cloning, patterns of DNA methylation in vast realms of the genome can be substantially altered. There is very little information about the mechanisms and conditions of these alterations, and investigations into these areas could be highly informative. By the same token, a thorough understanding of these problems will be paramount and a precondition to fully grasp the plasticity of mammalian genomes. Moreover, it is hard to imagine that, without this vital information at hand, we will be able to apply successfully our knowledge in molecular genetics to the solution of medical problems. A vast amount of basic research still lies ahead. I suspect that, in the futile hope of making 'quick discoveries' and, consequently, in neglecting to shoulder our basic homework now, we will only delay the breakthroughs in biomedical research that all of us hope for.

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