Role of DNA methylation in the regulation of transcription

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DNA methylation plays an important role in the regulation of gene expression during development. Methyl moieties at CpG residues suppress transcription by affecting DNA–protein interactions, thus altering the accessibility of genes to *trans*-acting factors in the cell. Because it works in *cis*, this mechanism is important in the control of X inactivation and genomic imprinting.

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Introduction

5-methylcytosine, which is found exclusively at CpG residues, is an integral part of the animal cell genome. The cell type specific patterns of DNA methylation that are seen in the adult organism are established in a programmed manner during development. Although most of the DNA is unmethylated at the blastula stage, a wave of *de novo* activity following implantation of the embryo systematically remodifies the bulk of the genome, while leaving unmethylated all of the CpG islands associated with housekeeping genes [1•,2]. At later stages of embryogenesis, this bimodal modification profile is further refined by the demethylation of tissue-specific genes in the cell types in which they are expressed. As a result of these events, the final adult modification pattern is characterized by full methylation of those genes that are inactive and undermethylation of those genes that are active [3].

The inverse correlation between DNA methylation and gene expression is striking and suggests that modification itself may play a direct role in the inhibition of transcription. This has been verified by experiments demonstrating that *in vitro* methylation of a variety of gene sequences inhibits their expression following transfection into fibroblast cells [4]. Conversely, many individual endogenous genes can be activated from their repressed state by treatment with 5-azacytidine, a powerful inducer of demethylation [5]. Taken together, these studies indicate that methylation serves as a global suppressor of gene expression. Furthermore, this function is evidently required for normal development, as mice containing site-directed disruptions of the DNA methyltransferase gene are unable to maintain a proper methylation pattern and, as a result, fail to progress past midgestation [6••].

Methylation inhibits basal transcription

The influence of DNA modification on gene transcription is probably mediated through an effect of methyl moieties on DNA–protein interactions. DNA methylation produces a reversible protrusion from the major groove of DNA and is, therefore, probably capable of altering local protein recognition signals in much the same way as it can block the action of a restriction enzyme when present at the sequence-specific site of cleavage. Using gene constructs with hybrid methylation patterns, it has been possible to carefully map the sites of action of DNA modification. For many genes, the major target for this modification appears to be the upstream regulatory region [7–9]. This finding clearly suggests that methyl groups can interfere with the initiation of transcription by preventing the binding of cellular factors. Such interference in binding has indeed been demonstrated experimentally for a number of known proteins, such as AP-2 [10], c-Myc/Myn [11], E2F [12], and NF-κB [13], all of which recognize sequences that contain CpG residues. Furthermore, under certain conditions, DNA modification may directly inhibit the action of RNA polymerase itself [14]. This is not the case for all of the proteins that interact with the transcription machinery, however, and several common factors, including Sp1 [15] and CTF [16], are not at all affected by DNA methylation.

Whereas DNA methylation can clearly prevent specific protein binding and subsequent activation, it is striking that all of the factors affected by modification appear to be present in different cell types. An insight into the way in which this mechanism might operate has been provided by the example of the liver-specific tyrosine aminotransferase gene [17]. *In vivo* footprinting experiments have demonstrated the presence of at least three hepatocyte-specific regions upstream of this gene, which yield a DNase I protected pattern. However, when cloned DNA from the same region was footprinted *in vitro* using extracts from liver and fibroblast, identical blocking patterns were observed, indicating that the factors that interact with this fragment are present in both cell types. What then prevents the binding of these protein factors in fibroblast cells *in vivo*? The fact that this gene is fully methylated in fibroblasts, but not in hepatic cells, clearly suggests that DNA modification is involved, and this has been proven by the demonstration that methylation of *Hha*I sites eliminates the *in vitro* footprint at one of the upstream loci. A series of similar experiments has shown that this is also the mode of action of methylation in the testis-specific H2B histone gene [18]. Assuming that this mechanism is generally true for other tissue-specific genes, it is likely

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that one of the functions of DNA methylation *in vivo* is to suppress the low levels of basal expression that could result from exposure of these sequences to the multitude of general transcription factors used by the different RNA polymerase systems [19] present in each cell.

Effects of methylation on chromatin structure

In addition to its direct effect on factor binding, DNA methylation may also operate by altering overall chromatin structure, thus indirectly influencing gene accessibility. Following DNA-mediated gene transfer into animal cells in culture, unmethylated exogenous sequences always integrate into the genome in a DNase I sensitive conformation. In striking contrast, DNA methylated *in vitro* adopts an inactive chromatin structure, thus remaining resistant to nuclease digestion [20]. This phenomenon cannot be due to interactions with any sequence-specific proteins, as the same results are obtained with any sequence that is inserted into the cells, thus suggesting that DNA modification has a general influence on local chromatin structure. The inhibitory action of methylation may, in fact, be mediated through the formation of a chromatin complex. This can be observed when naked DNA is injected into cells [21]. Initially, both methylated and unmethylated templates are transcribed equally, but as soon as the DNA becomes packaged, the modified gene is rendered inactive. Furthermore, when the injection is carried out using preformed chromatin templates, the effect of methylation is immediate. In this manner, DNA modification may be involved in re-establishing local chromatin structure following DNA replication in each cell generation *in vivo*. Maintenance methylation is completed within 90 seconds of DNA synthesis [22], providing the newly made DNA with the signatures required to direct the generation of active or inactive chromatin, which is completed about 30 minutes later [23].

DNA methylation probably influences chromatin structure in a variety of ways. Early *in vivo* experiments indicated that nucleosomes are distributed preferentially over methylated DNA in the genome [24,25], and transfection studies have confirmed that modification can indeed alter nucleosome positioning, as measured by specific restriction enzyme accessibility in nuclei [20]. There is no doubt that methylation can also modulate the binding of individual chromatin proteins. Histone H1, for example, is known to be primarily associated with inactive DNA and, strikingly, is missing from the nucleosomes of active genes [26]. In addition, a significant body of data suggests that this protein acts as a global repressor of basal transcription [27,28]. When tested *in vitro*, histone H1 appears to bind preferentially to methylated DNA and in this manner more effectively inhibits the initiation of RNA synthesis [29•]. It should be noted that histone H1 interacts with DNA in a non sequence specific manner, and this may explain why the degree of repression can often be increased simply through the addition of more methyl groups at almost any position in the promoter region.

The methylCpG-binding protein, MeCP1, binds specifically to DNA sequences that contain multiple symmetrically methylated CpGs. This protein is abundant in somatic cells and is probably one of the factors that acts to inhibit transcription [30]. In transient transfection experiments, for example, the repression of expression from a methylated gene construct can be alleviated by titrating out the MeCP1 using non-specific methylated DNA. F9 embryonic cells appear to have an extremely low level of MeCP1, and transient transfection experiments show that, using these cells, transcription of the same vectors is not inhibited by the presence of DNA methylation in the region of the gene. Gene repression mediated by MeCP1 probably depends both on the density of modification and the strength of the promoter. Thus, even low levels of methylation may suppress a weak promoter, while a strong promoter may overcome the effect of methylation completely [31]. Other cellular proteins are known to bind methylated DNA: MeCP2, which is more abundant than MeCP1 and is associated with chromosomal satellite regions *in vivo* [32], and the methylated DNA binding protein, MDBP1, which appears to interact with a specific degenerate sequence containing either TpG or methylated CpG residues [33].

Another way in which DNA modification may alter gene function is by modulation of DNA conformation, which is independent of protein binding. It is known, for example, that methylation has a large effect *in vitro* on the formation of Z-DNA [34] and triplex structures [35], and on DNA bending [36]. It is not yet clear, however, whether these characteristics actually play a role in the regulation of gene expression *in vivo*.

Changes in DNA methylation during development

De novo methylation of the entire genome is an early event in embryogenesis, and this modification probably sets in motion a chain of reactions that direct most tissue-specific genes to an inactive conformation. Even when these genes are artificially demethylated by 5-azacytidine in a normally non-expressing cell type, they do not automatically undergo activation, probably because of irreversible changes in chromatin structure that took place during normal development [37]. When these same genes are transfected into a cell in an unmodified form, they by-pass this process and are able to carry out basal transcription. What, then, is the normal mechanism for turning on tissue-specific genes during development? One possibility is that these sequences undergo programmed demethylation as part of the differentiation process. The rat α actin gene, for example, is strongly inhibited by methylation when transfected into fibroblasts [38]. In myoblasts, however, *cis*-acting sequences near the gene are recognized by cellular factors that direct active demethylation, and kinetic experiments show that this gene is transcribed only after the demodification process is complete [39]. Furthermore, a mutant gene construct that lacks the specific elements required for demethylation remains permanently inactive in myo-

blasts, despite the fact that these cells contain all of the factors necessary for maximum transcription. In a similar manner, expression of the κ chain in B lymphocytes requires prior demethylation. In this case also, demodification is a programmed part of B-cell differentiation that is independently directed by discrete *cis*-acting sequences. The involvement of the intronic κ enhancer in this process suggests that the same DNA elements may play a dual role during development, first by inducing demethylation, and then by enhancing transcription [40].

Demethylation of CpG sites in tissue-specific genes does not always precede gene activation. The rat phosphoenolpyruvate carboxykinase gene, for example, is fully expressed in the liver at the time of birth, yet many CpG residues in the gene are still modified and the methyl groups are not removed from the DNA until the animals are a few days old [41]. A similar observation has been made for the rat albumin [42] and chicken vitellogenin [43] genes. In the latter case, genomic sequencing has established that 11 CpG sites in the promoter region are already fully demethylated at the time of transcriptional initiation, whereas four other loci are at an intermediate stage of hemi-methylation [44]. It is not known for any of these genes whether these particular methyl groups play any role in gene regulation, but, if this is the case, the data would indirectly suggest that activation is induced by factors that override the effects of methylation. In this scheme, only after the gene has been turned on in this temporary manner would it undergo demethylation and thus allow fluent transcription in the absence of the initial differentiation factors.

The overriding of methylation may also play a role in the transcription of viral sequences. The frog virus 3 genome, although completely methylated at every cytosine residue, encodes a factor that activates transcription and allows the virus to propagate in animal cells [45]. The adenovirus type 2 [46] and the human immunodeficiency virus (HIV) [47] also appear to be transcribed despite the presence of methyl moieties that would normally inhibit RNA synthesis. For HIV, it is probably the viral *tat* gene that mediates this overriding effect.

DNA methylation in imprinted genes

It is obvious that methylation plays a unique role in the control of gene expression by acting in *cis* to modulate the accessibility of DNA to the *trans*-acting factors present in the cell. The significance of this mechanism is most evident in the case of X chromosome regulation, in which housekeeping genes on one X chromosome are active, whereas their homologues on the other X chromosome are kept inactive in the same cell.

Although methylation does not appear to play a role in the initial inactivation process [48,49], which takes place in the blastula, modification is clearly responsible for maintaining the silent state of many genes on the inactive X chromosome [50]. The repressed DNA is fully methylated and the genes lack bound transcription factors in their upstream domains [51]. In addition, they are functionally inactive, as measured by transfection assays [52]. Once an X chromosome is marked by DNA modification in the post-implantation embryo, the normal semiconservative methyltransferase activity will automatically maintain its inactive state in all sister cells. It therefore represents an ideal mechanism for controlling allele-specific gene activity.

Allele-specific transcription is also characteristic of genomically imprinted genes in animal cells. As these genes exhibit allele-specific differential methylation patterns in late embryonic and adult cells, it has been suggested that modification plays some role in the regulatory process. For the mouse *H19* gene, for example, only the inactive paternal allele is uniformly methylated, and this appears to be consistent with the mode of action of DNA modification as a *cis*-acting suppressor [53,54]. In contrast, both the *Igf2* [55] and *Igf2r* [56•] genes contain small discrete domains that are preferentially methylated in an anomalous manner on the active allele. Assuming that these sites play some role in the transcription process, it is possible that such elements represent binding sites for a repressor and that, by preventing its interaction with this element, DNA methylation maintains these genes in an active state. This model is consistent with recent studies on the expression of these endogenous imprinted genes in a methyltransferase-deleted mouse [57••]. As a result of low levels of maintenance methylation in homozygous embryos, the *H19* gene becomes active at both alleles, but both *Igf2* and *Igf2r* are now completely silenced. An examination of *Igf2r* methylation in the mutant embryos confirmed that there was a lack of methyl groups at the appropriate sites in the gene, which is probably responsible for the repression of the normally active maternal allele.

Conclusions

The adult genome has a bimodal pattern of DNA methylation, in which most tissue-specific genes are modified, whereas all housekeeping genes contain unmethylated CpG islands. This profile is established in the early embryo and serves as a global mechanism for the regulation of gene expression. DNA methylation appears to inhibit RNA synthesis by preventing the binding of basal transcription factors and by altering chromatin structure. During differentiation, tissue-specific genes undergo programmed active demethylation which, in several cases, represents an essential step in their activation. DNA methylation may also play a role in genomic imprinting, both as a means for transmitting allele-specific signals from gamete to embryo, and as a *cis*-acting mechanism for maintaining the imprinted transcription pattern in each cell.

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