How does DNA methylation repress transcription?

STEFAN U. KASS (skass@janbclc1.ssw.jnj.com)

DMITRY PRUSS (pruss@online.ru)

ALAN P. WOLFFE (awlmc@helix.nih.gov)

DNA methylation has an essential regulatory function in mammalian development, serving to repress nontranscribed genes stably in differentiated adult somatic cells. Recent data implicate transcriptional repressors specific for methylated DNA and chromatin assembly in this global control of gene activity. The assembly of specialized nucleosomal structures on methylated DNA helps to explain the capacity of methylated DNA segments to silence transcription more effectively than conventional chromatin. Specialized nucleosomes also provide a potential molecular mechanism for the stable propagation of DNA methylation-dependent transcriptional silencing through cell division.

The covalent modification of DNA provides a direct and powerful mechanism to regulate gene expression. Considerable experimental evidence supports the existence of such a mechanism in the majority of plants and animals[1–3]. The genome of an adult vertebrate cell has 60–90% of the cytosines in CpG dinucleotides methylated by DNA methyltransferase[4]. This modification can alter the recognition of the double helix by the transcriptional machinery and the structural proteins that assemble chromatin[5,6]. How these events might together contribute to gene control is the major theme of this review article.

DNA methylation could control gene activity either at a local level through effects at a single promoter and enhancer, or through global mechanisms that influence many genes within an entire chromosome or genome. An attractive suggestion is that DNA methylation evolved as a host-defense mechanism in metazoans to protect the genome against genomic parasites, such as transposable elements[1]. An increase in methyl-CpG correlates with transcriptional silencing for whole chromosomes, transgenes, particular developmentally regulated genes and human disease genes[3,7–9]. All of these systems exhibit epigenetic effects on transcriptional regulation in which identical DNA sequences are differentially utilized within the same cell nucleus. These patterns of differential gene activity are clonally inherited through cell division. Because specific methyl-CpG dinucleotides are maintained through DNA replication, DNA methylation states also provide an attractive mechanism (epigenetic mark) to maintain a particular state of gene activity through cell division and, thus, to contribute to the maintenance of the differentiated state[10]. We discuss how the molecular mechanisms that accomplish this important goal might also involve the assembly of specialized chromatin structures on methylated DNA.

Why is DNA methylation important?

Saccharomyces cerevisiae and *Drosophila melanogaster* live without any detectable methyl-CpG in their genomes. DNA-methylation-dependent gene regulation is not necessarily essential for cell division or metazoan development, because other gene regulatory mechanisms can compensate for the lack of DNA methylation in these organisms. In the mouse, primordial germ cells, embryonal stem cells and the cells of the blastocyst also progress through the cell cycle and divide without detectable DNA methylation[11,12]. Nevertheless once embryonic stem cells begin to differentiate, normal DNA methylation levels are essential for individual cell viability[13]. The *de novo* methylation of CpG dinucleotides is a regulated process[3]. In the embryo, normal DNA methylation levels are essential for post-gastrulation development[14]. Jaenisch[12] proposed that DNA methylation has no role in cell viability in mammalian embryonic lineages including the germ line, but that it has an important role in the differentiation of somatic cells. In complex organisms, such as vertebrates, which contain a large number of tissue-specific genes, DNA methylation provides a mechanism to turn off permanently the transcription of those genes whose activity is not required in a particular cell type[2]. This stable silencing of a large fraction of the genome would allow the transcriptional machinery to focus on those genes that are essential for the expression and maintenance of the differentiated phenotype. Consistent with this hypothesis, the inhibition of DNA methyltransferase activity with 5-azacytidine leads to the activation of several repressed endogenous genes[15]. How might CpG methylation contribute to this global control of gene activity?

Molecular mechanisms for transcriptional repression by DNA methylation

The most direct mechanism by which DNA methylation could interfere with transcription would be to prevent the binding of the basal transcriptional machinery and of ubiquitous transcription factors to promoters. This is not a generally applicable mechanism because some promoters are transcribed effectively as naked DNA templates independent of DNA methylation[6,16]. Certain transcription factors (e.g. the cyclic AMP-dependent activator CREB) bind less well to methylated recognition elements; however, the reduction in affinity is often insufficient to account for the inactivity of promoters *in vivo*[17,18]. It seems unlikely that DNA methylation would function to repress transcription globally by modifying the majority of CpGs in a chromosome, if the only sites of action are to be a limited set of recognition elements for individual transcription factors.

The second possibility is that specific transcriptional repressors exist that recognize methyl-CpG and, either independently or together with other components of chromatin, turn off transcription. This mechanism would have the advantage of being substantially independent of DNA sequence itself, thereby offering a simple means of global transcriptional control. It would be especially attractive if the methylation-dependent repressors work in a chromatin context because then DNA could maintain the nucleosomal and chromatin fiber architecture necessary to compact DNA. Moreover, because chromatin assembly also represses transcription, methylation-dependent repression mechanisms would add to those already in place.

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Bird and colleagues have identified two repressors MeCP1 and MeCP2 that bind to methyl-CpG without apparent sequence specificity[19–21]. Like DNA methylation itself, MeCP2 is dispensable for the viability of embryonic stem cells; however, it is essential for normal embryonic development[22]. Consistent with the capacity of methylation-dependent repressors to operate in chromatin, recent studies indicate that MeCP2 is a chromosomal protein with the capacity to displace histone H1 from the nucleosome[5,23]. Moreover, MeCP2 contains a methyl-CpG DNA-binding domain, which might alter chromatin structure directly, and a repressor domain, which might function indirectly to confer long-range repression *in vivo*[5,24]. The capacity for MeCP2 to function in chromatin explains several phenomena connected with unique aspects of chromatin assembled on methylated DNA.

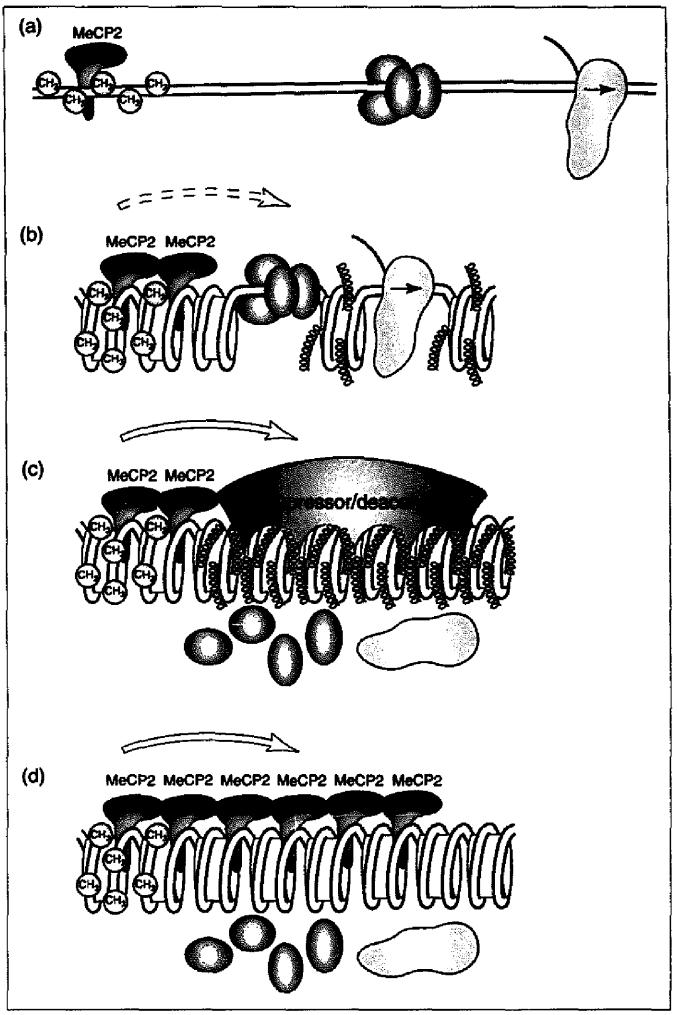

FIGURE 1. Molecular mechanisms by which chromatin assembly might facilitate transcriptional repression by MeCP2 (see the text for detailed discussion). The nucleosomes are shown in yellow, the histone tails as coils. (a) MeCP2 (purple) recognizes methylated DNA (CH_3) on naked DNA that is too far away from the basal transcriptional machinery (light blue) and RNA polymerase II (orange) to repress transcription. (b) The assembly of nucleosomes compacts the intervening DNA bringing the basal machinery and RNA polymerase closer to the repressive MeCP2. Transcription can now be repressed albeit weakly. (c) MeCP2 might recruit a transcriptional co-repressor and/or histone deacetylase, or other chromatin modification complex that might interact with nucleosomes, to assemble a more stable repressive chromatin structure, which allows strong repression. This structure erases the basal machinery and RNA polymerase from DNA. (d) MeCP2 might spread from the methylated DNA segment into adjacent chromatin, directly excluding the basal machinery and RNA polymerase from DNA, thus providing strong repression.

DNA methylation influences chromatin structure

A role for specialized chromatin structures in mediating transcriptional silencing by methylated DNA has been suggested by several investigators. High levels of methyl-CpG correlate with transcriptional inactivity and nuclease resistance in endogenous chromosomes[25]. Methylated DNA transfected into mammalian cells is also assembled into a nuclease-resistant structure containing unusual nucleosomal particles[26]. These unusual nucleosomes migrate as large nucleoprotein complexes on agarose gels. These complexes are held together by higher-order protein–DNA interactions despite the presence of abundant micrococcal nuclease cleavage points within the DNA. Individual nucleosomes assembled on methylated DNA appear to interact together more stably than on unmethylated templates[26]. Each nucleosome normally contains an octamer of core histones (H2A, H2B, H3 and H4) around which is wrapped approximately 160 bp of DNA, and a single molecule of histone H1, which constrains the linker DNA between adjacent nucleosomes. The replacement of histone H1 with MeCP2 is a possible explanation for the assembly of a distinct chromatin structure on methylated DNA (Refs 5, 26).

The accessibility of chromatin to nucleases could also be affected directly by the stability with which the histones interact with DNA within the nucleosome. DNA methylation does not influence the association of core histones with the vast majority of DNA sequences in the genome[27]. However, for certain specific sequences, such as those found in the promoter of the fragile X mental retardation gene 1, methylation of CpG dinucleotides can alter the positioning of histone–DNA

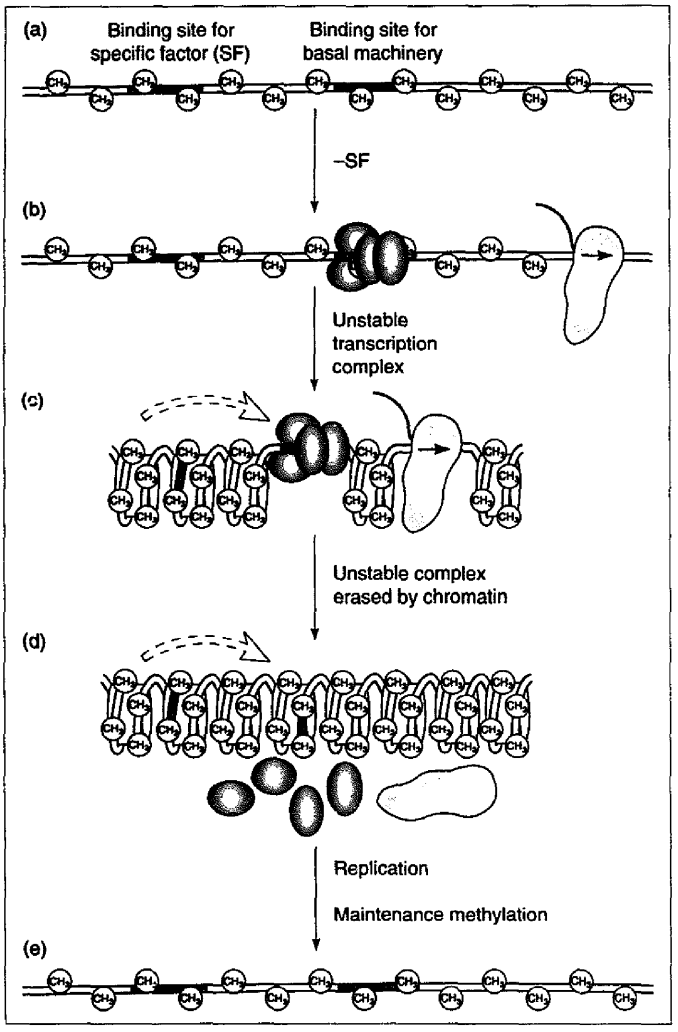

FIGURE 2. Methylation-dependent, dominant silencing of transcription. (a) A promoter containing binding sites for the basal transcriptional machinery (light blue) and for specific transcription factors (SF, red). Many potential sites of methylation of CpG are present (CH_3). (b) The basal transcriptional machinery (light blue) and RNA polymerase (orange) can potentially bind to methylated DNA and promote transcription in the absence of the specific factor. However, the transcription complex under these conditions is relatively unstable without the specific factor. (c, d) As chromatin is assembled on the methylated DNA any of the molecular events illustrated in Fig. 1 might help to exclude the transcriptional machinery from the promoter. (e) In the absence of transcription factor–DNA interactions the maintenance methyltransferase can gain access to DNA immediately after replication and methylate CpG sequences, such that the process (a–e) repeats itself with every round of replication.

contacts and the affinity with which these histones bind to DNA (Ref. 28). The exact chromatin structure found *in vivo* can also be a consequence of gene activity. Linker histones, such as H1, are relatively deficient on the transcribed region of genes[29]. So it is not surprising that transcriptionally inactive chromatin containing methyl-CpG should show an increase in the abundance of histone H1, whereas DNA sequences lacking methyl-CpG are deficient in H1 (Refs 30, 31). *In vitro* studies indicate that histone H1 can interact preferentially with methylated DNA under certain conditions, although there is no measurable preference for the assembly of H1 into a nucleosomal architecture containing methylated DNA (Refs 32–35). Recent *in vivo* studies indicate that rather than functioning as a general transcriptional repressor, histone H1 is highly specific with respect to the genes whose activity it regulates (reviewed in Ref. 36). It seems probable that the major differences between chromatin assembled on methylated versus unmethylated DNA will be determined by the inclusion of methylation-specific DNA-binding proteins, such as MeCP2.

DNA methylation influences chromatin function

There are features of transcriptional repression dependent on methylated DNA that can be explained by methylation-specific repressors operating more effectively within a chromatin environment. Transcriptional repression is strongly related to the density of DNA methylation[37,38]. There is a nonlinear relationship between the lack of repression observed at low densities of methyl-CpG and repression at higher densities. These results led to the demonstration that local domains of high methyl-CpG density could confer transcriptional repression on unmethylated promoters *in cis*[6,39]. This observation is consistent with MeCP2 containing DNA-binding and transcriptional repression domains[5]. Thus MeCP2 does not necessarily have to function by occluding regulatory elements from the transcriptional machinery (by binding to promoter sequences itself), but might bind to a methyl-CpG sequence at one place on a DNA molecule and then use the repression domain to silence transcription at a distance. Chromatin assembly itself might promote this 'action at a distance' by juxtaposing MeCP2 and the regulatory elements under control through the compaction of intervening DNA (see Ref. 40) (Fig. 1a, b).

Early experiments using the microinjection of templates into the nuclei of mammalian cells suggested that the prior assembly of methylated, but not unmethylated, DNA into chromatin represses transcription[41]. The importance of a nucleosomal infrastructure for transcriptional repression dependent on DNA methylation

was reinforced by the observation that immediately after injection into *Xenopus* oocyte nuclei, methylated and unmethylated templates both have equivalent activity[6]. However, as chromatin is assembled, the methylated DNA is repressed with the loss of DNase I hypersensitivity and the loss of engaged RNA polymerase. The requirement for nucleosomes to exert efficient repression can be explained in several ways. The repression domain of MeCP2 might recruit a co-repressor complex that directs the modification of the chromatin template into a more stable and transcriptionally inert state (Fig. 1c). One potential candidate co-repressor for MeCP2 is the SIN3 histone deacetylase complex, because inhibition of histone deacetylation can reverse some of the transcriptional repression conferred by DNA methylation[38,42]. Alternatively, like histone H1, MeCP2 might bind more efficiently to nucleosomal rather than to naked DNA (Ref. 36). Any cooperative interactions between molecules could propagate the association of MeCP2 along the nucleosomal array even into unmethylated DNA segments (Fig. 1d). This latter mechanism is analogous to the nucleation of heterochromatin assembly at the yeast telomeres by the DNA-binding protein Rap1p, which then recruits the repressors Sir3p and Sir4p that organize chromatin into a repressive structure[43]. All of these potential mechanisms could individually or together contribute to the assembly of a repressive chromatin domain. Although these molecular mechanisms are speculative, they illustrate the advantages of a nucleosomal infrastructure. It should be noted that MeCP2 can repress transcription in an *in vitro* extract, although this might be by direct occlusion of a transcription factor binding over the methylated promoter[5,20].

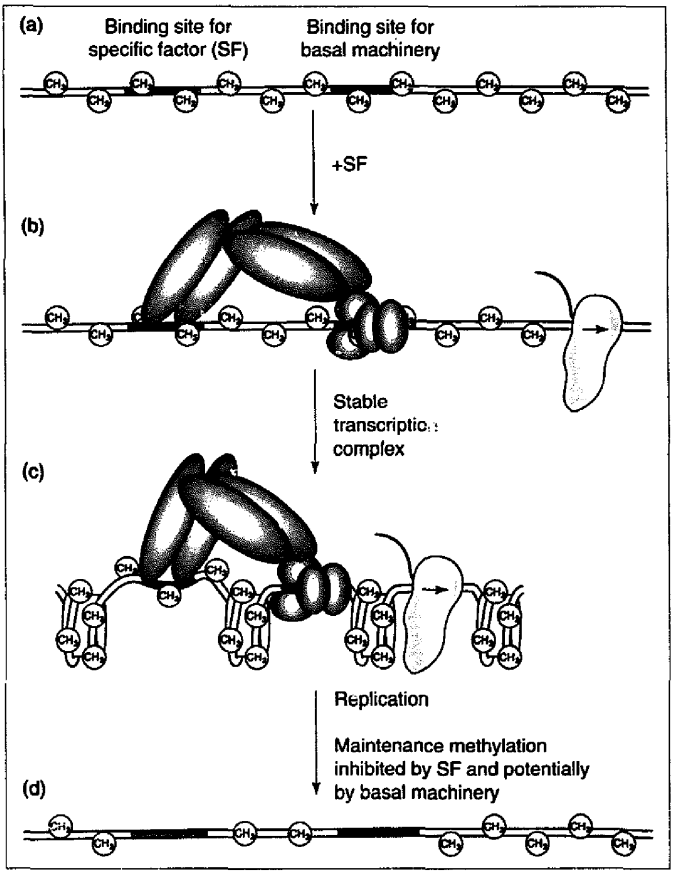

FIGURE 3. DNA demethylation that is dependent on sequence-specific transcription factors. (a) A promoter containing binding sites for the basal transcriptional machinery (light blue) and for specific transcription factors (SF, red). Many potential sites of methylation of CpG dinucleotides are present (CH_3). (b) The specific transcription factor (red) greatly stabilizes the interactions of the basal transcriptional machinery (light blue) with the promoter. This facilitates transcription of the gene by RNA polymerase II (orange). (c) Transcription is maintained in spite of the assembly of chromatin on methylated DNA either because the stable assembly of transcription factors and basal machinery resists displacement, and/or because these factors also recruit enzymatic activities that counteract the assembly of repressive chromatin structures. (d) The stable association of components of the transcriptional machinery with the promoter immediately after DNA replication might serve to exclude the maintenance of methyltransferase and direct progressive demethylation of the promoter.

If methylated DNA directs the assembly of a specialized repressive chromatin structure, it might be anticipated that the transcriptional machinery will have less access to such a structure than the orthodox chromatin assembled on unmethylated promoters and genes[25,26]. Activators, such as Gal4p–VP16, can normally penetrate a preassembled chromatin template to activate transcription, even in the presence of histone H1 (Ref. 40). However, once chromatin has been assembled on methylated DNA, Gal4p–VP16 can no longer gain access to its binding sites and activate transcription[6]. This suggests that the specialized features of chromatin assembly on methylated DNA provide a molecular lock to silence the transcription process permanently[44]. This capacity of DNA methylation to strengthen transcriptional silencing in a chromatin context could be an important contributor to the separation of the genome into active and inactive compartments in a differentiated cell[1,2].

The maintenance of DNA methylation and transcriptional repression in chromatin

DNA methyltransferase maintains the methyl-CpG content of both daughter DNA duplexes following replication[10]. Methyltransferase localizes to the chromosomal replication complex and maintenance methylation takes place less than one minute following replication[45,46]. By contrast, chromatin assembly takes 10–20 minutes in

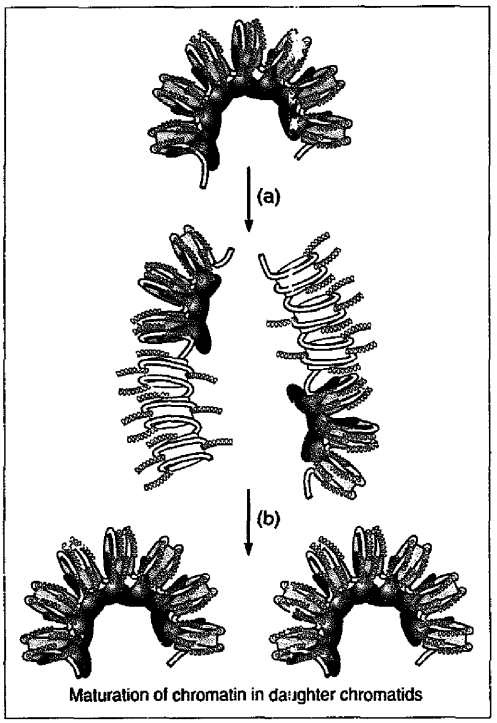

FIGURE 4. A model for the maintenance of specialized nucleosome structures on methylated DNA through replication. (a) Replication leads to the random distribution of parental nucleosomes (orange) in small groups to daughter chromatids. Methylated-DNA-binding proteins, such as MeCP2 (purple), might also be distributed to daughter chromatids. New nucleosomes (50% of total) are shown in yellow. (b) It is possible that methylated-DNA-binding proteins that segregate with parental nucleosomes help to re-establish a repressive chromatin structure. We show MeCP2 spreading to adjacent nucleosomes, although the re-establishment of transcriptional repression could be via any of the mechanisms illustrated in Fig. 1.

a mammalian tissue culture cell[47]. Histone deposition occurs in stages, and it is not until a complete histone octamer is assembled with DNA that histone H1 is stably sequestered[48]. Comparable limitations might restrict the stable association of methylation-specific repressors[5]. This would account for the lag time before methylated DNA is repressed following injection as a naked template into the nuclei of mammalian tissue culture cells or *Xenopus* oocytes[6,41].

A significant feature of transcriptional repression on methylated DNA is that it is not only time dependent but also potentially dominant[6]. Thus, at early times when chromatin assembly is incomplete, the transcriptional machinery has the potential to associate with methylated regulatory DNA. As chromatin structure matures, the basal transcriptional machinery is potentially erased from the template. This provides a general mechanism for the global silencing of transcription dependent only on DNA methylation state (Fig. 2). The strong activator Gal4p–VP16 cannot function if chromatin is assembled on methylated DNA before exposure to the activator, but if Gal4p–VP16 is present during chromatin assembly then transcriptional activity can resist methylation-dependent transcriptional silencing (Fig. 3; Ref. 6). Therefore, under certain circumstances, regulatory nucleoprotein complexes might be assembled that resist this powerful silencing mechanism. Such a mechanism has been suggested to be dependent on SP1 sites in the promoter of a housekeeping gene in the mouse (adenine phosphoribosyltransferase) that is maintained in a methylation-free state[49]. For example, if components of regulatory complexes could bind to DNA immediately after replication with reasonable efficiency and before DNA methyltransferase can begin to modify the template, then they might prevent DNA methylation around their binding sites. These sequences might then become progressively demethylated and eventually resist transcriptional repression (Fig. 3). This would provide a mechanism for the demethylation of regulatory DNA in particular differentiated cell lines.

Other mechanisms might contribute to the maintenance of transcriptional repression through DNA synthesis. The assembly of a specialized chromatin structure on methylated DNA might result in the presence of additional proteins (e.g. MeCP2) and histone modifications (e.g. histone deacetylation) that could be maintained in daughter chromatids. Nucleosomes segregate dispersively in small groups to daughter DNA molecules at the replication fork[50]. Particular modified histones and repressors, such as MeCP2, would be anticipated to segregate within the nucleosomal context[51]. These proteins could therefore provide at least 50% of the chromatin proteins necessary to restrict transcription (Fig. 4). Their continued presence on DNA could help to re-establish transcriptional repression on both daughter chromatids through any of the mechanisms illustrated in Fig. 1. Therefore, demethylation alone might be insufficient to relieve transcriptional repression until successive cell divisions eventually unravel the repressive chromatin structure.

Although we focus on molecular mechanisms that might influence DNA methylation and gene expression in dividing cells, DNA demethylation is also important in nondividing terminally differentiated cells. Under these circumstances, demethylation at particular promoters must occur in the absence of replication[52,53]. Presumably mechanisms must also exist to destabilize any repressive chromatin structure associated with methylated DNA in order to allow the demethylation machinery access to the template.

Outlook

The importance of DNA methylation and methylation-specific DNA-binding proteins for the viability of a differentiated mammalian somatic cell is well documented[14,22]. An attractive explanation for the importance of DNA methylation is that it helps to turn off transcription from the large number of genes not required in a particular differentiated cell through global mechanisms[2]. The major problem is in determining how this global repression might first be achieved and then maintained through successive cell generations. We have described evidence for methylation-specific and chromatin-dependent transcriptional repression mechanisms operating *in vivo*. Recent studies discussed here suggest that these two

mechanisms operate together to regulate gene expression more tightly[5,6]. There is now excellent precedent for transcriptional activators and repressors operating most effectively in a nucleosomal environment[42,43,54]. Clearly, future experiments should explore how MeCP2 is incorporated into a nucleosomal array, together with the physical and functional consequences of this inclusion. The potential interaction of the MeCP2 repression domain with co-repressor complexes that might modify chromatin is also an area of active interest. Finally, a mutual dependence on DNA methylation and chromatin assembly for transcriptional silencing provides a potential mechanism not only for the stable propagation of the repressed state through cell division, but also for the targeted demethylation of promoter DNA. The availability of replication systems capable of propagating methylation states as well as directing chromatin assembly[6,55] will allow this model to be tested directly.

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In a recent paper, Roundtree and Selker [*Genes Dev.* (1997) 11, 2383–2395] demonstrate that, in *Neurospora crassa*, DNA methylation inhibits the process of transcription elongation rather than initiation itself. This result is most easily explained by chromatin modifications dependent on DNA methylation inhibiting RNA polymerase II processivity.

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S.U. Kass, D. Pruss *and* ***A. P. Wolffe*** *are in the Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, National Institutes of Health, Building 18T, Room 106, Bethesda, MD 20892-5431, USA.*