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Regulation and Function of DNA Methylation in Vertebrates

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In vertebrates, genomic DNA is often methylated at the 5th position of cytosine in the sequence of CpG, and this is the only chemical modification that genomic DNA of vertebrates allows under physiological conditions. During evolution, vertebrates acquired CpG methylation as a new tool for controlling gene expression in addition to the varieties of transcription factors. In mammals, the methylation pattern of genomic DNA is erased and reset in germ line and at the early stage of embryogenesis. Maintenance-type methylation activity ensures clonal transmission of the lineage-specific methylation pattern in somatic cells. The methylation pattern is dynamic and changes during cell differentiation. Prior to the expression of tissue-specific genes, specific sites of the promoters are demethylated. In general, the methylation of a gene suppresses its expression. However, not much is known about the mechanisms that regulate the methylation state and the gene expression by DNA methylation.

Key words: DNA methylation, DNA methyltransferase, gene expression, vertebrates.

Methylation of cytosine residues in genomic DNA

A modification of cytosine methylation at its 5th position (5mC) was first found in 1948 in calf thymus DNA ("A chronicle of DNA methylation" is nicely reviewed by Weissbach in Ref. *1*) (Fig. 1). In 1951, the same modification was found in the DNA in wheat germ, and *N*-methylation at the 6th position of adenine also was found in bacterial DNA in 1955. As for bacterial DNA methylation, it has been elucidated that 5mC is utilized for a modification/restriction system and *N*-6-adenine methylation for the identification of a DNA strand to be repaired when mismatch base pairing occurs. The function of 5mC found in higher animals and plants, which was found about 50 years ago, is not fully understood yet.

When we look at the evolutionary tree, 5mC, which exists in bacteria (procaryotes), cannot be detected in yeasts, nematodes or insects (*2*). 5mC not *N*-6-methyladenine is found in echinoderms (*3*) and chordates (*4*). The spread of DNA methylation in the genome occurred in parallel with an abrupt increase in the gene number on the transition from invertebrates to vertebrates. The advent of DNA methylation in the genome was suggested to facilitate the evolution of vertebrates from an invertebrate ancestor by contributing to the regulation of transcription (*5*).

In animals, 5mC is found in the CpG (in plants 5mC is also found in CpXpG) sequence. In mouse, 80% of the CpG sequence is methylated (*6*). This CpG sequence is a sort of "forbidden sequence," and appears at only about 20% of the expected frequency. When the mammalian genome is scanned, some clusters of sequences that contain CpG at a high frequency are found. Such regions are called "CpG islands" (*6*). Many CpG islands are promoters of housekeeping genes, and they are usually undermethylated. The CpG islands may have escaped mutation during evolution, as these regions remain unmethylated.

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Does DNA methylation have a physiological meaning?

People have recognized the correlation between the methylation state and gene expression. The globin gene family is one of the first whose regulatory mechanisms as to tissue-specific transcription appear to involve DNA methylation (*7*). Usually, transcriptionally active genes are undermethylated and silent genes are heavily methylated.

Cultured cells undergo additional DNA methylation in the genes that are not necessary for survival during the establishment of a strain (*6*), where the methylation state of the genes may not necessarily mimic the *in vivo* state. In addition, *Drosophila*, which shows very complicated development, has no 5mC modification. The significance of DNA methylation had been looked upon with suspicion. DNA methylation might just be a result of already silenced genes, and a safe lock mechanism to keep the genes silent.

Recently, Yoder *et al.* (*8*) advocated that DNA methylation is a self-defense mechanism to silence the transposons and/or proviral DNAs that were integrated into the genome during evolution. In fact, when cells are treated with 5-azacytidine, a potent inhibitor of DNA methyltransferase (Dnmt), genome-wide demethylation occurs and this demethylation induces genes such as those of intra cisternal A particle-containing sequences, which are proviral DNAs (*9, 10*). At the same time, the expression of many tissue-specific genes is also induced on this treatment (Aoto, unpublished).

However, it is amazing that when fibroblast cells are treated with the reagent, 5-azacytidine, they start to differentiate into muscle cells, adipocytes, or chondrocytes (*11*). It is still fresh in our memory that MyoD was found in cells isolated with 5-azacytidine-treated myoblasts (*9*). Thus, MyoD is expressed through a demethylation stimu-

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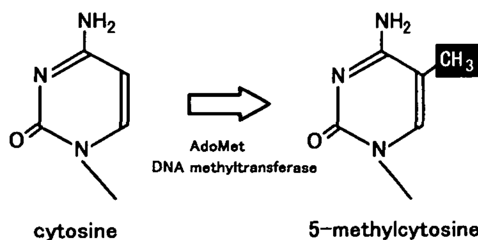

Fig. 1. **The cytosine residue in the CpG sequence is methylated at the 5 position in the presence of *S*-adenosylmethionine (AdoMet) and DNA methyltransferase.**

lus. This and related results raised the idea that regulation of the DNA methylation state could be a prerequisite mechanism for tissue-specific gene expression, that is, cell differentiation. We have identified a gene, *AZ1*, that is induced on the treatment of fibroblasts with 5-azacytidine (*10, 12*). As *AZ1* is specifically expressed in spermatocytes, and its promoter and first exon are rich in CpG sequence, the methylation state may contribute to its transcription *in vivo*.

Evidence that mammals undoubtedly require the.methylating enzyme, Dnmt, for the development of embryos has been provided. When Dnmt is destroyed, mouse embryos cannot survive past midgestation (*13*). In early embryos, a lack of Dnmt causes genome-wide demethylation and, at the same time, causes the loss of "genomic imprinting" (*14*). In mammals, some genes are expressed from only one allele, and this mono-allelic expression pattern depends strictly on from which germ line, male or female, the gene is inherited. Such genes whose expression is determined by the parental origin are called "imprinted genes." Genomic imprinting, an epigenetic phenomenon, was found to be due to DNA methylation (for a recent review see Ref. *15*). The marking of the imprinting genes is thought to be established in germ lines.

In mammalian female cells, one of the two X chromosomes is inactive. In parallel with the inactivation of one X chromosome, many genes on this inactive X chromosome (Xi) are methylated (*16*). Again, there is argument as to whether DNA methylation is a cause or a result of the silencing of genes on Xi. Recently, the gene regulating X-chromosome inactivation, called *Xist* (Xi specific transcript) (*XIST*), was identified (*16-18*). *Xist* is constantly expressed from the Xi chromosome, in which the *Xist* gene is demethylated. *Xist* in the mouse is also imprinted (*19*). *Xist* is preferentially expressed from the paternal X chromosome in female trophectoderm cells, and the paternal X chromosome is specifically inactivated. This imprinting is erased in the *Dnmt*-targeted (*Dnmt* −/−) mouse. After implantation, one of the two X chromosomes in the embryonic lineage that forms the adult female mouse undergoes random inactivation. Upon the differentiation of embryonic stem (ES) cells, the expression of *Xist* is suppressed. But in *Dnmt* −/− ES cells, the expression of *Xist* is not suppressed. This failure in the suppression of *Xist* expression, which may cause cell death, is restored on insertion of the *Dnmt*-mini gene into *Dnmt* −/− ES cells (*20*). Thus, DNA methylation regulates the expression of *Xist*, that plays a key role in X chromosome inactivation.

Cytosine methylation is thought to be one of the causes of oncogenesis through genetic and epigenetic changes (*21*). The epigenetic effect of DNA methylation on oncogenesis is explained by either the activation of a proto-oncogene due to its hypomethylation, or the inactivation of a tumor-suppressor gene due to its hypermethylation. In addition, cytosine residues in CpG sequences undergo mutation at a high rate. It is assumed that the thymine residue produced on the deamination of 5mC at the 4th position leads to a C to T transition. This type of mutation in a proto-oncogene or tumor-suppressor gene is expected to contribute to oncogenesis. The *p53* tumor-suppressor gene is a typical example that suffers frequent mutation. It has also been explained that an identical C to T transition mechanism acted during evolution to reduce the CpG sequences in the genome (*6*).

It should be emphasized that DNA methylation exists widely in chordates (*4*) and even in echinoderms (*3*), that include animals apparently having no such phenomena as genomic imprinting or X chromosome inactivation. *Dnmt* −/− mouse embryos die just after organogenesis starts (*13*), and the transcription activity of genes correlates with their methylation state (*22*). These results strongly support that DNA methylation may play a primary role, during development and/or differentiation, in tissue-specific gene expression. In vertebrates, in fact, important evidence that tissue-specific gene expression *in vivo* requires the regulation of DNA methylation has been reported. The distal enhancer of *myoD* that is responsible for its developmental stage-dependent expression is demethylated in somites prior to its expression (*23*). For *in vivo* expression of the keratin gene, Ets binding to its enhancer is necessary and is regulated by methylation near the binding motif (*24*). Prior to the transcription activation on estradiol treatment, the promoter region of the vitellogenin gene in the chicken is demethylated (*25*).

Although the DNA methylation mechanism has been acquired by chance, vertebrates may have chosen to utilize the modification as one of the control mechanisms for tissue-specific gene expression, just like mammals have adopted DNA methylation as a mechanism for genomic imprinting (*19*).

What does DNA methylation do?

Generally, heavily methylated genes are transcriptionally silent. DNA methylation usually prevents transcription initiation. Then, what is the mechanism for the suppression of a gene by DNA methylation? Two possible mechanisms have been proposed (*26*) (Fig. 2). The first one is a direct effect. The methylation of DNA directly affects the binding of some transcription factors, as 5mC protrudes into the major groove of the double-helix. If the binding motifs or near the binding sites is methylated, transcription factors, except for Spl (*27*), cannot bind to the motifs (*28*). Consequently, transcription is inhibited.

The second mechanism is an indirect effect. A typical example is the thymidine kinase gene promoter. The methylated promoter activity is inhibited only after the DNA has been assembled into chromatin (*29*). Proteins that specifically recognize the methylated state of DNA may contribute to the formation of transcriptionally inactive chromatin. Several DNA binding proteins that recognize 5mC (*28*), such as MDBP, MDBP2-H1, MeCP1, MeCP2, HMBP (*30*), MMBP-1 (*31*), MMBP-2 (*31*), and

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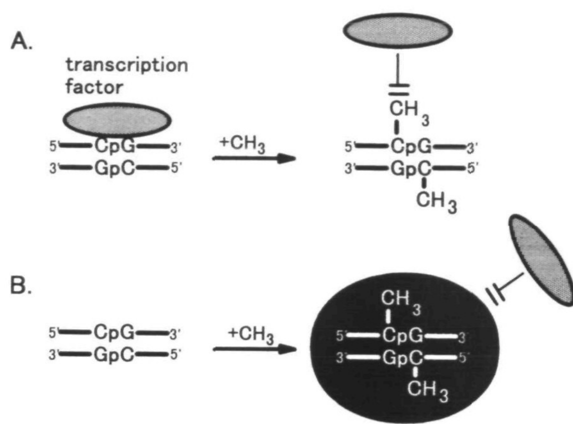

Fig. 2. **Possible mechanisms for the regulation of transcription by DNA methylation.** A: The methylation of DNA directly affects the binding of some transcription factors when the binding motifs or near the binding sites is methylated. As a consequence, transcription is inhibited. B: Proteins that specifically recognize the methylated state of DNA inhibit transcription by changing the chromatin structure.

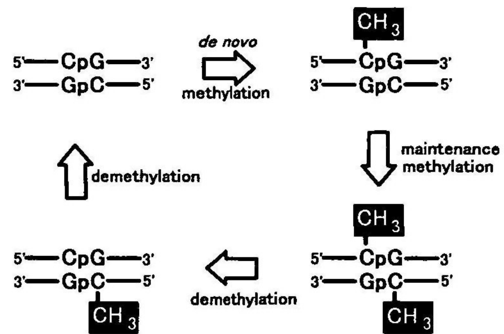

Fig. 3. **DNA methylation is regulated mainly through three steps:** (1) Formation of a new methylation pattern (*de novo* methylation), (2) maintenance of the methylation pattern during mitosis (maintenance methylation), and (3) erasing of the methylation pattern (demethylation).

MMBP-3 (*32*), have been identified in vertebrates. MDBP2-H1, which turned out to be a truncated form of histone H1 (*33*), is a factor that binds to the chicken vitellogenin gene. It was reported that histone H1 exhibits higher affinity for methylated than unmethylated DNA (*34*). MeCP1 and 2 were isolated from rat nuclei. Both proteins bind to methylated DNA regardless of its sequence. The content of MeCP1 is much lower than that of MeCP2, and recognizes heavily methylated DNA. Interestingly, *MeCP2*-targeted mouse embryos show a similar phenotype to that of *Dnmt*-targeted mouse (*35*). These three methylated DNA-binding proteins, MDBP2-H1, MeCP1, and MeCP2, inhibit transcription activity *in vitro* and/or *in vivo* (*26, 36, 37*). On the other hand, MDBP, HMBP, and MMBP-1, -2 and -3 recognize not only 5mC but also nucleotide sequences. HMBP binds to the Sp1 sites in a HIV-1 long terminal repeat when the element is methylated at CpGs (*30*). MMBP-1, -2 and -3 bind to the c-Myc binding motif when the core CpG in the E-box is methylated (*31, 32*). A similar type of methylated DNA binding proteins that recognizes methylated forms of the transcription factor binding motifs has been detected. One recognizes the methylated GABP binding motif (Uno, personal communication) in the enhancer of the *Cyp 2d-9* (P-450) gene (*38*), and the other binds to the methylated sequence that involves the Ets binding motif in the keratin gene enhancer (*24*, Umezawa, personal communication). Furthermore, there are cases in which DNA methylation creates the binding sites for transcription factors AP1 (*39*) and Sp1 (*40*), of which the binding apparently inhibits the transcription of the genes.

Regulation of DNA methylation

It has been proven that gene-specific methylation patterns are formed during development and differentiation (*41*). To be convinced that DNA methylation is a prerequisite mechanism for the transcription activity in vertebrates, it is important to elucidate how the methylation states are regulated and which factors are involved in the process *in vivo*. However, at present, very little evidence is available to explain their regulation. DNA methylation is regulated mainly through three steps, (i) formation of new methylation patterns, (ii) maintenance of the methylation patterns during mitosis, and (iii) erasing of the methylation patterns (Fig. 3).

Maintenance of methylation patterns by Dnmt. At present, only one factor, Dnmt, has been verified to be involved in the regulation of the methylation state. Dnmt preferentially methylates non-methylated cytosine in the CpG sequence complimentary to the methylated CpG sequence in double-stranded DNA (hemimethylated form). This hemimethylated CpG sequence-directed activity of Dnmt is responsible for the maintenance of the methylation pattern already formed in genomic sequences. cDNAs of *Dnmt* have been isolated from sea urchin (echinoderm) (*42*), frog (amphibian) (*43*), chicken (avian) (*44*), and mouse and man (mammals) (*20, 45, 46*). The molecules are composed of more than 1,500 amino acid residues. The predicted amino acid sequences of the Dnmts are highly homologous to each other (*43*) (Fig. 4), especially in the carboxyl-terminal 1/3 of the molecule, which is responsible for the catalytic activity (*45*). Ten motifs (I–X) are conserved in the bacterial cytosine-5-methyltransferases, and these motifs are also conserved in the Dnmts of those isolated from animals (*43, 47*). In addition to a catalytic domain, animal Dnmts possess a large amino-terminal domain composed of more than 1,000 amino acid residues. The two domains are connected through a repeated sequence of KG, which seems to be a hinge. The amino-terminal domain is thought to be the regulatory domain containing the nuclear localization signal (NLS), replication foci localization signal(s), and the Cys-rich (Zn finger-like) sequence that binds Zn^{2+}. It is not yet known what the Cys-rich domain does. Dnmt waits at replication foci and recognizes a newly replicated form of DNA that is hemimethylated in CpG to fully methylate the strand. Actually, Dnmt is localized to the replication foci in the middle to late S-phase (*48*), which coincides with the fact that methylated DNA replicates in the late S-phase. The sequence responsible for this localization has been identified in the amino-terminal domain (*48, 49*). Recently, another amino-terminal sequence, different from this region, was found to interact with proliferating cell nuclear antigen (PCNA) (*50*), an auxiliary factor for DNA replication and repair.

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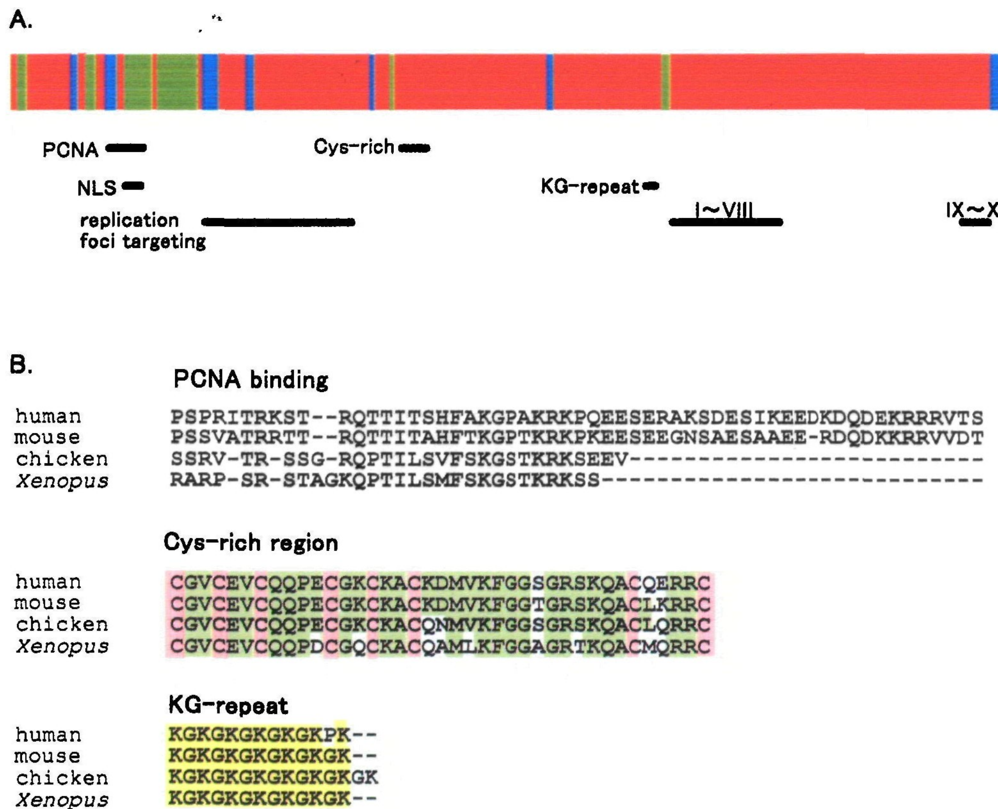

Fig. 4. **Schematic illustration of the homology in vertebrate DNA methyltransferases (A), and alignment of their motifs.** A: The amino acid sequences of human, mouse, chicken, and *Xenopus laevis* Dnmts were aligned, by means of the ClustalW program (DNA Databank of Japan). The homologous regions are in red and the non-homologous stretches longer than 5 amino acid residues are in blue. The regions in green are those which are homologous between man and mouse. The equivalent amino acid residues [(i) S and T, (ii) D and E, (iii) K and R, and (iv) L, I, and V] are included in the homologous residues. B: Alignment of the PCNA binding, cysteine-rich, and KG-repeat regions. Identical amino acid residues to in the human PCNA binding region, and the mouse Cys-rich and KG-repeat regions are in identical colors.

The interaction is specific to the early S-phase and the binding is competed for by the cell cycle related factor, $p21^{WAF1}$, which is a cyclin-dependent kinase (Cdk) inhibitor that is quickly expressed after the cell cycle has been arrested.

The expression of Dnmt is also under the control of the cell cycle. The Dnmt protein and activity are highest in the S phase and lowest in the G_1 phase. When the cell cycle is arrested by low serum conditions or differentiation is induced, Dnmt decreases (*51, 52*). This regulation of Dnmt is a posttranscriptional event, as the arrest of the cell cycle does not affect the transcription in isolated nuclei (*51–53*), and shortens the half lives of the Dnmt mRNA and protein through an unknown mechanism (*52*).

In somatic cells, Dnmt is usually localized in the nucleus. On the contrary, in early embryos Dnmt shows a strange distribution, that is, at the one- and two-cell stages Dnmt mainly exists in the cytoplasm near the plasma membrane, and becomes localized to nucleus only after the 8-cell stage (*54*). The genomic DNA tends to be demethylated until the beginning of organogenesis after implantation (*55*). The localization of Dnmt in the cytoplasm may contribute to the genome-wide demethylation. The mechanism underlying its localization to the cytoplasm, however, remains to be determined. Furthermore, mouse oocytes contain a 3,000 times higher amount of the Dnmt protein than murine erythroleukemia cells per cell (*54*), which contain about 6.5×10^4 molecules per cell (Suetake, unpublished observation). A tremendous amount of Dnmt is accumulated in oocytes. Similar accumulation of Dnmt has been found in *Xenopus laevis* oocytes (*43*).

Another strange feature of Dnmt regulation is its expression profile during spermatogenesis. At the stage of pachytene spermatocytes, the late stage of meiosis, the transcription start site of *Dnmt* changes from the somatic cell type to the testis-specific one. The transcript utilizing the testis-specific promoter does not produce functional Dnmt (*56, 57*). Thus, the Dnmt level becomes very low at this stage. This may be a mechanism that protects recombinating DNA from aberrant methylation by Dnmt, which has the ability to methylate partially gapped or stem looped DNA (*58, 59*). This meiotic stage is also thought to be the time of marking of the imprinted genes. In pachytene spermatocytes, many spermatocyte-specific genes are transcribed (*10, 12, 60*), maybe because of the demethylation events caused by such down regulation of Dnmt expression.

Creation of the methylation pattern. In the mouse life cycle, there are two major stages in which methylation patterns are formed. One is at the beginning of organogenesis, just after implantation, and the other is in the germ line (*55*). The creation of the specific methylation pattern in genomic DNA should consist of two steps, that is, the recognition of the site to be methylated and the actual methylation step. As for the information as to the site of methylation, several hundred base pair sequences have been identified in the 5′ upstream regions of the α-fetoprotein (*61*) and adenine phosphoribosyltransferase (*62*) genes as *cis*-acting elements that guide *de novo* methylation.

Then, which catalytic activity is responsible for the creation of the methylation pattern? Isolated Dnmt exhibits higher methylation activity toward hemimethylated than non-methylated DNA, that is, Dnmt is thought to

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contribute only to the maintenance of already formed methylation patterns. Furthermore, *Dnmt* −/− ES cells still exhibit *de novo* methylation activity (*63*). The existence of a novel DNA methyltransferase other than the maintenance-type Dnmt is expected. Somatic culture cells also possess *de novo* methylation activity (*64*). However, such a *de novo* DNA methyltransferase has not been identified yet in animals.

Recently, the *de novo* DNA methyltransferase gene, *masc1*, was identified in *Ascobolus* (fungi) (*65*). Targeting of this gene disrupts the DNA methylation in the reproductive stage at which methylation patterns are formed, but has no effect on the maintenance of the once formed methylation pattern in the vegetative stage. *Masc1* encodes a protein homologous to cytosine-5-methylase but has no large amino-terminal domain. On the other hand, maintenance-type Dnmt also exhibits *de novo* methylation activity *in vitro*, and methylates genomic DNA when ectopically expressed in cultured cells (*44, 66*). There is still a possibility that Dnmt contributes to the creation of the DNA methylation pattern.

Demethylation activity. Methylation patterns once formed and maintained should be demethylated prior to gene expression. It was reported that many tissue-specific genes are demethylated when cells are induced to differentiate (*22*). The erasing of the specific methylation patterns in genomic DNA should also consist of the steps of recognition of the site to be demethylated and the actual demethylation. Similar to the *de novo* methylation signal, *cis*-regulatory elements in the α-actin gene (*67*), the intronic κ chain enhancer sequence (*68*), and the downstream enhancer of the M-lysozyme gene (*69*) have been reported to guide the demethylation event.

The question is whether the demethylation step requires specific enzyme activities for the removal of 5mC from genomic DNA or not? If Dnmt activity is inhibited at the time of replication, after two rounds of replication, half the population of replicated strands is completely demethylated. The binding of a transcription factor, of which the binding to DNA is not affected by methylation, such as Sp1 (*27*), to the site to be demethylated may prohibit the methylation (*70, 71*). This replication-dependent demethylation mechanism could be an indication that the demethylation event is just the result of the transcription activation.

But, if demethylation mechanisms without a replication process exist, it is a sign that the methylation state positively contributes to the regulation of the gene expression process *in vivo*. In 1990, a specific site of the skeletal muscle α-actin promoter was proven to be demethylated without any replication process (*67*), and soon after that an *in vitro* demethylation assay system was reported (*72*). But now the situation is rather complicated. One group using a nuclear extract prepared from chicken embryos reported that the glycosylase that specifically recognizes 5mC is the major component of the demethylase (*72*). The glycosylase contains an RNA molecule as a necessary component for the activity. The RNA contains a sequence that is complementary to that of the DNA containing the methylated cytosine to be demethylated (*73*). That is, the RNA guides the glycosylase to the exact site to be demethylated. After excision of 5mC, an ordinary repair system fills the site with a nonmethylated cytidine. The other mechanism is more sensational. Another group reported that demethylase is an RNA enzyme just like ribozyme (*74*). Accessory protein(s) may endow the RNA enzyme with specificity as to where demethylation is to occur. At present, which (or both) mechanism contributes to the *in vivo* demethylation step is not clear. In any case, these results may strongly support that DNA methylation plays some important role in tissue-specific gene expression.

Conclusion

At present, we believe that most researchers, not only those in the DNA methylation field, accept the biological importance of DNA methylation in vertebrates. It took a long time for people to become confident that the DNA methylation of cytosine, 5mC, plays an important role *in vivo*. The purification, cloning and targeting of Dnmt was the first breakthrough in the DNA methylation field. The next breakthrough will be elucidation of the mechanisms underlying *de novo* methylation and demethylation. The finding of the factors that interact with those of *de novo* methylase, maintenance Dnmt and demethylase is also important to understand how the DNA methylation is regulated. Identification of the new factors will provide us with tools for elucidating the molecular mechanism underlying the regulation.

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