DNA methyltransferase in *Xenopus laevis*

（アフリカツメガエルにおけるDNAメチルトランスフェラーゼの研究）

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LIST OF ABBREVIATIONS

5mC	5-methylcytosine
bp	base pair
BSA	bovine serum albumin
BCIP	5-bromo-4-chloro-3-indoylphosphate
cDNA	complementary deoxyribonucleic acid
cDnmt1	chicken Dnmt1
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
Dnmt	DNA methyltransferase
DMEM	Dulbecco's modified Eagle's MEM
dNTP	deoxynucleoside triphosphate
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
FCS	fetal calf serum
GST	glutathione-*S*-transferase
hDNMT1	human Dnmt1
kb	kilo base
kDa	kilo Dalton
MBP	maltose-binding protein
MBT	midblastula transition
MEL	murine erythroleukemia
mDnmt1	mouse Dntm1

MMR	Marc's modified Ringer's solution
MEMFA	A buffer containing MOPS, EGTA, $MgSO_4$, formaldehyde
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger ribonucleic acid
NBT	*p*-nitro blue tetrazolium chloride
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
rDNA	ribosomal DNA
RNA	ribonucleic acid
RNase	ribonuclease
SAM	*S*-adenosyl-L-methionine
SDS	sodium dodecyl sulfate
TdT	terminal deoxynucleotidyl transferase
Tris	Tris (hydroxymethyl) aminomethane
TUNEL	TdT dUTP nucleotide end labeling
xDnmt1	*Xenopus* Dnmt1
X-gal	5-bromo-4-chloro-3-indoyl-*β-D*-galactoside
Xi	inactive X-chromosome
Xist	Xi specific transcript
xPCNA	*Xenopus* PCNA

The amino acid residues and their symbols

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

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CHAPTER 1. INTRODUCTION

1.1 DNA methylation

As a modified base in DNA, methylated cytosine at 5th position (5mC) was first found in calf thymus DNA (Hotchkiss, 1948), and thereafter, in bacteria. To date, 5mC is also identified in fungi, higher plants, annelids (marine polychaete annelid worm) (del Gaudio et al., 1997), arthropods (silk worm and cricket) (Patel and Gopinthan, 1987; Tweedie, 1999), echinoderms (sea urchin) (Fronk et al., 1992), and chordates (Tweedie et al., 1997). As exception, 5mC does not exist in *Saccharomyces cerevisiae* (Proffitt et al., 1984), and cannot be detected in *Saccharomyces pombe* (yeast), *Caenorhabditis elegans* (nematodes) (Simpson et al., 1986), and *Drosophila melanogaster* (insect) (Rae and Steele, 1979; Urieli-Shoval et al., 1982).

5mC found in the CpG dinucleotide is the only known chemical modification in the genomic DNA of animals under physiological conditions. In mouse, 80% of the CpG dinucleotide sequence in genomic DNA is methylated. The frequency of CpG is about 20% of the expected frequency, and thought be a forbidden sequence.

The frequency of 5mC in the genome is positively correlated with the gene number. That is, both the methylation frequency and the nucleotide numbers in genomic DNA are drastically increased during the evolution from invertebrates to vertebrates. All the long-lived multicellular animals with greater than $5x10^8$ bp as genome possess high frequency of 5mC. The utilization of DNA methylation for the regulation of gene expression could be a driving force of the evolution of vertebrates from invertebrates (Bird, 1995). A remarkable feature of DNA methylation is that it is not genetically fixed (epigenetic), but reversible. At the same time, the methylation patterns are inherited in somatic cells in a cell lineage specific manner.

1.2 Biological meaning of DNA methylation

In bacteria, DNA methylation is utilized to distinguish between their own genomic DNA and exogenously infected DNA, such as bacteriophage. A sequence-specific DNA methylating enzyme is accompanied with a restriction enzyme that recognizes an identical sequence. This is called "restriction modification" system, and thought to function as a kind of defense system for bacteria against bacteriophage. Bacteria can digest non-methylated DNA of bacteriophage, but not its own methylated DNA (Wilson and Murray, 1991). In addition to cytosine methylation, adenine methylation (N6-methyladenine) also exists in bacteria, and is utilized as a mark to identify the parent DNA strand just after replication. A mismatched base in the non-methylated strand is specifically repaired when mismatch base pairing occurs (Mordich, 1989).

Contrarily, in vertebrates, DNA methylation function as one of the transcriptional regulation mechanisms. Especially in mammals, it plays crucial roles in several physiological phenomena such as tissue-specific gene expression, genomic imprinting, X-chromosome inactivation, and cancer (Tajima and Suetake, 1998). Generally, tissue-specific genes are almost fully methylated in all the nonexpressing adult somatic tissues. Demethylation of the tissue-specific genes is usually observed only in the specific tissues, in which the gene is expressing. On the other hand, the promoter regions of constitutively expressing genes (housekeeping genes) that contain CpG islands in their promoter are undermethylated both in germ line and in somatic cells (Cedar, 1988; Bird, 1992; Razin and Cedar, 1991).

In mammals, both maternally and paternally inherited genomes are indispensable for normal development of embryo. The expression of certain genes

depends on their parental origin (Surani, 1998). This epigenetic phenomenon is called "genomic imprinting". To date, over 20 imprinted genes have been identified in human and mouse. Many of the genes play roles in growth and differentiation (Tilghman, 1999). The embryological and genetic studies predict the existence of distinct imprinted loci, in which the two parental alleles are differently marked. Actually, the imprinted genes possess sequence elements that are methylated only in one of the two parental alleles (Razin and Cedar, 1994).

In mammalian female, one of the two X-chromosomes is inactive. This X-chromosome inactivation contributes to the dosage compensation of X-linked genes (Marahrens, 1999). Many genes on this inactive X-chromosome (Xi) are methylated. The gene regulating X-chromosome inactivation, called *Xist* (Xi specific transcript), has been identified (Borsani et al., 1991; Brockdorff et al., 1992; Brown et al., 1992). The promoter of *Xist* in Xi chromosome is completely unmethylated, and is transcriptionally active. The expression of *Xist* is also under the control of DNA methylation (Norris et al., 1994).

DNA methylation is also thought to be one of the many causes of tumorigenesis (Jones and Laird, 1999). It was demonstrated that in some of the patients, an activation of a proto-oncogene is due to its hypomethylation, and inactivation of a tumor-suppressor gene to its hypermethylation. In addition, CpG sites in the coding regions of some tumor suppressor genes are hotspots for mutation leading to aberrant functions of the gene products. It is assumed that the creation of thymine residue by the deamination at 4th position of 5mC leads to a C to T transition. The *p53*, a tumor-suppressor gene, often suffers this type of mutation (Greenblatt et al., 1994; Magewu and Jones, 1994).

1.3 Possible mechanisms of the regulation of gene expression via DNA methylation

In vertebrates, heavily methylated genes are, in general, transcriptionally silent. DNA methylation usually inhibits transcription initiation (Bird and Wolffe, 1999). As for silencing of transcription, three mechanisms are proposed (Fig. 1). (i) The first one involves a direct inhibition of the binding of transcription factors to their binding motifs that are methylated. Some transcription factors such as E2F, c-Myc, c-Myb, and Ets cannot bind to their methylated motifs. (ii) The second mechanism is due to a group of proteins that contain methyl-CpG binding domain (MBD), which is responsible for the recognition of 5mC. Among them, MeCP2 and MBD1 contain transcription repression domain (TRD) in addition to MBD domain, and this TRD directly suppresses the transcription (Ng et al., 2000; Yu et al., 2000). (iii) The third mechanism is an indirect one. Among the MBD containing proteins, MeCP2, MBD2 and MBD3 recruit a histone deacetylase containing co-repressor complex (Wade et al., 1999; Zhang et al., 1999; Knoepfler and Eisenman, 1999). Recently, it has been reported that DNA methyltransferase (Dnmt1, see next section) directly binds HDAC (Fuks et al, 2000; Robertson et al., 2000; Rountree et al., 2000). Histone deacetylase removes acetyl-group from acetylated core histones H3 and H4 that are in transcriptionally active chromatin. The deacetylation of histones suppresses the transcription by altering the nucleosomal formation (Ng and Bird, 2000). Chromatin modification induced by DNA methylation and histone deacetylation is an important mechanism for gene silencing.

1.4 DNA methyltransferase in vertebrates

Under physiological conditions, two types of DNA methyltransferase activities are expected to exist (Fig. 2). One activity that introduces a new methyl-group to unmethylated CpG is referred to as "*de novo*-type" DNA methylation activity. The

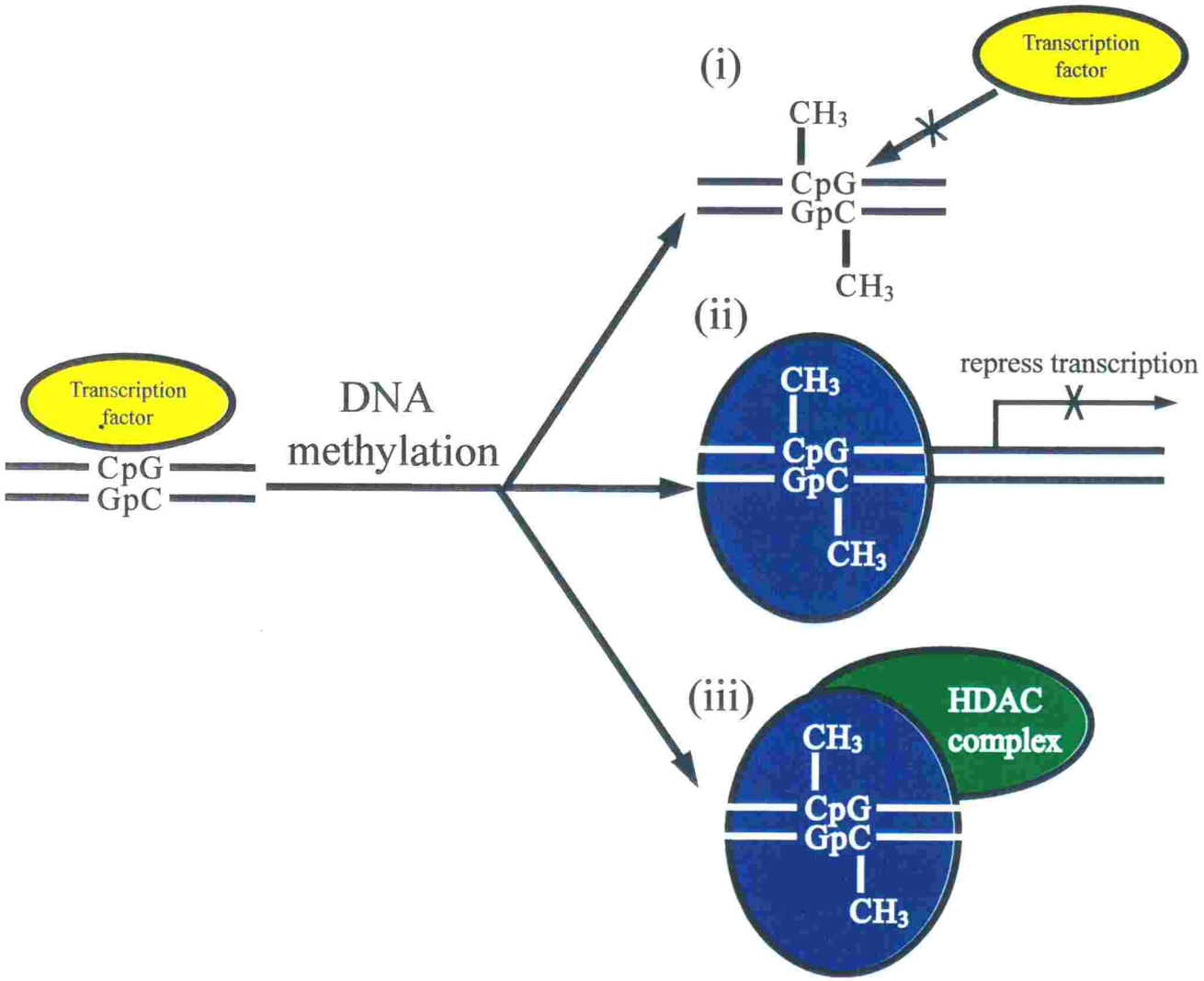


Figure 1. Transcription repression via DNA methylation. Possible models for transcription repression via DNA methylation are shown. (i) DNA methylation inhibits binding of a transcription factor (yellow oval). (ii) Methyl-CpG binding protein (MBD) (blue oval) binds to methylated DNA and inhibits transcription. (iii) Dnmt1 and MBD recruits histone deacetylase (HDAC) complex (green oval) and the methylated genes are, in due course, induced to form inactive chromatin structure.

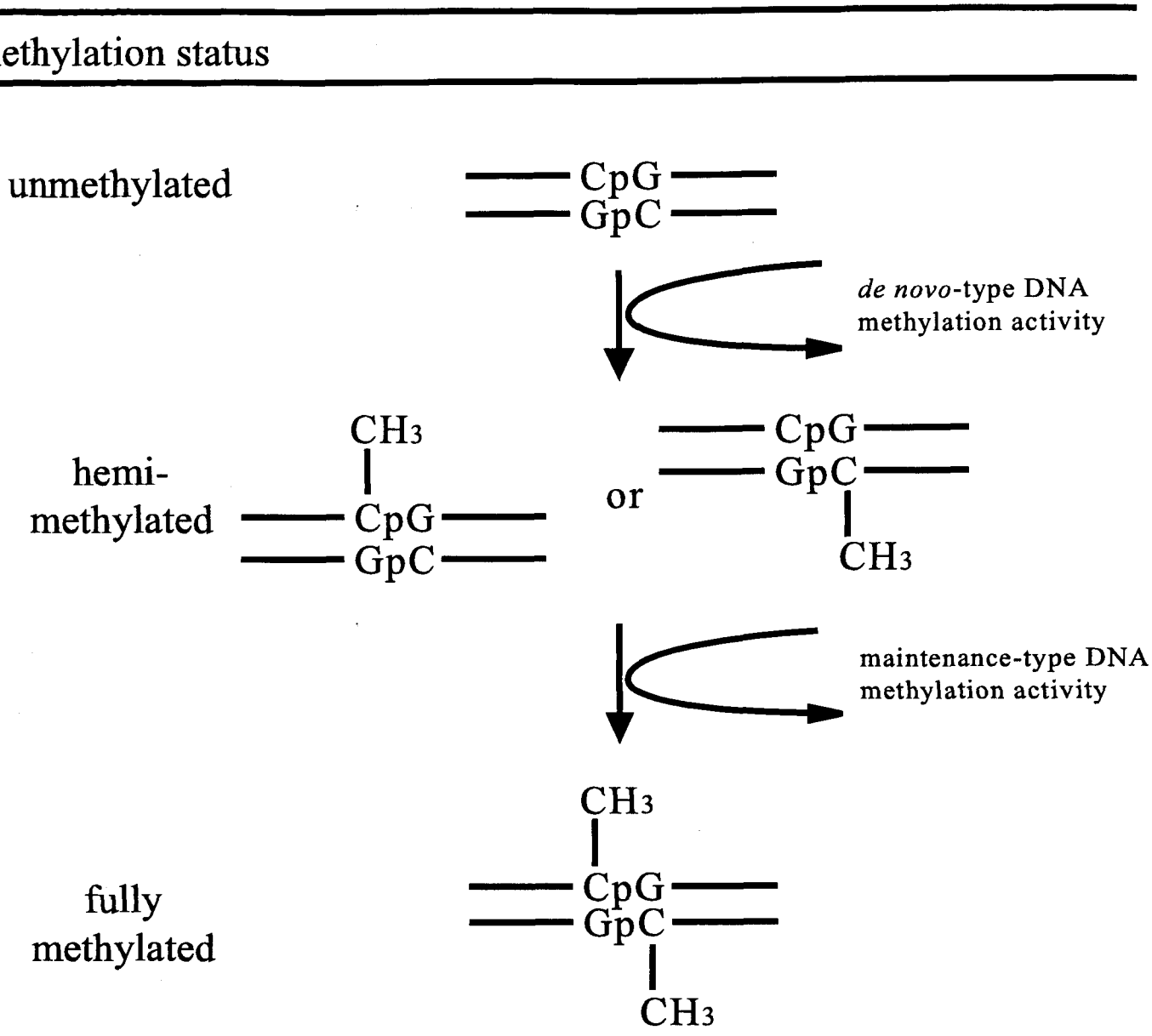


Figure 2. Two types of DNA methylation activities. DNA methylation pattern is established by *de novo*-type DNA methylation activity, and then the C in a symmetrical position in the opposite strand is methylated by maintenance-type DNA methylation activity.

methylation patterns once established are maintained through DNA replication and inherited in cell linage specific manner. This activity is referred to as "maintenance-type" DNA methylation activity. These two activities are thought to establish and maintain cell-type specific methylation pattern during development and differentiation.

DNA 5-cytosine methyltransferase (Dnmt) catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to the 5th position of cytosine. To date, four Dnmt-related genes have been identified in vertebrates (Fig. 3). Dnmts contain conserved motifs (I-X) that are responsible for methylation activity similar to those of bacterial type II DNA-(cytosine-5) methylases in the carboxyl-terminal region (Kumar et al., 1994). Dnmt1 is responsible for the maintenance of DNA methylation patterns. Dnmt1 specifically introduces a methyl group to hemimethylated CpG sequences in double-strand DNA. The cDNA of Dnmt1 has been isolated from human (Yen et al., 1992; Yoder et al., 1996), mouse (Bestor et al., 1988; Yoder et al., 1996), rat (Kimura et al., 1998), chicken (Tajima et al., 1995), and sea urchin (Aniello et al., 1996). One third of the carboxyl-terminal region of the molecule is a catalytic domain. A KG-repeat divides the amino- and carboxyl-terminal domains. The large amino-terminal domain connected with KG-repeat and contains Cys-rich zinc finger-like motif (Bestor, 1992) and polybromo1 homology domain (Bestor, 1996) is thought to be a regulatory domain. This amino-terminal domain contains the functional domains of nuclear targeting signals (Leonhardt et al., 1992), replication foci-targeting sequence (Leonhardt et al., 1992; Liu et al., 1998), and the cytoplasmic retention sequence (Cardoso and Leonhardt, 1999). The amino-terminal domain interacts with proliferating cell nuclear antigen (PCNA) (Chuang et al., 1997), Rb (Robertson et al., 2000), HDAC (Fuks et al., 2000; Robertson et al., 2000; Rountree et al., 2000), and DMAP1 (Rountree et al., 2000). As

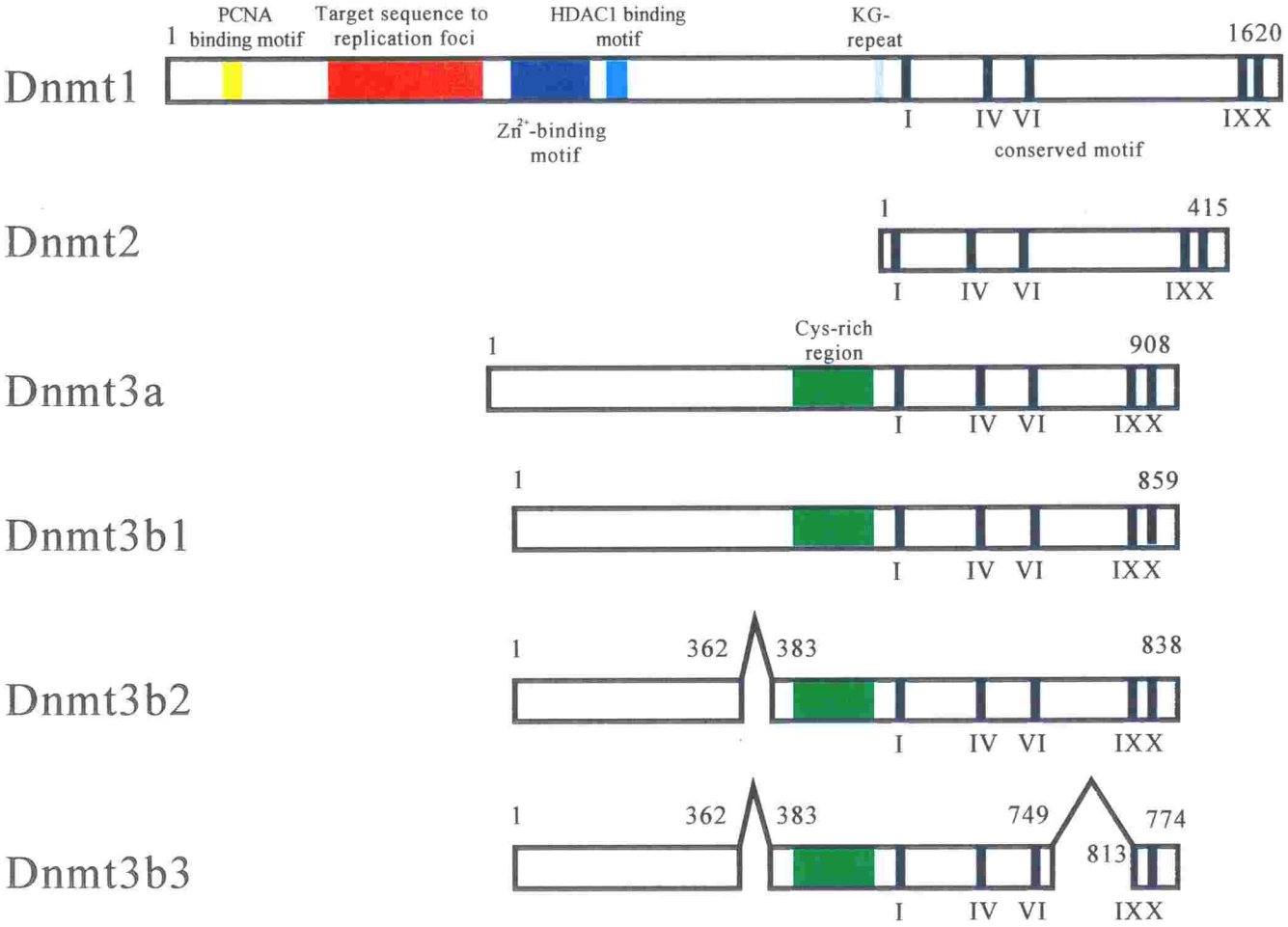


Figure 3. Mouse Dnmt family. Schematic illustration of mouse Dnmt1, Dnmt2, Dnmt3a and Dnmt3b. Black bars represent the five conserved motifs from bacterial type II DNA-(cytosine-5) methylase. PCNA binding motif (yellow box), the targeting sequence to replication foci (red box), Zn^{2+}-binding motif (blue box), HDAC1 binding motif (light blue box) and KG-repeat (gray box) are demonstrated in Dnmt1. The Cys-rich regions (green boxes) in Dnmt3 family are indicated. Alternatively spliced site of Dnmt3b (amino acid residues 362-383 and 749-813) are also indicated.

the mRNA and protein stability of Dnmt1 are under the control of cell cycle (Liu et al., 1996; Suetake et al., 1998), it is interesting that Dnmt1 directly interacts with PCNA, a prerequisite component for replication and repair, and Rb, a key component of cell cycle checkpoint. As mentioned above, DNA methylation is a signal to silence gene expressions. However, the fact that Dnmt1 interacts directly with HDAC and DMAP1, members of co-repressor complex, indicates that Dnmt1 by itself functions to suppress gene expressions as soon as it methylates the gene.

Dnmt2 has been isolated from human (Yoder and Bestor, 1998; van den Wyngaert et al., 1998), mouse (Yoder and Bestor, 1998; Okano et al., 1998a) by EST cloning. Although Dnmt2 contains 6 of the 10 conserved motifs, it lacks the large amino-terminal domain that Dnmt1 has. Dnmt2 protein, strangely, has no DNA methylation activity. Since mouse embryonic stem cells that lack Dnmt2 possess normal *de novo*- and maintenance-type methylation activities (Okano et al., 1998a), Dnmt2 may not contribute to the methylation of DNA.

The cDNAs of Dnmt3a and Dnmt3b encoded in different genes have been isolated from mouse (Okano et al., 1988b) and human (Xie et al., 1999). As Dnmt3a and Dnmt3b are able to introduce a methyl group to unmethylated DNA *in vitro*, expressed abundantly in embryonic stem cells, in which stage the methylation pattern is expected to be established, and low in differentiated embryoid bodies and adult tissues, they are thought to be responsible for *de novo*-type DNA methylation (Okano et al., 1998b). Targeting of either *Dnmt3a* or *Dnmt3b* gene does not show any severe defects in mouse early embryo as that of Dnmt1. Targeting of both *Dnmt3a* and *Dnmt3b* genes shows as severe phenotype as that of Dnmt1, and died before 11.5-day embryo (Okano et al., 1999). The DNA methylation level is decreased in the targeted mouse. Interestingly, in Dnmt3b deficient mouse, the methylation level of minor satellite

repeats is decreased, which is a typical feature of ICF syndrome. In fact, it is proven that mutations in human *DNMT3B* are responsible for ICF syndrome (Hansen et al., 1999; Okano et al., 1999). ICF syndrome is a rare autosomal recessive disease characterized by a variable immunodeficiency, centromeric instability, and mild facial anomalies. Dnmt3b has three alternative-splicing variants called Dnmt3b1, 3b2, and 3b3. Interestingly, Dnmt3b3 lacks a part of motif IX in the catalytic domain, and has no DNA methylation activity (Aoki et al., unpublished).

1.5 Regulation of DNA methylation in germ line and early development

DNA methylation dramatically changes during development. In mouse early embryo, before preimplantation stage, genomic DNA undergoes genome-wide demethylation. This demethylation wave partially erases the parental methylation pattern (Monk, 1987). The genomic DNA in blastocysts is relatively undermethylated (Monk, 1990; Chaillet et al., 1991). After implantation, genomic DNA of the embryo suffers the wave of *do novo* methylation, and establishes the tissue-specific methylation pattern (Monk, 1990). The rest of the genomic DNA methylation pattern involving demethylation and *de novo* methylation occurs in germ line cells during gametogenesis. This DNA re-methylation process is a crucial step in establishing the parental-specific methylation marks in imprinted genes (Chaillet et al., 1991).

In mouse, Dnmt1 is under the interesting regulation during developmental stage of germ line cells. Unique alternative 5' exons are chosen in male and female gametocytes (Mertineit et al., 1998). The Dnmt1 in growing oocytes, female germ line cells, utilizes an oocyte-specific exon, of which amino acids sequence is 118 amino acid residues shorter than that of somatic-type Dnmt1. The Dnmt1 is highly expressed and is excluded from nucleus (germinal vesicle). The mouse Dnmt1 in oocytes and early

embryos shows very strange localization pattern. In fully-grown oocytes and embryos till four-cell stage, Dnmt1 remains in the cytoplasmic compartment. In eight-cell embryos, some Dnmt1 molecules are translocated into nuclei. At blastocysts, most of the Dnmt1 molecules are again found in cytoplasm (Carlson et al., 1992; Mertineit et al., 1998). Retention of Dnmt1 in the cytoplasm during early development may contribute to the genome-wide demethylation that occurs at this stage. A long stretch including Cys-rich region in the amino-terminal domain is demonstrated to be responsible for Dnmt1 to retain in the cytoplasmic compartment (Cardoso and Leonhardt, 1999). In addition, mouse oocytes contain 3,000 times higher amount of Dnmt1 than murine erythroleukemia cells (MEL) per cell basis (Carlson et al., 1992). In male germ cells, during spermatogenesis, first exon of Dnmt1 is alternatively selected. At the stage of pachytene spermatocytes, the late stage of the first meiosis, the transcription of *Dnmt1* starts from the testis-specific first exon (Jue et al., 1995). This testis-specific type transcript is not translated to Dnmt1.

Several lines of genetic evidence demonstrate that the regulation of DNA methylation is essential for normal development in mammal. As for Dnmt1, mouse homozygous for a loss-of-function mutation in Dnmt1 gene (*Dnmt1* $^{-/-}$) dies at midgestation and embryos show developmental delay and aberrant expression of imprinted gene (Li et al., 1992 and 1993). As for *de novo*-type DNA methyltransferase, *Dnmt3b* (-/-) mouse is embryonic lethal, and *Dnmt3a* (-/-) is normal in development but becomes runtish and dies about 4 weeks after birth (Okano et al., 1999). By targeting both *Dnmt3a* and *Dnmt3b*, the embryo dies before 11.5-day embryo, of which feature is similar as that of Dnmt1 targeted mouse.

In zebrafish and *Xenopus laevis* early development, no such genome-wide demethylation in DNA methylation pattern as in mouse early development (Kass and

Wolffe, 1998; Macleod et al., 1999; Martin et al., 1999). Zebrafish embryos treated with 5-azacytidine (5-azaC) or 5-aza-2-deoxycytidine (5-azadC), a potent inhibitor of DNA methyltransferase (Jones and Taylor, 1980; Santi et al., 1983) exhibit DNA hypomethylation and abnormal development (Martin et al., 1999). In *Xenopus* early embryos, the suppression of Dnmt1 translation by microinjecting anti-sense Dnmt1 RNA decreases the methylation and induces the abnormal development of embryos (Stancheva and Meehan, 2000).

It is reported that *Drosophila* has no detectable amount of 5mC in the genomic DNA. Interestingly, ectopically expressed mouse Dnmt1 and Dnmt3a in *Drosophila* embryo introduces 5mC in the genomic DNA and causes abnormal development (Lyko et al., 1999).

1.6 DNA methylation in *Xenopus laevis*

During *Xenopus* early development, no change in the DNA methylation level of genomic DNA is detected (Bird and Southern, 1978; Kass and Wolffe, 1998). On the other hand, recently, Stancheva and Meehan (2000) have reported that the 5mC content drops to about 50% of the genomic DNA at stage 7 embryos (early blastula). The DNA methylation activity is detected in *Xenopus* embryos and culture cells (Adams et al., 1981). When either methylated or unmethylated gene is injected into nuclei of *Xenopus* oocytes, transcription starts immediately. Interestingly, the transcription from methylated template soon becomes silent and that from unmethylated template remains active (Kass et al., 1997). In *Xenopus*, DNA methylation contributes to gene silencing as well. In addition, the result indicates that gene silencing process via DNA methylation requires chromatin structure as the formation of nucleosome and chromatin takes a while. Similar phenomenon is previously reported in mammalian culture cells.

When methylated (*TK*) gene is injected, transcription lasts for 8 h and then silenced. But, when chromatin structure is pre-formed *in vitro*, the transcription of *TK* gene is inhibited as soon as the gene is injected (Buschhausen et al., 1987).

Ribosomal RNA genes (*rDNA*) of *Xenopus* are rich in CpG and the methylation sites were mapped (Bird and Southern, 1978). Most of their CpG sites are methylated except for the enhancer of *rDNA*. The enhancer regions are fully methylated in sperm *rDNA*. Demethylation of the enhancer region positively correlates with the transcription activity of *rDNA* during embryonic development (Bird et al., 1981).

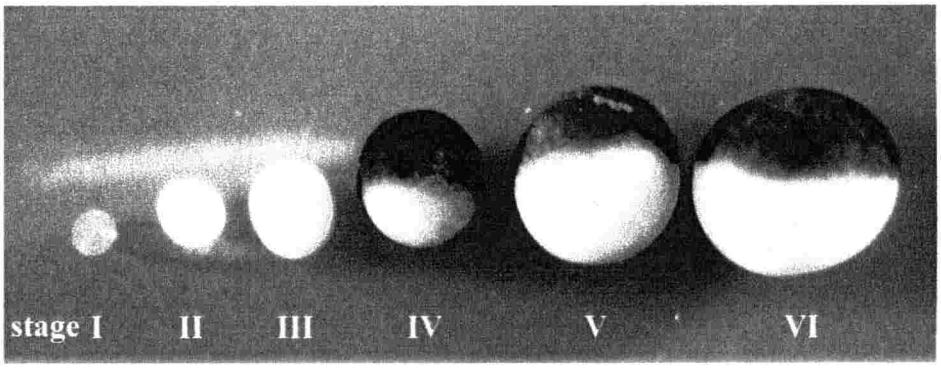
Recently, two methylated DNA binding proteins of mammalian orthologues, xMeCP2 and xMBD3, have been identified in *Xenopus* (Jones et al., 1998; Wade et al., 1999). These proteins are found in histone deacetylase containing co-repressor complexes and, similar to mammalian orthologues, favor to bind to the methylated DNA *in vitro*.

It is clear that *Xenopus* has a CpG methylation system that is responsible for gene silencing similar to that of mammals. However, quite little is known about the function of DNA methylation during development.

In studying the function of DNA methylation during early development, *Xenopus* has several advantages. Because of its large size (more than 1 mm in diameter) and easily obtained in large quantity, embryos at early developmental stage can be easily manipulated and visualized under microscope.

Development and fate mapping of embryos have been established. Dumont (1972) has classified *Xenopus* oogenesis into the six stages largely according to the size of oocytes; the smallest designated as stage I and the largest as stage VI (Fig. 4A). During the stages, oocytes are arrested at the deplotene stage of meiotic prophase and maternal stocks of RNA (ribosomal RNA and messenger RNA (mRNA)), mitochondria,

A



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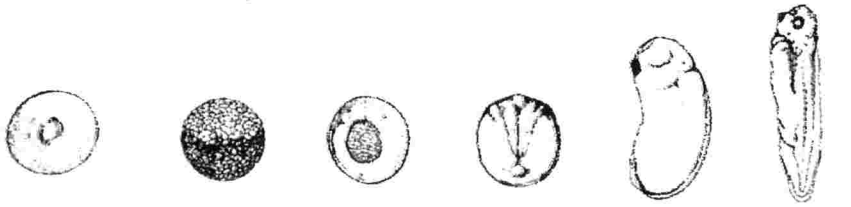


Figure 4. *Xenopus* oocytes and embryos. (A) *Xenopus* oocytes of different stages. The smallest transparent oocyte is at stage I (Dumont, 1972), while oocytes of increasing size represent stage II-VI, respectively. (Quoted from Smith et al., 1991.) (B) Early *Xenopus* development. Significant stages of *Xenopus* development are presented. (Quoted from Nieukoop and Faber, 1994.) Midblastura transition (MBT) is at stage 8.5.

and yolk are accumulated for the early development. Nieukoop and Feber (1994) classified the *Xenopus* embryos into the stages according to their morphological features (Fig. 4B). Until first 12 cleavages, *Xenopus* embryos cleave synchronously. From the 13th cleavage, synchrony is lost concomitant to the onset of zygotic transcription (stage 8.5). This has been termed as "midblastula transition" (MBT) (Newport and Kirschner, 1982). Gastrulation (stage 9-12) is a complex step involving morphogenic transformations and cellular movements. At this stage, the embryos form clear anteroposterior polarity, three germ layers of ectoderm, endoderm, and mesoderm, and establishes presumptive gut cavity. Neurulation (stage 13-20) is a step that involves the formation and folding the neural plate, and then the formation of the neural tube.

In addition to the morphological advantages in manipulating the embryos, the molecular tools are also available to study in detail (Sive et al., 2000a). Thus the microinjection technique using *Xenopus* embryos is useful in studying the role and regulation of specific gene products that play important roles in embryonic development.

Although genomic imprinting discovered in mammals is not reported in *Xenopus*, the elucidation of the function of DNA methylation in *Xenopus* may specifically shed light on the basic function of DNA methylation in vertebrates.

CHAPTER2. Cloning and expression of *Xenopus laevis* DNA methyltransferase cDNA

2.1 INTRODUCTION

DNA methylation plays important roles in various biological phenomena such as genomic imprinting and X-chromosome inactivation in mammals, tissue-specific gene expression, and carcinogenesis (Tajima and Suetake, 1998). The common mechanism underlying these phenomena is the regulation of expression of the related genes by DNA methylation. In animals, there are two types of methylation activities, *de novo-* and maintenance-type methylation activities. *De novo*-type methylation contributes to the establishment of tissue-specific methylation patterns at the implantation stage of embryogenesis (Monk, 1990), and maintenance-type methylation activity ensures clonal transmission of lineage-specific methylation patterns in somatic cells (Razin and Cedar, 1991; Toth et al., 1990).

Maintenance-type DNA methyltransferase (Dnmt1) favors to introduce a methyl group into the hemimethylated state of double-stranded DNA, which appears just after replication. Dnmt1 is believed to be responsible for maintaining the methylation pattern once formed in somatic cells. cDNA clones of Dnmt1 have been isolated from mouse (mDnmt1) (Bestor et al., 1988; Yoder et al., 1996), rat (Kimura et al., 1998), human (hDNMT1) (Yen et al., 1992; Yoder et al., 1996), and chicken (cDnmt1) (Tajima et al., 1995). An orthologous cDNA of Dnmt1 has also been isolated from sea urchin (uDnmt1) (Aniello et al., 1996).

mDnmt1 can be divided into two distinct domains. The carboxyl-terminal domain, comprised of about 500 amino acid residues, contains the catalytic site, which is conserved from bacterial type II DNA–(cytosine-5) methylases (Kumar et al., 1994).

The amino-terminal domain is thought to be a regulatory domain that recognizes the hemimethylated CpG sequence (Bestor et al., 1992), targets the molecule to replication foci (Leonhardt et al., 1992; Liu et al., 1998), and is responsible for the cytoplasmic retention in oocytes (Cardoso and Leonhardt, 1999). The amino-terminal domain contains a Cys-rich, zinc finger-like motif (Bestor, 1992). A Lys-Gly repeating sequence (KG-repeat) divides the amino- and carboxyl-terminal domains (Bestor et al., 1988). Both the Cys-rich region and the KG-repeat are conserved in hDNMT1, cDnmt1, and uDnmt1 (Yen et al., 1992; Tajima et al., 1995; Aniello et al., 1996).

As a first step to study the roles of Dnmt1 in early development, I cloned xDnmt1 cDNA from *Xenopus* oocytes cDNA library. The predicted amino acids sequence of xDnmt1 was compared with those of other animals. Isolated xDnmt1 cDNA will be a useful tool for examining the function of xDnmt1 during amphibian embryogenesis.

2.2 MATERIALS AND METHODS

2.2.1 Library screening and cDNA sequencing

A *Xenopus* oocyte cDNA library constructed in λgt10 (Rebagliati et al., 1985) was kindly provided by Dr. D. A. Melton (Harvard University). The library of $4x10^5$ plaque forming units was screened with a 2 kbp fragment of the 3' region of mDnmt1 cDNA (Bestor et al., 1988) as a probe. In the primary screening, more than 50 positive clones were detected. Among them, 20 clones were isolated and subcloned into the *Eco*RI site of pUC19. Of these clones, the XMT10 clone, which contained the largest insert, was analyzed. Using the 5'-end *Eco*RI fragment of the XMT10 clone as a probe, the library was rescreened, and the XMT5 clone was isolated. The two clones, XMT10 and XMT5, covered the entire coding region of xDnmt1 (Fig. 5B). A series of overlapping deletions was generated using exonuclease III (Sambrook et al., 1989), and sequenced by the dideoxy method (Sanger et al., 1977) using T7 DNA polymerase (Sequenase ver. 2.0, USB). The sequence was determined for both strands. 10% formamide was added to the polyacrylamide sequencing gels to improve the separation of GC -rich sequences (Tajima et al., 1995).

2.2.2 Construction of xDnmt1 plasmid and its expression

The XMT5 and XMT10 clones were combined into a single cDNA, and inserted into an expression vector, pKCRH2PL (Mishina et al., 1984), which was provided by Dr. Y. Morimoto (Mitsubishi Kagaku). mDnmt1 and cDnmt1 cDNAs subcloned into the identical vector were also used (Tajima et al., 1995; Takagi et al., 1995). Plasmids were transfected to COS1 cells as described (Takagi et al., 1995), using the calcium-phosphate method (Chen and Okayama, 1987), except that the cells were

recovered at 32 °C with the growth medium for 48 h. Post nuclear fractions and nuclear extracts were prepared as described (Bestor and Ingram, 1983), and the latter was also used as the enzyme source for activity measurements.

2.2.3 Cells

COS1 cells were maintained in Dulbecco's modified Eagle's MEM (DMEM) (Sigma Chemical Co., MO), supplemented with 10% fetal calf serum (FCS), 100 units/ml of penicillin, and 100 µg/ml of streptomycin, and were cultivated in plastic dishes at 37 °C in a 5% CO_2 atmosphere. *Xenopus* A6 cells were maintained in modified L-15 medium (Smith and Tata, 1991), containing 61% Leibovitz L-15 medium (Sigma Chemical Co., MO), 10% FCS, 100 units/ml of penicillin, and 100 µg/ml of streptomycin, and were cultivated at 22 °C.

2.2.4 Antibodies

The antiserum reactive with xDnmt1 was raised against a glutathione-*S*-transferase (GST) fusion protein containing amino acids 389-1,490 of xDnmt1 expressed in *Escherichia coli*. To this end, the 3' end *Eco*RI fragment of xDnmt1 was ligated into pGEX2TH, kindly provided by Dr. H. Maruta (Ludwig Institute for Cancer Research, Melbourne), and expressed in *Escherichia coli* strain NM522 in the presence of isopropyl-*β*-D-thiogalactopyranoside. The expressed GST fusion protein accumulated in inclusion bodies. Inclusion bodies were purified (Sambrook et al., 1989) and the GST fusion protein was further purified by electroelution from an SDS-PAGE (Hunkapiller et al., 1983). The antibodies raised against the protein in a rabbit were immunoselected using antigen-coupled Sepharose CL-4B (Amersham Pharmacia Biotech AB, Sweden) as an affinity matrix. Anti-mouse

Dnmt1 antibodies were raised and immunoselected as described (Takagi et al., 1995).

2.2.5 Western blotting

Post-nuclear fractions, or nuclear extracts was electrophoresed in a 7% SDS-polyacrylamide gel according to the method described in Laemmli (1970). After electrophoresis, protein bands were electrophoretically transferred onto nylon membrane. Anti-mDnmt1 antibodies, a primary antibody, were reacted in PBS containing 1% bovine serum albumin (BSA), 1% Triton X-100, 0.1% SDS, and 0.05% sodium azide. The xDnmt1-antibody complex was detected with goat anti-rabbit IgG antibodies conjugated with [^{125}I]-labeled protein A and was detected by exposing to X-ray film with intensifying screen at –70 °C.

2.2.6 DNA methyltransferase activities

DNA methyltransferase activities were determined as described (Takagi et al., 1995). The protein concentrations were determined as described by Lowry et al. (1951), using BSA as a standard. The supernatant fractions were used as the source of the enzyme. The reaction mixture contained 0.1 μg of poly(dI·dC)-poly(dI·dC) (Amersham Pharmacia Biotech AB, Sweden) and 2 μCi of [^{3}H]SAM (15.0 Ci/mmol, Amersham Pharmacia Biotech AB, Sweden) in a volume of 25 μl reaction buffer (Takagi et al., 1995).

2.2.7 Immunocytochemistry

A6 cells were fixed in 0.7x PBS containing 3.7% formaldehyde for 10 min at room temperature and washed three times for 5 min each with PBS containing 0.1% Triton X-100. For xDnmt1 staining, the fixed cells were washed twice for 10 min each

with PBS containing 2% Triton X-100, 0.4% SDS, 1% BSA and 10% FCS and incubated for overnight at 4 °C with anti-xDnmt1 polyclonal antibodies in an identical buffer. The specimens were washed three times for 5 min each with PBS containing 0.1% Triton X-100 and incubated with ALEXA 488 conjugated anti-rabbit IgG antibodies (Molecular Probes Inc., OR) for 2 h at room temperature in an identical buffer. After the incubation, the samples were washed with PBS containing 0.1% Triton X-100 and then immersed in PBS containing 50% glycerol..

For the staining of lamin, the fixed cells were washed twice for 10 min each with PBS containing 0.1% Triton X-100 and incubated for overnight at 4 °C with anti-rat lamin monoclonal antibody (clone MY 95) in an identical buffer. The specimens were washed three times for 5 min each with PBS containing 0.1% Triton X-100 and incubated with TRITC conjugated anti-mouse IgM antibodies (Chemicon International Inc., CA) for 2 h at room temperature in an identical buffer. After washing with PBS containing 0.1% Triton X-100, the samples were prepared as above.

2.2.8 Microscopy

An Olympus Epi-fluorescence microscope BX50 with an Olympus UPlanAPO 20x objective lens was used for examining the stained samples. Photographs were taken with an Olympus PM-30 camera using Provia 400 (Fuji Photo Film Co. Ltd., Tokyo). All the samples were exposed and printed under identical conditions.

2.3 RESULTS

2.3.1 Isolation and sequencing of the xDnmt1 cDNA

As the chicken (avian) Dnmt1 sequence is highly homologous to those of mammalian Dnmt1s, I expected that the *Xenopus* (amphibian) Dnmt1 (xDnmt1) sequence would also be similar to those of mammalian Dnmt1s. Thus, I first screened the *Xenopus* oocyte cDNA library with the labeled fragment of mouse Dnmt1 (mDnmt1) cDNA coding the catalytic domain of the enzyme as a probe and cloned XMT10. Using 5'-end fragment of the XMT10 clone, I then cloned XMT5. The two overlapping clones contained the entire coding sequence of xDnmt1 (Fig. 5A and B). The size of the deduced nucleotide sequence of xDnmt1 cDNA was 5,033 bp with poly(A). The A of initiation methionine residue (ATG) was at the nucleotide position 260 and the T of the stop codon (TAA) was at the nucleotide position 4,730. The elucidated nucleotide sequence contained a 4,470-nucleotide open reading frame that encoded a protein of 1,490 amino acids residues, the calculated molecular weight of which is 167,981.

2.3.2 Encoded amino acids sequence of xDnmt1 cDNA is highly homologous to those of other animal Dnmt1 cDNAs

The carboxyl-terminal domain composed of about 500 amino acids residues contains the motifs that are responsible for the catalytic activity. These motifs are conserved from bacterial type II DNA-(cytosine-5) methylases. Motif I is expected to contribute to SAM binding and motif IV contains the invariant Pro-Cys dipeptide sequence that is known to be a part of the catalytic center. When the motifs of xDnmt1 were aligned with those of mDnmt1, hDNMT1, cDnmt1 and uDnmt1, the sequences

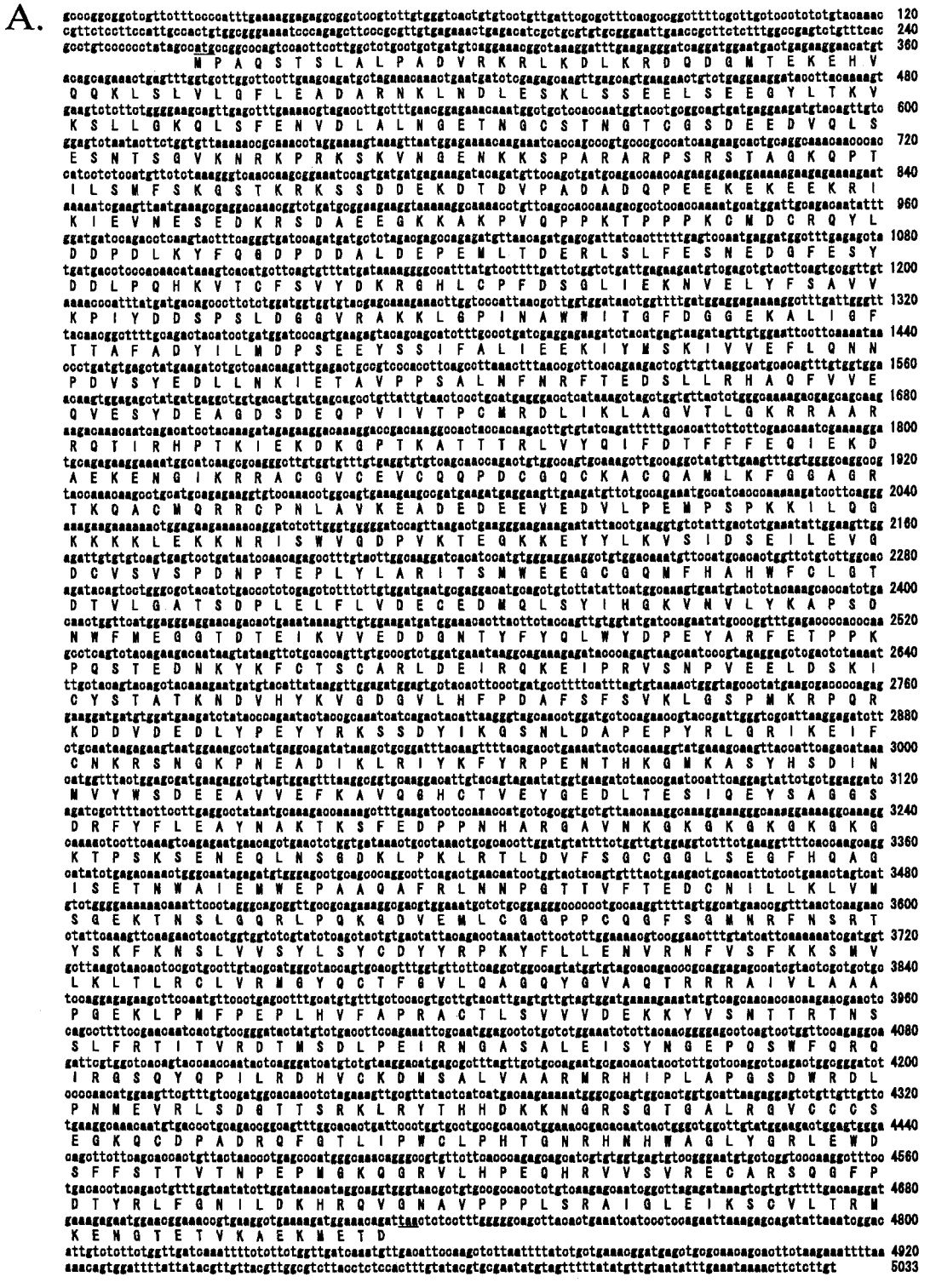


Figure 5. ***Xenopus*** **maintenance-type DNA methyltransferase (xDnmt1) cDNA.** (A) Nucleotide and deduced amino acids sequence of xDnmt1 (accession no. D78638). The predicted amino acids are shown in one letter code. The each site for initiation (ATG) and termination (TAA) codons is underlined. (B) Schematic illustration of xDnmt1 cDNA and clone XMT5 and XMT10. The coding region is shown in a black box. The restriction sites of *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), and *Xho*I (X) are indicated.

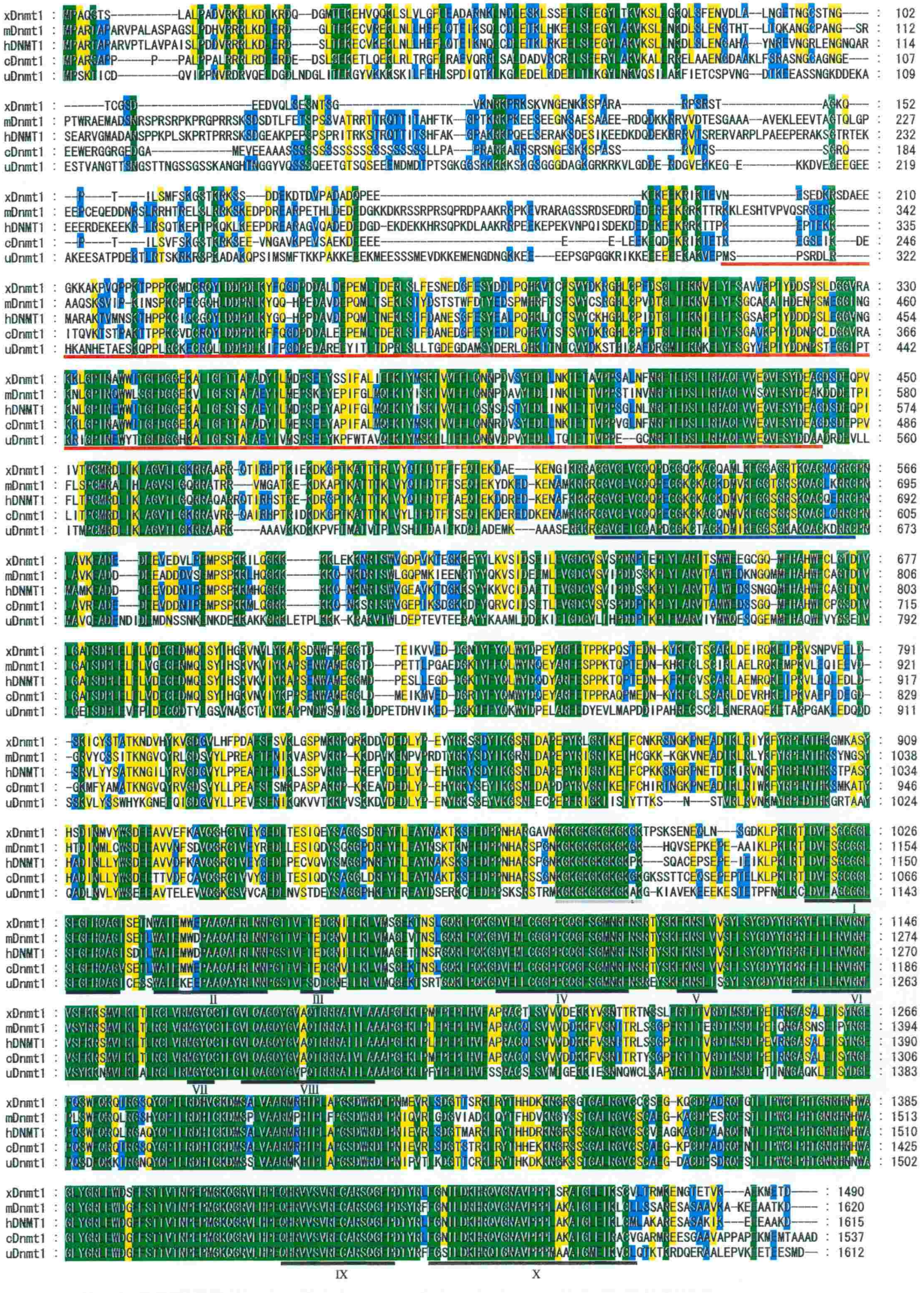
were highly conserved among the species (Fig. 6A, black bars). More than 85% of the amino acid residues were matched among the Dnmt1s. The two motifs first found in mDnmt1, a zinc-binding region (Fig. 6A, blue bar), which resides in the middle of the amino-terminal domain, and the KG-repeat (Fig. 6A, gray bar), which divides the carboxyl- and amino-terminal domains, were also conserved in xDnmt1. Another sequence of about 200 amino acids residues in the amino-terminal domain, which is responsible for localizing mDnmt1 in the replication foci at late S phase, showed about 63% matching among the Dnmt1s (Fig. 6A, red bars).

It has been reported that hDNMT1 interacts with PCNA at its amino-terminal sequence (Chuang et al., 1997). Moreover, Suetake found that xDnmt1 also bound to PCNA at amino-terminal region (Suetake, unpublished). Interestingly, however, the alignment of PCNA binding motif revealed that His170, which is the critical amino acid residue for the binding of PCNA in hDNMT1, was substituted with Met in xDnmt1 (Fig 6B, asterisk).

2.3.3 xDnmt1 cDNA encodes active DNA methyltransferase

The similarity of the deduced amino acid sequence of the xDnmt1 with those of the other vertebrate Dnmt1s indicates that the xDnmt1 cDNA encodes Dnmt1. To confirm that the cloned cDNA actually encodes Dnmt1, the xDnmt1 clones covering the entire coding sequence were combined into a single cDNA, which was inserted into an expression vector pKCRH2PL containing SV40 promoter, and expressed in COS1 cells (Fig. 7). DNA methyltransferase activities of the nuclear extracts from xDnmt1 transfected cells were lower than that from mDnmt1 or cDnmt1, but were significantly higher than that from *lacZ* transfected cells (Table I).

A.


xDnmt1
mDnmt1
hDNMT1
cDnmt1
uDnmt1
I
II
III
IV
V
VI
VII
VIII
IX
X

B.

Figure 6. Alignment of amino acid sequence of *Xenopus*, mouse, human, chicken, and sea urchin Dnmt1s. (A) Alignment of full sequence of Dnmt1s. Amino acids sequences were aligned using the Clustal W multiple sequence algorism. The targeting sequence of replication foci (red), Cys rich region (blue), and KG-repeat (gray) are underlined. The motifs I-X (black) are also underlined. The conserved amino acid residues were colored using GeneDoc program. When the amino acid residues in all 5 Dnmt1s are identical or similar, then are colored green. When 4 out of 5 are identical or similar, then are colored yellow. When 3 out of 5 are identical or similar, then are colored blue. (B) Alignment of PCNA binding motif of xDnmt1, mDnmt1, hDNMT1 and cDnmt1. Asterisk indicates the position of amino acid residue (His) that is critical for PCNA binding in hDNMT1. The conserved amino acid residues were colored using GeneDoc program. When the amino acid residues in all 4 Dnmt1s are identical or similar, then are colored green. When 3 out of 4 are identical or similar, then are colored yellow.

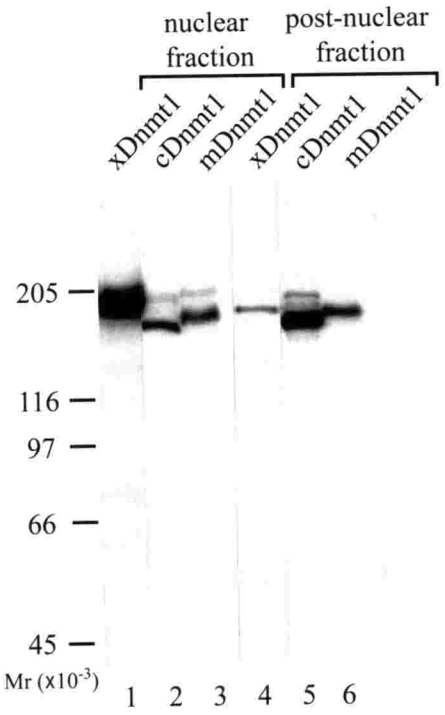


Figure 7. Expression of xDnmt1 cDNA in COS1 cells. xDnmt1, cDnmt1, and mDnmt1 cDNA were ligated into the expression vector pKCRHP2L in a sense orientation, and transfected into COS1 cells. After the recovery, nuclear extracts and post-nuclear fractions were separately recovered, electrophoresed, blotted onto nylon membrane, and immunodetected with goat anti-rabbit IgG conjugated with [^{125}I]-labeled protein A. The bands were detected by exposing to X-ray film at –70 °C. Molecular weight standard (Mr) were indicated.

Table I. DNA methyltransferase activities of transiently expressed xDnmt1 in COS1 cells. The nuclear extracts were prepared from COS1 cells transfected with pKCR-xDnmt1, pKCR-cDnmt1, pKCR-mDnmt1 and pKCR-*lacZ*. The activities were measured using poly(dI·dC)-poly(dI·dC) as a substrate.

clones	DNA methyltransferase activity (pmol/h/mg protein)
pKCR-xDnmt1	8.8
pKCR-cDnmt1	148.2
pKCR-mDnmt1	263.2
pKCR-*lacZ*	1.9

2.3.4 Localization of xDnmt1 in A6 cells

To see the localization of endogenous xDnmt1, I performed immunostaining. *Xenopus* kidney cell line A6 cells were immunostained with the specific antibodies raised against xDnmt1. Endogenous xDnmt1 was localized exclusively in the nuclei of A6 cells (Fig. 8A). Nuclear marker protein lamin immunostained with anti-lamin (MY95) monoclonal antibody showed identical localization as xDnmt1 (Fig. 8C).

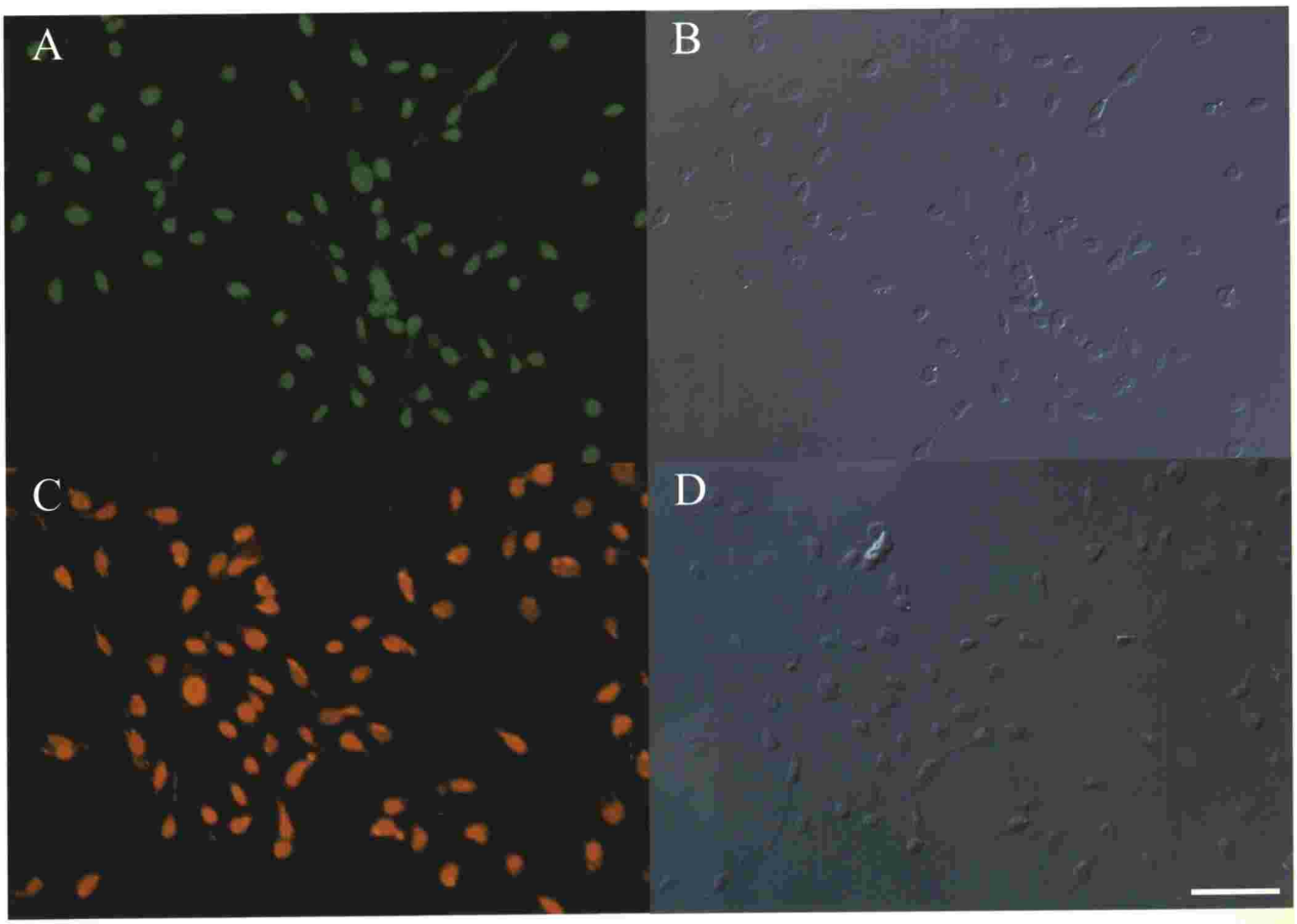


Figure 8. Localization of xDnmt1 in the nuclei of A6 cells. A6 cells were fixed for 10 min in 3.7% fromaldehyde and stained with anti-xDnmt1polyclonal antibodies (A) or anti-mouse lamin (MY 95) monoclonal antibodies (C). Right panel, the cells observed under Nomarski optics (B and D). Bar indicates 100 μm.

2.4 DISCUSSION

2.4.1 xDnmt1 was highly homologous to other vertebrate maintenance-type Dnmt1.

I isolated and sequenced the *Xenopus* Dnmt1 cDNA, and found that the predicted amino acids sequence was highly homologous to those of other animals' Dnmt1. Together with the results that the ectopically expressed xDnmt1 showed significant level of DNA methyltransferase activity, I concluded that xDnmt1 encodes *Xenopus* maintenance-type DNA methyltransferase.

The predicted amino-acid sequences of initially isolated cDNAs of mouse (Bestor et al., 1988) and human (Yen et al., 1992) Dnmt1s lacked 118 and 120 amino-acid sequences, respectively. Comparison of the amino-acids sequences of xDnmt1 with those of the short form mDnmt1 or hDNMT1 showed almost no homology in the amino-terminal region, upstream of replication foci targeting sequence (Kimura et al., 1996), while the amino-terminal region of cDnmt1 is highly homologous to that of xDnmt1 (Tajima et al., 1995; Kimura et al., 1996). Later on, the full length sequences of mDnmt1 and hDNMT1 are reported (Yoder et al., 1996), and the alignment revealed that their amino-terminal region, specially the 100 amino acids, are homologous to each other (Fig. 6A). In mouse, the previously reported short form is oocyte-specific type. This oocyte-type mDnmt1 is a product of alternatively spliced variant (Mertineit et al., 1998). The oocyte-type mDnmt1 is expressed and accumulated during oogenesis and is the major population of mDnmt1 during early development (Mertineit et al., 1998). All the xDnmt1 cDNA clones I isolated encoded no short form such that found in mouse, even if I screened the oocyte library of *Xenopus* cDNA. Furthermore, I could not detect any alternatively spliced variant in the RT-PCR products from A6 cell (data not shown). These results suggest that, in *Xenopus*, no short form

like mDnmt1 in oocytes exists. There could be a unique regulating mechanism of Dnmt1 in *Xenopus,* which is different from mouse, during early development

The predicted amino-acid sequence of xDnmt1 was highly homologous to those of the full sequences of mDnmt1, hDNMT1, cDnmt1, and uDnmt1. Among the functional domains reported, however, one exception was found in the PCNA binding motif (Chuang et al., 1997). His170 in the PCNA binding motif of hDNMT1 is the critical amino acid residue for binding to PCNA. Changing of His170 to Val disrupts the binding activity of hDNMT1 to PCNA (Chuang et al., 1997). Alignment analysis of xDnmt1 revealed that His in mDnmt1 and hDNMT1 is changed to Met in xDnmt1 (Fig. 6B). Nevertheless, Suetake pulled out PCNA molecule from *Xenopus* cDNA library by two-hybrid system using xDnmt1 as a "bait" (Suetake, unpublished). It would be useful to use *Xenopus* system to elucidate the interaction mode of xDnmt1 and xPCNA.

Considering that the highly homologous sequence of xDnmt1 to those of other animals, and that the endogenous xDnmt1 is specifically localized to the nuclei of A6 cells (Fig. 8), it can be estimated that xDnmt1 is also under the control of similar mechanism as those in other animals.

The xDnmt1 exogenously expressed in the nuclear fractions of COS1 cells showed extremely low DNA methyltransferase activity, though significant (Table I). This is partly due to the low translocation efficiency of xDnmt1 into nucleus. As shown in Fig. 7, lanes 1 and 4, most of the translated xDnmt1 in COS1 cells remained in the post-nuclear fraction, that is, in the cytoplasmic fraction. In A6 cells, however, endogenous xDnmt1 is localized in nuclei. The difference would be due to the difference of translocation machinery in mammalian COS1 cells and *Xenopus* A6 cells, or the folding of xDnmt1 after translation may be a temperature sensitive process. The latter possibility may be working. Neither *Xenopus* nor culture cells can survive at high

temperature, such as 37 °C, and poly(A) polymerase from *Xenopus* is not active at 37 °C, though it is active below 25 °C (Gebauer and Richter, 1995). Even 32 °C, at which temperature the cells transfected with xDnmt1 were recovered in the present study, may be too high for xDnmt1 to form a proper conformation to be translocated into the nucleus and/or to express full activity.

2.5. SUMMARY

In this chapter, I isolated xDnmt1 cDNA from a *Xenopus* oocytes cDNA library by screening with the mDnmt1 cDNA as a probe. The elucidated nucleotide sequence gave a 4,470 nucleotide open reading frame, and the predicted protein was composed of 1,490 amino acid residues, showing high homology to the animal Dnmt1s. The carboxyl-terminal domain of xDnmt1 contains the conserved motifs that are conserved from bacterial type II DNA-(cytosine-5) methylase. The cloned xDnmt1 expressed in COS1 cells showed a significant DNA methyltransferase activity. The endogenous xDnmt1 localized in the nuclei of A6 cells such as mammalian Dnmt1. These results suggest that the cloned xDnmt1 cDNA encodes maintenance-type DNA methyltransferase.

CHAPTER 3. Expression and localization of xDnmt1 during oogenesis

3.1 INTRODUCTION

Various Dnmt1 cDNAs are isolated from mouse (Bestor et al., 1988; Yoder et al., 1996), rat (Kimura et al., 1998), human (Yen et al., 1992; Yoder et al., 1996), chick (Tajima et al., 1995), frog (*Xenopus*) (Chapter 2), and sea urchin (Aniello et al., 1996). The predicted amino acid sequences of those Dnmt1s are highly homologous to each other. Dnmt1 possesses a carboxyl-terminal domain closely related to bacterial type II DNA-(cytosine-5) methylases and a large amino-terminal domain that has multiple regulatory functions (Bestor and Verdine, 1994; Bestor, 1996).

In mouse somatic cells, Dnmt1 is localized in the nuclei and specifically accumulates at the replication foci at late S-phase nuclei (Leonhardt et al., 1992; Liu et al., 1998). Different from somatic cells, expression and localization of Dnmt1 are specifically regulated in germ lines. During spermatogenesis in mouse, the transcription starts from the testis-specific site at the stage of pachytene spermatocytes, the late stage of meiosis. The transcript does not produce functional Dnmt1 (Jue et al., 1995, Mertineit et al., 1998). In growing oocytes, Dnmt1 was first localized in the nucleus. As the oogenesis proceeds, Dnmt1 is accumulated in the cytoplasm, and at ovulation Dnmt1 is excluded from the nuclei (germinal vesicles) (Mertineit et al., 1998). At the one- and two-cell stages, Dnmt1 is in the cytoplasm near plasma membrane, and only after the 8-cell stage it is translocated into the nuclei (Carlson et al., 1992). The amount of accumulated Dnmt1 in mouse mature oocytes and one-cell embryos is 3,000 times higher than that of murine erythroleukemia (MEL) cells per cell basis (Carlson et al., 1992). During oogenesis and early development, the transcription starts from the specific site in mouse *Dnmt1* gene, of which mRNA encodes the oocyte-specific Dnmt1

lacking 118 amino acids from its amino-terminus of somatic-type (Mertineit et al., 1998). Similar to mouse, *Xenopus* DNA methylation activity, possibly due to Dnmt1, is reported to be higher in whole cell extracts than in the nuclear extracts at stage V and VI oocytes (Adams et al., 1981).

In the present study, I examined the expression and distribution of xDnmt1 protein during oogenesis. The synthesis and accumulation of xDnmt1 sharply increased in the late stage of oogenesis. Different from mouse, at stage VI oocytes, significant amount of xDnmt1 was translocated into nuclei. The results suggest that xDnmt1 in oocytes is differently regulated from that of somatic cells and may play an important role in *Xenopus* early embryogenesis.

3.2 MATERIALS AND METHODS

3.2.1 Collection of oocytes and isolation of nuclei

Female *Xenopus laevis* were purchased from Kita-Nihon-Doubutsu (Aomori, Japan), and kept as described (Wu and Gerhart, 1991). Ovaries were obtained surgically from adult female *Xenopus* anesthetized in ice-cold water, and follicle cells were removed either manually with watchmaker's forceps or enzymatically with collagenase (Wako Pure Chemical Industries, Ltd., Osaka) treatment (Smith et al., 1991). Stages of oocytes were determined according to Dumont (Dumont, 1972). Nuclei at stage VI oocytes were manually isolated as described (Evans and Kay, 1991). To obtain unfertilized eggs, female *Xenopus* was injected 750 units of human chorionic gonadotropin (Sigma Chemical Co., MO). The laid unfertilized eggs were dejellied in 2% cysteine-HCl (pH 7.5) (Peng, 1991).

3.2.2 Metabolic labeling of oocyte

Ten manually defolliculated oocytes at each stage were incubated at 22 °C for the indicated time periods with 400 μCi/ml of EXPRE^{35}S^{35}S (New England Nuclear, MA) in L-methionine- and L-cysteine-free 0.7x DMEM (Sigma Chemical Co., MO), supplemented with 10 mM HEPES-NaOH (pH 7.4) and 10% FCS. The FCS used was extensively dialyzed against PBS.

3.2.3 Immunoprecipitation of xDnmt1

Whole oocytes, enucleated oocytes, and isolated nuclei were briefly sonicated in 870 μl of 0.575% SDS, 2.9 mM EDTA, and 57.5 mM triethanolamine-HCl (pH 7.4), and incubated at 95 °C for 10 min. To the mixtures were added 120 μl of 20% (w/v)

Triton X-100, 20 µl of 100 units/µl Trasylol, and 10 µl of 0.5 M iodoacetamide. The immunoselected anti-xDnmt1 polyclonal antibodies (Chapter 2) were added to the reaction mixture, and the reaction mixture was incubated at 4 °C overnight, then protein A-Sepharose was added to precipitate the xDnmt1-antibodies complex. The protein A-Sepharose was washed three times with 0.1% Triton X-100 and 0.75 unit/ml Trasylol in PBS. Proteins were solubilized in a sample buffer.

The immunoprecipitated radio-labeled xDnmt1 protein bands were analyzed by fluorography (Bonner and Laskey, 1974) after SDS-PAGE (Laemmli, 1970).

3.2.4 Western blot analyses of xDnmt1, α-tubulin, and proliferating cell nuclear antigen (PCNA)

For detecting xDnmt1 by Western blotting, the immunoprecipitated samples were separated by SDS-PAGE (Laemmli, 1970) and electrophoretically transferred onto nitrocellulose membrane. Anti-xDnmt1 antibodies, a primary antibody, were incubated in PBS containing 1% BSA, 1% Triton X-100, 0.1% SDS and 0.05% sodium azide. The xDnmt1-antibody complex was detected with alkaline phosphatase (E.Y. Laboratory Inc., CA) or horseradish peroxidase (HRP) (Dako A/S, Glostrup) coupled second antibodies, and a color reaction buffer containing 0.05 mg/ml BCIP and 1 mg/ml NBT (Takagi et al., 1995), or ECL Western blotting detection regents (Amersham Pharmacia Biotech AB, Sweden), respectively.

For detecting α-tubulin or PCNA, whole oocytes, enucleated oocytes or isolated nuclei were homogenized in 50 mM Tris-HCl (pH 6.8) containing 100 µg/ml phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged at 12,000 rpm for 10 min and were added 8 volumes of ice-cold acetone to precipitate proteins. The samples were electrophoresed in a 10% SDS-polyacrylamide gel (Laemmli, 1970).

After the proteins were electrotransferred, α-tubulin and PCNA bands were immunodetected by ECL using a monoclonal anti-α-tubulin antibody (DM1A) (Sigma Chemical Co., MO), and a monoclonal anti-PCNA antibody (PC10) (Transduction Laboratories, KY) as first antibodies, respectively.

3.2.5 Immunocytochemistry

Manually isolated nuclei were fixed in Dent's fixative (Dent et al., 1989) at –20 °C for overnight, embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetechnical Co. Ltd., Tokyo), 14 μm cryosections were prepared by HM400 (Microm Laborgeräte GmbH, Germany) at –17 °C, and then collected on 0.1% (w/v) gelatin (Iwaki Glass, Tokyo) coated slides.

For immunofluorescent staining, sections were washed three times with PBS containing 0.1% Triton X-100, and incubated for 2 h in PBS containing 2% Triton X-100, 0.4% SDS, 1% BSA and 10% FCS. The sections were incubated with anti-mDnmt1 antibodies (Takagi et al., 1995) as primary antibodies at 4 °C for overnight. As the titer of anti-mDnmt1 antibodies against xDnmt1 showed higher than that of anti-xDnmt1 antibodies, I used the anti-mDnmt1 antibodies for the immunocytochemical studies. Control sections were incubated either with anti-mDnmt1 antibodies pre-absorbed with the fused protein of mDnmt1 with GST or maltose-binding protein (MBP), or without anti-mDnmt1 antibodies. As for the preparation of the fusion protein of mDnmt1 with MBP, identical *Bam*HI/*Eco*RI fragment of mDnmt1 that was used to make the GST fusion protein was ligated into pMALc2 vector (New England Biolabs Inc., MA). The expressed fusion protein was purified as manufacturer's protocol using amylose coupled resin. The specimens were washed four times with PBS containing 0.1% Triton X-100, incubated with ALEXA

488 conjugated anti-rabbit IgG antibodies (Molecular Probes Inc., OR) for 2 h at room temperature, washed with PBS containing 0.1% Triton X-100, and immersed in PBS containing 50% glycerol. For the double staining, ALEXA 488 stained sections were further incubated with anti-rat lamin A/C (MY95) monoclonal antibody for 1 h at room temperature. The sections were then washed three times and incubated with TRITC conjugated anti-mouse IgM antibodies (Chemicon International Inc., CA). After washing four times, the sections were prepared as above.

For HRP staining, sections were treated for 30 min in PBS containing 0.3% H_2O_2 before blocking. The procedure for blocking and incubation with anti-mDnmt1 antibodies were basically the same as that for the immunofluorescent staining as described above. The sections were incubated at room temperature for 2 h with biotin-conjugated anti-rabbit IgG antibody (Chemicon International Inc., CA) as secondary antibodies. To visualize the localization of xDnmt1 protein, the sections were incubated at room temperature for 1 h with HRP conjugated streptavidin (Zymed Laboratories Inc., CA). After washing, the color development was performed with diaminobenzidin (Sigma Chemical Co., MO) (Guesdon et al., 1979).

An Olympus Epi-fluorescence microscope BX50 with an Olympus UPlanAPO 10x objective lens was used for examining stained sections. Photographs were taken as Chapter 2. For HRP staining, photographs were taken using Provia 100 (Fuji Photo Film Co. Ltd., Tokyo).

3.2.6 DNA methyltransferase activities

MEL cells, A6 cells and nuclei prepared from stage VI oocytes were homogenized with a buffer containing 0.3 M NaCl, 1 M sucrose, 3 mM $MgCl_2$, 0.3% (w/v) Triton X-100, 0.2 mM PMSF, 0.2 mM dithiothreitol, 20 mM Tris-HCl (pH 7.4)

(Takagi et al., 1995) and 5 μg/ml E64, centrifuged at $4x10^5$ g for 20 min at 4 °C, and supernatant fractions were used as the source of the enzyme. The reaction mixture contained 0.1 μg of poly(dI·dC)-poly(dI·dC) (Amersham Pharmacia Biotech AB, Sweden) and 2 μCi of [^{3}H]SAM (15.0 Ci/mmol, Amersham Pharmacia Biotech AB, Sweden) in a volume of 25 μl reaction buffer (Takagi et al., 1995). Whole cell extracts of $6x10^4$ cells for MEL cells and of $6x10^3$ cells for A6 cells, and nuclear extracts of 0.25 oocytes were added to a single reaction mixture as enzyme sources. After 1 h incubation at 37 °C, the DNA methyltransferase activities were determined as described (Takagi et al., 1995), except that after the reaction, the mixtures were added and incubated with 10 μg of DNase-free RNase A at 37 °C for 10 min, and then 10 μg of proteinase K (Nakarai Tesque, Kyoto) at 50 °C for 10 min in the presence of 0.5% SDS. The mixtures were then extracted once with phenol-chloroform and the specific radioactivities were determined (Takagi et al., 1995). The RNase A and proteinase K treatment dramatically decreased the background radioactivities in the absence of methyl acceptor, poly(dI·dC)-poly(dI·dC).

3.3 RESULTS

3.3.1 xDnmt1 protein is accumulated in oocytes at the late stage of oogenesis.

In mouse, the amount of Dnmt1 in a single mouse mature oocyte is 3,000 times higher than that in a single MEL cell (Carlson et al., 1992). I evaluated the amount of xDnmt1 in oocytes during oogenesis. To do this, xDnmt1 was concentrated from a whole cell extract of 100 oocytes by immunoprecipitation with anti-xDnmt1 antibodies, and then xDnmt1, of which apparent molecular weight is about 190 k (Chapter 2), was detected by Western blotting (Fig. 9A). The xDnmt1content rapidly increased in parallel with the maturation of oocytes. In the present case, the xDnmt1 protein could not be detected in 100 oocytes before stage III oocytes (data not shown). The xDnmt1 levels in stage III, IV, and V oocytes were calculated to be 1, 2 and 14%, respectively, of that in stage VI oocytes (Fig. 9B). The amount of xDnmt1 in a single mature oocyte was about 20 times higher than that in a single stage VI oocyte (Fig. 9A, right panel). A stage VI oocyte and a mature oocyte were calculated to contain about $1.2x10^4$ and $2.4x10^5$ times higher xDnmt1 protein than that in a single MEL cell, respectively. A *Xenopus* mature oocyte contained a two orders of magnitude higher amount of Dnmt1 than a mouse oocyte.

To determine whether the accumulation of the xDnmt1 proteins during oogenesis is caused by an increase in the net synthesis or inhibition of the degradation of xDnmt1, I next examined the changes in the rate of synthesis of xDnmt1 during oogenesis. Ten oocytes at each stage were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, whole extracts were immunoprecipitated with anti-xDnmt1 antibodies, and then the labeled xDnmt1 bands were detected (Fig. 10A). The incorporation of [^{35}S]amino acids into xDnmt1 increased linearly up to 18 h incubation (Fig. 10B). The

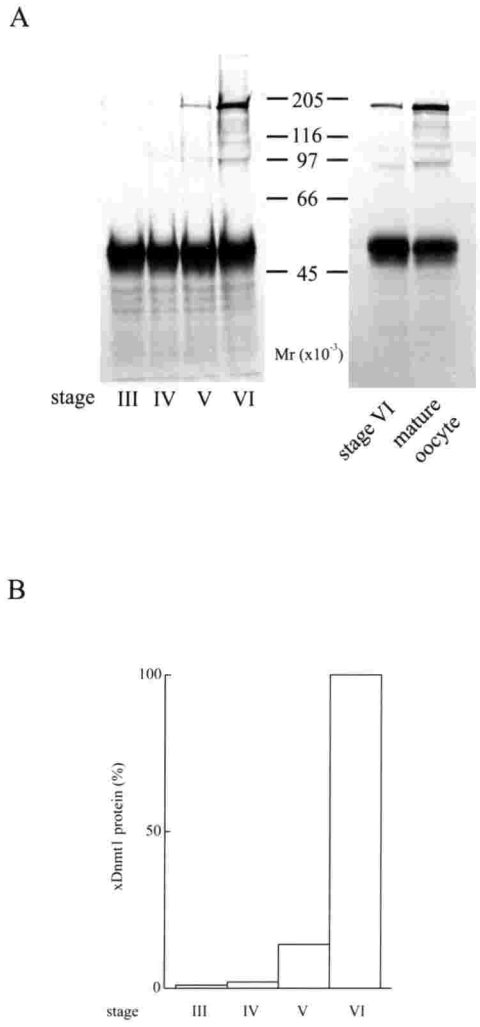


Figure 9. Accumulation of xDnmt1 during oogenesis. (A) Extracts of 100 *Xenopus* oocytes prepared from stage III to VI and mature oocytes were immunoprecipitated with anti-xDnmt1 antibodies and then analyzed by Western blotting using alkaline phosphatase coupled secondary antibodies. Intense bands at around 50 kDa are heavy chains of the anti-xDnmt1 antibodies. Molecular weight standards (Mr) are indicated. (B) The protein content of xDnmt1 was analyzed during oogenesis. The xDnmt1 bands (Fig. 9A) were quantified by a image analyzer, MCID (Imaging Research Inc., Canada). The amount of xDnmt1 obtained in stage VI oocytes was taken as 100%.

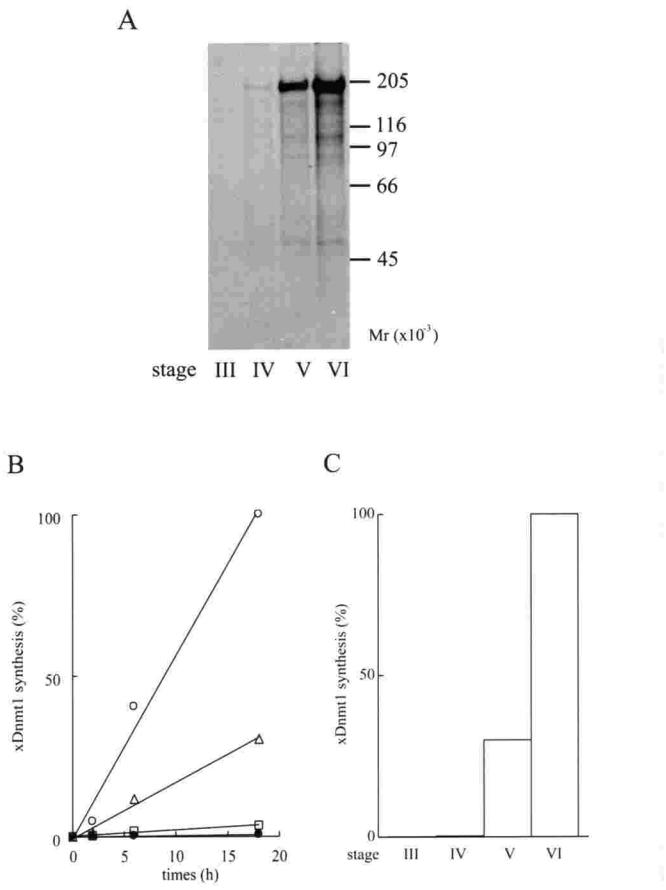


Figure 10. Synthesis of xDnmt1 during oogenesis. (A) Ten *Xenopus* oocytes at each stage (III-VI) were incubated with EXPRE^{35}S^{35}S for 18 h, immunoprecipitated, electrophoresed, and then fluorographed. Molecular weight standards (Mr) are indicated. (B) Ten *Xenopus* oocytes at each stage were incubated with EXPRE^{35}S^{35}S for 2, 6, and 18 h. After immunoprecipitation, the proteins were electrophoresed and the radio-labeled xDnmt1 was quantified by an image analyzer, BAS2000 (Fuji Photo Film Co. Ltd., Tokyo). The radioactivities of xDnmt1 bands were normalized to that of stage VI oocytes after 18 h incubation. The symbols indicated stage III (●), stage IV (□), stage V (Δ), and sage VI (○), respectively. (C) A histograms presentation of the *de novo* synthesis of xDnmt1 in each stage oocytes, after 18 h incubation shown in panel A. The radioactivities of xDnmt1 bands in panel A were determined by an image analyzer BAS2000 (Fuji Photo Film Co. Ltd., Tokyo). The values of xDnmt1 were normalized to that in stage VI oocytes as 100%.

radio-labeled xDnmt1 bands at each stage after 18 h incubation with [^{35}S]amino acids are shown in panel A. Radio-labeled xDnmt1 was significantly detected only after stage III, and increased to a maximum level in stage VI oocytes. The amount of radio-labeled xDnmt1 in stage III, IV and V oocytes were calculated to be 0.1, 0.3 and 30 %, respectively, of that in stage VI oocytes (Fig. 10C). These results clearly indicate that an increase in the xDnmt1 synthesis rate contributed to the accumulation of the protein in late stage oocytes.

3.3.2 Translocation of xDnmt1 into the nucleus

In mouse, mDnmt1 is localized in the nuclei only at the very early stage of growing oocytes. As the oogenesis proceeds, mDnmt1 becomes undetectable in the nucleus and accumulates at a high level in the cytoplasm (Mertineit et al., 1998).

To study the localization of xDnmt1 in *Xenopus* oocytes, I performed both biochemical and immunocytochemical analyses on stage VI oocytes. For biochemical analysis, stage VI oocytes were manually enucleated, and then fractionated into post-nuclear and nuclear (germinal vesicle) fractions. xDnmt1 in each fraction was immunoprecipitated with anti-xDnmt1 antibodies and then detected by Western blotting (Fig. 11A). The results indicated that xDnmt1 was clearly localized in the nuclei as well as the post-nuclear fraction (Fig. 11A). α-Tubulin, a cytoplasmic marker (Gard, 1991), and PCNA, a nuclear marker (Leibovici et al., 1990), were properly fractionated into the respective fractions under the conditions used (Fig. 11B).

In mouse oocytes, the Dnmt1 is not detected in the nuclei but accumulated to a very high level in the cytoplasm (Mertineit et al., 1998). However, a present biochemical study indicated that xDnmt1 existed in both the cytoplasm and nucleus in almost equal amounts in stage VI oocytes. To examine the localization of xDnmt1 in

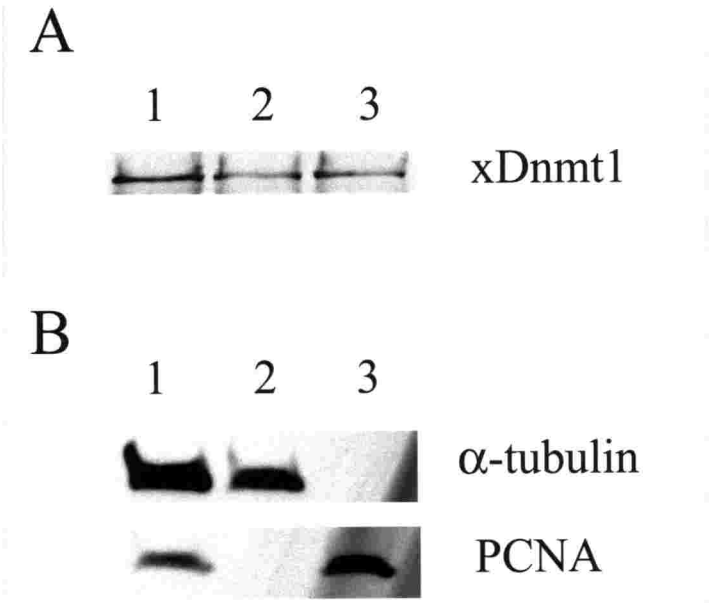


Figure 11. Distribution of xDnmt1 in stage VI oocytes. (A) Stage VI oocytes were fractionated into post-nuclear and nuclear fractions, and then the distribution of xDnmt1 was examined. Total extracts of 100 oocytes (lane 1), and post nuclear fraction (lane 2) and nuclear fraction (lane 3) were separately immunoprecipitated with anti-xDnmt1 antibodies, and electrophoresed, and then the xDnmt1 bands were detected by Western blotting. (B) Stage VI oocytes were fractionated as shown in panel A. Samples equivalent to three oocytes were immunodetected with anti-α-tubulin and anti-PCNA antibodies as post-nuclear and nuclear fraction markers, respectively. Lane 1-3 contained whole, post-nuclear, and nuclear fractions, respectively.

nuclei I next isolated nuclei from stage VI oocytes and then immunostained the xDnmt1. Manually isolated nuclei were cryosectioned and then stained with anti-mDnmt1 antibodies. Two different types of secondary antibodies that demonstrate the localization of xDnmt1, coupled with a fluorescent dye or peroxidase, uniformly stained the inside of the nuclei (Fig. 12A and E). When anti-mDnmt1 antibodies were pre-absorbed with excess GST-fused antigen, against which the antibodies were raised, the positively stained signals of xDnmt1 disappeared (Fig. 12B and F). When the antigenic sequence was fused to MBP and then used to pre-absorb the anti-mDnmt1 antibodies, the stained signals again disappeared (data not shown). Therefore, the immunostained signals observed in Fig. 12A and E, demonstrated the specific signals for xDnmt1. The periphery of the sections was stained relatively more densely than the rest of them (see Fig. 12A and E). The double-staining of xDnmt1 and lamin, which is known to be localized underneath the nuclear envelope (Fig. 12I, red), showed that the dense signals of xDnmt1 at the periphery of oocytes was completely co-localized with that of nuclear lamin (Fig. 12G, yellow). Although not conclusive, xDnmt1 could be preferentially localized underneath the nuclear envelope.

3.3.3 DNA methyltransferase activity in the nuclei prepared from stage VI oocytes

In somatic cells, the nuclei of proliferating cells exhibit high Dnmt1 activity, but when the cell cycle is arrested and cells are in quiescent state, the activity is at an undetectable level (Adams, 1990; Szyf et al., 1991; Liu et al., 1996; Suetake et al., 1998). In unfertilized mouse oocytes, Dnmt1 is accumulated in the cytoplasm and exhibits DNA methyltransferase activity (Carlson et al., 1992).

To determine if the uniformly distributed xDnmt1 protein in nuclei of *Xenopus* stage VI oocytes possesses DNA methyltransferase activity, I isolated nuclei and

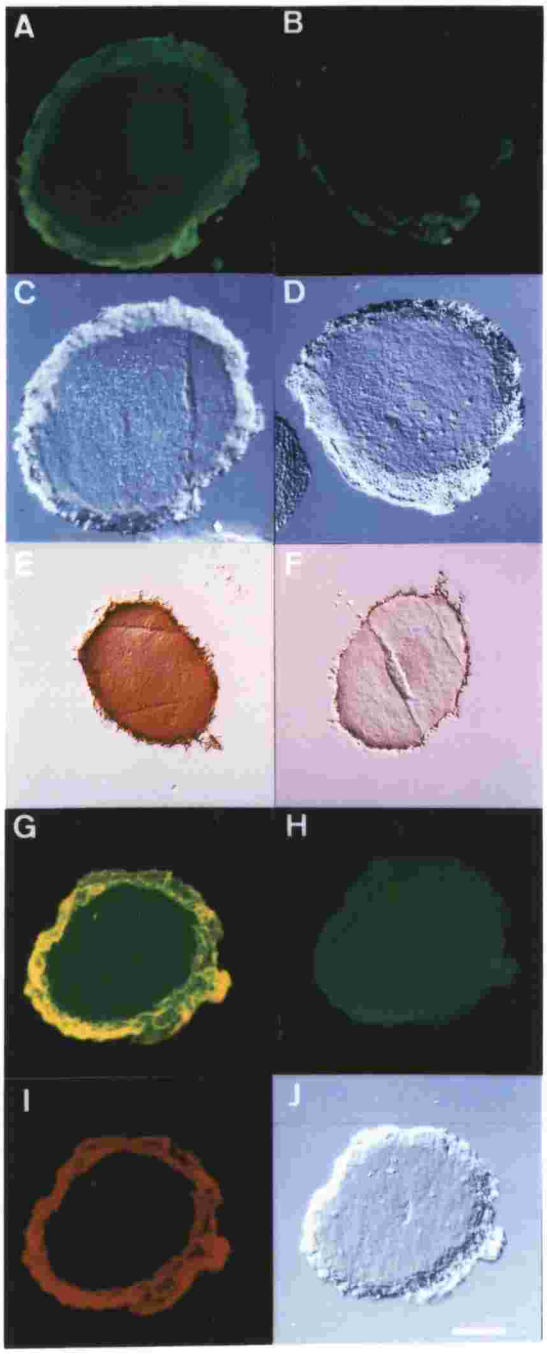


Figure 12. xDnmt1 in the nuclei of stage VI oocytes. The nuclei of stage VI oocytes were fixed in Dent's fixative and then cryosectioned at 14 μm. The sections were reacted with anti-mDnmt1 antibodies (A and E) or with the antibodies pre-absorbed with excess antigen against which the antibodies were raised (B and F), and then reacted with secondary antibodies coupled with ALEXA 488 (A and B), or HRP (E and F). The fields in panels A and B were also observed under Nomarski optics (C and D). The section was reacted with anti-lamin monoclonal antibody and anti-mDnmt1 antibodies, and then visualized with ALEXA 488 (H), and TRITC (I), respectively. The double-stained image with anti-lamin and anti-mDnmt1 antibodies is shown in panel G. The same section was also observed under Nomarski optics (J). Bar indicates 100μm.

determined the activity using poly(dI·dC)-poly(dI·dC) as a substrate (Table II). The DNA methyltransferase activity of nuclei prepared from stage VI oocytes was about $5x10^3$ times higher than that of A6 cell extract on a per cell basis. Since the xDnmt1 protein content in *Xenopus* stage VI oocytes was also about $1x10^4$ times higher than that in A6 cell extract on a per cell basis (Table II), the xDnmt1 that accumulated in the nuclei of stage VI oocytes during the course of oogenesis possess equivalent DNA methyltransferase activity to that in A6 cells.

When compared with DNA methyltransferase in mammalian cells, the xDnmt1 in the nuclei of stage VI oocytes again possess equivalent DNA methyltransferase activity as that in MEL cells (Table II).

Table II. DNA methyltransferase activities in the A6 cells, nuclei of stage VI oocytes, and MEL cells. The whole extracts of A6 cells and MEL cells, and nuclear extract from stage VI oocytes, were used to measure DNA methyltransferase activity using poly(dI·dC)-poly(dI·dC) as substrate. The activities were shown as the mean ±SD.

Enzyme source	Methyltransferase activity (fmol/h/cell)	Relative methyltransferase activity per cell	Relative methyltransferase protein per cell
Nuclear extracts of stage VI oocytes	160±42 (n=6)	1.1×10^{4}	0.6×10^{4}
Whole extracts of A6 cells	3.1×10^{-2} $\pm 1.5 \times 10^{-2}$ (n=3)	2.2	0.6
Whole extracts of MEL cells	1.4×10^{-2} $\pm 2.9 \times 10^{-3}$ (n=6)	1.0	1.0

3.4 DISCUSSION

3.4.1 xDnmt1 accumulates in oocytes during oogenesis

In the present study, I showed that xDnmt1 became detectable from stage III oocytes, and accumulated thereafter during the course of oogenesis (Fig. 9). *De novo* synthesis of xDnmt1 sharply increased from stage IV to VI (Fig. 10), and this increased synthesis seemed to cause the accumulation of xDnmt1. A similar event occurs in mouse oocytes; 3,000 times higher amount of Dnmt1 protein accumulates in an oocyte than in a single MEL cell (Carlson et al., 1992). In *Xenopus*, stage VI and mature oocyte were calculated to contain about $1.2x10^4$ and $2.4x10^5$ times higher xDnmt1 protein than in MEL cells on a per one cell basis, respectively. Considering the size of each oocyte of mouse (about 80 μm) and *Xenopus* (1.0 to 1.2 mm) oocytes, one to two orders of magnitude higher Dnmt1 content in *Xenopus* oocytes than in mouse is not surprising. The specific content per volume is rather low in *Xenopus* oocyte compared to in mouse oocytes, as the volume of *Xenopus* oocytes is about 10^3 times greater than that of mouse ones.

In *Xenopus* embryos, zygotic transcription starts after the midblastula transition (MBT) (Newport and Kirschner, 1982). Therefore, the gene products, *i.e.* mRNA and/or proteins, indispensable for early embryogenesis before MBT are generally accumulated in mature oocytes in large quantity, in order to prepare for the quick increase of cell number. PCNA, which plays important roles in replication and repair (Kelman, 1997), and has been reported to bind to Dnmt1 (Chuang et al., 1997), is accumulated in a $4x10^5$ times higher amount in mature oocytes than in an established *Xenopus* cell line, A6 cells, under proliferative conditions (Leibovici et al, 1990). In addition, histones (Woodland and Adamson, 1977), DNA polymerase α (Zierler et al., 1985), and *Xenopus* RNA

helicase, An3 (Longo et al., 1996), are also accumulated in oocytes during course of oogenesis. These maternal stockpiles support the first 12-round cell cleavage after fertilization before MBT (Kimelman et al., 1987). In analogy with other maternally accumulated proteins, the accumulated xDnmt1 is likely to play an important role in early embryogenesis, maybe in the regulation of gene expression.

3.4.2 xDnmt1 is translocated into nuclei of oocytes

xDnmt1 was accumulated in oocytes during oogenesis, which is similar to in mouse. In contrast to in mouse oocyte, in which almost all Dnmt1 is excluded from the nucleus, a significant amount, about 50%, of xDnmt1 was translocated into the nuclei. Why was mDnmt1 not but xDnmt1 was translocated into the nuclei of oocytes? Since *Xenopus* embryos undergo quick replication without endogenous transcription during early embryogenesis, the preexistence of xDnmt1 in the nucleus may contribute to the maintenance of the genomic DNA methylation state in the embryos. In concert with this, MeCP2, one of the methylated DNA binding proteins, is expressed in *Xenopus* oocytes, while it is not detected in mouse oocytes (Kass and Wolffe, 1998; Nan et al., 1998). MeCP2 specifically recognizes and binds to methylated DNA (Lewis et al., 1992), directly suppresses the expression of methylated gene (Nan et al., 1997; Yu et al., 2000), and recruits histone deacetylase (Jones et al., 1998; Nan et al., 1998). Thus, in *Xenopus* the regulation of DNA methylation during early embryos might be different from in mouse. The genes that are methylated by xDnmt1 in the nuclei of *Xenopus* early embryos might effectively be suppressed through MeCP2 before MBT.

In *Xenopus* oocytes, maternally expressed proteins that function in nuclei generally translocate into nuclei. PCNA (Leibovici et al., 1990), histones, and DNA polymerase α belong to this group (Davidson, 1986). On the other hand, the

proto-oncogene product c-Myc, and high mobility group-1-like protein, HMG-A, a nonhistone component of chromatin, which are exclusively localized in the nuclei of somatic cells, remain in cytoplasm of *Xenopus* oocytes (Gusse et al., 1989; Kleinschmidt et al., 1983). The localization of An3 dynamically changes during oogenesis, in stage I to V oocytes, An3 is distributed in both the cytoplasm and nuclei, and in stage VI oocytes it is excluded from nuclei (Longo et al., 1996).

The fact that not all but specific proteins are excluded from nuclei either completely or partially suggests that a machinery exists not in somatic cells but in oocytes, which recognizes a specific sequence in the proteins to determine their localization, *i.e.* whether they should be excluded from nuclei or not. Recently, such the sequence that retains Dnmt1 to the cytoplasm of oocytes has been identified in amino-terminal region of mDnmt1 (Cardoso and Leonhardt, 1999). On the other hand, species-specific exclusion in *Xenopus* oocytes has been reported (Rupp et al., 1994). When *Xenopus* MyoD, one of the four myogenic factors that control the myogenic process, is expressed in *Xenopus* early embryo, it is excluded from nuclei. But, ectopically expressed mouse MyoD is translocated into the nuclei of *Xenopus* oocytes (Rupp et al., 1994). *Xenopus* MyoD sequence, thus, contains the information not to be translocated into nuclei. Different from MyoD, ectopically expressed mDnmt1 as well as xDnmt1 was translocated into nuclei (see Chapter 4, Fig. 17). This result indicates that not the xDnmt1 but the oocytes contains the determinant for the localization of the molecule. *Xenopus* oocytes may contain the factors that retain Dnmt1 to cytoplasmic compartment, but may be incomplete, either in quality or in quantity to do so.

3.4.3 The xDnmt1 accumulated in the nuclei of oocytes has DNA methyltransferase activity

In the nucleus of stage VI oocytes, the DNA methyltransferase activity was $0.5x10^4$ times higher (Table II), and the total amount of xDnmt1 protein was $2x10^4$ times higher than the A6 cells on a per cell basis (Table II). Considering that the xDnmt1 protein was equally distributed to post-nuclear fractions and nuclei (Fig. 11A), meaning that the xDnmt1 protein in nucleus of a stage VI oocyte was $1x10^4$ times higher than a single A6 cell, the specific methyltransferase activity of the xDnmt1 in the nuclei of stage VI oocytes was almost identical to that in proliferating A6 cells. Moreover, the DNA methyltransferase activity in *Xenopus* oocyte was also almost identical to that in MEL cells, indicating that the specific activity of xDnmt1 is almost identical to that of mDnmt1.

My present result that significant amount of active xDnmt1 existed in the nuclei of stage VI oocytes conflicts with that reported by Adams *et al.* (Adams et al., 1981). Adams *et al.* reported that most of the DNA methylation activity in stage VI oocytes existed in the post-nuclear fraction, and the isolated nuclei showed negligible amount of the activity. The only difference I could point out is the way how the nuclei were prepared. Adams *et al.* used protease and nonionic detergent when they lyse oocytes. DNA methyltransferase might leak from the nuclei by the treatment. While, I manually took out nuclei directly form oocytes under microscope. At moment, I do not know the reason why Adams *et al.* could not detect DNA methylation activity in oocyte nuclei and I could detect it. However, the important point is that I confirmed the existence of xDnmt1 in the nuclei of stage VI oocytes by three different methods of immunoprecipitation followed by SDS-PAGE, immunocytochemistry, and enzyme activity.

3.5 SUMMARY

In vertebrate, DNA methylation plays an important role in the regulation of gene expression and embryogenesis. mDnmt1 is highly accumulated in mouse oocytes and is excluded from nuclei (Carlson *et al.*, 1992, *Genes Dev.* 6, 2536-2541). In this chapter, I examined the expression level and localization of xDnmt1 in oocytes during oogenesis. The xDnmt1 protein was detectable in the stage III oocytes and increased thereafter, until the oocytes had maturated. The rate of xDnmt1 synthesis rapidly increased after stage IV oocytes. Different from in mouse oocytes, xDnmt1 was equally distributed in the nuclear and post-nuclear fractions in stage VI oocytes. xDnmt1 translocated into nuclei was uniformly localized in nuclear matrix, and the accumulated xDnmt1 in stage VI nuclei had DNA methylation activity.

CHAPTER 4. Effects of overexpression of xDnmt1 on *Xenopus* early embryogenesis

4.1 INTRODUCTION

In *Xenopus*, maternally inherited gene products accumulated during oogenesis play crucial roles in early development. These proteins are utilized to accomplish the first 12 cleavages after fertilization. In *Xenopus* early embryogenesis, the cleavage cycle is synchronous and lacks G1 and G2 phases, thus rapid. Before 12 cell cycles, neither zygotic transcription nor cell growth in size occurs. After the twelfth cell cleavage, zygotic transcription starts, of which stage is called midblastula transition (MBT). Elongation of cell cycle intervals and asynchronous cell division accompany with the MBT (Kimelman et al., 1987).

The importance of Dnmt1 during mouse embryogenesis is clearly shown by the targeting experiment of *Dnmt1* (Li et al., 1992 and 1993). The homozygous mutant mice die at 8.5-day embryo. I should take notice that in mouse, maternally inherited Dnmt1 protein and mRNA are extremely rich (Carlson et al., 1992). Therefore, the phenotype observed in homozygous Dnmt1 -/-, which completely lacks zygotic transcription of the gene, does not directly show the effect of complete loss of Dnmt1 in the beginning of development, as the oocyte-type Dnmt1 is shown to be dominant until the stage of blastocyst (Mertineit et al., 1998).

To further study the function of Dnmt1 in early embryo, it is difficult to use mouse embryo, as it is difficult to manipulate and difficult to prepare in biochemical quantity. I decided to use *Xenopus* embryos for analyzing the function of xDnmt1 during early development. During the course of the present study, Stancheva and Meehan (2000) reported that the depletion of xDnmt1 from 16-cell stage (stage 5) by

injection of the antisense RNA induced aberrant demethylation in the specific genes that plays crucial roles in early events of cell differentiation at the onset of gastrulation.

In the present study, I employed a technique to inject mRNA to express loss-of-function xDnmt1 in *Xenopus* embryos. According to previous reports demonstrating crucial roles of Dnmt1 in early development, I expected a dominant-negative effect of the overexpressed loss-of-function xDnmt1. However, neither inactive xDnmt1, intact xDnmt1, nor mDnmt1 affected any of the early developmental events in *Xenopus* embryogenesis. The exogenous Dnmt1 became dominant only after gastrula (stage 10), after MBT. Therefore, the present result suggests that the susceptibility of embryos to the down regulation of active xDnmt1 is stage-dependent, and after gastrula the *Xenopus* embryos are insensitive to such change.

4.2 MATERIALS AND METHODS

4.2.1 Plasmid construction and *in vitro* transcription

pCS2+MT vector and *lacZ* inserted plasmid pCS2+MT-cBgal were kindly provided by Drs. H.B. Sucic and D. Turner (University of Michigan). The *Eco*RI (1,424) and *Dra*I (down stream of termination) 3.5 kb fragment of XMT10 clone (Chapter 2) was subcloned into the *Eco*RI and *Stu*I sites in pCS2+MT (pCS2+XMT10). To obtain 0.8 kb 5' end sequence of xDnmt1, I performed PCR using a pair of primers, XEATG (5'-TTGAATTCCATGCCGGCCCAGTCCACTTCC-3') containing initiation codon ATG, and XpvuII (5'-CCTTCTCTTCTGGTTGGTCTGCATCAGCTGG-3') containing *Pvu*II site at 792, and xDnmt1 cDNA as template (Chapter 2). The PCR product was subcloned into the *Eco*RI and *Pvu*II sites in pCS2+MT to produce pCS2+MT-XEATG-PvuII. The *Pvu*II fragment (792-2,348) of XMT5 clone (Chapter 2) was subcloned into the *Pvu*II site of pCS2+MT-XEATG-PvuII to produce pCS2+MT-XEATG. Finally, the *Eco*RI fragment excised from pCS2+MT-XEATG, a 5'fragment of xDnmt1, was subcloned into the *Eco*RI site of pCS2+MT-XMT10 to produce pCS2+MT-xDnmt1.

A loss-of-function xDnmt1 (mut-xDnmt1, C1,101A) cDNA was generated as follows according to the method described elsewhere (Maruta et al., 1991). The DNA fragment of 393 bp encoding amino acid residues from 969 to 1,099 of xDnmt1 protein was amplified with a primer set of XHs (5'-AGACCAAAAGCTTTGAAGAT-3') and XMa (5'-GGGCCCTCCGCAGAGCAT-3'). The DNA fragment of 317 bp encoding residues 1,094 to 1,199 of xDnmt1, in which the codon TGC coding Cys1,101 was changed to GCC coding Ala was amplified with a primer set of XMs (5'-ATGCTCTGCGGAGGGCCCCCCGCCCAAGGT-3') and XHa

(5'-CATTGGAAGCTTCTCTCC-3'). After purification of each PCR product on 1.6 % agarose gel, second PCR was performed using primers XHs and XHa, and a mixture of the two PCR products as a template to produce the *Hin*dIII/*Hin*dIII DNA fragment of 693 bp encoding catalytic center of loss-of-function xDnmt1. The mutation C1,101A was confirmed by sequencing the *Hin*dIII/*Hin*dIII DNA fragment subcloned into pUC19. The wild-type *Hind*III fragment was replaced with the mutated *Hin*dIII fragment to produce pCS2+MT-mut-XMT10. Then, pCS2+MT-mut-xDnmt1 was produced as described above.

To obtain pCS2+MT-mDnmt1, 4.9 kb blunt-ended *Hin*dIII-*Pma*C1 fragment excised from pCDNA3-mDnmt1 (Shimamura, 1999) was subcloned into blunt-ended *Xho*I site of pCS2+MT.

All the constructs were linearized with *Not*I and transcribed *in vitro* with SP6 RNA polymerase (Boehringer Mannheim GmbH, Germany) under manufacture's instruction to obtained capped mRNAs. The transcribed mRNAs were electrophoresis in 1.0% agarose/formaldehyde denaturing gel and stained with SYBR® Green II (Molecular Probes, OR). The stained mRNAs was quantified in a FluorImager system (Molecular Dynamics, CA) with known amount of antisense Krox20 RNA as a standard (kindly gifted from Dr. M. Taira, University of Tokyo) (Taira et al., 1994).

4.2.2 Oocytes, embryos, and microinjection

Stage VI oocytes were obtained as described in Chapter3. Unfertilized eggs of *Xenopus* were manually ovulated from a female frog injected with 300 units of human chorionic gonandotropic hormone, Gonatoropin (Teikoku Zouki, Ltd., Tokyo). Eggs were fertilized, and dejellied in 2% cysteine-HCl (pH 7.5) for 10 min at room temperature. Embryos were then kept at 20, 18 or 14 °C in 0.1x Steinberg's solution (6

mM NaCl, 0.067 mM KCl, 0.034 mM $Ca(NO_3)_2$, 0.083 mM $MgSO_4$, and 1 mM HEPES-NaOH (pH 7.4)) until they were served for microinjection (Sive et al., 2000b).

For standard injection, 5ng of mRNAs for the test were co-microinjected with 0.5 ng β-galactosidase mRNA in 5 nl of distilled RNase-free water into animal-pole side of 2- or 4-cell stage embryos settled in 5% Ficoll 400-0.33x Marc's modified Ringer's solution (MMR) (0.1 M NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES-NaOH (pH 7.4)) (Peng, 1991). Injected embryos were kept for an hour in 5% Ficoll 400-0.33x MMR and transferred into 0.1x MMR with 10 μg/ml gentamycin for further culture.

For the microinjection into oocytes, isolated stage VI oocytes were microinjected with 5 ng mRNA per oocytes in 5% Ficoll 400-0.3x MMR (Mg^{2+}, Ca^{2+} free). Injected oocytes were kept for overnight in 0.1x MMR (Mg^{2+}, Ca^{2+} free) with 10 μg/ml gentamycin. All the oocytes and embryos were kept in an incubator at 18 °C.

4.2.3 Metabolic labeling of embryo

Five embryos injected with Dnmt1 mRNAs were incubated at 18 °C for 24 h with 400 μCi/ml of EXPRE^{35}S^{35}S (New England Nuclear, MA) in 0.1x MMR with 10 μg/ml gentamycin.

4.2.4 Immunoprecipitation

One to five embryos were homogenized in 1 ml of a buffer containing 150 mM NaCl, 1% (w/v) NP-40, 0.01% sodium deoxycholate, 50 mM Tris-HCl (pH7.6), and 0.02 tablet of Complete®-mini/ml, a cocktail protease inhibitors (Boehringer Mannheim GmbH, Germany). The homogenate were kept on ice for 30 min and centrifuged at 15,000 rpm for 15 min at 4 °C to recover supernatant fractions.

For the detection of exogenous myc-tagged Dnmt1, the supernatants were incubated with Sepharose 4B (Amersham Pharmacia Biotech AB, Sweden) conjugated with anti-myc (9E10) monoclonal antibody for overnight at 4 °C. As for the detection of endogenous xDmnt1 and exogenous Dnmt1, the supernatants were incubated with anti-xDnmt1 polyclonal antibodies (Chapter 2, MATERIALS AND METHODS) for overnight at 4 °C, and then the Dnmt1-antibody complex was incubated with protein-A Sepharose (Amersham Pharmacia Biotech AB, Sweden) for 2 h at room temperature.

The immunoprecipitated proteins were separated by SDS-PAGE (Laemmli, 1970) and electrophoretically transferred onto nitrocellulose membrane. For the detection, the membrane sheet was incubated with anti-myc monoclonal antibody (9E10) in PBS containing 1% BSA, 0.1% (w/v) Triton X-100, and 0.05% sodium azide, or anti-xDnmt1 polyclonal antibodies in PBS containing 1% BSA, 1% (w/v) Triton X-100, 0.2% SDS, and 0.05% sodium azide. The Dnmt1s-antibody complexes were detected with the second antibodies coupled with alkaline phosphatase (E.Y. Laboratory Inc., CA) and a color reaction buffer containing 0.05 mg/ml BCIP and 1 mg/ml NBT (Takagi et al., 1995).

The immunoprecipitated radio-labeled Dnmt1s protein bands were analyzed by fluorography (Bonner and Laskey, 1974) after SDS-PAGE (Laemmli, 1970).

4.2.5 β-galactosidase staining assay

Embryos injected with mRNAs encoding bacterial β-galactosidase were fixed with 2% formamide, 0.2% glutaraldehyde, 0.02% (w/v) Triton X-100 and 0.01% sodium deoxycholate in PBS on ice for 1 h. Embryos were washed four times for 10 min each with PBS and then stained at room temperature with 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml 5-bromo-4-chloro-3-indoyl-*β-D*-galactoside (Wako Pure Chemical

Industries, Ltd., Osaka) and 2 mM $MgCl_2$ in PBS. Embryos obtained blue were re-fixed as above and stored in methanol (Sive et al., 2000c).

4.2.6 Microscopy

All the embryos were observed under Nikon SMZ-U stereo microscope and photographs were taken with Nikon FX35 camera using SUPERIA 400 film (Fuji Photo Film Co. Ltd., Tokyo).

4.3 RESULTS

4.3.1 Expression level of xDnmt1 protein was kept constant during early development

I first determined the expression level of xDnmt1 protein from the 2-cell stage embryo to the tailbud stage (Fig. 13). The amount of xDnmt1 protein per embryo was quantified by immunoprecipitation followed by Western blotting using anti-xDnmt1 polyclonal antibodies. During the early development, the expression level of xDnmt1 was kept constant per embryo and no significant change in the expression level was observed.

4.3.2 Expression of loss-of-function xDnmt1 had no effect on embryogenesis

To evaluate the role of Dnmt1 in the *Xenopus* embryogenesis, I employed a technique to overproduce loss-of-function xDnmt1 (mut-xDnmt1) to inhibit endogenous xDnmt1 activity. For the construction of mut-xDnmt1, the Cys at 1,101 in motif IV that plays a crucial role in the catalytic activity, was change to Ala (Fig. 14) (Wyszynski et al., 1992). The *in vitro* transcribed mRNA of mut-xDnmt1 was injected into the animal-pole side at 2-cell stages. The injected embryos normally developed (Fig. 15B, stage 11-12), and hatched. The activity staining of β-galactosidase, of which mRNA was co-injected with mut-xDnmt1 mRNA, indicates the cells that were expressing mut-xDnmt1 (Fig. 15F). The portion of embryos injected with the mut-xDnmt1 mRNA seemed to be normal.

The results obtained from several injection experiments were summarized in Table III.

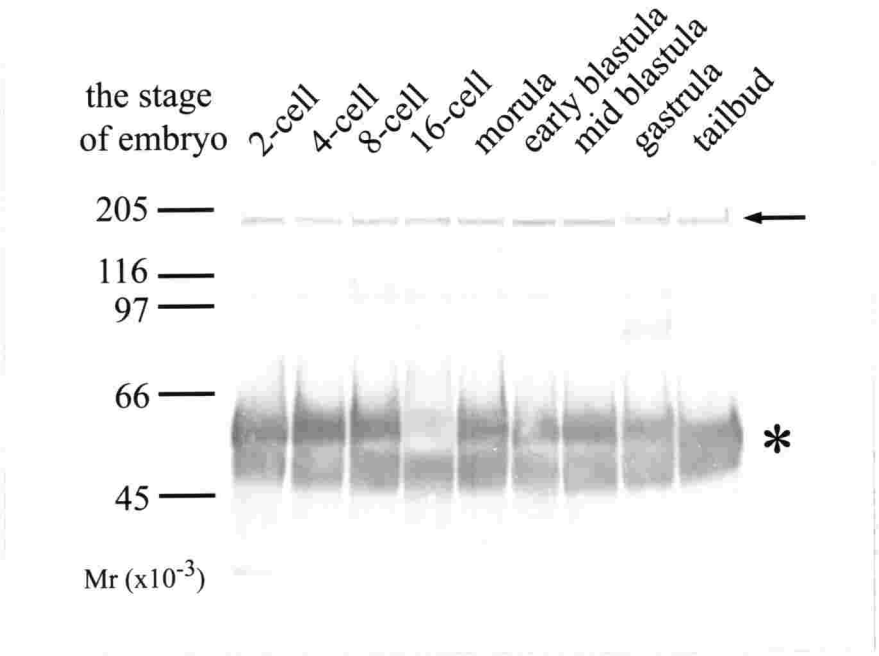


Figure 13. Expression of xDnmt1 during early development. Whole extracts of indicated stage embryos were immunoprecipitated with anti-xDnmt1 polyclonal antibodies and then analyzed by Western blotting using alkaline phosphatase-coupled secondary antibodies. An arrow indicates xDnmt1. Asterisk indicates IgG heavy chain. Molecular weight standards (Mr) are indicated.

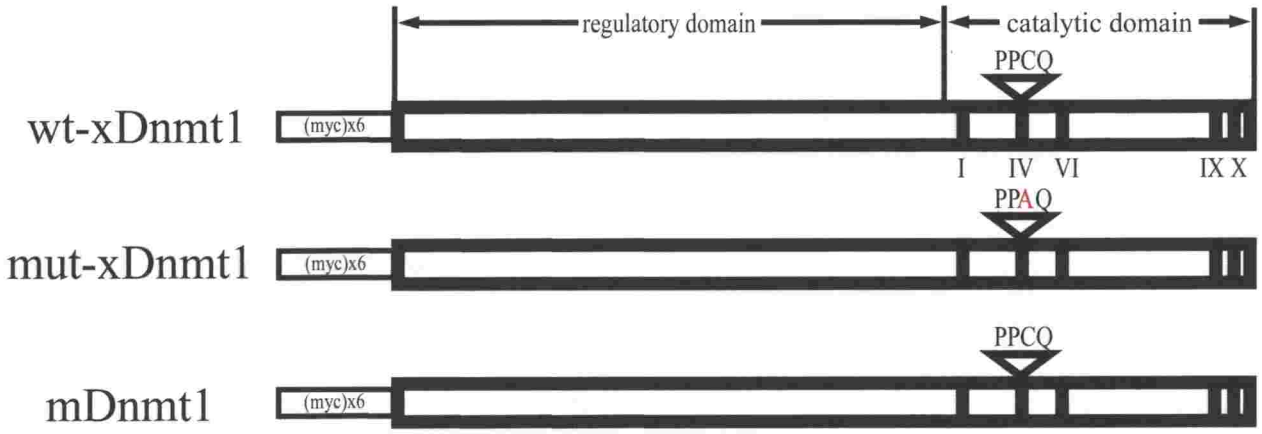


Figure 14. The constructs of wt-xDnmt1, mut-xDnmt1, and mDnmt1. Proteins encoded in the injected mRNAs are schematically illustrated. All the cDNAs were subcloned into pCS2+MT vector containing six myc-tag {(myc)x6} at the amino-terminal region, and transcribed *in vitro* with SP6 polymerase. I-X are the conserved motifs of the bacterial type-II DNA-(cytosine-5) methylase. The conserved sequence (PPCQ) of catalytic center in motif IV is shown in a single amino acid letter, and the changed amino acid residue is shown in red.

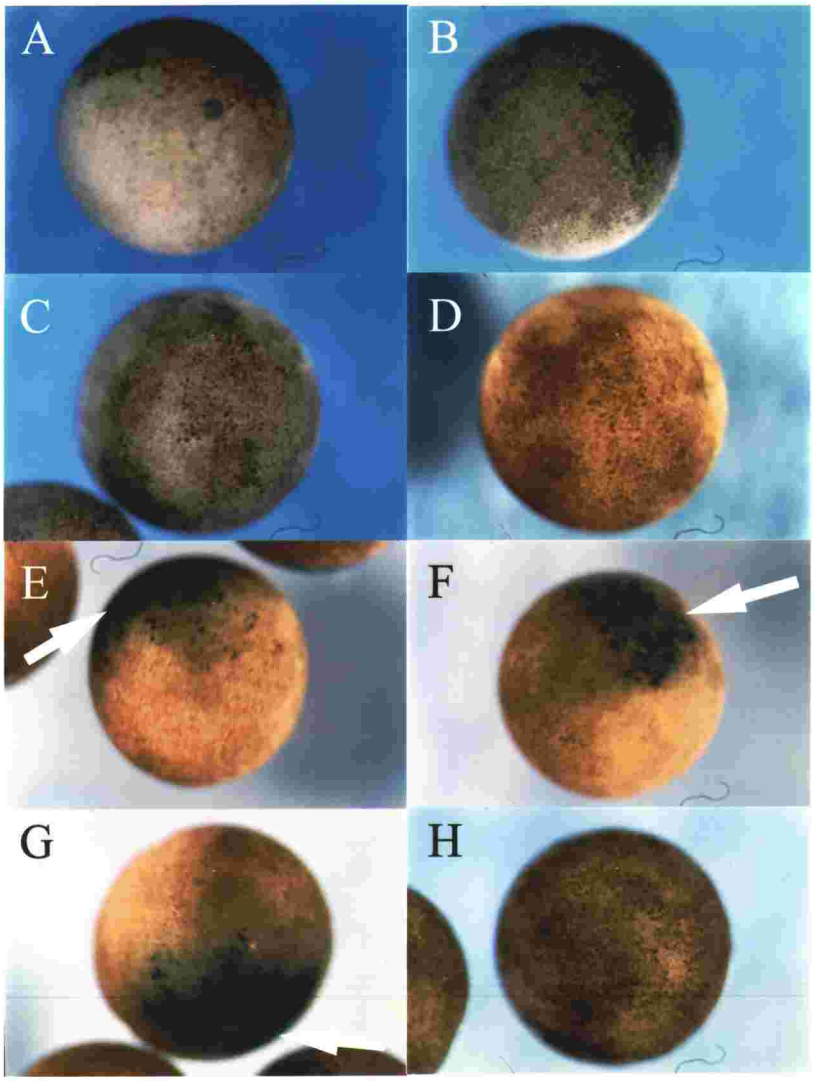


Figure 15. Exogenous expression of wt-xDnmt1, mut-xDnmt1, or mDnmt1 in *Xenopus* embryos. Embryos were injected with 5ng wt-xDnmt1 (A and E), mut-xDnmt1 (B and F), or mDnmt1 (C and G), and 0.5ng each of β-galactosidase mRNA into animal pole-side of 2-cell stage embryos. As controls no mRNA was injected in (D and H). Embryos were developed until stage 11-12, and were fixed and activity stained for β-galactosidase (E-H). White arrows indicate the cells stained by β-galactosidase staining assay.

Table III. Phenotypes of embryos that were overexpressed the Dnmt1s. Embryos were injected with 5ng each of indicated mRNAs into the 2- and 4-cell stage embryos and were cultured until stage 11-12. Results obtained from four independent experiments were summarized. The numbers of total embryos, and of the normal embryo are shown.

mRNA	total (n)	normal (n)
uninjected	53	53
wt-xDnmt1	41	41
mut-xDnmt1	41	41
mDnmt1	32	32
β-galactosidase	46	46

4.3.3 Expression of xDnmt1 or mDnmt1 had no effect on embryogenesis

Next, I examined the effect of overexpression of xDnmt1 (wt-xDnmt1) and mouse somatic-type Dnmt1 (mDnmt1). The embryos injected with wt-xDnmt1 mRNA showed normal development (Fig. 15A). Moreover, when embryos were injected with mDnmt1 mRNA also showed no morphological abnormalities (Fig. 15C). The results were also summarized in Table III.

4.3.4 Expression of exogenous xDnmt1

To see the injected mRNAs were effectively translated into proteins, The embryos that were cultured to blastula, gastrula, and tailbud stages were immunoprecipitated with anti-xDnmt1 polyclonal antibodies, and immunodetected by Western blotting (Fig. 16). Endogenous xDnmt1, of which molecular weight is 170k, was distinct from the exogenously expressed Dnmt1s in SDS-PAGE, as the exogenous Dnmt1s contain myc-tag at their amino-terminus (Fig. 14). In the injected embryos, both injected wt-xDnmt1 and mut-xDnmt1 mRNAs were translated almost to an equal level to the endogenous xDnmt1 at the blastula stage, and were ten-times higher than the endogenous xDnmt1 at the gastrula and tailbud stages (Fig. 16). As the β-galactosidase activity staining of co-injected embryos showed unequal distribution in the embryos (Fig. 15E-H), the wt-xDnmt1 or mut-xDnmt1 protein was expressed at limited part or the embryos. This means that ectopically expressed Dnmt1s are much higher concentration in the limited numbers of cells near the injected place than in the other cells.

Nevertheless, not any phenotypic effect was observed by injection with either mut-xDnmt1, or wt-xDnmt1 and mDnmt1.

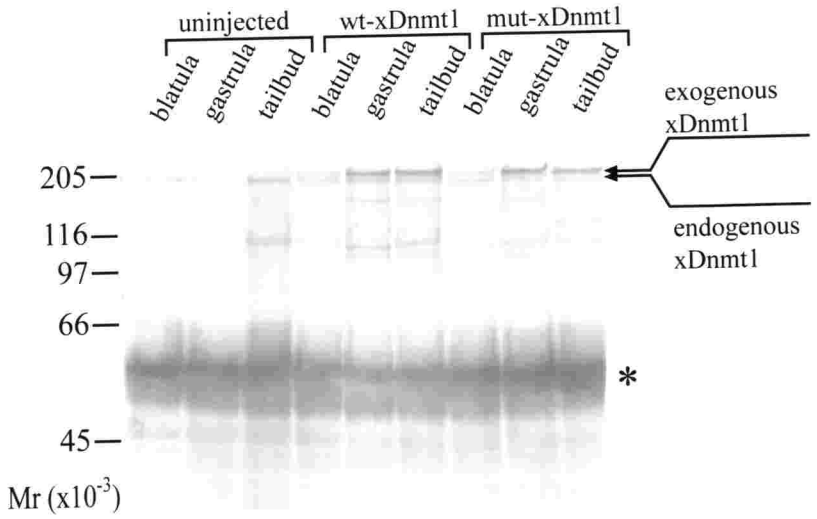


Figure 16. Translation of injected mRNAs in embryos. Whole extract of a single injected embryo at indicated stage was immunoprecipitated with anti-xDnmt1 polyclonal antibodies, and then analyzed by Western blotting using alkaline phosphatase-coupled secondary antibodies. Endogeneous and exogeneous xDnmt1 are indicated as arrows. Asterisk indicates IgG heavy chain. Molecular weight standards (Mr) are indicated.

4.3.5 Exogenous mut-xDnmt1, wt-xDnmt1, and mDnmt1 were normally localized both in nuclei and cytoplasm

Although the injected Dnmt1 mRNAs were effectively translated in embryos, they did not show any phenotypes. There is a possibility that exogenous Dnmt1s may not be recognized as members by the machineries in embryos, *i.e.*, exogenous Dnmt1 may not be localized properly, or may not be integrated into the preexisting complex. To test the former possibility whether the exogenous Dnmt1s were localized to proper places or not, I injected Dnmt1 mRNAs into stage VI oocytes. After 24 h incubation, the oocytes were subfractionated into nuclear and post-nuclear fractions. Then each fraction was immunoprecipitated with anti-myc monoclonal antibodies, electrophoresed, and then detected by Western blotting (Fig. 17). Exogenously expressed wt-Dnmt1, mut-xDnmt1, and mDnmt1 equally distributed in nuclear and post-nuclear fractions. The subcellular distribution of the exogenously expressed Dnmt1s were quite similar to that of endogenous xDnmt1 (see Chapter 3, Fig. 11A).

4.3.6 Immunoprecipitation of endogenous and exogenous xDnmt1

A possibility that endogenous Dnmt1s is forming functional complex and is not replaced with exogenous xDnmt1 may exist. To test this, next I looked for the protein bands that may co-precipitate with Dnmt1 by anti-xDnmt1 antibodies and anti-myc monoclonal antibody immunoprecipitations. When endogenous xDnmt1 is in the complex and the exogenous one cannot be integrated into it, I may see some difference in the constituents between the proteins bands co-precipitated with anti-xDnmt1 and anti-myc antibodies.

As shown in Fig. 18, I could not detect any significant extra band even in the lanes of immunoprecipitated the endogenous xDnmt1, suggesting that endogenous

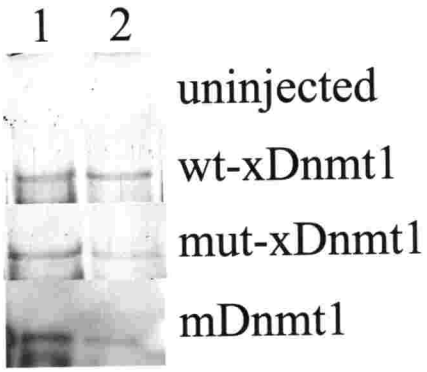


Figure 17. Exogenous Dnmt1s were localized to both nuclear and post-nuclear fractions in the injected stage VI oocytes. Five oocytes injected with wt-xDnmt1, mut-xDnmt1 or mDnmt1 were manually subfractionated into nuclear and post-nuclear fractions. The post-nuclear (lane 1), and nuclear (lane 2) fractions were separately immunoprecipitated with anti-myc (9E10) monoclonal antibodies, and then analyzed by Western blotting using alkaline phosphatase coupled secondary antibodies.

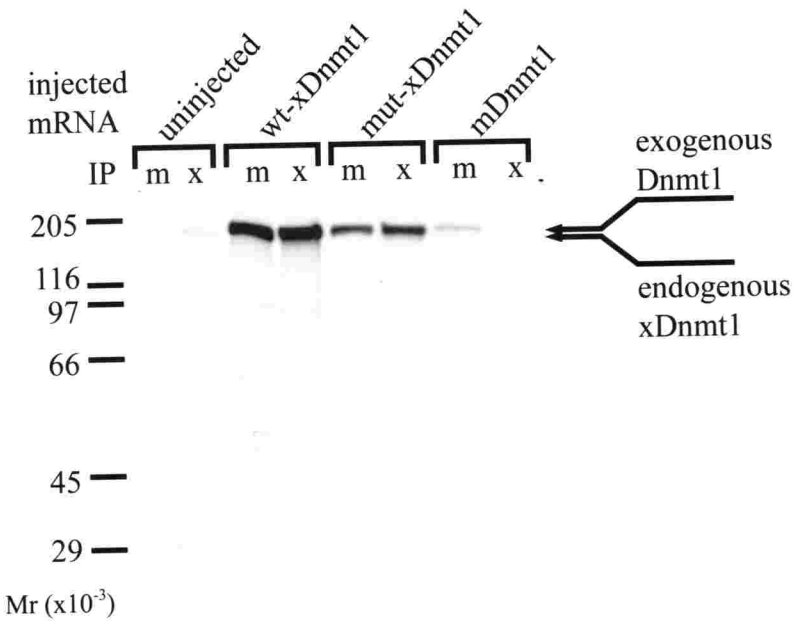


Figure 18. Immunoprecipitation of exogenous Dnmt1s and endogenous xDnmt1. Five embryos injected with indicated mRNA were incubated with EXPRE^{35}S^{35}S for 24 h and whole extracts of injected embryos were immunoprecipitated with anti-myc monoclonal (m) or anti-xDnmt1 (x) polyclonal antibodies, respectively. The immunoprecipitated proteins were electrophoresed, and then fluorographed. Exogenous Dnmt1s and endogenous xDnmt1 were indicated with arrows. Molecular weight standards (Mr) are indicated.

xDnmt1 in early embryos may not be integrated into a firm complex.

4.4 DISCUSSION

4.4.1 xDnmt1 protein during early development

Mouse embryo is not a suitable material for biochemical study of Dnmt1 in early embryogenesis, because it is difficult to manipulate and difficult to prepare in large quantity. Therefore, I have chosen *Xenopus* embryo for the purpose. As a first step, I determined the expression of xDnmt1 during early development.

The xDnmt1 proteins were expressed at a fixed level per embryo from 2-cell stage to tailbud stage (Fig. 13), indicating that Dnmt1 content is decreased per cell basis. Recently, Stancheva and Meehan demonstrated that the xDnmt1 protein level decreases at stage 11 (gastrula) compared with that at stage 5.5 (blastula) when normalized to the PCNA level (Stancheva and Meehan, 2000). Their result contradicts to my present result as the PCNA level is fixed to a constant level per embryo during early development (Leibovici et al., 1990). The discrepancy between the results could be due to the protein extraction procedures from embryos. While I checked the solubilization efficiency and solubilized all the xDnmt1 proteins present in embryos, Stancheva and Meehan extracted the proteins from embryos by an organic solvent, with which the extraction of xDnmt1 is insufficient (Kimura. unpublished).

Among the maternally accumulated proteins, not *Xenopus* c-Myc (Gusse et al., 1989) but *Xenopus* PCNA (xPCNA) (Leibovici et al., 1990) shows a similar expression profile as xDnmt1 during development. xPCNA protein starts to accumulate from the late stage of oogenesis and the level of xPCNA protein per embryo is fixed to a constant level throughout the early development. PCNA is an essential component of the DNA replication machinery, and also required for DNA recombination and repair. Human PCNA is reported to bind to the amino-terminal sequence of hDNMT1 (Chuang et al.,

1997). Recently, Suetake found that xPCNA also binds to xDnmt1 by using yeast two hybrids system (Suetake, unpublished). Considering the reported fact and my present result, xDnmt1 and xPCNA may functionally be interacting and are under a similar control during early development.

4.4.2 Overexpression of native and mutated xDnmt1

To understand the biological function of DNA methylation especially in early development, I attempted to perturb the DNA methylation pattern. I injected mRNA of Dnmt1s that were active (wt-xDnmt1), inactive (mut-xDnmt1), and of other species (mDnmt1) to overproduce in embryos.

There are so many reports showing that overexpression or suppression of Dnmt1 changes or switches the phenotypes. For examples, overexpression of active Dnmt1 induces cellular transformation (Wu et al., 1993) and terminal differentiation (Takagi et al., 1995). As for the phenotypes of the inhibition of endogenous Dnmt1, the effect is more severe. When the DNA methyltransferase activity is inhibited, the cells are transformed (Hsiao et al., 1986), differentiated (Taylor and Jones, 1979; Szyf et al., 1992), and/or inhibited the replication (Knox et al., 2000). When Dnmt1 in embryos is inhibited, in most of the cases embryos die or show abnormal development (Li et al., 1992 and 1993; Martin et al., 1999; Stancheva and Meehan, 2000). Underlying mechanism for those phenotypes may be due to a creation of aberrant methylation, either hyper- of hypo-methylation in the crucial genes.

Therefore, I expected that overexpression of xDnmt1 and mDnmt1 may induce genomic hypermethylation, and mut-xDnmt1 hypomethylation. Consequently, the overexpression may interfere normal cellular differentiation. However, neither wt-xDnmt1, mut-xDnmt1, nor mDnmt1 induced abnormal phenotypes or morphological

abnormalities. The reason why the overexpression of various types of Dnmt1 did not show any phenotype is uncertain. The injected mRNAs were actively translated into Dnmt1 proteins and was accumulated in embryos (Fig. 16). In addition, oocyte injection experiment revealed that Dnmt1 expressed was normally localized (Fig. 17).

4.4.3 A possible explanation for the phenotypes

I propose two possible mechanisms to explain that the embryos expressed with the exogenous Dnmt1 showed no morphological abnormalities. First one is that exogenous Dnmt1 was not recognized by the complex (if exists) and thus was not integrated to it as a component. The second one is that the expression timing of exogenous Dnmt1 was out of susceptible stage.

(i) Exogenous Dnmt1 was not recognized as endogenous Dnmt1: It is reported that Dnmt1 interacts with PCNA (Chuang et al., 1997), Rb (Robertson et al., 2000), HDAC (Fuks et al., 2000; Robertson et al., 2000; Rountree et al., 2000) and DMAP1 (Rountree et al., 2000). PCNA is the prerequisite component of replication machinery, and is abundantly expressed in *Xenopus* embryos (Leibovici et al., 1990), of which expression profile was similar to xDnmt1 (this chapter). Rb is also a key component of cell-cycle regulation and is known to interact with many cell-cycle related components. HDAC is a key component of huge complex of co-repressor. DMAP1, a new member of co-repressor, could also be a component of huge complex. Considering that Dnmt1 interacts with those components that are the members of large complexes, it is reasonable to speculate that Dnmt1 is also a key component of a large complex. In *Xenopus* early development, *Xenopus* cells divide synchronously in cell cycles lacking G1 and G2 phases for first 12 cleavages. The cell cycles are consisted of only S and M phases and one cycle accomplishes within 20-30 min. Accordingly, it could be possible

that endogenous xDnmt1 has already integrated in a complex to deal with this rapid cell cycles.

Therefore, it was likely that exogenous Dnmt1 was not able to be integrated into the preexisting complex containing xDnmt1 during *Xenopus* early development. If the hypothesis was correct, immunoprecipitation of endogenous xDnmt1 and exogenous Dnmt1 may pull down different factor(s) other than Dnmt1. But, I could not detect any extra band in the lanes of immunoprecipitated the endogenous xDnmt1. This was the same for those of immunoprecipitated the exogenous Dnmt1. I conclude that this possibility may not be the reason for "no-phenotype".

(ii) Timing of expression of exogenous xDnmt1: It has been reported that the loss or inhibition of Dnmt1 during early development induces either lethal or leads to abnormal development in mouse and zebrafish (Li et al., 1992 and 1993; Martin et al., 1999). In zebrafish, the treatment of 5-azaC, an inhibitor of DNA methyltransferase, effectively affects the development only by the time of early gastrula stage (Martin et al., 1999). The embryos are not susceptible after gastrulation. This result indicates that the timing of inhibiting DNA methyltransferase activity is important to exhibit abnormal development.

Recently, depletion of maternal xDnmt1 mRNA by injecting an antisense RNA has been reported (Stancheva and Meehan, 2000). Depletion of maternal xDnmt1 mRNA leads to complete reduction of the maternal xDnmt1 protein at 16-cell stage (stage 5), and embryos injected with antisense RNA exhibits delay in closing the blastopore. When they are allowed to develop further, antisense RNA injected embryos exhibit microcephalic phenotype in some cases accompanied by axis truncation. Moreover, some injected embryos die during gastrulation and neurulation. Depletion of xDnmt1 causes a decrease of 5mC contents in genomic DNA and the activation of

zygotic transcription starts earlier than midblastula transition. The results suggest that depletion of xDnmt1 at early stage of development may cause aberrant gene expression via hypomethylation to induce abnormal development or death in early development.

In the present experiment, wt-xDnmt1, mut-xDnmt1, and mDnmt1 mRNA injected embryos exhibited no phenotypes and normally developed. The exogenously expressed xDnmt1, wt-xDnmt1 and mut-xDnmt1 were accumulated during early development from the blastula to tailbud stage (Fig. 16). Although the injected mRNAs are translated immediately after their introduction, the level of exogenously expressed wt-xDnmt1, mut-xDnmt1 and mDnmt1 might be too low for changing the gross methylation level before blastula stage. Thus, it is likely that the level of exogenous Dnmt1s, especially the loss-of-function mut-xDnmt1, are not enough for effecting on the development by the blastula stage. The high level of exogenous Dnmt1 after blastula stage was too late for affecting the phenotype, by analogy with that in zebrafish (Martin et al., 1999).

4.5 SUMMARY

In vertebrate, DNA methylation plays an important role in early development. Mouse disrupted the Dnmt1 dies at midgestation. Moreover, inhibition of DNA methyltransferase by the treatment of 5-aza-cytidine and 5-aza-2-deoxycytidine in zebrafish embryos causes abnormal development. On the other hand, it has been reported that overexpression of Dnmt1 causes gene suppression in tumor cells. The results show that the proper regulation of DNA methyltransferase is necessary for normal development, and precise regulation of gene expression in vertebrate. In this chapter, I tried to perturb DNA methylation patterns in *Xenopus* embryos to understand the role of DNA methylation during early development. Wild-type xDnmt1, loss-of-function mut-xDnmt1, and mDnmt1 mRNAs were microinjected into *Xenopus* embryos. Although the injected Dnmt1s were expressed in high level, injected embryos exhibited no phenotypes. Possible mechanisms are discussed.

CHAPTER 5. Overexpression of mouse *de novo* DNA methyltransferases induces apoptosis in *Xenopus* embryo at gastrulation

5.1 INTRODUCTION

In mouse early development, DNA methylation patterns dramatically changes, involving genome-wide demethylation and *de novo* methylation (Monk, 1990). The fertilized egg first undergoes demethylation during preimplantation development, which erases a part of the inherited parental methylation pattern. After implantation, the embryo undergoes *de novo* methylation that establishes a new embryonic methylation pattern (Monk, 1987; Howlett and Reik, 1991; Kafri et al., 1992).

The enzymes that catalyze *de novo*-type methylation activity have been identified. Complementary DNAs for Dnmt3a and Dnmt3b have been isolated for mouse and human (Okano et al., 1998b; Xie et al., 1999). Mouse Dnmt3a and Dnmt3b are highly expressed in undifferentiated embryonic stem cells and expressed at low levels in adult somatic tissues (Okano et al., 1998b). From the genetic studies in mouse, the mice with *Dnmt3b* $^{-/-}$ genotype are embryonic lethal, whereas those with *Dnmt3a* $^{-/-}$ genotype are normal in development but become runt and die at about 4 weeks of age (Okano et al., 1999). Double knockout mutant [*Dnmt3a* $^{-/-}$, *Dnmt3b* $^{-/-}$] shows no increase of global DNA methylation levels after implantation (Okano et al., 1999). These results indicate that Dnmt3a and Dnmt3b are responsible for *de novo*-type DNA methylation activity *in vivo*.

Recently, it has been reported that the aberrant introduction of 5mC into genomic DNA causes abnormal development using *Drosophila melanogaster* (Lyko et al., 1999), which has no endogenous DNA methylation. Ectopic expression of mouse Dnmt1 and/or Dnmt3a in *Drosophila* demonstrates that mDnmt1 can act as

maintenance-type methyltransferase and mDnmt3a as *de novo*-type methyltransferase *in vivo*. The transgenic flies that express both mDnmt1 and mDnmt3a show developmental defects maybe by the presence of 5mC in the genomic DNA (Lyko et al., 1999).

To address the role of DNA methylation in early development, I attempted to perturb DNA methylation pattern, by utilizing *de novo*-type DNA methyltransferase, mDnmt3a, mDnmt3b, and bacterial type II DNA-(cytosine-5) methylase *M.Hpa*II. I injected mDnmt3a, mDnmt3b, or *M.Hpa*II mRNA into *Xenopus* embryos and observed their development. Expression of mDnmt3a and mDnmt3b induced apoptosis at gastrulation stage, and, to my surprise, the effect was DNA methyltransferase activity independent.

5.2 MATERIALS AND METHODS

5.2.1 Plasmid construction and *in vitro* transcription

mDnmt3a, mDnmt3b1, mDnmt3b2, and mDnmt3b3 cDNA was kindly provided by Dr. T. Chijiwa and Prof. H. Sasaki (National Institute of Genetics) and bacterial type II DNA-(cytosine-5) methylase *M.Hpa*II cDNA was a kind gifted from Dr. S. Klimasauskas (Institute of Biotechnology, Lithuania). They were subcloned into pCS2+MT vector.

A loss-of-function mDnmt3a (mut-mDnmt3a) cDNA was generated as follows. The DNA fragment of 566 bp encoding amino acid residues from 525 to 713 of mDnmt3a, in which the codon TGC coding Cys706 was changed to GCC coding Ala was amplified with a primer set of mD3aF (5'-CGACGATGGGTACCAGTCCTATTGC-3') containing *Kpn*I site and mD3aHinc (5'-GGTTGACAATGGAGAGGTCATTGGCGGG-3') containing *Hinc*II. The wild-type *KpnI*/*Hinc*II fragment was replaced with the mutated *KpnI*/*Hinc*II fragment to produce the mut-mDnmt3a cDNA. Finally, the mut-mDnmt3a cDNA was subcloned into pCS2+MT vector.

Loss-of-function mDnmt3b1 (mut-mDnmt3b1), 3b2 (mut-mDnmt3b2), and 3b3 (mut-mDnmt3b3) cDNAs were generated as described in Chapter 4 by using a primer set of m3bf (5'-GTCAATGACGTCCGGAAAATCACC-3'), m3bmut (5'-GGATTGACGTTAGAGAGATCATTGGCTGGGCTTCCACC-3'), m3bn (5'-CAATGATCTCTCTAACGTCAATCC-3'), and m3br (5'-TTGATGGCATCGATCATCACTGGG-3').

All the constructs obtained were linearized at the *Not*I site and transcribed *in vitro* with SP6 RNA polymerase (Boehringer Mannheim GmbH, Germany) under

manufacturer's instruction to obtained capped mRNAs. The size of transcribed mRNAs were confirmed by electrophoresis in 1.0% agarose/formaldehyde denatured gel and the gel was stained with SYBR® Green II (Molecular Probes, OR). The quantification of stained mRNAs was performed as described in Chapter 4.

5.2.2 Embryos and microinjection

Unfertilized eggs of *Xenopus* and embryos were obtained as described in Chapter4. Embryos were kept at 20, 18 or 14 °C in 0.1x Steinberg's solution until used for microinjection.

mRNAs (0.3-3 ng) were co-microinjected with 0.5 ng β-galactosidase mRNA in 5 nl of distilled RNase-free water into animal-pole side of 2-cell stage embryos in 5% Ficoll 400-0.33x MMR (Sive et al., 2000b). Injected embryos were kept for an hour in 5% Ficoll 400-0.33x MMR. Injected embryos were transferred into 0.1x MMR with 10 μg/ml gentamycin for further culture. All the embryos were incubated at 18 °C.

5.2.3 Immunoprecipitation and Western blotting analysis of exogenous mDnmt3s

One injected embryo each was homogenized in 1 ml of buffer containing 150 mM NaCl, 1% (w/v) NP-40, 0.01% sodium deoxycholate, and 0.02 tablet of Complete®-mini protease inhibitor cocktail/ml (Boehringer Mannheim GmbH, Germany), 50 mM Tris-HCl (pH 7.6) and kept on ice for 30 min. The whole extracts were centrifuged at 15,000 rpm for 15 min at 4 °C and the supernatant fractions were incubated with Sepharose 4B (Amersham Pharmacia Biotech AB, Sweden) conjugated with anti-myc monoclonal antibody (9E10) for overnight at 4 °C. The immunoprecipitated mDnmt3s proteins were separated by SDS-PAGE (Laemmli, 1970) and electrophoretically transferred onto nitrocellulose membrane. Anti-myc monoclonal

antibody (9E10), a primary antibody, was reacted in a PBS buffer containing 1% bovine serum albumin (BSA), 0.1% (w/v) Triton X-100, and 0.05% sodium azide. The mDnmt3-antibody complex was detected with alkaline phosphatase conjugated secondary antibodies (E.Y. Laboratory Inc., CA) and a color reaction buffer (4 mM $Mg(OAc)_2$, 50 mM Glycine-NaOH (pH 9.6)) containing 0.05 mg/ml BCIP and 1 mg/ml NBT.

5.2.4 β-galactosidase staining assay.

Embryos injected with mRNAs for β-galactosidase were analyzed as described in Chapter4.

5.2.5 TdT dUTP nucleotide end labeling (TUNEL) staining

TUNEL staining of whole mount *Xenopus* embryos was carried out using a protocol reported by Hensey and Gauiter (1997). Injected embryos were fixed for 1 h in MEMFA (100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 4% formaldehyde) and washed twice with methanol for 30 min each, and stored in methanol at –20 °C. For rehydration, the embryos were washed twice with PBT (PBS containing 0.2% (w/v) Tween 20) for 15 min each, and then washed twice with PBS for 15 min each. The embryos thus treated were preincubated with terminal deoxynucleotidyl transferase (TdT) buffer (GIBCO BRL, Life Technologies, Inc., MD) for 30 min. End labeling was carried out for overnight at room temperature in TdT buffer containing 150 U/ml TdT (GIBCO BRL, Life Technologies, Inc., MD) and 40 mM digoxygenin-dUTP (Boehringer Mannheim GmbH, Germany). The embryos were washed twice with PBS containing 1 mM EDTA for 1 h each at 65°C and then four times with PBS for 1 h each at room temperature. The embryos were incubated for 1 h in PBT containing 20% goat

serum (GIBCO BRL, Life Technologies, Inc., MD) and incubated for overnight at 4 °C with anti-digoxygenin antibody coupled with alkaline phosphatase (Boehringer Mannheim GmbH, Germany). To remove excess antibody, the embryos were washed six times with PBS for 1 h each at room temperature and then for overnight at 4 °C. The chromogenic reaction was carried out in alkaline phosphatase buffer (50 mM $MgCl_2$, 100 mM NaCl, 0.1% (w/v) Tween 20, 5 mM levamisol (Sigma Chemical Co., MO), 100 mM Tris-HCl (pH 9.6)), containing 338 µg/ml NBT and 175 µg/ml BCIP. The reaction was stopped within 15 min by transferring the embryos to MEMFA. The embryos were observed after dehydration in methanol and mounted in benzyl benzoate/benzyl alcohol 2:1 (Dent et al., 1989).

5.2.6 Microscopy

The embryos were observed under Nikon SMZ-U stereo microscope and photographs were taken with Nikon FX35 camera using SUPERIA 400 film (Fuji Photo Film Co. Ltd., Tokyo).

5.3 RESULTS

5.3.1 Effects of injection of the mRNAs of *de novo*-type methyltransferases on early development

Several lines of studies indicate that mDnmt3a and mDnmt3b can act as *de novo*-type DNA methyltransferase *in vivo*. To see the effect of perturbation of normal DNA methylation patterns in genomic DNA, I injected 3ng of mouse *de novo* methyltransferase, mDnmt3a mRNA (Fig. 19) into the animal-pole side of a blastomere at 2-cell stage and cultured until stage 11. The translation of the injected mRNAs was confirmed with immunoprecipitation of stage 11 embryo (Fig. 20, lane 2). The embryos injected with mRNA encoding mDnmt3a cleaved and normally developed until late blastula stage (data not shown). At early gastrula stage, the region where the mDnmt3a mRNA were injected started to come apart, became round shape, and detached from the embryo (Fig. 21A). The size of dissociated cells was obviously larger than those of normal cells. The dissociated cells were autolysed thereafter (data not shown).

To see whether the phenotype was specific to mDnmt3a, I next injected the other *de novo*-type DNA methyltransferases into *Xenopus* embryos. mDnmt3b1, 3b2, 3b3, and bacterial type II DNA-(cytosine-5) methylase *M.Hpa*II that recognize CCGG sequence (Fig. 19) were injected into the animal-pole side of a blastomere at 2-cell stage, and injected embryos were cultured until stage 11. The translation of injected mRNAs was confirmed with immunoprecipitation of stage 11 embryo with anti-myc monoclonal antibody (Fig. 20, lane 3-6). The phenotype of embryos injected the mDnmt3b1 and 3b2 mRNAs exhibited a very similar phenotype (Fig. 21B and C) as that by mDnmt3a, *i.e.* cell dissociation at early gastrula stage. On the contrary, mDnmt3b3 and *M.Hpa*II injected embryos exhibited no morphological abnormalities (Fig. 21D and E). All the

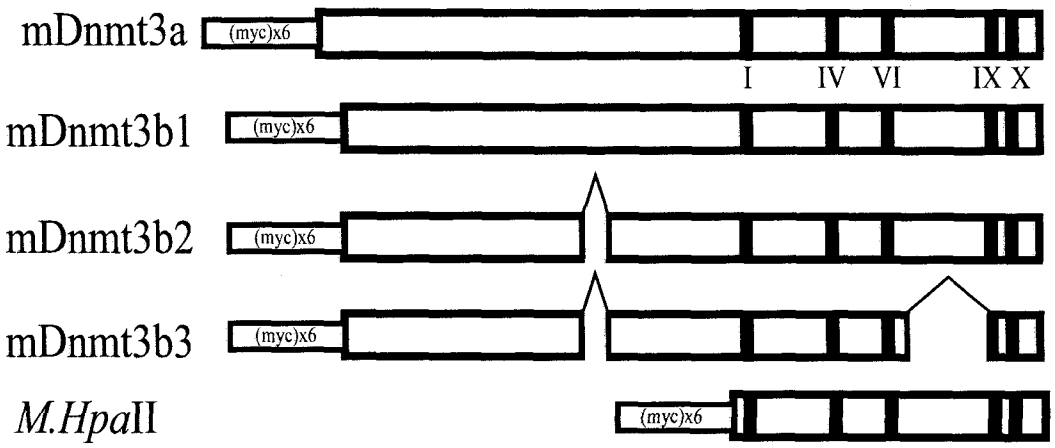


Figure 19. The constructs of mDnmt3a, 3b1, 3b2, 3b3, and *M.Hpa*II. Proteins encoded by injected mRNA are schematically illustrated. All the cDNAs were subcloned into pCS2+MT vector containing six repeated myc-tag epitope {(myc)x6} at the amino-terminus and transcribed *in vitro* with SP6 polymerase. I-X are the conserved motifs of the bacterial type-II cytosine methylase.

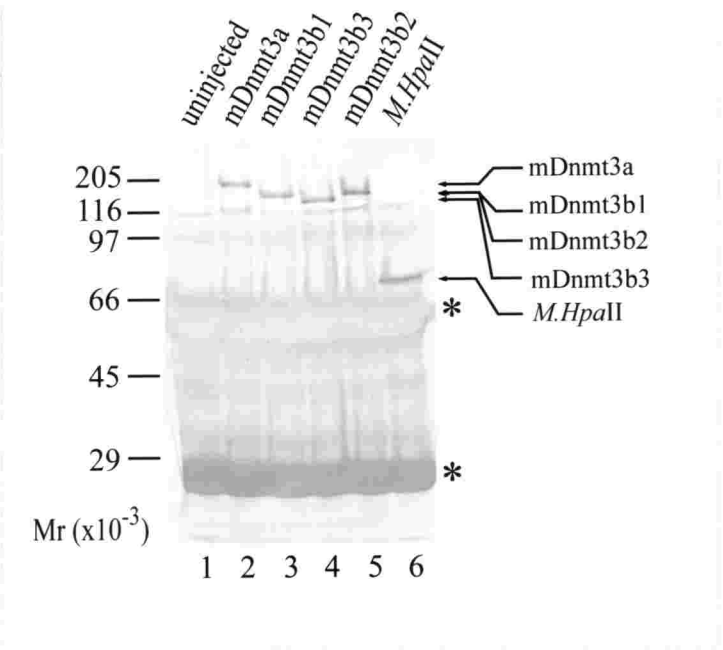


Figure 20. Expression of injected mRNA at stage11-12 (gastrula) embryos. Whole extract of single injected embryo at stage 11-12 was immunoprecipitated with anti-myc monoclonal antibody (9E10) and then analyzed by Western blotting using alkaline phosphatase coupled secondary antibodies. Each protein is indicated by arrow. Asterisks indicated mouse IgG heavy and light chains. Molecular weight standards (Mr) are indicated.

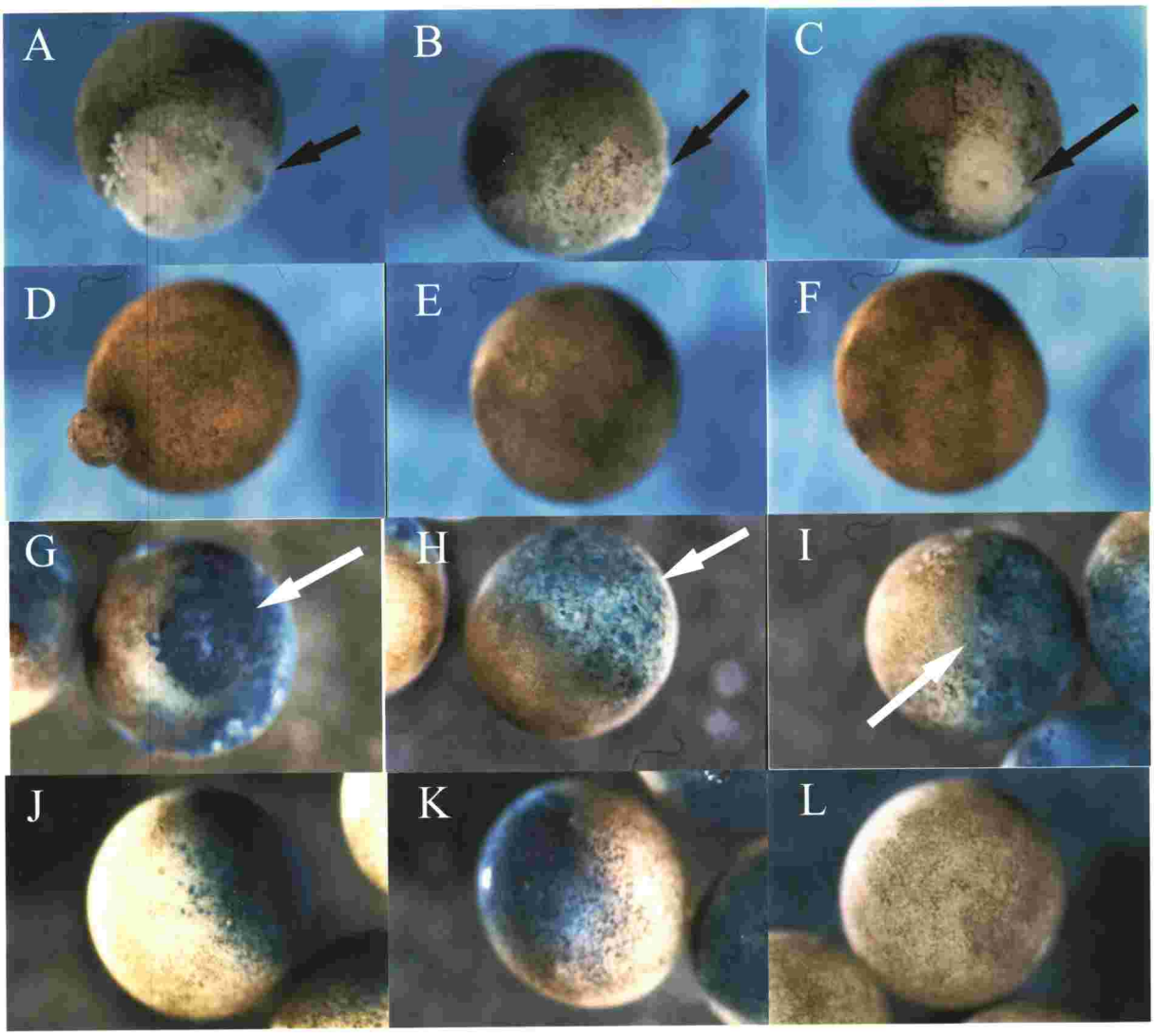


Figure 21. Typical phenotype of the embryos that were overexpressed mDnmt3a, 3b1, 3b2, 3b3, or *M.Hpa*II. Embryos were injected with 3ng each mRNA of mDnmt3a (A and G), mDnmt3b1 (B and H), mDnmt3b2 (C and I), mDnmt3b3 (D and J), or *M.Hpa*II (E and K), and 0.5ng mRNA of β-galactosidase into animal-pole side of 2-cell stage embryos. Panel F and L shows uninjected control embryos. Each embryo at stage 11-12 was fixed and stained the β-galactosidase (G-L). Black arrows indicate the region of cell dissociation (A-C), and white arrows indicate the dissociated cells stained with β-galactosidase activity (G-I).

cells that exhibited abnormal morphology were β-galactosidase staining positive, indicating that the abnormal morphologies were due to the direct effect of injected mRNAs (Fig. 21G-L).

The results obtained from four independent microinjection experiments were summarized in Table IV.

5.3.2 mDnmt3a, 3b1 or 3b2 injected embryos showed an apoptotic feature

The morphological feature of the dissociated cells appeared in the embryos injected with mDnmt3a, 3b1 or 3b2 mRNA resembled to that of apoptotic cells (Hensey and Gautier, 1997). To test this possibility, the mRNA-injected embryos were further analyzed by the TdT-mediated dUTP digoxygenin nick end labeling (TUNEL) staining technique, which demonstrates *in situ* DNA fragmentation in the cells died of apoptosis (Gravieri, et al., 1992). Whole embryos at stage 11-12, at which stage the injected embryos showed cell dissociation, were fixed and TUNEL stained (Fig. 22). The dissociated cells caused by injection of mDnmt3a, 3b1 and 3b2 were intensely stained by TUNEL staining (Fig. 22A-C). On the other hand, the cells of mDnmt3b3 and *M.Hpa*II injected embryos, which exhibited no morphological abnormalities, were obviously not stained (Fig. 22D-F). Thus, the apoptotic features of dissociated cells were biochemically proven.

To eliminate the side-effects due to high dose of injected mRNAs, I injected different amounts of mDnmt3a, 3b1, 3b2, 3b3 and *M.Hpa*II mRNA into 2-cell stage embryos and cultured until stage 11. When the injected dose was reduced to 0.3 ng per blastomere of 2-cell stage embryos, mDnmt3a, 3b1, and 3b2 injected embryos showed identical apoptotic phenotype as that injected with 3 ng per embryo (Fig. 23). Even when the dose was reduced, cell dissociation took place constantly at the gastrula stage

Table IV. Phenotypes of the embryos that were injected with mDnmt3a, 3b1, 3b2, 3b3, or *M.Hpa*II mRNA. Embryos were injected with 3ng each of indicated mRNA into the 2-cell stage embryos and were cultured until stage 11-12. Results obtained from four independent experiments were summarized. The numbers of total embryos that were injected, of the normal embryos, and of the embryos that showed cell dissociation are shown. The percentage of the embryos showed cell dissociation is also shown.

mRNA	total (n)	normal (n)	cell dissociation (n)	cell dissociation/ total (%)
uninjected	65	65	0	0
mDnmt3a	65	0	65	100
mDnmt3b1	64	0	64	100
mDnmt3b2	63	0	63	100
mDnmt3b3	62	58	4	6
*M.Hpa*II	50	46	4	8

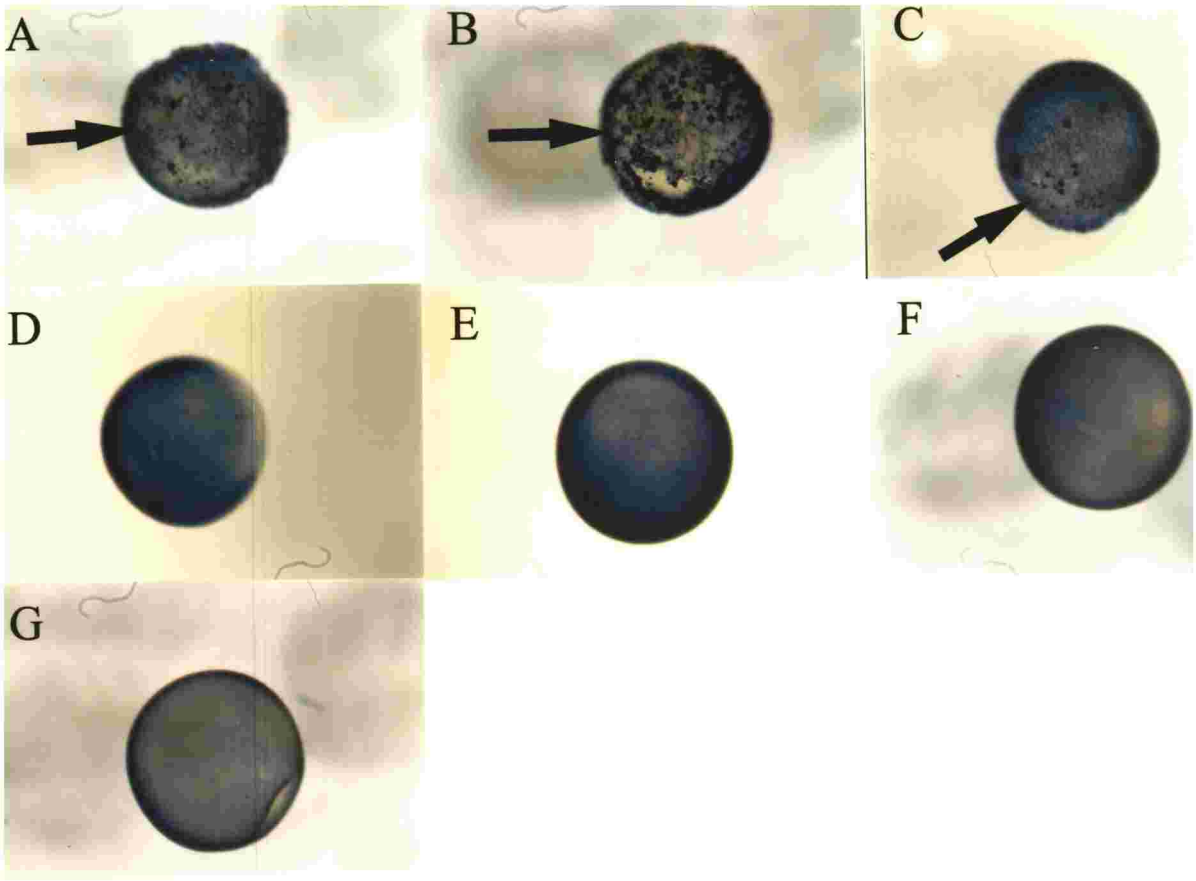


Figure 22. Embryos that were injected with mDnmt3a, 3b1, or 3b2 showed apoptotic features. Embryos injected with 1 ng each of the mRNA into animal-pole side of 2-cell stage embryos. Injected embryos were cultured until stage 11-12 and then fixed in MEMFA for 1 h. Fixed embryos were served for TUNEL assay. (A) mDnmt3a, (B) mDnmt3b1, (C) mDnmt3b2, (D) mDnmt3b3, (E) *M.Hpa*II, (F) β-galactosidase mRNA injected and (G) uninjected control embryos are shown. Arrows indicate the region showing cell dissociation.

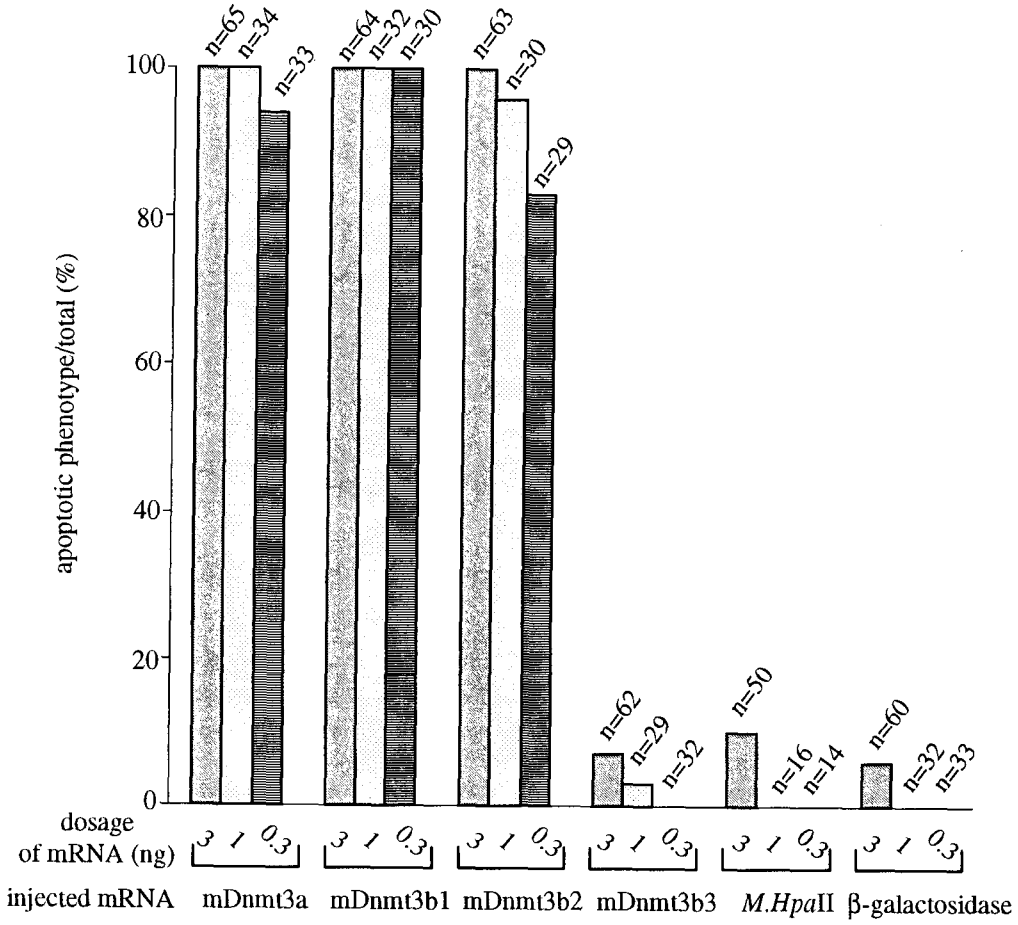


Figure 23. Effect of mDnmt3a, 3b1, 3b2, 3b3 or *M.Hpa*II mRNA on the phenotype. Indicated mRNA was microinjected at 3-0.3 ng per embryo into animal-pole side of 2-cell stage embryo. β-galactosidase mRNA was used as control. The percentage of resulting apoptotic phenotype scored at stage 11-12 was plotted. (n), number of injected embryos.

(data not shown). Injection of up to 3 ng per blastomere of β-galactosidase mRNA did not exhibit any developmental abnormality, and injected embryos did not show cell dissociation (Fig. 23). Therefore, I concluded that autonomous dissociation of the cells observed was induced by the specific function of the injected mDnmt3a, 3b1 and 3b2 mRNA.

5.3.3 Apoptotic effect of mDnmt3a and 3b was independent of DNA methylation activities

Apoptotic effect was induced by microinjection not with mDnmt3b3 but with mDnmt3a, 3b1 or 3b2, suggesting that *de novo*-type DNA methylation activity may induce the phenotype, as mDnmt3b3 lacks methyltransferase activity (Aoki, unpublished). But, at the same time, active *de novo*-type DNA methyltransferase, *M.Hpa*II, also exhibited no effect on the development.

To examine if the *de novo*-type DNA methylation activity itself is the cause for the abnormal development, I prepared and injected a loss-of-function mDnmt3s into embryos. For this, I introduced a site-directed mutagenesis, changing Cys to Ala at Pro-Cys dipeptide in motif IV, which abolishes the DNA methyltransferase activity (Wyszynski et al., 1992) (Fig. 24). I injected those mut-mDnmt3a, mut-mDnmt3b1, mut-mDnmt3b2, or mut-mDnmt3b3 mRNA into a blastomere of 2-cell stage embryos. Surprisingly, mut-mDnmt3a, mut-mDmnt3b1, and mut-mDnmt3b2 mRNA injected embryos exhibited identical phenotypes as the wild-type mRNA injected embryos. Again mut-mDnmt3b3 induced no abnormal development (Fig. 25, Table V). The results suggest that the cell dissociation observed in injected embryos is independent of DNA methyltransferase activities of Dnmt3a, 3b1, and 3b2. All the mut-mDnmt3s showed no DNA methylation activity and were efficiently translated into protein in

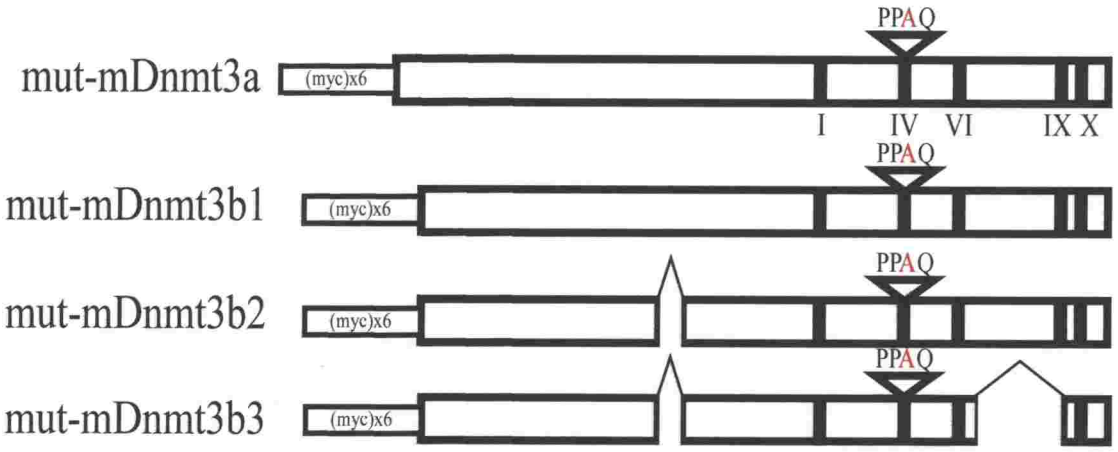


Figure 24. The constructs of mut-mDnmt3a, 3b1, 3b2, and 3b3. Proteins encoded by injected mRNA are schematically illustrated. All the cDNAs were subcloned into pCS2+MT vector containing six repeated myc-tag epitope {(myc)x6} at the amino-terminus and transcribed *in vitro* with SP6 polymerase. I-X are the conserved motifs of the bacterial type-II cytosine methylase. The sequence of catalytic center in motif VI is shown in a single letter and the substituted amino acids are shown in red.

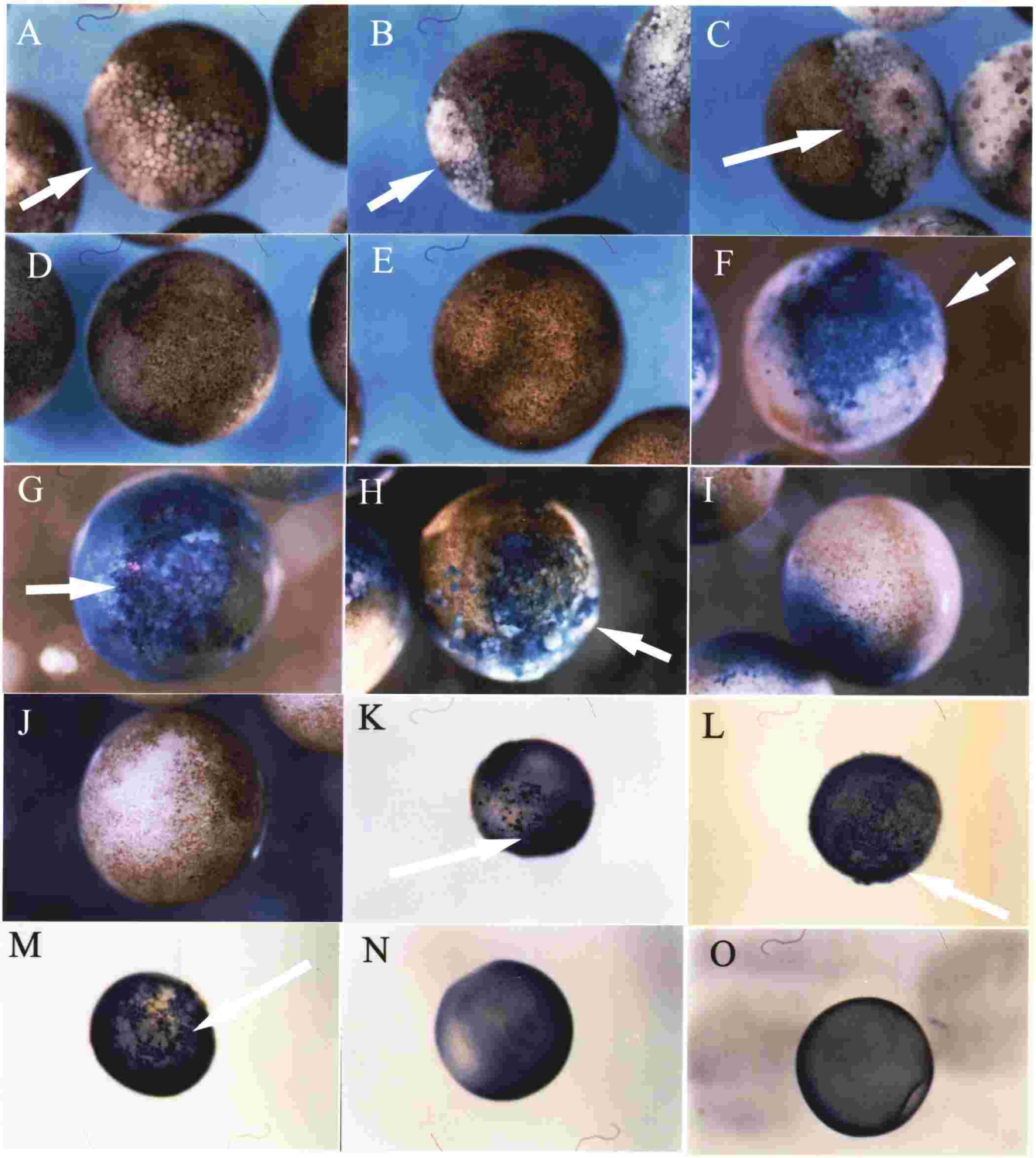


Figure 25. Typical phenotype of embryos that were overexpressed mut-mDnmt3a, 3b1, 3b2, or 3b3. Embryos were injected with 1ng each mRNA of mut-mDnmt3a (A, F, and K), mut-mDnmt3b1 (B, G, and L), mut-mDnmt3b2 (C, H, and M), or mut-mDnmt3b3 (D, I, and N), and 0.5 ng of β-galactosidase mRNA into animal-pole side of 2-cell stage embryos. Panels E, J and O show uninjected control embryos. Each embryo at stage 11-12 was fixed and performed β-galactosidase staining (F-J) and TUNEL (K-O) staining. White arrows indicate the region of apoptosis.

Table V. Phenotypes of the embryos injected with mut-mDnmt3a, 3b1, 3b2, or 3b3 mRNA. Embryos were injected with 1ng each of indicated mRNA into the 2-cell stage embryos and were cultured until stage 11-12. Results obtained from four independent experiments were summarized. The numbers of total embryos that were injected, of the normal embryos, and of the embryos that showed cell dissociation are shown. The percentage of the embryos showed cell dissociation is also shown.

mRNA	total (n)	normal (n)	cell dissociation (n)	cell dissociation/ total (%)
uninjected	45	45	0	0
mut-mDnmt3a	40	0	40	100
mut-mDnmt3b1	34	0	34	100
mut-mDnmt3b2	28	0	28	100
mut-mDnmt3b3	41	38	3	7

injected embryos (data not shown).

5.4 DISCUSSION

5.4.1 Apoptotic phenotype of embryos injected with mDnmt3a, 3b1, and 3b2 mRNA

Overexpression of wild-type mDnmt3a, 3b1, and 3b2, caused cell dissociation at early gastrula stage, while that of wild-type mDnmt3b3 and *M.Hpa*II, did not show any morphological abnormalities (Fig. 21). Moreover, overexpression of loss-of-function mDnmt3a, 3b1, and 3b2 also caused cell dissociation, while that of loss-of-function mDnmt3b3 showed no morphological abnormalities (Fig. 25). All the cells dissociated from embryos exhibited an apoptotic feature that was confirmed by TUNEL staining (Fig. 22 and 25).

The methyltransferase activities of mDnmt3a and 3b were confirmed *in vitro* (Aoki and Miyagawa, unpublished). The amino acid change from Cys to Ala at Pro-Cys in motif IV completely abolished the DNA methyltransferase activity in mDnmt3. This was confirmed with the products transiently expressed in 293T cells (data not shown). These results clearly indicate that the apoptotic phenotype observed in the present experiment is independent of DNA methyltransferase activity.

This is further supported by the overexpression experiment of *M.Hpa*II, a bacterial type II DNA-(cytosine-5) methylase that possesses *de novo*-type DNA methylation activity, in *Xenopus* embryos. *M.Hpa*II did not induce any phenotype on *Xenopus* early development. DNA methyltransferase activity itself cannot induce the apoptotic phenotype.

Then, how does the mDnmt3a, 3b1, or 3b2 induce the apoptotic phenotype? The only structural difference between mDnmt3b2 and 3b3 is that mDnmt3b3 lacks 62 amino acid residues covering motif VIII and a part of motif IX, which are responsible

for DNA methyltransferase activity. As the DNA methyltransferase activity was shown to be independent of the apoptotic phenotype. I expect that the deletion of 62 amino acid residues in mDnmt3b3 may grossly change the three-dimensional structure of the entire molecule. I further speculate that not mDnmt3b3 and *M.Hpa*II, but mDnmt3a, 3b1 and 3b2 interact with a protein(s) or DNA that plays crucial roles in the apoptotic phenotype. To identify the mechanism of the induction of phenotype, I am planning to pull down a specific factor(s) interacting specifically with mDnmt3a, 3b1, or 3b2.

5.4.2 A possible target of mDnmt3a, 3b1 and 3b2 in apoptotic phenotype

As for the apoptotic phenotype, induced by the overexpression of mDnmt3a, 3b1, or 3b2, inhibition of cadherin synthesis could be a possible target. In *Xenopus*, E-cadherin expression starts at the time of zygotic transcription begins, just before gastrulation. At the early stage of development, E-cadherin is expressed only in the ectoderm, not in the mesoderm or endoderm (Choi and Gumbiner, 1989; Levi et al., 1991). Embryos injected with the mRNA encoding loss-of-function mutant E-cadherin show no detectable effect until gastrulation. At stage 11.5, injected embryos show cell dissociation in the outer ectodermal layer (Levine et al., 1994). This phenotype is quite similar to the present phenotype induced by mDnmt3a, 3b1, or 3b2. It may be possible that overexpression of mDnmt3a, 3b1, and 3b2, affected the expression of E-cadherin to induce apoptotic phenotype.

The abnormal development associated with cell dissociation observed in the present experiment by Dnmt3 reveals that the dissociated cells exhibits an apoptotic feature confirmed with TUNEL staining (Fig. 22 and 25). The apoptotic cell dissociation during early gastrulation has been reported in a few other cases. The inhibition of DNA replication (Stack and Newport, 1997), transcription, or translation

(Hensey and Gauiter, 1997) causes apoptotic cell death at early gastrulation. At present, it is uncertain that what is the target of mDnmt3a, 3b1, and 3b2, and what occurs thereafter. Further investigation about the target(s) of mDntm3 family may reveal the regulation system of *de novo*-type DNA methyltransferases.

In this chapter, I showed the effects of ectopic expression of mDnmt3 family on early *Xenopus* development. As ectopic expression of mDnmt3a, 3b1, or 3b2 induced abnormal development in *Xenopus* embryos, it was expected that Dnmt3 family should be involved in some common machinery for development that might endogenously exist in *Xenopus*.

5.5 SUMMARY

Recently, mammalian *de novo*-type DNA methyltransferases, Dnmt3a and 3b, has been cloned in mouse and human. From genetic studies of mouse and ectopic expression in *Drosophila*, Dnmt3a and 3b are proven to be responsible for *de novo* DNA methylation *in vivo*. In this chapter, I intended to perturb DNA methylation patterns in *Xenopus* embryos with ectopic expression of *de novo*-type DNA methyltransferases. Embryos that were overexpressed mouse Dnmt3a, 3b1, and 3b2 showed apoptotic phenotypes at early gastrula stage, while embryos that were overexpressed mouse Dnmt3b3 and bacterial methylase *M.Hpa*II did not show any morphological abnormalities. However, I revealed that this apoptotic phenotype was independent of DNA methyltransferase activity. It was expected that endogenous regulating machineries in *Xenopus* was modulated by the ectopic expression of Dnmt3 family.

CHAPTER 6. CONCLUSION

In this study, I firstly reported the cloning of xDnmt1 cDNA, compared with mammalian and other vertebrate Dnmt1, and shown that cloned xDnmt1 cDNA encodes maintenance-type DNA methyltransferase (Chapter 2). Then, I showed the accumulation and localization of xDnmt1 during *Xenopus* oogenesis. The xDnmt1 existed $1x10^4$ times higher in stage VI oocyte than that in somatic cells (Chapter 3). This high level of xDnmt1 in oocyte resembles to that in mouse case. Surprisingly, xDnmt1 was positively translocated into nuclei and distributed equally to nuclei in the stage VI oocyte. The localization of xDnmt1 in nuclei was confirmed by the immunochemical staining of xDnmt1 in isolated nuclei of stage VI oocyte.

To elucidate the physiological meaning of DNA methylation in *Xenopus* early development, I attempted to change the DNA methylation patterns during development by injecting xDnmt1 mRNA and its loss-of-function one into *Xenopus* embryos. Although both xDnmt1 proteins were expressed to high level in injected embryos, they could not induce any abnormalities in development (Chapter 4). As for the discrepancy between my present results and that reported by Stancheva and Meehan (2000) who reported that the depletion of xDnmt1 induced abnormal development, I discussed about the developmental stage-dependent effect of the perturbation of DNA methylation.

Next, I injected mouse Dnmt3 family, *de novo*-type DNA methyltransferases. Interestingly, I found that Dnmt3a, 3b1, and 3b2 injected embryos show apoptotic phenotypes, but Dnmt3b3 injected embryos showed no morphological abnormalities. When the loss-of-function Dnmt3a, 3b1, 3b2, or 3b3, which lack DNA methyltransferase activities, was injected, the embryos showed an identical phenotype as those injected the wild-type constructs. The apoptotic phenotype was shown to be independent of their DNA methyltransferase activities. The result suggests that the

ectopic expression of mDnmt3a, 3b1, and 3b2 disturbed the machinery involved in the endogenous regulation system, possibly related to *de novo* DNA methylation (Chapter 5).

Hopefully, I could be able to demonstrate that *Xenopus* embryo is a useful model system to investigate the role(s) of DNA methylation in early development.

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RELATED PUBLICATIONS

Kimura, H., Ishihara, G., and Tajima, S. (1996) Isolation and expression of a *Xenopus laevis* DNA methyltransferase cDNA. *J. Biochem.* **120**,1182-1189

Kimura, H., Suetake, I., and Tajima, S. (1999) *Xenopus* maintenance-type DNA methyltransferase is accumulated and translocated into germinal vesicles of oocytes. *J. Biochem.* **125**, 1175-1182

ABSTRACTS

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