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Xenopus Eggs Express an Identical DNA Methyltransferase, Dnmt1, to Somatic Cells[1]

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In mouse, an oocyte-specific short isoform of DNA methyltransferase-1 (Dnmt1) lacking amino terminal 118 amino acid residues exists and plays a crucial role in maintaining the methylation state of imprinted genes during early embryogenesis [Howell *et al.* (2001) *Cell* 104, 829–838]. To address the question of whether or not *Xenopus* oocyte expresses such a short isoform, we raised monoclonal antibodies against the amino-terminal portion of *Xenopus* Dnmt1. Two of the isolated monoclonal antibodies, 3C6 and 4A8, were determined to recognize (1–32) and (115–126) of *Xenopus* Dnmt1, respectively. The amounts of Dnmt1 in *Xenopus* eggs were determined to be similar, 10.0 ± 2.5, 8.0 ± 0.8, and 8.2 ± 0.2 ng per egg with monoclonal antibodies 3C6 and 4A8, and polyclonal antibodies, respectively. This indicated that Dnmt1 in *Xenopus* mature eggs had an identical amino-terminal sequence to the amino acid sequence deduced from the cDNA. Together with the fact that Dnmt1 in A6 cells immuno-reacted with all the monoclonal antibodies isolated and with the polyclonal antibodies, we concluded that Dnmt1 expressed in *Xenopus* mature eggs possesses an identical amino-terminal sequence to that in somatic cells. Immuno-purified *Xenopus* Dnmt1 in mature eggs showed similar specific activity to that in proliferating A6 cells and that of mouse recombinant Dnmt1.

Key words: DNA methylation, DNA methyltransferase, mature eggs, monoclonal antibody, *Xenopus laevis*.

In vertebrates, the 5th position, of the cytosine residue in the CpG sequence in genomic DNA is often methylated (*1*). Genomic DNA methylation contributes to physiological or pathological phenomena such as tissue-specific gene expression (*2–4*), genomic imprinting (*5*), X-chromosome inactivation (*6, 7*), and carcinogenesis (*8*). In vertebrates, two types of DNA methyltransferase activities have been reported, *i.e., de novo*– and maintenance-type activities. *De novo*–type DNA methyltransferase activity is thought to create the tissue-specific DNA methylation patterns at the implantation stage of embryogenesis, and the maintenance-type DNA methyltransferase activity ensures the clonal transmission of the cell type–specific methylation pattern during mitosis.

A maintenance-type DNA methyltransferase, designated as Dnmt1, has been isolated from various animals, such as mouse (*9, 10*), man (*10*), chick (*11*), *Xenopus* (*12*), and sea urchin (*13*). The predicted amino acid sequences are highly homologous to each other (*11*). Dnmt1 possesses a carboxyl-terminal domain closely related to those of bacterial DNA (cytosine-5) methyltransferases (*14*), and a large amino-terminal domain that has multiple regulatory functions such as the recognition of replication foci at the late S-phase in somatic cells (*15*) and the retention in the cytoplasmic compartment in mouse oocytes (*16*). This amino-terminal domain has been reported to bind many biologically important molecules such as proliferating cell nuclear antigen (PCNA), tumor suppressor Rb, transcription factor E2F, histone deacetylase 1 (HDAC1), HDAC2, and transcription repressor DMAP1 (*17–20*).

In mouse, when the *Dnmt1* gene is knocked out, homozygous mutant embryos cannot survive past midgestation (*21*), and their genomic imprinting is canceled (*22*). Similar to in mouse, depletion of the embryonic Dnmt1 with the antisense RNA decreases the methylation level in *Xenopus* embryos and leads to the abnormal development of the embryo (*23, 24*). The amount of Dnmt1 accumulated in mouse mature oocytes and one-cell embryos is 3,000 times higher than that in murine erythroleukemia cells on a per cell basis (*25*), and interestingly Dnmt1 was not translocated into germinal vesicles (nuclei) but was localized exclusively in the cytoplasmic compartment in mouse oocytes (*26*). Again, similar to in mouse, in *Xenopus*, Dnmt1 is accumulated during oogenesis (*27*). However, different from in mouse, half of the Dnmt1 in *Xenopus* oocytes is translocated into germinal vesicles (nuclei) (*27*). The Dnmt1 in mouse oocytes was the translation product from the unique transcript utilizing oocyte-specific exon1 (*26*). As a consequence, the translated Dnmt1 in mouse oocytes

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Abbreviations: Dnmt, DNA methyltransferase; DTT, dithiothreitol; FCS, fetal calf serum; GST, glutathione-*S*-transferase; HDAC, histone deacetylase; mDnmt1, mouse Dnmt1; PAGE, polyacrylamide-gel electrophoresis; PBS, Dulbecco's phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; xDnmt1, *Xenopus* Dnmt1.

lacks 118 amino acid residues from the amino-terminus of the somatic-type Dnmt1 (*26*). This oocyte-type Dnmt1 is dominant in mouse early embryos until the blastocyst stage (*26*), and has been reported to play a crucial role in maintaining the methylation imprinting during embryogenesis (*28*).

It is interesting to address the question of whether or not such a short form of Dnmt1 exists in *Xenopus* early embryos, like in mouse. To characterize Dnmt1 in early *Xenopus* embryos, we prepared four types of monoclonal antibodies specific to the amino-terminal portion of Dnmt1. After identifying their epitopes, we examined the existence of the short form of Dnmt1 utilizing the antibodies. Different from in the case of mouse, we found that the Dnmt1 expressed in *Xenopus* mature oocytes had an identical amino-terminal structure to that in somatic cells.

MATERIALS AND METHODS

Cells—Xenopus A6 cells were maintained in medium containing 61% Leibovitz L-15 medium (Sigma Chemical, MO), 10% fetal calf serum (FCS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 26°C (*29*). Murine myeloma cells, P3U1 cells, were maintained in RPMI 1640 medium supplemented with 10% FCS, 5 μg/ml MC210 (Dainippon Pharmaceutical), 1 mM sodium pyruvate, 0.1 mM non essential amino acids (Gibco BRL, MD), 100 u/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C under a 5% CO_2 atmosphere.

Plasmids—The cDNAs encoding the amino-terminal regions of *Xenopus* Dnmt1 (xDnmt1) were obtained either by amplification by PCR using the xDnmt1 cDNA plasmid harboring the entire sequence (*12*) as a template, or by oligonucleotide synthesis. The PCR was performed with Pfu polymerase (Stratagene, CA), using the primer sets listed in Table I. The PCR products were cloned into the pGEX-4T2 vector (Amersham Pharmacia Biotech, UK) to produce glutathione *S*-transferase (GST)–fused protein. The oligonucleotides listed in Table II were annealed and then directly subcloned into the pGEX-4T2 vector. All the sequences subcloned were determined by the dideoxy termination method (*30*).

Recombinant Proteins—The amino-terminal portions of GST-fused xDnmt1 were expressed in BL21(DE3), and the expressed recombinant proteins were purified on a glutathione-Sepharose column according to the manufacturer's directions (Amersham Pharmacia Biotech).

For epitope mapping, purified fusion proteins were used. As for immunization, the glutathione-Sepharose purified fusion protein harboring the amino-terminal (1–142) of xDnmt1 (10 mg in total) was thoroughly dialyzed against 0.15 M NaCl, 50 mM Tris-HCl (pH 8.0). Then the GST-fused protein was mixed with $CaCl_2$ to a final concentration of 25 mM and 3 units of thrombin (Nakalai Tesque, Kyoto), and then incubated at 25°C for 6 h. The cleaved protein was again loaded onto a glutathione-Sepharose column equilibrated with 0.15 M NaCl, 50 mM Tris-HCl (pH 8.0), and the unbound fraction was collected. The pooled fraction was dialyzed against 50 mM Tris-HCl (pH 7.4), and further purified on a MonoQ column (Amersham Pharmacia Biotech) with a linear gradient of NaCl, from 0 to 0.5 M, in 50 mM Tris-HCl (pH 7.4).

Antibodies—Balb/c mice were immunized with the purified amino-terminal (1–142) of xDnmt1, and then cell fusion, cell culture, and selection were performed as described (*31*). Four positive clones were isolated after two rounds of cloning. The antibodies were purified from ascites

TABLE I. **PCR primer sets used for the construction of GST fusion proteins.** The primers were synthesized for PCR amplification of the sequence encoding the amino-terminal region of xDnmt1 (Acc. No., D78638). The names of the constructs (#), the corresponding amino acids numbers (Coding seq.), and the nucleotide sequences of primers (Forward and Reverse) are summarized. The underlined sequences indicate the *Eco*RI and *Sal*I linker sequences utilized for in-frame ligation into the expression vector.

#	Coding seq		
1	1-142	Forward	5'-GGAATTCATGCCGGCCCAGTCCACTTC-3'
		Reverse	5'-GGTCGACCTAGGCACGGGCTGGTGATTTCTTG-3'
2	1-84	Reverse	5'-CGTCGACCTAAAAGCTCAACTGCTTCCCCAG-3'
3	1-68	Reverse	5'-CGTCGACCTATTCCTCAGACAGTTCTTCACTG-3'
5	1-32	Reverse	5'-CGTCGACCTATTCCTTCTCAGTCATTCCATCC
6	1-16	Reverse	5'-CGTCGACCTATTTCCTGACATCAGCAGGCAG-3'
7	17-32	Forward	5'-GGAATTCCGGCTAAAGGATTTGGAGAGG-3'
9	85-142	Forward	5'-GGAATTCGAAAACGTAGACCTTGCTTTG-3'
10	85-120	Reverse	5'-CGTCGACCTAACCAGAAGTATTAGACTCCGA-3'
12	121-142	Forward	5'-GGAATTCGTTAAAAACCGCAAACCTAGG-3'

The forward primers for #2, 3, 5, and 6 were same as that for #1, and that for #10 the same as for #9. The reverse primer for #7 was the same as that for #5, and those for #9 and #12 the same as for #1.

TABLE II. **Oligonucleotides used to construct GST fusion proteins.** Chemically synthesized complementary oligonucleotides encoding the amino-terminal region of xDnmt1 are shown. The names of the constructs (#), the corresponding amino acids numbers (Coding seq.), and the synthesized complementary sequences are summarized. Both ends of each cDNA were designed to create *Eco*RI and *Sal*I sites for subcloning into the expression vectors.

#	Coding seq		
4	69–85	5'	-AATTCGGATACCTTACAAAAGTGAAGTCTCTTCTGGGGAAGCAGTTGAGCTTTGAATAGG-3'
		3'	-GCCTATGGAATGTTTTCACTTCAGAGAAGACCCCTTCGTCAACTCGAAACTTATCCAGCT-5'
8	33–51	5'	-AATTCCATGTACAGCAGAAACTGAGTTTGGTGCTTGGCTTCCTTGAAGCAGATGCTAGATAGG-3'
		3'	-GGTACATGTCGTCTTTGACTCAAACCACGAACCGAAGGAACTTCGTCTACGATCTATCCAGCT-5'
11	115–126	5'	-AATTCGAGTCTAATACTTCTGGTGTTAAAAACCGCAAACCTTAGG-3'
		3'	-GCTCAGATTATGAAGACCACAATTTTTGGCGTTTGGAATCCAGCT-5'
13	12–21	5'	-AATTCGCTGATGTCAGAAAACGGCTAAAGGATTTGGAGTAGG-3'
		3'	-GCGACTACAGTCTTTTGCCGATTTCCTAAACCTCATCCAGCT-5'
14	17–24	5'	-AATTCCGGCTAAAGGATTTGGAGAGGGATTAGG-3'
		3'	-GGCCGATTTCCTAAACCTCTCCCTAATCCAGCT-5'
15	21–28	5'	-AATTCTTGGAGAGGGATCAGGATGGAATGTAGG-3'
		3'	-GAACCTCTCCCTAGTCCTACCTTACATCCAGCT-5'
16	25–32	5'	-AATTCCAGGATGGAATGACTGAGAAGGAATGG-3'
		3'	-GGTCCTACCTTACTGACTCTTCCTTACCAGCT-5'
17	26–36	5'	-AATTCGATGGAATGACTGAGAAGGAACATGTACAGCAGTAGG-3'
		3'	-GCTACCTTACTGACTCTTCCTTGTACATGTCGTCATCCAGCT-5'
18	121–131	5'	-AATTCGTTAAAAACCGCAAACCTAGGAAAAGTAAAGTTTAGG-3'
		3'	-GCAATTTTTGGCGTTTGGATCCTTTTCATTTCAAATCCAGCT-5'
19	126–136	5'	-AATTCCCTAGGAAAAGTAAAGTTAATGGAGAAAACAAGTAGG-3'
		3'	-GGGATCCTTTTCATTTCAATTACCTCTTTTGTTCATCCAGCT-5'
20	132–142	5'	-AATTCAATGGAGAAAACAAGAAATCACCAGCCCGTGCCTAGG-3'
		3'	-GTTACCTCTTTTGTTCTTTAGTGGTCGGGCACGGATCCAGCT-5'

using a Hi-trap protein G column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The isotype of each monoclonal antibody was determined with a mouse screening isotype kit (Zymed, CA).

Polyclonal antibodies against xDnmt1 (389–1490) antibodies were prepared as described (*27*). The antibodies were affinity purified with antigen-coupled Sepharose CL-4B.

Western Blotting—The full-length and fusion proteins of xDnmt1 were separated by SDS–polyacrylamide-gel electrophoresis (PAGE) in 7.5 and 12.5% polyacrylamide gels, respectively (*32*). Electro-blotting onto nitrocellulose membranes (Schleicher & Schuell, NH), blocking, and immunostaining were performed basically as described previously (*33*). The blotted membranes were incubated with 1/1,000 diluted anti-xDnmt1 monoclonal antibodies or anti-xDnmt1 polyclonal antibodies in PBS containing 1% BSA and 0.1% (w/v) Triton X-100. The xDnmt1 band was detected by means of alkaline phosphatase-coupled second antibodies (E.Y. Laboratories, CA).

Preparation of Cell Extracts—For the preparation of cell extracts of A6 cells, cells at the proliferating stage were homogenized with a Potter homogenizer in 5 volumes of ice-cold 50 mM Tris-HCl (pH 7.6) buffer containing 1 M sucrose, 3 mM $MgCl_2$, 1 mM EDTA, 0.3 M NaCl, 1 mM DTT, and 1/50 (v/v) Protease Inhibitor Cocktail (Nacalai Tesque). The homogenate was ultracentrifuged at 400,000 ×*g* for 20 min at 4°C, and the supernatant fraction was used.

To prepare egg extracts, mature eggs were homogenized in 3 volumes of 50 mM Tris-HCl (pH 6.8) buffer containing 1/50 (v/v) Protease Inhibitor Cocktail with a Potter homogenizer. The homogenate was centrifuged at 12,000 ×*g* for 10 min at 4°C, and the cytoplasmic fraction was used (*34*).

Peptide Synthesis and Purification—Polypeptides of the amino terminal (1–32) and (115–126) sequence of xDnmt1 were synthesized on Fmoc-SAL-resin in a ACT440Ω peptide synthesizer (Advanced Chemtech, KY). The synthesized peptides were purified on a reverse phase YMC-Pack ODS-AM column (YMC, Kyoto). The purity and mass number of the peptides were confirmed by MALDI-TOF MS using Voyger-^{TM}DE (Perseptive Biosystems, MA).

Immunoprecipitation—The monoclonal antibodies were coupled to CNBr-activated Sepharose CL-4B (Amersham Pharmacia Biotech) to obtain an affinity matrix (5 mg IgG/ml matrix). For the immuno-precipitation of xDnmt1, the IgG-matrix (20 μl) was incubated for 3 h at 4°C with either cell extracts prepared from 1×10^8 cells or egg ex-tracts prepared from about 600 mature eggs, with or without a 500 molar excess of synthesized peptides. The matrix was centrifuged at 2,000 ×*g* for 2 min at 4°C and then washed four times with 50 mM Tris-HCl (pH 7.6) containing 0.1% (w/v) Triton X-100 and 0.3 M NaCl. The unbound, wash, and bound fractions were examined by Western blotting.

For the elution of xDnmt1, the matrix was washed as described above, and then incubated with 1 mg/ml of synthesized peptides in 50 mM Tris-HCl (pH 7.2), 0.3 M NaCl, and 0.1% (w/v) Triton X-100. The elution was repeated three times.

DNA Methyltransferase Activities—The amount of xDnmt1 protein eluted from the immuno-complex with the synthesized polypeptide (115–126) was determined with an image analyzer MCID (Imaging Research, Canada) after immuno-detection. The purified recombinant xDnmt1, generously provided by Mr. D. Hayata (Institute for Protein Research, Osaka University), was used as a standard. The reaction mixture contained 5 ng of immuno-purified xDnmt1, 0.1 μg of poly(dI-dC)-poly(dI-dC) or poly(dG-dC)-poly(dG-dC) (Amersham Pharmacia Biotech), and 2 μCi of [^{3}H]SAM (15.0 Ci/mmol; Amersham Pharmacia Biotech, UK) in a volume of 25 μl reaction buffer, and then the radioactivity of ^{3}H-labeled DNA was measured as described (*33*), except that after the reaction, the mixtures were supplemented with 10 μg of proteinase K (Nacalai Tesque) Kyoto) and incubated at 50°C for 10 min in the presence of 0.5% SDS.

RESULTS

Alignment of Xenopus Dnmt1 with Mouse and Human Dnmt1—The exon-intron boundary of xDnmt1 in the 5′ region was estimated by alignment of xDnmt1 cDNA nucleotide sequences with those of mouse Dnmt1 (mDnmt1) (*35*). This estimation could be supported by the homology alignment of amino acid sequences with mDnmt1 and human Dnmt1 (hDnmt1). There are three Met residues in the first 160 amino acid sequence in xDnmt1 (Fig. 1B). If the first Met is in exon1, the second and third ones are expected to be in putative exon2 and exon5, respectively, of xDnmt1 (Fig. 1). In mouse oocytes, it has been proven that the transcription starts from oocyte-specific exon1 and that the codon of Met in exon4 is utilized as the initiation ATG. This isoform lacking 118 amino acid residues is dominant until the blastocyst stage, and plays a crucial role in the maintenance of methylation imprinting (*28*). Interestingly, the amino-terminal sequence of mouse Dnmt1 lacking in the oocyte-type is highly homologous to that of xDnmt1 (Fig. 1B). In xDnmt1, the corresponding Met residue in exon4 of *mDnmt1* is absent, and two Met residues other than the first one are present in putative exon2 and exon5. The Met in exon2 matches Kozak's rule, but that in exon5 does not.

Preparation of Monoclonal Antibodies—To address the question of whether or not *Xenopus* oocytes express a similar Dnmt1 isoform to that in mouse oocytes that lacks the amino-terminal sequence, we raised monoclonal antibodies against the amino-terminal segment of xDnmt1. The amino-terminal segment coding (1–142) of xDnmt1 was expressed as a GST fusion protein (Fig. 2A, lane 1). The fusion protein was cleaved with thrombin, and the xDnmt1 fragment was separated from GST (Fig. 2A, lane 2) and utilized for immunization. Four clones producing antibodies, 3C6, 4A8, 5A8, and 5C9, were isolated. The antibodies produced by these four clones specifically immuno-reacted with xDnmt1 prepared from *Xenopus* A6 cells (Fig. 2B, lanes 2–5), and the mobilities of the immuno-reacted bands were identical to that in the case of anti-xDnmt1 polyclonal antibodies raised against (389–1490) of xDnmt1 (Fig. 2B, lane 6) (*27*). The result that monoclonal antibodies raised against (1–142) immuno-reacted with the xDnmt1 in A6 cells, which gave a single band with the polyclonal antibodies, clearly indicated that xDnmt1 expressed in A6 cells was not utilizing the Met158 in exon5 as an initiation site.

Epitope Mapping of the Monoclonal Antibodies—All four monoclonal antibodies were determined to consist of IgG_1 heavy and κ light chains. To map the epitopes of the mono-

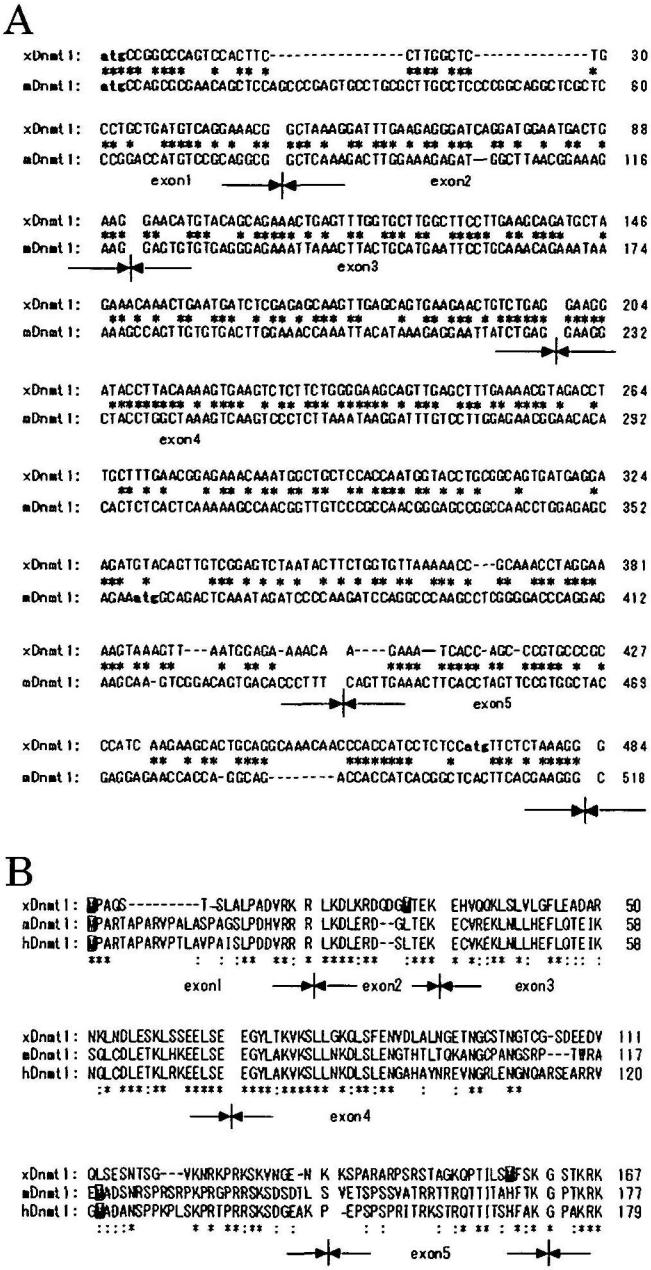

Fig. 1. **Alignment of xDnmt1 with mDnmt1 and hDnmt1.** The about 500 nucleotide sequences of xDnmt1 and mDnmt1 cDNAs starting from initiation codon (ATG) were aligned (A). The exon boundaries of mDnmt1 (*35*) and the putative exon boundaries of xDnmt1 are indicated by arrows. The codons for Met residues are indicated in lower case letters (atg). Asterisks (*) between two sequences show identical nucleotides. The numbers on the right of the panel are the nucleotide numbers. The about 180 amino acid sequences from the initiation Met of the reported somatic-type xDnmt1, mDnmt1, and hDnmt1 were aligned with the program described (*40*) (B). The corresponding sites for exon boundaries are indicated by arrows as in panel A. The Met residues are boxed. Asterisks (*) and colons (:) under amino acid residues indicate identical and similar [(i) Q, R, K, E, D, and N, (ii) S, T, and A, and (iii) M, L, V, F, and I] amino acid residues, respectively. The numbers on the right of the panel are the amino acid sequence numbers.

clonal antibodies, we constructed a series of GST-fused polypeptides (Fig. 3A). An equal amount of each fusion

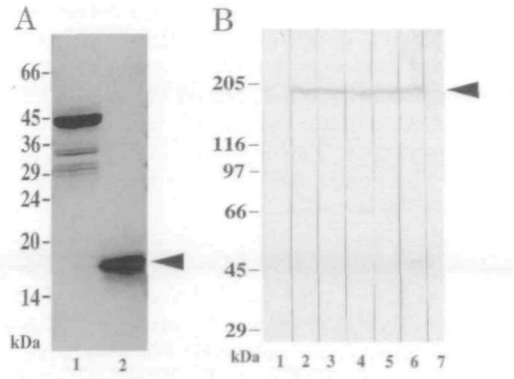

Fig. 2. **Preparation of the antigen and the specificity of the isolated monoclonal antibodies.** Amino terminal (1–142) was expressed as a GST fusion protein and purified on a glutathione-Sepharose column (A, lane 1). The purified protein was cleaved with thrombin and then further purified on a MonoQ column (lane 2). The arrowhead indicates amino terminal (1–142) fragment of xDnmt1. Whole cell extracts of A6 cells were subjected to SDS-PAGE and then blotted onto a nitrocellulose membrane (B). Strips of the membrane were reacted with anti-xDnmt1 monoclonal antibody 4A8 (lane 2), 3C6 (lane 3), 5A8 (lane 4), 5C9 (lane 5), or polyclonal antibodies raised against (389–1490) xDnmt1 (lane 6). Membrane strips without first antibodies are also shown (lanes 1 and 7). The bands were detected with alkaline phosphatase-conjugated antibodies specific for mouse (lanes 1–5) or rabbit (lanes 6 and 7) IgG. Molecular weight standards are indicated.

fragment purified on glutathione-Sepharose was subjected to SDS-PAGE and immuno-reacted with the four monoclonal antibodies (Fig. 3B). 4A8 immuno-reacted with #1, #9, and #11, but not with the others. Thus, its recognizing sequence was identified as (115–126) of xDnmt1. Monoclonal antibody 3C6 specifically bound to #1, #2, #3, and #5, but not with the others. The epitope of 3C6 was in sequence (1–32). Since none of the sub-regions of (1–32), such as #6, 7, and 16–20, immuno-reacted with 3C6, we could not restrict the antigenic site to a shorter sequence. The other two monoclonal antibodies, 5A8 and 5C9, immuno-reacted with two distinct peptides in the amino terminal region, #5 and #9 for 5C9, and #5 and #11 for 5A8, respectively.

To confirm the epitopes of the monoclonal antibodies, we examined the effect of the chemically synthesized polypeptides, (1–32) and (115–126), which corresponded to the identified antigenic sequences for the immuno-reactivity of 3C6 and 4A8, respectively. Whole cell extracts of A6 cells were immuno-precipitated with the monoclonal antibody-coupled Sepharose in the presence or absence of either the synthesized polypeptide of (1–32) or (115–126) to examine whether the polypeptides interfered with the interaction of antibodies and xDnmt1 or not. In the absence of a competitor polypeptide, all four monoclonal antibodies completely immuno-precipitated the xDnmt1 (Fig. 4, A–D, lane 1). When xDnmt1 was immuno-precipitated with 4A8 in the presence of polypeptide (115–126), about 70% of the xDnmt1 was recovered in the unbound fraction (Fig. 4A, lanes 8–10). While polypeptide (1–32) did not affect the recovery of xDnmt1 in the bound fraction (Fig. 4A, lanes 5–7). On the other hand, the immuno-precipitation of xDnmt1 was considerably inhibited by polypeptide (1–32) but not by polypeptide (115–126) for 3C6, 5A8, or 5C9 (Fig. 4, B–D). In addition, with all monoclonal antibodies, no additive inhibi-

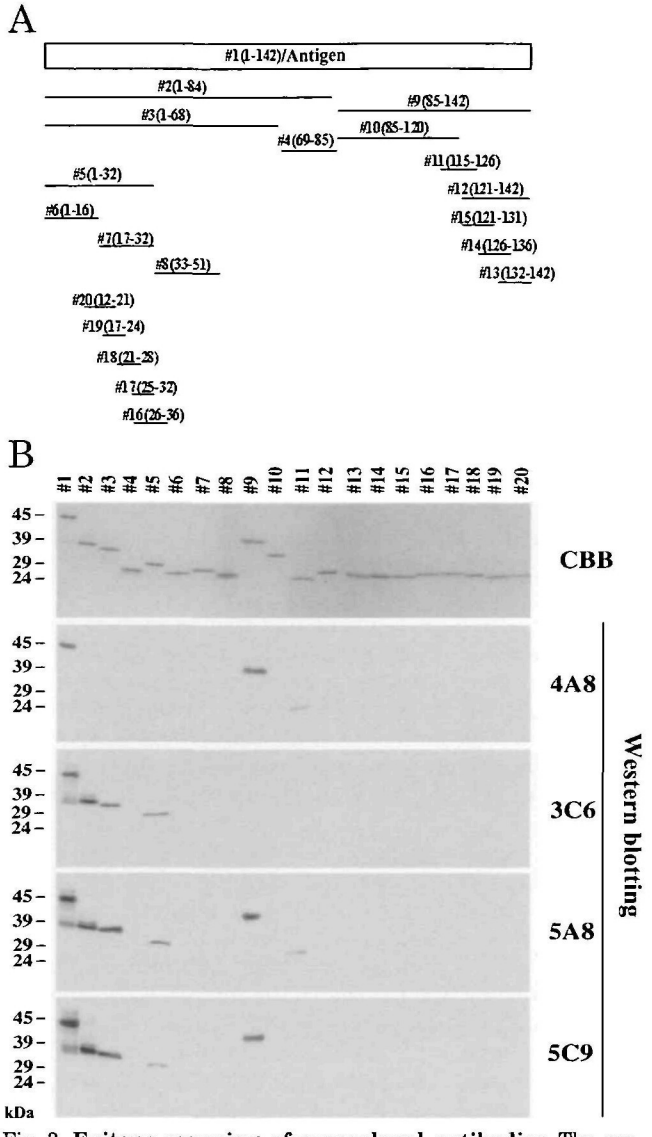

Fig. 3. **Epitope mapping of monoclonal antibodies.** The construction of GST-fused polypeptides is illustrated (A). The numbers of the constructs (#1–#20) and their encoding sequences corresponding to xDnmt1 in parentheses are shown. The immuno-reactivity of the antibodies was detected by Western blotting (B). The GST-fused polypeptides (0.2 μg) expressed from the constructs shown in panel A were electrophoresed and stained with CBB, or immunoblotted with each monoclonal antibody. Molecular weight standards are indicated.

tory effect on the xDnmt1 binding was observed in the presence of both polypeptides (1–32) and (115–126) (Fig. 4, A–D, lanes 11–13). This demonstrated that the ability to recognize epitope (1–32) of 5A8 and 5C9 was strong enough

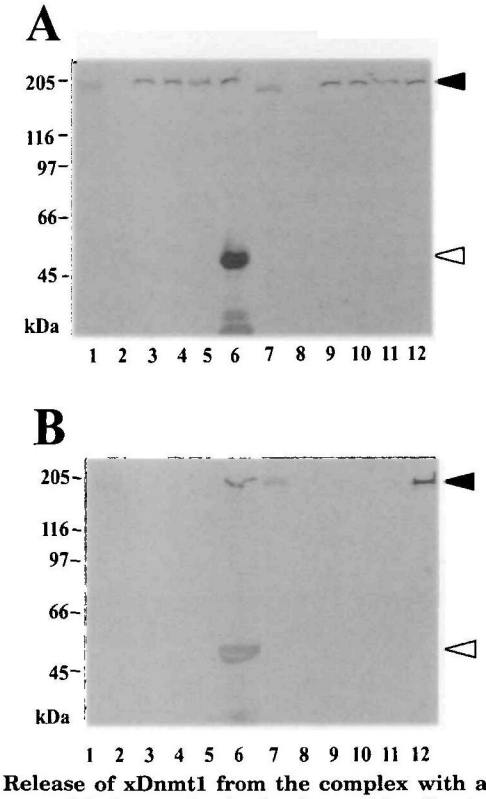

Fig. 5. **Release of xDnmt1 from the complex with antibody-Sepharose with the synthesized polypeptides.** One tenth of nuclear extracts (input) of the sample used for the immunoprecipitation (lanes 1 and 7), the unbound fractions (lanes 2 and 8), the fractions obtained on three sequential elutions with 1 mg/ml each of polypeptide (115–126) (lanes 3 and 9 for the first, lanes 4 and 10 for the second, and lanes 5 and 11 for the third elution), and the remaining fraction on the matrix (lanes 6 and 12) were subjected to SDS-PAGE, and xDnmt1 was immuno-blotted. The membranes were immuno-detected with 4A8 (panel A, lanes 1–6), 3C6 (panel B, lanes 1–6), and anti-xDnmt1 polyclonal antibodies (panels A and B, lanes 7–12). The closed and open arrowheads indicate xDnmt1 and the immunoglobulin heavy chain, respectively. Molecular weight standards are also indicated at the left of the panel.

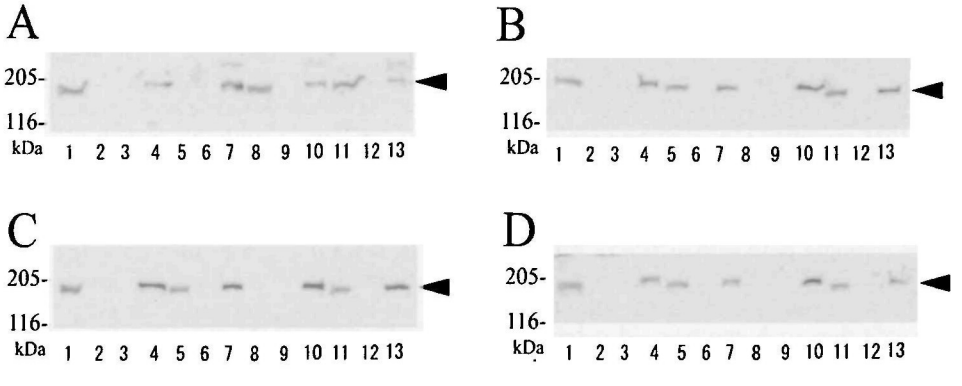

Fig. 4. **Inhibition of the binding of monoclonal antibodies to xDnmt1 with synthesized polypeptides.** Whole cell extracts prepared from A6 cells were immunoprecipitated without (lanes 2–4), or with chemically synthesized polypeptides (1–32) (lanes 5–7), (115–126) (lanes 8–10), or both (1–32 and 115–126) (500 molar excess of the polypeptide of coupled IgG). In lane 1 in each panel, one half of the extract used for immuno-precipitation was used. The unbound (lanes 2, 5, 8, and 11), washed (lanes 3, 6, 9, and 12), and bound (lanes 4, 7, 10, and 13) fractions were subjected to SDS-PAGE. xDnmt1 was immuno-detected with monoclonal antibody 4A8 (A), 3C6 (B), 5A8 (C), or 5C9 (D), respectively. The arrowheads indicate the positions of xDnmt1. Molecular weight standards are also indicated.

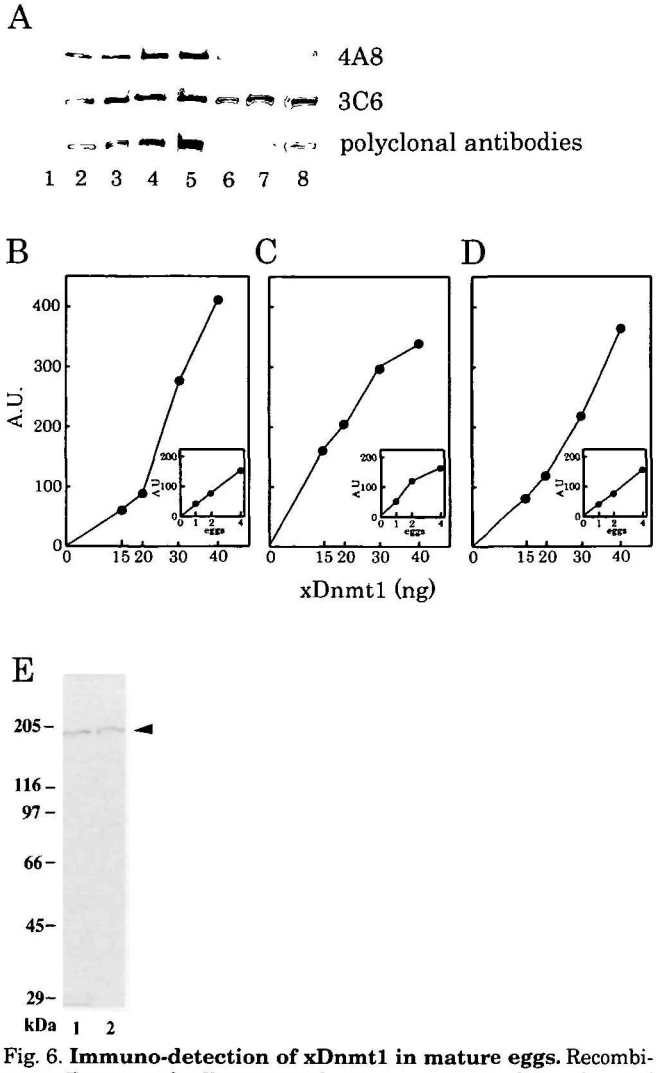

Fig. 6. **Immuno-detection of xDnmt1 in mature eggs.** Recombinant xDnmt1 and cell extracts of mature eggs were electrophoresed in the same gel, and xDnmt1 protein bands were determined. Recombinant xDnmt1, 0 (lane 1), 15 (lane 2), 20 (lane 3), 30 (lane 4), and 40 ng (lane 5), and cell extracts of mature eggs prepared from 1 (lane 6), 2 (lane 7), and 4 eggs (lane 8) were used. xDnmt1 was immuno-detected with 3C6, 4A8, and polyclonal antibodies (A). The areas of xDnmt bands were densitometrically determined (Density, A.U.; arbitrary units), and plotted against the content of recombinant xDnmt1 for 4A8 (B), 3C6 (C), and polyclonal antibodies (D). From these standard curves the amounts of xDnmt1 in mature eggs were determined (B–D, insets). The extracts of mature eggs (lane 1) and A6 cells (lane 2) were immuno-blotted with anti-xDnmt1 monoclonal antibody 3C6 (E). Arrowheads indicate the bands of xDnmt1. Molecular weight standards are indicated.

and that epitope (115–126) was too weak to pull-down the xDnmt1.

We next examined if synthesized polypeptide (1–32) or (115–126) could release xDnmt1 from the immuno-precipitated complex with either 3C6 or 4A8, respectively. A total of about 70% of the xDnmt1 bound to the 4A8-coupled matrix was released from the immuno-complex on three-times elution with synthesized polypeptide (115–126) (Fig. 5A). On the other hand, synthesized polypeptide (1–32) could not release xDnmt1 from the immuno-complex with 3C6 (Fig. 5B).

TABLE III. **Specific activities of the immuno-purified xDnmt1.** The specific activities of purified Dnmt1, with poly(dIdC)-poly(dIdC) and poly(dGdC)-(dGdC) as methyl acceptors, were determined. The xDnmt1 was immuno-purified from mature eggs and A6 cells, using 4A8-coupled Sepharose.

Enzyme source	Methyl acceptor	
	poly(dI-dC)-poly(dI-dC)	poly(dG-dC)-poly(dG-dC)
xDnmt1 from eggs	202 ± 32.2[1] (4)[2]	15.9 ± 6.6 (4)
xDnmt1 from A6 cells	170 ± 12.1 (4)	14.7 ± 1.4 (4)
mDnmt1[3]	188 ± 24.7 (3)	15.7 ± 3.3 (3)

[1]The activities (nmol/h/mg protein) are shown as means ± standard deviation. [2]The numbers in parentheses are the numbers of measurements performed using different preparations. [3]Recombinant mDnmt1 was prepared from recombinant baculovirus-infected insect cells, and was provided by Mr. D. Hayata (Institute for Protein Research, Osaka University).

xDnmt1 in Mature Eggs Was Mainly Translated from the Identical Met to the Somatic-Type—Using two monoclonal antibodies, 3C6 and 4A8, we next addressed the question of whether or not the xDnmt1 in mature eggs lacks the amino-terminal short segment like that in mature mouse eggs. There are two Met residues, at 28 and 158, after the expected initiation Met1, as shown in Fig. 1. Both 3C6 and 4A8 should not recognize the xDnmt1 starting at Met 158, since the molecule does not contain the epitope. In addition, 3C6, which recognized (1–32), is expected not to react with the xDnmt1 starting at Met 28, since sequence (29–32) composed of four amino acids (the initiation Met being omitted) is too short to act as an antibody recognition site.

Using these two monoclonal antibodies and polyclonal antibodies recognizing (389–1490) of xDnmt1, we semi-quantified the xDnmt1 in eggs. Firstly, 3C6 clearly recognized the xDnmt1 in mature eggs, indicating that the xDnmt1 utilizing Met1 exists in the mature eggs, which is identical to the isoform expressed in A6 cells (see Fig. 1B). The xDnmt1 content was determined to be about 10.0 ± 2.5, 8.0 ± 0.8, and 8.2 ± 0.2 ng per egg with 3C6, 4A8, and polyclonal antibodies, respectively (Fig. 6, A–D). This clearly indicates that in *Xenopus* oocytes, xDnmt1 utilizes an identical initiation Met1, which is expected to be in exon1, to that in A6 cells, somatic cells. In addition, as shown in Fig. 6E, the mobilities of xDnmt1 extracted from A6 cells and mature eggs were identical, indicating that no isoforms produced from a large deletion or insertion exist in mature eggs.

DNA Methylation Activity of xDnmt1 in Mature Eggs—In the previous study, we found that the specific activity of xDnmt1 is almost identical to that of mDnmt1 in MEL cells, using a crude extract (27). To see whether or not the specific activity of xDnmt1 in mature eggs is comparable to that in somatic cells, we measured the DNA methylation activity of the immuno-purified xDnmt1 from both mature eggs and A6 cells. The specific activities of xDnmt1 from mature eggs and A6 cells were almost identical with either poly(dIdC)-poly(dIdC) or poly(dGdC)-poly(dGdC) as a methyl acceptor (Table III). The activity of xDnmt1 prepared from mature eggs was similar to that of the recombinant His-tagged mDnmt1. The xDnmt1 activity for poly(dIdC)-poly(dIdC) was about twelvefold higher than that for poly(dGdC)-poly(dGdC), which is again similar to that for mDnmt1 (Table III).

DISCUSSION

In mouse oocytes and mature eggs, a distinct exon1 is utilized for the transcription of Dnmt1 (*26*), which is 118 amino acid residues shorter than that expressed in somatic cells. This short form is the major Dnmt1 in early embryogenesis and plays a crucial role in maintaining the methylation state of imprinted genes (*28*). In mouse oocytes, interestingly, Dnmt1 is excluded from germinal vesicles (nuclei). Different from mouse, *Xenopus laevis* may not have genomic imprinting, which has only been reported for mammals, and Dnmt1 is positively translocated into germinal vesicles in oocytes (*27*). In the present study, we aimed to determine if *Xenopus* has the oocyte-type, *i.e.* a short isoform of Dnmt1 in mature eggs. To address this question, we isolated four hybridoma cell lines producing monoclonal antibodies against the amino-terminal region of xDnmt1.

Based on the homology around the sequence of Met1 (ATG) (Fig. 1, A and B), and that monoclonal antibody 3C6, which recognizes (1–32) of xDnmt1, immuno-detected an identical sized xDnmt1 in A6 cells to that by other monoclonal and polyclonal antibodies with similar intensity (Fig. 2B), we concluded that the cDNA reported (*27*) is the somatic-type.

If the first exon distinct from that of somatic-type xDnmt1 was utilized in oocytes and changed the amino terminal sequences, two possibilities exist; utilization of the initiation Met occurring in the unknown oocyte-specific exon or the downstream Met after exon2. In the former case, the amino-terminal sequence is expected to be different from the reported one, and thus monoclonal antibody 3C6 may not cross react with such a xDnmt1. As for the latter case, Met28 in putative exon2 or Met158 in putative exon5 may be utilized. As described, Met158 may not be used since neither 3C6 nor 4A8, of which the epitope was within (1–142), immuno-reacted with xDnmt1 in mature eggs (Fig. 6), and the mobility of the band of immuno-detected xDnmt1 in mature eggs on SDS-PAGE was identical to that for A6 cells (Fig. 6E).

Considering the nucleotide sequence around the ATG (Met) in exon2 satisfies Kozak's rule, this Met28 was the most possible candidate for the alternative initiation Met. However, the following results do not indicate that Met28 was utilized as the initiation methionine. 3C6 immuno-reacted with xDnmt1 in mature eggs and the amount of xDnmt1 determined with 3C6 in mature eggs was almost identical to that determined with 4A8 or polyclonal antibodies against (389–1490) (Fig. 6). The supposed oocyte-type xDnmt1 starting at Met28 contains only a four amino acid residue-sequence of the antigenic sequence for 3C6, which is too short to recognize, at least in quantitative manner. Thus we concluded that *Xenopus* eggs express the somatic-type xDnmt1, which is distinct from that in mouse eggs (*26*). But, this result does not mean that in *Xenopus*, the transcriptional regulation is the same in oocytes and somatic cells. *Xenopus* may utilize a specific promoter and/or enhancer other than that in somatic cells for the high expression of xDnmt1 in mature oocytes (*27*).

The reason for the difference in the choosing mechanism for the amino-terminal sequence between mouse and *Xenopus* oocytes is open to question. But it is interesting that in mouse oocytes, Dnmt1 was positively excluded from germinal vesicles (*16, 26*), and this localization was maintained basically until the blastocyst stage except for a short period of time at the 8 cell-stage (*26, 28*). Concomitant with this, genome-wide demethylation occurs during early embryogenesis (*36, 37*). The translocation of the oocyte-type mDnmt1 into nuclei at the 8-cell stage was proposed to be important for maintaining the methylation imprinting (*28*). On the contrary, in *Xenopus*, about half the xDnmt1 is translocated into germinal vesicles in mature oocytes (*27*), genome-wide demethylation is not detected during the early stage of embryogenesis (*38, 39*), and a short oocyte-type does not exist, as shown in the present study.

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