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Xenopus Maintenance-Type DNA Methyltransferase Is Accumulated and Translocated into Germinal Vesicles of Oocytes[1]

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In vertebrates, DNA methylation plays an important role in the regulation of gene expression and embryogenesis. DNA methyltransferase, which catalyzes the introduction of a methyl group at the 5th position of cytosine in the CpG sequence, is highly accumulated in mouse oocytes and is excluded from nuclei [Carlson *et al.* (1992) *Genes Dev.* 6, 2536-2541]. In this study, we examined the expression level and localization of *Xenopus* DNA methyltransferase in oocytes during oogenesis. The DNA methyltransferase protein was detectable in stage III oocytes and increased thereafter, until the oocytes had matured. The rate of DNA methyltransferase synthesis rapidly increased after stage IV oocytes. Different from in mouse oocytes, DNA methyltransferase was equally distributed in the nuclear and post-nuclear fractions, in stage VI oocytes. DNA methyltransferase translocated into nuclei was uniformly localized in the nuclear matrix, and the accumulated DNA methyltransferase in stage VI nuclei had DNA methylation activity.

Key words: DNA methyltransferase, nuclear localization, oogenesis, *Xenopus laevis*.

In vertebrates, the 5th positions of the cytosine residues in the CpG sequences in genomic DNA are often methylated (*1*). Dynamic regulation of DNA methylation is known to contribute to physiological phenomena (*2*), such as tissue-specific gene expression (*3*), genomic imprinting (*4*), X chromosome inactivation (*5*), and carcinogenesis (*6*). In vertebrates, two types of DNA methyltransferase activities have been reported, *i.e. de novo*- and maintenance-type DNA methyltransferase activities. In mouse, *de novo*-type DNA methyltransferase activity contributes to the establishment of tissue-specific methylation patterns at the implantation stage of embryogenesis (*7*), and maintenance-type methyltransferase activity ensures clonal transmission of lineage-specific methylation patterns in somatic cells (*8*). The latter type of DNA methyltransferase, designated as Dnmt1, has been characterized (*2*). Recently, putative *de novo*-type DNA methyltransferases, Dnmt2 (*9, 10*) and Dnmt3 (*11*), were reported, but their physiological significance has not been determined yet. When *Dnmt1* is destroyed in mouse, the homozygous mutant embryos cannot survive past midgestation (*12*), their genomic imprinting being canceled (*12*). Embryonic stem cells harboring a homozygous mutation of *Dnmt1* are able to proliferate. However, when the cells are induced to differentiate, they cannot survive (*12*). Dnmt1 activity is indispensable for the differentiation of embryonic stem cells, as well as for the maintenance of genomic imprinting in somatic cells.

Previously, various Dnmt1 cDNAs have been isolated from mouse (*13-15*), man (*15, 16*), chick (*17*), *Xenopus* (*18*), and sea urchin (*19*). The predicted amino acid sequences of these Dnmt1s are highly homologous to each other. Dnmt1 possesses a carboxyl-terminal domain closely related to those of bacterial DNA-(cytosine-5) methyltransferases and a large amino-terminal domain that has multiple regulatory functions (*20, 21*).

In mouse somatic cells, Dnmt1 is localized in nuclei and specifically accumulates at replication foci in late S-phase nuclei (*22, 23*). Different from in somatic cells, the expression and localization of Dnmt1 are specifically regulated in germlines. During spermatogenesis in mouse, transcription starts from the testis-specific site at the pachytene spermatocyte stage. The transcript does not produce functional Dnmt1 (*24, 25*). In growing oocytes, Dnmt1 is first localized in the nucleus. As oogenesis proceeds, Dnmt1 is accumulated in the cytoplasm, and at ovulation Dnmt1 is excluded from nuclei (germinal vesicles) (*25*). At the one- and two-cell stages, Dnmt1 is in the cytoplasm near the plasma membrane, and only after the 8-cell stage it is translocated into nuclei (*26*). The amount of accumulated Dnmt1 in mouse mature oocytes and one-cell embryos is 3,000 times higher than that in murine erythroleukemia (MEL) cells on a per cell basis (*26*). During oogenesis and early development, the transcription starts from a specific site in the mouse *Dnmt1* gene, of which the mRNA encodes

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Abbreviations: BSA, bovine serum albumin; Dnmt, DNA methyltransferase; FCS, fetal calf serum; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; MBP, maltose binding protein; MBT, midblastula transition; MEL, murine erythroleukemia; PBS, Dulbecco's phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride; SAM, *S*-adenosyl-L-methionine; XDnmt1, *Xenopus* Dnmt1.

an oocyte-specific Dnmt1 lacking 121 amino acids from its amino-terminus of the somatic-type (*25*). Similar to in mouse, *Xenopus* Dnmt1 (XDnmt1) accumulates in oocytes during oogenesis (*18*), and DNA methylation activity, possibly due to XDnmt1, has been reported to be higher in whole cell extracts than in nuclei (germinal vesicle) in stage V and VI oocytes (*27*). Since the genomic imprinting phenomenon has not been reported in amphibians, including *Xenopus*, it is reasonable to speculate that the accumulation of Dnmt1 in oocytes may contribute to other important processes in embryogenesis. Therefore, the characterization of XDnmt1 may hopefully shed light on the function of DNA methylation in vertebrates.

In the present study, we have chosen *Xenopus laevis* as a model to examine the expression and distribution of the Dnmt1 protein during oogenesis. *Xenopus* oocytes, which can be classified into the I to VI stages (*28*), have the advantages that they are easy to obtain in large quantities and easy to manipulate under a microscope. The synthesis and accumulation of XDnmt1 sharply increased in late stages of oogenesis. Different from in mouse, in stage VI oocytes, a significant amount of XDnmt1 was translocated into nuclei (germinal vesicles). The results suggest that XDnmt1 in oocytes is differently regulated from that in somatic cells and may play an important role in *Xenopus* early embryogenesis.

MATERIALS AND METHODS

Collection of Oocytes and Isolation of Nuclei (Germinal Vesicles)—Female *X. laevis* were purchased from Kita-Nihon-Doubutsu (Aomori), and kept as described (*29*). Ovaries were obtained surgically from adult females anaesthetized in ice-cold water, and follicle cells were removed either manually with watchmaker's forceps or enzymatically by collagenase (Wako Pure Chemical Industries, Osaka) treatment (*30*). The stages of oocytes were determined according to Dumont (*28*). Nuclei (germinal vesicles) in stage VI oocytes were manually isolated as described (*31*). To obtain mature oocytes, females were injected with 750 units of human chorionic gonadotropin (Sigma Chemical, MO). The laid mature oocytes were dejellied in 2% cysteine-HCl (pH 7.5) (*32*).

Antibodies—An antiserum reactive with XDnmt1 was raised against a glutathione-*S*-transferase (GST) fusion protein containing amino acids 389-1490 of XDnmt1 expressed in *Escherichia coli*. To this end, the 3′ end *Eco*RI fragment of XDnmt1 (*18*) was ligated into pGEX2TH, kindly provided by Dr. H. Maruta (Ludwig Institute for Cancer Research, Melbourne), and expressed in *E. coli* strain NM522 in the presence of isopropyl-β-D-thiogalactopyranoside. The expressed GST fusion protein accumulated in inclusion bodies. Inclusion bodies were purified (*33*) and the GST fusion protein was further purified by electroelution from an SDS-PAGE gel (*34*). The antibodies raised against the protein in a rabbit were immunoselected using antigen-coupled Sepharose CL-4B as an affinity matrix (*35*).

Anti-mouse Dnmt1 antibodies were raised and immunoselected as described (*35*). Anti-rat lamin A/C monoclonal antibody (clone #TiM 92) was kindly provided by Drs. Y. Yoneda and Y. Matsuoka (Osaka University, Suita).

Labeling of Oocyte Proteins—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 0.7×Dulbecco's modified Eagle's MEM, supplemented with 10 mM Hepes-NaOH (pH 7.4) and 10% fetal calf serum (FCS). FCS was extensively dialyzed against Dulbecco's phosphate-buffered saline (PBS).

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 then 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 mixtures were mixed and immunoprecipitated with anti-XDnmt1 antibodies as described (*18*). The immunoprecipitated XDnmt1 protein bands were analyzed by fluorography (*36*) after SDS-PAGE (*37*).

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 (*37*) and then electrophoretically transferred to a nitrocellulose membrane. The XDnmt1 protein bands were immunodetected as described (*18*), with slight modifications. Anti-XDnmt1 antibodies, the primary antibodies, 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 alkaline phosphatase (E.Y. Laboratory, CA) or horseradish peroxidase (HRP) (Dako A/S, Glostrup) coupled second antibodies, and a color reaction buffer containing 0.05 mg/ml indoyl phosphate and 1 mg/ml *p*-nitro blue tetrazolium chloride (*35*), or ECL Western blotting detection reagents (Amersham Pharmacia Biotech UK, Buckinghamshire), 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 then 8 volumes of ice-cold acetone were added, to precipitate proteins. The samples were electrophoresed in a 10% SDS-polyacrylamide gel (*37*). After the proteins had been electrotransferred, the α-tubulin and PCNA bands were immunodetected by ECL using monoclonal anti-α-tubulin antibody, clone # DM1A (Sigma Chemical, MO), and monoclonal anti-PCNA antibody (Transduction Laboratories, KY) as first antibodies, respectively.

Immunocytochemistry—Manually isolated nuclei were fixed in Dent's fixative (*38*) at −20°C overnight and embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo), and then 14 μm cryosections were prepared with HM400 (Microm Laborgeräte GmbH, Germany) at −17°C, and then collected on gelatin (Iwaki Glass, Tokyo)-coated slides.

For immunofluorescent staining, sections were washed three times with PBS containing 0.1% Triton X-100, and then 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-mouse Dnmt1 antibodies as primary antibodies at 4°C overnight. As the titer of anti-mouse Dnmt1 antibodies against XDnmt1 was higher than that of anti-XDnmt1 antibodies, we used the anti-mouse Dnmt1

antibodies for the immunocytochemical studies. Control sections were incubated with either anti-mouse Dnmt1 antibodies pre-absorbed with the fused protein of mouse Dnmt1 with gluthathione *S*-transferase (GST) or maltose binding protein (MBP), or without anti-mouse Dnmt1 antibodies. As for the preparation of the fusion protein of mouse Dnmt1 with MBP, an identical *Bam*HI/*Eco*RI fragment of mouse Dnmt1 to that used to make the GST fusion protein (*35*) was ligated to the pMAL-c2 vector (New England Biolabs, MA). The expressed fusion protein was purified according to the 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, OR) for 2 h at room temperature, washed with PBS containing 0.1% Triton X-100, and then immersed in PBS containing 50% glycerol. For double-staining, ALEXA 488 stained sections were further incubated with anti-rat lamin A/C 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, 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 procedures for blocking and incubation with anti-mouse Dnmt1 antibodies were basically the same as those for the immunofluorescent staining described above. The sections were incubated at room temperature for 2 h with biotin-conjugated anti-rabbit IgG antibodies (Chemicon International) as secondary antibodies. To visualize the localization of the XDnmt1 protein, the sections were incubated at room temperature for 1 h with HRP-conjugated streptavidin (Zymed Laboratories, CA). After washing, color development was performed with diaminobenzidin (Sigma Chemical, MO) (*39*).

An Olympus Epi-fluorescence microscope, BX50, with an Olympus UPlanAPO×10 objective lens was used to examine stained sections. Photographs were taken with an Olympus PM-30 camera using Provia 400 (Fuji Photo Film, Tokyo) for the immunofluorescent and Provia 100 (Fuji Photo Film) for the HRP staining studies. For all the samples the exposure and printing were performed under identical conditions.

DNA Methyltransferase Activity—MEL cells and nuclei prepared from stage VI oocytes were homogenized in a buffer comprising 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) (*35*), and 5 μg/ml E64, and then centrifuged at $4\times10^5\times g$ for 20 min at 4°C, and the resulting supernatant fractions were used as enzyme sources. The reaction mixture contained 0.1 μg of poly (dI•dC)-(dI•dC) (Amersham Pharmacia Biotech UK, Bukinghamshire) and 2 μCi of [^{3}H]-*S*-adenosyl-L-methionine (SAM) (15.0 Ci/mmol; Amersham Pharmacia Biotech-UK) (*40*) in a volume of 25 μl reaction buffer (*35*). Whole cell extracts of 6×10^4 MEL cells and nuclear extracts of 0.25 oocytes were added to single reaction mixtures as enzyme sources. After 1 h incubation at 37°C, the DNA methylation activity was determined as described (*35*), except that after the reaction, the mixtures were supplemented and incubated with 10 μg of DNase-free RNase A at 37°C for 10 min, and then with 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-chlorofolm and then specific radioactivities were determined (*35*). The RNase A and proteinase K treatments dramatically decreased the background radioactivities in the absence of the methyl acceptor, poly (dI•dC)-poly (dI•dC).

RESULTS

The XDnmt1 Protein Is Accumulated in a Late Stage of Oogenesis—In mouse, the amount of Dnmt1 in a single mature oocyte is 3,000 times higher than that in a single murine erythroleukemia (MEL) cell (*26*). In *Xenopus*, oocytes can be classified into stages I to IV, largely according to size (*28*). Overall growth from stage I to stage IV requires at least 8 months. Stage I-III oocytes are less than 500 μm in diameter. Stage V oocytes have a diameter of 1,000-1,200 μm, whereas stage VI oocytes have diameters of greater than 1,200 μm. There are significant metabolic differences between stages IV, V, and VI oocytes (*30*). We

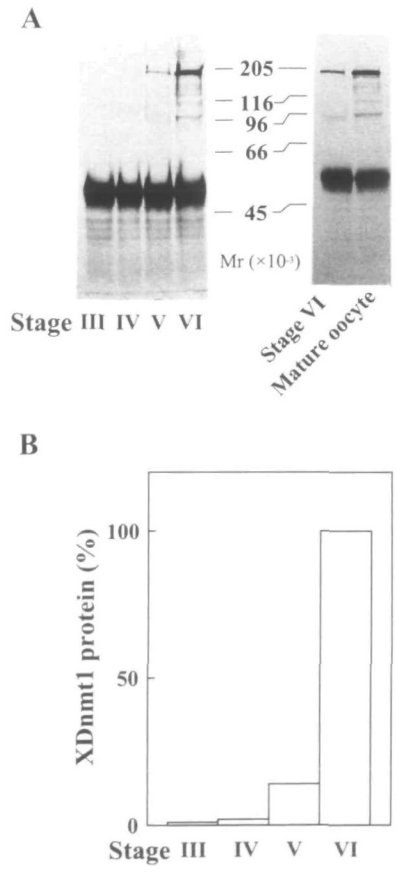

Fig. 1. **Accumulation of XDnmt1 during oogenesis.** A: Extracts prepared from 100 oocytes each of stages III, IV, V, and VI, and mature oocytes were immunoprecipitated with anti-XDnmt1 antibodies and then analyzed by Western blotting using alkaline phosphatase coupled secondary antibodies. The intense bands at around 50 kDa represent the heavy chains of anti-XDnmt1 antibodies. Molecular weight standards (M_r) are indicated. B: Relative XDnmt1 content. The XDnmt1 bands (A) were quantified with an image analyzer, MCID (Imaging Research, Canada). The amount of XDnmt1 in stage VI oocytes was taken as 100%.

determined 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 the apparent molecular weight is about 190 k (*18*), was detected by Western blotting (Fig. 1A)[3]. The XDnmt1 content rapidly increased in parallel with the maturation of oocytes. In our 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. 1B). The amount of XDnmt1 in a single mature oocyte was about 20 times higher than that in a single stage VI oocyte (Fig. 1A, right panel). A stage VI oocyte and a mature oocyte were calculated to contain about 1.2×10^4 and 2.4×10^5 times higher levels of XDnmt1 protein than a single MEL cell, respectively. A *Xenopus* mature oocyte contained a two orders of magnitude higher amount of Dnmt1 than a mouse oocyte (*26*).

To determine whether the accumulation of the XDnmt1 protein during oogenesis is caused by an increase in net synthesis or inhibition of the degradation of XDnmt1, we 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. 2). The incorporation of [^{35}S]amino acids into XDnmt1 increased linearly up to 18 h incubation (Fig. 2B). The 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 amounts 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. 2C). These results clearly indicate that an increase in the XDnmt1 synthesis rate contributed to the accumulation of the protein in late stage oocytes.

Translocation of XDnmt1 into the Nucleus—In mouse, Dnmt1 is localized in the nuclei only at very early stage of the growing oocytes. As oogenesis proceeds, Dnmt1 becomes undetectable in the nucleus and accumulates at a

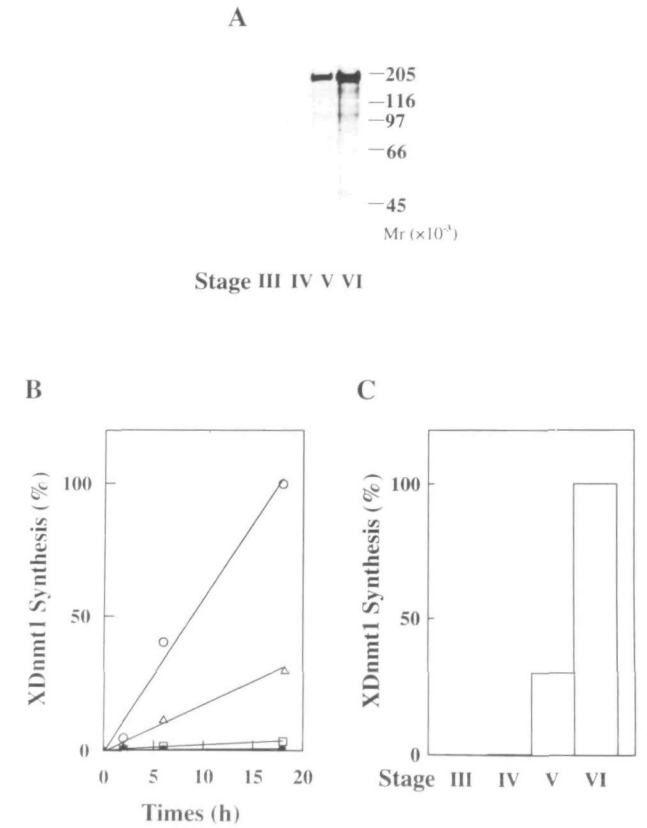

Fig. 2. **Synthesis of XDnmt1 during oogenesis.** A: Ten *Xenopus* oocytes at each stage (III–VI) were incubated with EXPRE^{35}S^{35}S for 18 h, immunoprecipitated, electrophoresed, and then fluorographed. Molecular weight standards (M_r) are indicated. B: Ten *Xenopus* oocytes at each stage were incubated 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 with an image analyzer, BAS2000 (Fuji Photo Film, Tokyo). The radioactivities of XDnmt1 bands were normalized as to that of stage VI oocytes after 18 h incubation. The symbols indicate stage III (●), stage IV (○), stage V (△), and stage VI (□), respectively. C: A histogram 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 with an image analyzer BAS2000 (Fuji Photo Film, Tokyo). The XDnmt1 values were normalized as to that in stage VI oocytes taken as 100%.

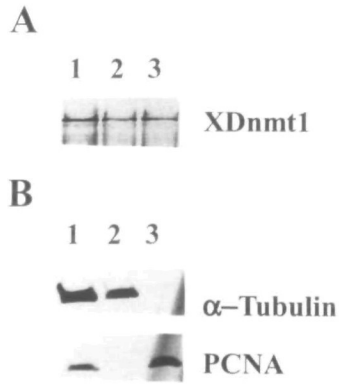

Fig. 3. **Distribution of XDnmt1 in stage VI oocytes.** A: Stage VI oocytes were fractionated into post-nuclear and nuclear fractions, and then the distribution of XDnmt1 was examined. Total extracts of 100 oocytes (lane 1), and the post-nuclear fraction (lane 2) and nuclear fraction (lane 3) were separately 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. Lanes 1–3 contained whole, post-nuclear and nuclear fractions, respectively.

[3] During the course of this study, we found that the 100 kDa protein band observed for stage IV–VI oocytes immunoprecipitated with anti-mouse Dnmt1 antibodies reported in Ref. *18* (Figs. 1A and 2A) did not represent XDnmt1 but a component of *Xenopus* oocytes other than XDnmt1. The 100 kDa protein band resulted from the non-specific binding of anti mouse Dnmt1 antibodies. In addition, the XDnmt1 bands detected for stages I to III oocytes in Ref. *18* were likely to be due to contamination by follicle cells of stage I to III oocytes, since we could not detect these prominent bands for stage III (Fig. 1) and stage I to II oocytes (data not shown), when follicle cells were removed carefully by means of collagenase treatment (Fig. 1).

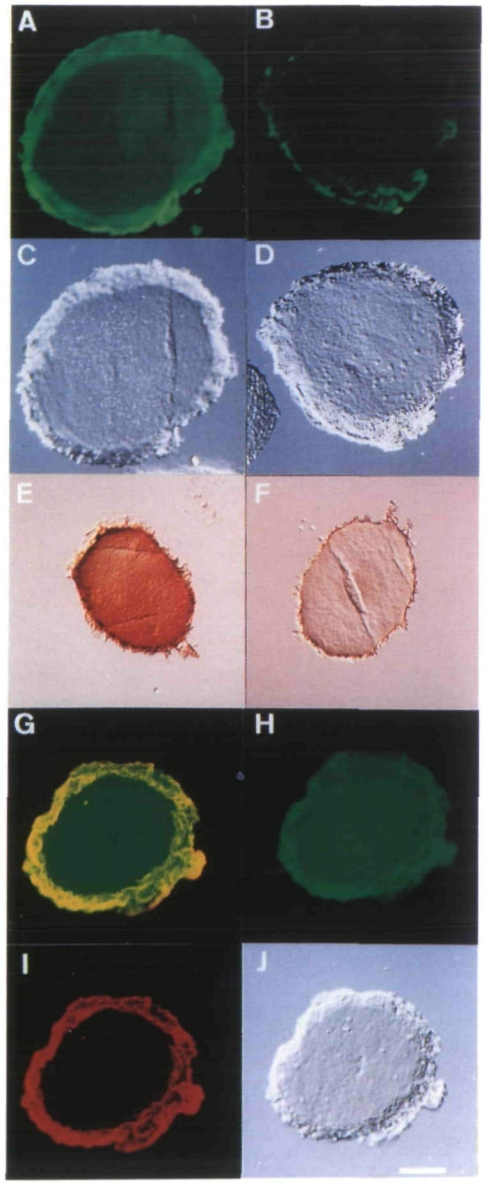

Fig. 4. **XDnmt1 in the nuclei of stage VI oocytes.** The nuclei of stage VI oocytes were fixed in Dent's fixative and then cryosectioned at 14 μm. The sections were reacted with anti mouse Dnmt1 antibodies (A and E) or with the antibodies pre-absorbed with excess antigen against which the antibodies 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). A section was reacted with anti-lamin monoclonal and anti-mouse Dnmt1 antibodies, and then visualized with ALEXA 488 (H), and TRITC (I), respectively. The double-stained image with anti-lamin and anti mouse Dnmt1 antibodies is shown in panel G. The same section was also observed under Nomarski optics (J). Bar, 100 μm.

high level in the cytoplasm (*25*).

To study the localization of XDnmt1 in *Xenopus* oocytes, we 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. 3A). The results indicated that XDnmt1 was clearly localized in the nuclei as well as the post-nuclear fraction (Fig. 3A). α-Tubulin, a cytoplasmic marker (*41*), and PCNA, a nuclear marker (*42*), were properly fractionated into the respective fractions under the conditions used (Fig. 3B).

In mouse oocytes, the Dnmt1 is not detected in the nuclei but accumulated to very high levels in the cytoplasm (*25*). However, our 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 nuclei we next isolated nuclei from stage VI oocytes and then immunostained XDnmt1. Manually isolated nuclei were cryosectioned and then stained with anti-mouse Dnmt1 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. 4, A and E). When anti-mouse Dnmt1 antibodies were pre-absorbed with excess GST-fused antigen, against which the antibodies were raised, the positively stained signals of XDnmt1 disappeared (Fig. 4, B and F). When the antigenic sequence was fused to MBP and then used to pre-absorb the anti-mouse Dnmt1 antibodies, the stained signals again disappeared (data not shown). Therefore, the immunostained signals observed in Fig. 4, A 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. 4, A and E). The double-staining of XDnmt1 (Fig. 4H, green) and lamin, which is known to be localized underneath the nuclear envelope (Fig. 4I, red), showed that the dense signal of XDnmt1 at the periphery of oocytes was completely co-localized with that of nuclear lamin (Fig. 4G, yellow). Although not conclusive, XDnmt1 could be preferentially localized underneath the nuclear envelope.

DNA Methyltransferase Activity in 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 (*43–46*). In unfertilized mouse oocytes, Dnmt1 is accumulated in the cytoplasm and exhibits DNA methyltransferase activity (*26*).

To determine if the uniformly distributed XDnmt1 protein in the nuclei of *Xenopus* stage VI oocytes possesses DNA methyltransferase activity, we isolated nuclei and

TABLE I. **Methyltransferase activities in MEL cells and the nuclei of stage VI oocytes.** Whole cell extracts prepared from MEL cells and nuclear extracts of stage VI oocytes were used to measure DNA methyltransferase activity, with poly(dI·dC)-poly(dI·dC) as the substrate. The activities are expressed as means ± SD.

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

[a]The values were calculated from Figs. 1 and 3A. The stage VI oocytes contained 1.2×10^{4} times as much Dnmt1 protein as that in MEL cells on a per cell basis (Fig. 1), and the nuclei contained about half (Fig. 3A).

determined the activity using poly(dI·dC)-poly(dI·dC) as a substrate (Table I). The DNA methyltransferase activity of nuclei prepared from stage VI oocytes was about 1×10^4 times higher than that of an MEL cell extract on a per cell basis. Since the XDnmt1 protein content in *Xenopus* stage VI oocytes was also about 1.2×10^4 times higher than that in an MEL cell extract on a per cell basis (Fig. 1), the XDnmt1 that accumulated in the nuclei of stage VI oocytes during the course of oogenesis possess equivalent DNA methyltransferase activity to that in MEL cells.

DISCUSSION

In the present study, we utilized *Xenopus laevis* to examine the expression, accumulation, and distribution of XDnmt1 in oocytes. *Xenopus* is a laboratory animal that has been extensively studied, especially as to oogenesis and embryogenesis. *Xenopus* oocytes are advantageous for biochemical studies on Dnmt1; they can be classified into I to VI stages (*28*), are easy to obtain in large quantities, and are easy to manipulate under a microscope. In the previous study, we isolated the cDNA of XDnmt1 (*18*), and showed that the predicted amino acid sequence was highly homologous to those of mammalian Dnmt1s. The results suggest that XDnmt1 may play important roles other than genomic imprinting, which has not previously been reported in amphibians. The characterization of XDnmt1 may shed light on the function of DNA methylation in vertebrates.

XDnmt1 Accumulates in Oocytes during Oogenesis—We showed that the XDnmt1 protein became detectable from stage III, and accumulated thereafter during the course of oogenesis (Fig. 1). *De novo* synthesis of XDnmt1 sharply increased from stage IV to VI (Fig. 2), and this increased synthesis seemed to cause the accumulation of XDnmt1. A similar event occurs in mouse oocytes; a 3,000 times higher amount of the Dnmt1 protein accumulates in an oocyte than in a single MEL cell (*26*). In *Xenopus*, stage VI and mature oocytes were calculated to contain about 1.2×10^4 and 2.4×10^5 times higher amounts of the XDnmt1 protein than in MEL cells on a per cell basis, respectively. Considering the sizes of mouse (about 80 μm) and *Xenopus* (1.0 to 1.2 mm) oocytes, the one to two orders of magnitude higher Dnmt1 content in *Xenopus* oocytes than in mouse oocytes is not surprising. The specific content per volume is rather low in *Xenopus* oocytes 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, transcription starts after the midblastula transition (MBT) (*47*). Therefore, the gene products, *i.e.* mRNA and/or proteins, indispensable for early embryogenesis before MBT are generally accumulated in mature oocytes in large quantities, in order to prepare for the quick increase in cell number. PCNA, which plays important roles in replication and repair (*42*), and has been reported to bind to Dnmt1 (*48*), is accumulated in a 4×10^5 times higher amount in mature oocytes than in an established *Xenopus* cell line, A6 cells, under proliferative conditions (*42*). In addition, histones (*49*), DNA polymerase α (*50*), and *Xenopus* RNA helicase, An3 (*51*), are also accumulated in oocytes during the course of oogenesis. These maternal stockpiles support the first 12-round cell cleavage after fertilization before MBT (*52*). 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.

Translocation of XDnmt1 into Nuclei of Oocytes—XDnmt1 was accumulated in oocytes during oogenesis, which is similar to in mouse. In contrast to in mouse oocytes, in which almost all Dnmt1 is excluded from the nucleus, a significant amount, about 50%, of XDnmt1 was translocated into the nuclei. Why was mouse Dnmt1 not but XDnmt1 was translocated into the nuclei of oocytes? In mouse, genome-wide demethylation occurs after fertilization until the implantation stage (*7*). On the other hand, it has been reported that the methylated state of the *Xenopus* genome is maintained throughout early embryogenesis (*53*). 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 (*53, 54*). MeCP2 specifically recognizes and binds to methylated DNA (*55*), recruits histone deacetylase (*56, 57*), and eventually suppresses the expression of methylated genes (*58*). Thus, in *Xenopus* the regulation of DNA methylation during early embryogenesis 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 are generally translocated into nuclei. PCNA (*59*), histones, and DNA polymerase α belong to this group (*60*). 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 the cytoplasm of *Xenopus* oocytes (*61, 62*). 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 (*51*). 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. In addition to this, species-specific exclusion in *Xenopus* oocytes has been reported (*63*). When *Xenopus* MyoD, one of the four myogenic factors that control the myogenic process, is expressed in early *Xenopus* embryos, it is excluded from nuclei. But ectopically expressed mouse MyoD is translocated into the nuclei of *Xenopus* oocytes (*63*). In analogy with these proteins, XDnmt1 may also have a signal sequence that is recognized by the machinery that exists in oocytes to determine its localization.

The XDnmt1 Accumulated in the Nuclei of Oocytes Has DNA Methyltransferase Activity—In the nuclei of stage VI oocytes, the DNA methyltransferase activity was 1×10^4 times higher (Table I), and the total amount of the XDnmt1 protein was 1.2×10^4 times higher than in MEL cells on a per cell basis (Fig. 1). Considering that the XDnmt1 protein was equally distributed in the post-nuclear fractions and nuclei (Fig. 3A), meaning that the XDnmt1 protein in the nucleus of a stage VI oocyte was 0.6×10^4 times higher than

that in a single MEL cell, the specific methyltransferase activity of XDnmt1 in the nuclei of stage VI oocytes was almost identical to that in proliferating MEL cells.

Our present result that a significant amount of active XDnmt1 exists in the nuclei of stage VI oocytes is in conflict with the report by Adams *et al.* (*27*). Adams *et al.* reported that most of the DNA methylation activity in stage VI oocytes existed in the post-nuclear fraction, and that isolated nuclei showed a negligible amount of the activity. The only difference we can point out is the way the nuclei were prepared. Adams *et al.* used a protease and a nonionic detergent when they lysed oocytes. DNA methyltransferase might leak from the nuclei with such treatment. While we manually removed nuclei directly from oocytes under a microscope. At the moment, we do not know why Adams *et al.* could not detect DNA methylation activity in oocyte nuclei but we could detect it. However, the important point is that we confirmed the existence of XDnmt1 in the nuclei of stage VI oocytes by means of three different methods: immunoprecipitation, immunocytochemistry, and enzyme activity determination.

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