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Isolation and Expression of a *Xenopus laevis* DNA Methyltransferase cDNA[1]

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A *Xenopus* DNA methyltransferase cDNA was isolated from a *Xenopus* oocyte cDNA library by screening with the mouse DNA methyltransferase 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 animal DNA methyltransferases, especially in the catalytic domain in the carboxyl-terminal region. The cysteine-rich region and the Lys-Gly repeat which were first found in the mouse sequence were conserved in *Xenopus*. However, 200 amino acid residues at the amino-terminus of *Xenopus* DNA methyltransferase were quite different from those of mouse and human, but showed 70% homology with those of chicken. The cloned *Xenopus* DNA methyltransferase cDNA expressed in COS1 cells showed a significant DNA methyltransferase activity. The size of the translation product of *Xenopus* DNA methyltransferase cDNA expressed in COS1 cells was identical with that of the endogenous DNA methyltransferase in *Xenopus* A6 cells and also with the size of newly synthesized DNA methyltransferase in *Xenopus* oocytes. However, a slightly larger immunoreactive band of about 205 kDa, and a small immunoreactive band of about 100 kDa, which were poorly labeled by short incubation with radiolabeled amino acids, were the main bands in stage I-III and stage IV-VI oocytes, respectively.

Key words: cDNA cloning, DNA methyltransferase, *Xenopus laevis*.

In vertebrates, the CpG dinucleotide sequence in genomic DNA is often methylated at the 5th position of the cytosine residue, and this methylation plays important roles in various biological phenomena such as genomic imprinting and X chromosome inactivation in mammals, and carcinogenesis (*1, 2*). The common mechanism underlying these phenomena is regulation of the expression of related genes by DNA methylation. In animals, there are two types of methylation activities, *de novo* and maintenance methylation activities. In mouse, *de novo* methylation contributes to the establishment of tissue specific methylation patterns at the implantation stage of embryogenesis (*3*), and maintenance-type methylation activity ensures clonal transmission of lineage-specific methylation patterns in somatic cells (*2, 4*). *De novo* methylation activity is physiologically important, but the enzyme that catalyzes the activity has not yet been identified.

Up to the present, DNA methyltransferase (MT) that favors introduction of a methyl group into the hemimethylated state of double-stranded DNA is the only methylase that has been discovered in vertebrates. This MT is believed to be responsible for maintaining the methylation pattern once formed in somatic cells. cDNA clones of MT that catalyze maintenance-type methylation have been isolated from mouse (MMT) (*5*), human (HMT) (*6*), and chicken (CMT) (*7*). A homologous cDNA of MT has also been isolated from sea urchin (UMT) (*8*). MMT can be divided into two distinct domains. The carboxyl-terminal domain, composed of about 500 amino acid residues, contains the catalytic site, which is conserved from bacterial type II DNA cytosine methylases (*5, 9*). The amino-terminal domain is thought to be a regulatory domain that recognizes the hemimethylated CpG sequence (*9*) and replication foci (*10*). The amino-terminal domain contains a cysteine-rich, zinc finger-like motif (*5, 9*). A Lys-Gly repeating sequence (KG-repeat) divides the amino- and carboxyl-terminal domains (*5, 9*). Both the cysteine-rich region and the KG-repeat are conserved in HMT, CMT, and UMT (*5-8*).

When the MT gene is destroyed in mouse, the homozygous mutant embryo cannot survive past midgestation (*11*), and in the embryo, imprinting of *Igf2, Igf2r, H19* (*12*), and even of *Xist* (*13*), which is thought to play a crucial role in X chromosome inactivation (*14*), is canceled. The embryonic stem cells harboring a homozygous mutation for MT gene are able to proliferate as normal cells. However, when the cells are induced to differentiate, they do not survive (*15*). MT activity is indispensable for the cells to differentiate, as well as for the establishment of genomic imprinting in germ cells. In the mouse oocyte, a 3,000-fold larger amount of MT protein exists on the per

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Abbreviations: DMEM, Dulbecco's modified Eagle's MEM; FBS, fetal bovine serum; MT, DNA methyltransferase; SDS, sodium dodecylsulfate.

cell basis (*16*). On the other hand, a negligible amount of MT activity exists in the *Xenopus* oocyte. The MT activity in *Xenopus* oocytes increases after fertilization (*17*).

In the present study, we report cloning of *Xenopus* MT (XMT) cDNA. The predicted amino acid sequence was compared with those of other animal MTs. The isolated XMT will be a useful tool for examining the function of MT during amphibian embryogenesis.

MATERIALS AND METHODS

Library Screening and Sequencing—A *Xenopus* oocyte cDNA library constructed in λgt10 (*18*) was kindly provided by Dr. D.A. Melton (Harvard University). The library of 4×10^6 plaque-forming units was screened with a 2 kb fragment of the 3′ region of MMT cDNA (*5*) 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 XMT (Fig. 1B).

A series of overlapping deletions was generated using exonuclease III (*19*), and sequenced by the dideoxy method (*20*) using T7 DNA polymerase (Sequenase ver. 2.0, USB). The sequence was determined for both strands. Ten percent formamide was added to the polyacrylamide sequencing gels to improve the separation of GC-rich sequences (*7*).

Construction of XMT Expression Vector and Its Expression—The XMT5 and XMT10 clones were combined into a single cDNA, and inserted in an expression vector, pKCRH2PL (*21*), which was provided by Dr. Y. Morimoto (Mitsubishi Kagaku). MMT and CMT cDNAs subcloned into the identical vector were also used (*7, 22*).

Plasmids were transfected to COS1 cells as described (*22*), using the calcium-phosphate method (*23*), 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 (*24*), and the latter was also used as the enzyme source for activity measurements.

Cells—COS1 cells were maintained in Dulbecco's modified Eagle's MEM (DMEM), supplemented with 10% fetal bovine serum (FBS), 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 (*25*), containing 61% Leibovitz L-15 medium, 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, and were cultivated at 22°C.

Oocytes in different stages were surgically prepared from female *Xenopus* anesthetized in ice-cold water (*26*). The follicle cells were removed and oocytes were washed with modified Barth's medium.

Immunoprecipitation—*Xenopus* cells and oocytes were incubated with 32 and 390 μCi/ml of EXPRE^{35}S^{35}S (NEN), respectively, in methionine and cysteine-free 0.7×DMEM, supplemented with 10% dialyzed FBS and 10 mM Hepes buffer (pH 7.4) at 22°C for 5 h. COS1 cells transfected with the plasmids containing MT cDNA were cultivated at 32°C for 48 h in the growth medium and then either harvested or further incubated with 32 μCi/ml of EXPRE^{35}S^{35}S in methionine and cysteine-free DMEM, supplemented with 10% dialyzed FBS at 32°C for 5 h. Whole oocytes, post nuclear fractions, or nuclear extracts were briefly sonicated in 870 μl of 0.575% SDS, 2.9 mM EDTA, and 57.5 mM triethanolamine buffer, pH 7.4, and boiled for 2 min. To the sonicated mixtures were added 100 μl of 20% (w/v) Triton X-100, 20 μl of 100 units/μl Trasylol, and 10 μl of 0.5 M iodoacetamide. The solution was mixed, the immunoselected anti-MT antibodies (*22*) were added, and the reaction mixture was incubated at 4°C overnight, then protein A-Sepharose was added to precipitate the XMT-antibodies complex. The protein A-Sepharose was washed three times with 0.1% Triton X-100 and 0.75 unit/ml Trasylol in phosphate-buffered saline. Proteins were solubilized in a sample buffer and were electrophoresed in a 7% SDS-polyacrylamide gel (*27*). Protein bands were detected by staining with Coomassie Brilliant Blue R-250 or/and by fluorography (*28*).

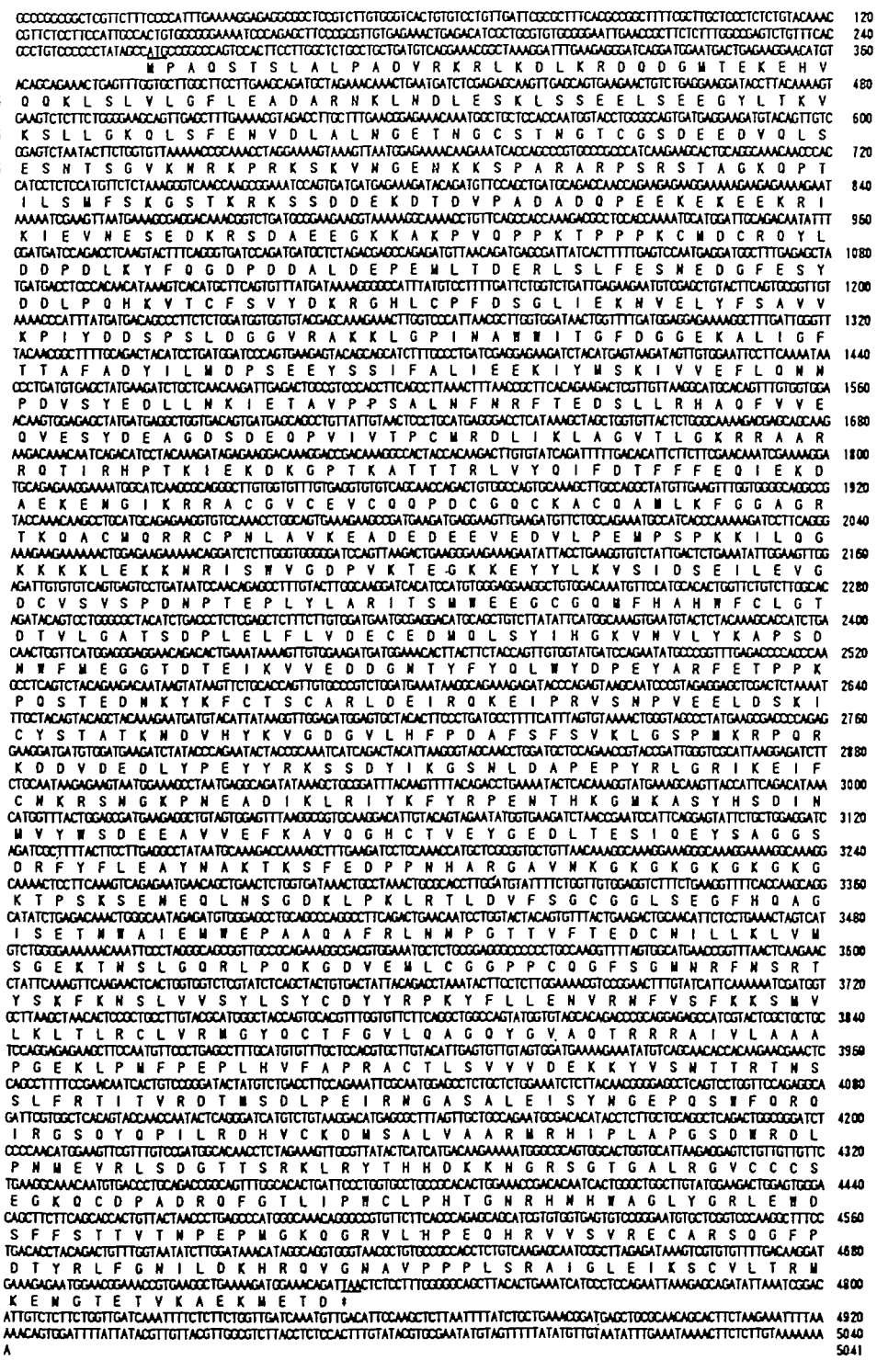
MT Activity—MT activities were determined as described (*22*) except that the reaction mixtures were incubated at 30°C. The protein concentrations were determined as described by Lowry *et al.* (*29*), using bovine serum albumin as a standard.

RESULTS

Isolation and Sequencing of the Xenopus MT cDNA—As the chicken (avian) MT sequence is highly homologous to those of mammalian MTs (*7*), we expected that the *Xenopus* (amphibian) MT (XMT) sequence would also be similar to those of mammalian MTs. Thus, we first screened the *Xenopus* oocyte cDNA library with the labeled fragment of mouse MT (MMT) cDNA coding the catalytic domain of the enzyme as a probe, and we cloned XMT10. Using the 5′-end fragment of the XMT10 clone, we then cloned XMT5. The two overlapping clones contained the entire coding sequence of XMT (Fig. 1). The size of the deduced nucleotide sequence of XMT cDNA was 5,033 bp with poly(A). The A of the initiation methionine residue (ATG) was at nucleotide position 260 and the T of the stop codon (TAA) was at nucleotide position 4,730. The elucidated nucleotide sequence contained a 4,470-nucleotide open reading frame that encoded a protein of 1,490 amino acid residues, the calculated molecular weight of which is 167,981. The predicted molecular weight of XMT is similar to those of other vertebrate MTs (*5-7*).

Encoded Amino Acid Sequence of XMT cDNA Is Homologous to Those of Other Animal MT cDNAs—The carboxyl-terminal domain composed of about 500 amino acid residues contains the motifs which are responsible for the catalytic activity (*5*). These motifs are conserved from bacterial type II DNA cytosine methylases (*5*). Motif I is expected to contribute to *S*-adenosyl-L-methionine binding and motif IV contains the invariant Pro-Cys dipeptide sequence that is known to be a part of the catalytic center (*30, 31*). When the motifs of XMT were aligned with those of MMT, HMT, CMT, and UMT, the sequences were highly conserved among the species (Fig. 2A). More than 85% of the amino acid residues were matched among the MTs (Table I). The two motifs first found in MMT (*5*), a zinc-finger-like cysteine-rich region, which resides in the middle of the amino-terminal domain, and the KG-repeat,

A



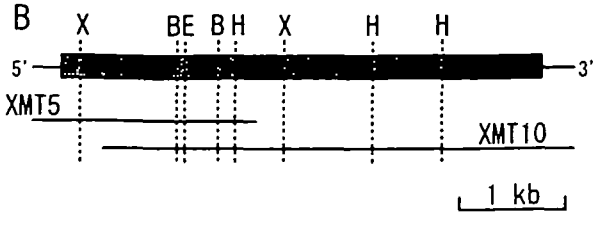


Fig. 1. **A: Nucleotide and deduced amino acid sequences of XMT cDNA.** The predicted amino acid sequence is shown below the nucleotide sequence. Amino acids are shown in one letter code. The sites for the initiation (ATG) and the termination codons (TAA) are underlined. **B: Schematic illustration of XMT cDNA and clones XMT5 and XMT10.** The coding region is shown as a shaded box. The restriction sites of *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Xho*I (X) are indicated.

A.

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          I                    II                   IV                   VI
MMT  DVFSGCGGLSEGFHQAGI 1045  TLWAIEMWDPAAQAFRLNNP 1067  DVEMLCGGPPCQGFSGMNR 1119  RPRFFLLENVRNFVSYRRSMV 1164
HMT  ******************** 1039  ********D*********** 1061  ******************** 1113  ****************FK**** 1158
CMT  *******************V 1075  ********E*********** 1097  ******************** 1149  ****************FK**** 1194
XMT  ******************** 1035  *N******E*********** 1057  ******************** 1109  **KY***********FKK*** 1154
UMT  ***A**************** 1152  SS****KEE*****Y***** 1174  ***L**************** 1226  ****************KKN** 1271

          VII                 VIII                    IX                    X
MMT  MGYQCT 1180  VLQAGQYGVAQTRRRAIILA 1202  HRVVSVRECARSQGFPDSY 1447  GNILDRHRQVGNAVPPPLAKAIGL 1474
HMT  ****** 1174  ********************** 1196  ******************T* 1442  *****K****************** 1469
CMT  ****** 1210  *******************V** 1232  ******************T* 1477  *****K****************** 1504
XMT  ****** 1170  *******************V** 1192  ******************T* 1437  *****K***********SR**** 1464
UMT  ****** 1287  I*********P********** 1309  ******************T* 1554  *S***K***I*******M*A***M 1581
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B.

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MMT  CGVCEVCQQPECGKCKACKDMVKFGGTGRSKQACLKRRC  575    KGKGKGKGKGKGK  1006
HMT  ****************************S********QE***  570    ***********P*  1000
CMT  *******************QN******S********Q***  603    **************  1032
XMT  **********D**Q****QA*L****A**T****MQ***  564    **************   995
UMT  ******I**A*D****T******I****S*KA****KD***  671    ************A*  1110
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C.

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MMT  KKLESHTVPVQSRSE--RKAAQSKSVI-PKINSPKCPECGQHLDDPNLK-YQQHPEDAVDEPQMLTSEKLSIYDSTSTWFDTYEDSPMHRFTSFS  298
HMT  *E----------PT*--K*M*RA*T*MNS*THP***IQ*G*Y****D**-*G***P**********N*****F*ANESG*ES**AL*Q*KL*C**  290
CMT  KEGSEIK---DEITQ--V*TSTP-----A*TTP***VD*R*Y****D**FF*GD*D**LE**E***D*R***F*ANEDG*ES***L*Q*KV****  322
XMT  NESEDKRSDAEEGKK--A*PVQP-----**TPP***MD*R*Y****D**YF*GD*D**L***E***D*R**LFE*NEDG*ES*D*L*Q*KV*C**  286
UMT  EEEEKAKVEPMSPSRDLRHKANHETAE-S*QPPLR*K**R*L****D**IFPGD****RE*YIT**DPR**LLTGDEGDAMS*DERLQ*KI*N*C  398

MMT  VYCSRGHLCPVDTGLIEKNVELYFSGCAKAIHDENPSMEGGINGKNLGPINQWWLSGFDGGEKVLIGFSTAFAEYILMEPSKEYEPIFGLMQEKI  393
HMT  ***KH*****I********I**F***S**P*Y*DD**L***V*********E**IT*******A******S*******D**P**A**********  385
CMT  **DK******F******R*I******AV*P*Y*D**CLD**VRA*K*****A**IT*******A****T****D*******E**A***A******  417
XMT  **DK******F*S*************AVV*P*Y*DS**LD**VRA*K*****A**IT*******A****T****D****D**E**SS**A*IE***  381
UMT  **DKST*I*AF*R*M****K******YV*P*Y*D***T****PT*RI****E*YTT*****H*A*************V*S**E**K*FWTAV****  493

MMT  YISKIVVEFLQNNPDAVYEDLINKIETTVPPSTINVNRFTEDSLLRHAQFVVSQVESYDEAK  455
HMT  ***********S*S*ST***************GL*L***************E********G  447
CMT  *M***********R*VS****L*********VGL*F***************E********G  479
XMT  *M**************VS****L*****A****AL*F***************E********G  443
UMT  *M***L*I*******V*P*****LTQ*******EG--C***************E******D*A  553
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D.

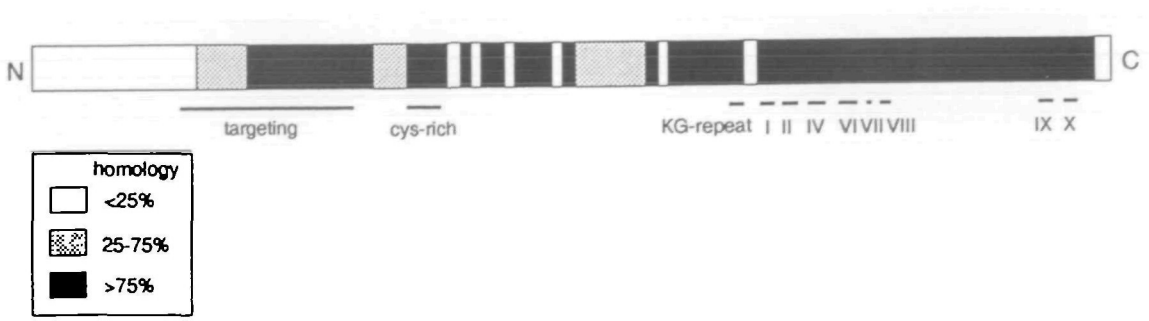

Fig. 2

which divides the carboxyl- and amino-terminal domains (*5, 9*), were also conserved in XMT (Fig. 2B and Table I). Another sequence of about 200 amino acid residues in the amino-terminal domain, which is responsible for localizing MMT in the replication foci at late S phase (*10*), showed about 63% matching among the MTs (Table I). The amino-terminal portion of this targeting sequence was poorly conserved, but the carboxyl-terminal portion was highly conserved (more than 75% matching) among the animal MTs (Fig. 2C). The amino-terminal 207 amino acid residues of MMT showed no significant homology with those of the four animal MTs (Table I). However, when 207 and 208 amino acid residues of MMT and HMT, and 200 and 239 amino acid residues of CMT and XMT (Fig. 3) were separately aligned, the percentages of equivalent amino acid residues were 70%. The homology of the entire molecule of MTs is schematically illustrated in Fig. 2D.

XMT cDNA Sequence Encodes Active Methyltransferase—The similarity of the deduced amino acid sequence of XMT with those of the other vertebrate MTs indicated that the XMT cDNA encoded MT. To confirm that the cloned cDNA actually encodes MT, the XMT clones covering the entire coding sequence were combined into a single cDNA, which was inserted in an expression vector containing SV40 promoter (pXMT), and expressed in COS1 cells. Methyltransferase activities of the nuclear extracts from cells transfected with the three separately constructed pXMT clones were lower than that from cells transfected with plasmids containing CMT or MMT, but were significantly higher than that from cells transfected with the *lacZ*-containing plasmid (Table II).

Post nuclear fraction and nuclear extract of COS1 cells transfected with the plasmids containing XMT, CMT, MMT, or *lacZ* were immunoprecipitated with the specific antibodies raised against MMT. As shown in Fig. 4, lanes 1 and 5, the antibodies precipitated a single major band with a size similar to those of the bands precipitated from the cells transfected with pCMT (lanes 2 and 6) and pMMT (lanes 3 and 7). The amount of the expressed XMT band

TABLE I. **Equivalent amino acid residues in the sequence motifs among MTs.** Predicted amino acid residues of MMT (*5*), HMT (*6*), CMT (*7*), XMT (this work), and UMT (*8*) were aligned using a program, ODEN/align, developed by Dr. Ina at the National Institute of Genetics, Japan, and the motifs found and the amino-terminal 207 amino acid sequence in MMT were compared with the corresponding sequences in the other MTs.

	Numbers of amino acid residues				Homology (%)
	Identical (i)	Similar[a] (s)	Sum (i)+(s)	Total	
Whole molecule[b]	654	233	887	1,502[b]	59.1
Eight motifs in catalytic domain					
I	16	2	18	18	100
II	14	3	17	20	85.0
IV	18	1	19	19	100
VI	15	5	20	21	95.2
VII	6	0	6	6	100
VIII	17	3	20	20	100
IX	18	1	19	19	100
X	17	6	23	24	95.8
Cysteine-rich region	26	6	32	39	82.1
KG-repeat	12	1	13	13	100
Targeting signal for replication foci	113	43	156	248	62.9
Amino-terminus	6	12	18	207	8.7
	(10	18	28		13.5)[c]

[a]The following amino acid residues in each group were considered as equivalent. (i) A, S, T, P, and G; (ii) N, D, E, and Q; (iii) H, R, and K; (iv) M, L, I, and V; (v) F, Y, and W. [b]Taken from the amino acid sequence of MMT (*5*). [c]Numbers in parentheses are those calculated after UMT was omitted.

TABLE II. **Methylation activities of transiently expressed XMT in COS1 cells.** The nuclear extract was prepared from COS1 cells transfected with pXMT (clones #1, #7, and #8), pCMT, pMMT, or p*lacZ*. The activities were measured using poly(dI-dC)-poly(dI-dC) as the substrate.

Clone	DNA methyltransferase activity (pmol/h/mg protein)
pXMT #7	7.5
pXMT #8	8.8
pXMT #1[a]	5.4
pCMT	148.2
pMMT	263.2
p*lacZ*	1.9

[a]To construct pXMT #1 clone, *Eco*RI fragment of XMT6A clone was used instead of that of XMT5. XMT6A is 203 nucleotides shorter in the 5′ region than the XMT5 sequence shown in Fig. 1A.

Fig. 2. **A: Eight highly conserved motifs of MTs coding catalytic domain.** The conserved motifs I, II, IV, VII, VIII, IX, and X are presented. **B: Alignment of the cysteine-rich region and the KG-repeat of animal MTs. C: Alignment of the sequence of MMT that is reported to function as a targeting sequence to the replication foci with the corresponding sequences of MTs.** A-C: Each of the alignments was taken from the alignment of the entire MTs, using a program, ODEN/align, developed by Dr. Ina at the National Institute of Genetics, Japan. The identical amino acid residues are indicated as (*), and non-identical amino acid residues, compared with those of MMT, are shown. **D: Schematic illustration of the homology in MT molecule.** The regions of the eight conserved motifs in the catalytic domain (I, II, IV, VI, VII, VIII, IX, X), cysteine-rich region (cys-rich), KG-repeat, and a targeting sequence to the replication foci (targeting) are indicated by lines under the bar. The equivalent amino acid residues defined in Table I were used to calculate percentages of homology.

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XMT  MPAQS--TSLALPADVRKRLKDLKRDQDGNTEKEHVQQKLSLVLGFLEADARNKLNDLESKLSSEELSEEGYLTKVKSLL  78
     *** * .. ***. .*.**.** **.*...*** .*.** * *** *.  .* *.. .  ***** **.***.**
CMT  MPARSAPPPPALPPALRRRLRDLERDEDSLSEKETLQEKLRLTRGFLRAEVQRRLSALDADVRCRELSEERYLAKVKALL  80

XMT  GKQLSFENVDLA--LNGETNGC----------STNGTCGSDEEDVQLSESNTSGVKNRKPR 127
      ..*. ** * *      .***                *. . . .*      * *  .. . **.*
CMT  RRELAAENGDAAKLFSRASNGCAGNGEEEWERGGRGEDGAMEVEEAAASSSSSSSSSSSSSSSSSSSSLLPAPRARKAR 160

XMT  KSKVNGENKKSPARARPSRSTAGKQPTILSMFSKGSTKRKSSD-------DEKDTDVPADADQPEEKEKEEKRIKIEVNE 200
     .*. *** ***** .* . ** .*.******.********** .        .  . ..  **** .******* *
CMT  RSRSNGESKKSPASSRVTRS-SGRQPTILSVFSKGSTKRKSEEVNGAVKPEVSAEKDEEEEEELEEKEQDEKRIKIETKE 239
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Fig. 3. **Alignment of amino-terminal sequences of MTs.** The amino-terminal sequences of 200 and 239 amino acid residues of XMT and CMT, respectively, are aligned. The identical amino acids are marked (*) and similar amino acids as defined in Table I are marked (.).

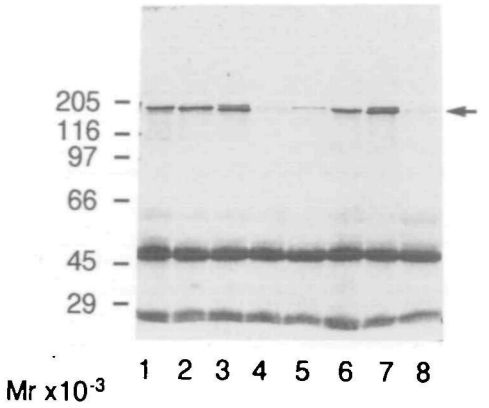

Fig. 4. **Immunoprecipitation of transiently expressed MTs in COS1 cells.** The plasmid pXMT #8 (lanes 1 and 5), pCMT (lanes 2 and 6), pMMT (lanes 3 and 7), or p*lacZ* (lanes 4 and 8) was transfected into COS1 cells. After the recovery of the cells in growth medium at 32°C for 48 h, post nuclear fractions (lanes 1-4) and nuclear extracts (lanes 5-8) were prepared for immunoprecipitation. The immunoprecipitated protein bands were analyzed in SDS-polyacrylamide gel stained with Coomassie Brilliant Blue R250. The arrow indicates the bands for MT. Intense bands at around 50 k and 29 k are the heavy and light chains of the anti-MT antibodies, respectively. Molecular weight standards ($M_r \times 10^{-3}$) are indicated.

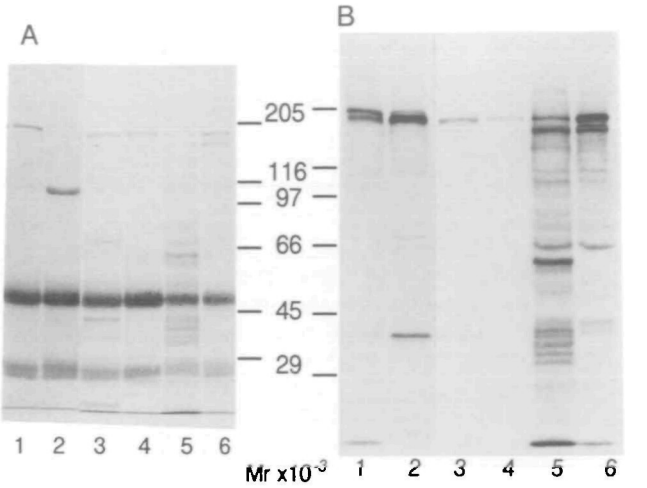

Fig. 5. **Immunoprecipitation of endogenous XMT in oocytes and cultivated cells.** *Xenopus* oocytes in stage I-III (14 oocytes, lane 1) or stage IV-VI (11 oocytes, lane 2), A6 cells (lanes 3 and 4), or COS1 cells transfected with pXMT #8 (lanes 5 and 6) were labeled with EXPRE^{35}S^{35}S, immunoprecipitated, and analyzed in an SDS polyacrylamide gel. As for the cultivated cells, post nuclear fractions (lanes 4 and 6) and nuclear extracts (lanes 3 and 5) were separately prepared and immunoprecipitated. A. Coomassie Blue staining. Intense bands at around 50 k and 29 k are heavy and light chains of the anti-MT antibodies, respectively. B: Fluorography. The sheet was exposed overnight (lanes 1 and 2) or for 1 h (lanes 3-6). Molecular weight standards ($M_r \times 10^{-3}$) are indicated.

that remained in the post nuclear fraction was comparable to that of CMT or MMT, but the amount of XMT translocated into nuclei was low. A band of similar size was scarcely detected in cells transfected with p*lacZ*, suggesting that the detected band in the cells transfected with pXMT was insert-dependent. We concluded that the isolated clone encodes DNA methyltransferase.

XMT Protein Expressed from the cDNA in COS1 Cells Is Identical in Size with Endogenous XMT—*Xenopus* oocytes, which are arrested at an early stage of miosis, can be classified into stages I to VI, largely according to size (*32*). Overall growth from stage I to stage VI requires at least 8 months. Stage I-III oocytes are less than 500 μm and stage V-VI oocytes are larger than 1,000 μm. The size difference between the full-grown stage VI oocyte, which is ready for fertilization, and the stage V oocyte is not easy to discern by simple observation. Oocytes in stage I-III or stage IV-VI, *Xenopus* A6 cells, or pXMT-transfected COS1 cells were radiolabeled, immunoprecipitated, and electrophoresed. Coomassie Blue staining showed that the size of the major XMT in stage I-III oocytes (Fig. 5A, lane 1) was distinctly larger than those in nuclear and post nuclear fractions of A6 cells (lanes 3 and 4), which were identical in size to the XMT expressed in COS1 cells (lanes 5 and 6). In stage IV-VI oocytes, the size of the immunoprecipitated major band was about 100 kDa, and this product seemed to be a degradation product of intact MT. In addition to the 100 kDa major band, two weakly stained bands that were identical to those of stage I-III oocytes and A6 cells were also detected (lane 2). Fluorography of the same gel, however, detected significant amounts of radiolabeled bands in stages I-III and IV-VI oocytes that were identical in size with that in A6 cells. Interestingly, the 100 kDa band found in stage IV-VI oocytes was not radiolabeled, and the bands identical in size with those in A6 cells became major bands in both stages I-III and IV-VI oocytes (Fig. 5B, lanes 1-4). These results, especially the fact that XMT expressed in A6 cells was identical in size with that from XMT cDNA expressed in COS1 cells, indicate that the cloned XMT cDNA was the maintenance-type MT of *Xenopus laevis*.

DISCUSSION

We have isolated and sequenced the *Xenopus* MT cDNA, and we found that the predicted sequence was highly homologous to those animal MT sequences. From this, together with the results that the size of the immunodetected protein band of ectopic XMT was identical to that of the endogenous XMT in A6 cells, and that the ectopic XMT showed significant MT activity, we concluded that XMT cDNA encodes *Xenopus* MT. The cloned XMT may function as a maintenance MT. The amino-terminal region, recognizing hemimethylated DNA or replicating foci at late S phase (*10*), is thought to be a regulatory domain of the enzyme. The sequence of the amino-terminal domain of XMT, including the Cys-rich region and the KG-repeat, except for an about 200 amino-acid-residue sequence at the amino terminus, was highly homologous to those of MMT, HMT, CMT, and UMT. Even the 200 amino-acid-residue sequence at the amino terminus showed 70% homology when the XMT sequence was aligned with that of CMT. This amino-terminal sequence, which was conserved among mammals, amphibian and avian, might play a role in interacting with the animal class-specific factors that modulate MT activity or in the localization of MT.

In the present study, the molecular weight of XMT was calculated to be 167,981 from the predicted amino acid sequence. The apparent molecular weight of XMT in A6

cells and that expressed in COS1 cells was estimated to be about 190k from the mobility in SDS polyacrylamide gel. This apparent size of XMT is similar to those of MMT and CMT (Fig. 4). *Xenopus* oocytes at stage I-III expressed a larger XMT band than that in A6 cells. In the advanced stage, *Xenopus* oocytes contained a much smaller MT of about 100 kDa as a major band. Interestingly, the large MT band in stage I-III oocytes was radiolabeled rather weakly, and the 100 kDa band in stage IV-VI oocytes was scarcely radiolabeled. This labeling property of MT in oocytes suggests that the abundant MT proteins found in oocytes were translated in early-stage oocytes or even earlier. In oocytes in both stages I-III and IV-VI, the major labeled bands were at about 190 kDa, being identical in mobility to that in A6 cells or ectopic XMT in COS1 cells. As the stage I-III oocytes actively synthesize the 190 kDa MT species, the larger size MT in stage I-III oocytes could be a modification product of the 190 kDa species. However, there is still a possibility that the 205 kDa species was not produced from the 190 kDa band, but was the direct translation product. Consequently, the small 100 kDa band enriched in stage IV-VI could be a degradation product of either 190 or 205 kDa species of MT. As stage I-VI oocytes are arrested at the early stage of miosis, it is reasonable to speculate that the genomic DNA may be partly exposed and, thus, susceptible to methylation when an active MT is near by. Since the DNA methylation state of the gene directly affects the transcription activity, the localization and/or activity of MT should be strictly regulated. During mouse spermatogenesis, spermatocytes shut off the transcription of MT mRNA that encodes active enzyme at the pachytene stage where cross-over is occurring (*33*). In the present study, interestingly, unusual MT proteins of different sizes accumulated in different stages of oocytes, though synthesis seemed to have occurred in early stage oocytes or even earlier. The appearance of apparently large- and small-size MTs in stage I-III and in advanced stage IV-VI oocytes, respectively, could reflect the physiological processes involved in the regulation of MT activity.

Like the mouse oocyte, the *Xenopus* oocyte contains large amounts of MT protein. Coomassie Blue staining of XMT in Fig. 5A, lanes 1 and 2, shows samples that were immunoprecipitated from 14 and 11 oocytes in stages I-III and IV-VI, respectively, while XMT in each of lanes 3 and 4 was from 10^6 A6 cells. Based on the Coomassie Blue staining, *Xenopus* oocytes contain roughly 10^5 times more MT than A6 cells on the per cell basis. In mouse, an about 3,000-fold larger amount of MT is detected in oocytes than in mouse erythroleukemia (MEL) cells on the per cell basis (*16*). Since the genomic imprinting phenomenon has not been reported in amphibians, including *Xenopus*, it is reasonable to speculate that the curious size changes and abundance of MT in oocytes may contribute to other important processes in embryogenesis.

The XMT expressed in COS1 cells showed extremely low, though significant, MT activity. This is partly due to low translocation efficiency of XMT into the nucleus. As shown in Fig. 4, in lanes 1 and 5, most of the translated XMT in COS1 cells remained in the post nuclear fraction, that is, in the cytoplasmic fraction. A significant amount of XMT was located in the cytoplasm as revealed by immunofluorescence microscopy (data not shown). When pXMT-transfected COS1 cells were recovered at 37°C, MT protein and the activity were hardly detected (data not shown). Folding of XMT after translation may be a temperature-sensitive process. Neither *Xenopus* nor cultivated cells can survive at high temperature, such as 37°C (*25, 34*), and poly(A)polymerase from *Xenopus* is not active at 37°C, though it is active below 25°C (*35*). Even 32°C, at which temperature the cells transfected with XMT cDNA were recovered in the present study, may be too high for XMT to form a proper conformation to be translocated into the nucleus and/or to express full activity. On cultivation at 37°C, where almost no translation product of XMT was detected, XMT may not form a proper conformation and thus may be immediately eliminated by the cell machinery.

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