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Exogenous Expression of Mouse Dnmt3 Induces Apoptosis in *Xenopus* Early Embryos[1]

Hironobu Kimura, Isao Suetake, and Shoji Tajima[2]

Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871

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Mouse DNA (cytosine-5) methyltransferases Dnmt3a and Dnmt3b are expected to be *de novo*–type DNA methyltransferases. In the present study, we found that exogenously expressed mouse Dnmt3a or Dnmt3b induced abnormal cell clusters at the gastrulation stage in *Xenopus* embryos. The abnormal cells were judged to be apoptotic from the positive staining with the TdT dUTP nucleotide end–labeling method and the rescue by hBcl-x_L, a Bcl-2 homologue. On the other hand, neither bacterial DNA (cytosine-5) methyltransferase nor Dnmt3b3, one of the three isoforms of Dnmt3b that has no DNA methylation activity, induced apoptosis. In addition, mutant Dnmt3a and the other two Dnmt3b isoforms, Dnmt3b1 and Dnmt3b2, which have no DNA methylation activity due to a change of the cysteine residue in the catalytic center to an alanine residue, retained the ability to induce apoptosis. This indicates that the apoptosis was not induced by DNA methylation activity. The domain of Dnmt3b1 (3b2) responsible for the apoptosis is the catalytic domain in the carboxyl-terminal half.

Key words: apoptosis, DNA methyltransferase, embryogenesis, *Xenopus laevis*.

In vertebrates, the 5th positions of the cytosine residues in CpG sequences in genomic DNA are often methylated (*1*). This modification functions as a transcriptional regulation mechanism (*2*). Generally, tissue-specific genes are almost fully methylated in all non-expressing adult somatic tissues. Demethylation of tissue-specific genes is usually only observed in specific tissues, in which the genes are expressed. The promoter regions of constitutively expressed genes (housekeeping genes) that possess CpG islands in their promoters are undermethylated (*2–4*). In vertebrates, two types of DNA methyltransferase activity have been reported: *de novo*–type DNA methylation activity, which creates a tissue-specific methylation pattern; and maintenance-type activity, which ensures clonal transmission of lineage-specific methylation patterns during replication. Dnmt1 is responsible for the latter activity (*4*). Recently, two DNA methyltransferases, Dnmt3a and Dnmt3b, were identified (*5*), and this Dnmt3 family is expected to be responsible for the creation of methylation patterns at an early stage of embryogenesis (*6, 7*).

In early mouse development, DNA methylation patterns dramatically change, involving genome-wide demethylation and *de novo* methylation (*8*). In preimplantation embryos, parts of the inherited parental methylation patterns are erased. After implantation, the embryo undergoes *de novo* methylation, which establishes a new methylation pattern (*9–11*). On the other hand, reports on the methylation states in early *Xenopus* embryos have been controversial. No methylation change in *Xenopus* genomic DNA has been detected in early embryos (*12, 13*). On the other hand, Stancheva and Meehan have reported that the methylated cytosine content of *Xenopus* genomic DNA decreases to 50% in stage 7 embryos (early blastula stage) (*14*). In addition, Stancheva *et al.* obtained evidence that *de novo* methylation activity does exist in early *Xenopus* embryos (*15*).

Aberrant expression or suppression of Dnmt1 affects terminal differentiation and embryogenesis by disturbing DNA methylation (*16–19*). Gene targeting of Dnmt3a and/or Dnmt3b in mouse (*6*) and aberrant expression of mouse Dnmt3a in *Drosophila* (*7*) severely affect embryogenesis. The effect of this exogenously expressed mouse Dnmt3a on *Drosophila* development depends upon its *de novo* methylation activity, since a non-active Dnmt3a construct did not affect the development (*7*). Recently, it was reported that Dnmt3a associates with co-repressor RP58 and thus inhibits transcription (*20*). Interestingly, a mutant Dnmt3a that lacks methylation activity can also effectively inhibit the transcription *via* interaction with RP58, suggesting that DNA methylation activity is not prerequisite for the transcriptional silencing mediated by Dnmt3a (*20*).

To address the question of whether or not exogenously expressed *de novo*–type DNA methyltransferases affect the early embryogenesis of *Xenopus laevis*, we injected mouse Dnmt3a and Dnmt3b mRNAs into two-cell stage *X. laevis* embryos. Overexpression of the enzymes induced apoptosis. Surprisingly, the effect was independent of the DNA methylation activity of Dnmt3a or Dnnmt3b. It was suggested

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[2] To whom correspondence should be addressed. Tel: +81-6-6879-8627, Fax: +81-6-6879-8629, E-mail: tajima@protein.osaka-u.ac.jp

Abbreviations: β-gal, β-galactosidase; hBcl-x_L, human Bcl-x_L; HDAC, histone deacetylase; *M. Hpa* II, *Hpa* II methylase; PBS, Dulbecco's phosphate buffered saline; TUNEL, TdT dUTP nucleotide end labeling.

that Dnmt3 interacts with a key component involved in the apoptosis cascade.

MATERIALS AND METHODS

Plasmid Construction and In Vitro Transcription—The expression vectors pCS2+, pCS2+MT, pCS2+NLSMT, and pCS2+cBgal (*lacZ* harboring plasmid) were kindly provided by Drs. H.B. Sucic and D. Turner (University of Michigan, MI). Mouse Dnmt3a, Dnmt3bs, *i.e.*, Dnmt3b1, Dnmt3b2, and Dnmt3b3, and bacterial type-II DNA (cytosine-5) methylase *Hpa* II (*M. Hpa* II) cDNAs were ligated into the multi-cloning site of pCS2+MT, and human Bcl-x_L (hBcl-x_L) cDNA into that of pCS2+. Mouse cDNAs for Dnmt3a and Dnmt3bs were kindly provided by Drs. Takahide Chijiwa and Hiroyuki Sasaki (Natl. Inst. of Genet., Mishima), *M. Hpa* II by Dr. Saulius Klimasauskas (Inst. of Biotechnology, Lithuania), and hBcl-x_L by Drs. Yutaka Eguchi and Yoshihide Tsujimoto (Osaka University, Osaka).

Loss-of-function Dnmt3a (mut-Dnmt3a), Dnmt3b1 (mut-Dnmt3b1), Dnmt3b2 (mut-Dnmt3b2), and Dnmt3b3 (mut-Dnmt3b3) were generated by ordinary oligonucleotide-directed mutagenesis (*21*), the Cys706, Cys657, Cys637, and Cys637 residues (TGC), respectively, in motif IV being changed to Ala (GCC), and then ligated into pCS2+MT.

Truncated forms of Dnmt3b1, which encodes the amino-terminal sequence including the Cys-rich region (1–535) and the carboxyl-terminal sequence (537–859), were separated by *Eco*47III digestion and then ligated into the *Eco*RI site of pCS2+NLSMT. The partial sequences of Dnmt3b1 that encode the amino-terminal sequence (1–429) and the carboxyl-terminal amino acid sequence including the Cys-rich region (430–859), and the 63-amino-acid sequence (750–812) in the carboxyl-terminal half of Dnmt3b1, the sequence missing in Dnmt3b3, were PCR amplified with Pfu (Stratagene, CA), and then subcloned into pCS2+NLSMT. All the PCR-amplified fragments were subcloned and then their nucleotide sequences were determined by the dideoxy method (*22*).

All the constructs obtained were linearlized at the *Not*I site and transcribed *in vitro* with SP6 RNA polymerase (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions to obtain capped mRNA. The size of transcribed mRNA was confirmed by electrophoresis in a 1.0% agarose/formaldehyde denatured gel and the gel was stained with SYBR Green II (Molecular Probes, OR). The stained mRNA was quantified with a FluoroImager system (Molecular Dynamics, CA) with a known amount of antisense Krox20 RNA as a standard (kindly provided by Dr. M. Taira, University of Tokyo, Tokyo) (*23*).

Embryos and Microinjection—Fertilized *Xenopus* eggs were prepared as described elsewhere (*24*). For the standard injections, 1–3 ng of mRNA for the test was co-injected with 0.5 ng of β-galactosidase (β-gal) mRNA in 5 nl of distilled RNase-free water into the animal-pole side of 2- or 4-cell stage embryos. All the embryos were incubated at 18°C after injection.

Immunoprecipitation and Western Blotting—Injected embryos were homogenized in 1 ml of 50 mM Tris-HCl (pH 7.6) buffer containing 150 mM NaCl, 1% (w/v) NP-40, 0.01% sodium deoxycholate, and 0.02 tablet/ml of Complete-mini protease inhibitor cocktail (Roche Diagnostics GmbH, Germany), and kept on ice for 30 min. The whole extracts were centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant fractions were recovered and incubated with anti-Myc monoclonal antibody (9E10)–coupled Sepharose overnight at 4°C. The immunoprecipitated proteins were separated by SDS-PAGE (*25*), electrophoretically transferred to a nitrocellulose membrane, and detected with anti-Myc monoclonal antibody as the first antibody and alkaline phosphatase–conjugated antibodies as the second antibody (*19, 26*).

β-Gal Staining—Embryos injected with mRNA encoding bacterial b-gal were fixed and stained as described by Sieve *et al.* (*24*). In brief, embryos were fixed with a solution comprising 0.2% glutaraldehyde, 2% formamide, 0.02% Triton X-100, and 0.01% sodium deoxycholate in Dulbecco's phosphate-buffered saline (PBS) at 0°C for 1 h. The fixed embryos were washed four times for 10 min each with PBS, and then stained with 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside (Wako Pure Chemical Industries, Osaka), and 2 mM $MgCl_2$ in PBS. The stained embryos were re-fixed and stored in methanol.

TdT dUTP Nucleotide End Labeling (TUNEL) Method—TUNEL staining of whole-mount *Xenopus* embryos was carried out as described (*27*). Fixed embryos were end-labeled with 150 U/ml TdT (Gibco BRL, MD) and 40 mM digoxygenin-dUTP (Roche Diagnostics GmbH, Germany). The labeled end was detected with anti-digoxygenin antibodies coupled with alkaline phosphatase (Roche Diagnostics GmbH, Germany). The embryos were observed after dehydration in methanol and mounted in benzyl benzoate/benzyl alcohol 2:1 (*28*).

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

DNA Methylation Activity—Myc-tagged Dnmt3s and mutant Dnmt3s were expressed in HEK 293T cells. Transfection with Lipofectoamine Plus (Gibco BRL, MD) was performed according to the manufacturer's instructions. The expressed enzymes were prepared with anti-Myc monoclonal antibody (9E10)–coupled Sepharose, and the DNA methylation activities were determined as described (*29*) using poly(dG-dC)-poly(dG-dC) (Amersham Pharmacia Biotech, UK) as the methyl acceptor.

RESULTS

Exogenous Expression of Dnmt3a or Dnmt3b1 (3b2) in Early Xenopus Embryos Induced Abnormal-Cell Morphologies—To determine the effect of *de novo*–type DNA methyltransferase, we injected the mRNAs of mouse Dnmt3a, and the three isoforms of Dnmt3b, *i.e.*, Dnmt3b1, Dnmt3b2 and Dnmt3b3, produced through alternative splicing (*5*), and *M. Hpa* II (Fig. 1) into two-cell *Xenopus* embryos. The embryos injected with all the mRNAs normally developed until the late brastula stage. At the early gastrula stage (stage 11), the cells in the region where the Dnmt3a or Dnmt3b1 (3b2) mRNA was injected started to become round in shape, and almost detached from the embryos (Fig. 2, A, B, C, G, H, and I). The abnormal cells were obviously larger than the normal cells. These abnormal cells expressed the co-injected β-gal (Fig. 2, G–K), indicating that the effect was due to the injected Dnmt3a or Dnmt3b1 (3b2) mRNA.

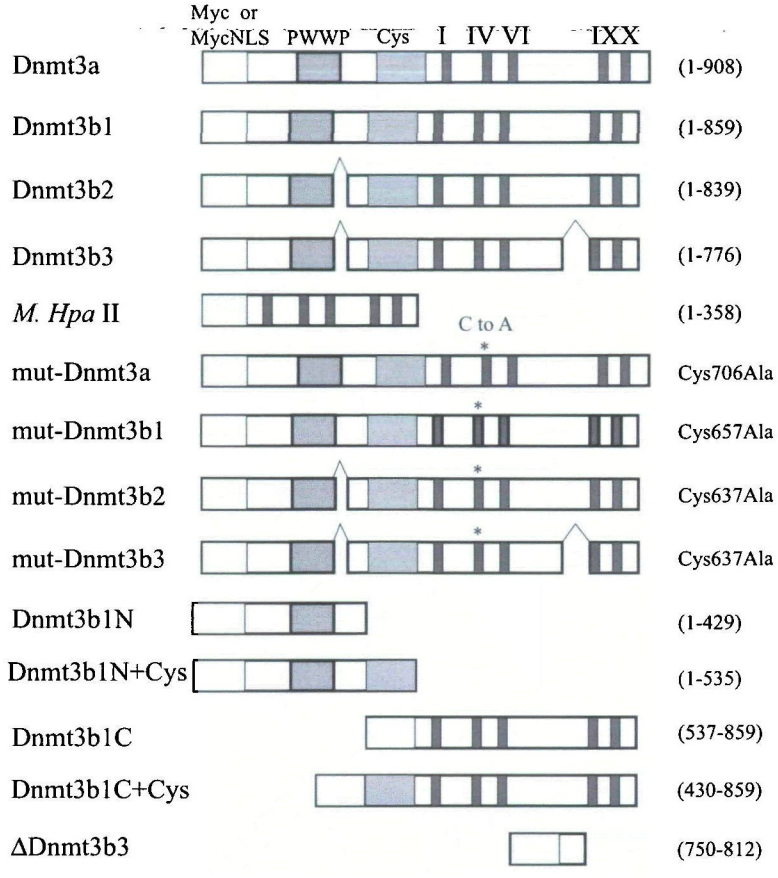

Fig. 1. **Schematic illustration of the constructs used in this study.** The translation products expressed in *Xenopus* embryos are schematically illustrated. Dnmt3b2 lacks amino acid residues 363–382, and Dnmt3b3 lacks amino acid residues 363–382 and 731–813 of Dnmt3b1. "mut-" indicates the mutation of the cysteine residue at the catalytic site in motif IV to an alanine residue (indicated by asterisks). The amino-terminal (N) or carboxyl-terminal (C) half of Dnmt3b with or without the Cys-rich region (Cys) was also constructed. ΔDnmt3b3 is the construct consisting of the sequence that is missing in the carboxyl-terminal region of Dnmt3b3. All the constructs contain a Myc-tag (Myc) or Myc-tag with a nuclear localization signal (MycNLS) in the amino-terminal portion. "PWWP" and "Cys" indicate the PWWP domain and the cysteine-rich region, respectively. I, IV, VI, IX, and X are the conserved motifs of DNA (cytosine-5) methylases.

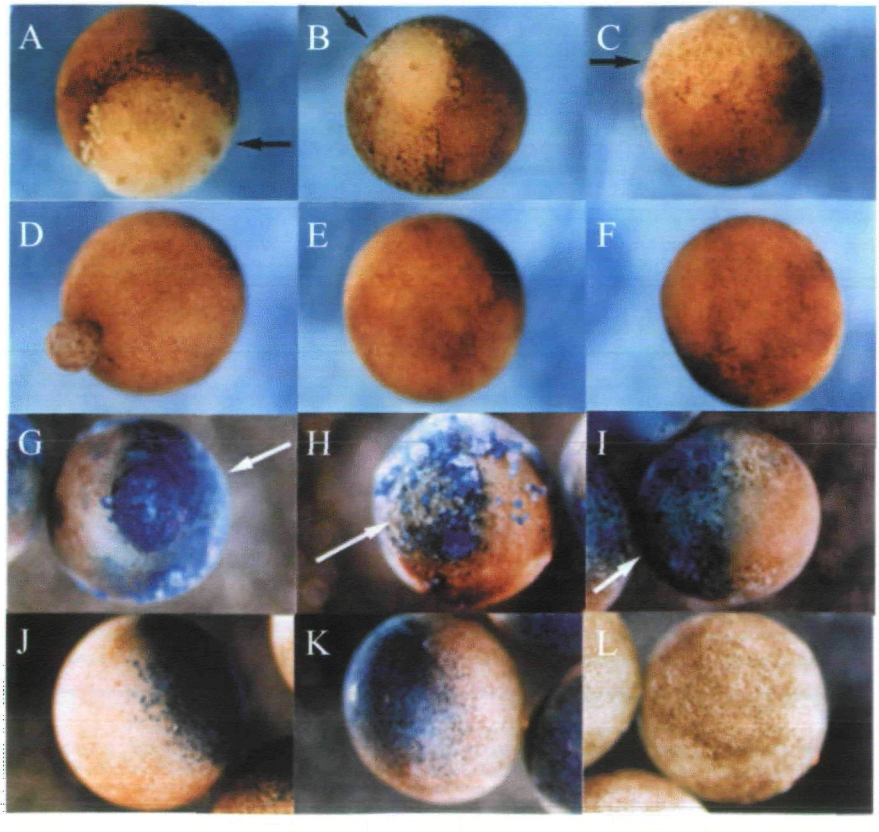

Fig. 2. **Appearance of the embryos that exogenously expressed Dnmt3a, Dnmt-3b1, Dnmt3b2, Dnmt3b3, and *M. Hpa* II.** Two-cell stage embryos were injected with 0.5 ng of β-gal and 3 ng of Dnmt3a (A and G), Dnmt3b1 (B and H), Dnmt3b2 (C and I), Dnmt3b3 (D and J), or *M. Hpa* II (E and K) mRNA. Embryos were incubated until stages 11–12. Panels F and L show uninjected embryos. Each embryo was fixed, then β-gal activity was detected (G-K; stained blue). Black arrows indicate the region where abnormal cells appeared, and white arrows indicate the abnormal cells that are stained blue.

The embryos injected with Dnmt3b3 or *M. Hpa* II showed no morphological abnormality (Fig. 2, D, E, J, and K). The results obtained in four independent microinjection experiments are summarized in Table I. Under the conditions used, all the injected mRNAs were effectively translated into proteins (Fig. 3), indicating that it was not simply a difference in the expression level of Dnmt3a or Dnmt3b1 (3b2) that induced the phenotype.

Abnormal Cells Showed Apoptotic Features—The morphological features of the abnormal cells that appeared in the embryos injected with Dnmnt3a or Dnnmt3b1 (3b2) resembled apoptotic cells (*27, 30*). To examine this possibility, mRNA-injected embryos were analyzed by the TUNEL method, which detects the *in situ* fragmentation of nuclear DNA of cells that have died due to apoptosis (*31*). Whole embryos at stages 11–12 were fixed and then TUNEL stained (Fig. 4). The abnormal cells in embryos injected

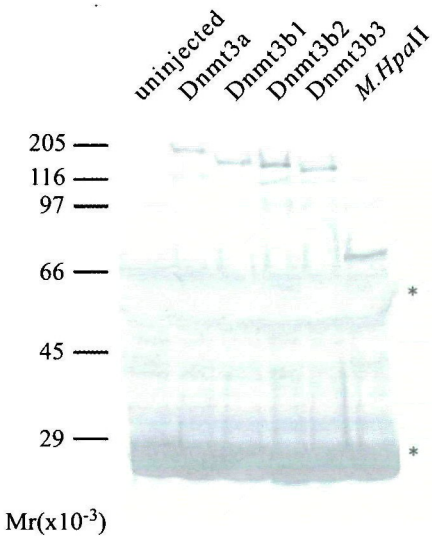

Fig. 3. **Injected mRNA was effectively translated into proteins in *Xenopus* embryos.** Whole-cell extracts prepared from injected embryos at stages 11–12 were immuno-precipitated with anti-Myc monoclonal antibody (9E10), then analyzed by Western blotting with alkaline phosphatase-coupled secondary antibodies. Asterisks indicate the IgG heavy and light chains used for immunoprecipitation. Molecular weight standards (M_r) are indicated.

TABLE I. **Effects of Dnmt3a, Dnmt3b1, Dnmt3b2, Dnmt3b3, and *M. Hpa* II expression on embryogenesis.** Embryos were injected with 3 ng of the indicated mRNA at the two-cell stage and then incubated until stages 11–12. The results obtained in four independent experiments are summarized. The numbers of total embryos (total), and normal embryos (normal), embryos with abnormal cell clusters showing cell dissociation (abnormal), and ones that died of an unidentified cause (other) are shown. The percentages of the embryos that showed abnormal cell dissociation are also shown (%).

mRNA	Total	Normal	Abnormal	Other	%
Uninjected	65	65	0	0	0
Dnmt3a	65	0	63	2	97
Dnmt3b1	65	0	57	8	88
Dnmt3b2	65	0	54	11	83
Dnmt3b3	65	55	1	9	2
M. Hpa II	50	46	0	4	0
β-Gal	65	56	0	9	0

TABLE II. **Dose-dependency on Dnmt3a, Dnmt3b1, Dnmt3b2, Dnmt3b3, and *M. Hpa* II mRNA of the apoptotic phenotype.** Each mRNA (0.3–3 ng per embryo) was injected into 2-cell stage embryos. β-Gal mRNA was a negative control for the apoptotic effect. The percentages of the embryos in which apoptosis was induced at stages 11–12 are shown.

MRNA	0.3	1	3[a]
	(ng mRNA injected/blastomere)		
Dnmt3a	88 (35)[b]	97 (35)	97 (65)
Dnmt3b1	84 (35)	91 (35)	88 (65)
Dnmt3b2	70 (35)	80 (35)	83 (65)
Dnmt3b3	0 (35)	2 (35)	2 (65)
M. Hpa II	0 (35)	0 (35)	0 (50)
β-Gal	0 (35)	0 (35)	0 (65)

[a]Values taken from Table I. [b]Numbers of injected embryos (n) are indicated.

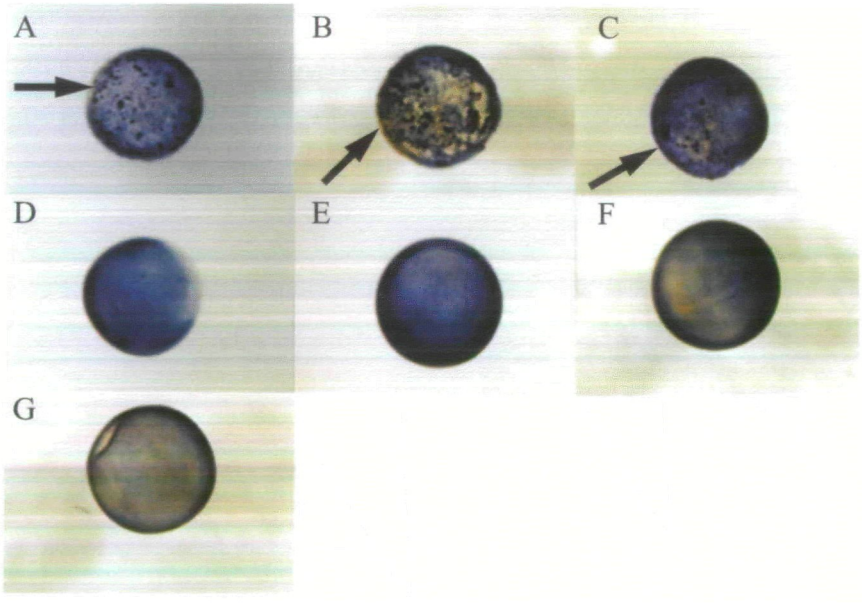

Fig. 4. **The abnormal cells in the embryos injected with Dnmt3a, Dnmt3b1, and Dnmt3b2 were TUNEL-positive.** Embryos were injected with 1 ng of Dnmt3a (A), Dnmt3b1 (B), Dnmt3b2 (C), Dnmt3b3 (D), *M. Hpa* II (E), or β-gal (F) mRNA into the animal-pole side at the two-cell stage. Injected or uninjected (G) embryos were incubated until stages 11–12, fixed, and then stained. Arrows indicate the region where abnormal cells appeared.

with Dnmt3a or Dnmt3b1 (3b2) mRNA were TUNEL-positive, which is a typical feature of apoptosis (Fig. 4, A–C). On the other hand, the embryos injected with Dnmt3b3 or *M. Hpa* II, which exhibited no morphological abnormalities, were TUNEL-negative (Fig. 4, D and E).

To eliminate possible side-effects of high dose injection of mRNAs, we injected different amounts of Dnmt3a, Dnmt3b1, Dnmt3b2, Dnmt3b3, or *M. Hpa* II mRNA into two-cell embryos. Even when the injected dose was reduced to 0.3 ng per blastomere, Dnmt3a or Dnmt3b1 (3b2) significantly induced the apoptotic phenotype (Table II). Injection of β-gal mRNA did not cause any developmental abnormality up to 3 ng per blastomere.

Apoptosis Induced by Dnmt3b1 (3b2) Was Rescued by a Bcl-2 Homologue—Many types of apoptosis, although not all, activate caspases *via* cytochrome *c* export from mitochondria, and this step is suppressed by Bcl-2 or its homologues (*27, 30, 32*). To determine if the apoptosis induced by *de novo* methyltransferases was suppressed by Bcl-2, we co-injected Dnmt3b1 mRNA with hBcl-x_L mRNA, a Bcl-2 homologue (*33*). As shown in Fig. 5, hBcl-x_L effectively suppressed the apoptosis induced by Dnmt3b1 (Fig. 5F). After further incubation until stage 28, the rescued embryos co-injected with hBcl-x_L formed an axis (Fig. 5J), although their development was slightly retarded compared to that of normal embryos. The expression level of the Dnmt3b1 protein at stage 12 was not affected by co-injection of hBcl-x_L (data not shown). This indicates that exogenously expressed Dnmt3b1 activated the apoptosis cascade that induces cytochrome *c* export from mitochondria. The results are summarized in Table III.

The Induced Apoptosis Was Not Due to the DNA Methyltransferase Activity of Exogenously Expressed Dnmt3—The apoptosis was not induced by injected Dnnmt3b3 or *M. Hpa* II, but it was induced by Dnmt3a, Dnbmt3b1, or Dnmt3b2 (Fig. 2). Since Dnmt3b3 possesses no DNA methyltransferase activity (*29*), it is conceivable that the *de novo*

TABLE III. **Apoptosis induced by Dnmt3b1 was prevented by co-injection of hBcl-x_L.** Embryos were injected with 0.5 ng of Dnmt3b1 with or without 4 ng of hBcl-x_L, 4 ng hBcl-x_L alone, or 0.5 ng β-gal mRNA at the two-cell stage, and then incubated until stage 28. The numbers of total embryos (total), and embryos with a normal appearance (normal), ones with apoptosis (apoptosis), and ones that died of an unidentified cause (other) at stage 12, and the numbers of embryos that showed termination of development (arrested) and normal axis formation (axis) at stage 28, from which the numbers for "other" at stage 12 were omitted, are shown.

		Stage 12			Stage 28	
mRNA	Total	Normal	Apoptosis	Other	Arrested	Axis
Dnmt3b1	20	0	15	5	11	4
Dnmt3b1 + hBcl-x_L	20	16	4	0	0	20
hBcl-x_L	20	15	0	5	0	15
β-Gal	20	17	0	3	0	17
Uninjected	20	19	0	1	0	19

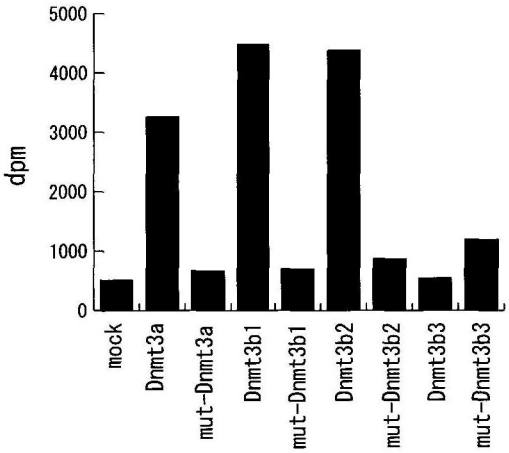

Fig. 6. **Mutant Dnmt3a and Dnmt3b1 (3b2) lack DNA methylation activity.** Plasmids harboring Dnmt3a and Dnmt3bs were expressed in HEK293T cells. The expressed enzymes were immunopurified with anti-Myc monoclonal antibody-coupled Sepharose, then DNA methylation activity was measured. The radioactivity of the methyl group transferred to poly(dG-dC)-poly(dG-dC) was determined (dpm).

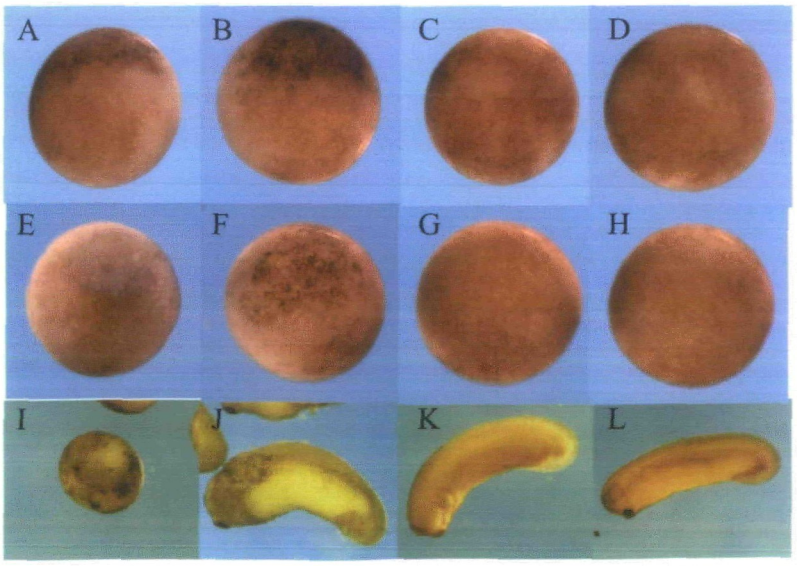

Fig. 5. **Bcl-x_L effectively suppressed the apoptosis induced by Dnmt3b1.** Embryos were injected with 0.5 ng of Dnmt3b1 (A, E, and I), 0.5 ng of Dnmt3b1 and 4 ng of hBcl-x_L (B, F, and J), or 4 ng of hBcl-x_L (C, G, and K). Panels D, H, and L show uninjected embryos. The photographs were taken at stages 10.5 (A–D), 12 (E–H), and 28 (I–L).

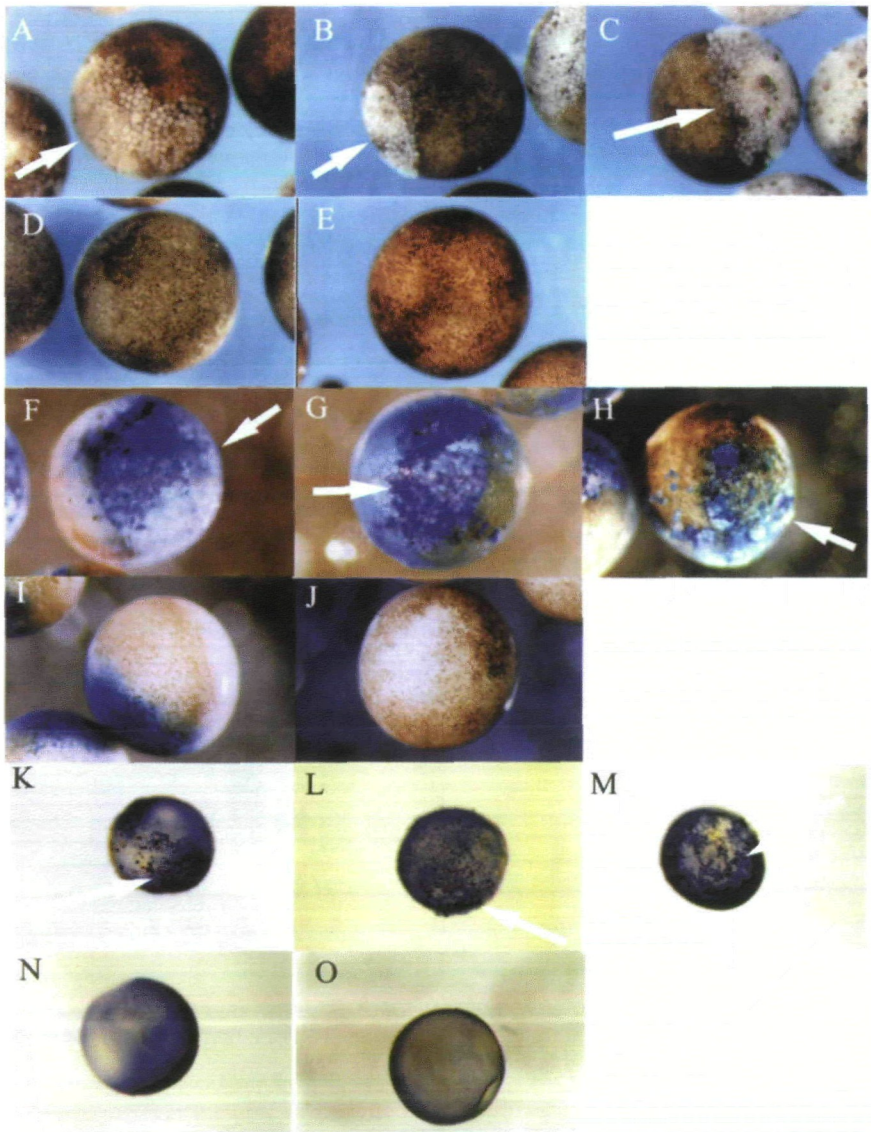

Fig. 7. **Mutated Dnmt3a and Dnmt3b1 (3b2) still induced apoptosis.** The appearance of embryos that exogenously expressed mutant (mut-) Dnmt3s. Embryos were injected with 0.5 ng of β-gal and 1 ng of mut-Dnmt3a (A, F, and K), mut-Dnmt3b1 (B, G, and L), mut-Dnmt3b2 (C, H, and M), or mut-Dnmt3b3 (D, I, and N). Panels E, J, and O show uninjected embryos. Each embryo was fixed, its β-gal activity was detected (F–J), and then it was TUNEL-stained (K–O). Arrows indicate the cells showing apoptotic features.

TABLE IV. **Effects of mutant (mut-) Dnmt3a, Dnmt3b1, Dnmt3b2, and Dnmt3b3 expression on embryogenesis.** Embryos were injected with 1 ng of the indicated mRNA at the two-cell stage and then incubated until stages 11–12. The numbers of embryos with a normal appearance (normal), with an apoptotic appearance (apoptosis), and with a dead or abnormal appearance, other than apoptosis (other), are shown. The percentages of embryos that showed apoptosis are also shown (%)

mRNA	Total	Normal	Apoptosis	Other	%
Uninjected	45	45	0	0	0
mut-Dnmt3a	44	0	40	4	91
mut-Dnmt3b1	45	0	34	11	76
mut-Dnmt3b2	30	0	28	2	93
mut-Dnmt3b3	45	38	3	4	7
β-Gal	45	37	0	8	0

DNA methyltransferase activity of Dnmt3a and Dnmt3b1 (3b2) was the cause of the apoptosis. However, bacterial *de novo* DNA methyltransferase *M. Hpa* II, which specifically methylates the CCGG sequence, did not affect early embryogenesis (Fig. 2, E and K).

To determine if the DNA methyltransferase activity was the cause of the apoptosis, we constructed mutants of Dnmt3a and three Dnmt3b isoforms, of which cysteine residues in the Pro-Cys dipeptide in motif IV were changed to alanine residues (Fig. 1), which were expected to abolish the catalytic activity (*34, 35*). As expected, none of the mutants exhibited methylation activity (Fig. 6). Surprisingly, the injection of mutated Dnmt3a and Dnmt3b1 (3b2) still induced apoptosis (Fig. 7, A, B, C, F, G, H, K, L, and M). Again mutated Dnmt3b3 did not induce apoptosis (Fig. 7, D, I, and N). All the mutants were efficiently translated in injected embryos at the gastrula stage (data not shown). The results are summarized in Table IV. The results clearly indicate that the apoptotic phenotype was independent of the DNA methyltransferase activity of Dnmt3a or Dnmt3b1 (3b2).

Apoptosis Was Caused by the Carboxyl-Terminal Catalytic Domain of Dnmt3b1—The injection of Dnmt3b3, which lacks the 63 amino acid residues from after motif VIII to the middle of motif IX in the carboxyl-terminal region in the catalytic domain, did not induce apoptosis. This suggests that the carboxyl-terminal half of Dnmt3b

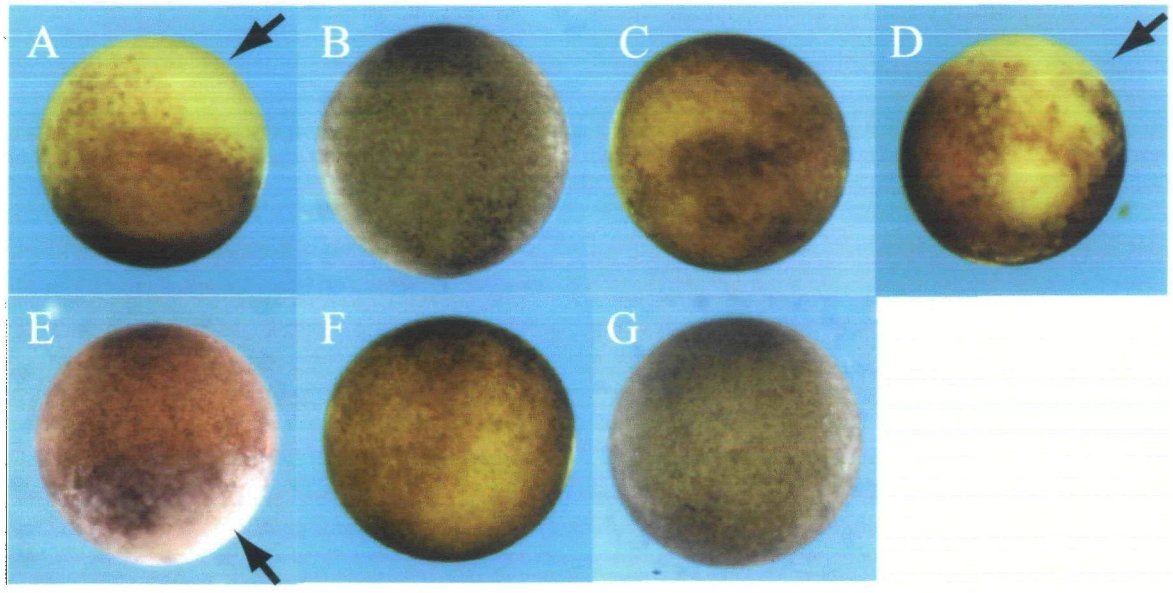

Fig. 8. **Carboxyl-terminal half of Dnmt3b1 induced apoptosis.** Embryos were injected with 1 ng of Dnmt3b1 (A), the amino-terminal half with (Dnmt3b1N+Cys) (B) or without the Cys-rich region (Dnmt3b1N) (C), the carboxyl-terminal half with (Dnmt3b1C+Cys) (D) or without the Cys-rich region (Dnmt3b1C) (E), and the sequence in the carboxyl-terminal region that is missing in Dnmt3b3 (ΔDnmt3b3) (F) into the animal-pole side at the two-cell stage, and then incubated until stages 11–12. Panel G shows an uninjected embryo. Arrows indicate the cells that showed apoptotic features.

TABLE V. **Effects of injection of the amino- and carboxyl-terminal domains of Dnmt3b1.** Embryos were injected with 1 ng of each mRNA construct into the animal-pole side at the two-cell stage, and then incubated until stages 11–12. The results obtained in several experiments are summarized. The numbers of embryos with a normal appearance (normal), with an apoptotic appearance (apoptosis), and that died of other than apoptosis (other) are shown. The percentages of embryos that showed apoptosis are also shown (%).

mRNA	Total	Normal	Apoptosis	Other	%
Uninjected	70	70	0	0	0
Dnmt3b1	65	0	63	2	97
Dnmt3b1N	20	19	0	1	0
Dnmt3b1N+Cys	31	27	1	3	3
Dnmt3b1C	50	2	41	7	82
Dnmt3b1C+Cys	20	0	19	1	95
DnmtΔ3b3	20	19	0	1	0
β-Gal	50	36	0	14	0

could be responsible for the phenotype. The carboxyl-terminal region is highly homologous between Dnmt3a and Dnmt3b. We thus examined the responsible domain, which may exist in the carboxyl-terminal half, as to the induction of apoptosis using Dnmt3b1. Constructs encoding the amino- and carboxyl-terminal halves of Dnmt3b1 with or without the Cys-rich region were constructed (Fig. 1) and injected into embryos. As expected, only the injection of the 3′ half encoding the carboxyl-terminal half induced apoptosis (Fig. 8B, C, D, and E, and Table V). Apoptosis was also induced by the construct that lacked the Cys-rich region. mRNA coding the 63-amino-acid sequence of Dnmt3b1 and Dnmt3b2 that is missing in Dnmt3b3 was constructed and injected into embryos, but it did not induce apoptosis (Fig. 8F and Table V).

DISCUSSION

In the present study, we exogenously expressed mouse *de novo* DNA methyltransferases Dnmt3a and Dnmt3b in *Xenopus* embryos and found that they induced apoptosis at the gastrulation stage. In *Drosophila*, it has been demonstrated that the exogenous expression of mouse Dnmt3a induces a developmental defect, and a truncated Dnmt3a that lacks the catalytic domain does not give any phenotype (*7*). Unlike in *Drosophila*, the apoptotic phenotype observed in *Xenopus* embryos on Dnmt3 expression was not due to the *de novo* methylation activity of the Dnmt3 family. This was shown by the facts that bacterial cytosine methylase *M. Hpa* II, which possesses *de novo* methylation activity, did not induce apoptosis, and, more directly, that the Dnmt3s in which the cysteine residue in the catalytic center had been changed to an alanine residue, and which were enzymatically inactive, also induced apoptosis.

The possibility that exogenously expressed Dnmt3s nonspecifically triggered apoptosis is unlikely. Firstly, although no homologue of Dnmt3s has yet been identified in *Xenopus*, such a homologue may exist, since early *Xenopus* embryos exhibit *de novo* methylation activity (*15*), and zebra fish has Dnmt3 that is highly homologous to the mouse or human Dnmt3 family (*36*). Secondly, the expression of Dnmt3b3, which has an identical sequence to Dnmt3b2 except for lacking 63 amino acid residues in the carboxyl-terminal half (see Fig. 1), did not give any abnormal phenotype on embryogenesis. As the short sequence missing in Dnmt3b3 by itself could not induce apoptosis (Fig. 8 and Table V), the apoptotic phenotype caused by Dnmt3b1 (3b2) is due to the higher order structure of its carboxyl-terminal region.

Recently, Stancheva *et al.* reported that the DNA demethylation caused by the depletion of Dnmt1 induces apopto-

sis (*15*). Similar to in our present work, the apoptosis induced by DNA demethylation is rescued by co-expression of Bcl-2, indicating that both apoptotic events are triggered upstream of the Bcl-2 signal. In Bcl-2 rescued embryos, however, abnormal cell proliferation is induced (*15*). In our study, however, co-expression of hBcl-x_L not only prevented the apoptosis caused by exogenously expressed Dnmt3s but also, importantly, rescued normal axis formation (see Fig. 5J). Therefore, the trigger of apoptosis induced by exogenously expressed Dnmt3s may not be the same as that caused by genome-wide demethylation due to the depletion of Dnmt1 (*15*).

An apoptotic phenotype at the early gastrulation stage in *Xenopus* has been reported when DNA replication, transcription, or translation is inhibited (*27, 37*). Recent reports have shown that Dnmt3a and/or Dnmt3b repress transcription in a manner that does not require DNA methylation activity (*20, 38*). Dnmt3a inhibits the transcription by interacting with co-repressor RP58 and histone deacetylase (HDAC) (*20*). RP58 and HDAC have been reported to bind to Dnmt3a through the Cys-rich region (Fig. 1). On the other hand, since the Cys-rich region is not responsible for the apoptosis observed in the present study (Fig. 8 and Table V), the transcription repression caused by RP58 and HDAC may not be involved in the apoptotic event.

Since Dnmt3s is a DNA-binding protein, the possibility that the exogenously expressed Dnmt3s may occupy some part of genomic DNA to inhibit transcription should be considered. This possibility is supported by the fact that Dnmt3b3, which lacks 63 amino acid residues between catalytic motifs VIII and IX, the corresponding site of which is known as the part of target DNA sequence recognition domain (TRD) in bacterial cytosine methyltransferases (*39*), did not induce apoptosis. Recently, the amino-terminal half of Dnmt3b has been shown to bind DNA through the PWWP domain (*40*). The amino-terminal half of Dnmt3b1 (3b2), however, did not induce apoptosis. Therefore, it is unlikely that the DNA binding of Dnmt3s alone induced the apoptosis.

Considering varieties of possible mechanisms, the overexpressed Dnmt3s seem to deplete the endogenous factor that plays a crucial role in the apoptotic pathway in *Xenopus* embryos. In any case, the specific conformation of the catalytic domain is expected to be required for such an interaction. Further characterization of the target of Dnmt3s needs to be performed.

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