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and Intracellular Localization during Oogenesis and Preimplantation Development

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The imprinting of mammalian genes depends on the maintenance of DNA methylation patterns during pre- and postimplantation development. Dnmt1o is a variant form of the somatically expressed Dnmt1 cytosine methyltransferase that is synthesized and stored in the oocyte cytoplasm and trafficks to the eight-cell nucleus during preimplantation development, where it maintains DNA methylation patterns on alleles of imprinted genes. Transcripts encoding Dnmt1 are present in preimplantation embryos, suggesting that Dnmt1 protein is also expressed in the preimplantation embryo, and may account for maintenance methylation at preimplantation stages other than the eight-cell embryo. However, using an antibody that detects Dnmt1, but not Dnmt1o, no Dnmt1 protein was detected on immunoblots or by immunocytochemical staining in wildtype preimplantation embryos. Moreover, Dnmt1 protein produced in the oocyte from a modified Dnmt1 allele, *Dnmt1*$^{1s/1o}$, trafficked to nuclei of eight-cell embryos, but not to nuclei of other stages. The highly restricted nuclear localization patterns of oocyte-derived Dnmt1o and Dnmt1 during preimplantation development add further support to the notion that DNA methyltransferases other than Dnmt1 are required for maintaining imprints during preimplantation development.

Key Words: **DNA methyltransferase; Dnmt1; DNA methylation; genomic imprinting; oocyte growth; preimplantation embryo; mouse.**

INTRODUCTION

The predominant form of DNA (cytosine-5)-methyltransferase in mammals is Dnmt1, a protein of relative molecular weight 190,000 (M_r 190,000) composed of a large amino-terminal regulatory domain and a smaller carboxy-terminal catalytic domain that is closely related to bacterial C5-specific restriction methyltransferases (Bestor, 2000). Sequences within the N-terminal domain are required for the localization of Dnmt1 to the nucleus and to replication foci during S-phase (Leonhardt *et al.,* 1992). Although Dnmt1 catalyzes both *de novo* and maintenance methylation *in vitro*, it has a 5- to 30-fold preference for hemimethylated DNA substrates over unmethylated substrates (Yoder *et al.,* 1997), suggesting that its main role, or only role, in mammalian organisms is to maintain DNA methylation patterns. Homozygous mutant ES cells and embryos derived from matings between parents heterozygous for Dnmt1 null alleles have marked reductions in Dnmt1 and in the level of CpG methylation (Lei *et al.,* 1996). The residual methylation appears to be confined to repetitive DNA sequences, whose methylation may be due to other DNA (cytosine-5)-methyltransferases.

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Genes encoding Dnmt1-like proteins, such as Dnmt3a and Dnmt3b, have recently been cloned and their methyltransferase activities measured. *In vitro*, Dnmt3a and Dnmt3b primarily possess *de novo* methyltransferase activities (Okano *et al.*, 1998, 1999; Xu *et al.*, 1999). Moreover, the majority of genomic methylation is retained when Dnmt3a and Dnmt3b are removed from the developing embryo, consistent with a lack of any significant contribution to maintaining methylation patterns (Okano *et al.,* 1999). These observations on Dnmt1 and Dnmt1-related proteins in the mouse have raised the possibility that Dnmt1 provides all of the maintenance methyltransferase activity in the mammalian organism.

The Dnmt1 protein is abundantly expressed in cells of the developing embryo, and it is readily detected in nuclei of all cells of the early postimplantation embryo (Trasler *et al.*, 1996; Yoder *et al.*, 1997). However, Dnmt1 protein has not been detected in oocytes and in cells of preimplantation embryos (Cardoso and Leonhardt, 1999; Carlson *et al.*, 1992; Mertineit *et al.*, 1998). In these stages, there is an abundance of a variant Dnmt1 protein called Dnmt1o, with a relative molecular weight of 175,000 (M_r 175,000). Dnmt1o protein is synthesized from an oocyte-specific transcript, which is translated into a truncated version of Dnmt1 that is missing its N-terminal 118 amino acids (Mertineit *et al.*, 1998). Dnmt1o protein is in the cytoplasm of the mature metaphase II (MII) oocyte, and in the cytoplasm of all preimplantation cleavage stages, but is only found in the nucleus of blastomeres at the eight-cell stage (Cardoso and Leonhardt, 1999; Carlson *et al.*, 1992; Howell *et al.*, 2001; Mertineit *et al.*, 1998). Studies in mice homozygous for a targeted disruption of the Dnmt1 oocyte-specific promoter indicate that preimplantation Dnmt1o protein is derived from the oocyte's cytoplasmic Dnmt1o (Howell *et al.*, 2001). Heterozygous mutant embryos derived from homozygous mutant oocytes lack Dnmt1o protein during their preimplantation development and have a 50% reduction in methylated alleles of imprinted genes, most likely because the loss of nuclear Dnmt1o protein at the eight-cell stage leads to a loss of maintenance methylation in eight-cell blastomeres. The proteins that supply the maintenance methyltransferase activity for the other S phases of preimplantation development are unknown.

Genetic experiments to examine the role of Dnmt1 in maintaining methylation patterns during preimplantation development have not been described. The possibility of an oocyte Dnmt1 contribution cannot be easily tested because of the lethal nature of the constitutive Dnmt1 null mutation (Lei *et al.*, 1996) and the difficulty in devising a conditional Dnmt1 mutation that selectively removes Dnmt1 protein from all stages of oogenesis. In addition to oocyte sources of Dnmt1, embryonically expressed Dnmt1 might also be involved in maintaining imprints during preimplantation development. It would be feasible, although quite difficult, to test this possibility. Dnmt1 is known to maintain virtually all DNA methylation in the postimplantation embryo, and is an essential protein for postimplantation embryonic development (Lei *et al.*, 1996). Therefore, a genetic test of whether or not Dnmt1 protein that is made in the preimplantation embryo contributes to the maintenance of methylation patterns on imprinted genes would require the measurement of methylation patterns in preimplantation embryos that are homozygous for a null Dnmt1 allele. The presence of unmethylated copies of parental alleles that normally maintain their gametic methylation would implicate Dnmt1 in the maintenance of imprinted methylation patterns during preimplantation development.

In lieu of genetic experiments to decipher the role of Dnmt1 in maintaining methylation patterns during preimplantation development, we measured Dnmt1 expression and intracellular localization in oocytes and cleavage-stage preimplantation embryos. In addition to measuring *Dnmt1* and *Dnmt1o* mRNAs, we used three different antibodies to determine the levels and intracellular locations of Dnmt1 and Dnmt1o proteins. One antibody detects epitopes found only in Dnmt1 and the other two detect epitopes found in both Dnmt1 and Dnmt1o. The antibodies were used to detect Dnmt1 and Dnmt1o on immunoblots or by immunostaining individual oocytes or preimplantation-stage embryos. Finally, we used the Dnmt1-specific antibody to follow the fate of a large maternal contribution of Dnmt1 protein produced in a mouse line containing a modified Dnmt1 locus.

MATERIALS AND METHODS

Animals

All wild-type tissues, oocytes, and embryos were obtained from CD-1 mice (Charles River Canada, St. Constant, QC). The engineered mouse line with a modified Dnmt1 allele, in which exon 1o was replaced with exon 1s sequences, is designated $Dnmt1^{1s/1o}$. Standard gene modification methods were used to produce the targeting vector for this allele (see Fig. 5), which was then transferred into W9.5 ES cells (gift of C. Stewart). Excision of the resistance cassette after electroporation of a Cre recombinase-expressing plasmid was confirmed by PCR with primers of sequence 5′-AGGAAAACAGTGGAGGAAAC-3′ and 5′-TACTTGCACAGGGCTGTCCT-3′. Oocytes and preimplantation embryos were obtained from $Dnmt1^{1s/1o}$ females on a mixed 129/Sv and C57BL/6 background. Noon of the day on which a vaginal plug was found was designated embryonic day 0.5 (E0.5) and the day of delivery as postnatal day 1 (D1). Embryos from timed pregnant females were removed from the uterine horns at E7.5 and at E17.5–E18.5. Testes and ovaries could be distinguished in embryos at E12.5 and later by the presence or absence of seminiferous cords, respectively (Hogan *et al.*, 1986).

Collection of Oocytes and Embryos

Immature oocytes in prophase I of meiosis were collected at E17.5–E18.5, D1 (at birth), D5, D10, and D15 (Eppig and Telfer, 1993). Ovaries from three or four mice were dissected in phosphate-buffered saline (PBS), pH 7.2, teased into smaller pieces, and transferred to 2 ml of PBS, 2 mg/ml collagenase (Sigma), 0.025% trypsin (Gibco BRL, Burlington, ON) and 0.02 mg/ml DNase (Sigma). The tube was capped tightly and shaken at high speed for

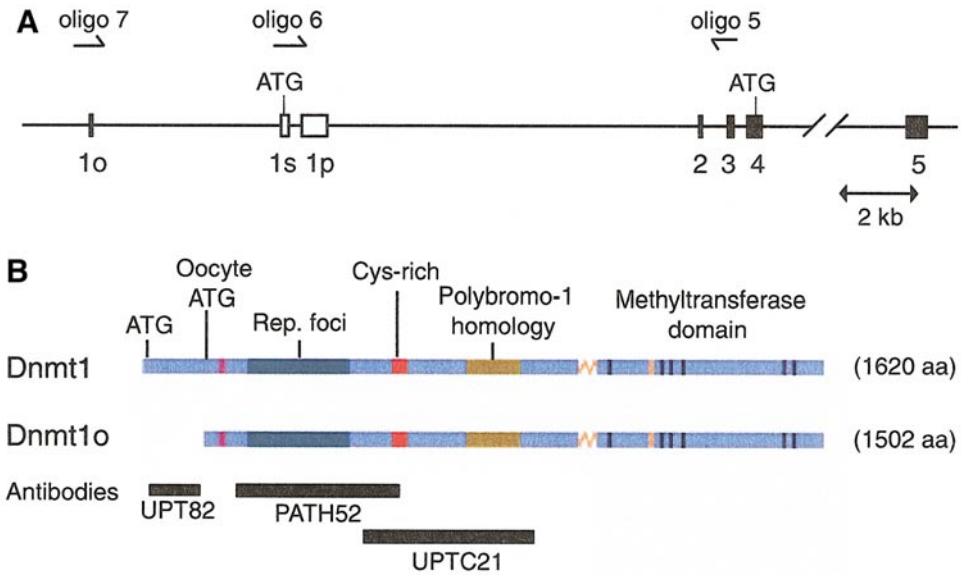

FIG. 1. Sex-specific exons and oocyte-specific species of Dnmt1. (A) Organization of sex-specific exons in the 5′ end of the *Dnmt1* gene. The oocyte-specific *Dnmt1* transcript initiates at exon 1o and is spliced to exon 2; Dnmt1o translation is initiated at the ATG in exon 4 (Mertineit *et al.*, 1998). The resulting protein is 118 amino acids shorter than the somatic form, whose ATG codon is located in exon 1s (also spliced to exon 2). (B) Comparison of Dnmt1 and Dnmt1o. The difference between the two forms of Dnmt1 proteins is not known to extend beyond the N-terminal truncation of Dnmt1o. The somatic and oocyte ATG codons are shown, as are a sequence that mediates interaction of Dnmt1 with replication foci during S phase of somatic cells (Rep. foci), a zinc-binding cysteine-rich domain (Cys-rich), and a domain that is related to a domain in Polybromo-1 from chicken. (Taken from Bestor, 2000). The epitopes specific to the three antibodies UPT82, UTC21, and PATH52 are shown in the figure.

10 min at 37°C, and the contents were subsequently diluted with one volume of Hepes-buffered minimal essential medium, MEM-H (Gibco BRL), modified as described (Clarke *et al.*, 1988; Schroeder and Eppig, 1984). Oocytes were collected individually by a micropipette from the dissociated ovaries.

Fully grown, germinal vesicle (GV)-stage oocytes were obtained by puncture of ovarian follicles of 21- to 35-day-old females in MEM-H supplemented with 100 μg/ml of dibutyryl cyclic AMP, dbcAMP (Sigma), as previously described (Clarke *et al.*, 1992). The dbcAMP prevents meiotically competent oocytes from undergoing germinal vesicle breakdown (GVBD) in culture (Cho *et al.*, 1974). GVBD oocytes were derived from GV oocytes that were isolated in MEM-H free of dbcAMP and incubated in 5-μl drops of bicarbonate-buffered MEM under oil at 37°C in an atmosphere of 5% CO_2 in air for 2–4 h.

Metaphase II (MII) oocytes were collected from 7-week-old females that were superovulated by injection of 7.5 IU of pregnant mares' serum gonadotropin, PMSG (Ayerst Vetlabs), followed 44–48 h later by 5 IU of human chorionic gonadotropin, hCG (Ayerst Vetlabs). These oocytes were recovered from the oviducts 20 h post-hCG, and the cumulus cells were dispersed with 1 mg/ml hyaluronidase (Roche Diagnostics, Laval, QC), as described (Hogan *et al.*, 1986).

Preimplantation embryos were obtained as previously described (Clarke *et al.*, 1992). Superovulated females were caged individually with stud males overnight, and examined for the presence of a vaginal plug the next morning. One-cell embryos were recovered from the oviducts at E0.5 in Hepes-buffered KSOM medium (Erbach *et al.*, 1994), and the cumulus mass was dispersed as described above. Two-cell embryos were obtained by flushing the oviducts of females killed 1.5 days after hCG administration. Four-cell embryos, eight-cell embryos, morula, and blastocysts were obtained by culturing two-cell embryos in 5-μl drops of bicarbonate-buffered KSOM or CZB media under oil at 37°C in an atmosphere of 5% CO_2 in air. Postimplantation embryos were dissected out of the uterine horns at E7.5.

For all isolation procedures, healthy-looking oocytes and preimplantation embryos were collected in a 35-mm Petri dish by using a mouth-controlled micropipette and washed free of any adhering somatic cells by transfer through two dishes of culture medium. Oocytes and embryos were either processed immediately for immunofluorescence or pooled and stored at −80°C in DEPC-PBS for RNA extraction; stored at −80°C in lysis buffer for immunoblotting; or stored at −80°C in culture medium for DNA isolation.

RNA Isolation and RT-PCR

Total RNA was extracted from homogenized tissues by using the Trizol reagent (Gibco BRL) according to the manufacturer's instructions. For isolated oocytes and preimplantation embryos (80 per tube), 10 μg of glycogen (Roche Diagnostics) was added to the Trizol and cellular RNA was recovered as described previously (Clarke *et al.*, 1997). RNA pellets obtained from these isolated cells were dissolved in 20 μl of DEPC-treated water. The SuperScript One-Step RT-PCR System (Gibco BRL) was used to analyze transcripts produced by exon 1o and exon 1s of *Dnmt1* (Mertineit *et al.*, 1998). Reactions were performed as directed by the manufacturer. For tissues, 0.5 μg of total

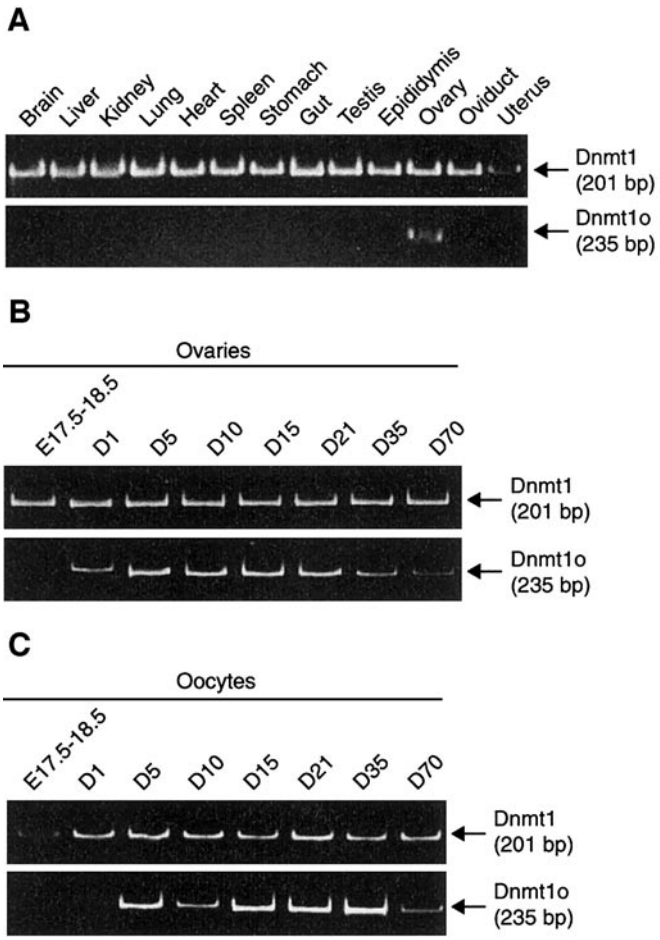

FIG. 2. RT-PCR analyses of *Dnmt1* mRNA splice variants in tissues, ovaries, and oocytes. (A) Reverse transcription PCR (RT-PCR) was performed on RNA samples isolated from different adult tissues. The *Dnmt1* transcript is identified as a 201-bp PCR fragment using oligo 6 and oligo 5 in the PCR (Fig. 1A). The *Dnmt1o* transcript is identified as a 235-bp PCR fragment using oligo 7 and oligo 5 in the PCR (Fig. 1A). (B) RT-PCR analysis of *Dnmt1* and *Dnmt1o* transcripts from ovaries isolated from embryos (E) at 17.5 to 18.5 days of development or ovaries isolated at different days (D) following birth. (C) RT-PCR analysis of oocytes isolated from different embryonic or postnatal stages.

RNA was used in a 25-μl RT-PCR. For isolated cells, 10 μl of the dissolved RNA pellet was added to the 25-μl RT-PCR. Oligo 5 (Mertineit *et al.*, 1998), 5′-GCAGGAATTCATGCAGTAAG-3′, is specific to exon 3, which is common to both *Dnmt1* and *Dnmt1o* transcripts. Oligo 6 (5′-GGGTCTCGTTCAGAGCTG-3′) is specific to exon 1s, and oligo 7 (5′-GGTTGATTGAGGGTCATT-3′) is specific to exon 1o (Mertineit et al., 1998). Reverse transcription was allowed to proceed for 10 min at 50°C then shifted to 55°C for 30 min. PCR involved 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. A final extension at 72°C was performed for 7 min. A portion of the reaction (8 or 10 μl) was analyzed by electrophoresis through 12% polyacrylamide gels.

Immunoblotting

Oocytes and preimplantation embryos were collected manually from washings of oviducts or uteri and pooled and lysed in 5% SDS, 2.5% glycerol, 2.5% β-mercaptoethanol, 0.01% bromophenol blue, 0.025 M Tris–HCl, pH 6.8 (1× sample buffer). Lysates of whole gonads, accessory reproductive tissues, and postimplantation embryos were prepared by homogenization in 0.15 M NaCl, 0.05 M Tris–HCl (pH 7.5), 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 5 mM phenylmethylsulfonylfluoride, centrifuged, and the supernatant was added to an equal volume of 2× sample buffer. The amount of protein in the tissue lysates was quantified by using the amido black protein spot assay (Dieckmann-Schuppert and Schnittler, 1997). All samples were denatured by heating at 95°C for 5 min, and then separated by electrophoresis on SDS-5% polyacrylamide gels. Afterwards, they were transferred to Hybond ECL nitrocellulose membranes (Pharmacia Biotech, Baie d'Urfé, QC). The gels were stained with Coomassie Blue to examine the efficiency of protein transfer and membranes were stained with Ponceau S to confirm the presence of the appropriate amount of protein in all lanes. Membranes were blocked in 5% Carnation dry skim milk in 10 mM Tris–HCl (pH 8.0), 140 mM NaCl, for a least 1 h and probed with UPT82 (see below) or preimmune serum diluted 1:3000 in blocking buffer overnight. Following three washes of 5 min each in 0.1% Tween-20 TBS, the membrane was incubated for 1 h at room temperature in biotinylated donkey anti-rabbit IgG (Jackson Research Labs) diluted 1:5000 in blocking buffer. The membrane was washed as above, incubated for 30 min in a 1:1000 dilution of streptavidin–horseradish peroxidase conjugate (Amersham), and washed as above. Bound antibody was detected by chemiluminescence (ECL Plus; Amersham). The same membranes were reprobed with a 1:15,000 dilution of PATH52 antibody, generated against epitopes between amino acids 255 and 753 of Dnmt1 (Carlson *et al.*, 1992).

Immunocytochemistry

Two new antibodies were generated to investigate the Dnmt1 and Dnmt1o expression in oocytes and preimplantation embryos. UPT82 was generated by immunizing a rabbit with a fusion protein that contained the full-length bacterial GST protein and the amino terminal 118 amino acids of Dnmt1. These 118 amino acids are not found in Dnmt1o. UPT82 was affinity purified on a column containing a 6-histidine–118-amino-acid fusion protein. UPTC21 was generated by immunizing a chicken with a fusion protein of 6-histidine plus a segment of Dnmt1 from amino acid 636 to amino acid 1108. These amino acids are present in both Dnmt1 and Dnmt1o.

Denuded oocytes and preimplantation embryos were freed of the zona pellucida by using acidified (pH 2.5) Tyrode's medium (Hogan *et al.*, 1986), and fixed for 10–15 min at room temperature in 3.7% formaldehyde in PBS. Alternatively, small ovarian follicles were fixed intact. All solutions for immunofluorescence of Dnmt1 were prepared in PBS and procedures were carried out at room temperature, unless specified otherwise. The fixed cells were blocked for at least 1 h in blocking buffer (3% BSA, 0.1% Triton X-100) and then incubated in either 1:100 preimmune serum, 1:100 UPT82, 1:100 UPTC21, or 1:500 PATH52 antibodies diluted in the same blocking buffer overnight at 4°C in a humidified chamber. The cells were washed three times for 5 min each in blocking buffer, then incubated in 1:250 of goat-anti-rabbit Bodipy-TMRX secondary antibody (Molecular Probes, Eugene, OR) or in 1:4000 of a rabbit-anti-chicken secondary antibody for 1 h, and washed as before. To

mount the cells for viewing, a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, CA) supplemented with 0.4 μg/ml of the DNA-binding dye DAPI (Roche Diagnostics) was placed on a glass microscope slide and the cells were transferred into the drop with a pipette. A cover slip was gently placed on the drop to spread the mounting medium and flatten the cells, and was subsequently sealed with nail polish. Immunofluorescence was visualized by using a Zeiss Axiophot, Zeiss LSM410 laser scanning confocal microscope. All images were recorded under identical laser settings. The specificity of the UPT82 antibody for the Dnmt1 protein was demonstrated by incubating the antibody with saturating amounts of the Dnmt1-specific 118-aa polypeptide (with a 6-histidine tag at the N terminus) at 4°C overnight and adding it to fixed embryos obtained from *Dnmt1*$^{1s/1o}$ homozygous females. Under these conditions, no staining was observed.

RESULTS

Expression of Dnmt1 mRNA Splice Variants in Tissues and Isolated Oocytes

The relative positions of the three alternative first exons (1o, 1s, and 1p) of the *Dnmt1* gene are shown in Fig. 1A. Exon 1s transcripts are translated into the 1620-aa M_r 190,000 Dnmt1 protein, whereas exon 1o transcripts are translated into the 1502-aa M_r 175,000 Dnmt1o protein (Fig. 1B). The initiation codon for Dnmt1 is in exon 1s, whereas the initiation codon for Dnmt1o is an internal methionine codon of Dnmt1, located in the common exon 4 (Mertineit *et al.*, 1998; Yoder *et al.*, 1996). Compared to Dnmt1o, Dnmt1 contains an additional 118 amino acids at its amino terminus (Fig. 1B). Portions of Dnmt1 used to generate PATH52 (Carlson *et al.*, 1992), UPT82, and UPTC21 polyclonal antisera are shown in Fig. 1B. To understand the temporal and spatial patterns of Dnmt1 and Dnmt1o expression, we measured Dnmt1 and Dnmt1o transcript and protein levels during development. Transcript expression was determined by RT-PCR, and protein expression by immunoblotting and immunocytochemistry.

Using a forward oligonucleotide primer (oligo 6) in exon 1s and a reverse primer (oligo 5) in exon 3, we measured levels of *Dnmt1* transcripts in adult tissues. Similarly, *Dnmt1o* transcripts were measured by using a forward oligonucleotide primer in exon 1o (oligo 7) and oligo 5. The 201-bp PCR product representing the *Dnmt1* transcript was present in all adult tissues examined. In contrast, the 235-bp *Dnmt1o* product was detected exclusively in postnatal ovaries (Figs. 2A and 2B). To determine whether transcript levels were associated with the growth and maturation of oocytes, we measured *Dnmt1* and *Dnmt1o* transcripts in isolated oocytes at different stages of development. As shown in Fig. 2C, *Dnmt1* transcripts were present at low levels in fetal oocytes and at higher levels in postnatal oocytes at all stages. In contrast, *Dnmt1o* transcripts were first detected in isolated oocytes as early as postnatal day 5 and were subsequently seen at increasing levels in all classes of growing oocytes. The amount of *Dnmt1o* transcripts in MII oocytes was significantly less than the level in GVBD-stage oocytes (data not shown). The absence of *Dnmt1o* transcripts in isolated D1 oocytes (Fig. 2C), but its presence in D1 ovaries (Fig. 2B) is likely due to the small number of manually collected oocytes used in the RT-PCR assay and the preponderance of nongrowing oocytes in the collection.

Immunoblotting and Immunostaining of Dnmt1 Protein Isoforms in Isolated Oocytes

The detection of *Dnmt1* and *Dnmt1o* transcripts in growing oocytes (D5 through D70 lanes in Fig. 2C) suggests that both forms of the Dnmt1 protein might be present in these germ cells. In MII oocytes, Dnmt1o was the only protein detected on immunoblots using the PATH52 antibody; earlier stages of oogenesis were not examined (Carlson *et al.*, 1992). To determine more precisely the forms of Dnmt1 protein present in oocytes at various stages of development, an antibody (UPT82) was produced in a rabbit immunized with the N-terminal 118 amino acids of Dnmt1. This antibody provides a means of directly detecting Dnmt1 protein in mouse germ cells and cleavage-stage embryos.

Immunoblot analysis of oocytes at different stages of growth showed no evidence of the M_r 190,000 form of Dnmt1 after probing with the UPT82 antibody (Fig. 3A). UPT82 detected only the Dnmt1 protein in lysates of somatic cells or lysates of tissues with a mixture of somatic and germ cells (D10 ovary, D10 Ut/Ovd, D49 Ov, and D70 Ts lanes in Fig. 3A). When the same membrane was reprobed with the PATH52 antibody, only Dnmt1o (M_r 175,000) could be detected in lysates of postnatal oocytes (Fig. 3B). No expression was seen in a lysate of 400 D1 oocytes. Dnmt1o protein was also detected in the D10 and D49 ovaries (Fig. 3B). Thus, the presence of both Dnmt1 protein species in the ovaries appears to be due to the full-length form in the proliferating somatic cells and the truncated isoform in oocytes.

The immunoblotting data do not exclude a low level of the M_r 190,000 form of Dnmt1 in one or more stages of growing oocytes. We previously reported dynamic changes in the amount and intracellular localization of one or more forms of Dnmt1 protein in oocytes of postnatal ovaries immunostained with the PATH52 antibody (Howell *et al.*, 2001; Mertineit *et al.*, 1998). To test the possibility that the M_r 190,000 Dnmt1 is one of the forms found in oocytes, but is present at levels not detected on immunoblots (Fig. 3), we immunostained small follicles and larger oocyte–granulosa cell complexes with the UPT82 antibody. Confocal microscopy showed no evidence of Dnmt1 protein within MII oocytes, whereas surrounding somatic granulosa cells were intensely stained (Fig. 4A). In comparison, both MII oocytes and granulosa cells were stained by the UPTC21 antibody (Fig. 4B) and by the pATH52 antibody (Mertineit *et al.*, 1998). Even fully grown germinal vesicle-stage oocytes did not stain with UPT82 (data not shown). These data are consistent with the absence of Dnmt1 protein in mature MII oocytes.

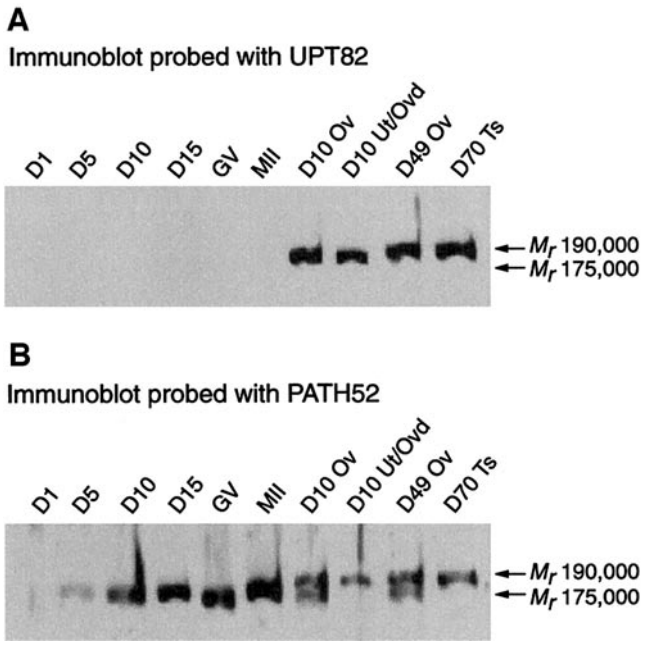

FIG. 3. Immunoblot of oocytes probed with Dnmt1 antibodies. (A) Immunoblot probed with the UPT82 antibody identifies the M_r 190,000 Dnmt1 protein in lysates of pooled oocytes or lysates of adult tissues. (B) Same blot as in (A), but probed with the PATH52 antibody that identifies both the M_r 175,000 Dnmt1o and the M_r 190,000 Dnmt1 proteins. Lysates from the following number of oocytes were loaded: 400 D1 oocytes, 400 D5 oocytes, 200 D10 oocytes, 200 D15 oocytes, 50 GV oocytes, and 50 MII oocytes. The following amount of protein from adult tissues were loaded: 10 μg of D10 ovary, 10 μg of D10 uterus/oviduct, 15 μg of D49 ovary, and 20 μg of D70 testis. The presence of protein in all lanes was verified by staining with Ponceau S. Shown is a one blot from two independent experiments. GV, germinal vesicle stage; MII, metaphase II.

Dnmt1 RNA and Protein Expression during Preimplantation Development

Despite the lack of Dnmt1 protein expression in oocytes, Dnmt1 protein might be expressed during preimplantation development, either from maternal transcripts or from transcripts produced *de novo* in the preimplantation embryo. To address these possibilities, we first measured *Dnmt1* and *Dnmt1o* transcripts in preimplantation cleavage-stage embryos. As shown in Fig. 5A, *Dnmt1* transcripts were present at all stages of preimplantation development, in steadily increasing amounts from the one-cell embryo to the blastocyst. In contrast, *Dnmt1o* transcripts were only found in the one-cell embryo. Immunoblot analysis of early embryos using the UPT82 antibody showed that the M_r 190,000 form of Dnmt1 was abundantly present in E7.5 embryos and in adult ovaries, but was absent from all preimplantation cleavage stages (Fig. 5B). When the same membrane was reprobed with the PATH52 antibody, only the M_r 175,000 Dnmt1o form was observed in all preimplantation embryos (Fig. 5C). Thus, Dnmt1 protein was not detected on immunoblots with an antibody that detects epitopes present only in Dnmt1 (UPT82), nor with an antibody that detects epitopes common to Dnmt1 and Dnmt1o (PATH52).

The UPT82 antibody was also used to stain cleavage-stage preimplantation embryos. As shown in Fig. 4A, there was no evidence of Dnmt1 protein in two, four-, and eight-cell embryos, morulae, and blastocysts. In contrast, when embryos of these stages were stained with UPTC21, a staining pattern similar to PATH52 staining was observed (Fig. 4B). These findings add further support to the notion that Dnmt1o protein is the sole form of Dnmt1 in the preimplantation embryo.

The possibility that Dnmt1 might provide maintenance activity at preimplantation stages other than eight-cell embryos was further tested by forcing the expression of Dnmt1 in preimplantation stages and examining its intracellular localization. There are two ways of forcing the expression of Dnmt1 in preimplantation development. It could be expressed in the oocyte's cytoplasm, from which it could traffic to different intracellular locations in preimplantation embryos, or *Dnmt1* transcripts could be made after fertilization, and then translated into Dnmt1 protein. We took the first approach and forced the expression of Dnmt1 protein in the oocyte. Using gene targeting techniques in ES cells, the *Dnmt1* locus was modified to express *Dnmt1* from the oocyte-specific promoter (Fig. 6). The 3′ end of exon 1o was replaced by exon 1s sequences, so that the modified exon 1o, designated exon 1s/1o, contains all coding information found in exon 1s (Figs. 6A and 6B). Oocyte-specific transcripts from this modified *Dnmt1* allele, designated $Dnmt1^{1s/1o}$, encode for Dnmt1 protein. Homozygous mutant $Dnmt1^{1s/1o}$ female mice produce MII oocytes containing both the M_r 190,000 and the M_r 175,000 form of Dnmt1, as shown on immunoblots stained with UPT82 and UPTC21 (Figs. 6E and 6F). Both forms of Dnmt1 protein are probably expressed because of the short 5′ UTR in the $Dnmt1^{1s/1o}$ transcript and thus frequent translation initiation from the methionine codon in exon 4. This codon is normally the initiation codon for Dnmt1o protein produced from wildtype *Dnmt1o* transcripts. Preimplantation embryos derived from these mutant oocytes also express Dnmt1 protein (Fig. 4C). Interestingly, the time course of nuclear localization of Dnmt1 protein in these embryos parallels that of Dnmt1o protein in wildtype embryos, with nuclear localization evident only at the eight-cell stage. Cytoplasmic Dnmt1 protein evident in two-, four-, and eight-cell embryos stained with UPT82 is different than the pattern of cytoplasmic localization of Dnmt1o protein seen in wildtype embryos (Carlson *et al.*, 1992; Howell *et al.*, 2001). Cytoplasmic Dnmt1 protein from homozygous $Dnmt1^{1s/1o}$ oocytes is aggregated, whereas wild-type cytoplasmic Dnmt1o protein is more evenly distributed (Figs. 4B and 4C). The distribution of both forms is seen after staining with the UPTC21 antibody (Fig. 4D). The difference in intracytoplasmic distribution of the two Dnmt1 forms is likely to be due to differences in the way the two

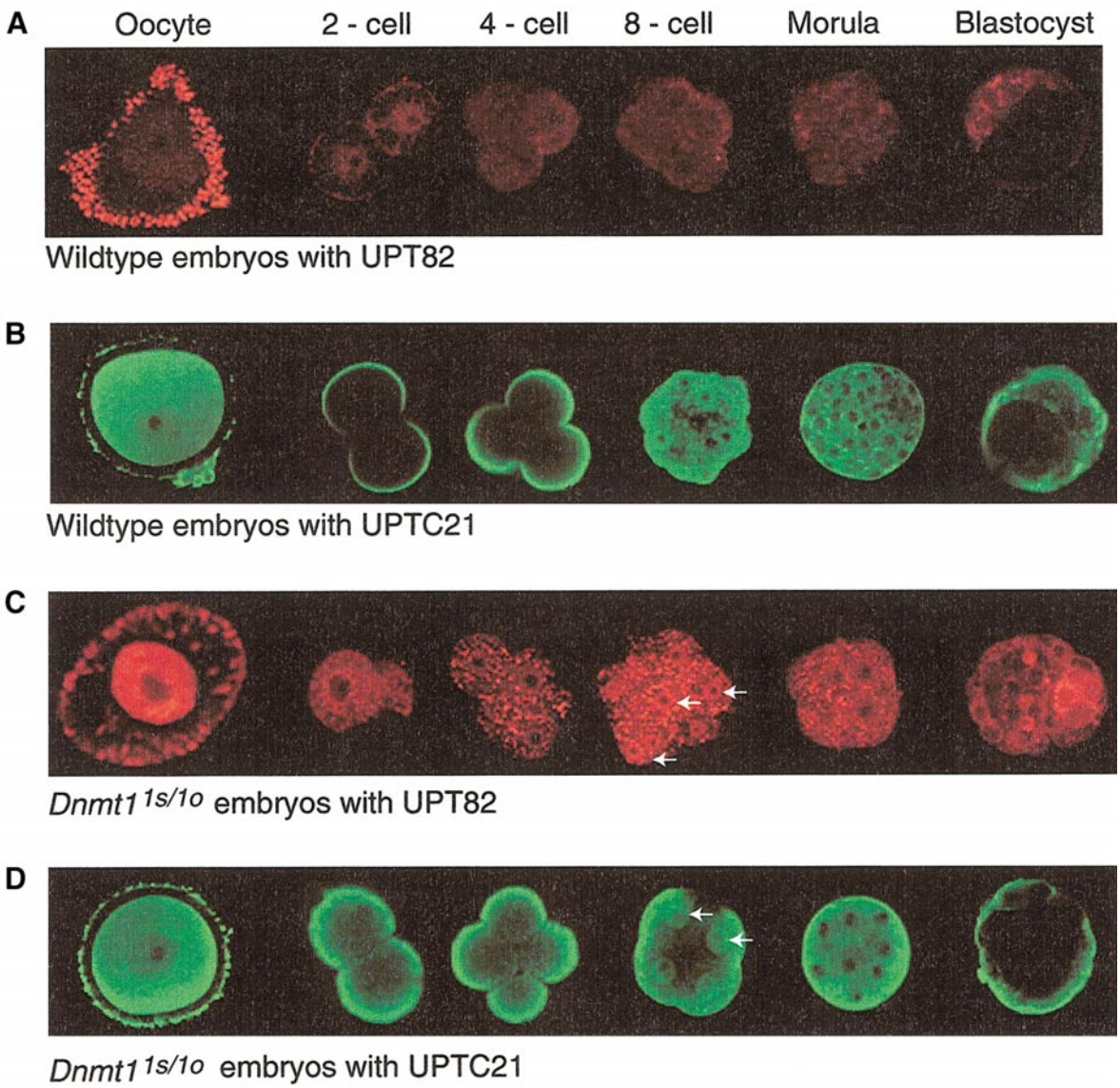

FIG. 4. Immunolocalisation and confocal microscopy of Dnmt1 isoforms in growing oocytes and ovarian follicles as well as preimplantation embryos (two-cell to blastocyst stages). All the panels represent confocal Z-sections. (A) Immunostaining with UPT82 to localize Dnmt1 in the wildtype embryos. (B) Immunostaining with UPTC21 to localize both Dnmt1 and Dnmt1o in the wildtype embryo. (C) Immunostaining with UPT82 to localize Dnmt1 in embryos derived from homozygous *Dnmt1*$^{1s/1o}$ mutant embryos. (D) Immunostaining with UPTC21 to localize Dnmt1 and Dnmt1o in embryos derived from homozygous *Dnmt1*$^{1s/1o}$ mutant females.

proteins are stored in the oocyte's cytoplasm and the way they traffic after fertilization.

DISCUSSION

Dnmt1 and Dnmt1o in Oocytes and Preimplantation Embryos

The process of genomic imprinting involves two crucial steps: the establishment of DNA methylation patterns in male and female gametes, and the subsequent maintenance of these patterns following fertilization (Chaillet *et al.*, 1991). These steps occur in settings of dramatic changes in the intracellular composition and localization of forms of the Dnmt1 methytransferase (Carlson and Bestor, 1992; Mertineit *et al.*, 1998). In MII oocytes and in all preimplantation cleavage-stage embryos, there are large amounts of the M_r 175,000 Dnmt1o protein, approximately 50,000-fold higher on a per-nucleus basis in the oocyte as compared to the amount of Dnmt1 protein present in a cycling somatic

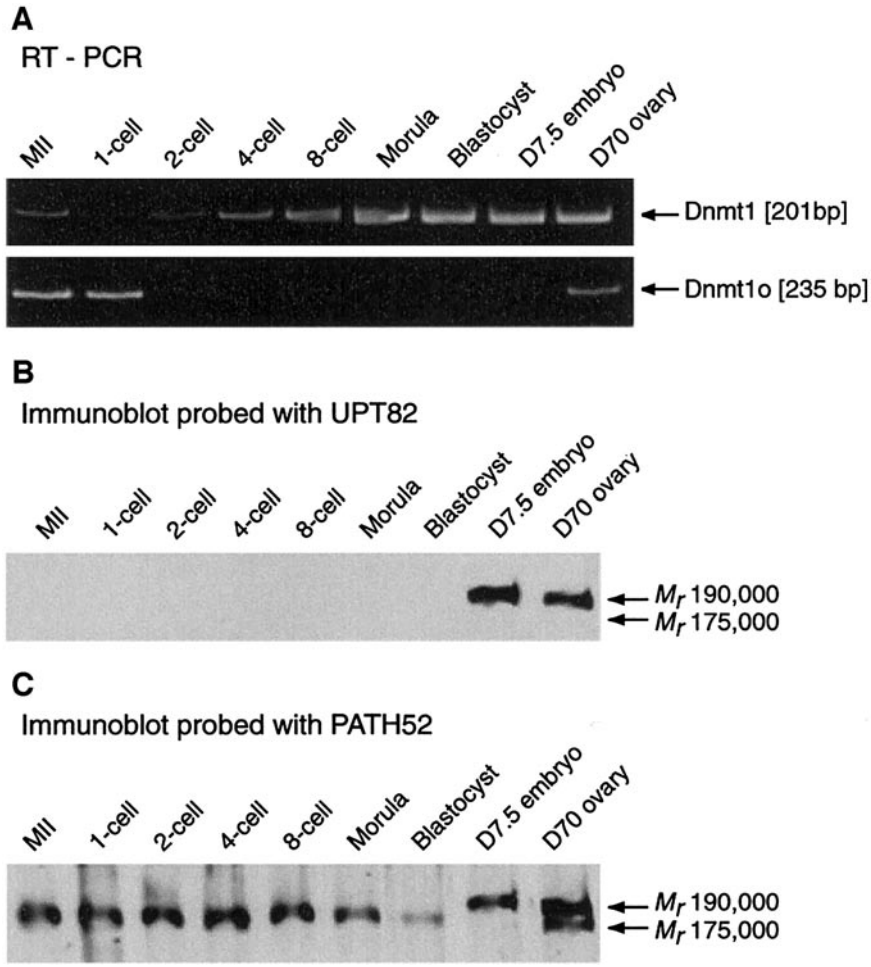

FIG. 5. Time course of Dnmt1 and Dnmt1o mRNA and protein expression during preimplantation development. (A) RT-PCR analyses of *Dnmt1* mRNA splice variants in preimplantation embryos, including morulae and blastocysts. Expression in metaphase II (MII) oocytes, embryos at 7.5 days of development (D7.5 embryo), and adult ovary (D70 ovary) are also shown. (B) Immunoblot of lysates from different preimplantation stages, probed with UPT82. A total of 30 mature oocytes and 30 embryos of each preimplantation stage were loaded. The following amount of protein from embryonic tissues were loaded: 15 μg of D7.5 postimplantation embryo and 15 μg of D70 ovary. (C) Same immunoblot as shown in (B), but probed with PATH52.

cell (Carlson *et al.*, 1992). Dnmt1o's only known function during the period spanning oocyte maturation and preimplantation development is to maintain methylation patterns on imprinted genes during a single S phase of preimplantation development (Howell *et al.*, 2001). Our conclusion from the experiments presented here is that methylation patterns at other S phases are not maintained by the M_r 190,000 Dnmt1. Therefore, DNA cytosine methyltransferases other than Dnmt1 are required for maintaining imprints during preimplantation development.

The nucleocytoplasmic trafficking of oocyte-derived Dnmt1o (in wildtype mice) and Dnmt1 (in *Dnmt1*$^{1s/1o}$ mice) proteins throughout preimplantation development is similar. Both proteins are primarily found in the cytoplasm of cleavage-stage embryos, with exclusion from all nuclei except those of eight-cell embryos. Despite their similar nucleocytoplasmic trafficking, Dnmt1o and Dnmt1 (in *Dnmt1*$^{1s/1o}$ mice) proteins are found in different cytoplasmic locations within MII oocytes, and within two- and four-cell embryos. Whereas cytoplasmic Dnmt1o protein is uniformly concentrated near the plasma membrane in oocytes and embryos, Dnmt1 protein is diffusely distributed in MII oocytes, and found in cytoplasmic concentrates in both two- and four-cell embryos (Fig. 4). The location of Dnmt1o protein near the plasma membrane of mature oocytes, two-cell embryos, and four-cell embryos may be due to binding to annexin V, a calcium-sensitive phospholipid binding protein (Howell *et al.*, 2001; Ohsawa *et al.*, 1996). If Dnmt1 protein does not bind to annexin V because of the additional 118 N-terminal amino acids, then it might

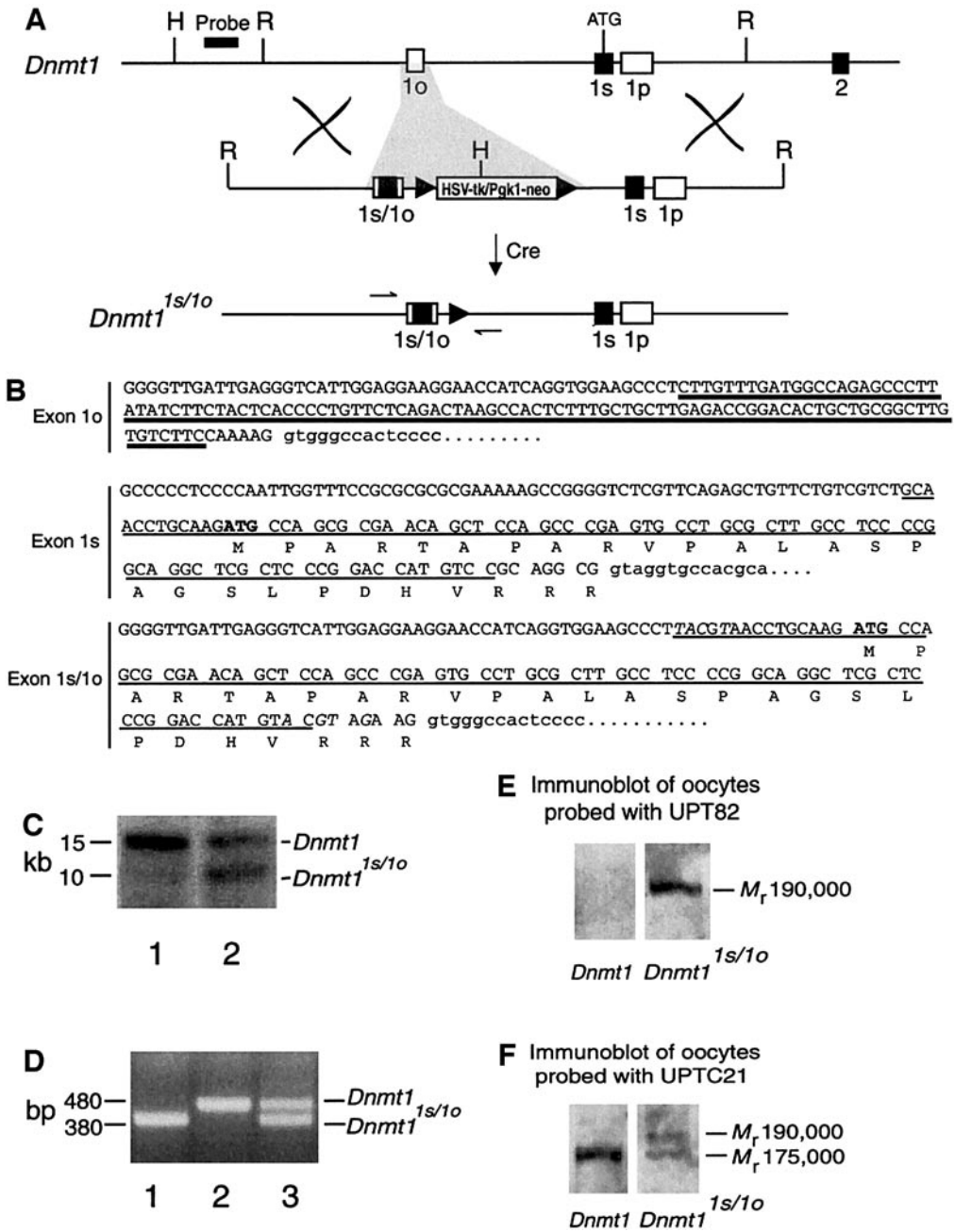

FIG. 6. *Dnmt1*$^{1s/1o}$ allele. (A) Targeted modification of the Dnmt1 locus by insertion of Dnmt1 1s exon sequences into exon 1o and Cre-mediated excision of resistance cassette placed approximately 100 bp 3′ of the modified exon 1o. H, *Hin*dIII; R, *Eco*RI; X, *Xba*I. Wildtype Dnmt1 locus, targeting construct and recominant *Dnmt1*$^{1s/1o}$ allele. (B) Sequences of exon 1o, exon 1s, and hybrid exon 1s/1o. The underlined sequences in exon 1s replace the underlined sequences in exon 1o to give rise to the mutant exon 1s/1o. The amino acid codons for exon 1s and exon 1s/1o are shown. The nucleotides represented in italics are the changes made in exon 1s to create *Sna*BI restriction sites (TACGTA). These sites were used to replace a portion of exon 1o with exon 1s sequences. (C) Confirmation by Southern blot that homologous recombination occurred in ES cells. DNA was cleaved with *Hin*dIII and the probe used is shown in (A). Lane 1, Wildtype ES cells. Lane 2, Heterozygous recombinant ES cells. (D) PCR of tail DNA using primers flanking exon 1o and exon 1s/1o (shown by arrows in A) distinguishes wildtype *Dnmt1* and mutant *Dnmt1*$^{1s/1o}$ alleles following Cre-mediated excision of the resistance cassette. Lane 1, Homozygous mutant *Dnmt1*$^{1s/1o}$. Lane 2, Homozygous wildtype *Dnmt1*. Lane 3, Heterozygous *Dnmt1*$^{1s/1o}$/*Dnmt1*. (E) Immunoblot probed with the UPT82 antibody shows the M_r 190,000 Dnmt1 somatic isoform in oocytes obtained from homozygous *Dnmt1*$^{1s/1o}$ females. (F) Immunoblot probed with the UPTC21 antibody reveals the M_r 175,000 Dnmt1o variant in addition to the M_r 190,000 *Dnmt1* isoform in oocytes obtained from homozygous *Dnmt1*$^{1s/1o}$ females.

become sequestered in organelles or in aggresomes. This sequestration suggests that the majority of oocyte-derived Dnmt1 protein is not available for subsequent trafficking to nuclei of eight-cell embryos. Therefore, it is very likely that a much greater fraction of the cytoplasmic store of Dnmt1o protein moves to the nuclei in eight-cell embryos than does the cytoplasmic store of Dnmt1 protein in *Dnmt1*$^{1s/1o}$ oocytes.

Although Dnmt1 and Dnmt1o proteins have very similar *in vitro* DNA methyltransferase activities (Carlson *et al.*, 1992), their DNA methyltransferase activities may differ in the nuclei of preimplantation embryos. One way this might occur is through the interaction of Dnmt1 and Dnmt1o with different proteins. The only known protein that interacts with the 118 amino acids at the amino terminus of Dnmt1 is DMAP1 (Rountree *et al.*, 2000). The function of DMAP1 is not known, but it is feasible that specific interactions with the amino terminus of Dnmt1 by DMAP1 or other unknown proteins would regulate its DNA methyltransferase activity.

We can make important inferences about the origins of the Dnmt1o protein and the Dnmt1 protein (from the *Dnmt1*$^{1s/1o}$ allele) seen in oocytes and preimplantation cleavage stages. Because *Dnmt1o* transcripts are not detected after division of the one-cell embryo, all preimplantation Dnmt1o protein is probably derived from oocyte stores of Dnmt1o protein. This interpretation is consistent with the maternal effect inheritance pattern of the *Dnmt1*$^{\Delta 1o}$ mutation (Howell *et al.*, 2001). Interestingly, no Dnmt1 protein was detected in wildtype MII oocytes despite the presence of *Dnmt1* transcripts, yet Dnmt1 protein can be made in the oocyte from the *Dnmt1*$^{1s/1o}$ allele. These observations suggest that the protein translation machinery of fully grown oocytes distinguishes *Dnmt1* transcripts from *Dnmt1o* and *Dnmt1*$^{1s/1o}$ transcripts. Differences in the 5′ UTRs of these transcripts (Fig. 5) may determine the observed differences in oocyte-specific translation.

Relevance to Cloning of Mammals

Our findings may have implications for the success of the cloning of mammals. The cloning of mammals by transplantation of somatic nuclei into enucleated zygotes or ooplasts has been plagued by low recoveries of viable clonal offspring and unpredictable phenotypic variation among the survivors (Humpherys *et al.*, 2001; Tamashiro *et al.*, 2000; Young and Fairburn, 2000). While this has been explained by reference to failures to reprogram unidentified aspects of chromatin structure, specific differences between nuclei of somatic cells and preimplantation embryos have been difficult to identify. We report here that preimplantation embryos of certain cleavage stages are completely free of Dnmt1 protein, which is present in nuclei of cycling somatic cells, and likely to be present in the donor nuclei used in cloning experiments. Transplantation of somatic nuclei into ooplasts therefore introduces a form of Dnmt1 protein that is foreign to preimplantation embryos and which might be responsible in part for the abnormal genomic methylation (Kang *et al.*, 2001; Ohgane *et al.*, 2001) and developmental abnormalities commonly seen in offspring derived by transplantation of somatic nuclei.

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