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Sex-specific exons control DNA methyltransferase in mammalian germ cells

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SUMMARY

The spermatozoon and oocyte genomes bear sex-specific methylation patterns that are established during gametogenesis and are required for the allele-specific expression of imprinted genes in somatic tissues. The mRNA for Dnmt1, the predominant maintenance and de novo DNA (cytosine-5)-methyl transferase in mammals, is present at high levels in postmitotic murine germ cells but undergoes alternative splicing of sex-specific 5′ exons, which controls the production and localization of enzyme during specific stages of gametogenesis. An oocyte-specific 5′ exon is associated with the production of very large amounts of active Dnmt1 protein, which is truncated at the N terminus and sequestered in the cytoplasm during the later stages of oocyte growth, while a spermatocyte-specific 5′ exon interferes with translation and prevents production of Dnmt1 during the prolonged crossing-over stage of male meiosis. During the course of postnatal oogenesis, Dnmt1 is present at high levels in nuclei only in growing dictyate oocytes, a stage during which gynogenetic developmental potential is lost and biparental developmental potential is gained.

Key words: DNA methyltransferase, Gametogenesis, Alternative RNA splicing

INTRODUCTION

The genomes of the male and female gametes contain very different methylation patterns that are established during gametogenesis (Sanford et al., 1987; Monk et al., 1987; Driscoll and Migeon, 1990; reviewed by Yoder et al., 1997a). Sex-specific differences in methylation patterns are most prominent at imprinted loci (Tremblay et al., 1997) and parasitic sequence elements (the location of most genomic m^5C; Yoder et al., 1997a). Gametic methylation patterns undergo some alterations after fertilization (Sanford et al., 1987; Monk et al., 1987) but imprinted loci retain characteristic methylation differences throughout development (Tremblay et al., 1997; Olek and Walter, 1997).

The predominant form of DNA (cytosine-5)-methyltransferase in mammals is Dnmt1, which is composed of a C-terminal domain closely related to bacterial C5-specific restriction methyltransferases and a large N-terminal domain that has multiple regulatory functions (Bestor et al., 1988; Bestor and Verdine, 1994; Bestor, 1996). Homozygous mutations of the *Dnmt1* gene in mice lead to severe abnormalities in genomic imprinting and X inactivation (Li et al., 1993a,b; Panning and Jaenisch, 1996), and mutant embryos die prior to midgestation (Li et al., 1992). There is also genetic evidence for a second specialized DNA methyltransferase that participates in the silencing of newly integrated retroviral DNA (Lei et al., 1996; Lengauer et al., 1997), and a candidate DNA methyltransferase that may serve this function has been identified (Yoder and Bestor, 1998). However, Dnmt1 is the predominant de novo and maintenance DNA methyltransferase in all tissues and cell types examined to date (Yoder et al, 1997b). Early predictions (Holliday and Pugh, 1975; Jähner and Jaenisch, 1984) of distinct classes of de novo and maintenance DNA methyltransferases have not been confirmed, and the extant data indicate that Dnmt1 is the major de novo and maintenance DNA methyltransferase in mammals (Yoder et al., 1997b).

Dnmt1 mRNA has been reported to be expressed at high levels in mitotic and postmitotic male germ cells (Singer-Sam et al., 1990; Trasler et al., 1992; Numata et al., 1994) and Dnmt1 protein has been found in large amounts in mature oocytes and early embryos (Monk et al., 1991; Howlett and Reik, 1991; Carlson et al., 1992). We report here that Dnmt1 is subject to unusual transcriptional and post-transcriptional regulatory mechanisms in germ cells that depend on alternative splicing of sex-specific 5′ exons. Dnmt1 protein in oocytes within antral follicles is almost exclusively cytoplasmic and is truncated at the N terminus due to alternative splicing of a 5′ exon, while a different 5′ exon in pachytene spermatocytes

leads to the production of a non-translated mRNA. The control of Dnmt1 expression in germ cells shows a number of unusual features: alternative splicing of sex-specific exons, the use of fundamentally different mechanisms (cytoplasmic sequestration in oocytes versus translational down-regulation in pachytene spermatocytes) to reduce the amount of Dnmt1 in the vicinity of chromosomes in male and female germ cells, and large changes in amount, size, and localization of the enzyme in postmitotic germs cells and in early embryos.

MATERIALS AND METHODS

Isolation of gonads and germ cells

CD-1 mice were obtained from Charles River Canada, Inc. (St Constant, Quebec, Canada). Genital ridges were isolated from day-11 mouse embryos. Ovaries and testes were distinguished in embryos 12.5 days postcoitum and later by the presence of seminiferous cords in the testes. Purified populations of male germ cells were obtained from enzymatically dissociated mouse testes by sedimentation at unit gravity on 2-4% BSA gradients (Bellvé, 1993). Isolated type A spermatogonia (average purity=86%, *n*=2 cell separations) and type B spermatogonia (85% pure, *n*=2) were obtained from the testes of 8-day-old mice. Preleptotene spermatocytes (87% pure, *n*=3), leptotene/zygotene spermatocytes (90% pure, *n*=3) and prepubertal pachytene spermatocytes (82% pure, *n*=3) were obtained from the testes of 17-day-old mice. Pachytene spermatocytes (83% pure, *n*=3) were obtained from 70-day-old mice. Female mice were superovulated and oocytes recovered from oviducts by established methods (Hogan et al., 1986).

Immunocytochemistry

Mice were perfused through the heart with physiological saline followed by Ste Marie's fixative (95% ethanol/glacial acetic acid, 99:1). Ovaries and testes were dissected, post-fixed for 1 hour on ice, dehydrated through graded ethanols, cleared in xylene and embedded in paraffin. Gonads were sectioned at 5 μm thickness, placed on slides coated with 0.5% gelatin, heated to 60°C for 1 hour, and stored at 4°C prior to use. All solutions for immunofluorescence of Dnmt1 were prepared in phosphate-buffered saline (PBS), pH 7.2, and procedures were carried out at room temperature, unless otherwise specified. The polyclonal rabbit anti-Dnmt1 antibody (anti-pATH52) has been described previously (Bestor, 1992; Li et al., 1992). Sections were deparaffinized, rehydrated through graded ethanols, blocked for 30 minutes in blocking buffer (0.2% cold-water fish skin gelatin (Sigma Chemical Co., St. Louis, MO), 5% goat serum (Gibco) and 0.2% Tween 20) and incubated with a 1:200 dilution of anti-pATH52 or preimmune serum in blocking buffer overnight at 4°C in a humidified chamber, then incubated for 1 hour with a 1:200 dilution of fluorescein-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA). Slides were counterstained with 0.5 μg/ml propidium iodide (Molecular Probes, Eugene, OR) for 5 minutes, washed three times in PBS and mounted with an antifade solution containing 0.025% 1,4-diazobicyclo-(2,2,2)-octane (DABCO, Sigma) in 90% glycerol (Laird et al., 1995). The specimens were examined with a Zeiss Axiophot or Zeiss LSM410 confocal microscope.

RNA blot analysis

Total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987), electrophoresed on 1.5% agarose formaldehyde gels and transferred to Zetabind nylon membranes (CUNO, Meriden, CT). Blots were hybridized under the conditions of Church and Gilbert (1984) with Dnmt1 cDNA probes (Bestor et al., 1988) or cDNA probes for exon 1p labelled to specific activities of $5\text{-}8\times10^8$ cpm/μg of DNA by random priming (Feinberg and Vogelstein, 1984). An end-labelled oligonucleotide probe for 18S rRNA was used to assess RNA loading. Blots were analyzed by a phosphorimager (Fuji Medical Systems USA, Inc., Stamford, CT).

Immunoblot analysis

Lysates of mouse ovaries, superovulated oocytes and purified male germ cells 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 100 μg/ml PMSF. Proteins (20 μg except where noted) were denatured by heating at 100°C in 2% SDS, separated by electrophoresis on SDS-5% polyacrylamide gels, and transferred to nitrocellulose membranes (Protran; Schleicher & Schuell). Blots were stained with Ponceau S to confirm equal loadings and probed with anti-pATH52 as described (Li et al., 1992).

RNase H mapping, 5′ RACE cloning and RT-PCR analysis

The oligonucleotides used for RNase H mapping were oligo 1 (5′ TTGGCGGGACAACCGTTGG 3′), oligo 2 (5′ AATTTCT-CCCTCACACAC 3′) and oligo 3 (5′ ATCCCTCACTCCTCGAA 3′). Oligonucleotides (250 pmol) were annealed to 20 μg of total RNA. RNase H treatment, electrophoresis, blotting and probing were as described (Yoder et al., 1996).

RACE cloning was based on techniques described by Troutt (1992), Frohman (1993) and Edwards et al. (1991). The Dnmt1-specific oligos 1 or 5 (5′ GCAGGAATTCATGCAGTAAG 3′) were annealed to 1.0 μg of total RNA in 10 mM Tris, pH 8.3. cDNA synthesis was performed with 10 units of AMV-Reverse Transcriptase (Promega) in 50 mM Tris, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT and 0.3 mM dNTP. The reaction was incubated at 42°C for 20 minutes and then at 50°C for 60 minutes. RNA was degraded with 0.5 N NaOH for 10 minutes at 37°C. The cDNA was precipitated with ethanol and resuspended in 10 μl RNA ligase buffer (10 mM Tris, pH 8.0, 25% polyethylene glycol-8000, 1 mM hexamine cobalt chloride, 10 mM $MgCl_2$, 1 mg/ml BSA and 10 mM ATP) supplemented with an anchor oligo that was blocked at the 3′ end (Durand et al., 1990) and phosphorylated at the 5′ end. The sequence of the anchor oligo was 5′ CACGAATTCACTATCGATTCTGGAACCTTCAGAGG 3′. Ligation involved 10 units T4 RNA ligase (New England Biolabs) and incubation for 16 hours at 22°C. A portion (0.5 μl) of the mixture was added to a 25 μl primary PCR reaction (10 mM Tris, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 0.2 mM dNTP, 2.5 units Taq polymerase (Promega) and 1 mM anchor primer-1 (5′ GAAGGTTCCAGAATCGATAG 3′) and Dnmt1 oligo 5. Incubation conditions were 93°C for 15 seconds, 55°C for 15 seconds, 72°C for 15 seconds for 30 cycles. A final extension at 72°C was performed for 10 minutes. A portion (0.5 μl) of the primary reaction was added to a 25 μl secondary nested PCR. The reaction conditions were as for the primary PCR, except anchor primer-2 (5′ GAATCGATAGTGAATTCGTG 3′) and nested Dnmt1 oligos 2 or 4 (5′ ACACAAGCCGCAGCAGTGT 3′) were used.

The complete sequence of exon 1o was obtained by analysis of multiple RACE clones from ovaries of 7-day and adult animals. 427 nucleotides of exon 1p were identified by RACE. The remaining sequence was deduced by RT-PCR analysis of testis RNA with primers complemenary to genomic sequence.

The Superscript One-Step RT-PCR System (Life Technologies) was used to analyze splicing variants of Dnmt1. Reactions were performed as directed by the manufacturer. 1 μg of total RNA was seeded into a 50 μl RT-PCR reaction with oligo 5 as the RT primer. Oligo 6 (5′ GGGTCTCGTTCAGAGCTG 3′) was used to amplify the somatic transcript and oligo 7 (5′ GGTTGATTGAGGGTCATT 3′) was used to amplify the oocyte transcript. Reverse transcription was allowed to proceed for 10 minutes at 50°C then shifted to 55°C for 30 minutes. PCR involved 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds. A final extension at 72°C was performed for 7 minutes. A portion of the reaction (10 μl) was analyzed by electrophoresis through a 3% wide-range agarose gel (Sigma).

RESULTS

Localization of Dnmt1 in male and female germ cells

Ovaries and testes were sectioned and stained for Dnmt1 with the antibody pATH52, which is highly specific for Dnmt1 and recognizes multiple epitopes within the region between amino acids 255-753 (Bestor, 1992; Li et al., 1992; Leonhardt et al., 1992). Spermatogenesis occurs in synchronous waves that pass down the seminiferous tubule; the distinctive morphology of the haploid spermatids present at a given stage allows accurate identification of cell types in situ. Spermatogonia are mitotically active stem cells that lie at the basal side of the seminiferous epithelium, and migrate towards the lumen of the tubule as they differentiate into spermatocytes, undergo meiosis and begin spermiogenesis. Meiotic prophase lasts about 12 days in the mouse and is conventionally divided into the leptotene, zygotene, pachytene and diplotene phases according to the state of synapsis of meiotic chromosomes; crossing-over occurs during the pachytene stage, which occupies more than 6 days of meiotic prophase. Spermatogonia, preleptotene and leptotene spermatocytes all express high levels of nuclear Dnmt1, while pachytene spermatocytes are not detectably stained (Fig. 1), despite the fact that they have been reported to contain large amounts of Dnmt1 mRNA (Singer-Sam et al., 1990; Trasler et al., 1992; Numata et al., 1994). This suggests that Dnmt1 protein is sharply down-regulated at the pachytene stage of spermatogenesis via post-transcriptional mechanisms.

Male germ cells undergo mitosis and meiosis throughout adult life, but germ cells in ovaries complete the last round of mitosis and most of meiotic prophase prior to birth. The growth of arrested primary oocytes commences after birth, and gonadotropin stimulation causes fully grown oocytes to resume meiosis. As shown in Fig. 2, dramatic changes in the amount and localization of Dnmt1 in oocytes were observed in postnatal ovaries. Non-growing oocytes do not stain detectably (arrows in Fig. 2A), while growing oocytes show intense staining of nuclei and substantial cytoplasmic staining (Fig. 2B,C). As the oocytes continue to grow Dnmt1 is no longer detectable in nuclei but accumulates to very high levels in the cytoplasm (Fig. 2C,D). Late-stage dictyate oocytes in Graafian follicles show very intense and uniform cytoplasmic staining (Fig. 2D). At the time of ovulation all Dnmt1 staining is cytoplasmic and is associated with the oocyte cortex (Fig. 2E); this indicates that Dnmt1 is actively retained near the oocyte cortex, as exclusion from the nucleus or the vicinity of the spindle would produce a uniform cytoplasmic distribution. Mature oocytes and early preimplantation embryos contain very high levels of Dnmt1

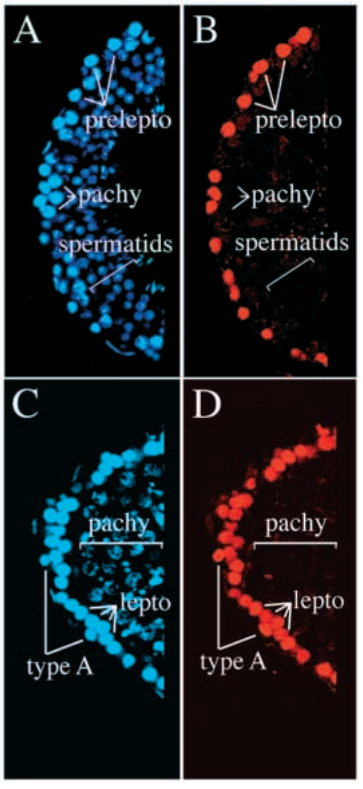


Fig. 1. Dnmt1 in spermatogenesis. Pseudocolor confocal laser scanning micrographs of a sector of a seminiferous tubule viewed down the long axis of the tubule; (A and C) are stained for DNA (blue) and (B and D) for Dnmt1 (red). In B, preleptotene spermatocytes stain intensely for Dnmt1, while pachytene spermatocytes show no detectable staining. C and D show that type A spermatogonia and leptotene spermatocytes contain large amounts of Dnmt1 while later pachytene spermatocytes are free of Dnmt1 staining.

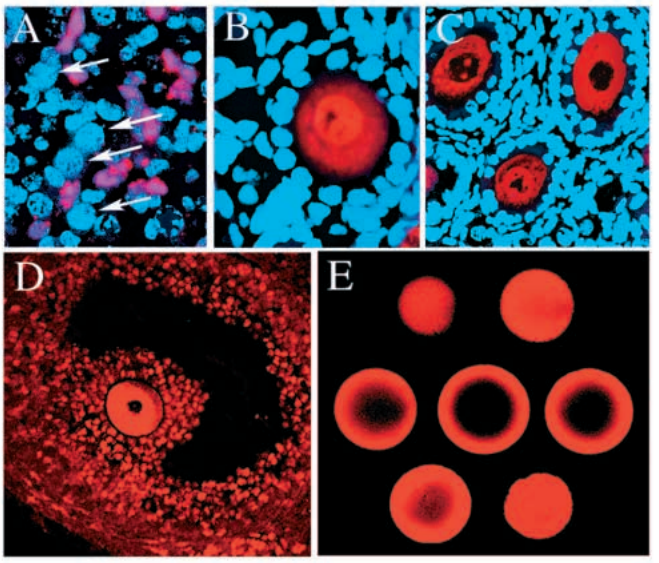


Fig. 2. Dnmt1 in oogenesis. As in Fig. 1, blue is DNA and red is Dnmt1. (A) Non-growing oocytes in the ovary of a newborn mouse show no sign of Dnmt1 staining (arrows), although neighboring supporting cells show significant staining. (B) The growing oocyte stains intensely in both nucleus and cytoplasm; in (C) the growing oocytes at the top of the field show predominantly cytoplasmic staining, while the oocyte at the bottom of the field shows staining in both nucleus and cytoplasm. Oocytes from B and C are from a 7-day-old animal. (D) A fully grown oocyte in a Graafian follicle from a 70-day-adult has intense, uniform cytoplasmic staining. (E) Optical sections show that Dnmt1 is associated with the oocyte cortex in an ovulated oocyte arrested in metaphase of meiosis II. DNA in nuclei of oocytes stains faintly with intercalating dyes, in part because the nuclei are large and their DNA concentration low. Late oocytes contain such large amounts of Dnmt1 protein that staining of somatic cells is not apparent at this exposure.

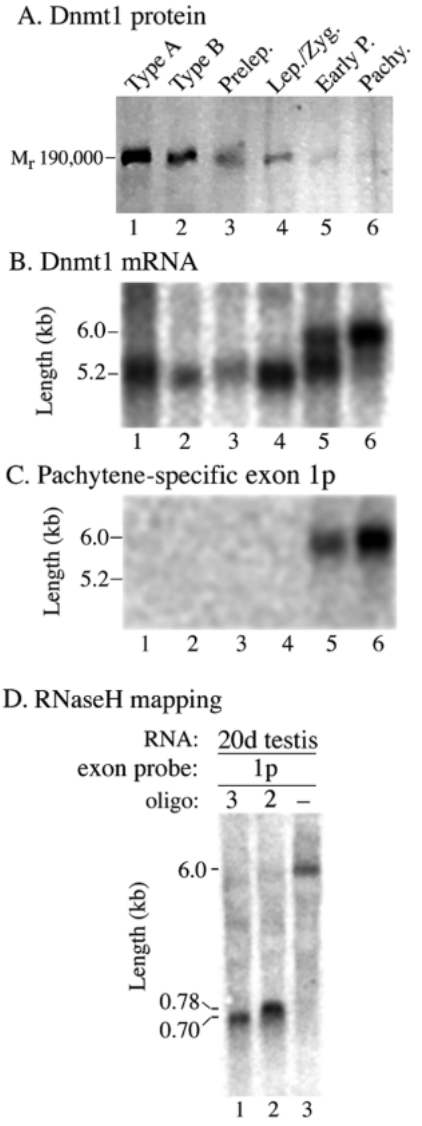


Fig. 3. Sizes and amounts of Dnmt1 protein and mRNA in spermatogenic cells. Male germ cells were fractionated by unit-gravity sedimentation in gradients of bovine serum albumin prior to analysis. (A-C) Lanes 1 and 2, type A and type B spermatogonia, respectively; lane 3, preleptotene spermatocytes; lane 4, leptotene and zygotene spermatocytes; lane 5, early pachytene spermatocytes from testes of prepubertal 17-day-old mice; lane 6, pachytene spermatocytes from a 70-day-old animal. (A) An immunoblot stained with the anti-Dnmt1 antiserum pATH52 demonstrates the presence of Dnmt1 in postmitotic leptotene/zygotene spermatocytes and the absence of Dnmt1 in pachytene spermatocytes. Protein quantities were determined by BCA assay (Pierce) and equal amounts of total protein were loaded onto each lane. (B) The RNA blot shows that the total amount of Dnmt1 mRNA is nearly constant within the stages examined, but the mRNA in pachytene spermatocytes can be seen to contain additional sequences, which were localized to the 5′ end and cloned; (C) shows the blot in B stripped and probed with the alternative 5′ sequences. Equal loadings were confirmed by rehybridization of the blot with a ^{32}P-labeled oligonucleotide complementary to 18S ribosomal RNA (Trasler et al., 1992). (D) RNase H mapping of the 5′ sequences. Oligos 2 and 3 (see Fig. 5 and Materials and methods) targeted RNase cleavage to a site near the splice junction and indicated that the pachytene-specific sequences total approx. 770 nucleotides. RNA was from testes of 20-day-old animals.

protein: at least 10,000-fold more per cell than does a cycling somatic cell (Carlson et al., 1992).

In summary, immunolocalization data reveal that pachytene spermatocytes contain no detectable Dnmt1 protein, but other studies have shown that they contain large amounts of Dnmt1 mRNA. The growing oocyte contains very large amounts of Dnmt1 protein, which during the growth phase largely disappears from the nucleus and comes to be localized first throughout the cytoplasm and, at ovulation, in a shell just within the oocyte cortex.

Sizes and amounts of Dnmt1 protein and mRNA in male and female germ cells

Male germ cells at specific stages of spermatogenesis were isolated from enzymatically dissociated testes by sedimentation at unit gravity in gradients of bovine serum albumin as described (Bellvé, 1993). As shown in Fig. 3A, Dnmt1 protein is abundant in Type A and Type B spermatogonia, easily detectable in preleptotene and leptotene/zygotene spermatocytes, but undetectable in pachytene spermatocytes. These findings are consistent with the immunocytochemical data of Fig. 1.

As shown in Fig. 3B, the total amount of Dnmt1 mRNA does not vary more than twofold during prophase of meiosis I, while the amount of protein decreases from high in spermatogonia to undetectable in pachytene spermatocytes (Fig. 3A). A larger form of Dnmt1 mRNA is present in pachytene spermatocytes (Fig. 3B) and has been observed only in these cells (Trasler et al., 1992). Data that will be described later indicate that the larger (6.0 kb) mRNA is the result of sex-specific alternative splicing of a 5′ exon.

Analysis of RNA purified from pre- and post-natal ovaries showed only a band of approx. 5.2 kb (data not shown), which is characteristic of the somatic form of Dnmt1 (Bestor et al., 1988; Yoder et al., 1996). However, immunoblot analysis revealed that ovaries contain a species of Dnmt1 of M_r 190,000 and a second species of M_r 175,000; this latter species was the only detectable form in ovulated oocytes but was undetectable in ovaries of newborn mice, which contain predominantly non-growing oocytes (Fig. 4A). Only the M_r 175,000 form is detectable in isolated oocytes, which indicates that the M_r 190,000 form is derived from supporting cells in the ovary. These data identify a smaller, oocyte-specific form of the Dnmt1 protein and agree with the immunolocalization data of Fig. 2, where growing oocytes were seen to stain intensely with Dnmt1 antibodies while non-growing oocytes were unstained.

Complex alternative splicing of sex-specific exons in Dnmt1 mRNA

The discovery of additional 5′ sequences in the somatic Dnmt1 mRNA that had been refractory to cloning (Tucker et al., 1996; Yoder et al., 1996; Glickman et al., 1997), together with evidence of a larger Dnmt1 mRNA in testis and a smaller protein in oocytes, prompted a search for alternatively spliced exons in male and female germ cells. RNase H mapping (Hake and Hecht, 1993) identified alternative exons at the 5′ end of both male (Fig. 3D) and female (Fig. 4C) germ cells. The mRNA in pachytene spermatocytes was found to have about 800 additional nucleotides at the 5′ end (Fig. 3D), and the oocyte-specific form was found to be about 100 nucleotides shorter than the somatic mRNA (Fig. 4C). RACE (rapid

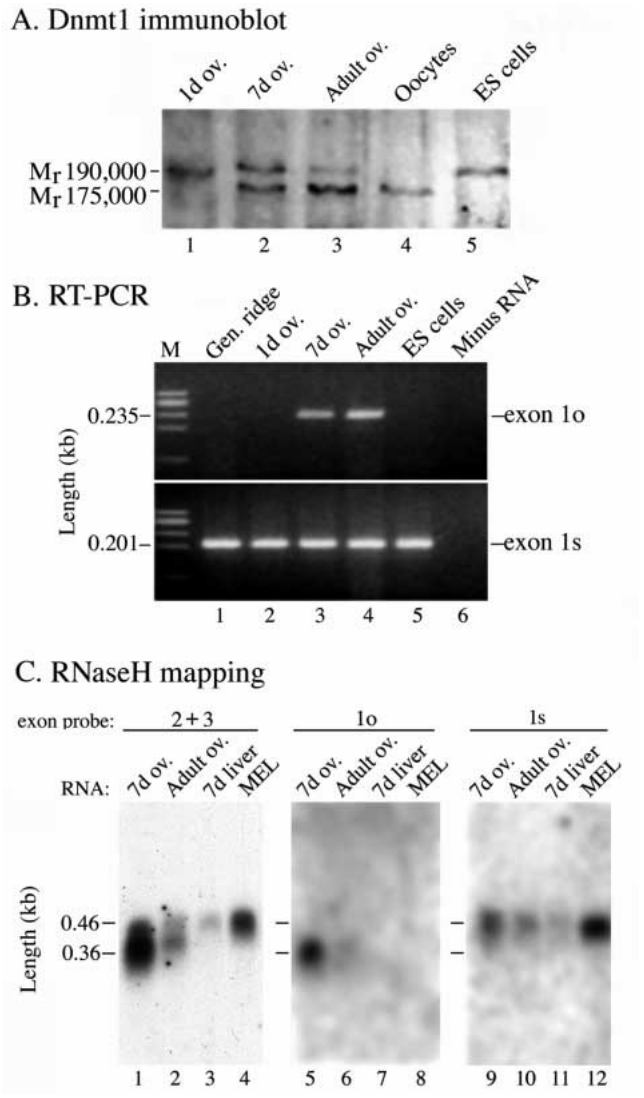


Fig. 4. Size, distribution, and amount of Dnmt1 mRNA and protein in ovaries and oocytes. (A) Immunoblot analysis of Dnmt1 in ovaries and oocytes. A smaller (M_r 175,000) form of Dnmt1 appears in ovaries at the time when oocyte growth has begun (lane 2) and is the sole form in ovulated oocytes (lane 4). The oocyte contains very large amounts of Dnmt1; lanes 4 and 5 have similar band intensities, but lane 4 contained 30 oocytes, and lane 5 contained approx. 10^5 embryonic stem cells. (B) RT-PCR analysis of an oocyte-specific 5′ exon reveals that oocyte-specific sequences are present only at stages where the truncated protein is also found. The oligos were 5 and 7 (see Fig. 5 and Materials and Methods). The lower panel in B shows that oligos specific to the somatic form of the Dnmt1 mRNA (oligos 5 and 6 in Fig. 5 and Materials and methods) gave a PCR product with RNA from all tested sources. (C) RNase mapping of the oocyte-specific exon 1o and somatic exon 1s. Oligo 1 was used to target RNase H cleavage to exon 4; the probes used are indicated. Exon 1o can be seen to be present at high levels only in ovaries from 7-day mice, when most oocytes are in the growth phase.

amplification of cDNA ends; Frohman, 1993) was used to clone the germ cell-specific sequences.

Comparison of pachytene spermatocyte-specific exon sequences with the sequence of a clone of genomic DNA spanning the first exon of the somatic Dnmt1 mRNA (the gift of K. L. Tucker and R. Jaenisch) revealed that the pachytene spermatocyte-specific sequences lie in a single exon of approx. 780 nucleotides, whose 5′ boundary is approx. 80 nucleotides downstream of the 3′ end of exon 1 of the somatic mRNA (Figs 3C and 5A). The TSSG and TSSW algorithms of the Gene finder suite (http://defrag.bcm.tmc.edu:9503/gene-finder/gf.html) identified a high-probability promoter immediately 5′ of the spermatocyte-specific exon. The pachytene-specific exon was named exon 1p, and the first exon of the somatic form was named exon 1s (Fig. 5A). These were concluded to represent the first exons as there were no splice acceptor consensus sequences within the range delimited by RNase H mapping of 5′ ends (Fig. 3 and Yoder et al., 1997b).

Exon 1p contains seven ATG codons (Fig. 5B), five of which are in a favorable context for translation initiation (Kozak, 1996). The first ATG that can yield active Dnmt1 is the tenth in the sequence, and according to Kozak (1996) initiation from such codons does not occur or is very inefficient. The pachytene spermatocyte form of Dnmt1 mRNA is only weakly associated with polysomes (Trasler et al., 1992), and all data indicate that the form of Dnmt1 mRNA present in pachytene spermatocytes is not actively translated. The inactive mRNA appears to be as stable as the productive mRNA, as their steady-state levels are similar (Fig. 3B). Untranslated mRNA has been observed to be stable in male germ cells in other cases (reviewed by Kleene, 1996).

RACE cloning and cDNA library screens were used to identify the oocyte-specific 5′ sequences. These were found to reside in a single exon (1o) of 157 nucleotides that lies approx. 6 kb 5′ of the exon 1s (Fig. 5A). No splice acceptor sites were within the 300 nucleotides of genomic sequence upstream of the 5′ end of the cDNA, and exon 1o is concluded to represent an oocyte-specific 5′ exon. The first in-frame ATG codon is in exon 4 (Fig. 5A); it had been found earlier that in vitro transcription-translation from that ATG codon yields a protein that precisely comigrates with Dnmt1 protein from oocytes upon SDS-polyacrylamide gel electrophoresis (Carlson et al., 1992). There are three additional ATG codons upstream of the one that can yield a protein of the size observed in oocytes (Fig. 5A,B), but as will be described later the likelihood that this mRNA is translated is much greater than for the male pachytene mRNA. As shown in Fig. 4B, exon 1o is found only when the truncated form of Dnmt1 protein is also present.

Developmental regulation of Dnmt1 localization in germ cells and early embryos

Fig. 6 summarizes expression and localization data for the Dnmt1 protein and mRNA during gametogenesis and early development. Dnmt1 is present at high levels in spermatogonia and spermatocytes until the pachytene stage, where it falls to undetectable levels (Jue et al., 1995). The transient drop at the pachytene stage coincides with the disappearance of the 5.2 kb Dnmt1 mRNA and the accumulation of a larger 6.0 kb Dnmt1 mRNA, which is not translated.

Oocytes accumulate very large amounts of Dnmt1 protein during the growth phase; this enzyme is nuclear in growing oocytes (the only point during postnatal oogenesis where nuclear Dnmt1 has been observed) but becomes cytoplasmic towards the end of the growth phase and is confined to a shell near the oocyte cortex in ovulated oocytes (Carlson et al., 1992, and this report). The cytoplasmic Dnmt1 is encoded by an

alternatively spliced mRNA that has a different 5′ exon and which is lacking N-terminal sequences present in the somatic enzyme. Dnmt1 remains cytoplasmic in the preimplantation embryo (except for a brief period at the 8-cell stage; Carlson et al., 1992) until implantation. After implantation Dnmt1 is exclusively nuclear in all tissue types and cell lines examined to date (Trasler et al., 1996).

DISCUSSION

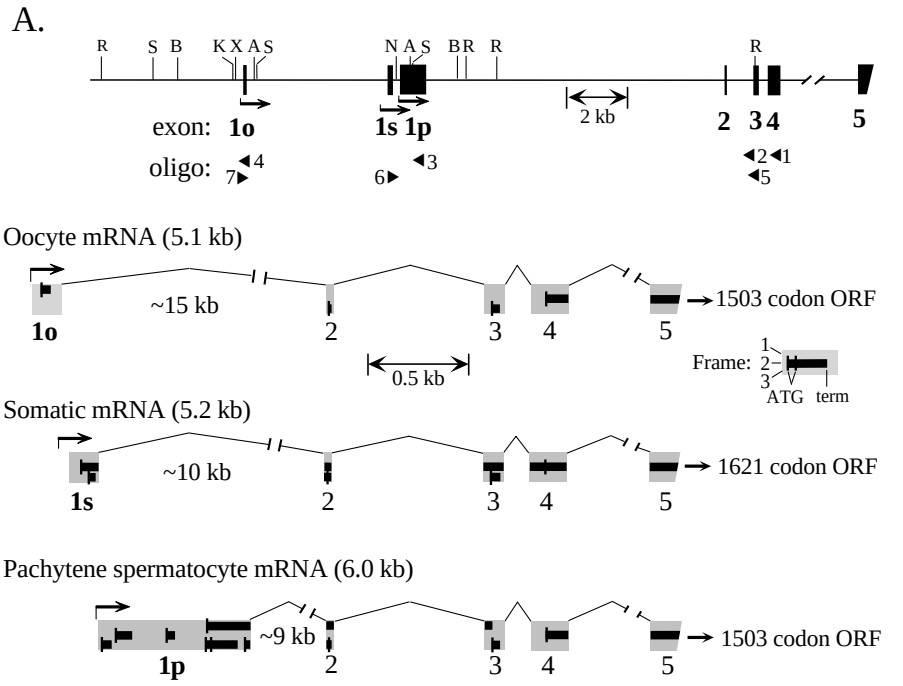
The Dnmt1 mRNA in germ cells undergoes alternative splicing of sex-specific exons, which controls production and localization of Dnmt1 protein, and fundamentally different mechanisms (cytoplasmic sequestration versus translational down-regulation) act to reduce the amount of nuclear Dnmt1 protein at specific stages of gametogenesis. There are also large changes in the total amount of Dnmt1 and repeated reversals of the ratio of protein in nucleus versus cytoplasm in oocytes and early embryos.

Alternative splicing of sex-specific exons

Three alternative 5′ exons have been identified in the Dnmt1 gene: one is specific to the growing oocyte, one to the pachytene spermatocyte, and one to all somatic cells and other germ cell types of both sexes. Extensive characterization of the 5′ region of the Dnmt1 mRNA of somatic cells has failed to show evidence of alternative splicing or multiple transcriptional start sites in this region (Yoder et al., 1996; Tucker et al., 1996). While alternative splicing and alternative transcriptional start sites in mammalian germ cells have been observed in many cases (reviewed by Kleene, 1996), this is the first case in which each sex has been found to use a different 5′ exon. It is also unusual that one 5′ splice site should accept three different 3′ splice sites; this had previously been documented in only a few cases (Rotwein and Hall, 1991; Bermingham and Scott, 1988).

Cytoplasmic sequestration versus translational repression

Dnmt1 behaves in somatic cells as a replication factor: it is nucleoplasmic through most of the cell cycle and associates with replication foci during S-phase (Leonhardt et al., 1992), as do other replication factors (Spector, 1993). Cytoplasmic Dnmt1 has not been observed in any somatic cell during interphase (Trasler et al., 1996). The situation in oocytes and early embryos is quite different. As summarized in Fig. 6, Dnmt1 shows a nuclear distribution in early growing oocytes, a lack of nuclear staining and an uniform cytoplasmic distribution in later growing oocytes, and a subcortical distribution in ovulated oocytes. Maternal stores of Dnmt1 protein in mature oocytes are very large (Carlson et al., 1992).



B.

Exons 1o and 2

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GGGGTTGATTGAGGGTCATTGGAGGAAGGAACCATCAGGTGGAAGCCCTCTTGTTTGATG
GCCAGCCCTTATATCTTCTACTCACCCCTGTTCTCAGACTAAGCCACTCTTTGCTGCTTG
AGACCGGACACTGCTGCGGCTTGTGTCTTCCAAAAGGCTCAAAGACTTGGAAAGAGATGG
CTTAACAGAAAAG
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Exons 1s and 2

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GCCCCTCCCAATTGGTTTCCGCGCGCGCGAAAAAGCCGGGGTCTCGTTCAGAGCTGTTCT
GTCGTCTGCAACCTGCAAGATGCCAGCGCGAACAGCTCCAGCCCGAGTGCCTGCGCTTGC
CTCCCCGGCAGGCTCGCTCCCGGACCATGTCCGCAGGCGGCTCAAAGACTTGGAAAGAGA
TGGCTTAACAGAAAAG
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Exons 1p and 2

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CTGACAGCCAGGGCGCGCATGCGCGGGGCAGCGTTTGGCGCGGGCTGTGCTTCCTGTCGC
GCGTGCCCTTAGCTGTTGGCGCCAAAATGGGTGGGGGCCGGGGTGCTGGCAAGCCCATTG
ACTGGGAGCTTGAGGGAGCAAGGGGATCAGCCCTCTGAAATAGAACAAGCTTGGGGACAC
CCTCAGGGGTTCCCCTCTTTGGTCAGCCAGGCTCCTGGGTGTGCAGGAAGTGCCCCTCTC
CCGGCCTGTGGACACCCTGAAGCCCCCACTCCGCTCCAGCCTCCACTTCCGGTGCCCTGC
AGATCCCGCCGCGCCCACCCGAGGGTCCCTCCGCCGGTTCCCCTGGCCCTGGTTCAGAAG
GGCCCTCCTTCCCAGCCCCGCTGTCAAGTCCTAGGACCTTTTCTCTCTCATCCTGCCGGG
CCGGGCCTGGGCAGCTTGAAGCCCTCGTGGGATTTTGTTTTGTTTTCATCCTAACGTAAA
GGAGCTCCTGCGATCCTGGAGCTATCAAAGAGACTCTGCCTGGAAAGAGCTGGTGCTGCA
GAATGGGCCCTCCACTTTGGCAGTGACATAACTCTGTCCCGAGATCCCAGAGCAATCGAG
GAAACCTTTAACTCCATTCAAAGTGTTTATCAGGGGGAAGGAAAGATGATGGTAGTGAG
ATGTCTCGGCCCCCGCCCTATTATTTTAGCCCCTGTAAACCAGTTTTTTCGGTCCTGGCG
CCATCGAGGAAGCTTTTTACATTGTCCAGTGTTTATTTCGAGGAGTGAGGGATGGCAGGC
TCAAAGACTTGGAAAGAGATGGCTTAACAGAAAAG
```

Fig. 5. Organization of Dnmt1 exons in genomic DNA and mRNA in somatic cells, pachytene spermatocytes and oocytes. (A) The positions of exons 1o, 1s and 1p in the Dnmt1 genomic locus on proximal mouse chromosome 9 are shown at top; splicing patterns and distribution of open reading frames are shown below. (B) Sequences of the alternatively spliced exons; exon 2, which is common to all three transcripts, is underlined, and ATG codons are boxed. The restriction endonuclease sites shown at the top of A are: R, *Eco*RI; S, *Sac*I; B, *Bam*HI; K, *Kpn*I; X, *Xba*I; A, *Avr*II; N, *Not*I.

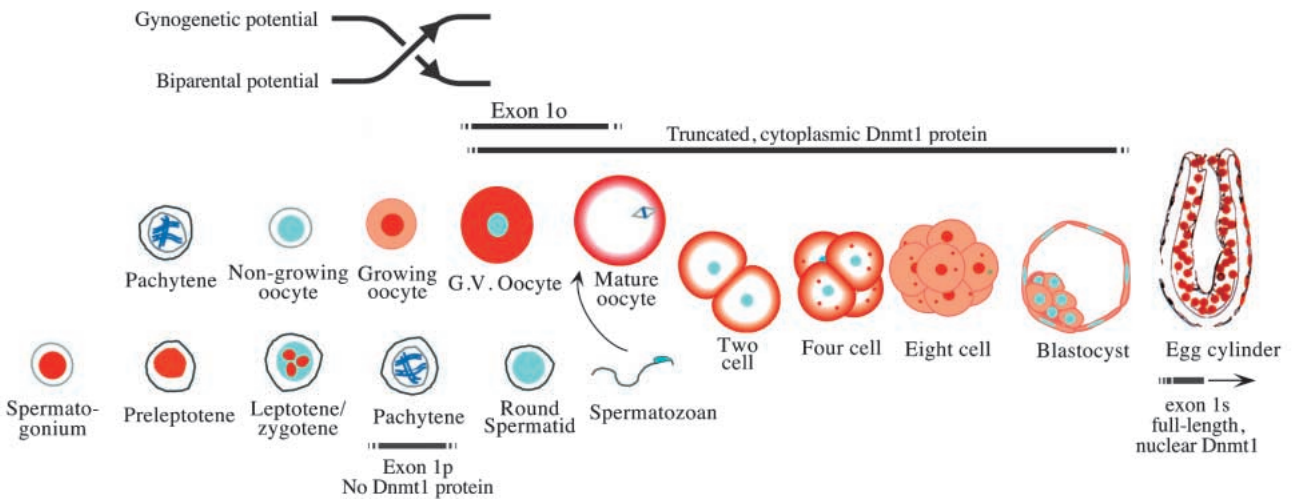

Fig. 6. Dnmt1 protein and mRNA in male and female germ cells and in early mouse embryos. The amount and location of Dnmt1 is represented by the intensity of red shading; nuclei are shown in blue when free of detectable Dnmt1 staining. Dnmt1 is present in postnatal oocyte nuclei only during the growth phase, during which time nuclei lose gynogenetic developmental potential and gain biparental potential (Kono et al., 1996). The accumulation of large amounts of truncated cytoplasmic Dnmt1 coincides with the appearance of the alternative 5′ exon. Nuclear foci that stain intensely for Dnmt1 appear briefly in leptotene/zygotene spermatocytes; staining of nucleoplasm during this stage is very faint outside of the foci (Jue et al., 1995). Dnmt1 is not detectable in pachytene spermatocytes, in which another alternative exon prevents translation of Dnmt1 mRNA. Dnmt1 is briefly in nuclei at the 8-cell stage but is found in the cytoplasm of blastocysts. After implantation Dnmt1 protein is localized to nuclei in all somatic cell types.

Staining remains cytoplasmic in embryos until the 8-cell stage, where nuclei stain intensely (Carlson et al., 1992). Nuclei in the blastocyst are again free of Dnmt1, but after implantation all staining is found in nuclei (Trasler et al., 1996).

Dnmt1 contains a functional nuclear localization sequence (NLS) near the N terminus. This NLS is not deleted from the oocyte-specific Dnmt1, and the cytoplasmic localization of the truncated Dnmt1 found in oocytes cannot be explained by a lack of the appropriate nuclear import signal. Furthermore, the truncated form of Dnmt1 has full enzymatic activity and is capable of entering nuclei, as shown by the fact that it briefly does so in 8-cell embryos (Carlson et al., 1992). Dnmt1 appears to be actively retained near the cortex of the mature oocyte, as the enzyme is confined to a shell just under the surface of the oocyte. It is of interest that the extreme N terminus of the somatic form of Dnmt1 was identified as a factor that binds to Annexin V with high affinity (Ohsawa et al., 1996). Annexin V or a related membrane-associated protein may be actively involved in the cytoplasmic sequestration of truncated Dnmt1 in the oocyte. The complete cytoplasmic sequestration of a replication factor appears to be unprecedented. It is also of interest that the amount of Dnmt1 per cell in oocytes and early embryos is several thousand-fold higher than in any somatic cell type, and the possibility of a cytoplasmic function unrelated to DNA methylation cannot be excluded.

Translational repression of Dnmt1 in pachytene spermatocytes

Translation of many mRNAs depends on a ribosome scanning mechanism: the ATG closest to the 5′ cap site is the sole initiation codon, if it conforms to the RXXATGG consensus (Kozak, 1996). However, there are numerous exceptions to this rule; short upstream open reading frames inhibit translation in some cases but not others. The Drosophila Antp mRNA is translated despite 15 short upstream open reading frames (Ye et al., 1997), and the BiP and FGF2 mRNAs are also translated from mRNAs that contain upstream ORFs (reviewed by Geballe and Morris, 1994; Morris, 1997). In some cases, notably that of S-adenosyl L-methionine decarboxylase, tissue-specific translation depends on the presence of an upstream ORF (reviewed by Morris, 1997).

Dnmt1 mRNA is expressed at high levels in pachytene spermatocytes (Singer-Sam et al., 1990; Trasler et al., 1992; Numata et al., 1994), but there is no detectable Dnmt1 protein at this stage. It was found that pachytene spermatocyte Dnmt1 mRNA has an alternative 5′ exon that contains seven ATG codons, five of which conform to the consensus for favorable translation (Kozak, 1996). Additional small ORFs in exons 2 and 3 cause the ATG of the first ORF that can yield active enzyme to be the 10th in the sequence. The multiple upstream ORFs appear to strongly inhibit translation, as the mRNA is only weakly associated with ribosomes in pachytene spermatocytes (Trasler et al., 1992) and there is no detectable Dnmt1 protein at stages where the pachytene-specific exon is present (Figs 1B and 3A). It is concluded that the pachytene-specific exon interferes with translation of the downstream ORF that can produce enzymatically active Dnmt1. The oocyte-specific exon contains a single ORF of 14 codons, and there is an ORF of three codons in exon 2 and another of eight codons in exon 3 before the long ORF. Because the oocyte contains very large amounts of truncated Dnmt1 protein, the upstream ORFs in the oocyte-specific mRNA are clearly compatible with translation (reviewed by Gabelle and Morris, 1994; Morris, 1997).

Sex-specific exons and cytosine methylation in gametogenesis

It has been suggested that genomic methylation patterns might be established by a family of sequence-specific de novo methyltransferases during gametogenesis and early

development, and that a maintenance methyltransferase dependent on hemimethylated sites might perpetuate the resulting methylation patterns in somatic cells (Holliday and Pugh, 1975; Jähner and Jaenisch, 1984). However, no specialized de novo methyltransferase has been identified, there is no discernible sequence context that distinguishes methylated and unmethylated sites in genomic DNA (Tremblay et al., 1997; Olek and Walter, 1997), and while Dnmt1 does prefer hemimethylated substrates by a factor of 10- to 30-fold it is the predominant de novo methyltransferase in extracts of all cell types and tissues examined (Yoder et al., 1997b). The de novo methylation activity of Dnmt1 is cued by factors other than direct digital sequence recognition (Bestor and Tycko, 1996; Yoder et al., 1997b). There are high levels of Dnmt1 protein in nuclei of postmitotic germ cells at specific stages, and we suggest that this may identify the stages at which de novo methylation occurs in germ cells.

While little is known of the timing of de novo methylation during gametogenesis, nuclear transplantation studies have shown that the nuclei of non-growing oocytes can support the development of gynogenetic mouse embryos to day 13.5, while gynogenetic embryos containing nuclei of later oocytes do not develop past day 9 (Kono et al., 1996). However, nuclei of more mature oocytes were much more effective in supporting the development of biparental embryos than were nuclei of non-growing oocytes (Kono et al., 1996). This indicates that gynogenetic developmental potential is lost and biparental developmental potential gained during oocyte growth. As shown in Fig. 6, oocyte growth is the only stage of postnatal oogenesis at which nuclei contain large amounts of Dnmt1 protein, and this finding indicates that the regulatory regions of imprinted genes may undergo de novo methylation at this time. The large amounts of truncated, cytoplasmic Dnmt1 protein appear to function as a maternal store of DNA methyltransferase during early development, as homozygous mutant embryos develop to day 8.5, while embryonic stem cells of the same genotype (which do not have large stores of Dnmt1) promptly undergo apoptosis when induced to differentiate (Lei et al., 1996).

Meiotic chromosomes present several features that make them vulnerable to de novo methylation during crossing over. Structural features associated with recombination are preferred targets of Dnmt1 (Bestor and Tycko, 1996), and DNA in perichiasmate regions is exposed to diffusible proteins (Narayan and Raman, 1995). Germ cells may protect their meiotic DNA from dysregulated de novo methylation by excluding Dnmt1 from the vicinity of meiotic chromosomes at all stages except for the growing oocyte, where it is suggested that imprinted genes undergo de novo methylation. The concentration of Dnmt1 into large nuclear foci during an interval in the development of leptotene/zygotene spermatocytes suggests that de novo methylation of paternally imprinted loci might occur at this time (Trasler et al., 1990; Jue et al., 1995).

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