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DNA methyltransferase is developmentally expressed in replicating and non-replicating male germ cells

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ABSTRACT

Genomic methylation patterns are established during maturation of primordial germ cells and during gametogenesis. While methylation is linked to DNA replication in somatic cells, active *de novo* methylation and demethylation occur in post-replicative spermatocytes during meiotic prophase (1). We have examined differentiating male germ cells for alternative forms of DNA (cytosine-5)-methyltransferase (DNA MTase) and have found a 6.2 kb DNA MTase mRNA that is present in appreciable quantities only in testis; in post-replicative pachytene spermatocytes it is the predominant form of DNA MTase mRNA. The 5.2 kb DNA MTase mRNA, characteristic of all somatic cells, was detected in isolated type A and B spermatogonia and haploid round spermatids. Immunoblot analysis detected a protein in spermatogenic cells with a relative mass of 180,000–200,000, which is close to the known size of the somatic form of mammalian DNA MTase. The demonstration of the differential developmental expression of DNA MTase in male germ cells argues for a role for testicular DNA methylation events, not only during replication in premeiotic cells, but also during meiotic prophase and postmeiotic development.

INTRODUCTION

Gamete DNA contains sex- and sequence-specific patterns of methylated cytosine residues that have been suggested to be important in the regulation of development and in the phenomenon of genomic imprinting. These methylation patterns are established by *de novo* methylation and demethylation in primordial germ cells and also in later stages of gametogenesis. Trasler et al. (1) have shown that post-replicative spermatocytes carry out *de novo* methylation or demethylation of certain testis-specific genes while housekeeping genes remain unmethylated. There are also differences in the location of methylated sites in certain genes in the testis as compared to the ovary, and the overall level of methylation of oocyte DNA is lower than that of spermatozoal DNA (2,3). These and other data demonstrate that sex- and sequence-specific methylation patterns are established during gametogenesis (4,5,6).

The methylation of cytosine residues, a post replication process in which a methyl group is transferred from S-adenosyl-L-methionine to the 5-position of cytosine in DNA, is catalyzed by the enzyme DNA (cytosine-5)-methyltransferase (DNA MTase, EC 2.1.1.37) (7). The enzyme is strongly stimulated by the hemimethylated sites that result from semiconservative DNA replication and this property ensures the clonal transmission of methylation patterns (8,9,10). Sequence analysis has shown that DNA MTase contains a C-terminal domain of about 500 amino acids that is closely related to bacterial cytosine-specific restriction methyltransferases (7). This domain is attached by a run of alternating Gly and Lys residues to a 1,000 amino acid N-terminal domain that has been suggested to play an as yet undefined regulatory role. The protein has an apparent relative mass (M_r) of 190,000 in both mouse and human cells; smaller forms can be produced in certain cell types by proteolytic cleavage of peptide sequences near the N-terminus (10,11,12). Surprisingly, somatic cell types with different methylation patterns contain identical or very similar forms of DNA MTase protein or mRNA (7) and it is not known how *de novo* methylation might be regulated.

De novo methylation is rare in somatic cells under most conditions but common in gametogenesis and has been shown to occur in post-replicative spermatocytes. We have used nucleic acid probes and specific antibodies to examine differentiating male germ cells for evidence of alternative expression or forms of DNA MTase that might be involved in specific *de novo* methylation. We report here that a testis-specific DNA MTase mRNA, about 1,000 nucleotides larger than the DNA MTase mRNA of somatic cells, is present in pachytene spermatocytes, a cell type that is known to be active in *de novo* methylation.

MATERIALS AND METHODS

Animals and Chemicals

CD-1 mice of different ages were obtained from Charles River Laboratory (Wilmington, MA). Guanidine isothiocyanate, agarose, restriction endonucleases and protein molecular weight

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standards were purchased from Bethesda Research Laboratories (Gaithersburg, MD). For labelling probes, dCTP (α-^{32}P-deoxycytidine 5'-triphosphate tetra (triethylammonium) salt, 3000 Ci/mmole) was obtained from Amersham Corp. (Arlington Heights, IL). Unless indicated in the text, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Tissues and Cells for RNA extraction

Total RNA was purified from adult mouse tissues (ovary, seminal vesicle, epididymis, lung, liver, kidney, heart, brain, whole blood, spleen, intestine) and the the testes of prepubertal and adult mice by disruption of tissue in a guanidine isothiocyanate solution, followed by centrifugation in CsCl and extraction with phenol as previously described (13).

Isolated populations of male germ cells from the testes of 8-, 17- and 70-day old mice were obtained by the unit gravity sedimentation procedure (14,15,16,17). Type A spermatogonia (average purity, 84%; n=2 cell separations), type B spermatogonia (average purity, 81%; n=2) and interstitial cells (30−50% Leydig cells; 5−10% germ cells; 5−10% erythrocytes; n=2) were isolated from 240 8-day old mice (16). Preleptotene spermatocytes (average purity, 86%, n=2), leptotene-zygotene spermatocytes (average purity, 81%; n=2), and prepubertal pachytene spermatocytes (average purity, 82%; n=2) were isolated from 100 17-day old mice (16). Pachytene spermatocytes (average purity, 82%; n=2), round spermatids (average purity, 90%; n=2) and residual body/cytoplasts (average purity, 84%; n=2) were isolated from the testes of 10 70-day old mice (14,17). RNA was isolated from the purified spermatogenic cells by the guanidine isothiocyanate-CsCl-phenol procedure (13).

RNA was also isolated from polysomal gradients of purified male germ cells. Polysomal gradients were performed as described by Kleene et al. (18) with modifications introduced by Hake et al. (19). Briefly, pellets of isolated spermatogenic cells, isolated as described above, were resuspended in $MgCl_2$ buffer (19). The cell suspensions were homogenized and the post-mitochondrial supernatants centrifuged through 10−35% sucrose gradients containing a 60% sucrose cushion. Eight fractions were collected from each gradient. Control fractions, using EDTA to release the polysomes, were isolated by replacing the $MgCl_2$ in the resuspension solution with 10mM EDTA.

DNA Probes and Northern Blot Analysis

Two mouse DNA MTase cDNA probes, pR5 and pR2K, were used (7). pR5 encodes the Cys-rich region of DNA MTase and corresponds to nucleotides 1614−2055 in the cDNA sequence. pR2K contains a 2,057 bp insert (nucleotides 2979−5063 in the cDNA) that includes all the sequences that are conserved between mammalian and bacterial DNA cytosine methyltransferases. The cDNA probes were labeled, by the random priming method of Feinberg and Vogelstein (20), to specific activities of 5×10^8 to 1×10^9 cpm/μg of DNA.

Denatured RNAs were electrophoresed in 1.0−1.5% agarose-formaldehyde gels, transferred to nitrocellulose filters and hybridized using previously published procedures (13).

Antibodies and Immunoblotting

A polyclonal rabbit antibody (anti-pATH5) was raised against a TrpE fusion protein that contained amino acids 137 to 635 of DNA MTase. The fusion protein was purified from *E.coli* and injected into rabbits according to standard procedures. The antibodies recognized purified DNA MTase and a single protein of the appropriate size in whole cell lysates of murine erythroleukemia cells (see Fig. 5).

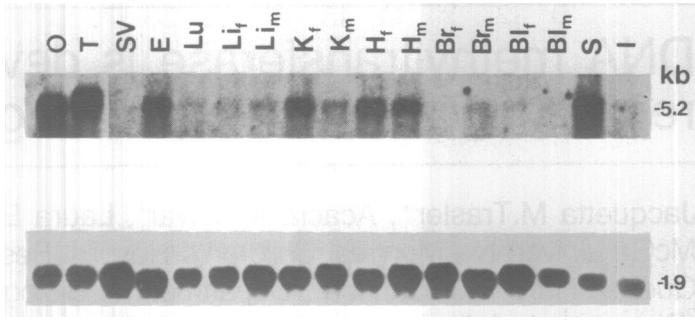

Figure 1. Northern blot analysis of the tissue distribution of DNA methyltransferase in the adult mouse. Top panel was probed with the DNA methyltransferase probe PR5. Bottom panel shows the same filter after hybridization with a probe for 18S ribosomal RNA. O, ovary; T, testis; SV, seminal vesicle; E,epididymis; L,lung; Li$_f$ or Li$_m$, liver (female or male); K, kidney; H, heart; BR, brain; BL, blood; S, spleen; I, intestine. Ten μg aliquots of total RNA were loaded in each lane of a 1.5% agarose/formaldehyde gel.

Protein extracts from the testes of prepubertal and adult mice were prepared. The testes were removed and homogenized immediately in tissue suspension buffer (0.15 M NaCl, 0.05 M Tris Cl (pH 7.5), 100 μg/ml PMSF, 2 μg/ml aprotinin and 2 μg/ml leupeptin). The protein concentrations of the extracts were determined using a Bio-Rad protein assay following the manufacturer's instructions. A whole-cell lysate of 10^5 murine erythroleukemia cells, a cell type in which DNA MTase has been extensively studied (7), was used as a basis of comparison for testicular DNA MTase proteins. Twenty-five and 100 μg aliquots of the testicular protein extracts were heated at 60°C for 10 minutes in non-reducing gel-loading buffer (0.05 M Tris Cl-pH 6.8, 2% SDS, 30% glycerol, 0.05% bromophenol blue) (21), electrophoresed in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (22,23,24). The membrane was incubated overnight with the anti-pATH 5 DNA MTase antibody (1:15,000) in borate Blotto buffer (0.1 M boric acid, 0.025 M sodium borate, 0.08 M NaCl, pH 7.4 containing 5% Carnation instant nonfat dried milk) followed by a goat anti-rabbit IgG conjugated with alkaline phosphatase (1:5,000) from Promega. The colour was developed following instructions supplied with the Promega ProtoBlot kit and using the reagents 5-bromo-4-chloro-3-indolyl phosphate and nitrotetrazolium blue chloride.

RESULTS

Tissue Distribution of DNA Methyltransferase Transcripts

The pattern of RNA expression of DNA MTase was analyzed on Northern blots of RNA from adult mouse ovary, testis, seminal vesicle, epididymis, lung, liver, kidney, heart, brain, whole blood, spleen and intestine (Fig. 1). A DNA MTase mRNA transcript of 5.2 kb was found in all the tissues assayed. When differences in the amount of RNA loaded (as determined by probing the blot with an 18S ribosomal RNA probe) were taken into account, the 5.2 kb DNA MTase mRNA was most abundant in mouse ovary and testis. DNA MTase transcripts were also abundant in RNA from the epididymis, kidney, heart and spleen

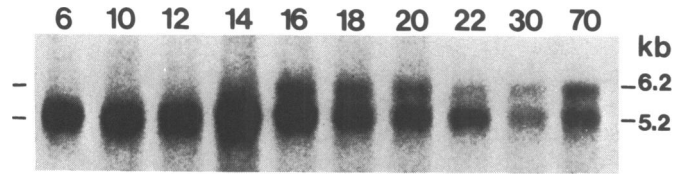

Figure 2. Developmental expression of DNA methyltransferase mRNAs in prepubertal mouse testes. Total testis RNA was prepared from testes of mice aged 6, 8, 10, 12, 14, 16, 18, 20, 22, 30 and 70 days of age and 15 μg aliquots of RNA were loaded in each lane of a 1.5% agarose gel. The blot was hybridized with the PR5 DNA methyltransferase probe. Note the presence of 5.2 and 6.2 kb transcripts.

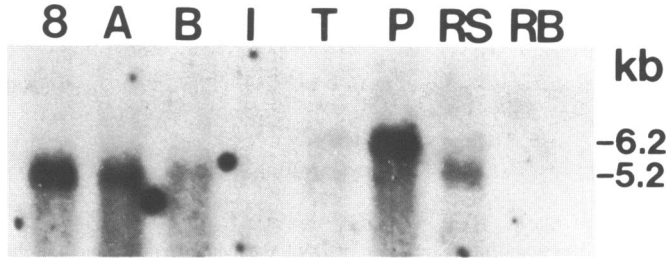

Figure 3. Expression of DNA methyltransferase mRNAs in isolated testicular cells from the mouse. Ten μg aliquots of RNA were loaded in each lane, transferred to nylon membranes and hybridized with the PR5 DNA Metase probe. 8, total testis RNA from an 8 day old mouse; A, type A spermatogonia; B, type B spermatogonia; I, interstitial cells; T, total testis RNA from an adult mouse; P, pachytene spermatocytes; RS, round spermatids; RB, residual bodies.

and were least abundant in the seminal vesicle, lung, liver, brain, whole blood and intestine. Examination of the autoradiogram, shown in Fig. 1, after overexposure suggested the presence of a slower migrating mRNA transcript that was only present in testis RNA. The results indicate that the mRNA for DNA MTase is abundantly expressed in the testis.

Expression of DNA Methyltransferase mRNAs During Male Germ Cell Development

Since the timing and temporal appearance of successive cell types during the first spermatogenic wave after birth is precise and well known (25,26), prepubertal mice of various ages were used to examine the developmental pattern of expression of DNA MTase mRNAs during spermatogenesis (16,27). The temporal appearance of DNA MTase transcripts in total testicular RNA from mice aged 6 through 70 days is shown in Fig. 2. The 5.2 kb DNA MTase transcript is present in the testes of mice as early as 6 days after birth and is more abundant in young mice than in adult mice. When the abundance of the 5.2 kb transcript was corrected for RNA loading using an 18S rRNA probe and laser densitometry and compared to the 70 day value (set at 100%), a distinct developmental pattern was seen—6 days (180%), 10 days (113%), 12 days (131%), 14 days (290%), 16 days (252%), 18 days (162%), 20 days (151%), 22 days (120%) and 30 days (60%). The highest levels of accumulated 5.2 kb DNA MTase transcript coincided with the developmental appearance in the seminiferous epithelium of spermatogonia and spermatocytes.

Interestingly, a second slower migrating transcript is present, first appearing in the mouse testis between 14 to 16 days after birth and decreasing substantially by 22 days after birth. Although we have reservations about exact mRNA sizing of transcripts in the 5–6 kb region of the gel, we estimate that the slower migrating transcript is approximately 6.2 kb. The timing of appearance of the 6.2 kb DNA MTase transcript coincides with the appearance of pachytene spermatocytes in the seminiferous epithelium (16). Identical results were obtained when the blot shown in Fig. 2 was hybridized with the DNA MTase pR2k probe (data not shown).

Germ Cell Localization of DNA Methyltransferase mRNAs in the Testis

RNA was prepared from isolated populations of male germ cells at different stages of their development to determine the cellular localization of the two testicular DNA MTase transcripts (Fig. 3). Since about 16% of the cells in the seminiferous epithelium of 6-day-old mice are type A spermatogonia (84% are Sertoli cells),

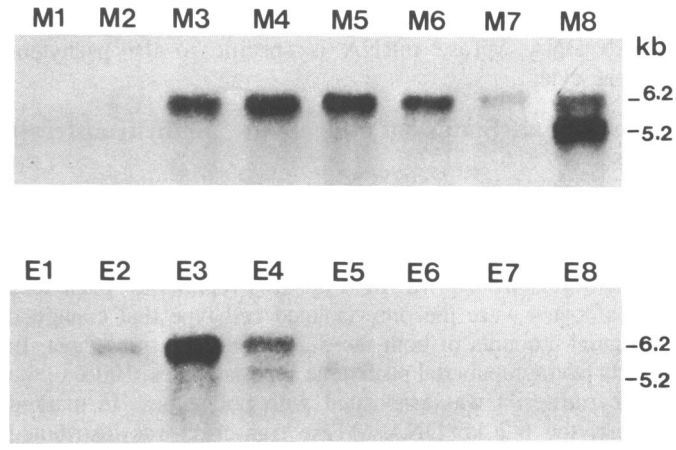

Figure 4. Polysomal distribution of DNA methyltransferase mRNAs from prepubertal pachytene spermatocytes. The top panel shows RNA fractions from a gradient containing Mg++. The bottom panel shows RNA from a gradient containing EDTA in place of Mg++. M1, top of gradient. One hundred percent of the 5.2 kb transcript is associated with polysomes; in contrast, the 6.2 kb transcript is distributed throughout the gradient.

it was unclear if DNA MTase mRNAs were expressed in early germ cells. Purification of type A and B spermatogonia from 8 day old mice indicated that the 5.2 kb DNA MTase transcript was present in RNA from both type A and type B spermatogonia but was not detected in interstitial cells (Fig. 3). The 5.2 kb transcript was present at low levels in pachytene spermatocytes; the predominant transcript in these cells was the 6.2 kb transcript. In the postmeiotic spermatids, very little of the 6.2 kb transcript was detected and the 5.2 kb transcript was the predominant mRNA species present.

The results from the analysis of isolated spermatogenic cells suggested that the 6.2 kb DNA MTase transcript was specific to meiotic male germ cells. To determine if the 6.2 kb transcript was also present in early meiotic prophase cells, RNA was prepared from isolated populations of preleptotene spermatocytes, leptotene/zygotene spermatocytes and prepubertal pachytene spermatocytes from the testes of 17-day old mice. The 5.2 kb transcript was present in all meiotic cell types; in contrast, the 6.2 kb transcript was only found in prepubertal pachytene

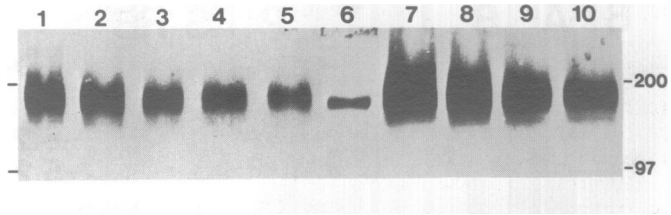

Figure 5. Developmental expression of DNA methyltransferase protein in the mouse testis. Twenty-five (lanes 1–5) or 100 μg (lanes 7–10) aliquots of several stages of prepubertal mouse testes (6 day testis—lanes 1,7; 16 day testis—lanes 2,8; 22 day testis—lanes 3,9; 30 day testis—lane 4) or adult mouse testis (70 day testis—lanes 5,10) protein were loaded on 10% SDS/PAGE gels. A total cell lysate of 10^5 murine erythroleukemia cells (lane 6) was used as a basis of comparison for testicular DNA methyltransferase proteins. After transfer to nitrocellulose membranes, DNA methyltransferase proteins were detected using the pATH5 DNA methyltransferase antibody and an alkaline phosphatase-labelled second antibody.

spermatocytes (data not shown). These results suggest that the 6.2 kb DNA MTase mRNA is specific to the pachytene spermatocyte.

Translational Status of the DNA Methyltransferase Transcripts

Polysome analysis was performed to monitor the translational status of the two DNA MTase transcripts. A polysomal gradient of cell homogenates from purified prepubertal pachytene spermatocytes is shown in Fig. 4. Prepubertal pachytene spermatocytes were the only isolated cell type that contained substantial amounts of both the 5.2 and 6.2 kb transcripts. In extracts from prepubertal pachytene spermatocytes, 100% of the 5.2 kb transcript was associated with polysomes. In marked contrast, the 6.2 kb DNA MTase transcript was distributed throughout the gradient with approximately 15% of it associated with polysomes. The fact that both mRNAs were released from polysomes when EDTA was substituted for Mg^{++}, confirmed that the DNA MTase transcripts were on polysomes and not just nonspecifically sedimenting in the polysomal region of the gradient (Fig. 4, lower panel).

DNA Methyltransferase Protein Expression in the Developing Testis

Western blot analysis was used to determine the presence, relative abundance and size of DNA MTase protein(s) in extracts from the prepubertal and adult testis (Fig. 5). More specifically, we wanted to determine if the 6.2 kb testis-specific DNA MTase mRNA transcript coded for a larger size protein than the 5.2 transcript. In testis extracts from prepubertal mice (Fig. 5., lanes 1–3, 7–9) and adult mice (lanes 5, 10), the pATH 5 DNA MTase antibody detected a wide band of protein(s) of molecular mass 180,000–190,000. The DNA MTase protein recognized by the pATH5 antibody in testis was similar in size to that from murine erythroleukemia cells (Fig. 5, lane 6). DNA MTase protein was abundant in extracts of the testes of both young (6, 16, 22 and 30 days of age) and adult mice (70 days of age). There was no evidence of a DNA MTase protein of a higher molecular mass than 180,000–190,000 in testis extracts of mice aged 16, 22, 30 or 70 days of age when either 25 μg (Fig. 5., lanes 1–5) or 100 μg (lanes 7–10) of protein were loaded on the gels. These results suggest that the 6.2 kb mRNA transcript that is detected in testes of mice aged 16–70 days does not encode a DNA MTase protein of molecular mass greater than 180,000–190,000.

DISCUSSION

The results show that DNA MTase is expressed in the testis at a very high level and that pachytene spermatocytes contain a DNA MTase mRNA that is about 1 kb longer than the mRNA found in all other cells. The slower migrating mRNA appears to be unique to the testis and is largely restricted to pachytene spermatocytes. While the high level of expression of DNA MTase in premeiotic germ cells is consistent with the rapid rate of division of these cells (28), expression of a new form of DNA MTase mRNA in a post-replicative cell type is surprising.

A DNA MTase mRNA of 5.2 kb, similar in size to the transcript found in RNA from many different somatic tissues, was expressed throughout spermatogenesis. A 5.2 kb DNA MTase transcript has also been found in RNA from a number of different mouse cultured cell types (7). Interestingly, in the present study, a DNA MTase transcript of 6.2 kb, apparently unique to the testis, was detected in the mouse testis 16 days after birth and was shown in cell separation studies to be present in meiotic pachytene spermatocytes. It is clear therefore, from our results, that DNA MTase mRNA is abundantly expressed not only in the rapidly dividing spermatogonia but also in non-replicating spermatogenic cells such as pachytene spermatocytes and round spermatids. A recent study by Singer-Sam and colleagues (29), using reverse transcriptase-mediated quantitative PCR, also detected the presence of mRNA for DNA MTase during meiosis; the study did not analyze transcript size.

The presence of a different DNA MTase transcript in spermatocytes than in spermatogonia suggests that the product, function or regulation of DNA MTase may be different in non-replicating than in replicating spermatogenic cells. Previous restriction enzyme studies from our laboratory, in which the protamine genes were found to become progressively methylated during meiotic prophase in the mouse testis, indicated that *de novo* methylation was occurring after DNA replication ceased in the testis (1). The present study supports the results of our previous work by providing evidence that the enzyme that methylates DNA, and presumably the protamine genes during meiotic prophase, is expressed in post-replicating (post-spermatogonial) male germ cells. Ariel and colleagues (6) have reported that another testis-specific gene, phosphoglycerate kinase 2, becomes methylated in postmeiotic male germ cells; this study provides further evidence of gene-specific *de novo* methylation during the post-replicative phase of spermatogenesis. It is possible that DNA MTase mRNA encodes a protein, in non-replicating cells, that plays a role in *de novo* methylation of DNA and may be one of the reasons spermatozoal DNA is more methylated than oocyte DNA (3). DNA MTase in pachytene spermatocytes may also play a role in DNA repair that occurs at this time during spermatogenesis. The great decrease in the levels of the 6.2 kb DNA MTase mRNA in round spermatids suggests a meiosis-specific function for the transcript.

The fact that both the 5.2 and 6.2 kb transcripts are associated with polysomes is good evidence that both transcripts are being translated. The presence of a portion of the 6.2 kb DNA MTase mRNA in the nonpolysomal fraction suggests that the the 6.2 kb transcript is translationally regulated, like other mouse testicular genes (30), or perhaps that it is less efficiently translated than the 5.2 kb transcript. Since probing with two different cDNA probes, one oriented to the 5′ end and the other to the 3′ end of the gene, detected the same two transcripts and the mouse has only one DNA MTase gene (7), we are confident that the

5.2 and 6.2 kb transcripts arise from the same gene. When the poly (A)+ tails were removed with RNase H, two distinct DNA MTase transcripts were evident in RNA from pachytene spermatocytes as compared to round spermatids (data not shown); the 1.0 kb difference between the two transcripts cannot be ascribed solely to a difference in poly (A)+ tail length. The difference in transcript size is thus likely to be due to an alternative start site, alternate splicing or an alternate poly (A)+ addition site; any of these mechanisms could provide a way to differentially regulate the expression of DNA MTase in replicating versus non-replicating spermatogenic cells (31).

The protein data provide further evidence that DNA MTase is expressed throughout spermatogenesis. In the adult mouse, the seminiferous tubules in the testis are composed predominantly of meiotic pachytene spermatocytes (15% of cells) and postmeiotic spermatids (71% of cells) (17); thus these meiotic and postmeiotic cells are most likely to be the site of the DNA MTase protein detected in the adult mouse testis extract. The demonstration of testicular DNA MTase mRNA and protein in the present study and previous results showing increases in DNA methylation during and after meiotic prophase in the testis (1,6) argue for a role for testicular DNA methylation events, not only during replication in premeiotic germ cells, but also during meiotic prophase and postmeiotic development. The temporal appearance during testicular development of the 6.2 kb DNA MTase transcript suggests a novel role or regulation for DNA methylating enzyme(s) at the time that mouse male germ cells undergo chromosome pairing and recombination. It is possible that the additional sequences present in the 6.2 kb meiosis-specific DNA MTase mRNA are involved in regulation of sequence-specific *de novo* methylation, and characterization of the protein product of this mRNA is underway.

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