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Sex-specific promoters regulate *Dnmt3L* expression in mouse germ cells

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BACKGROUND: Dnmt3L, a member of the DNA methyltransferase 3 family, lacks enzymatic activity but is required for de-novo methylation of imprinted genes in oocytes and for transposon repression in male germ cells. METHODS: We used northern blots, RT–PCR, 5′ rapid amplification of complementary DNA (cDNA) ends (RACE), RNase H mapping, real-time/quantitative RT–PCR and *in situ* hybridization to identify and characterize *Dnmt3L* transcripts produced during germ cell development. RESULTS: Mouse *Dnmt3L* uses three sex-specific promoters, not the single promoter previously thought. A promoter active in prospermatogonia drives transcription of an mRNA encoding the full-length protein in perinatal testis, where de-novo methylation occurs. Late pachytene spermatocytes activate a second promoter in intron 9 of the *Dnmt3L* gene. After this stage, the predominant transcripts are three truncated mRNAs, which appear to be non-coding. We could also detect similar adult testis transcripts in humans. In the mouse ovary, an oocyte-specific promoter located in an intron of the neighbouring autoimmune regulator (*Aire*) gene produces a transcript with the full open reading frame (ORF). This is the only *Dnmt3L* transcript found in growing oocytes and is absent in the oocytes of *Dnmt3L*–/– females. CONCLUSIONS: Sex-specific promoters control *Dnmt3L* expression in the mouse germ line, mirroring the situation at the *Dnmt1* and *Dnmt3A* loci.

Key words: Dnmt3L/imprinting/oocyte/promoter/testis

Introduction

DNA methylation is a heritable epigenetic modification which plays an important role in transcriptional repression of imprinted genes (Li *et al*., 1993; Bourc'his *et al*., 2001; Kaneda *et al*., 2004), transposons (Yoder *et al*., 1997; Walsh *et al*., 1998; Bourc'his and Bestor, 2004; Webster *et al*., 2005) and genes on the inactive X chromosome (Panning and Jaenisch, 1996). There is also evidence to suggest that methylation is important for maintaining the stability of pericentromeric satellite repeats, as mutations in the DNMT3B methyltransferase are found in patients suffering from immunodeficiency-centromeric instability-facial anomaly (ICF) syndrome (OMIM 242860) and are accompanied by demethylation of classical satellite repeats and the formation of branched and multiradiate chromosomes (Hansen *et al*., 1999; Okano *et al*., 1999; Xu *et al*., 1999). Aberrant DNA methylation is often seen in cloned embryos (Dean *et al*., 2001; Humpherys *et al*., 2001), tumours (Jones and Laird, 1999) and disease syndromes involving imprinted genes (Reik and Walter, 2001b).

Methylation undergoes reprogramming in mouse germ cells, which is essential to allow removal of methylation inherited with the parental alleles and establishment of new patterns of modification (Yoder *et al*., 1997; Reik *et al*., 2001). Most imprinted genes carry a methylation mark on the maternal

allele at the differentially methylated region (DMR), but a small number inherit a mark on the paternal allele (Reik and Walter, 2001a; Bestor and Bourc'his, 2006). Loss of methylation occurs shortly after the germ cells colonize the embryonic gonad (Davis *et al*., 2000; Hajkova *et al*., 2002). Establishment of new imprints by de-novo methylation of the DMR occurs in the prospermatogonia in the male germ line and during the oocyte growth phase in the ovary (Davis *et al*., 2000; Ueda *et al*., 2000; Lucifero *et al*., 2004; Li *et al*., 2004). Methylation of intracisternal A particles (*IAP*), LINE1 elements (*L1*) and minor satellite repeats also decreases in post-migratory germ cells but is not wholly removed (Hajkova *et al*., 2002; Lees-Murdock *et al*., 2003), which is important for maintaining transcriptional repression of transposons (Bourc'his and Bestor, 2004; Webster *et al*., 2005) and probably for the stability of the minor satellite DNA; remethylation of any demethylated elements also occurs in the prospermatogonia in males and the growing oocyte in females (Lees-Murdock *et al*., 2003; Lucifero *et al*., 2004).

Three enzymatically active DNA methyltransferases have been characterized in mice (Goll and Bestor, 2005). Dnmt1 is thought to maintain methylation by converting newly replicated hemimethylated DNA strands to fully methylated DNA. In germ cells, *Dnmt1* produces an oocyte-specific isoform important for maintaining methylation on both paternally and maternally imprinted genes in the early embryo (Howell *et al*., 2001), whereas in the male germ line it is down-regulated at the pachytene stage of meiosis by a switch in promoter usage which results in the production of an untranslated mRNA (Mertineit *et al*., 1998). Dnmt3a and Dnmt3b are more important for de-novo methylation (Okano *et al*., 1999) and both proteins are localized to the germ cell nuclei during periods of de-novo methylation (Lees-Murdock *et al*., 2005). Deletion of *Dnmt3a* specifically in the male germ line results in a failure in spermatogenesis, accompanied by a loss of methylation on two of the three paternally methylated imprinted genes examined (Kaneda *et al*., 2004). In the female germ line, deletion of *Dnmt3a* prevents methylation of imprinted genes in the oocyte.

Mice with germ line-specific mutations in *Dnmt3a* show some similarity to mice carrying a deletion in the *Dnmt3L* gene (Bourc'his *et al*., 2001; Hata *et al*., 2002; Bourc'his and Bestor, 2004). Dnmt3L has homology to the other members of the Dnmt3 family but is missing crucial residues in the conserved catalytic motifs and has not been shown to have enzymatic activity *in vitro* (Aapola *et al*., 2001; Bourc'his *et al*., 2001; Hata *et al*., 2002). Offspring of *Dnmt3L–/–* females show loss of methylation of imprinted genes in the oocyte and subsequent loss of imprinting, but methylation on other sequences tested was normal (Hata *et al*., 2002; Bourc'his and Bestor, 2004).

Mutant males lack viable sperm because of failure of spermatogenesis. Methylation of *L1* and *IAP* elements was also decreased, and very high levels of transcription from these elements were seen. Partial demethylation of some imprinted genes was seen, although this was not accompanied by loss of imprinting; methylation of pericentromeric satellites was unchanged (Bourc'his and Bestor, 2004; Webster *et al*., 2005). Spermatogenetic failure in these mice appears to be the result of arrest in the pachytene stage of meiosis and is accompanied by abnormal chromosome synapsis.

The similarity in phenotypes of the *Dnmt3L–/–* and *Dnmt3a–/–* mice, together with the lack of conservation of catalytic motifs in Dnmt3L, suggests that the latter may act as a cofactor for Dnmt3a. Dnmt3L has also been shown to enhance methylation by Dnmt3a in *in vitro* assays (Chedin *et al*., 2002; Suetake *et al*., 2004).

Previous work had indicated that the *Dnmt3L* locus has only one promoter driving transcription of a single long transcript (Aapola *et al*., 2001, 2004a,b; Bourc'his *et al*., 2001; Hata *et al*., 2002). We show here that mouse *Dnmt3L* produces sex-specific transcripts from three different promoters in germ cells. One transcript that encodes the full-length protein is the predominant form in prospermatogonia but not in spermatocytes: instead, an internal promoter is activated in late pachytene spermatocytes to produce three short transcripts that lack significant open reading frame (ORFs). A third promoter located in an intron of the upstream autoimmune regulator (*Aire*) gene is activated in growing oocytes and produces a transcript that is capable of coding for a full-length Dnmt3L protein. These results indicate an important role for promoter switching in regulating the production of active Dnmt3L protein in mouse germ cells, in agreement with a requirement for precise programming of methylation events in this lineage.

Materials and methods

Mice

C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) (for the laboratories of T.H.B. and J.M.T.), and Swiss To mice were purchased from Harlan UK Ltd (Oxon, England) (C.P.W. laboratory) and bred in-house. *Dnmt3L–/–* mice have been previously described (Bourc'his *et al*., 2001). Natural matings were used to produce embryos: the day of the vaginal plug was taken as 0.5 days post coitum (dpc) (Hogan *et al*., 1995). Animal work was carried out according to Institutional regulations and with ethical approval from the relevant local licensing authority.

Isolation of RNA

All tissues used were dissected in phosphate-buffered saline (PBS) according to standard methods (Hogan *et al*., 1995). RNA was extracted following homogenization of tissue in a Dounce homogenizer and/or syringe using Trizol reagent (Invitrogen) as per the manufacturer's instructions. The RNA was dissolved in diethylpyrocarbonate-treated water and final concentrations were measured using a spectrophotometer. Human adult testis RNA was purchased from BD Biosciences.

Northern blot analysis

Total RNA was electrophoresed on a 1.2% formaldehyde gel and transferred to Nytran Plus nylon membranes (Schleicher and Schuell). Blots were hybridized using the conditions of Church and Gilbert (1984). Probes specific to the various transcripts were generated using PCR (see Table I for primers). Blots were analysed after exposure to Kodak film.

5′ and 3′ rapid amplification of complementary DNA ends PCR

5′ and 3′ rapid amplification of complementary DNA (cDNA) ends (RACE) was performed on total RNA using the FirstChoice™ RLM-RACE Kit (Ambion), exactly as according to the manufacturer's protocol, using primers specific for *Dnmt3L* (see Table I). PCR products were cloned into the TOPO-TA vector (Invitrogen) and sequenced in-house. Novel transcripts have been deposited with Genbank (Round

Table I. Details of primers for *Dnmt3L* used for PCR, rapid amplification of complementary DNA ends (RACE), RNase H and generation of probes for northern analysis

Exon	Primers
1a	F: 5′-CATAGGCTCCATCCAGCATT-3′
1b	F: 5′-GAAACTCAGCCTTTGGGACA-3′
1c	F: 5′-GGCTTCCTGGAGAACACCAC-3′
1c	R: 5′-TTCTCAGGAAGCCCATTTTG-3′
2	F: 5′-ACCCTTCTAGCCGATTACATCA-3′
2	R: 5′-CCAGAGGACTGTCAGCATCA-3′
3	F: 5′-CCTGAAGGAGGACAGTGTGG-3′
4	F: 5′-AAGTGAACCGACGGAGCAT-3′
4	R: 5′-GTTCACTTTGACTTCGTACCTGAT-3′
5	F: 5′-CCTCTGCTGTGGAACTCTCC-3′
6	F: 5′-GATAAGTTCCTGGAGTCCCT-3′
6	R: 5′-AGTCGGGGCTCTCACAGAT-3′
7	F: 5′-GGGACCTCAGAGAGGATCAA-3′
8	F: 5′-ATGGAAGAGACAGCCAGTGC-3′
8	R: 5′-GCACTGGCTGTCTCTTCCAT-3′
9a	F: 5′-AACGCTGAAGTACGTGGAAGAT-3′
9a	R: 5′-CATTTGTGACATCTTCCACGTACT-3′
9b	F: 5′-CAAGCAGCCAAACATCTTGA-3′
9b	R: 5′-CTGGGGTCTAGCCAAAAGTG-3′
Large 9c	F: 5′-GGATTGTTTATGGCCACCAC-3′
Large 9c	R: 5′-AGGGAGCACGGAAACTCATT-3′
Small 9c	R: 5′-TGACCCAGACAGACCTCCTA-3′
10	R: 5′-AGCGATCACAAGAGCTGCCTA-3′
11	R: 5′-AGGAAGCGGGTAGTTGTCTCTT-3′
12	R: 5′-ACCCGCATAGCATTCTGGTA-3′
13	R: 5′-GCAAAGTGAGCTGCACAGAG-3′
Human 9b	F: 5′-CGAGGCACCACTGACATACAG-3′
Human 10	R: 5′-GTTCAGCACCAGATTGTCCACG-3′
Human β-actin	F: 5′-AGAAAATCTGGCACCACACC-3′
Human β-actin	F: 5′-CCATCTCTTGCTCGAAGTCC-3′

spermatid forms 1-3 as EF051621-EF051623 respectively; oocyte form as EF051624) (accession numbers pending).

PCR, cDNA synthesis and RT–PCR

Primers are as listed in Table I, except for the mouse β-actin primers, which were as previously described (Obata and Kono, 2002). Synthesis of cDNA from the RNA was carried out using standard procedures (Sambrook *et al*., 2001). In brief, 5 μg of total RNA was added to a 50 μl reaction containing 10 mM Tris–HCl (pH 8.3), 0.2 μg Oligo(dT)$_{15}$ primer (Promega), 1.5 mM deoxynucleoside triphosates, 1× Moloney murine leukemia virus (MMLV)-RT buffer and 20 U MMLV-RT (Ambion). PCR was performed in 50 μl containing 2 μl cDNA or 100 ng DNA, 1× Taq buffer, 1.5 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.4 μM each primer and 1.25 U *Taq* polymerase. The mixture was left for 3 min at 94°C for initial denaturation, followed by 30 (β-actin) or 35 (*Dnmt3L*) cycles consisting of 30 s at 94°C, 1 min at 63°C and 1 min at 72°C, followed by a final extension of 7 min at 72°C. Human cDNA was synthesized from testis total RNA (BD Biosciences) using the Superscript II RT method (Invitrogen). Briefly, 4 μg of total RNA was added to a 20 μl reaction consisting of 0.2 μg Oligo(dT)$_{12–18}$ primer, 0.5 mM deoxynucleoside triphosphates, 40 U RnaseOUT, 200 U Superscript II RT, 4 μl 5× First-strand buffer and 0.1 M dithiothreitol (DTT) and the reaction carried out according to the manufacturer's guidelines.

RNase H mapping

Oligonucleotides used for the RNase H mapping are listed in Table I (exon 10R and small exon 9cR). The oligonucleotides (250 pmol) were annealed to 20 μg of total RNA then incubated with RNase H, which cleaves RNA in the primer : mRNA duplex. The cleaved products were fractionated on an agarose/formaldehyde gel, transferred to a nylon membrane and probed to detect the fragments corresponding to the 5′ ends of transcripts. RNase H treatment, electrophoresis, blotting and probing were exactly as previously described (Yoder *et al*., 1996).

In situ *hybridization*

Probes for *in situ* hybridization were generated using PCR, cloned into the TOPO-TA cloning vector (Invitrogen) and sequenced to confirm their identity. Primers for *Dnmt3L* are given in Table I and primers for *Mvh* were as described (Toyooka *et al*., 2000). RNA probes were transcribed *in vitro* using the DIG RNA labelling kit (Roche). Cryosections were prepared and *in situ* hybridization carried out as described (Laufer *et al*., 1997) using anti-DIG antibody from Roche for detection.

Real-time/quantitative RT–PCR (QRT–PCR)

Germ cells were collected on two separate occasions and fractions enriched in specific cell populations isolated as previously described (Trasler *et al*., 1992). RNA was isolated from these using the RNeasy extraction kit with DNaseI treatment and further concentrated using the RNA MinElute kit (both Qiagen Inc.). Samples were diluted to 10 ng/μl, aliquoted in single-use volumes and stored at –80°C. QRT–PCR was carried out using the Quantitech SYBR Green RT–PCR kit (Qiagen) on an Mx4000 QPCR system (Stratagene) as previously described (La Salle *et al*., 2004). In brief, 10 ng or 100 pg of total RNA were used for the transcripts and 18S, respectively, and one-step QRT–PCR reactions were performed in a 25 μl volume for 40 cycles. The standard curve for each transcript was generated using total RNA from 70 days post-partum (dpp) testes. In all cases, reactions were performed in triplicate on the same sample of germ cell RNA. Specificity was assessed with the melting curve analysis and confirmed on a 3% agarose gel after each QRT–PCR experiment. Results were normalized to their corresponding 18S rRNA content and calibrated accordingly to the lowest consistently expressing time point (residual bodies). Analysis of expression in the two batches of enriched germ cells gave almost identical results. Primer sequences are indicated in Table II except for 18S, which were as previously described (La Salle *et al*., 2004).

Results

Alternative transcripts of Dnmt3L *are found in the post-natal testis*

Alternative transcripts of the *Dnmt3L* gene were identified in the adult testis by carrying out 5′ and 3′ RACE with primers to all the known exons of the gene. This approach recovered several clones corresponding to shorter transcripts from the *Dnmt3L* locus specifically in adult mouse testis. Using the BLAT alignment tool (http://genome.ucsc.edu/cgi-bin/hgBlat) to compare the sequences to the mouse genome, we found that these clones all originated from a novel exon located between exons 9 and 10, which we have designated exon 9b (Figure 1A). Alternative splicing of transcripts arising from this promoter gives rise to three transcripts, which we have designated forms 1, 2 and 3. Form 1 contains exon 9b, together with a second novel exon, designated exon 9c. Form 2 also contains exons 9b and 9c, but use of a second splice acceptor site in 9c results in a shorter version of this exon (9cS) being included in the final transcript. Form 3 only contains exon 9b.

To confirm the presence of these transcripts in the adult testis, we carried out northern blotting using total RNA and probes specific for the different transcripts of the gene (Figure 1B). A probe containing exons 3–12 detected a transcript of ~1.7 kb in

Table II. Details of *Dnmt3L* primers used for real time/quantitative RT–PCR

Transcript	Primers	Product size (bp)	Annealing temperature (°C)
Form 1 (9b and large 9c)	F: 5′-TTATACAGACAATTGGGATGTTGG-3′ R: 5′-CATAAACAATCCCCACTTAGATCA-3′	147	60
Form 2 (9b and small 9c)	F: 5′-AGGTGAACATCAGATATCAGGGCT-3′ R: 5′-ACCCAGACAGCAGCAACTCT-3′	127	67
Form 3 (9b only)	F: 5′-GAACATCAGATATCAGGTGGAGAA-3′ R: 5′-ATCCGGTGGAACTGGAACAT-3′	120	60

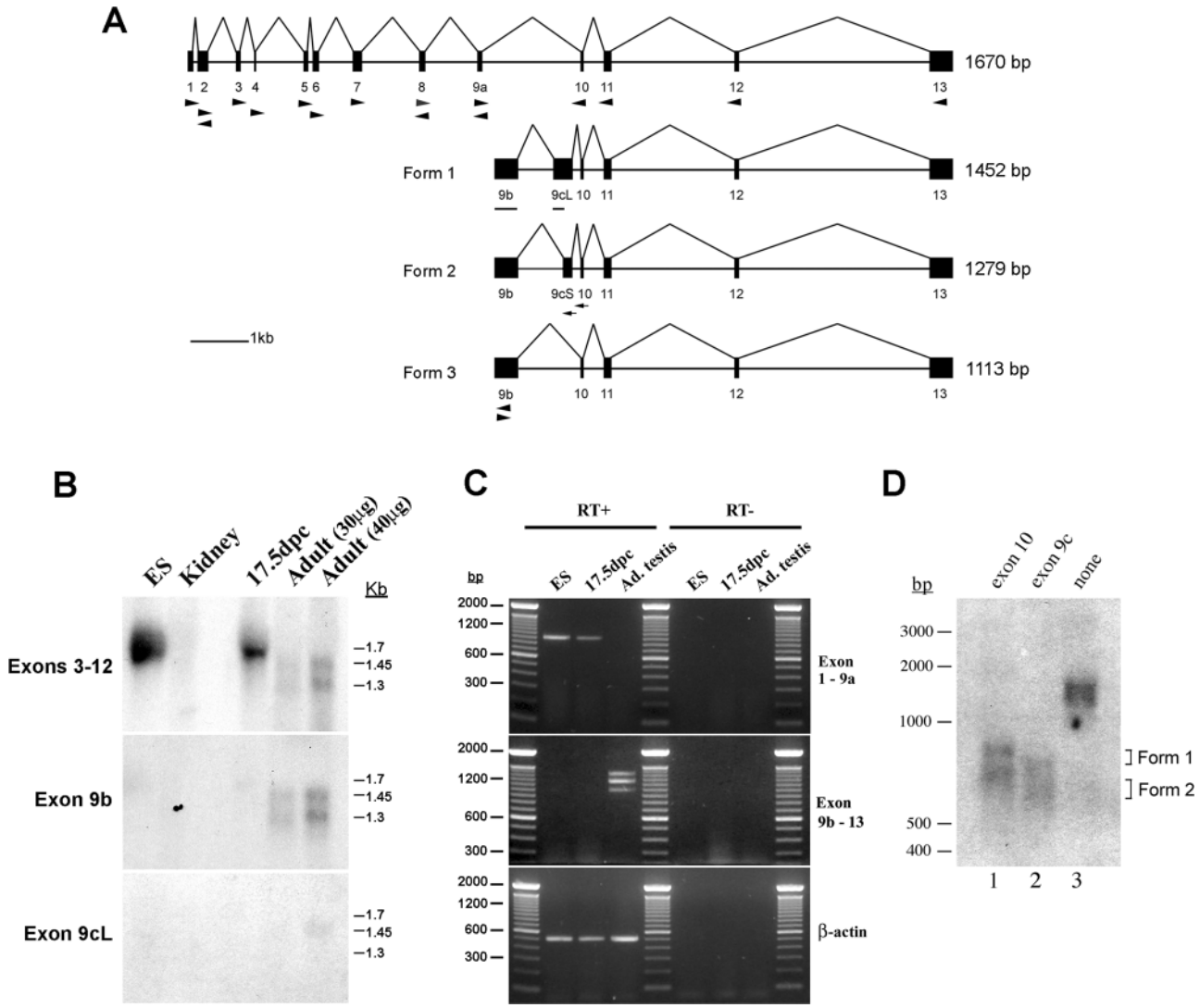

Figure 1. Alternative transcripts of *Dnmt3L* are produced in the mature testis. (**A**) Structure of the alternative transcripts present in the male germ line. Three transcripts were detected in adult testis, indicated as forms 1–3. Forms 1 and 2 differ only in the use of an internal splice acceptor site in exon 9c, which results in a shorter version of the exon (exon 9cS) being included in the mature transcript. The location of primers used in rapid amplification of complementary DNA ends (RACE) and RT–PCR are indicated by arrowheads and those used for RNase H mapping as arrows. The regions in the novel transcripts used as probes in the northern and RNase H assays are underlined and transcript sizes are indicated at right. (**B**) Northern blot indicating transcript abundance in different tissues. Total RNA from embryonic stem (ES) cells (5 µg), kidney (10 µg), 17.5 days post-coitum (dpc) testis (20 µg) and adult testis was electrophoresed and blotted to nylon, then hybridized with probes corresponding to the indicated regions. The sizes of the major transcripts are indicated at right. Only the larger of the two major adult transcripts was detected using a probe specific for the long version of exon 9c (9cL, bottom). (**C**) RT–PCR to confirm transcript size and composition. Exons 1–9a were undetectable in adult testis in this assay (top panel). Transcripts arising from the novel promoter at exon 9b produce transcripts including exons 10–13 (second panel). β-actin was used as a positive control and negative controls that lack RT are shown on the right for each reaction. The size standard at left increases in 100 bp increments and the positions of some major bands have been indicated. (**D**) RNase H analysis of 5′ ends. Total RNA from adult testis was hybridized to primers in exon 10 or the 3′ end of 9c, digested with RNase H, then run and blotted on a gel as for the northern, using exon 9b as a probe. Two bands were seen corresponding to the sizes for the 5′ ends of forms 1 and 2 predicted from 5′ RACE. Lane 3 is an undigested control (no primer added), with forms 1 and 2 appearing as a doublet.

embryonic stem (ES) cells and 17.5 dpc testis corresponding to the full-length transcript. In the adult testis, the 1.7 kb transcript was absent but two smaller, weaker bands were detected (more clearly visible when more input RNA was used) at ~1.45 and 1.3 kb. A probe corresponding to exon 9b detected both of these bands, but not the 1.7 kb transcript, whereas a probe for the long form of exon 9c (9cL) detected the larger of these two bands alone, indicating that the top band (1.45 kb) in the adult testis lane corresponds to form 1, whereas the bottom band (1.3 kb) corresponds to form 2. Form 3 was not detected using this assay, suggesting that this transcript is more weakly expressed or less stable.

RT–PCR using primers specific for exons 1 and 9a confirmed that this region was present in transcripts produced by ES cells and 17.5 dpc testis but not detectable in adult testis (Figure 1C). Using primers in exons 9b and 13, three transcripts corresponding to adult testis forms 1–3 were detected in the adult but not in ES cells or 17.5 dpc testis, with form 3 consistently giving a weaker signal than forms 1 and 2. These products were isolated from agarose gels, cloned and sequenced and confirmed that adult testis transcripts 1–3 contain exons 10–13 as well as the alternative 5′ exons detailed above. The negative controls for each primer set, done in the absence of RT, are shown in Figure 1: these were uniformly negative in all experiments and are omitted from subsequent figures.

RNase H mapping (Figure 1D) with RNA from adult testis confirmed that exon 9b represents the true 5′ end of the adult transcripts and that no further 5′ exons exist in this tissue. Primers used for protection were in the 3′ region of exon 9c and in exon 10 (see Figure 1A), and exon 9b was used as a probe to detect the fragments corresponding to the 5′ ends of the 9b-containing transcripts. Two major products were detected, corresponding to the sizes of the transcripts containing either exon 9b and the long form of exon 9c (form 1) or 9b and the short form of 9c (form 2) as determined by 5′ RACE and no larger bands were detected, indicating that no further upstream exons exist. No band clearly matching form 3 (exon 9b alone) could be detected in this assay.

Cell-type specificity of transcripts in the testis

Our results above indicated that a switch to the 9b-containing transcripts occurs during testis maturation. We isolated total RNA from testis at different times during development and carried out multiplex RT–PCR using forward primers in exons 1 and 9b and a common reverse primer in exon 13. Figure 2A shows that the 9b-containing transcripts were detected from 25 dpp onwards. The transcript containing exons 1–13 appeared to be strongly expressed in ES and 17.5 dpc testis but was very weak compared with the 9b transcripts in later stages. Germ cells in the testis start to enter meiosis at around 12–14 dpp, suggesting that the appearance of the short adult transcripts of *Dnmt3L* may coincide with the entry of the germ cells into a particular meiotic stage.

As antibodies to Dnmt3L were not available, we carried out RNA *in situ* hybridization using probes specific for exons 1–9a or for exon 9b on cryosections taken from mouse testis at the 17.5 dpc and adult stages to determine which cell types express the various transcripts. As a positive control and to identify the location of the germ cells, we used a probe for mouse *Vasa* homologue (*Mvh*), which labels all of the germ cells at 17.5 dpc, but predominantly the spermatogonia and spermatocytes in the adult stages (Figure 2B) (Toyooka *et al*., 2000). The probe corresponding to *Dnmt3L* exons 1–9a showed strong staining in the prospermatogonia at 17.5 dpc and some weak staining in the spermatogonia in the adult. In contrast, the exon 9b probe showed no staining at 17.5 dpc but strongly stained a population of germ cells in the intermediate layer of the testis corresponding to stages from late pachytene to round spermatid in germ cell development.

To clarify which cell types expressed the shorter forms in the adult, we fractionated germ cells using unit gravity sedimentation and carried out QRT–PCR using primers specific for each transcript in enriched germ cell populations (Figure 2C). The 9b-containing transcripts were absent in the spermatogonia but were present at high levels in the pachytene and round spermatid fractions. All three of the adult transcripts showed a similar distribution. It is not possible to compare levels of transcription of one message with the other in this experiment; the expression profiles were, however, in good agreement with the data presented in Figure 2A and B.

An ovary-specific transcript indicates the presence of a third promoter

The presence of testis-specific transcripts of *Dnmt3L* led us to examine the ovary for evidence of specific transcripts in this tissue as well. A *Dnmt3L* expressed sequence tag (EST) (CA599020) derived from ovary contained two novel 5′ exons (Figure 3A) spliced directly to exon 2. The sequence of 5′ RACE products generated from adult mouse ovary RNA using primers in exon 2 matched this EST with slightly more 5′ sequence. We confirmed that no further 5′ sequence was present using RNase H (data not shown). These novel 5′ exons were designated exons 1b and 1c, with the first exon in the prospermatogonia transcripts being exon 1a. Using the BLAT alignment tool, we mapped exon 1b mapped between exons 3 and 4 of the *Aire* gene, which is proximal to the *Dnmt3L* locus and transcribed in the opposite direction (Figure 3A).

To assess whether the transcript containing these novel 5′ sequences is only found in the ovary, we carried out RT–PCR using forward primers in exons 1b and 1c and a reverse primer in exon 4 and found that the messenger RNA (mRNA) could be detected in ovary but not in ES cells or testis (Figure 3B). Importantly, we could not detect exon 1a-containing transcripts in ovary (Figure 3C). These results, together with the 5′ RACE, show that the ovary contained a novel transcript which originates at a promoter close to exon 1b that is not active in prospermatogonia and ES cells and that this upstream promoter gave rise to all the *Dnmt3L* transcripts present in ovary.

We isolated total RNA from ovaries at various stages of development and carried out RT–PCR to determine when the oocyte-specific transcript started to appear. Transcripts containing exons 1b and 1c could be detected from 7 dpp, which corresponds to the entry of the first cohort of oocytes into the

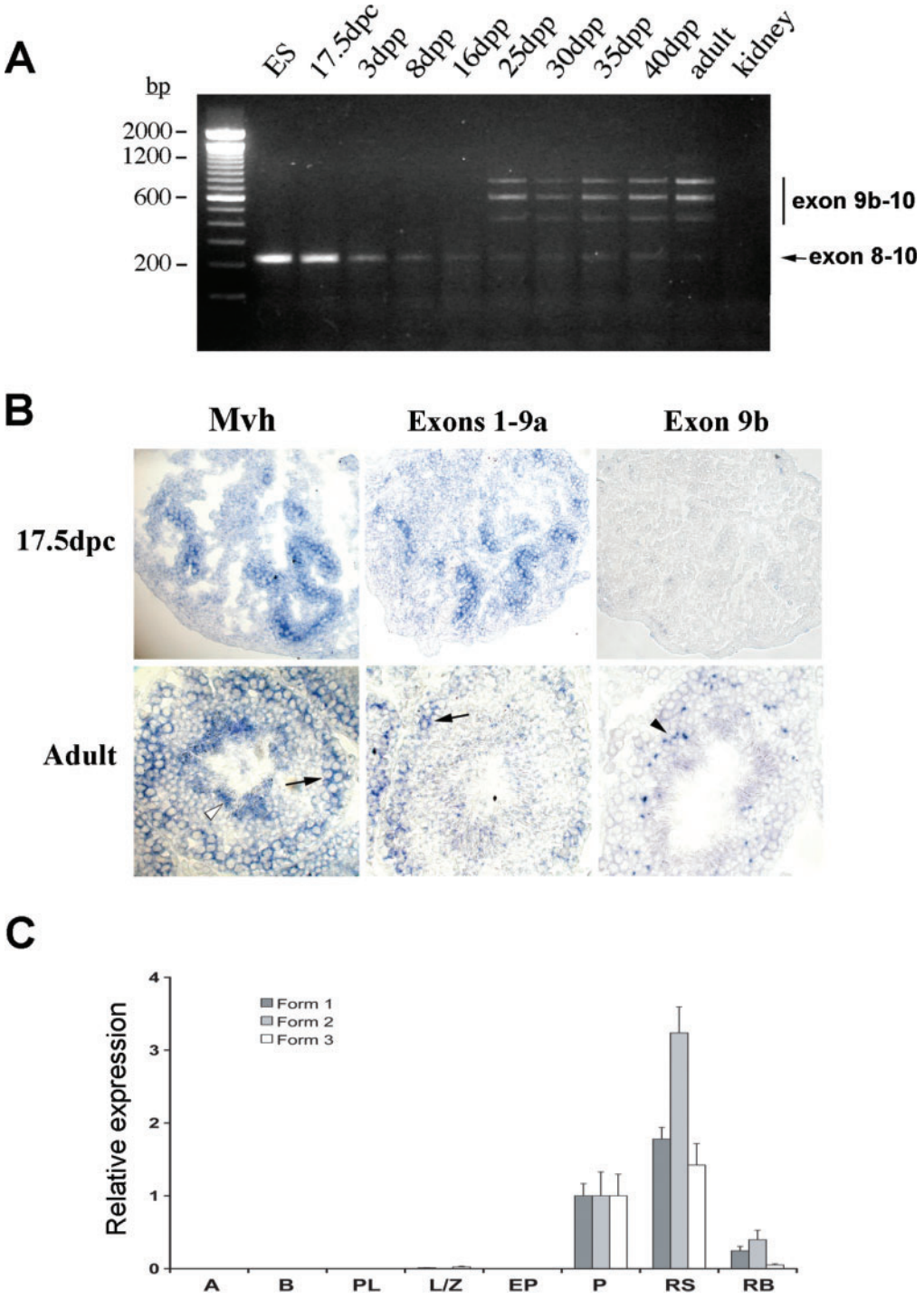

Figure 2. Cell-type specificity of *Dnmt3L* transcripts in the testis. (**A**) Promoter switching during development. Multiplex RT–PCR was carried out using the primers indicated at right. Transcripts originating at exon 1 (exons 8–10) were found in embryonic stem (ES) cells and perinatal testis but declined shortly after birth, whereas those originating at exon 9b (exons 9b–10) could be detected from 25 days post-partum (dpp) to adult stages. *Dnmt3L* is not expressed in kidney (right). Size ladder as for Figure 1. (**B**) RNA *in situ* showing transcript localization. Cryosections from 17.5 days post-coitum (dpc) (top) and adult testis (bottom) were hybridized to the indicated probes. Mouse *Vasa* homologue (*Mvh*) was transcribed in prospermatogonia at 17.5 dpc but was found mainly in spermatogonia (arrow) and spermatids (open arrowhead) in adult testis. Transcripts containing exons 1–9a of *Dnmt3L* were abundant in prospermatogonia but could also be weakly detected in spermatogonia of adult testis (arrow). Transcripts containing exon 9b were absent from prospermatogonia (top) and spermatogonia but present at high levels in cells nearing the end of spermatogenesis (arrowhead). (**C**) Confirmation of cell-type specificity by real-time/quantitative RT–PCR (QRT–PCR). Total RNA from enriched germ cell populations was prepared and QRT–PCR carried out with primers specific for the different transcripts. The relative expression of each transcript was normalized to the corresponding 18S content in that fraction, then calibrated according to its expression in the pachytene spermatocytes (set at 1). Each reaction was performed in triplicate and data are presented as the mean plus SD. A, type A spermatogonia; B, type B spermatogonia; PL, preleptotene spermatocytes; L/Z, leptotene/zygotene spermatocytes; EP, early pachytene spermatocytes; P, pachytene spermatocytes; RS, round spermatids; RB, elongating spermatids and residual bodies.

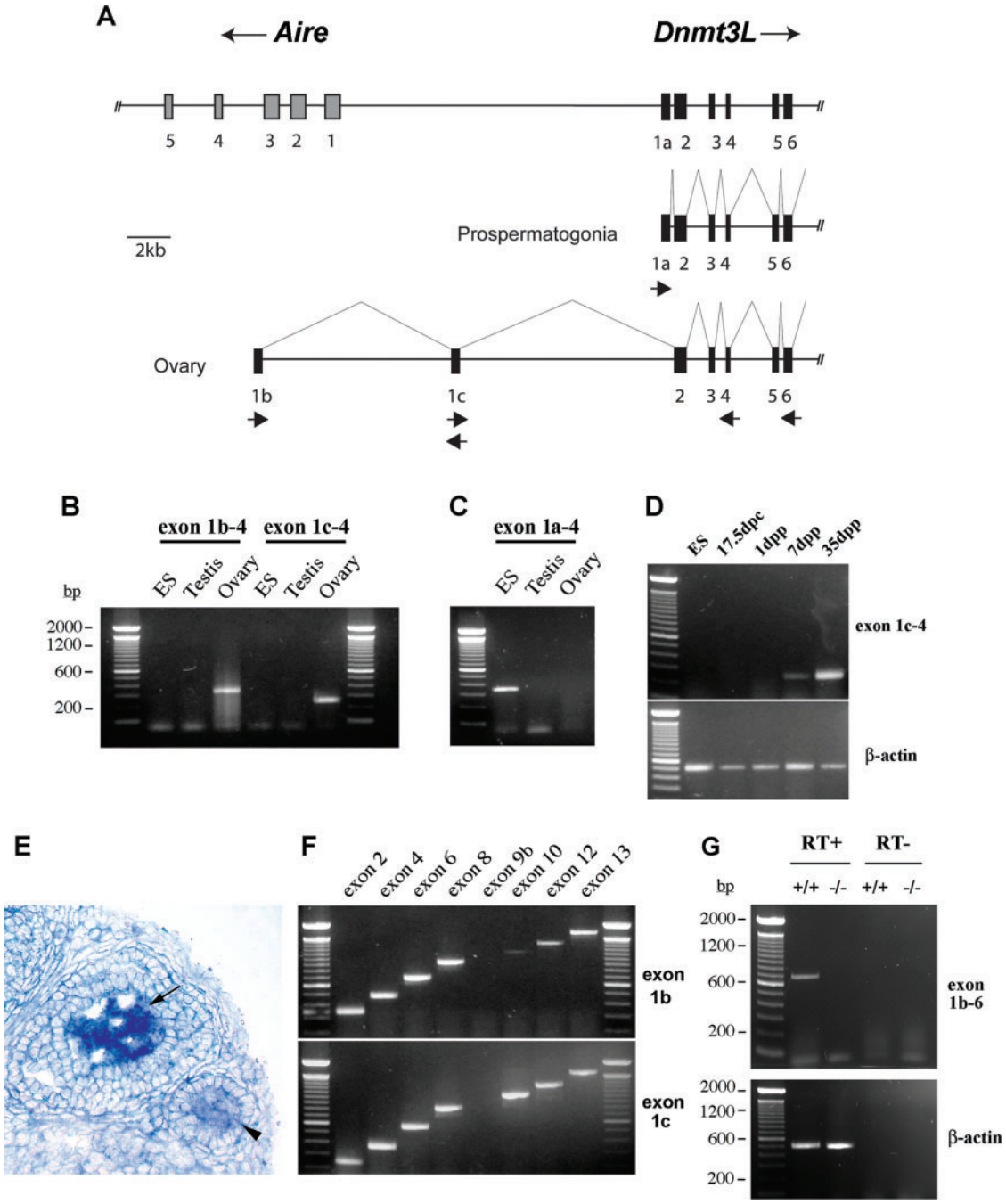

Figure 3. Transcription of *Dnmt3L* in the ovary. (**A**) The 5′ end of the *Dnmt3L* locus and that of the neighbouring autoimmune regulator (*Aire*) gene, indicating the structure of the *Dnmt3L* transcript found in ovary compared with prospermatogonia. Two novel *Dnmt3L* exons, 1b and 1c, were found spliced directly to exon 2 in the ovary transcript. Exon 1b is located between exons 3 and 4 in the *Aire* gene. The positions of primers used for RT–PCR are indicated by arrows. The direction of transcription of the two genes is indicated at the top. (**B**) RT–PCR on total RNA using the primers indicated at top of figure. Transcripts containing exons 1b and 1c could be detected in ovary but not in embryonic stem (ES) cells or testis. (**C**) Absence of transcripts containing exon 1a in ovary by RT–PCR. PCR was carried out with primers in exons 1a and 4. (**D**) Stage-specific activation of the ovary promoter. Total RNA was isolated from ovaries at the indicated stages of development and RT–PCR carried out with primers in exons 1c and 4 (top panel). Transcripts originating from the ovary promoter could be detected from 7 days post-partum (dpp) when the first wave of oocytes enters the growth phase. β-actin was used as a positive control. (**E**) *In situ* hybridization of 5-week-old mouse ovary using a probe generated from the exons 1b and 1c. Messenger RNA was present in growing oocytes in the unilaminar primary follicle (arrowhead) and had accumulated to high levels in the germ cells by the early vesicular secondary follicle stage (arrow). (**F**) Oocyte-specific transcripts contain all the coding exons. RT–PCR was carried out using forward primers in exon 1b (top) or 1c (bottom) and reverse primers in the indicated exons. Note the absence of transcripts containing exon 9b. (**G**) The oocyte-specific transcript is absent in *Dnmt3L* mutant mice. Total RNA from the ovary was isolated from mice with a targeted deletion of exons 3–5 of *Dnmt3L* (–/–), or their wild-type littermates (+/+), and subjected to RT–PCR with primers in exons 1b and 6 (top panel). As a positive control, β-actin was amplified from the same RNA (bottom panel) and a negative control reaction with no RT is shown at right (RT–).

growth phase (Figure 3D). *In situ* hybridization with a probe spanning exons 1b and 1c showed that the transcripts were confined to the germ cells and confirmed that mRNA started to accumulate as the unilaminar primary oocyte began to grow, with high levels detected in early secondary follicles (Figure 3E). We also carried out RT–PCR on 35 dpp ovary RNA with reverse primers in downstream exons (Figure 3F), which confirmed that the oocyte-specific transcript contained all the protein-coding exons 2–13 present in the prospermatogonia transcript but lacked the testis-specific exon 9b.

These results suggested that the effect on the female germ line in the *Dnmt3L* knockout mice may be due to disruption of the transcript which originates from the oocyte-specific promoter. To confirm this, we carried out RT–PCR on RNA from ovaries of wild-type and *Dnmt3L*–/– mice (Bourc'his *et al*., 2001), in which exons 3–5 have been replaced with a β-geo cassette, using primers in exons 1b and 6. It can be seen from Figure 3G that no oocyte-specific transcripts were detected in the adult knockout mice (–/–), but they were present in their wild-type littermates (+/+). Primers for β-actin resulted in amplification in both wild-type and mutant mice (Figure 3G), indicating that intact mRNA was present in all samples. This also suggests that the β-galactosidase staining pattern seen in the growing oocyte in mutant mice arises from a transcript which initiates at the oocyte-specific promoter described here (Bourc'his *et al*., 2001).

Analysis of the coding potential and conservation of the mRNA

Analysis of the ORFs indicates that it is likely that the ovary transcript uses the same ATG as the prospermatogonia transcript and yields an identical protein. The longest ORF in each round spermatid transcript began at the ATG found in exon 11 and is not in a favourable context for translation initiation (Kozak, 1996), suggesting that these transcripts may be non-coding. In human, five short ESTs (BQ028660, AI631758, AW237277, BF222724 and AI468252) mapped to the intron between exons 9 and 10 of *DNMT3L* (Aapola *et al*., 2004b), some of which were spliced to exon 10 and continued to the last exon, suggesting that they represent transcripts from the round spermatid promoter in humans. Three of the five ESTs were from cDNA libraries derived from germ cell sources. To verify the presence of these transcripts in human adult testis, we carried out RT–PCR using a forward primer based on these ESTs and a reverse primer in exon 10 (Figure 4A). This generated a band of 654 bp from the genomic DNA and a 468-bp band from the cDNA, indicating the presence of a spliced transcript originating in exon 9b and splicing into exon 10 in this tissue in humans (Figure 4B). Sequencing indicated that splice donor and acceptor sites match those of ESTs AI631758 and AW237277. Comparison of the mouse, human and predicted rat (Lees-Murdock *et al*., 2004) *Dnmt3L* sequences reveals a lack of conservation of the start methionine in adult testis isoforms. These analyses indicate that although the production of short transcripts in adult testis is conserved between mouse and human, indicating they may have some function, these transcripts are unlikely to be protein coding.

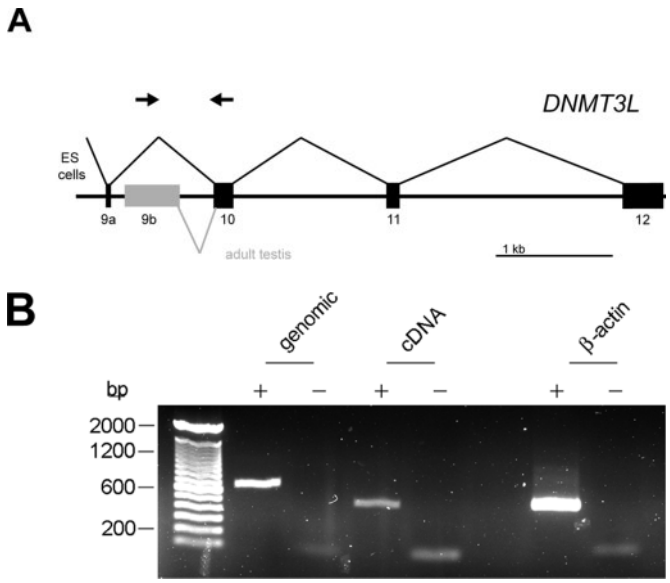

Figure 4. Production of short isoforms in adult human testis. (**A**) Map of the human *Dnmt3L* locus showing the exons normally present in the full-length transcript (black) and the position of exon 9b utilized in adult testis (grey) together with the location of the primers for RT–PCR. (**B**) Confirmation of an adult testis transcript in human. A forward primer in intron 9 and reverse primer in exon 10 gave the expected product from genomic DNA of 654 bp (lane 1), which is absent from a negative control reaction (lane 2). RT–PCR from adult testis RNA with the same primers generates a product of 468 bp (lane 3), absent from an RT-minus control (lane 4), indicating the presence of a transcript utilizing exon 9b in adult human testis which is spliced to exon 10. Sequencing confirmed the identity of the PCR product and the presence of a small intron between exons 9b and 10 (data not shown). The RT–PCR reaction for human β-actin (435 bp) is also shown as a positive control.

Figure 5 summarizes the data regarding the location of the sex-specific exons and the splicing events occurring at the mouse *Dnmt3L* locus. Exon 1a is used to drive transcription predominantly in the prospermatogonia in the male, in ES cells and to a much lesser extent in adult spermatogonia. An alternative promoter upstream of exon 1b drives expression in the oocyte, with the transcript including a second unique non-coding exon as well as the coding exons 2–13. In the post-natal testis, there is a decrease in transcription from the prospermatogonia promoter, and as cells enter meiosis, transcription from this promoter ceases. At the end of prophase I of meiosis, a third promoter becomes active, producing truncated transcripts that are likely to be non-coding and contain one or two unique 5′ exons. This situation is very similar to that for the *Dnmt1* and *Dnmt3a* loci in mouse, which also produce sex-specific transcripts from unique promoters, and the structure of these loci are shown here for comparison.

Discussion

Gene knockout experiments have demonstrated the importance of the Dnmt3L protein in male germ cell development (Bourc'his *et al*., 2001; Hata *et al*., 2002; Bourc'his and Bestor., 2004; Webster *et al*., 2005). A transcript coding for a full-length version of the protein is initially transcribed in

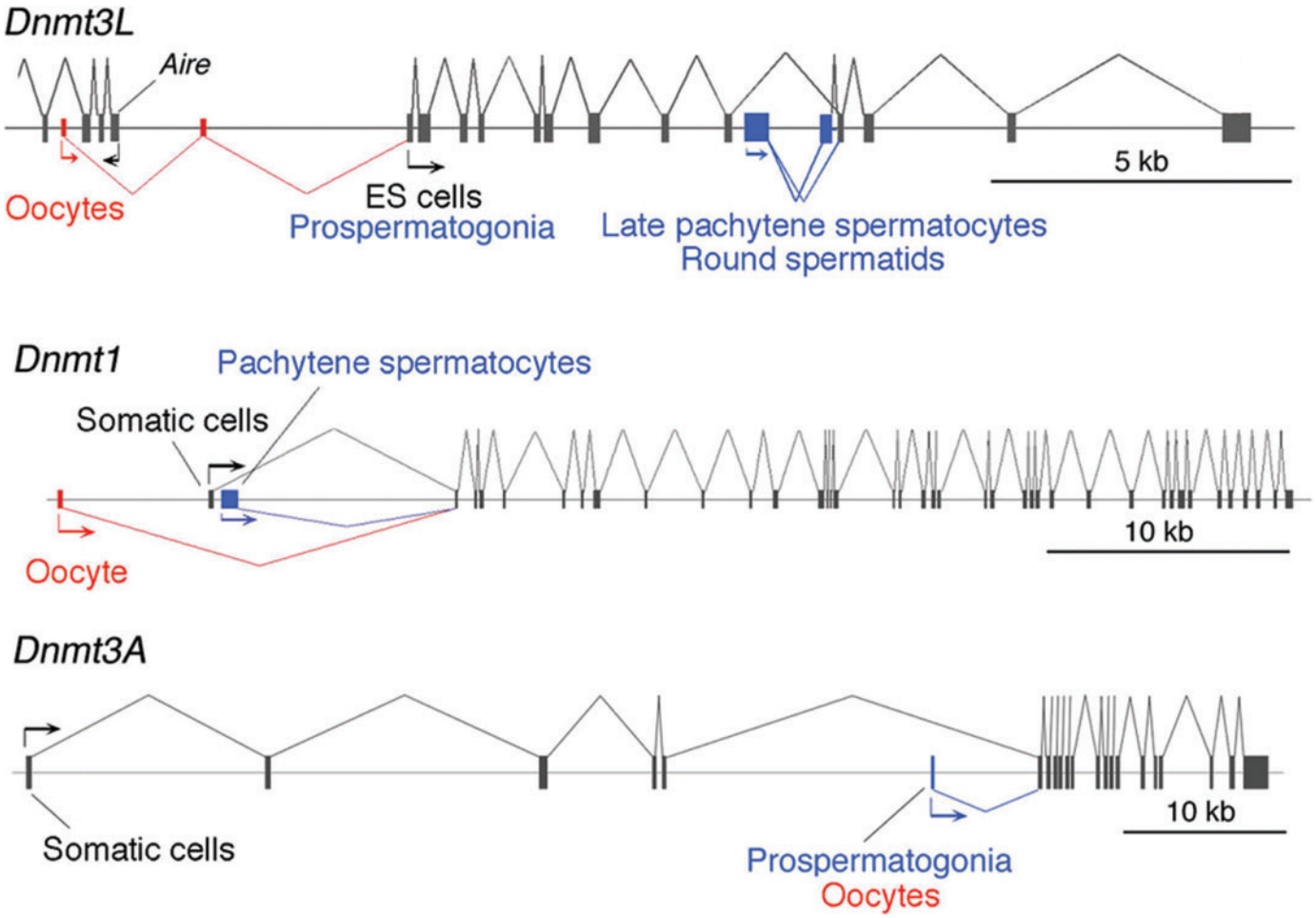

Figure 5. Schematic showing promoter usage at the mouse *Dnmt3L*, *Dnmt1* and *Dnmt3a* loci. Exons used in the female germ line are shown in red and those used in the male germ line are shown in blue. Transcriptional start sites are indicated by arrows.

prospermatogonia in the perinatal period, when de-novo methylation occurs. Transcription from this promoter declines as prospermatogonia differentiate into type A spermatogonia during sexual maturation, as we show here and elsewhere (Bourc'his and Bestor, 2004; La Salle *et al*., 2004), and ceases as the cells progress through spermatogonial proliferation and meiosis. At this point, an alternative promoter is activated and three short transcripts are produced from late pachytene stage onwards from this promoter in the 3′ end of the gene. These transcripts are unlikely to produce a functional protein, because the only ORF in-frame with the Dnmt3L protein has an ATG which does not match the Kozak consensus sequence, is preceded by multiple upstream ORFs and is not conserved between mice and humans. There are a number of other genes that utilize an alternative promoter to produce a different transcript during late meiosis or round spermatid stages in male germ cells (see Kleene, 2001 for a recent review). For many of these genes, such as *SOD-1* (Gu and Hecht, 1996), *Cytochrome C* (Hake and Hecht, 1993), *Proenkephalin* (Rao and Howells, 1993) and *Dnmt1* (Trasler *et al*., 1992; Mertineit *et al*., 1998), the presence of additional sequences in the 5′ untranslated region has been shown to correlate with low or undetectable protein. As in the case of *Dnmt3L*, production of a pachytene-specific transcript for α-tubulin lacking any ORF is conserved across species, suggesting that these non-coding transcripts have important functions (Kleene, 2001). Conservation of adult testis transcript production for *Dnmt3L* between humans and mice strongly suggest that these may play an important role in regulating production of the full-length protein in round spermatids.

In the mouse ovary, a third promoter at the *Dnmt3L* locus, upstream of those used in prospermatogonia and round spermatids, is used to drive transcription of the gene in growing oocytes. This transcript is likely to utilize the same initiation codon as the prospermatogonia transcript and thus produce a full-length Dnmt3L protein containing all of the conserved motifs. This appears to be the only transcript present in the ovary, which would indicate that all of the protein produced in the oocyte must be derived from this transcript.

Targeted disruptions of the *Dnmt3L* gene have replaced exons 3–5 (Bourc'his *et al*., 2001) and exons 3–8 (Hata *et al*., 2002) with a β-geo cassette containing a splice acceptor sequence followed by multiple stops in all frames and the gene for a β-galactosidase/neomycin resistance fusion protein. Because neither of these mutations disrupt the round-spermatid promoter between exons 9a and 9b, the phenotype seen in the male mice, including formation of aberrant chromosome pairing structures, activation of selfish DNA elements, partial demethylation of *H19* and *Rasgrf1* and loss of germ cells in the

adult, must be due to the absence of the prospermatogonia transcript. Male germ cells do not progress to late pachytene in *Dnmt3L*–/– mutants and so cannot be tested for the presence of transcripts containing exon 9b. In contrast, because the oocyte-specific transcript described here is the only one produced in the ovary and is absent in the mutant mice, the phenotype seen in existing female knockout mice and their offspring must be due to disruption of this transcript *in vivo*, supporting the prediction that the ovary-specific transcript codes for a functional protein.

Alternative promoter usage has also been reported for the *Dnmt3a* (Chen *et al*., 2002) and *Dnmt1* (Mertineit *et al*., 1998) genes (Figure 5). A second promoter at the *Dnmt3a* locus produces a shorter, more active form of the protein known as Dnmt3a2 specifically in tissues showing de-novo methylation, including the germ cells of both sexes: however, no evidence has been found so far of any sex-specific transcripts of this gene. There are clearer parallels however between the *Dnmt3L* and *Dnmt1* loci in terms of structure and germ cell expression (see Figure 5). Both genes use an oocyte-specific promoter to drive protein production in the egg and use promoter switching in the male germ line to produce transcripts with low translation potential. The oocyte-specific promoter of *Dnmt1* produces a longer transcript but a slightly shorter protein, which is stored in the oocyte and required for maintaining methylation on imprinted genes in the early embryo. Disruption of the *Dnmt3L* oocyte transcript (Bourc'his *et al*., 2001; Hata *et al*., 2002) results in a failure to establish methylation on the same genes: both promoters are also active in growing oocytes, suggesting that they may be controlled in parallel. In the male germ line, the switch from the coding form of *Dnmt1* to the non-coding form appears to occur in the early pachytene stage, where both transcripts can be detected (Trasler *et al*., 1992; Mertineit *et al*., 1998). By the late pachytene stage only the non-coding form is seen, whereas for *Dnmt3L* the switch appears to occur in the late pachytene stage, with the non-coding transcripts predominating in round spermatids. Although the function of the non-translated form of *Dnmt1* that is present in pachytene spermatocytes and the truncated forms of *Dnmt3L* that are present in round spermatids are not known, a role in the suppression of the full-length protein by transcriptional or translational interference is likely.

In conclusion, our results show that Dnmt3L production is controlled by three separate promoters, not just one as previously thought. These ensure production of the full-length protein is restricted to times when de-novo methylation is known to occur, i.e. the prospermatogonia and growing oocyte stages.

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