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Developmental and Cell Type Specificity of LINE-1 Expression in Mouse Testis: Implications for Transposition

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The LINE-1, or L1, family of interspersed repeated DNA constitutes roughly 10% of the mammalian genome. Its abundance is due to duplicative transposition via an RNA intermediate, L1-encoded proteins, and reverse transcription. Although, in principle, transposition may occur in any cell type, expression and transposition of a full-length functional element in the germ line are necessary to explain the evolutionary genetics of L1. We have found differential expression of L1 protein and RNA in germ and somatic cells of the mouse testis during development. Of particular interest is the coexpression of full-length, sense-strand L1 RNA and L1-encoded protein in leptotene and zygotene spermatocytes at postnatal day 14 of development. Expression in meiotic prophase precedes the strand breakage that occurs during chromosomal recombination; this offers an avenue for L1 insertion into new locations in chromosomal DNA in a cell type that ensures L1 propagation in future generations.

LINE-1, or L1, is an interspersed repeated DNA present at ~100,000 copies per mammalian genome. L1 achieved this high copy number by duplicative transposition involving an RNA intermediate; hence, it is a retrotransposon. The longest copies of L1 that have been characterized from the mouse genome are ~6.8 kb in length and contain two long open reading frames (ORFs), termed ORF 1 and ORF 2 (Fig. 1) (reviewed in reference 9). Both ORFs encode basic proteins; ORF 2 is a reverse transcriptase (16). The mechanism of L1 transposition is not known in detail, but the necessary intermediates must include a full-length, sense-strand transcript and the proteins encoded by ORF 1 and ORF 2. ORF 1 may serve a packaging function for the RNA, since it copurifies with the full-length L1 RNA found in ribonucleoprotein particles (14). ORF 2, the reverse transcriptase, is required for conversion of the RNA intermediate into DNA before or during integration. These L1-encoded proteins may also provide the machinery for generation of a variety of processed pseudogenes (23).

Transposition of L1 occurs in both somatic and germ cells; known insertions of L1 into human genes provide examples of both. Somatic transposition and insertion of L1 into the *APC* tumor suppressor gene (17) and the *myc* gene (18) have been implicated in humans with colon cancer and breast carcinoma, respectively. Two independent examples of germ line or early embryo transposition with insertion into the human factor VIII gene have been described as causing hemophilia (10). Although L1 transposition appears to occur in many cell types, the evolution of L1 and the processed pseudogenes absolutely require L1 expression and transposition in cells that are destined for the next generation (9). In mammals, this means germ cells, primordial germ cells, or early embryos before the germ line becomes a distinct lineage. There is evidence for production of L1 RNA and ORF 1 protein in embryonal carcinoma cells that may represent early developmental stages (6, 14, 15, 22). However, with the exception of one report describing L1 transcripts in mouse blastocysts (19), there is no evidence for L1 expression in developmentally relevant cell types in vivo. Because of the relative ease of accessing the germ line in males, we chose to examine L1 expression in the testis as a first step in a screen for potential transposition intermediates of L1 in vivo.

The testis contains both somatic and germ cells. In contrast to the female germ cell lineage, in which the proliferation of oogonia and early phases of meiosis I occur in utero, the seminiferous tubules of adult testis contain all three of the intermediate cell types on the pathway to the male gamete. These cell types are present continuously throughout adulthood, so it is theoretically possible to survey all of the cell types involved in male germ cell production by examining the adult testis. Spermatogonia are the proliferative cells, spermatocytes are the cells involved in meiosis, and spermatids are the haploid cells that undergo dramatic morphological differentiation to give rise to mature spermatozoa. Spermatogonia line the basement membrane of the seminiferous tubules. As germ cells begin meiosis and then differentiation, they move towards the lumen, where mature sperm is released. Before release, spermatids shed their excess cytoplasm in the form of a residual body. The only other cell type found within the seminiferous tubules is the (somatic) Sertoli cell. Other somatic cell types lie under the basement membrane of the tubules and fill the interstitial spaces between tubules. Among these are myoid cells and the hormone-producing Leydig cells.

Our results provide evidence for both germ line and somatic cell expression of L1 RNA and ORF 1 protein in the mouse testis. In adult testis, L1 ORF 1 protein is detected in spermatids as well as in Leydig and myoid cells by immunohistochemistry. However, only a shortened L1 RNA is detected in these adult cells, raising the possibility that L1 expression occurs in the absence of productive transposition. In contrast, ORF 1 protein is detected by immunohistochemistry in prepuberal mouse testis, primarily in leptotene and zygotene spermatocytes, and there is coexpression of a full-length, sense-strand L1 RNA. These early meiotic cell types may therefore produce active intermediates in L1 transposition, fulfilling the evolu-

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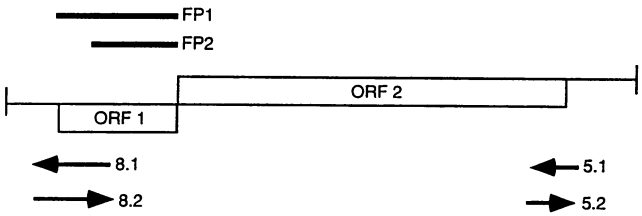


FIG. 1. Map of a genomic copy of mouse L1 and location of probes. ORF 1 and ORF 2 are the ORFs. Target site direct duplications are indicated by vertical bars at the ends of the structure. Fusion proteins FP1 and FP2, used for antibody production, are schematized above the structure. Locations of probes used to detect the sense (8.1 and 5.1) and antisense (8.2 and 5.2) strands of L1 are indicated below the structure; arrows indicate the direction of transcription from the L1 subclones described previously (14).

tionary requirement for transposition in cells that spawn the next generation.

MATERIALS AND METHODS

Mice. Adult C57BL/6 mice were purchased from Taconic Farms (Germantown, N.Y.) and used for immunohistochemistry. ICR and CD1 mice were used for all other experiments; no significant differences in L1 expression were found among these different strains of mice. ICR mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.); CD1 mice were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). Prepuberal time course experiments were done with mice designated exact age by the vendor (born within 24 h of stated date of birth).

Primary antibodies. Independent affinity-purified antibodies were prepared against either the entire first ORF of L1 (FP1) or its carboxyl-terminal two-thirds (FP2) as described previously (15) and shown in Fig. 1.

Immunohistochemistry. Testes from adult and prepuberal mice were fixed in Bouin's solution for 1 to 4.5 h, depending on tissue size, and then embedded in paraffin and sectioned at 5 μm. For immunoperoxidase staining, sections were deparaffinized and treated with 1.0% peroxide for 15 min to quench endogenous peroxidases. Sections were extracted with a 1.0-mg/ml solution of bovine pancreatic trypsin, type 3 (Sigma), in phosphate-buffered saline (PBS; pH 7.4) for 0 to 4 min. L1 antibodies, FP1 and FP2, or preimmune immunoglobulin G (IgG) was added at a concentration of 1.0 μg/ml in PBS containing 2% normal goat serum and 0.02% azide (blocking solution) and then incubated for 15 h at 4°C. Sections were washed in PBS between all incubations. A Vectastain Elite ABC kit (Vector Laboratories) was used to locate antibody labelling by following the manufacturer's instructions for detection with diaminobenzidine. Sections were counterstained with toluidine blue before dehydration and mounting in Permount and then monitored and photographed with a Zeiss Axiophot microscope and Ektachrome 64T film (Kodak).

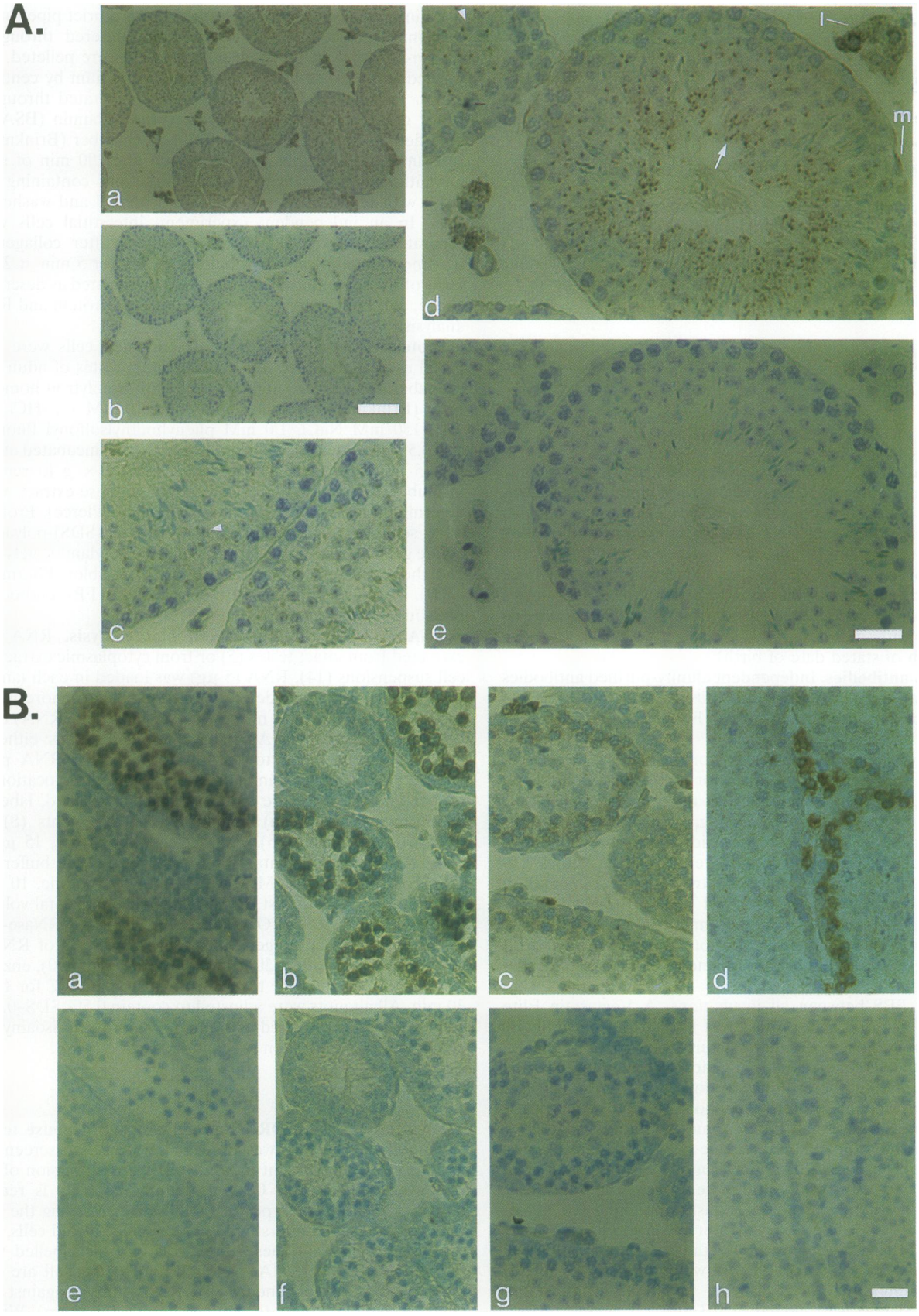
Cell fractionation. Spermatogenic cells were fractionated from adult mouse testis by a modification of the procedure of Romrell et al. (20). Briefly, testes were excised and decapsulated and then transferred to a flask containing 0.6 mg of collagenase CLS3 (Worthington) per ml in modified Eagle's medium (GIBCO) supplemented with 60 μg of ampicillin per ml. Testes were shaken in a 35°C water bath for 5 min to dissociate interstitial cells. Bovine pancreatic trypsin and DNase I (Sigma) were added at 0.3 and 1.0 mg/ml, respectively; the flask was shaken again in a 35°C water bath for 4 min to dissociate the germ cells from the seminiferous tubules. The resulting cell suspension was broken up by a brief pipetting in a silanized Pasteur pipet before being filtered through a 70-μm-pore-size nylon mesh screen. Cells were pelleted, and washed three times in modified Eagle's medium by centrifugation at 500 × *g* for 5 min and then separated through a linear gradient of 2 to 4% bovine serum albumin (BSA) in modified Eagle's medium in a Cell-Sep chamber (Brinkmann Instruments). Fractions were collected after 90 min of sedimentation at unit gravity at 22°C. Fractions containing cell types with identical morphology were pooled and washed in PBS. In an independent experiment, interstitial cells were separated from the seminiferous tubules after collagenase treatment by allowing the tubules to settle for 5 min at 22°C. Cells of the seminiferous tubules were dissociated as described above, and both preparations were used for protein and RNA analysis.

Protein analysis. Extracts of fractionated cells were prepared as described previously (15). Intact testes of adult and prepuberal mice were homogenized with a polytron homogenizer (Brinkmann) in buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40. The homogenate was incubated at 0°C for 15 min and then centrifuged at 12,000 × *g* to remove insoluble debris. Protein concentrations in these extracts were determined by the bicinchoninic acid assay (Pierce). Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide gels (11) and then transferred to nitrocellulose (Novablot; Pharmacia LKB). L1 ORF 1 proteins were detected with FP1 antibody as previously described (15).

RNA extraction and Northern blot analysis. RNA was extracted from intact testes (5) or from cytoplasmic extracts of cell suspensions (14). RNA (5 μg) was loaded in each lane of 1% agarose-formaldehyde gels. Following electrophoresis, the RNA was transferred to nitrocellulose. Northern (RNA) blots were hybridized to ^{32}P (Amersham)-labelled probes: either in vitro transcripts made to portions of L1 (SP6 RNA polymerase; Boehringer Mannheim) (see Fig. 1 for location of probes and the structure of L1) or random-primed, labelled (Boehringer Mannheim) human β-actin fragments (8), as described previously (15). For nuclease digestions, 15 μg of testis RNA from postnatal day 14 was mixed with buffer (40 mM Tris [pH 7.5], 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl) and 40 μg of yeast tRNA (as carrier) in a total volume of 90 μl. Before RNase One (1.0 U; Promega) or RNase-free DNase (4.0 U; Boehringer Mannheim) and 80 U of RNasin (Promega) were added, 30 μl was withdrawn (time 0), enzyme was added, and then the tube was incubated at 37°C for 10 or 30 min. All aliquots were adjusted to contain 0.5% SDS–0.3 M sodium acetate, extracted with phenol-chloroform-isoamyl alcohol (50:49:1), and then precipitated with ethanol.

RESULTS

Localization of L1 ORF 1 protein in adult mouse testis. Immunohistochemistry was used at the outset to screen the various cell types present in adult testis for expression of the ORFs encoded by L1. ORF 1 immunoreactivity is readily detected in three cell types of the adult testis, using the FP1 antibody (Fig. 2A): interstitial (Leydig) cells, myoid cells, and spermatids. None of these three cell types is labelled with preimmune IgG (Fig. 2A, panels b and e), and all are also labelled with the independent L1 ORF 1 antibody against FP2 (Fig. 2A, panel c, shows the round spermatid pattern). Within the seminiferous tubules, L1 ORF 1 labelling appears as a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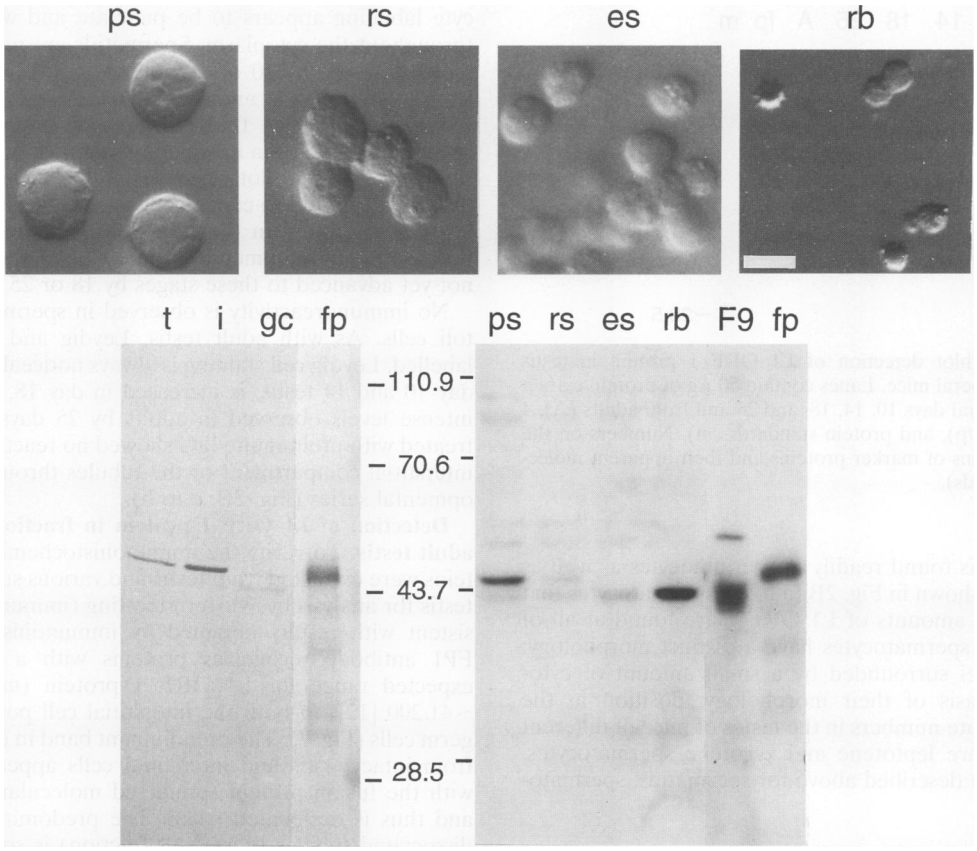

FIG. 3. Western blot detection of L1 ORF 1 protein in extracts of fractionated cells from adult mouse testis. Nomarski photomicrographs (magnification, ×670; bar, 10 μm) are shown for each fraction of spermatogenic cells: pachytene spermatocyte (ps), round spermatid (rs), elongating spermatid (es), and residual body (rb). Protein extracts from whole testis (t), interstitial cells (i), homogenized seminiferous tubules (gc), fractionated testis cells (largely spermatogenic, except for Leydig cells from the interstitium cofractionating with pachytene spermatocytes [ps]), F9 cells (F9), and purified FP1 (fp) were separated by SDS-PAGE and transferred to nitrocellulose. L1 ORF 1 proteins were detected with alkaline phosphatase-conjugated secondary antibody (left panel) and with ^{125}I-protein A (right panel). Protein (50 μg) was loaded in each lane except (25 μg), fp (4 ng), and F9 (12.5 μg). Numbers indicate the positions of marker proteins and their apparent molecular size (in thousands). The larger band (~53,000) seen in the F9 lane is nonspecific (15).

single, discrete dot in round spermatids. Immunoreactivity is also found in elongating spermatids and residual bodies, where it appears to redistribute into several smaller aggregates. There is no immunoreactivity in mature spermatozoa, Sertoli cells, or spermatogonia. Rarely, tubules with faintly immunoreactive spermatocytes are found. Spermatocyte labelling is detected so infrequently in the adult sections that it is attributed to a prepachytene cell type, such as leptotene or zygotene spermatocytes, each of which represents only about 2% of the cells within seminiferous tubules of adult mice (2). In these early spermatocytes, Leydig and myoid cells, L1 ORF 1 appears to be located throughout the cytoplasm in a punctate pattern. This pattern is reminiscent of the distribution of L1 ORF 1 seen in embryonal carcinoma cells (15) and contrasts with the single cytoplasmic structure seen in spermatids.

No ORF 2 immunoreactivity could be detected with either of two antibodies prepared against ORF 2 fusion proteins. Since these antibodies react strongly with ORF 2 expressed either in *Escherichia coli* or by in vitro transcription/translation (10a), we believe that ORF 2 expression is simply too low to detect in the testis.

Localization of L1 ORF 1 protein in prepuberal mouse testis. Prepachytene spermatocytes are a small proportion of the total cells in the adult; however, earlier in development, these cell types are found as a much higher proportion of the total cells within seminiferous tubules (2). For this reason, sections of testis taken from prepuberal mice at postnatal days 10, 14, 18, and 25 were examined for L1 ORF 1 expression by immunohistochemistry with the FP1 antibody.

Within seminiferous tubules of prepuberal mice, L1 ORF 1

FIG. 2. Immunoperoxidase detection of L1 ORF 1 expression in the testis from adult and prepuberal mice. Serial sections of adult (A) and prepuberal (B) mouse testis were treated with the indicated antibodies. (A) Affinity-purified FP1 antibody (panel a), the corresponding preimmune IgG (panel b), and affinity-purified FP2 antibody (panel c) are shown. Panels d and e show enlargements of the central tubule of panels a and b. Examples of L1 labelling in spermatids and residual bodies are indicated by arrowheads and arrows, respectively. Leydig (l) and myoid (m) cells are also labelled. Magnification, ×78, and bar, 100 μm (panels a and b); magnification, ×352, and bar, 25 μm (panels c to e). (B) L1 ORF 1 expression in prepuberal mouse testis was detected with FP1 antibody. Testis sections were from postnatal days 10, 14, 18, and 25 (panels a to d, respectively). Corresponding serial sections treated with preimmune IgG are shown below (panels e to h). Magnification, ×275; bar, 25 μm.

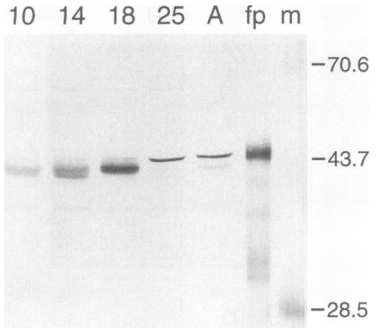

FIG. 4. Western blot detection of L1 ORF 1 protein in testis extracts from prepuberal mice. Lanes contain 50 μg of protein extract from testis at postnatal days 10, 14, 18, and 25 and from adults (A), 4 ng of purified FP1 (fp), and protein standards (m). Numbers on the right indicate positions of marker proteins and their apparent molecular size (in thousands).

immunoreactivity is found readily in spermatocytes as well as in spermatids. As shown in Fig. 2B (a to d), spermatocytes that express significant amounts of L1 ORF 1 are found at all of these ages. These spermatocytes have a distinct morphology: large, round nuclei surrounded by a small amount of cytoplasm. On the basis of their morphology, position in the tubules, and absolute numbers in the testes of mice of different ages, these cells are leptotene and zygotene spermatocytes. Unlike the pattern described above for spermatids, spermatocyte labelling appears to be punctate and widely distributed throughout the cytoplasm. Spermatids are never found in the sections from day 10 or 14 animals, are rare in the sections from day 18 animals, and become increasingly abundant by day 25. The spermatids that are present in the sections from prepuberal mice stain in a manner identical to that of adults: a single cytoplasmic dot adjacent to the nucleus. The staining pattern attributed to condensing spermatids and residual bodies was not found in these sections. This result is expected, because the developmental progression of spermiogenesis has not yet advanced to these stages by 18 or 25 days postnatal.

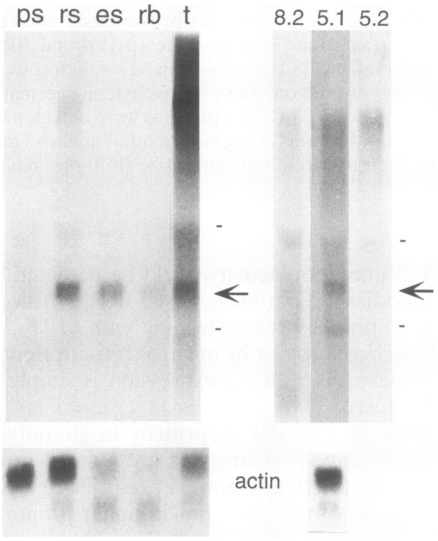

FIG. 5. Northern blot detection of L1 RNA in extracts of fractionated cells from adult mouse testis. Autoradiograms are the result of hybridization to 8.1 (left set) or other single-stranded probes (right set) as indicated (Fig. 1). Two sets of blots were used; one set was first hybridized to 8.2, to 8.1, and then to actin; the other was hybridized first to 5.2, to 5.1, and then to actin. Upper and lower dashes indicate positions of 28S and 18S rRNA, respectively; arrows indicate the 2.8-kb L1 transcript. Lanes on the left contain RNA from pachytene spermatocytes (ps), round spermatids (rs), elongating spermatids (es), residual body (rb), and whole testis (t). In this experiment, interstitial cells were removed prior to dissociation of seminiferous tubules; therefore, Leydig cells are not contaminating the pachytene spermatocyte fraction. Lanes on the right contain RNA from round spermatids. Two forms of actin are detected: 2.1 kb (upper band) and 1.6 kb (lower band).

No immunoreactivity is observed in spermatogonia or Sertoli cells. As with adult testis, Leydig and myoid cells are labelled. Leydig cell staining is always noticeably less intense in day 10 and 14 testis, is increased in day 18, and reaches the intense levels observed in adults by 25 days. Serial sections treated with preimmune IgG showed no reactivity in either the interstitial compartment or the tubules throughout the developmental series (Fig. 2B, e to h).

Detection of L1 ORF 1 protein in fractionated cells from adult testis. To verify the immunohistochemistry results, proteins were extracted from testis and various subfractions of the testis for analysis by Western blotting (immunoblotting). Consistent with results obtained by immunohistochemistry, the FP1 antibody recognizes proteins with a mobility in the expected range for L1 ORF 1 protein (molecular weight, ~41,200 [12]) in both the interstitial cell population and the germ cells (Fig. 3). The predominant band in extracts prepared from intact testis and interstitial cells appears to comigrate with the fusion protein (predicted molecular weight, 44,600) and thus is designated p44.6. The predominant band in the dissociated tubules (germ cell fraction) is somewhat smaller, with an apparent molecular size of ~43,000 (p43).

The cells of the testes were dissociated and further fractionated to yield separate cell populations consisting of Leydig cells with pachytene spermatocytes, round (early) spermatids, elongating (late) spermatids, and residual bodies (Fig. 3). Other cell types found in the testis are present at a low frequency in the adult and result in only minor contamination of the fractions (2). In these fractions, the p44.6 form of L1 ORF 1 that appears to be present in pachytene spermatocytes is much less abundant in round spermatids and not detected in elongating spermatids or residual bodies. In contrast, the p43 form increases in relative amount throughout the developmental progression from its lowest levels in pachytene spermatocytes, to reach its highest level in residual bodies (Fig. 3). Neither of these L1 ORF 1 proteins was detected with the preimmune IgG (not shown). From these results, we conclude that p44.6 is the form of L1 ORF 1 protein detected in Leydig cells by immunohistochemistry and that p43 is the form in spermatids. Since L1 ORF 1 protein is not detected in pachytene spermatocytes by immunohistochemistry, immunoreactivity on the Western blot is likely to be due to Leydig cells that cofractionate with pachytene spermatocytes. This interpretation is consistent with the known migration of Leydig cells in these gradients (2), as well as with the presence of p44.6. In fact, in a subsequent experiment, several washes were performed during the collagenase treatment prior to fractionation through the BSA gradient. This treatment, which serves to lower the number of Leydig cells in the cell suspension, dramatically reduced the amount of p44.6 present in the pachytene spermatocyte cell fraction.

Detection of L1 ORF 1 protein in extracts of prepuberal mouse testis. Protein extracts were prepared from the developmental series of mouse testis and compared with the proteins present in adult testis by using Western blots to detect L1

ORF 1 protein. Immunoreactive proteins are present at all ages, but at least three forms are observed and their relative proportions differ among the different ages (Fig. 4). p44.6 is present in all of the extracts, although it is barely detectable in day 10 mice. The relative amount of this protein increases with age, reaching adult levels by day 25. p44.6 appears to account for Leydig cell immunoreactivity. This increase in intensity with developmental age observed by Western blotting correlates with the developmental increase in intensity of Leydig cell staining by immunohistochemistry. The germ cell-specific p43 is also detected throughout the series. It is present in the 10- and 14-day testis; thus, it is expressed in spermatocytes as well as in spermatids. Finally, a new form of L1 ORF 1 with an apparent molecular size of 42,000 (p42) is observed in the testis extracts prepared from postnatal day 10 and 14 mice; this form is also attributed to spermatocytes, on the basis of the intense staining observed in 10- and 14-day testis by immunohistochemistry.

Expression of L1 RNA in adult testis. RNA was isolated from cell fractions similar to those used for the protein analysis described above, except that the interstitial cells were removed before the seminiferous tubules were dissociated. RNA preparations were analyzed for the presence of L1 transcripts by Northern blotting. Of particular interest are potential transposition intermediates; these should be full-length (≥6.8-kb), sense-strand RNA. Such transcripts are rare. In all cell and tissue types examined, RNA species that hybridize to L1 probes are found; only rarely, however, are they full length and sense stranded (7, 14, 15, 22). More often, L1-containing transcripts cover a wide size range and contain both strands of L1. These are believed to be due to nonspecific expression arising from L1 insertions in other transcription units (9).

In RNA isolated from adult testis, the sense-strand-specific ORF 1 probe, 8.1, detects a broad band (or collection of bands) centered around 2.8 kb (Fig. 5, arrow), in addition to the typical smear. The discrete transcript is barely detectable in pachytene spermatocytes, is most prominent in round spermatids, and then decreases in amount in elongating spermatids and residual bodies. The representation of the ~2.8-kb transcript in the pachytene fraction could be due to a small amount of cross-contamination with round spermatids and interstitial cells. Rehybridization of the same blot to an actin probe provides independent verification of the fractionation procedure as well as a control for the amount of RNA loaded in each gel lane. Two forms of actin mRNAs are known to be made in the testis: the 2.1-kb form decreases throughout spermiogenesis, whereas the 1.6-kb form is expressed only in postmeiotic germ cells (26). In these fractions, as expected, the amount of the 2.1-kb actin mRNA decreases as the cells mature and the 1.6-kb actin mRNA is present only in postmeiotic cells, increasing in amount as the cells mature (Fig. 5).

Since RNA isolated from round spermatids showed the greatest intensity of hybridization to the 8.1 probe, it was also hybridized to the sense-strand-specific probe for the 3′ end of L1 and both antisense-specific probes. The 3′ end, sense-strand-specific probe (5.1) also detects an RNA species of about 2.8 kb. No discrete transcripts are detected with the antisense-specific probe (5.2) from the 3′ end. The 5′ end, antisense-specific probe (8.2), however, detects several discrete RNA species, including one with a mobility similar to those of the transcripts detected with the sense-strand-specific probes. Clearly, no full-length, sense-strand L1 transcript is detected in the adult testis.

Detection of the 2.8-kb L1 RNA complements the detection of L1 ORF 1 protein by immunocytochemistry and Western blot analysis and could account for translation of the germ cell-specific form of ORF 1 protein, p43, in postmeiotic cells of adult testis. In contrast to the results of Western blotting, which demonstrate distinct species of L1 ORF 1 protein in germ cells and interstitial cells, analysis of RNA extracted from the same cell separations shown in Fig. 3 revealed no difference in the hybridization pattern of transcripts detected (data not shown).

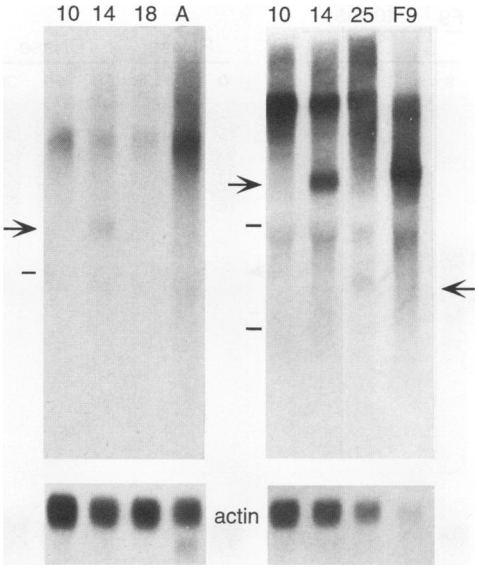

FIG. 6. Northern blot detection of L1 RNA in extracts from prepuberal mice. Autoradiograms from two blots show hybridization to 8.1 and actin on the same blots following rehybridization. The day 10 and 14 RNA preparations used on the two blots were isolated from different animals in order to confirm the results. Lanes are identified by numbers showing the age of the animal; A, adult; F9, F9 cells. The position of 28S rRNA is indicated by the dash on the left blot and the upper dash on the right blot; 18S is shown on the right blot only (lower dash). Arrows to the side of each blot mark the position of the 7-kb (left) and 2.8-kb (right) transcripts.

Expression of L1 RNA during testicular development. RNA was prepared from testes from prepuberal mice of different ages and analyzed for expression of L1 by Northern blotting. As shown in Fig. 6, day 14 testes contain a readily detectable transcript of about 7 kb that hybridizes to the sense-strand-specific ORF 1 probe, 8.1. This transcript is not detected in testis RNA preparations from any other age. The best explanation for this result is that the proportion of the ~7-kb RNA is high in postnatal day 14 testis because of a combination of the high proportion of cells that express it (leptotene and zygotene spermatocytes) along with an elevated level of expression at that specific time in development. At other times, that transcript cannot be detected above the background by Northern analysis. The ~2.8-kb RNA found in adult testis is not detected in the extracts from day 10, 14, or 18 or F9 cells but is observed in the extract prepared from day 25 animals. F9 RNA, known to contain a full-length, sense-strand L1 transcript, is shown on the same gel for comparison. The ~7-kb L1 transcript present at day 14 appears to be slightly shorter than the F9 transcript but slightly longer than the longest characterized L1 elements in the mouse genome (6.8 kb [12]).

The ~7-kb transcript from day 14 testis was hybridized to sense and antisense probes from the 5′ and 3′ ends of L1 to determine whether it is full length and sense stranded. This transcript is detected with both the 5′ and the 3′ probes that detect the sense strand but with neither of the probes that

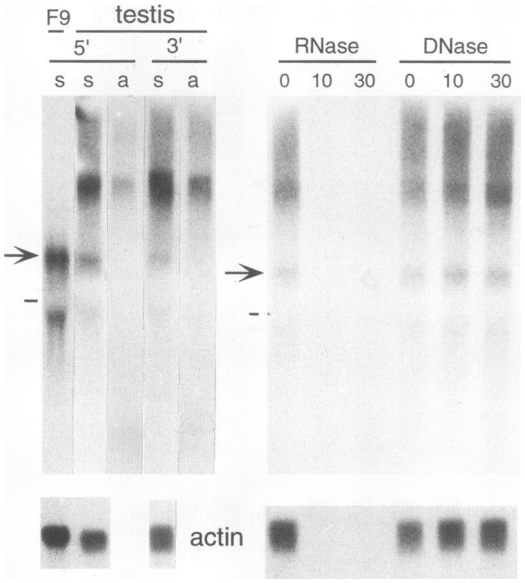

FIG. 7. Characterization of L1 RNA in the testis on postnatal day 14. Autoradiogram on the left shows hybridization of RNA from F9 cells or postnatal day 14 testis to probes that detect the 5′ end sense (s; 8.1) and antisense (a; 8.2) and 3′ end sense (s; 5.1) and antisense (a; 5.2) strands of L1. Two sets of blots were hybridized sequentially, as described in the legend to Fig. 5. Autoradiogram on the right shows RNA treated with RNase and DNase for the indicated times; hybridization was to 8.1 and then to actin. Dashes indicate the position of 28S rRNA; arrows indicate the position of the 7-kb L1 RNA.

detect the antisense strand (Fig. 7). In addition, the RNA preparation was treated with RNase and DNase. All of the L1 hybridization signal disappears with RNase treatment and is unaffected by DNase (Fig. 7), including the transcript(s) that is larger than 7 kb. The larger transcripts are most likely due to heterogeneous, nonspecific L1 expression in heterogeneous nuclear RNA since both strands are present and since the proportion of the higher-molecular-weight material is far less in cytoplasmic preparations (e.g., compare the total testis RNA to the cytoplasmic RNA from fractionated germ cells [Fig. 5]). Thus, we conclude that the 7-kb transcript isolated from postnatal day 14 testis is a full-length, sense-strand L1 RNA and hence a strong candidate to be a bona fide transposition intermediate.

DISCUSSION

Expression and transposition. L1 RNA and ORF 1 protein are expressed in mouse testis in both germ and somatic cells. Expression of L1 RNA and protein products in testis varies with developmental age and cell type, forming a complex pattern. The evolutionary genetics of the L1 family requires L1 transposition in cells that are destined for the next generation (9). This criterion is met by testicular germ cells. Expression of full-length RNA and L1-encoded proteins is a necessary step in the transposition of L1 and occurs in leptotene and zygotene spermatocytes. The *I* element of *Drosophila melanogaster* provides an example of a direct correlation between the expression of a LINE-like retrotransposon and its transposition frequency: transcription of the full-length *I* element is restricted to the ovary of dysgenic females, where retrotransposition is known to occur (4).

The most significant L1 expression pattern observed in this study, with regard to L1 transposition in cells destined for the next generation, was that detected in extracts prepared from testis of postnatal day 14 mice. In these extracts alone, there is clear evidence for a full-length, sense-strand L1 RNA and its coexpression with ORF 1 protein, i.e., coexpression of L1 transposition intermediates. At the same time in development, strong staining of L1 ORF 1 is observed in the leptotene and zygotene spermatocytes by immunohistochemistry. This leads us to the conclusion that expression of L1 ORF 1 protein in the day 14 testis is due to the 7-kb L1 RNA in leptotene and zygotene spermatocytes. At this early stage of testicular development, leptotene and zygotene spermatocytes compose 13 and 14% of the total cells in the seminiferous epithelium, respectively. The relative numbers of these cell types decline steadily as the animals mature, until they each compose only 2% of the total cells in adult testis (2). In our immunohistochemistry screens for L1 ORF 1 expression, strong spermatocyte staining was observed in the day 10 and 14 animals. Although spermatocyte staining also was found easily in sections from day 18 and 25 animals, it appeared to diminish in intensity. Spermatocyte staining was extremely difficult to find in any sections from adult animals and was always just barely above background. We attribute both the difficulty in locating immunoreactive spermatocytes in adult testis by immunohistochemistry and the inability to detect the ~7-kb transcript at times other than postnatal day 14 to the significantly reduced proportion of leptotene and zygotene spermatocytes in combination with lower levels of expression at other times.

Variations in L1 expression during testicular development. In adult testis, L1 ORF 1 protein is expressed as two forms. Both of these appear to be larger than the predicted size of the primary translation product of ORF 1, on the basis of the genomic consensus sequence (21). At least one additional form, p42, is present in testis from prepuberal mice. Multiple forms of mouse L1 ORF 1 protein have been encountered previously in proteins extracted from the embryonal carcinoma cell lines F9 and C44 (15). As with expression in embryonal carcinoma cells, multiple forms of L1 ORF 1 in testis may be due to differences in posttranslational modification or to the expression of different polymorphic variants of L1 known to be present in the genome.

Taken together, results of immunohistochemistry and Western blotting on protein extracts from prepuberal and adult testis lead to tentative identification of the three forms. p44.6 is responsible for Leydig cell staining because it fractionates with interstitial cells. Furthermore, it is present throughout development and increases in amount between days 10 and 25, when it finally reaches the high-level expression characteristic of adults. This exactly mirrors the intensity of Leydig cell staining observed by immunohistochemistry. p43 and p42 are restricted to germ cells. p43 fractionates with round and elongating spermatids and residual bodies from adult testis and accounts for the staining of these cell types observed by immunohistochemistry. Since it is also present in day 10 and 14 testis, it must be found in pre- as well as postmeiotic cells. The final form, p42, was detected in extracts prepared from prepuberal mice but not from adults. It also appears to be germ cell specific and must account for at least some of the spermatocyte staining observed by immunohistochemistry, because it is readily detected by Western blotting of extracts from day 10 and 14 mice.

In RNA isolated from adult testis, there is one discrete transcript which could account for the translated proteins. No difference is detected in the amount of this ~2.8-kb RNA in interstitial versus germ cell preparations, even when the same cell extracts that were used for Western blots in which protein differences are obvious are used. One interpretation of this

result is that both the Leydig cell and the spermatid proteins (p44.6 and p43, respectively) are translated from the 2.8-kb transcript, and the difference in the proteins is due to either differences in posttranslational modification of the same primary translation product or to sequence (or small-length) polymorphisms within the RNA population. An alternative possibility is that the L1 RNA(s) responsible for the different protein forms found in one or both of these two cell types of the adult testis is not sufficiently abundant to be detected as a discrete transcript but rather is hidden within the smear extending throughout the Northern blots.

The relationship between the adult protein and RNA expression patterns and those of the leptotene and zygotene spermatocytes in prepuberal testis is not clear at this time. At least two explanations are possible: either the expression in leptotene and zygotene spermatocytes in prepuberal mice is unrelated to expression in adult spermatids (e.g., comes from an independent locus of L1), or the expression in spermatocytes is a precursor to the expression in spermatids. We favor the former possibility because of the length difference between the two transcripts and because there is no evidence for expression of RNA or protein in the intermediate cell type, pachytene spermatocytes. Although it is possible that the 7-kb RNA in spermatocytes is processed to give the 2.8-kb transcript of spermatids, one or the other RNA species should be present in pachytene cells. Furthermore, since ORF 1 protein is found in prepachytene spermatocytes and in spermatids, it should persist and be detectable in pachytene spermatocytes. As this is not the case, it appears likely that the control and source of L1 expression differs in the two cell types.

There are ~10,000 copies of the 5′ end of L1 (i.e., the region that contains ORF 1) in the mouse genome. Although most of them are probably defective because of truncations, rearrangements, and frameshift and termination mutations (9), it is clear that the mouse genome contains multiple independent loci that could drive expression of an ORF 1 protein. The conditions for expression could vary such that one (or more) locus is specifically expressed in Leydig cells, another is expressed in spermatids, and another is expressed in leptotene and zygotene spermatocytes. Only one of these three distinct expression patterns, namely, the one leading to expression of full-length L1 RNA in leptotene and zygotene spermatocytes, is likely to be responsible for the type of transposition that explains the evolutionary genetics of L1. In contrast, the 2.8-kb transcript must be missing significant regions of ORF 2 even if the extreme 3′ end is present, as suggested from our Northern blotting results. In fact, a similar rearrangement of the consensus L1 structure as found in this testis transcript has been reported as an insertion into a γ-actin pseudogene and carries all of the hallmarks of a retrotransposed sequence (1). Thus, although the 2.8-kb RNA lacks sequences necessary for transposition in *cis*, it could be a substrate for the proteins acting in *trans*, as may be the case for other, non-L1 transcripts. Its expression in germ cells (spermatids) potentially allows its passage to the next generation. Thus, this expression pattern could be a model for the generation of other types of processed pseudogenes. Leydig cell expression, even if it leads to a new insertion event, would not persist in the next generation. However, this expression pattern may be a model for somatic cell transposition, of the type that is associated with cancer (17, 18); both of these latter RNA patterns would require reverse transcriptase in *trans* for transposition, because they are too short to encode a functional ORF 2 themselves.

Why transpose in meiotic prophase? The mechanism of L1 transposition is not known in detail. One particularly significant question concerns the priming of reverse transcription. Essentially, two models exist: reverse transcription could occur in a ribonucleoprotein particle, as with retroviruses (24) and yeast Ty1 (3), or in situ on the chromosome (9). Recently, strong evidence for in situ priming of reverse transcription has been obtained by using the related ORF from the LINE-like R2Bm element of *Bombyx mori* (13). Unlike mammalian L1, however, R2Bm contains an endonuclease activity that generates a break in a specific target DNA sequence. Given the apparent lack of target site specificity for integration of L1, it seems unlikely that it has such a cleavage activity. Thus, L1 may be able only to insert into DNA that already contains nicks or breaks.

During prophase of the first meiotic division, chromosomes are tightly paired and undergo recombination. Nicks and/or breaks must occur at this time, offering a perfect opportunity for L1 insertion. Our data demonstrate expression of L1 transposition intermediates in leptotene and zygotene spermatocytes. Zygotene spermatocytes are just forming the chiasmata between chromosomes. Expression of L1 transposition intermediates in the cytoplasm should precede the formation of breaks in the nucleus. A small amount of L1 RNA and protein may then find its way back into the nucleus for integration, while the rest is lost at that time or reorganized for later elimination with the residual body. It has been suggested that L1 functions as a "molecular glue" to repair breaks in chromosomal DNA (25); the observed expression of L1 in spermatocytes is consistent with this hypothesis.

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