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Tightly regulated, developmentally specific expression of the first open reading frame from LINE-1 during mouse embryogenesis

(gene expression/germ cells/repetitive DNA/retrotransposon/steroidogenic cells)

STEPHANIE A. TRELOGAN AND SANDRA L. MARTIN*

University of Colorado Health Sciences Center, Department of Cellular and Structural Biology and Program in Molecular Biology, Campus Box B111, 4200 East Ninth Avenue, Denver, CO 80262

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ABSTRACT **LINE-1 (L1) has achieved its status as a middle repetitive DNA family in mammalian genomes by duplicative transposition. Although transposition may occur in any cell type, expression and transposition of a full-length functional element in the germ line are necessary for evolutionarily significant propagation of L1. An immunohistochemical analysis of adult mouse ovaries and mouse postimplantation embryos revealed expression of L1 open reading frame 1 in the germ line as well as in steroidogenic tissues. These results demonstrate that L1 expression is controlled by a tightly regulated temporal and spatial program of events during development and imply that multiple loci of L1 in the mouse genome are active for expression.**

LINE-1 (L1) is an interspersed repetitive DNA sequence that constitutes an estimated 10% of the mammalian genome. A member of an evolutionarily conserved superfamily of non-long terminal repeat-containing mobile elements, L1 is thought to have attained its high copy number (≈100,000 in the mouse genome) by duplicative transposition involving an RNA intermediate, a process known as retrotransposition. The structure of L1 is consistent with its classification as a retrotransposon. Elements are flanked by target site duplications. In the mouse genome, most copies of L1 are truncated from the 5′ end and are therefore thought to be inactive for transposition; the 3′ end contains a poly(A) signal followed by an A-rich tail. Full-length elements are ≈6.8 kb and contain two long open reading frames (ORFs) (reviewed in ref. 1). ORF 2 of mouse L1 has homology to reverse transcriptase (2); reverse transcriptase activity has been demonstrated for human ORF 2 protein (3). ORF 1 protein, which cofractionates with the full-length L1 RNA found in ribonucleoprotein particles, may serve a packaging function for the RNA (4).

Although the specific mechanism of L1 transposition is not yet known, a model can be predicted from its structural features. First, a full-length, functional genomic element must be transcribed; although mouse L1 appears to contain its own promoter (5), transcription may also be affected by nearby enhancers in the genome. The full-length, sense-strand RNA transcript moves to the cytoplasm, where both ORFs are translated. ORF 1 (4) and possibly ORF 2 (6) proteins associate with L1 RNA, forming a ribonucleoprotein particle. Reverse transcription by ORF 2 protein must occur, resulting in L1 cDNAs, which are integrated into the genome (reviewed in ref. 7). According to this model, necessary intermediates for transposition of L1 must include a full-length, sense-strand transcript as well as the proteins encoded by ORF 1 and ORF 2. For this reason, expression of ORF 1 protein may serve as a hallmark for L1 transposition; however, it is important to keep in mind that expression is necessary, but not necessarily sufficient, for transposition.

It is clear that recent L1 insertion events have occurred in somatic and germ cells (8–11); however, from an evolutionary standpoint, the only significant transposition occurs in cells that are destined for the next generation. In mammals, these cells include primordial germ cells (PGCs), germ cells, and early embryos. The PGCs are first recognized in extraembryonic mesoderm at day 7.5 postcoitum (p.c.) (12). Between days 9 and 12 p.c., the PGCs migrate back into the embryo, settling in the genital ridge (13–15). Day 13 p.c. is the first stage at which the male and female gonads are histologically differentiable (16). At this point, germ cell development takes strikingly different paths in the male and female mouse. In the male, the PGCs differentiate into primitive spermatogonia between days 15 and 16 p.c., proliferate, and then arrest before birth. After birth, the spermatogonia begin to undergo the processes of mitosis and meiosis, which continue throughout life (17). In the female, the PGCs differentiate between days 15 and 16 p.c. and the oogonia complete mitotic proliferation *in utero*. At ≈16 days p.c., the oocytes begin the process of meiosis, arresting in the diplotene stage of prophase of meiosis I, where they remain until puberty. After the female reaches puberty, the oocytes mature individually, completing meiosis I in the ovary but not undergoing meiosis II until after fertilization (18).

Preliminary studies of evolutionarily significant L1 transposition focused on embryonal carcinoma cells, which are thought to represent early stages in embryonic development. Detection of L1 RNA and ORF 1 protein in these cultured cells (4, 19, 20) spurred the search for L1 transposition intermediates in developmentally relevant cell types *in vivo*. In one study, full-length, sense-strand L1 transcripts were detected in mouse blastocysts (21). In a separate investigation (22), a survey of L1 expression in the developing postnatal mouse testis was conducted. In adult mouse testis, L1 ORF 1 protein was detected along with a truncated L1 transcript in spermatids, Leydig cells, and myoid cells. Additionally, coexpression of L1 ORF 1 protein and a full-length, sense-strand RNA was found in prepuberal mouse leptotene and zygotene spermatocytes.

To continue the *in vivo* screen for potential L1 transposition intermediates during mouse germ cell development, we examined adult mouse ovaries and gonadal tissue of mouse postimplantation embryos for L1 ORF 1 expression. Because of the relatively few germ cells in these tissues, we used immunohistochemistry, the technique of choice for exploration of protein expression in small populations of cells. The pattern of immunoreactivity in gonadal tissue provides evidence for L1 ORF 1 expression in specific stages of germ cell development as well as in restricted somatic cells. L1 ORF 1 immunoreactivity in those cells that contribute to the next generation provides evidence for the first step in evolutionarily significant L1 transposition. In addition, we observed immunoreactivity in androgen-producing cells in the

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Abbreviations: L1, LINE-1 element; ORF, open reading frame; PGC, primordial germ cell; p.c., postcoitum.

*To whom reprint requests should be addressed.

testis and ovary. This result led us to examine other steroidogenic tissues, revealing L1 ORF 1 immunoreactivity in the syncytiotrophoblast cells of the placenta, cells that also produce androgens. Taken together, these results demonstrate that L1 expression is under the control of a tightly regulated temporal and spatial program of events during development and differentiation. An additional implication of these data is that multiple loci of L1 in the mouse genome are expressed and, therefore, potentially active for transposition.

MATERIALS AND METHODS

Mice. C57BL/6 mice were used. Pregnancy was established when vaginal plugs were observed the morning following mating, which was taken as day 0.5. Postimplantation embryos were harvested from the following stages of development: 8.5, 10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, and 17.5 days p.c. Adult ovaries were harvested from a nonpregnant female.

Primary Antibodies. Preimmune IgG and affinity-purified polyclonal antibody (FP1) against L1 ORF 1 were prepared as described (20). L1 ORF 1 immunoreactivity is specific for a 42-kDa protein, a size that corresponds to the translated sequence of ORF 1 (20, 22).

Immunohistochemistry. Postimplantation embryos and adult mouse ovaries were fixed in Bouin's fixative for 4–8 hr, depending on size. To facilitate fixation of the older embryos (days 14.5–17.5 p.c.), the specimens were cut sagittally. After fixation, embryos and ovaries were embedded in paraffin and sectioned serially at 5 μm. To locate tissues of interest, every 12th section was stained with hematoxylin/eosin to facilitate morphological examination; sections containing tissues of interest were then subjected to immunoperoxidase staining. Sections were deparaffinized and treated with 3% hydrogen peroxide for 1 hr to quench endogenous peroxidases. Permeabilization was then performed for 5 min to 1 hr with a 1 mg/ml solution of bovine pancreatic trypsin, type 3 (Sigma), in phosphate-buffered saline (PBS), followed by treatment for an equal amount of time with a 1 mg/ml solution of soybean trypsin inhibitor (Sigma) in PBS. Optimal trypsin conditions were determined experimentally for each block of tissue; optimal times were the longest treatments possible without destruction of tissue morphology. Sections were preblocked with 10% normal goat serum in PBS for 30 min at 37°C. Anti-L1 ORF 1 antibody or preimmune IgG was added at a concentration of 1 μg/ml in PBS containing 1.5% normal goat serum and 0.02% sodium azide and then incubated for 15 hr at 4°C. Sections were washed in PBS between all incubations. The Vectastain Elite ABC kit (Vector Laboratories) was used to locate antibody labeling, following the manufacturer's instructions for detection with diaminobenzidine. Immunostained sections were counterstained with 0.05% toluidine blue, dehydrated, and mounted in Permount. Results were observed with a Zeiss Axiophot microscope and photographed with Ektachrome 64T film (Kodak) using bright-field illumination.

RESULTS

This investigation began with a survey of the developing germ cells in mouse postimplantation embryos and ovaries. Postimplantation embryos of developmental stages 8.5, 10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, and 17.5 days p.c., as well as adult ovaries, were examined by immunohistochemistry using affinity-purified antibody against L1 ORF 1 protein. Prior to day 15.5 p.c., none of the embryonic tissues of the mouse exhibited immunoreactivity with the antibody against L1 ORF 1 protein. Fig. 1*A* shows the torso of a 10.5-day-p.c. embryo stained with anti-L1 ORF 1 antibody, which demonstrates this lack of immunoreactivity. Similarly, no immunoreactivity was observed in the undifferentiated gonad of a 12.5-day-p.c. embryo (Fig. 1*B*).

Germ-line immunoreactivity was first observed at day 15.5 p.c. Fig. 1*C* shows the lack of immunoreactivity in 14.5-day-p.c. testis stained with anti-L1 ORF 1 antibody. In contrast, the cytoplasm of primitive spermatogonia of the testis was immunoreactive for L1 ORF 1 at days 15.5, 16.5, and 17.5 p.c. (Fig. 1 *D–F*, respectively); staining with preimmune serum on an adjacent section revealed no specific immunoreactivity in these cells (Fig. 1*G*). The location of the immunoreactive cells inside the seminiferous tubules, coupled with the developmental stages of the embryos, led to their identification as primitive spermatogonia. A similar time line of germ-line immunoreactivity was observed in the embryonic ovary. Fig. 1*H* shows the 14.5-day-p.c. ovary, stained with anti-L1 ORF 1 antibody, which exhibited no immunoreactivity. At days 15.5, 16.5, and 17.5 p.c., the cytoplasm of developing oocytes was immunoreactive for L1 ORF 1 (Fig. 1 *I–K*); no immunoreactivity was observed in these cells in the adjacent section stained with preimmune serum (Fig. 1*L*). The classification of the immunoreactive cells as developing oocytes was based on their morphology: large cells with condensed chromosomes, indicating entrance into meiosis.

In the course of the survey of embryonic gonadal tissue, L1 ORF 1 immunoreactivity was also observed in some somatic cell types. Immunoreactivity was present in the cytoplasm of Leydig cells of the 17.5-day-p.c. mouse embryo testis (Fig. 1*F*) but was not observed in this cell type at earlier developmental stages; furthermore, staining an adjacent section with preimmune serum showed no immunoreactivity in these cells (Fig. 1*G*). Immunoreactivity was also present in the cytoplasm of theca cells of the adult mouse ovary (Fig. 2*A*); Fig. 2*B* shows the preimmune control. Because a characteristic shared by these cell types is their capacity to produce steroid hormones, we embarked on a survey of other steroidogenic tissues in the embryo: adrenal gland and placenta. No immunoreactivity was seen in the adrenal gland at any developmental stage examined (data not shown). In contrast, immunoreactivity was observed in cytoplasm of syncytiotrophoblast cells of the placenta at all developmental stages examined: 13.5, 14.5, 15.5, 16.5, and 17.5 days p.c. The 17.5-day-p.c. placenta stained with anti-L1 ORF 1 antibody is shown in Fig. 2 *C* and *E*; Fig. 2 *D* and *F* show the preimmune control. Notably, the steroidogenic cell types exhibiting L1 ORF 1 immunoreactivity are all characterized by active production of androgens; in the mouse embryo, the adrenal gland does not produce androgens or androgen precursors (23).

DISCUSSION

This investigation of mouse postimplantation embryos and adult ovaries revealed L1 ORF 1 immunoreactivity in several cell types. In all cell types examined, L1 ORF 1 immunoreactivity is confined to the cytoplasm. This pattern of staining is identical to results of previous studies, which reported a punctate pattern of ORF 1 immunoreactivity in embryonal carcinoma cell lines (20) and spermatocytes (22). These observations are consistent with the proposed mechanism of L1 transposition, in which ORF 1 protein complexes with L1 RNA following translation (7).

Because of our interest in evolutionarily significant transposition of L1, this investigation was originally focused on the developing germ cells of the mouse. Fig. 3 puts the results of this investigation, along with the results of previous studies, into the context of the chronology of mouse germ cell development. Expression of L1 during the earliest stages of PGC development, prior to migration to the genital ridges, is suggested by the results of two previous studies. A survey of six embryonal carcinoma cell lines, each possessing different developmental potentials, revealed co-expression of ORF 1 protein and full-length, sense-strand L1 transcripts in only two cell lines, F9 and C44. A link between these cell lines is the close relationship of both to

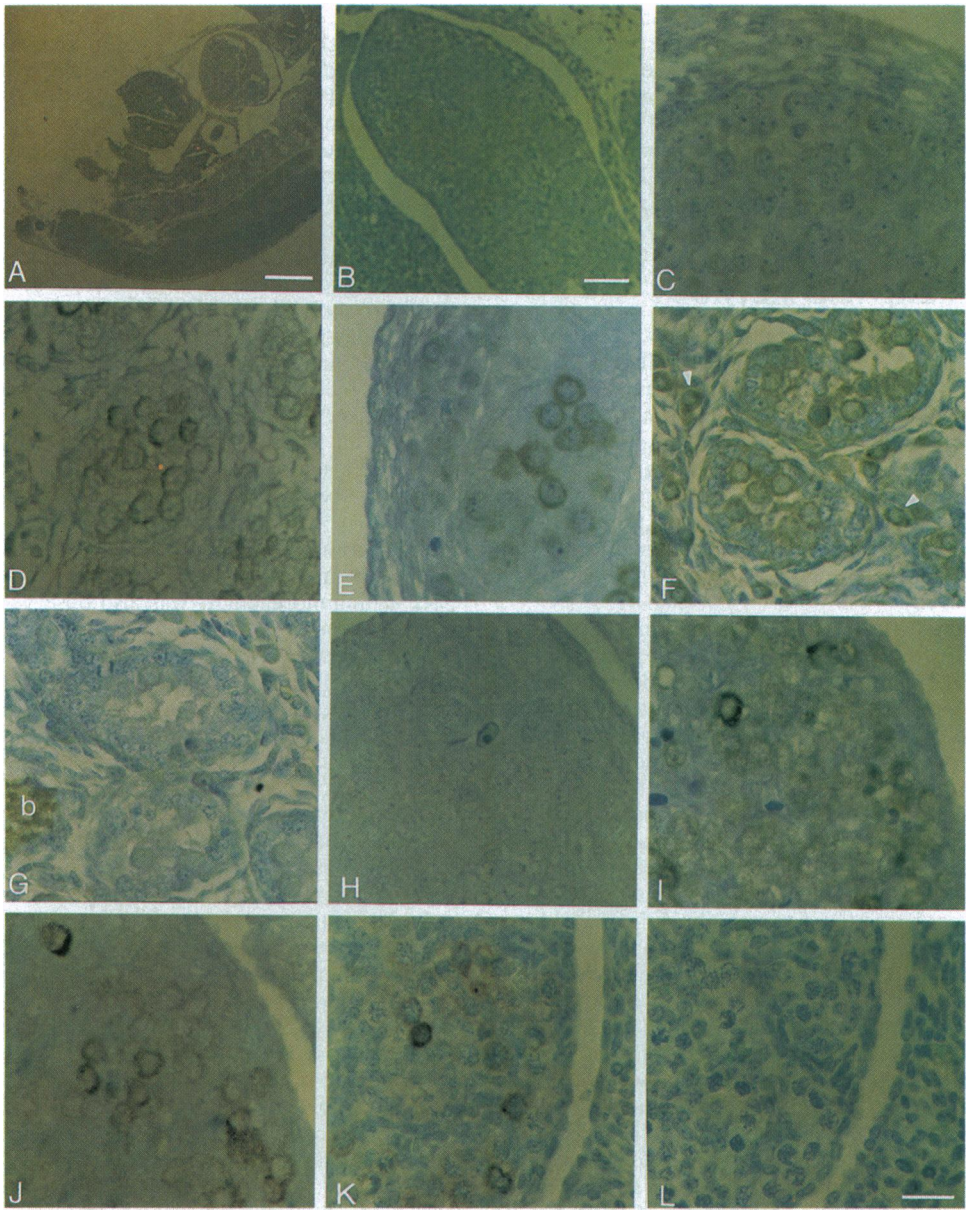

FIG. 1. Immunoperoxidase detection of L1 ORF 1 immunoreactivity in the developing gonads of mouse embryos. (*A*) Torso of a 10.5-day-p.c. embryo stained with anti-L1 ORF 1 antibody. (×14; bar = 450 μm.) (*B*) Genital ridge of a 12.5-day-p.c. embryo stained with anti-L1 ORF 1 antibody. (×79; bar = 75 μm.) (*C–F*) Testes of 14.5-, 15.5-, 16.5-, and 17.5-day-p.c. embryos, respectively, stained with anti-L1 ORF 1 antibody; arrowheads in *F* indicate Leydig cells. (*G*) Testis of 17.5-day-p.c. embryo stained with preimmune IgG; serial section adjacent to section shown in *F* (b, blood vessel). (*H–K*) Ovaries of 14.5-, 15.5-, 16.5-, and 17.5-day-p.c. embryos, respectively, stained with anti-L1 ORF 1 antibody; arrowheads in *F* indicate Leydig cells. (*G*) Testis of 17.5-day-p.c. embryo stained with preimmune IgG; serial section adjacent to section shown in *F* (b, blood vessel). (*H–K*) Ovaries of 14.5-, 15.5-, 16.5-, and 17.5-day-p.c. embryos, respectively, stained with anti-L1 ORF 1 antibody. (*L*) Ovary of 17.5-day-p.c. embryo stained with preimmune IgG; serial section adjacent to section shown in *K*. (*C–L*, ×315; bar=20 μm.).

primitive endoderm, the tissue from which PGCs might be derived (20). Additionally, full-length, sense-strand L1 transcripts were detected in mouse blastocysts (21).

In the male germ line, L1 expression has been detected in three different cell types, all of which are separated by intervening developmental stages (Fig. 3). This study revealed L1 ORF 1 immunoreactivity in the primitive spermatogonia of 15.5-, 16.5-, and 17.5-day-p.c. embryonic testis; however, a previous investigation revealed no L1 expression in the type A or B spermatogonia of postnatal mouse testis. In addition, coexpression of L1 ORF 1 protein and a full-length, sense-strand L1 transcript was observed in leptotene and zygotene, but not pachytene and diplotene, spermatocytes of the prepuberal mouse testis. Finally, L1 ORF 1 protein and a short, sense-strand L1 transcript were detected in round spermatids of the adult mouse testis (22).

In the female germ line, L1 expression has been detected in only one cell type to date (Fig. 3); however, because female germ cells do not undergo meiosis II until after fertilization, this particular developmental stage of the female germ line remains to be examined for L1 expression. L1 ORF 1 immunoreactivity was observed in the 15.5-, 16.5-, and 17.5-day-p.c. embryonic ovary, in oocytes entering prophase of the first meiotic division; these cells may be developmentally analogous to the spermatocytes of the prepuberal mouse testis in which L1 expression was detected.

In addition to these different cell types in the mouse germ line, this survey of mouse gonadal tissue revealed L1 ORF 1 immunoreactivity in somatic cells—specifically, Leydig cells of the embryonic testis and theca cells of the adult ovary. Interestingly, L1 ORF 1 expression in these cell types correlates

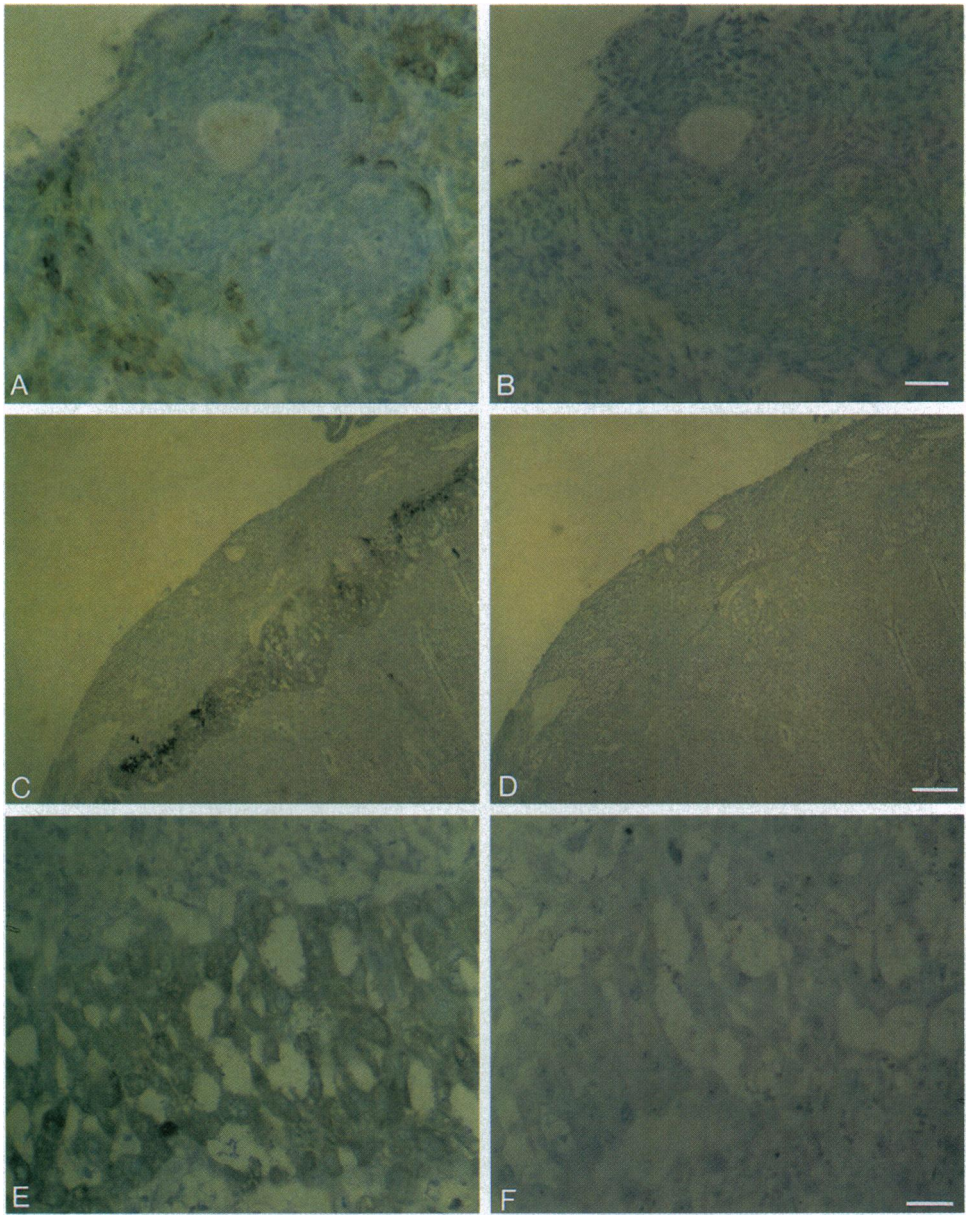

FIG. 2. Immunoperoxidase detection of L1 ORF 1 immunoreactivity in adult mouse ovary and placenta. (*A*) Follicle of adult mouse ovary stained with anti-L1 ORF 1 antibody. (*B*) Serial section adjacent to section shown in *A* stained with preimmune IgG. (*A* and *B*, ×200; bar=30 μm.) (*C* and *E*) Placenta from a 17.5-day-p.c. embryo stained with anti-L1 ORF 1 antibody. (*D* and *F*) Serial sections adjacent to sections shown in *C* and *E* stained with preimmune IgG. (*C* and *D*, ×20; bar = 300 μm; *E* and *F*, ×158; bar=40 μm.)

with active androgen production. Theca cells of adult ovarian follicles produce androgens, which are then transported to the granulosa cells, where conversion to estrogen occurs (24). Leydig cells undergo a burst of androgen production during embryogenesis and then return to quiescence until the male reaches puberty, at which point active androgen production begins anew (25). This pattern correlates with L1 ORF 1 expression in the developing testis of the mouse: in the survey of prenatal mouse testis, expression in the Leydig cells begins at day 17.5 p.c., whereas in the postnatal mouse testis, Leydig cells do not show intense L1 ORF 1 immunoreactivity until postnatal day 25 (22).

This apparent correlation of L1 ORF 1 expression with steroid hormone production in these gonadal somatic cell types suggested a common regulatory mechanism, leading to an examination of other steroidogenic embryonic tissues: embryonic adrenal gland and placenta. No L1 ORF 1 immunoreactivity was observed in adrenal gland of any of the post-implantation embryos screened (day 12.5 p.c. through 17.5 p.c.). In contrast to other mammals, the mouse embryonic adrenal gland does not contain 17-α-hydroxylase, one of the enzymes required to produce C19 steroids; thus, no androgen production occurs in this tissue (23). In the placenta, L1 ORF 1 immunoreactivity was confined to the syncytiotrophoblast cells, which do produce androgens (26). Thus, L1 ORF 1 immunoreactivity in these cells seems to correlate specifically with active production of androgens.

The results of this and other investigations indicate that L1 is expressed in several cell types in the mouse: in at least five different cell types in the germ line, as well as in somatic cells which are actively producing androgens. Because these cell types are all developmentally and functionally distinct, each is likely to represent a unique pattern of L1 expression. For example, the somatic androgen-producing cells are clearly very different from the germ cells. Additionally, expression in the germ line is not confined to a specific stage of development;

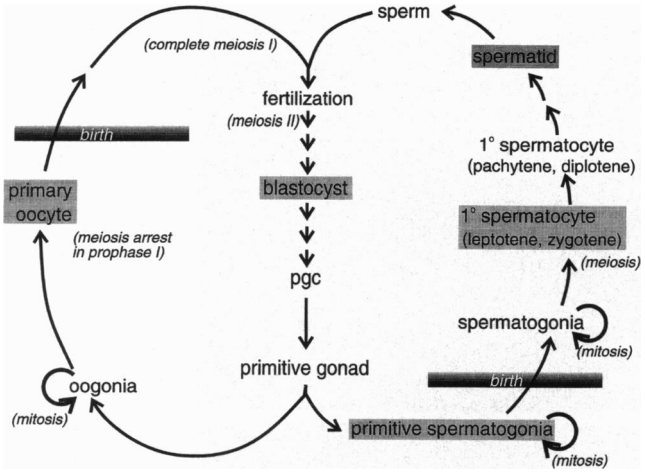

FIG. 3. Diagrammatic representation of mouse germ cell development. Stages in which putative L1 transposition intermediates have been identified are denoted by shaded boxes. Only those developmental stages in which L1 expression has been investigated are shown.

rather, L1 is expressed at various points in the gamete life cycle. In the testis, primitive spermatogonia, spermatocytes, and round spermatids all represent different stages in the development of the male germ cell lineage; furthermore, these cell types are separated by intermediate stages of development in which L1 expression cannot be detected (Fig. 3). This may be the result of the unique regulatory mechanisms for gene expression possessed by these different cell types (27).

These distinct patterns of L1 expression, coupled with previous detection of multiple forms of ORF 1 protein (20, 22, 30), imply that multiple loci in the mouse genome are active for expression and are therefore potentially active for transposition. The developmental stage and cell type specificity of L1 expression can be attributed to mechanisms that regulate gene expression; these could be due either to intrinsic regulatory elements within the L1 structure, to the surrounding environment, or both. For example, insertion of elements next to different genomic enhancers could result in differential expression of L1. Another potential regulator of L1 expression is DNA methylation (28, 29). Regardless of which of these mechanisms are involved, the developmental stage- and cell type-specific patterns of expression have implications for L1 transposition and evolution. If an active element is inserted into a region of DNA facilitating its expression, the result could be a burst of cell type-specific transposition. Such an event in the germ line would have evolutionary ramifications: the element responsible for the burst of transposition would, as long as it remained active, dominate the evolution of L1.

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