Cell Type-specific Expression of LINE-1 Open Reading Frames 1 and 2 in Fetal and Adult Human Tissues*

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The LINE-1 (L1) family of non-long terminal repeat retrotransposons is a major force shaping mammalian genomes, and its members can alter the genome in many ways. Mutational analyses have shown that coexpression of functional proteins encoded by the two L1-specific open reading frames, ORF1 and ORF2, is an essential prerequisite for the propagation of L1 elements in the genome. However, all efforts to identify ORF2-encoded proteins have failed so far. Here, applying a novel antibody we report the presence of proteins encoded by ORF2 in a subset of cellular components of human male gonads. Immunohistochemical analyses revealed coexpression of ORF1 and ORF2 in spermatogonia of fetal testis, in germ cells of adult testis, and in distinct somatic cell types, such as Leydig, Sertoli, and vascular endothelial cells. Coexpression of both proteins in male germ cells is necessary for the observed genomic expansion of the number of L1 elements. Peptide mass fingerprinting analysis of a ~130-kDa polypeptide isolated from cultured human dermal microvascular endothelial cells led to the identification of ORF2-encoded peptides. An isolated ~45-kDa polypeptide was shown to derive from nonfunctional copies of ORF2 coding regions. The presence of both ORF1- and ORF2-encoded proteins in vascular endothelial cells and its apparent association with certain stages of differentiation and maturation of blood vessels may have functional relevance for vasculogenesis and/or angiogenesis.

LINE-1 (long interspersed nuclear element) retrotransposons (L1)§ cover about 17% of the human genome (1) and play a significant role in shaping the mammalian genome, not only through their own expansion but also through the mobilization of non-L1 sequences. Although the average haploid human genome harbors ~516,000 L1 copies, the subgroup of active L1s is fairly small, encompassing 80–100 elements (2, 3). So far, 82 retrotransposition-competent, full-length L1 elements were isolated and characterized (2–6). Remarkably, 84% of the retrotransposition capability of those elements was shown to be present in six highly active L1s (3). L1s affected the genome by (i) insertion of truncated L1s into new sites, (ii) intrachromosomal homologous recombination between L1s, (iii) transduction of 3′-flanking sequences during retrotransposition, (iv) aiding trans generation of processed pseudogenes and retrotransposition of Alu elements (7), and (v) by causing genome instability through substantial deletions (8, 9).

A retrotransposition-competent, functional L1 element (RC-L1) covers ~6.1 kb and contains a 5′-untranslated region with an internal, CpG-rich promoter, a 1-kb ORF1 encoding a protein (p40) of approximately 40 kDa with RNA binding capability, followed by a 3.8-kb ORF2 coding for a protein (p150) with a predicted molecular mass of about 150 kDa with endonuclease (EN) and reverse transcription (RT) activities and a cysteine-histidine-rich domain. The 3′-end of L1 is terminated by a short 3′-untranslated region and a poly(A) tail (10) (Fig. 1A). L1 mRNAs are atypical of mammalian RNAs because they are bicistronic, and the mechanism of translation of L1 is not understood. The two ORFs are in-frame and separated by a 63-bp noncoding spacer region. Mutational analyses demonstrated that both ORF1- and ORF2-encoded functional proteins are required for retrotransposition (4, 11). At least three functions of the ORF2-encoded protein were shown to be essential for retrotransposition, RT activity, EN activity, and a function associated with the cysteine-histidine-rich motif. Insertion of a new L1 copy into the loose genomic target sequence 5′-TTTT/TTT-3′ (8, 11–13) is initiated by a process termed target-induced reverse transcription (14, 15). The structure of the target site duplications flanking de novo L1 integrants suggests a model for second strand synthesis of L1 termed “microhomology-driven single strand annealing” (9, 16).

To date the only L1-specific translational product identified in human cells is encoded by ORF1. Human ORF1 protein (ORF1p) could be detected in the cytoplasm of a number of testicular germ cell tumors, in breast carcinoma, medulloblastoma, and a variety of transformed cell lines (17–20). The only
nonmalignant human tissue ORF1p has been detected in so far is the epithelium of normal mammary gland (17). ORF1p encoded by L1s in mice was verified in embryonal carcinoma cell lines, male and female germ cells, Leydig cells of embryonic testis, theca cells of adult ovary, and a large variety of transformed cell lines (21–23). However, all efforts to detect ORF2 proteins (ORF2p) in murine or human tissues using antibodies that detect baculovirus-produced or bacterially expressed ORF2p failed so far (10), and no direct evidence for in vivo translation of ORF2 has been provided hitherto.

Although it is clear that L1 retrotransposition events have occurred in somatic and germ cells (for review, see Ref. 10), only those events that take place in cells destined for the next generation are evolutionarily successful. In mammals these cells include primordial germ cells, germ cells, and early embryos. Thus, if L1 retrotransposition takes place in these cell types, functional proteins encoded by ORF1 and ORF2 are expected to be present because of their absolute requirement for L1 retrotransposition (4).

During human fetal development, primordial germ cells and supporting somatic cells, the precursor Sertoli cells, are enclosed in seminiferous cords, where primordial germ cells differentiate into prespermatogonia. These multiply by mitosis, arrest in the G0/G1 phase of the cell cycle, and subsequently enter another mitotic phase. Between embryonic weeks 10 and 22, primordial germ cells and all three types of prespermatogonias (mitotic, quiescent transitional, and mitotic transitional cells) appear side by side. After developmental week 26, only quiescent and mitotic prespermatogonia remain in fetal testis (24). In the seminiferous tubules of adult testis (Fig. 2), all intermediate cell types on the pathway to male gametes, like spermatogonia, spermatocytes, and spermatids, are present continuously throughout adulthood and can thus be viewed in single sections. Spermatogonia line the basal membrane of the seminiferous tubules. As germ cells undergo meiotic divisions and differentiation, they move toward the lumen, where mature spermatids are released. In the human testis, testosterone-producing Leydig cells and seminiferous tubules are connected by the microvasculature, which plays an essential role in the distribution of hormones and nutritional factors.

To explore the potential of functional human L1s to expand during germ cell development, we examined gonadal and other tissues at different developmental stages for the presence of proteins encoded by ORF1 and ORF2. To this aim we raised a polyclonal antibody against polypeptides encoded by the 5′-end of ORF2 and employed a previously generated antibody directed against the p40 protein (25). Coexpression of ORF1- and ORF2-encoded proteins was detected in fetal prespermatogonia, spermatocytes, immature spermatids, as well as in somatic cell types, such as Leydig, Sertoli, and vascular endothelial cells and placental syncytiotrophoblasts. Identified ORF2-en-
capped proteins were characterized by peptide mass fingerprinting analysis. ORF1- and ORF2-encoded proteins that were detected in fetal early meiotic cell types destined for the next generation may represent the enzymatic machinery for L1 retrotransposition and genomic expansion. The existence of both proteins in human vascular endothelial cells and the apparent association with differentiation and maturation of these cells may also have functional relevance for vasculogenesis and/or angiogenesis.

EXPERIMENTAL PROCEDURES

Tissue Samples—Human adult testicular tissues were obtained from 40–70-year-old patients orchietomized because of prostate cancer. Paraffin-embedded fetal tissues were provided by the Department of Pathology and Gynecopathology of the University Clinic Hamburg-Eppendorf, Research on adult and fetal human tissues was approved by the ethics committee of the institution.

Yeast Expression—To construct plasmid pCBW12 expressing a fusion of the L1-EN domain to the C terminus of the Gal4 DNA-binding domain (Gal4-DBD-EN), the DNA encoding L1-EN was inserted in-frame between NdeI and BamHI restriction sites of yeast expression vector pGBK7T (Clontech). The empty parental vector pGBK7T or pCBW12 was transfected into yeast AH109 cells to express Gal4-DBD fusion proteins. Growth and manipulation of the yeast strain and preparation of cell extracts were performed according to standard procedures (26).

Primary Antibodies—The well characterized antibody AH40.1 directed against L1 ORF1-encoded p40 protein was a gift from Thomas G. Fanning, Armed Forces Institute of Pathology, Washington, D. C. (19, 29). To raise antibodies detecting the EN domain of ORF2p, peptides 1420 and 1419, covering amino acids 48–63 and 152–166 of ORF2p, respectively (see Fig. 1, A and B), were chosen, based on algorithms that predict antigenic sites (Lasergene Software, DNASTAR). Numbering corresponds to the amino acid positions in the active L1.2 element (27). Two chickens were immunized commercially (Eurogentec) by three boost injections of each of the two peptides. IgY antibodies were prepared from egg yolks (28) and purified by affinity chromatography. For that purpose peptides 1420 and 1419 (2.5 mg each) were covalently coupled to 1 g of CNBr-activated Sepharose 4B (Amersham Biosciences). The gel was packed into a column and washed using standard methods. After equilibration with storage buffer (0.1 m phoshate buffer, pH 7.6, 0.1 m NaCl, 0.1% NaN<sub>3</sub>), the IgY fraction was passed over the column twice. Subsequently, the column was washed with storage buffer, and bound antibodies were eluted with 10 bed volumes of 100 mM glycine, pH 2.5, and mixed with 1 bed volume of 1 M phosphate buffer, pH 7.6, 0.1 M NaCl, 0.1% NaN<sub>3</sub>, the IgY fraction was passed over the column twice. Subsequently, the column was washed with storage buffer, and bound antibodies were eluted with 10 bed volumes of 100 mM glycine, pH 2.5, and mixed with 1 bed volume of 1 M Tris-HCl, pH 8.0. The affinity-purified antibody, referred to as anti-EN antibody at a dilution of 1:1,000 for 45 min, washed with PBS, and incubated with rabbit anti-chicken IgG horseradish-peroxidase and avidin-biotin complex methods enhanced by a modified nickel-enhanced glucose oxidase technique (29). To test for the specificity of the anti-EN antibody, peptides 1419 and 1420 were immobilized separately on CNBr-activated Sepharose 4B and preincubated over-night with the antibody at a ratio of 1:3 (12.5 ng of anti-EN antibody and 37.5 ng of peptides/ml). The supernatant of this mixture containing the peptide-depleted antibody was used for immunohistochemistry in control experiments. The following controls were performed in addition: (i) primary or secondary antibody was replaced by PBS, (ii) peroxidase reaction alone was visualized without antibody pretreatment. Preabsorption experiments reexamining the specificity of the AH40.1 antibody were performed as described previously (19). Immunostained sections were counterstained with Calcein Red and photographed with a Leica microscope equipped with a digital camera.

Human dermal microvascular endothelial (HDME) cells, derived from dermis (PromoCell, Heidelberg, Germany) were cultured to confluence on gelatin-coated dishes in Endothelial Cell Growth Medium VM (PromoCell) and fixed in 4% formaldehyde for 30 min. After washing with PBS, cells were incubated overnight at room temperature with anti-EN antibodies. The peroxidase reaction was carried out as described above.

Immunoblot Analysis—Purified L1-EN and cell extracts were separated on an SDS-12% polyacrylamide gel and electroblotted onto a Hybond-P membrane (Amersham Biosciences). After transfer, membranes were blocked in phosphate-buffered saline (PBS) containing 4% bovine serum albumin, incubated with the affinity-purified polyclonal antibody and/or angiogenesis.

RESULTS

Anti-EN Antibodies Directed against L1 ORF2p—To investigate human germ line tissue lines for expression of L1 ORF2p we raised antibodies against peptides within the N-terminal domain of ORF2p (Fig. 1, A and B). These anti-EN antibodies recognized the bacterially expressed EN domain (amino acids 1–25) with an apparent molecular mass of 28 kDa (13) (Fig. 1, C, lane A) and Baculovirus-produced 150-kDa ORF2p. As a negative control we used whole cell extracts from chicken embryo fibroblasts because the chicken genome does not contain LINE-1 sequences. The chicken-specific retrotransposon CR1 is only distantly related to mammalian L1 elements and differs greatly in the relevant ORF2 sequences (30, 31). As expected, the anti-EN antibody failed to detect any protein in these
proteins are expressed in various cell types expressing L1-encoded ORF2-proteins in the adult testis. The demonstration of expression pattern in the different cell types is described under “Detection of ORF1p and ORF2p in Adult Testicular Tissue” and documented in Fig. 3. Cell types expressing L1-encoded proteins are boxed.

To test the anti-EN antibody for its ability to detect native ORF2p in situ, we transfected the episomal retrotransposition reporter plasmid pJM101/L1RP carrying an RC-L1 element under the control of a heterologous promoter (5) into HeLa cells and performed immunofluorescence experiments (Fig. 1D).

Although HeLa cells that were transfected with the empty vector pCEP4 (Fig. 1, b, d, and f) as well as untransfected HeLa cells (data not shown) displayed only weak background immunoreactivity that may be explained by endogenous ORF2p, transfection with pJM101/L1RP resulted in 48 h, in much more intense immunostaining of the cytoplasm (Fig. 1, a and c) of a subset of cells. 38 days of hygromycin selection of pJM101/L1RP-transfected cells resulted in strong fluorescence of the cytoplasm of each cell in the culture (Fig. 1D, e). In contrast, pCEP4-transfected hygromycin-resistant cells exhibited only weak background activity (f) that is also observed in untransfected HeLa cells. Because pJM101/L1RP differs from pCEP4 exclusively by an active RC-L1, the data strongly suggest that the difference in immunoreactivity is caused by the interaction of the affinity-purified anti-EN antibody with overexpressed L1RP ORF2p. There was no immunoreactivity detectable, when pJM101/L1RP-transfected hygromycin-resistant cells were immunostained with peptide-depleted primary anti-EN antibody, which was generated by preabsorption with peptide 1419 and 1420 immobilized on Sepharose beads (Fig. 1D, h) or when the primary antibody was omitted (g).

To evaluate the possibility that cellular toxicity caused by overexpressed functional ORF2 proteins or by retrotransposition events, and not the detection of the ORF2 protein itself, might be responsible for the significant fluorescence in pJM101/L1RP-transfected cells, an inactive L1-reporter construct was transfected into HeLa cells, and immunostaining with the anti-EN antibody was performed (data not shown). Transfection and 38 days of hygromycin selection of the inactive L1.2A-reporter construct harboring a missense mutation in an endonuclease active site (H230A) (11) resulted in strong staining of each transfected cell (data not shown) similar to the staining observed after transfection of the active L1RP-reporter construct (e). Additionally, the generation time of the L1.2A mutant-expressing cells did not differ from pJM101/L1RP-transfected cells. Taken together, these results indicate that the detected fluorescence signals are a consequence of the specific interaction between anti-EN antibody and ORF2p and are not caused by cells dying of any toxic effect of L1-encoded proteins or deleterious L1 retrotransposition events.

Our finding that pCEP4-transfected and untransfected cells are stained weakly by the anti-ORF2 antibody (Fig. 1, b, d, and f) is consistent with previous results from Skowronska and Singer (32), who detected heterogeneously sized L1 transcripts in various human cell lines including HeLa (32). Translation of at least a subset of those L1-specific transcripts in HeLa could be demonstrated by Leibold and co-workers (25), detecting low amounts of reactive L1-ORF1p in the same cell line.

Detection of ORF1p and ORF2p in Adult Testicular Tissue—To explain the evolutionary genetics of L1, L1 retrotransposition is expected to occur during germ cell development. Therefore, we first looked for ORF2 expression in the adult testis (Figs. 2 and 3). Within the seminiferous tubules, extensive immunostaining with the anti-EN antibody was observed in the cytoplasm of Sertoli cells, in a subset of secondary spermatocytes and immature spermatids, as well as in some residual bodies (Fig. 3, A and D, and Table I). There was no immunoreactivity detectable in spermatogonia at the basis of the seminiferous tubules, primary spermatocytes, peritubular myofibroblasts, vascular smooth muscle cells, and interstitial tissue. Unexpectedly, strong immunostaining was also observed in some somatic cell types: in endothelial cells of capillaries and large blood vessels, in interstitial cells covering the seminiferous tubules and blood vessels (covering cells (33)), and in the cytoplasm of Leydig cells (Fig. 3, A, C, and D). There was no specific immunostaining when the anti-EN antibody was incubated with peptide 1420 immobilized on Sepharose beads prior to its use for immunohistochemistry (Fig. 3D). In contrast, specific immunostaining was not affected by either preabsorption of the anti-EN antibody with immobilized peptide 1419 (Fig. 3C) or preincubation with Sepharose beads alone (Fig. 3D). These results indicate that the anti-EN antibodies are directed predominantly against the epitope represented in peptide 1420.

Staining with antibodies to ORF1p also revealed immunoreactivity in the cytoplasm of Sertoli cells, in secondary spermatocytes, immature spermatids, residual bodies, and in Leydig cell nuclei (Fig. 2).
dig cells but not in spermatogonia (Fig. 3E). Furthermore, anti-ORF1 antibodies stained endothelial cells of blood vessels, although the fraction of immunoreactive blood vessels was reduced compared with staining with anti-EN antibodies. Serial control sections exposed to the secondary antibody alone (Fig. 3F) and to preabsorbed anti-ORF1 antibody (data not shown) did not exhibit any specific immunostaining. Taken together, these results indicate that the pattern of cell types in...
immunoprecipitated ORF2p by antibodies specific for the EN domain of L1RP ORF2p. Because of the high molecular mass of the amino acids (Fig. 4A), the 10 tryptic peptides identified match a protein with a predicted molecular mass of 150 kDa. Peptide mass fingerprinting analysis of the ~45-kDa polypeptide (Fig. 4B) identified five tryptic peptides (Fig. 5A), which matched those encoded by the 5’-truncated ORF2 of a genomic L1PA2 copy (GenBank accession no. S23650) described previously (34) (Fig. 5B). This L1 copy contains two nonsense mutations within the central region of ORF2 and several one-nucleotide deletions. Two of the identified tryptic peptides (Fig. 5; peaks 4 and 5) were identical in sequence to those from L1RP, ORF2p (Fig. 5), whereas the remaining three peptides (peaks 1–3) contained several point mutations as compared with L1RP ORF2. By searching the Ensembl human genome data base (proteinview, contigview (35)) these peptides identified two closely related 5’-truncated L1PA2 copies located on chromosomes 1p32 and 1p35. Both L1 copies have coding capacity for the identified 45-kDa polypeptide, and their sequences are consistent with the L1PA2 sequence described by Hohjoh and co-workers (34).

Because primary HDME cells represent the same cell type as adult testicular vascular endothelial cells which also stained positive for ORF2p (Fig. 3), we concluded that ORF2-encoded proteins identified by MALDI-TOF MS are also responsible for the ORF2 immunoreactivity observed in vascular endothelial cells in the tissue sections. However, given the high copy number of L1s in the genome we do not know whether L1-encoded proteins detected in other somatic cell types and in germ cells are the result of the products of the same expressed L1 elements.

To sum up it can be said that all data obtained in the described experiments argue for the specific interaction of our anti-EN antibody with ORF2-encoded proteins and against any cross-reaction with unspecific cellular proteins.

Detection of ORF1p and ORF2p in Fetal Testicular and Placental Tissues—To obtain insight into the temporal and spatial regulation of L1 expression in the germ line we examined testicular tissue of an 18- and a 28-week fetus (Fig. 6). Immunostaining with the anti-EN antibody was observed mainly in the cytoplasm of prespermatogonia as well as in Leydig cells (Fig. 6, A–C and Table I). L1 expression in prespermatogonia was also confirmed by the presence of ORF1p (Fig. 6D). Furthermore, endothelial cells of more mature blood vessels exhibited immunostaining for both ORF1p and ORF2p (Fig. 6A), whereas those of small and immature capillaries were negative. Other fetal testicular cell types like vascular smooth muscle cells, peritubular myoid cells, and cells of the interstitial connective tissue did not exhibit any immunoreactivity with the applied antibodies (Table I). No immunoreactivity was detected when the peptide-depleted anti-EN antibody was used (Fig. 6E). All somatic cell types of the testicular tissue that were immunoreactive in our study are characterized by the presence of receptors for the steroid hormones, androgen and/or progesterone (36). This finding led us to examine other fetal tissues that contain cell types that are major targets of those steroids, such as epididymis and placenta. We observed ORF1 and ORF2 immunoreactivity not only in the columnar epithelium of the epididymis (Fig. 6, F and G), but also in syncytiotrophoblasts and endothelial cells of placental blood vessels (Fig. 6, I and K). All three cell types display receptors for androgen or progesterone. In contrast, neither of the two L1-specific antibodies revealed any immunoreactivity in interstitial tissue, vascular smooth muscle cells, epididymal peritubular myoid

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### Table I

| Analyzed tissue/cell type       | Expression of ORF1p | Expression of ORF2p |
|--------------------------------|---------------------|---------------------|
| Testis                         |                     |                     |
| Adult (40–70 years)            | −                   | −                   |
| Spermatogonia                  | +                   | +                   |
| Primary spermatocytes          | +                   | +                   |
| Secondary spermatocytes        | +                   | +                   |
| Immature spermatids with       | +                   | +                   |
| residual bodies                |                     |                     |
| Sertoli cells                  | +                   | +                   |
| Leydig cells                   | +                   | +                   |
| Covering cells                 | +                   | +                   |
| Vascular endothelial cells     | +                   | +                   |
| Peritubular myofibroblasts     | −                   | −                   |
| Interstitial connective tissue | −                   | −                   |
| Vascular smooth muscle cells   | −                   | −                   |
| Fetal (18/28 weeks)            |                     |                     |
| Prespermatogonia (primordial   | +                   | +                   |
| germ cells)                    |                     |                     |
| Leydig cells                   | +                   | +                   |
| Vascular endothelial cells     | +                   | +                   |
| Peritubular cells              | −                   | −                   |
| Interstitial tissue            | −                   | −                   |
| Vascular smooth muscle cells   | −                   | −                   |
| Epididymis, fetal (28 weeks)   |                     |                     |
| Columnar epithelium            | +                   | +                   |
| Vascular endothelial cells     | +                   | +                   |
| Peritubular myoid cells        | −                   | −                   |
| Interstitial connective tissue | −                   | −                   |
| Vascular smooth muscle cells   | −                   | −                   |
| Placenta                       |                     |                     |
| Syncytiotrophoblasts           | +                   | +                   |
| Vascular endothelial cells     | +                   | +                   |
| Cytotrophoblasts               | −                   | −                   |
| Interstitial connective tissue | −                   | −                   |
| Vascular smooth muscle cells   | −                   | −                   |

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* S. Ergün and G. G. Schumann, unpublished results.

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cells, and placental cytotrophoblasts (Table I). Serial control sections incubated with the peptide-depleted anti-EN antibody (Fig. 6, H and L) or with preabsorbed anti-ORF1 antibody (data not shown) did not lead to any immunoreactivity.

**DISCUSSION**

Although an intact ORF2 was demonstrated to be essential for L1 retrotransposition (4, 11), all efforts to detect ORF2 proteins have failed so far (10). Here we have generated an antibody to polypeptides of the EN domain of human L1 ORF2p to discern ORF2p immunoreactivity in human adult and fetal cells, and placental cytotrophoblasts (Table I). Serial control sections incubated with the peptide-depleted anti-EN antibody (Fig. 6, H and L) or with preabsorbed anti-ORF1 antibody (data not shown) did not lead to any immunoreactivity.

**FIG. 4.** Immunoprecipitation and peptide mass fingerprinting analysis of ORF2 proteins. A, immunolocalization of ORF2p in HDME cells using anti-EN antibodies (+ anti-EN). As a control, immunoperoxidase reaction was performed without primary anti-EN antibodies (−anti-EN). Magnification, ×650. B, immunoprecipitation of ORF2p from HDME cell lysates. The immunoprecipitate with anti-EN antibodies obtained from HDME cell lysates was resolved by SDS-PAGE and visualized by silver staining (lane 2). As a control, immunoprecipitation was performed without adding cell lysate (lane 1). Two bands with apparent masses of ~130 and ~45 kDa appear specifically.

**FIG. 5.** Peptide mass fingerprinting analysis of the immunoprecipitated 45-kDa polypeptide. A, MALDI-TOF MS revealed five mass peaks (peaks 1–5) of tryptic peptides corresponding to those encoded by L1PA2 copies on chromosomes 1p32 and 1p35 (GenBank accession no. S23650). The table beneath lists the deviations (Delta) between observed (mass_a) and calculated (assigned_b) masses with corresponding amino acid sequences. Amino acid residues differing from the peptide sequence of the functional L1RP ORF2p (Fig. 4D) are shaded. Peaks without numbering result from contaminating keratin-specific tryptic peptides; T, peaks resulting from trypsin autodigestion; m/z, mass-to-charge ratio. B, graphical representation of the data base hits. Matched tryptic peptides within L1PA2 ORF2p (GenBank accession no. S23650) are shown. Amino acid position 2 of the presented peptide sequence corresponds to amino acid position 665 of the functional L1RP ORF2p shown in Fig. 4D.

**FIG. 6.** Peptide mass fingerprinting analysis of the immunoprecipitated 130-kDa polypeptide. A, MALDI-TOF MS of the trypsin-digested 130-kDa polypeptide revealed 10 mass peaks (peaks 1–10) of tryptic peptides identical in sequence to tryptic peptides from L1.3 and L1RP. Mass peaks resulting from trypsin autodigestion are marked (T). The table beneath lists the deviations (Delta) between observed (mass_a) and calculated (assigned_b) masses with corresponding identified amino acid sequences (Sequence). Peaks without numbering result from contaminating keratin-specific tryptic peptides; m/z, mass-to-charge ratio. B, graphical representation of the data base hits. Matched tryptic peptides within L1RP ORF2p (GenBank accession no. S65824) are shown in red.
Fig. 6. Immunolocalization of ORF1p and ORF2p in human fetal tissues. Testicular tissues from an 18-week fetus (A) and a 28-week fetus (B–D), and epididymal (F and G) and placental (I and K) tissue of a 28-week fetus were stained with anti-EN (A–C, F, and I) or AH40.1 (D, G, and K) antibody. Immunostaining of p40 and ORF2p is found in prespermatogonia (arrowheads in A–D), fetal Leydig cells (long arrows in A and C), and vascular endothelial cells (short arrow in A), in the columnar epithelium of the epididymis (arrowheads in F and G), and in syncytiotrophoblasts (long arrows) and vascular endothelial cells (short arrows) of the placenta (I and K). As controls, immunohistochemical staining of serial sections of fetal testicular (E), epididymal (H), and placental (L) tissue was performed with peptide-depleted anti-EN antibodies. Magnification, ×650 (A, B, and E–L) and ×1000 (C and D).
tissues. We report the presence of both ORF1 and ORF2 translation products at different stages of the human male germ cell development. Coexpression of both ORFs was observed in basally as well as centrally localized prespermatogonia of seminiferous cords during the early fetal period (week 18) and was also as well as centrally localized prespermatogonia of seminiferous cords. Immunostaining indicates that p40 (18) as well as ORF2 proteins are localized in the cytoplasm of L1 expressing cells (Fig. 6, A–D).

Immunostaining of adult testicular tissue uncovered translation products from both ORFs in spermatocytes and immature spermatids, but not in spermatogonia, the stem cells of the germinal epithelium (Figs. 2 and 3 and Table 1). Thus, L1 expression seems to be activated after quiescent spermatogonia differentiated into primary spermatocytes that are entering meiosis I. These results are consistent with the previous finding that both full-length L1 RNA and ORF1 protein are present in spermatocytes but absent in spermatogonia of prepuberal mouse testis (21). Taken together, our data imply that L1 proteins are only expressed in differentiating and proliferating cells of the germinal epithelium. Provided that these proteins are functional, their presence would fulfill the requirement for transposition in cells that spawn the next generation. This is the first observation of proteins encoded by both L1-specific ORFs in nonmalignant human germ line tissue.

Factors that determine germ line specificity of L1 transcription have not been identified, but it has been suggested that the SOX family of transcription factors and the ubiquitous nuclear transcription factor YY1 may be involved (37, 38). The interpretation of DNA methylation patterns by methyl-CpG-binding proteins was shown to be a further potent regulator of L1 expression (39). Interestingly, MeCP2, a major repressor of transcription of methylated L1s, is widely expressed in mammalian tissues, but undetectable in human⁴ as well as in murine germ cells (40).

This study also revealed ORF1 and ORF2 expression in a variety of somatic cells, specifically, Sertoli, Leydig, and testicular covering cells, epididymal columnar epithelial cells, placental syncytiotrophoblasts, and vascular endothelial cells. Notably, our results are in line with previous studies, demonstrating mouse L1-ORF1 expression in the cytoplasms of mouse embryonal spermatogonia, Leydig cells, placental syncytiotrophoblasts, and in spermatocytes and spermatids (21, 23). Because ORF1 immunoreactivity was detected in Leydig cells of the mouse testis and in theca cells of the mouse ovary, Trelogan and Martin (23) noted a possible correlation between L1 expression and the production of steroid hormones. Besides Leydig cells and placental syncytiotrophoblasts, we observed human L1 expression in several cell types not producing steroids. Thus, there is no direct hint for an interrelationship between L1 expression and steroid production in human tissues. Nevertheless, it is worth mentioning that all immunostained somatic cell types identified in our study exhibit receptors targeted by either androgen or progesterone (36). It remains to be studied whether there is a connection between the presence of these steroid receptors and the up-regulation of L1 expression in certain somatic cell types.

Although it has been known for several years that high expression of human endogenous retroviruses is one of the characteristics of syncytiotrophoblasts (41), it is quite intriguing that significant protein expression of L1s in human and mouse is demonstrated in the same specific cell type. Although evidence has recently accumulated suggesting that endogenous retroviral expression may be involved in mediating the cell fusion observed in the placenta (42), it is completely unclear whether the expressed L1-encoded proteins have any function for the host cell.

In contrast to previous findings by Brathauer and Fanning (19), we observed immunostaining of different cell types in nontransformed adult testicular tissue with the AH40.1 antibody. This can be explained by differences in the fixation and staining methods between the two laboratories. Although the previous study used formalin for fixation resulting in a reduced staining (19), our tissues were fixed in Bouin’s solution as part of a more sensitive method to retain antigenic sites (29).

In the adult testis ORF2p was observed in all vascular endothelial cells, whereas ORF1p staining was restricted to endothelial cells of the microvasculature. This could be explained either by a lower expression level of ORF1p relative to ORF2p or by a lower affinity of the AH40.1 antibody to its antigen compared with the anti-EN antibody. Our findings indicating high expression levels of ORF2p in endothelial cells of mature but not of immature blood vessels in developing fetal testicular tissue and in human placenta suggest increasing expression during maturation of these cells. If the identified L1 proteins were functional and retrotransposition could occur in these cells, this finding would be surprising because endothelial cells are considered as genetically stable. It is also unclear what the purpose of retrotransposition would be in these cells because somatic retrotransposition is an evolutionary dead end. A new insertion in a somatic cell cannot be passed onto the next generation, and, if the insertion harms the host, the precursor of the insertion might not be passed on. The high amount of L1-encoded proteins in quiescent vascular endothelial cells and their obvious accumulation accompanying endothelial maturation implicate a functional relevance of L1 in vasculogenesis and/or angiogenesis. Because targeting the endothelial cells is a major focus of anti-angiogenic tumor therapy (43) it is of great interest to find out if and how L1-encoded proteins influence the behavior of endothelial cells, particularly of those in tumors.

Another explanation for the detection of ORF2p alone without ORF1p is the expression of mutated or 5’-truncated L1 copies that express ORF2p but lack coding capacity for a functional ORF1p. Although elements harboring a disrupted ORF1 gene, but a functional ORF2 gene, are not able to promote either their own transposition (4) or the generation of processed pseudogenes (44), they should still be competent for the mobilization of Alu sequences (45). Thus, the adventitious synthesis of ORF2p from even truncated L1 elements may be a potential source of RT which could catalyze both somatic or germ line retroposition of SINE elements. Therefore, not only RC-L1s may constitute a genetic load to their host, but also L1 copies devoid of a functional ORF1. This hypothesis is supported by the recent observation that less than full-length L1 elements, at times, were under negative selection (46). It was suggested that some long (>500 bp) non-full-length L1 elements could produce L1 products, such as L1 RNA or an active RT, which could be deleterious and make them subjects to purifying selection (46).

Peptide mass fingerprinting analysis that followed protein immunoprecipitation with our anti-EN antibody uncovered two polypeptides in HDME cell lysates with estimated molecular masses of 45 and 130 kDa featuring sequence identity in a number of tryptic peptides to ORF2 proteins. Although all data obtained on the amino acid sequence of the ~130-kDa peptide are so far consistent with a functional ORF2p, it is still possible that the peptide is encoded by a nonfunctional L1 copy because our analysis by mass spectrometry covers only 13% of the
predicted ORF2p sequence. Also, none of the tryptic peptides identified by mass spectrometry is unique to the so-called “hot” RC-L1 elements (50) because they correspond to sequences that are highly conserved in L1 ORF2p and are also present in very much less active members of the Ta (L1PA1) family. They even differ only by a single amino acid substitution from the corresponding coding regions of the older now extinct L1PA2 and L1PA3 families (47). Therefore, instead of representing functional L1 activity the ~130-kDa polypeptide may represent the adventitious translational product of nonfunctional read-through transcripts. Peptide mass fingerprinting analysis of the ~45-kDa protein indicates that it derived from the mutated ORF2 of nonfunctional L1PA2 elements on chromosome 1p32 and 1p55 (34). Because zinc finger domains have recently been shown to mediate protein-protein interaction (48, 49), interaction of the ~45-kDa protein with the ~130-kDa protein through the cysteine-histidine-rich, putative zinc-knuckle domains at their C termini may account for the fact that our anti-EN antibody, directed against the N-terminal end of ORF2p, pulled down a ~45-kDa protein representing the C-terminal portion of the protein. If this is the case and the ~45-kDa protein does not interact with the anti-EN antibody directly, as suggested by our MS data, immunohistochemical staining of HDME cells (Fig. 4A) should be derived from the ~130-kDa ORF2 polypeptide. At this point we cannot exclude the possibility that other EN domain including ORF2-specific polypeptides are at least in part responsible for the immunohistochemical observed in tests, epididymis, and placenta.

The first indirect evidence for in vivo translation of ORF2p was provided by Maxine Sassaman’s laboratory using an L1 expression vector with the L1-ORF2p coding region replaced by a lacZ reporter cassette in the teratocarcinoma cell line NTera2D1 (50). After transfection of the L1 reporter construct, β-galactosidase-expressing cells were detected, indicating ORF2 was translated. Although the expression of ORF2 was shown to be enhanced when the translation of ORF1 is decreased, the absence of ORF1 translation led to a decrease in the expression of ORF2. The authors presented several pieces of evidence indicating that the translation of L1Hs ORF2 is most likely to be initiated internally. It was suggested that translation of ORF2 may occur by some form of internal ribosomal entry site or by ribosomal shunting (10, 50). The fact that we identified exclusively ORF2- and not ORF1-specific peptides in our peptide mass fingerprinting analyses is consistent with the assumption that ORF1 and ORF2 are translated separately (50). Also, Ilves et al. (51) provided evidence that ORF2 of the rodent L1 is translated by reinitiation or internal initiation rather than frameshifting, even though, in contrast to L1Hs, the two ORFs overlap and are out of frame. Our data also show that immunoprecipitation combined with peptide mass fingerprinting analysis is a valuable tool to investigate the expression status of replication-competent and mutated, inactive L1 elements in individual cell types. Further investigation will fruitfully complement the genetic studies on L1 composition of the human genome.

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