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A Novel Transcript Encoding Truncated LIM Kinase 2 Is Specifically Expressed in Male Germ Cells Undergoing Meiosis

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LIM kinases, composed of LIMK1 and LIMK2, have unique structural features that contain two LIM motifs at the N-terminus and a catalytic domain at the C-terminus. We report evidence of a novel type of mouse LIMK2 (Limk2) transcript specifically expressed in testis. cDNA cloning showed this Limk2 variant, designated tLimk2, lacked LIM domains at the N-terminus, due to usage of a testis-specific, alternative initiation exon. In Northern blot analysis, tLimk2 was detected in intact adult testis, but not in germ-cell-deficient or immature testis, indicating the stage-specific expression of tLimk2 in spermatogenic cells. *In situ* hybridization clearly demonstrated that tLimk2 was restrictedly expressed in differentiated germ cells (pachytene spermatocytes to round spermatids) and not expressed in early stages of spermatogenic cells and somatic cells in testis. These results suggested the possibility that the tLimk2 product is involved in spermatogenesis, especially in meiotic and/or postmeiotic processes. © 1998 Academic Press

Protein phosphorylation is a common posttranslational protein modification and a fundamental mechanism for regulating almost all cellular events, including control of cell division, differentiation, metabolic control, cell to cell or cell to matrix interaction, and signal transduction. For fulfill these various functions, many protein kinases are needed and it has been postulated that over 1,000 kinases may exist in mammals. Conversely, yet to be identified kinases appear to be involved in a complex network system of signal transduction and in many physiological function in cells. LIM-kinase (LIMK), which was originally cloned by our group (1), is a dual specificity (serine/threonine and tyrosine) kinase that has unique structural features in that it is composed of two tandemly arrayed LIM domains at the N-terminal, a protein kinase domain at the C-terminal, and PDZ domain at the interposing region. The protein kinase domain shares highly conserved amino acid residues of known protein kinases, but the overall domain structure is not closely related to other members of the protein kinase family. The LIM domain is defined as double zinc-finger motif with a cysteine-rich consensus sequence (2, 3). The LIM-motif has been noted in a variety of cytoplasmic and nuclear proteins, which contribute to diverse and important biological functions, such as contact-dependent cell growth, cell fate determination and differentiation, and embryonic development (For review, see [3]). Both LIM and PDZ motifs are known to be modular functional units mediating specific protein-protein interactions (2-4).

In human, rat, and mouse, two types of related but distinct LIMKs, named as LIMK1 and LIMK2, have been identified (1, 5-10). LIMK1 and LIMK2 share about 70 % amino acid identity in kinase domains, and the overall genomic organization (exon-intron structure) is similar (5-7, 11). We also cloned a putative LIMK2 homologue gene in chicken (12), and LIMK1 (13) and LIMK2 (our unpublished data) orthologues in *Xenopus,* hence the important and essential role of LIMKs extends to various species. Since LIMK1 mRNA is predominantly expressed in brain and spinal cord (8-10), an important role in neural development and function has been suggested. In human, the hemizygosity of LIMK1 is implicated in impaired visuospatial constructive cognition in patients with Williams syndrome (14). Furthermore, LIMK1 is strongly expressed in developing neural tissues, such as brain, spinal cord, and anlagens of sensory organs in murine (15) and *Xenopus* (13) embryos, and overexpression of LIMK1 induce impaired neural differentiation of PC12 cells (16).

On the other hand, the biological significance of

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LIMK2 is not well understood. In Northern blot analysis, LIMK2 mRNA is ubiquitously expressed in various organs in mammals (5-7). At least, two alternative transcripts of LIMK2 gene exist in mammals (6, 7, 17); the ordinary transcript, LIMK2a, encodes a protein containing two LIM domains, but another one, LIMK2b, has only one and half LIM domains. LIMK2a protein is distributed in both the cytoplasm and the nucleus, but LIMK2b protein is sparse in the nucleus (17), suggesting that LIM domains are essential for proper regulation of LIMK2 activity and function. Furthermore, LIMK2b mRNA is predominantly expressed in brain (7). In addition, we recently found that another LIMK2 transcript was specifically expressed in testis (7). In the present study, we have identified this novel isoform of mouse LIMK2 gene transcripts. This variant, designated tLimk2, which lacks the whole LIM domains, is restrictedly expressed in differentiated testicular germ cells, suggesting the possible involvement of LIMK2 in regulation of spermatogenesis, especially in meiotic and/or post-meiotic processes.

MATERIALS AND METHODS

Cloning. For cDNA cloning of the testis-specific Limk2 variant, mouse LIMK2 (Limk2) cDNA fragment (corresponding to 321-1748 bp [7]) was labeled with [α-^{32}P] dCTP and used as a probe. Approximately 2×10^5 independent colonies of mouse testis cDNA library (18) were screened. Screening and cloning of mouse Limk2 genomic clones have previouly described in detail (7). Clones were subsequently sequenced by the dideoxy termination method and the following analysis was performed using DNASIS software (Hitachi Software Engineering).

Northern blot hybridization. Poly-A$^+$ RNA was prepared from various mouse tissues by standard protocol, electrophoresed, transferred onto nylon membranes, and then hybridized with the ^{32}P-labeled cDNA fragments of mouse Limk1 (corresponding to 204-809 bp [8]) or Limk2 (described above). Filters were washed under high stringency condition and subjected to autoradiography.

Antibody and immunoblotting. To prepare LIMK2-specific antiserum, a peptide corresponding to the C-terminal sequence of rat LIMK2 (amino acid residues of 623-638 [6]), which is completely the same as that of mouse Limk2 (7), was conjugated to keyhole limpet hemocyanin. Rabbits were first injected subcutaneously with the peptide mixed with Freund's complete adjuvant, followed by 6 times inoculations with the peptide conjugated with Freund's incomplete adjuvant at 1-month intervals. The IgG fraction was purified and the anti-LIMK2 antibody was affinity purified on a column of Tresyl-activated Sepharose 4B coupled to the antigenic peptide.

Cell suspensions were prepared from mouse testis and thymus, incubated in lysis buffer, and sonicated. Cell lysates were electrophoresed, blotted onto membrane, and then incubated with antibody. Reactive molecules were detected by using ECL enhanced chemiluminescence system (Amersham), according to manufacture's recommendation.

In situ hybridization. Freshly dissected testes were fixed in Bouin's solution, embedded in paraffin, and sectioned at 10 μm of thickness. Two mouse Limk2 cDNA fragments (K2L and K2PK, corresponding to 38-667 and 321-1748 bp, respectively [7]), which were subcloned into pBluescript SK(−), served as templates for *in vitro* transcription. *In situ* hybridization was essentially carried out as described previously (19). Briefly, hybridization was done using 1×10^6 cpm/slide of probe at 55°C for 16 hr. The slides were then washed under conditions of high stringency, dehydrated in a graded series of ethanol, and air-dried. Sections were coated with Kodak NTB-2 emulsion (Eastman Kodak Co.) and exposed for 3 weeks.

RESULTS

Identification of testis-specific Limk2 transcripts and products. In our previous study (7), we noted the presence of a novel Limk2 variant expressed in testis. By Northern blot analysis, a 3.5 kb major band of Limk2 mRNA was ubiquitously detected in all tissues examined, except for the testis (Fig. 1A). The 1.7 kb band was strongly expressed in testis, and it was never found in other tissues examined, through early stage of embryo (8 d.p.c.) to adult mice. The smaller transcripts were detected using the C-terminal region of Limk2 cDNA as a probe, but not when using the N-terminal (data not shown), suggesting that the testis-specific transcript is a variant, N-terminal truncated form of Limk2. As for Limk1, a 3.3 Kb band was apparently expressed in testis, as well as in brain, thymus, lung, and spleen (Fig. 1A).

By immunoblot analysis, in thymus, where intact size of Limk2 mRNA was expressed, the Limk2 product was detected as a major band of 72 kD (Fig. 1B), consistent with reported data (5). On the other hand, a specific band with estimated molecular mass of 55 kD was predominantly detected in testis. This smaller sized molecule was confirmed to be a Limk2 gene product, since preincubation of anti-LIMK2 antibody with an excess amount of peptide immunogen led to disappearance of the 55 kD band, as well as the 72 kD product (data not shown).

To identify cDNA encoding the testis-specific Limk2 variant, an adult mouse testis cDNA library was screened using a kinase region of Limk2 cDNA as a probe. Several positive clones, including the possible entire coding region, were isolated and most were identical to that of the mouse Limk2 cDNA (7). However, they had unique 89 bp of nucleotides in their 5′-terminal, which was not comparable to ordinary Limk2 cDNA (Fig. 2 A). It appeared that testis-specific Limk2 transcripts truncated most of the N-terminal half structures, including two tandemly repeated LIM domains and the subsequent PDZ domain (Fig. 2B). Therefore, we designated this variant as ***t***estis-specific, ***t***runcated Limk2, " tLimk2" hereafter. In tLimk2, the length of 3′-untranslated region (UTR) was much shorter than that of intact Limk2 (265 bp vs. 1476 bp). The total number of nucleotides in tLimk2 was 1717 bp, being consistent with size of the testis-specific transcripts detected by Northern blot analysis (Fig. 1A). As shown, there was only one potential ATG translational initiation codon upstream of a single, long open reading frame (451 amino acids). Although the apparent Kozak consensus sequence (20) was not confirmed, we num-

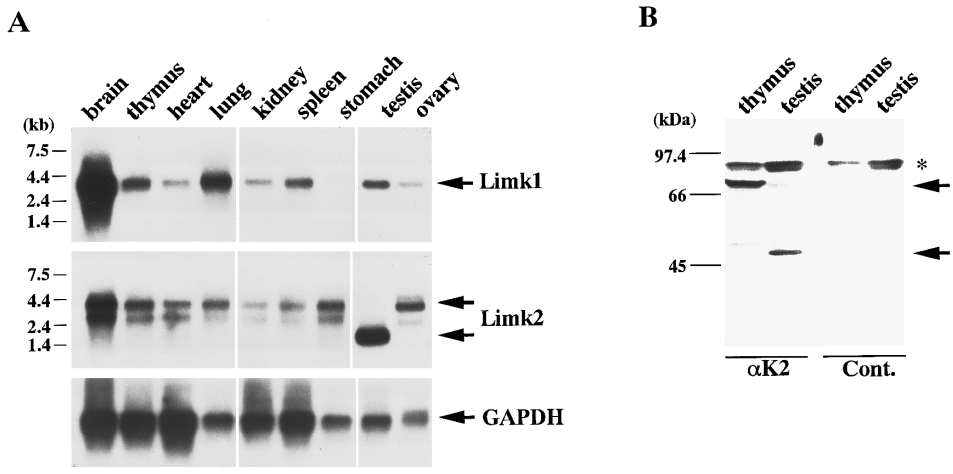

FIG. 1. Molecular detection of a novel, testis-specific isoform of mouse Limk2. (A) Expression of Limk2 mRNAs in adult mouse tissues. Poly(A)$^+$ RNA (2 μg each lane) from various tissues were subjected to Northern blot analysis, using the ^{32}P-labeled fragment of mouse Limk1 and Limk2 cDNA (top and middle panels, respectively). The blot was also hybridized with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe to establish the amount of RNA loaded (bottom panel). Positions of molecular weight markers are indicated to the left. (B) Detection of Limk2 products in testis and thymus. Cell lysates were subjected to immunoblotting, using anti-LIMK2 IgG (αK2) or control IgG (Cont.), respectively. *Nonspecific signals. Molecular mass (kDa) of marker proteins are indicated to the left.

bered the first ATG as a start codon (+1), since there was an in-frame stop codon upstream of the ATG, and the predicted size of tLimk2 product encoded by 451 amino acids was in good accord with the molecular mass of tLimk2 protein determined by immunoblotting (Fig. 1B).

Identification of genomic unit generating tLimk2 transcripts. We recently reported the genomic organization of the mouse Limk2 gene (7). The position of sequence divergence between Limk2 and tLimk2 cDNA (Fig. 2A) coincides with the junction of exons 5 and 6. Precisely sequenced, the region corresponding to the 5′-terminal sequence specific for tLimk2 cDNA was found in the intron region between exons 5 and 6 (Fig. 3A). Since this exon was considered to be an initiator exon in tLimk2 transcription, we designated it "exon 1t". Exon 1t can be accepted into exon 6 and sequences at exon-intron boundaries fit with the donor/acceptor splicing GT-AG rule (Fig. 3B) (21).

Fig. 3B shows the upstream and downstream sequence of exon 1t. Although this region did not contain the TATA or CAAT motif, we did find a consensus motif for Sp1-like binding site (GGAGGG), characteristic for many TATA-less promoters (22). The presence of Sp1-motif located closely proximal to putative exon 1t suggested that the region near here contained a transcription initiation site. Preliminary, our primer extension analysis also supported this notion (data not shown). We also identified several other consensus sequences for transcription factor binding sites in the promoter region of the tLimk2, such as GATA1 (23), Sox5 (24), and Ets1 (25).

Expression of tLimk2 transcripts in testicular germ cells. To determine if tLimk2 was expressed in germ cells or somatic cells, we examined expression of Limk2/tLimk2 mRNA in W/W^v, jsd/jsd, and cryptorchid testes, which were devoid of differentiated germ cells (26-28). In these agametic testes, tLimk2 mRNA was not detected at all (Fig. 4A). We then examined Limk2/tLimk2 mRNA expression in prepubertal mouse testes. The infantile first wave of spermatogenesis is well characterized by accumulation of germ cells at a progressively more advanced stages (29). As shown in Fig. 4B, the intact size of Limk2 mRNA was strongly expressed in testis of 12 days. This size of transcripts gradually decreased during development and could hardly be detected on 60 days. On the other hand, tLimk2 was first faintly expressed in testis of 20 days, then the expression drastically increased. At 30 days, tLimk2 was predominantly expressed, and in adult mice, ordinary Limk2 was scarcely evident. These results suggested that tLimk2 may be expressed in later stages of testicular germ cells.

To determine the germ cell types in which tLimk2 mRNA is expressed more precisely, *in situ* hybridization analysis was performed. Since the tLimk2-specific cDNA region was as short as 90 bp in length, we used subtractive cDNA fragments as probes; one of which (K2PK) contained a kinase domain and thus can recognize both intact-type Limk2 and tLimk2 mRNA, and another (K2L) encoding LIM domains and can hybridize with only intact-type Limk2. Using these probes, essentially the same hybridized signals were confirmed in embryonic tissues (data not shown). When hybridized with the K2L antisense probe, signals were rarely observed in adult mouse testis (Fig. 5B). On the other hand, strong signals were detected in ring-like layers of germ cells within the seminiferous tubules, when

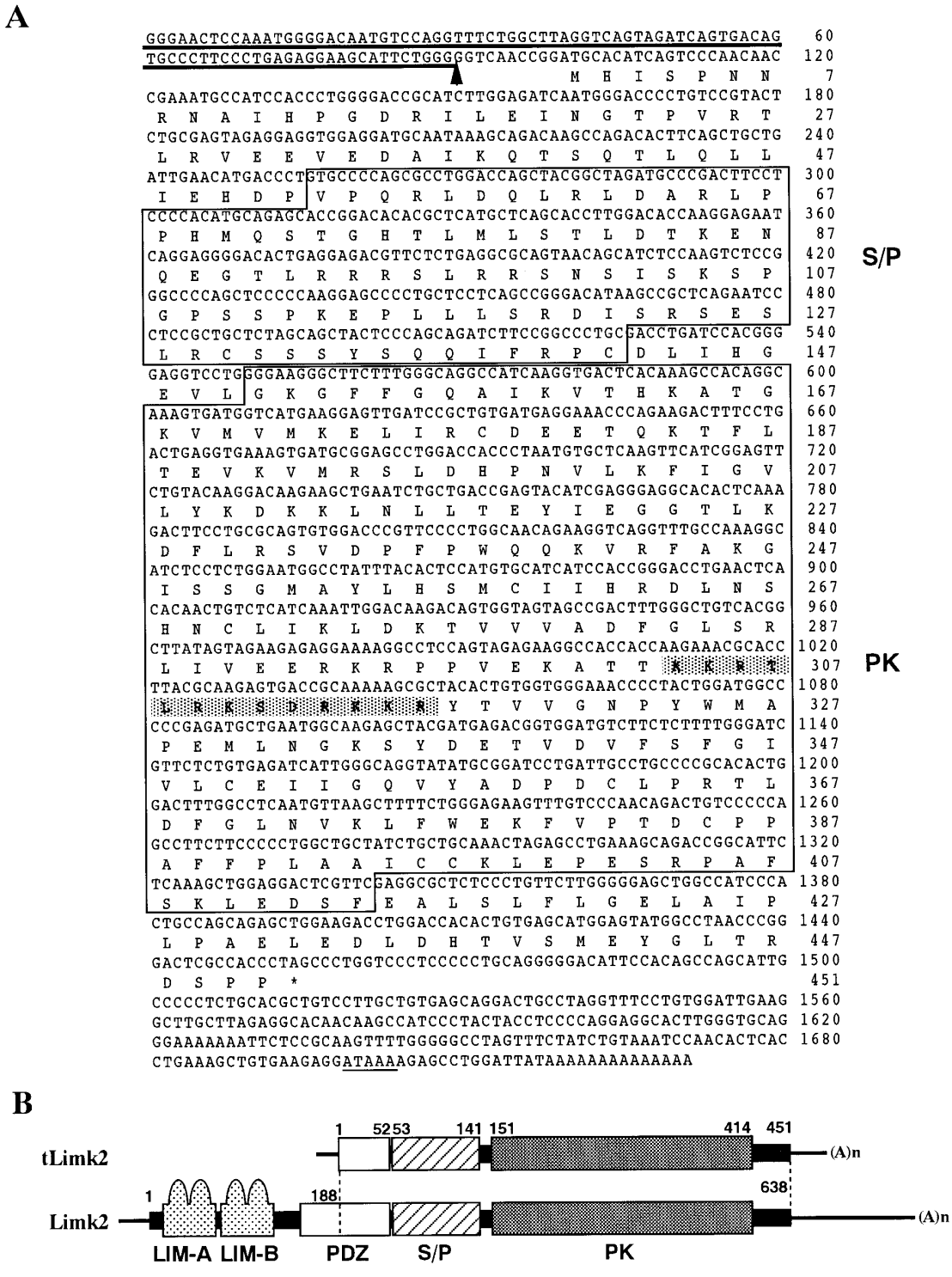

FIG. 2. Nucleotide and deduced amino acid sequences of mouse tLimk2 cDNA. (A) The nucleotides and deduced amino acids are numbered to the right. A serine/proline-rich region (S/P) and a protein kinase domain (PK) are boxed. A basic kinase insert, a putative nuclear localization signal, is shaded. A bold line indicates sequences specific for tLimk2 cDNA. An arrowhead indicates the junctional position of nucleotide substitution between Limk2 and tLimk2 cDNA. Positions of the putative polyadenylation signal in the 3′-UTR are underlined. The cDNA sequence has been submitted to the DDBJ/EMBL/GenBank DNA databases under Accession No. AB012291. (B) Schematic representation of structures of tLimk2, comparable to that of Limk2. Residue numbers of each product are shown. Thin lines are indicated the 5′- and 3′-UTR.

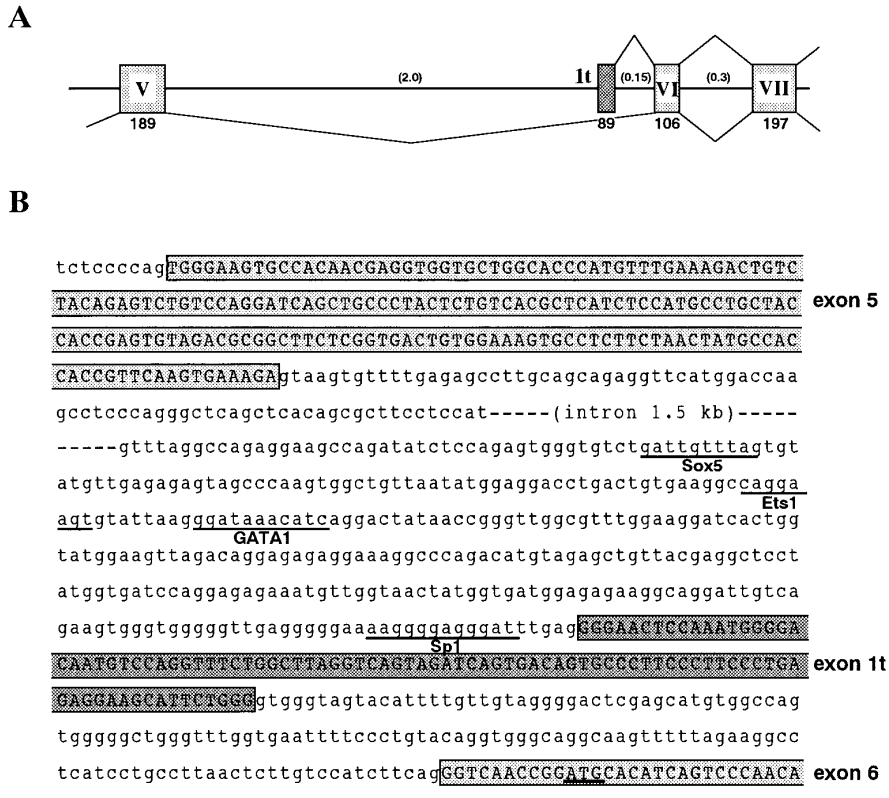

FIG. 3. Different exon usage between Limk2 and tLimk2 transcripts. (A) Partial genomic organization of the mouse Limk2 gene. Predicted splicing patterns for ordinary Limk2 and tLimk2 transcripts are indicated. Exons and introns are represented as numbered boxes and lines, respectively. The numbers of nucleotides in each exons are also indicated. The intron sizes (kb) are in the parentheses. (B) The nucleotide sequence of the surrounding region of exon 1t. A putative translation start site is underlined. Consensus binding motifs for Sox5, Ets1, GATA1, and Sp1 were identified during a computer search against the Transcription Factor Database (TRANSFAC). The DNA sequence has been submitted to the DDBJ/EMBL/GenBank DNA databases under Accession No. AB012292.

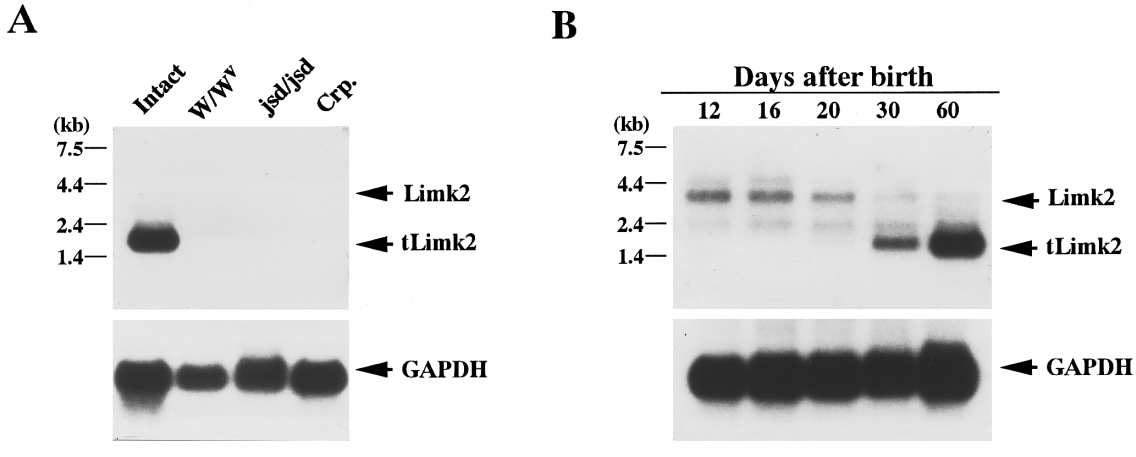

FIG. 4. Expression of tLimk2 mRNA in agametic and prepubertal mouse testes. Poly(A)$^+$ RNA (2 μg each lane) prepared from agametic testes (A) and various stages of mice testes (B) were subjected to Northern blot analysis using the ^{32}P-labeled fragment of mouse Limk2 cDNA. The blot was also hybridized with GAPDH probe to establish the amount of RNA loaded (bottom panel). Positions of molecular weight markers are indicated to the left.

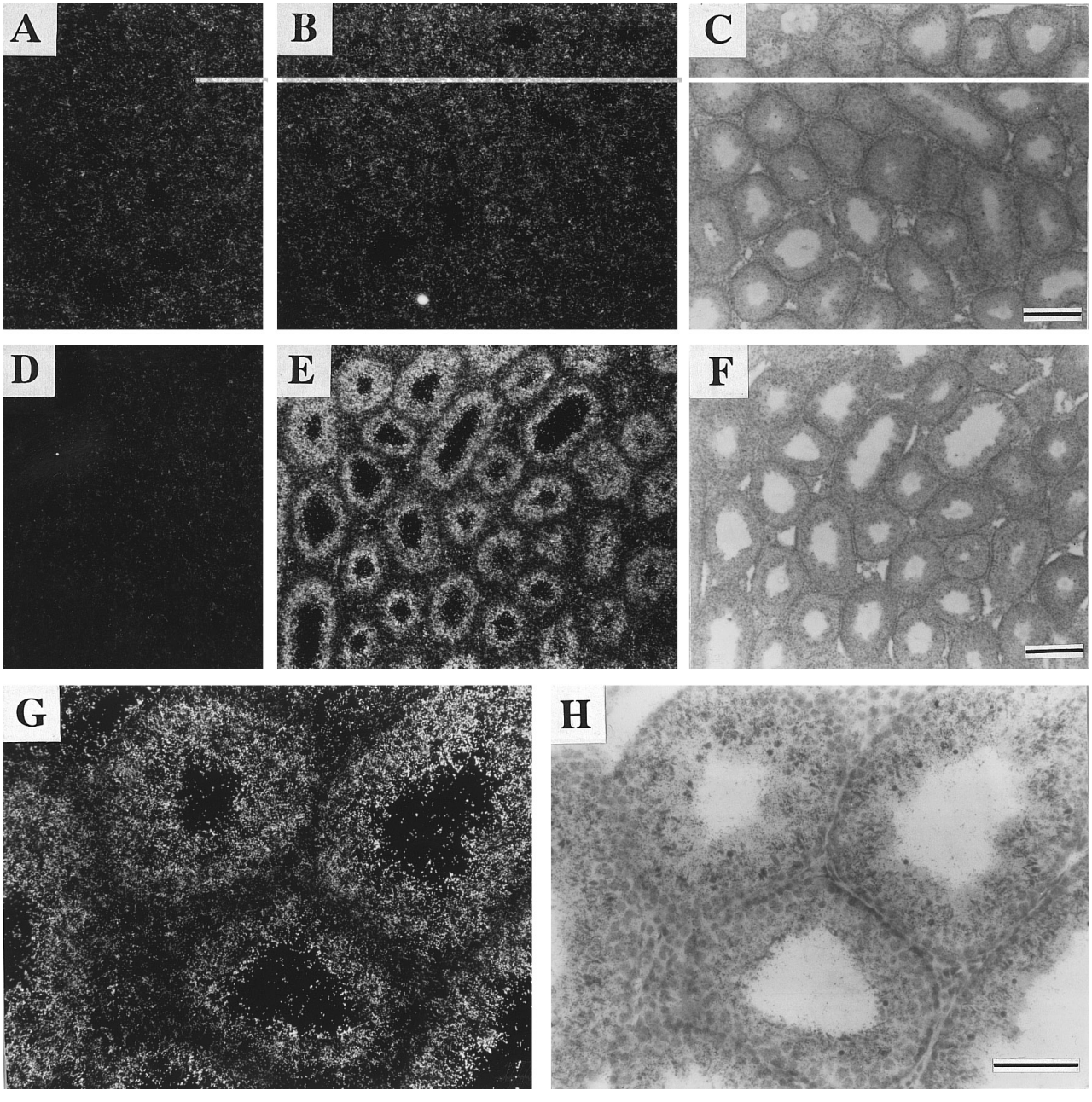

FIG. 5. Distribution of tLimk2 mRNA in mouse testis. The sections of adult mouse testis hybridized with riboprobes (A: K2L/sense; B,C: K2L/antisense; D: K2PK/sense; E-H: K2PK/antisense) are shown. C, F, and H are the corresponding bright-field images to B, E, and G, respectively. Bar, 150 μm in C and F, and 50 μm in H. In G and H, note that specific signals were not detected in cells located at the interstitial or peripheral regions of the seminiferous tubules.

we used the K2PK riboprobe (Fig. 5E). These findings indicat that tLimk2 mRNA, but not intact-type Limk2 mRNA, was specifically expressed in testicular germ cells of adult mice. Precisely observed, tLimk2 mRNA was detected in differentiated germ cells (Pachytene spermatocytes to round spermatids) in all seminiferous tubules, whereas tLimk2 were not detected at the periphery of seminiferous tubules, which contained spermatogonia, early spermatocytes, and Sertoli cells (Fig. 5G, H). These results are in good accord with data on Northern blot analysis (Fig. 4A).

DISCUSSION

LIMKs are the only known LIM-family molecules containing kinase or other catalytic domains. Based on their unique structure and biochemical characteristics, the possible involvement of LIMKs in a crucial, and

maybe a novel intracellular signaling pathway has to be considered. Available evidence suggests that the important role of LIMKs in neural development and function (See, Introduction). However, yet much less is known of the biological significance of LIMKs in various organs and tissues. We therefore examined the expression of LIMKs in mouse embryo and adult organs, and we found that a novel type of Limk2 transcript with a different mRNA size was specifically expressed in testis. cDNA cloning showed that this Limk2 variant, designated tLimk2, lacked most of the N-terminal half of Limk2 and two LIM domains were truncated (Fig. 2).

Northern blot (Fig. 4) and *in situ* hybridization (Fig. 5) analyses clearly demonstrated that tLimk2 mRNA is expressed in differentiated testicular germ cells, i.e. Pachytene spermatocytes to round spermatids, not in early stages of germ cells and somatic cells (Sertoli cells, Leydig cells, and so on). Spermatogenesis, a unique and complex process of germ cell differentiation in testis, is accompanied by extensive morphological changes together with increasing complexity and diversification of cellular constituents. In the meiotic and spermiogenic phase of germ cells, stage-specific expressions of a variety of genes and their products are well known (30-32). The expression pattern of tLimk2 is similar to other testis-specific or relatively highly expressed kinases, such as the phosphoglycerate kinase (PGK) -2 (33), serine/ threonine kinase c-MOS (34) and MAK (35), and the dual specificity kinase NEK-1/2 (36). Considering not only their expression pattern but also structural features and substrate specificities, the possible involvement of these kinases in meiotic and postmeiotic processes has been suggested. However, we have now little evidence showing their functional significance during spermatogenesis. Thus, tLimk2 could be an alternative, useful tool for studying the molecular mechanism of meiosis and spermiogenesis in vertebrates.

Generation of different mRNAs from a single genomic transcription unit has been observed in a variety of testis-specific genes (30-32). Such multiple transcripts can be generated by independent promoters, or differential processing of precursor RNA. Indeed, a structural difference between Limk2 and tLimk2 cDNA is that 3′-UTR of tLimk2 is much shorter than that of intact Limk2 (Fig. 2), perhaps related to differential RNA processing. However, we find that generation of testis-specific tLimk2 transcripts and products mainly results from differential splicing. It appears that N-terminal tLimk2-specific cDNA sequences extending from 1 to 89 bp locate in the interposing region between exons 5 and 6 of the mouse Limk2 locus (as exon 1t), and the site of divergence between Limk2 and tLimk2 also corresponds to an intron-exon junction of exon 6 (Figs. 2 and 3), which means that tLimk2 cDNA is generated by a different promoter and initiation exon usage. A similar testis-specific splicing pattern was noted in tyrosine kinase fer/ferT (37). ferT, testis-specific transcript of fer, is also generated by an alternative splicing mode through utilization of a different promoter, and the product contains a unique N-terminal domain that is absent from fer protein. Another example for male germ cell-specific splicing is a gene for the cAMP-responsive transcriptional factor CREB (38). Alternative splicing of an additional exon into the CREB mRNA in Pachytene spermatocytes results in a translational switch from an mRNA encoding intact CREB (activator) to an mRNA encoding novel inhibitor CREB isoform (repressor).

As a result of specific splicing, the tLimk2 products are likely to lack two tandemly arrayed LIM domains. In some LIM-family molecules, such as Xlim-1 (39), LIM domains interact with their homeodomain intramolecularly and to inhibit DNA-binding. This inhibition by the LIM domain is released by association of the LIM domain with the activator protein Ldb1/NLI (40, 41). As for LIMK1, overexpression of the LIM domain in PC12 cells inhibited activated Ras-induced neuronal differentiation, thereby suggesting the LIM domains of LIMK1 also acts as a functional repressor (16). It may be that the LIM domain of Limk2 protein interacts with the kinase domain to regulate catalytic activity. Furthermore, it was reported that truncation of the LIM domain of human LIMK2 led to accumulation of products in the nucleus (17). Accordingly, it could be that tLimk2 protein is, due to loss of the LIM domain, constitutively active and exclusively acts in the nucleus of spermatogenic cells.

In conclusion, our data demonstrate that the Limk2 variant form lacking LIM domains, tLimk2, is highly exressed in male germ cells undergoing and after meiosis. This finding will be useful to elucidate the relationship between structure and function of Limk2 kinase. Our ongoing studies will examine the functional importance of tLimk2 in spermatogenesis, and for this, biochemical characterization of tLimk2 protein, identification of cellular substrates, and *in vivo* disruption of tLimk2 function using transgenic mice or knockout mice strategy will be needed. Such analyses will shed light on not only the role of tLimk2 protein in the differentiation process of spermatogenic lineage, but also on common cellular functions of the Limk2 product in other tissues and organs.

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