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Gene 267 (2001) 243–253

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

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The mouse lp_{A3}/*Edg7* lysophosphatidic acid receptor gene: genomic structure, chromosomal localization, and expression pattern

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Received 8 December 2000; received in revised form 2 February 2001; accepted 27 February 2001
Received by A. Dugaiczyk

Abstract

The extracellular signaling molecule, lysophosphatidic acid (LPA), mediates proliferative and morphological effects on cells and has been proposed to be involved in several biological processes including neuronal development, wound healing, and cancer progression. Three mammalian G protein-coupled receptors, encoded by genes designated *lp* (lysophospholipid) receptor or *edg* (endothelial differentiation gene), mediate the effects of LPA, activating similar (e.g. Ca^{2+} release) as well as distinct (neurite retraction) responses. To understand the evolution and function of LPA receptor genes, we characterized lp_{A3}/*Edg7* in mouse and human and compared the expression pattern with the other two known LPA receptor genes (lp_{A1}/*Edg2* and lp_{A2}/*Edg4non-mutant*). We found mouse and human lp_{A3} to have nearly identical three-exon genomic structures, with introns upstream of the coding region for transmembrane domain (TMD) I and within the coding region for TMD VI. This structure is similar to lp_{A1} and lp_{A2}, indicating a common ancestral gene with two introns. We localized mouse lp_{A3} to distal Chromosome 3 near the varitint waddler (*Va*) gene, in a region syntenic with the human lp_{A3} chromosomal location (1p22.3-31.1). We found highest expression levels of each of the three LPA receptor genes in adult mouse testes, relatively high expression levels of lp_{A2} and lp_{A3} in kidney, and moderate expression of lp_{A2} and lp_{A3} in lung. All lp_A transcripts were expressed during brain development, with lp_{A1} and lp_{A2} transcripts expressed during the embryonic neurogenic period, and lp_{A3} transcript during the early postnatal period. Our results indicate both overlapping as well as distinct functions of lp_{A1}, lp_{A2}, and lp_{A3}.

Keywords: Lysophospholipid; vzg-1; Development; G protein-coupled receptor; Polymorphism

1. Introduction

Lysophosphatidic acid (LPA) was first recognized as a component of serum that induces increased proliferation and stress fiber formation on fibroblast cells (Moolenaar et al., 1986; van Corven et al., 1989; Ridley and Hall, 1992). Later, LPA was shown to induce proliferative and morphological changes on numerous other types of cells, such as neuroblasts (Jalink et al., 1993; Hecht et al., 1996; Fukushima et al., 1998), kidney mesangial cells (Gaits et al., 1997), and spermatids (Garbi et al., 1999). These effects are mediated through the activation of specific G protein-coupled receptors (GPCRs) (Hecht et al., 1996; Fukushima et al., 1998; Ishii et al., 2000; reviewed in Contos et al., 2000b). The first identified LPA receptor (encoded by lp_{A1}/*vzg-1/Edg2/Gpcr26*), cloned from a mouse embryonic cerebral cortical cell line (Hecht et al., 1996), mediated neurite retraction and increased proliferation (Fukushima et al., 1998; Ishii et al., 2000). Unlike many other GPCR genes, which are intronless, mouse lp_{A1} contains introns upstream of transmembrane domain (TMD) I and within TMD VI (Contos and Chun, 1998). The gene is localized at central Chromosome 4 near vacillans (*vc*), although in some mouse strains, part of the gene is duplicated on Chromosome 6 (Contos and Chun, 1998). Recent analysis of lp_{A1} knockout mice demonstrates multiple developmental functions for this gene and suggests that null mutations in lp_{A1} may have contributed to the *vc* phenotype (Contos et al., 2000a).

A second LPA receptor gene (lp_{A2}/*Edg4non-mutant*), initially identified in human genomic databases by homology searches (An et al., 1998; Contos and Chun, 1998), is similar to a human mutant form (EDG4) possibly involved

Abbreviations: E, embryonic day; EST, expressed sequence tag; GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; P, postnatal day; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RFLP, restriction fragment length polymorphisms; RT-PCR, reverse transcriptase-PCR; TMD, transmembrane domain; UTR, untranslated region

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0378-1119/01/$ - see front matter
PII: S0378-1119(01)00410-3

in ovarian cancer (An et al., 1998; Contos and Chun, 2000). Both human and mouse LP_{A2} have ~55% amino acid identity with LP_{A1}. As with lp_{A1}, mouse lp_{A2} contains an intron upstream of TMD I and another within TMD VI (Contos and Chun, 2000). Mouse lp_{A2} is localized on Chromosome 8 near 'kidney anemia testes (*kat*)', although mutations in lp_{A2} have been ruled out as a cause of this phenotype (Contos and Chun, 2000).

A third LPA receptor gene in mouse was suggested by our finding of another cerebral cortical cell line GPCR cDNA clone with substantial similarity (~50% amino acid identity) to the initial two LPA receptor genes. To understand LPA signaling in the organism more fully, we undertook a complete characterization of this gene (lp_{A3}), similar to analyses of lp_{A1} and lp_{A2}. Unlike LP_{A1} and LP_{A2}, heterologously expressed LP_{A3} does not produce LPA-dependent neurite retraction (Ishii et al., 2000). However, other LPA-dependent responses mediated by LP_{A3} are similar to LP_{A1} and LP_{A2}, such as increased MAP kinase activation, inositol phosphate production, and inhibition of adenylate cyclase (Ishii et al., 2000). Here we present additional information regarding lp_{A3}, including its genomic structure, chromosomal location, and expression pattern.

2. Materials and methods

2.1. cDNA clone isolation and rapid amplification of cDNA ends (RACE)

Cerebral cortical cell lines were generated from the BALB/cAnNCrlBR strain (Chun and Jaenisch, 1996) and used to identify novel GPCR genes possibly involved in cerebral cortex development. Reverse transcriptase-polymerase chain reaction (RT-PCR) identification of the initial lp_{A3} cDNA fragment was accomplished with degenerate oligonucleotides designed to TMD II and TMD VII of GPCRs, in a protocol identical to that used to isolate lp_{A1} (previously called *vzg-1*) cDNA fragments (Hecht et al., 1996). Briefly, cDNA was reverse-transcribed from poly(A)$^+$ RNA isolated from cell line, TSM. PCR products resulting in amplification of the lp_{A3} clone were performed with 40 ng of cDNA, using the same primer combinations as for lp_{A1} (Hecht et al., 1996). The PCR product was gel-purified, T/A subcloned into pBluescript, and sequenced. This lp_{A3} fragment, labeled using ^{32}P and random hexamers, was used to isolate a cDNA clone from a total mouse embryo day 15 (mouse strain Swiss/Webster-NIH) cDNA library (Clontech). This clone was subsequently sequenced entirely in both directions. For RACE experiments, nested primers were designed 100–500 nt from the ends of this initial cDNA clone, and products generated from the Clontech Marathon-Ready cDNA templates (strain: Swiss/Webster-NIH) using PCR protocols detailed elsewhere (Contos and Chun, 2000). The entire mouse lp_{A3} cDNA sequence is available through GenBank (accession #AF272366).

Table 1
Oligonucleotide sequences

Edg6a′	5′-CGAGACCATCGGCTTTTTCTATA-3′
Edg6b′	5′-CCCAGAATGATGACAACCGTCTT-3′
513A	5′-GCTATCGAGAGGCACATCAC-3′
513B	5′-CAATAAAGGCACCAAGCACAATGA-3′
501A	5′-GGTTATTGCTGTGGAAAGAC-3′
501B	5′-CTTGCAGTTCAGGCCGTCCAG-3′
501EL	5′-GCCGAGATGTTGCAGAGGCAATTC-3′
501F	5′-GCTGGTTCCTGCTGCTCGCA-3′
501I	5′-CGCCTAAGACGGTCATCACT-3′
501J	5′-GCGCTTTACTAAACGCCGT-3′
501O	5′-CCAAAGGCAGACAGCTCAA-3′
501N	5′-GAAAAAGTCCATGCGCTTGT-3′
501A	5′-GGTTATTGCTGTGGAAAGAC-3′
edg5KO1	5′-AACGTCTCCTCCAACTGCACT-3′
lpA3mhe0g	5′-GCGGGCGAGCGACAGCGGA-3′
lpA3mhe1d	5′-GAATTGCCTCTGCAACATCTC-3′
lpA3mhe2a	5′-GAGTAGATGATGGGGTTCA-3′

2.2. Genomic Southern blot analysis

Genomic DNA (20 μg) from a *Mus musculus* mixed background strain (C57BL/6J × BALB/cAnNCrlBR)F1 was 10-fold overdigested with various restriction enzymes (indicated in Fig. 2). Conditions for making and probing the Southern blots are as detailed elsewhere (Contos and Chun, 1998). The 501AB probe was a 439 bp PCR product amplified from cDNA using the primers 501A and 501B (Table 1) and contains 372 bp from exon 2 and 67 bp from exon 3. The 501FJ probe was a 402 bp PCR product amplified from cDNA using the primers 501F and 501J (Table 1) and is located entirely within exon 3.

2.3. Genomic clone isolation and restriction mapping

A PCR strategy (detailed in Contos and Chun, 1998) was successfully used to isolate λ clones from a mouse 129/SvJ genomic library in λ FIX II (Stratagene). For clones containing exon 2, we used primers 501A and 501I (PCR product size: 378 bp). A total of approximately 4×10^6 clones were screened. To isolate the three clones containing exon 3, we used primers 501F and 501J (PCR product size: 404 bp) and screened a total of 1×10^6 independent phage. Restriction maps were constructed as previously described (Contos and Chun, 1998), using 501AB, 501FJ, T3, and T7 probes, as well as PCR between T3 or T7 and various primers located within the insert.

2.4. Subcloning and sequencing

All mouse clones isolated by PCR or library screening were manually sequenced (Sanger et al., 1977). These clones were the initial mouse PCR product isolated by degenerate oligonucleotide PCR, the 1.6 kb mouse cDNA

clone, several RACE products, and two subcloned mouse 129/SvJ genomic DNA fragments: a 5.5 kb *Xho*I/*Not*I fragment including exon 2 (XN5.5; accession #AF272364) and a 2.3 kb of a *Xho*I/*Not*I fragment (7.5 kb total) including exon 3 (XN7.5; accession #AF272365).

2.5. DNA sequence analysis

Raw sequence data were read into files and assembled into contigs using the DNasis software program (Hitachi). Repetitive elements were determined using RepeatMasker (Smit, 1996). Exon 1 sequence (i.e. cDNA sequence upstream of exon 2) is from a mouse kidney expressed sequence tag (EST) (accession #AW107032). Mouse C57BL/6J genomic sequence containing lp_{A3} was deposited as part of the Mouse Genome Project (accession #AC068947). Human genomic sequence containing lp_{A3} was deposited as part of the Human Genome Project (accession #AL139150 and #AL139822). Previously deposited human cDNA sequence (accession #AF127138, AF186380, AF236117, NM_012152, XM_002057) was compared with human genomic sequence to determine the boundary between exons 2 and 3. One of the deposited human cDNA clones apparently has 21 bp of genomic intron sequence at its 5′ end based on comparison with mouse sequence and the presence of a putative intron donor site that conforms strongly to consensus donor sites. However, it remains possible that this intron site is not used. Human exon 1 was located by homology searches to mouse exon 1, with the intron boundary placed in the same relative location. Human ESTs (accession #AA446859, AI223077, AI288165, AA446859, AA568130, AI223077, AI288165, AI432375, AI567144, AW172356, AW274018, BF359477, BF439156) confirmed that transcription through the indicated poly(A) site occurs *in vivo*.

2.6. RFLP detection and chromosomal mapping

To find restriction fragment length polymorphisms (RFLPs), primers were used to amplify products from *M. musculus* (C57BL/6JEi) or *M. spretus* (SPRET/Ei) genomic DNA. These two strains are referred to as B or S samples,

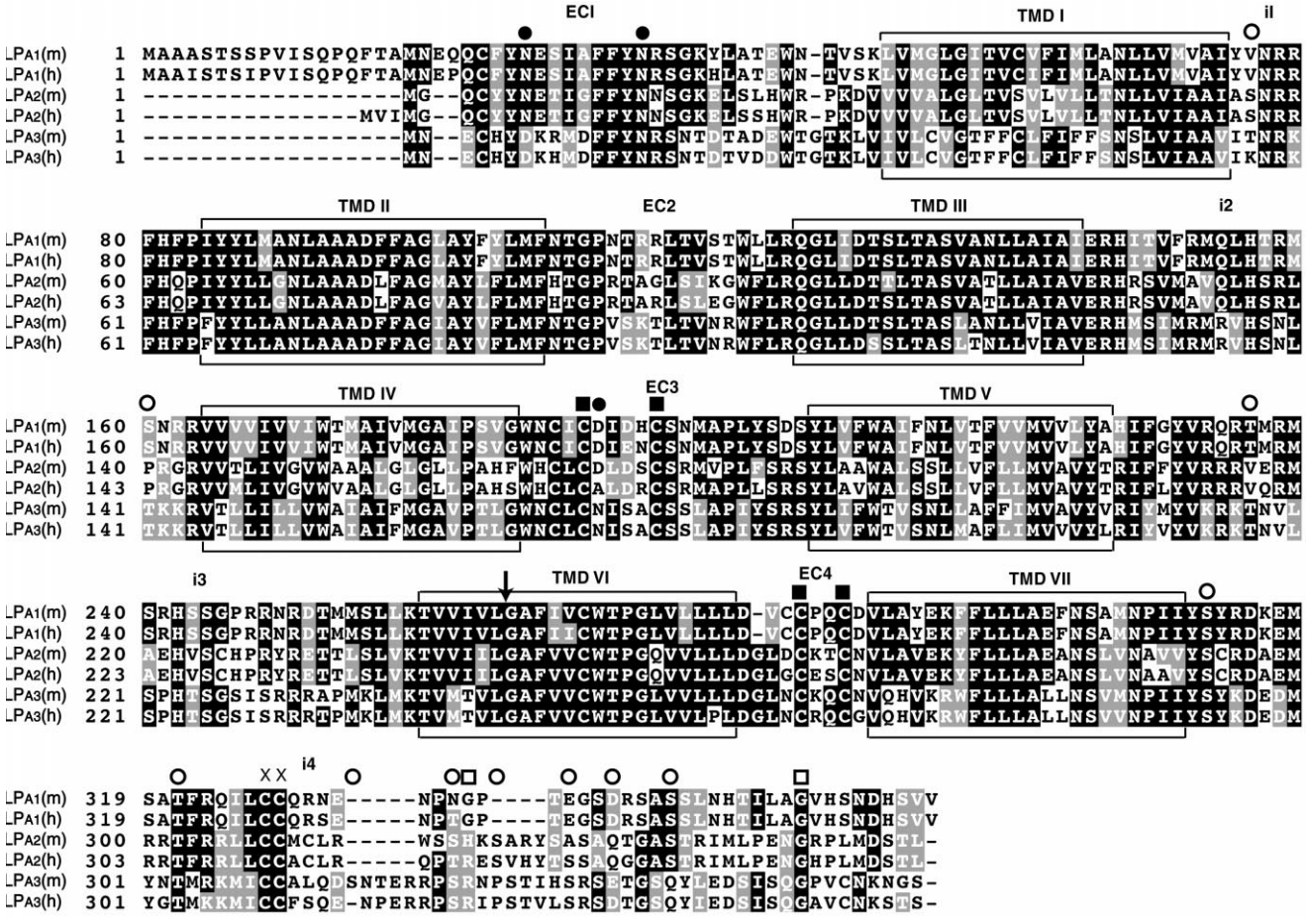

Fig. 1. Amino acid alignment of mouse (m) and human (h) LP_{A1}, LP_{A2}, and LP_{A3} receptor sequences. Residues identical in ≥50% of the sequences are shaded black. Conservative residue changes in ≥50% of the sequences are shaded gray. Approximate locations of the seven transmembrane domains (TMDs) are bracketed (based on hydrophobicity plot analyses). Putative sites of post-translational modifications in one or more of the receptors are indicated above the modified residue: N-linked glycosylation (●), Ser/Thr phosphorylation (○), cysteine palmitoylation (×), and cysteines involved in disulfide bonds (■). The arrow in TMD VI indicates the conserved exon-intron boundary in the coding region of the genes. Partially adapted from (Contos et al., 2000b).

respectively. PCR conditions were the same as outlined above for RT-PCR, except 1 μl of genomic DNA (50 ng) was used as template. The 501O/501N reaction products were digested by adding 10 μl of a mixture consisting of 7.5 μl H_2O, 2 μl 10× NEB2 (New England Biolabs restriction digest buffer #2), and 0.5 μl (5 U) *Eco*RV. Tubes were incubated at 37°C for 2 h, then 6× loading buffer was added and 20 μl electrophoresed on a 1.4% agarose gel containing ethidium bromide. The 501A/edg5KO1 products were treated similarly except buffer and enzymes were replaced with NEB1, 0.5 μl (10 U) *Sac*I, and 0.5 μl (10 U) *Nco*I. The formal names of the crosses are The Jackson Laboratory interspecific backcross panels (C57BL/6J × *M. spretus*)F_1 × C57BL/6J, called Jackson BSB, and (C57BL/6JEi × SPRET/Ei)F_1 × SPRET/Ei, called Jackson BSS (Rowe et al., 1994). Raw data were submitted to The Jackson Laboratory for comparison to other markers typed to the panel. It can be viewed at http://www.jax.org/resources/documents/cmdata. The human chromosomal position of lp_{A3} is based on contig alignment and/or localization of neighboring genes with fluorescence in situ hybridization (see GenBank flatfile with accession #AL139150).

2.7. Tissues, cell lines, and northern blots

For RNA isolation, 6 month old C57BL/6J mice (purchased from The Jackson Laboratory) were sacrificed by cervical dislocation and organs immediately dissected, frozen in liquid nitrogen, and stored at −80°C. Cell lines, P19 and 3T3 (ATCC, Rockville, MD), were maintained in DMEM supplemented with 10% fetal-calf serum (FCS) and 1× penicillin/streptomycin. Mouse cell lines, TR, TSM, and V (Chun and Jaenisch, 1996), were maintained in Opti-MEM supplemented with 2.5% FCS and 1× penicillin/streptomycin. Cell lines, TR and TSM, displayed neuroblast properties, whereas V displayed glial properties. Total RNA was prepared using the guanidine isothiocyanate method (Ausubel et al., 1994). Northern blotting was performed with standard protocols (Ausubel et al., 1994). Probes for lp_{A1}, lp_{A2}, and lp_{A3} were generated by PCR from plasmid templates, gel-purified using the Qiaquick gel-extraction kit (Qiaqen), then labeled using random hexamers and ^{32}P-dCTP. Primers used to amplify the various lp_A gene probes were lp_{A1}: 513A/513B, lp_{A2}: edg6a'/6b', and lp_{A3}: 501A/501B (Table 1). Probes never contained residual vector sequence. Blots were incubated at 55°C overnight with 5×10^6 dpm/ml in hybridization solution (25% formamide, 0.5 M Na_2HPO_4, 1% BSA, 1 mM EDTA, 5% SDS), followed by successive 20 min 20°C washes in 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, 0.5× SSC/0.1% SDS, 0.2× SSC/0.1% SDS, and 0.2× SSC/0.1% SDS at 65°C. Blots were stripped by rocking for 20 min successively in 2× SSC at 20°C, 10 mM Tris (pH 8.0)/1% SDS at 80°C, and finally 2× SSC at 20°C. The blots were then rehybridized with another probe (order of hybridization: lp_{A1}, lp_{A2}, lp_{A3}, then cyclophilin).

2.8. RT-PCR

Generation of the cDNA template, conditions for PCR, and the β-actin primers have been described (Contos and Chun, 2000). Primer pairs used, their locations, and expected PCR product sizes were lpA3mhe0g/501EL; exon 1/2; 562 bp, and lpA3mhe1d/lpA3mhe2a; exon 2/3; 382 bp. The lpA3mhe1d/lpA3mhe2a primers recognized sequences identical in the mouse and human genes.

3. Results

3.1. Isolation and sequencing of mouse lp_{A3} cDNA clones

Degenerate oligonucleotides designed to conserved sequences within GPCRs were used to amplify novel GPCR cDNA sequences from a cerebral cortical cell line library (Hecht et al., 1996) resulting in the isolation of a full-length mouse lp_{A3} cDNA sequence (2205 bp) consisting of 173 bp of 5′ untranslated region (UTR), 1064 bp of coding region, and 968 bp of 3′ UTR, of which 25 nt are poly(A); see Section 2.1. This sequence has been deposited with

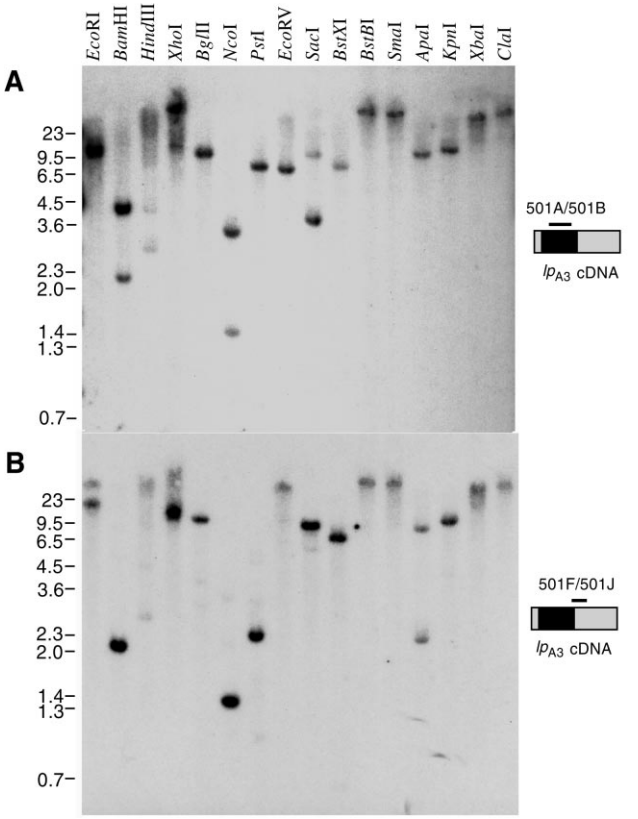

Fig. 2. Southern blot analysis of mouse lp_{A3}. C57BL/6J genomic DNA (20 μg per lane) was digested with the indicated restriction enzymes, electrophoresed, blotted, and hybridized with lp_{A3} probes from either the coding region (A) or the 3′ UTR (B). Relative locations of the probes are indicated to the right in the cDNA schematic (black indicates open reading frame). The 501AB probe is located primarily in exon 2 but also contains some of exon 3. The 501FJ probe is located entirely within exon 3.

GenBank (accession #AF272366). The human LP_{A3} cDNA and protein sequence is known (Bandoh et al., 1999), which allowed us to compare all mouse and human LP_A receptor sequences. The mouse and human LP_{A3} protein sequences are 90.7% identical. Compared to mouse/human LP_{A1} or LP_{A2} protein sequences, the mouse/human LP_{A3} protein sequences are 45.4–49.7% identical, and contain several of the same putative post-translational modification sites (Fig. 1).

3.2. Genomic structure of mouse lp_{A3}

We first used Southern blotting to determine if mouse lp_{A3} was present as a multi-exon, single copy gene (Fig. 2). Using several restriction enzymes as well as probes corresponding to two different parts of the cDNA, the mouse gene (including exon 3) was determined to be single-copy and to contain two primary coding exons 10 kb apart. To precisely determine the genomic structure of mouse lp_{A3}, we isolated and characterized 129/SvJ genomic clones using restriction mapping, subcloning, and sequencing (accession #AF272364, AF272365). Restriction maps showing relative locations of these genomic clones in the gene are shown in Fig. 3A. Two exons of 754 (termed 'exon 2') and 1288 bp (termed 'exon 3') were found on these genomic clones with introns located in the 5′ UTR (22 bp upstream of the start codon) and within the coding region for TMD VI (Fig. 4B,C). The remaining cDNA sequence in the 5′ UTR was likely encoded by another upstream exon. This hypothesis was confirmed recently when we found C57BL/6J mouse lp_{A3} genomic sequence deposited with GenBank (accession #AC068947), in which the remaining upstream cDNA sequence was present as a single exon (termed 'exon 1'; Figs. 3A and 4A). Intron donor/acceptor sites and the poly(A) site correspond to consensus sequences (Fig. 5A,B). By comparing all of the analyzed mouse clones (Swiss/Webster-NIH cDNA, 129/SvJ genomic DNA, and C57BL/6J genomic DNA), we found only a few sequence differences (Figs. 4A–C and 5C), though the predicted protein sequence remains identical.

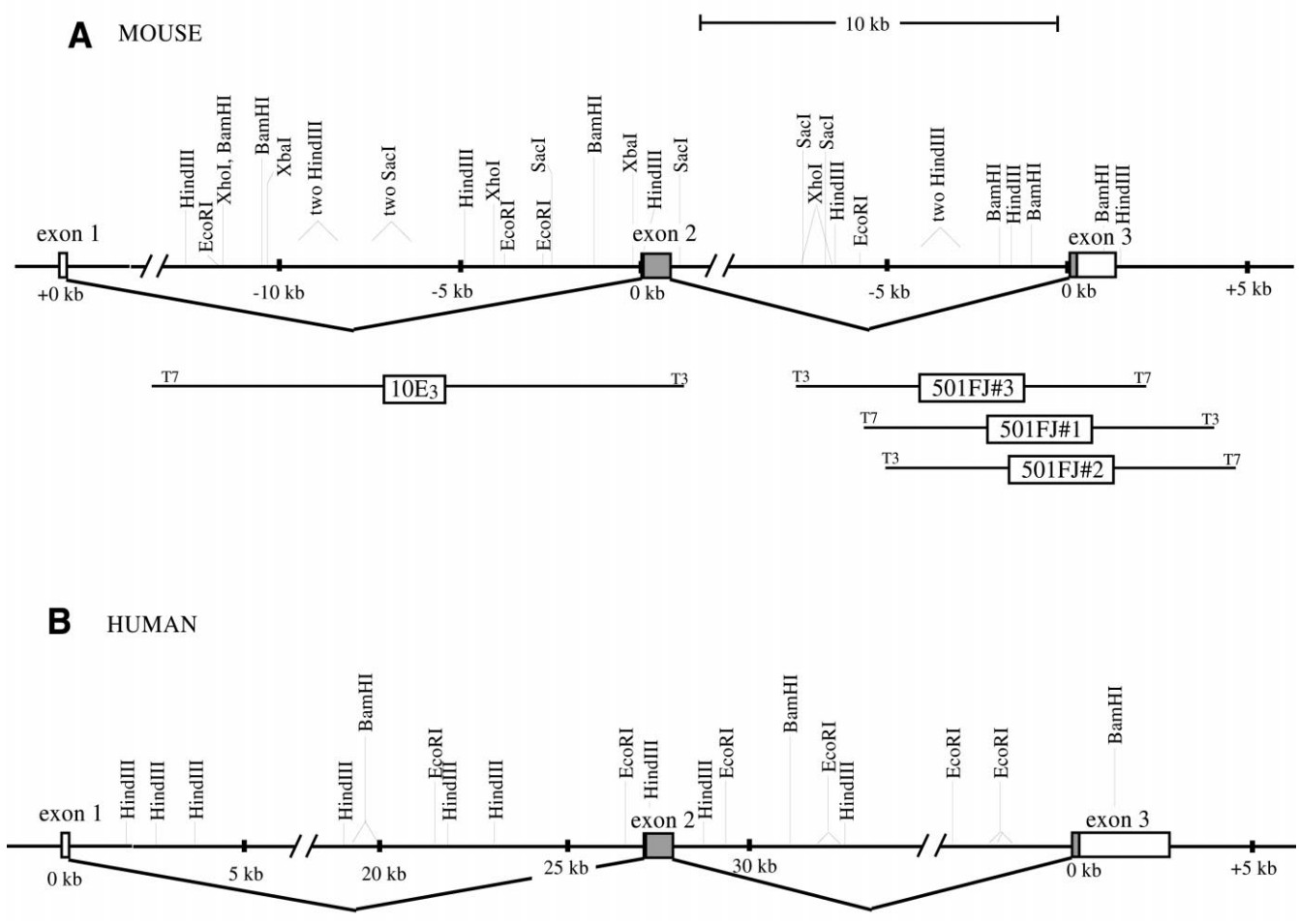

Fig. 3. Genomic organization and restriction maps for (A) mouse lp_{A3} and (B) human lp_{A3}. Shown below the mouse genomic map are the relative locations of the four λ genomic clones isolated. Boxes indicate lp_{A3} exons and shaded areas indicate coding regions. Contiguous genomic DNA sequences are numbered such that 0 kb is the start of the exon in that contig. A 10 kb scale bar is shown at the top, with hatched bars indicating additional genomic sequence not shown.

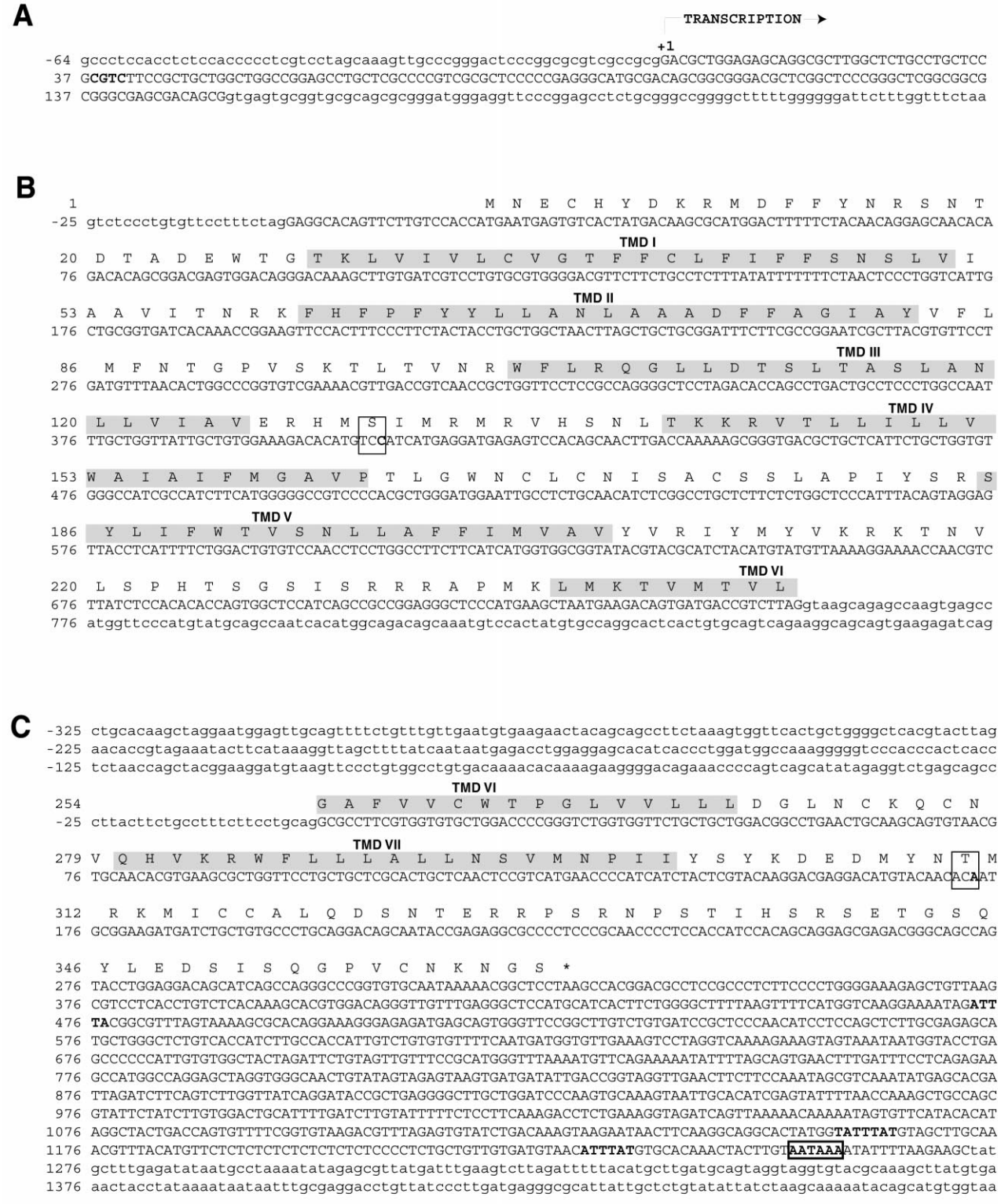

Fig. 4. Genomic sequence of mouse lp_{A3}. (A) Exon 1 sequence. (B) Exon 2 sequence. (C) Exon 3 sequence. Exon 1 sequence is C57BL/6J and exons 2 and 3 are 129/SvJ. Nucleotides are numbered such that the first basepair of each exon is +1. Encoded amino acids are independently numbered. Exon sequence is shown in uppercase with open reading frame translated above. Putative transmembrane domains in the translation product are shaded. The polyadenylation consensus sequence is boxed and three mRNA destabilization consensus sequences in the 3′ UTR are shown in bold (there are eight such sites in the human 3′ UTR). In addition, two codons are boxed where there are nucleotide sequence differences with the cDNA, with the variable nucleotide in bold.

3.3. Genomic structure of human lp_{A3}

Analysis of human lp_{A3} genomic clone sequences deposited with GenBank (Fig. 3B; accession #AL139150 and AL139822) revealed 2.3 kb of additional transcript sequence (total transcript size ~3495 bp), of which only 1148 bp had been previously known (Bandoh et al., 1999). Because the 5′ end of the transcript has not been mapped, the actual transcript size may be larger. Additional 3′ UTR sequence was confirmed by finding multiple overlapping ESTs in this region (data not shown). Human lp_{A3} is divided amongst three exons of ~169, 757, and 2569 bp (Fig. 3B). Intron boundaries and polyadenylation sites correspond to consensus sequences (Fig. 5A, B) and are located in conserved locations relative to all known mouse and human lp_A genes (Fig. 1). At the DNA sequence level, mouse and human lp_{A3} 5′ UTR sequence is 74% identical, the coding region sequence is 84% identical, and the 3′ UTR

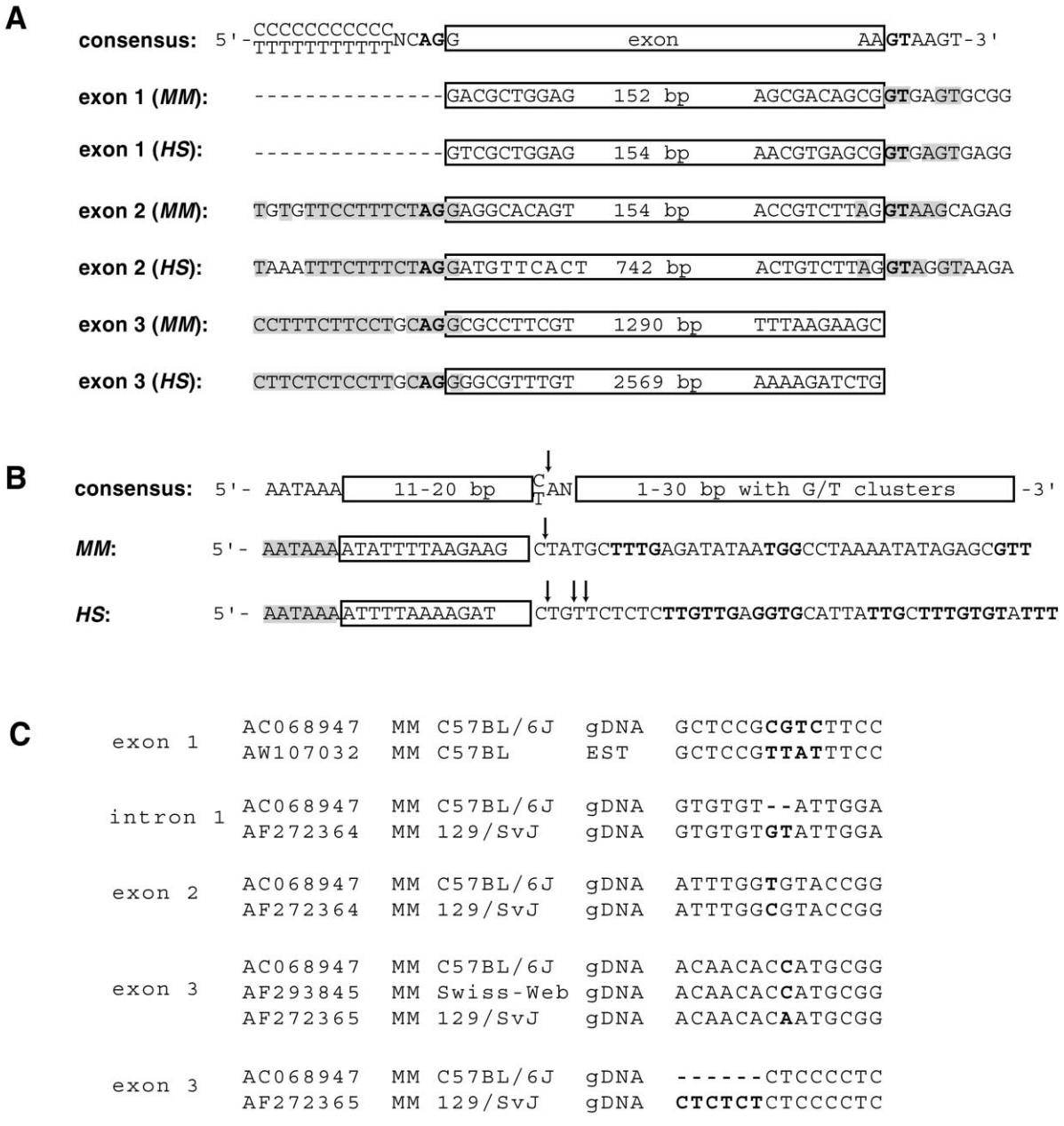

Fig. 5. Intron boundaries, poly(A) sites, and sequence polymorphisms in human and mouse lp_{A3}. (A) Mammalian consensus intron donor/acceptor sequence aligned with human and mouse lp_{A3} exon boundaries. The nearly invariant AG and GT of all such sequences are shown in bold, whereas additional residues that align with the consensus are shaded. Boxes represent sequences present in the spliced mRNA transcript (for exon 1 in human, this is putative). *MM, Mus musculus; HS, Homo sapiens*. (B) Mammalian consensus polyadenylation sequence aligned with the mouse and human lp_{A3} polyadenylation regions. Polyadenylation sites actually found in cDNAs are indicated with arrows, and GT clusters are shown in bold. (C) Sequence polymorphisms between various mouse clones. The relative location, accession number, strain, type of sequence, and the actual sequences are shown.

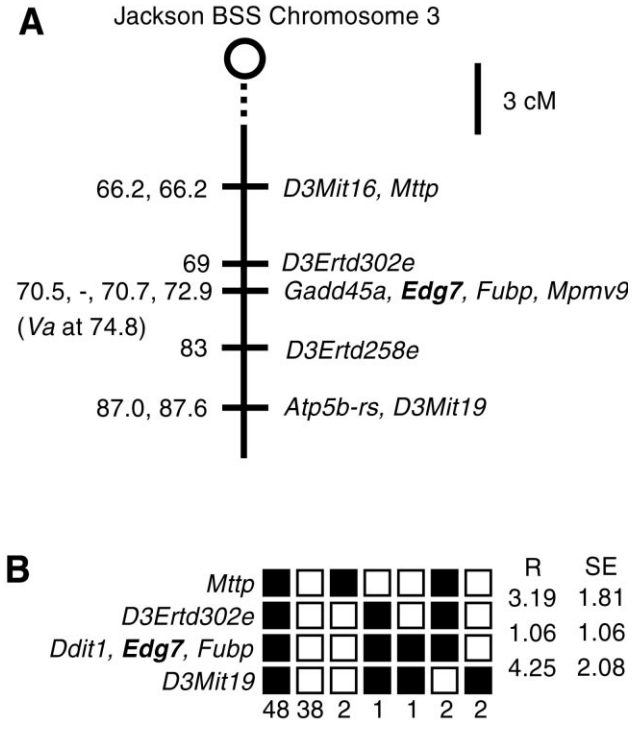

Fig. 6. Linkage map of the BSS backcross panel placing *Edg7* (lp_{A3}) at distal mouse Chromosome 3. (A) A 3-cM scale bar is shown to the right, which refers only to the genes mapped in the BSS backcross panel. To the left, relative map positions (in cM) from the Mouse Genome Database are shown. *Va*, varitint waddler. (B) Haplotype from The Jackson Laboratory BSS backcross showing part of Chromosome 3 with loci linked to *Edg7*. Loci are listed in order with the most proximal at the top. The black boxes represent the C57BL/6JEi allele and the white boxes the SPRET/Ei allele. The number of animals with each haplotype is given at the bottom of each column of boxes. The percentage recombination (R) between adjacent loci is given to the right, with the standard error (SE) for each R.

sequence is ~60% identical. There are several sequence polymorphisms between the human clones (data not shown), though these polymorphisms do not alter the encoded protein. A recently identified human lp_{A3} cDNA clone variant (termed HOFNH30; accession #AF236117) was hypothesized to be encoded by alternatively spliced exons, based on numerous sequence differences with previously published clones (Fitzgerald et al., 2000). We found that all of the differences in AF236117 were located in exon 3 (in exon 3, DNA sequence identity with genomic DNA clones is 87%; in exon 2, DNA sequence identity with genomic DNA clones is 100%). However, no EST or genomic sequences currently in the database contain the same sequence variations as the AF236117 clone.

3.4. Chromosomal locations of mouse and human lp_{A3}

We mapped the chromosomal position of mouse lp_{A3} (official gene symbol *Edg7*) by determining segregation of RFLPs in a *M. musculus* (C57BL/6JEi) × *M. spretus* (SPRET/Ei) backcross panel. In 94 progeny from one of the backcross panels (BSS), an easily discernable *SacI* RFLP near exon 2 cosegregated with the *D3Ertd330e*, *D3Xrf112*, *D3Xrf408*, *Gadd45a* (growth arrest and DNA-damage-inducible, alpha), and *Fubp* (fucose binding protein) (Fig. 6). This allowed localization of *Edg7* to distal mouse Chromosome 3, near the varitint waddler (*Va*) locus (Fig. 6). The chromosomal location was confirmed in a separate backcross panel (BSB) using a distinct (*Eco*RV) polymorphism located 1.5 kb away. The human lp_{A3} genomic clone is from chromosome 1p22.2-1p32.1, which is syntenic to the distal Chromosome 3 mouse location.

3.5. Northern blot detection of the mouse lp_{A1}, lp_{A2}, and lp_{A3} transcripts

To determine the expression pattern of lp_{A3} and to compare it to the expression patterns of lp_{A1} and lp_{A2}, northern blots with total RNA from eleven different adult mouse tissues were probed with fragments from lp_{A1}, lp_{A2}, and lp_{A3}. We found that the 3.8 kb mouse lp_{A1} transcript was expressed widely with approximately equivalent abundance in brain, heart, lung, testes, and intestine (Fig. 7A). A smaller (2.0 kb), more intense transcript was also observed in testes, the smaller size of which probably reflects use of an alternate polyadenylation site (Contos and Chun, 1998). The distribution pattern of the 2.8 kb lp_{A2} and 2.4 kb lp_{A3} transcripts was nearly identical, with both being most abundant in testes, kidney, and lung (Fig. 7A). Low levels of the lp_{A2} transcript were observed in spleen, thymus, and stomach. In addition, a larger lp_{A2} transcript form (~6 kb) of lesser intensity was apparent wherever the smaller transcript was observed. Low levels of the lp_{A3} transcript were observed in intestine, heart, thymus, and stomach. We also probed a northern blot containing total brain RNA from various developmental ages (Fig. 7B). As previously determined (Weiner et al., 1998), the lp_{A1} transcript was found to be expressed in a biphasic manner, with peak expression levels during the neurogenic (embryonic day (E) 11-E17) and myelination (postnatal day (P) 7-P30) periods. The lp_{A2} transcript was only observed in the embryonic and early postnatal brain, with the ~6 kb transcript form of approximately half the intensity of the 2.8 kb form. The lp_{A3} transcript was expressed primarily during the perinatal and postnatal period (E18-P18).

3.6. RT-PCR detection of the lp_{A3} transcript

RT-PCR confirmed the expression pattern of the lp_{A3} transcript determined by northern blot and demonstrated expression in glial-like (V) and teratocarcinoma (P19) cell lines (Fig. 8). RT-PCR also demonstrated that a transcript consisting of spliced exons 1, 2, and 3 is the predominant form in cells.

4. Discussion

The expression pattern of mouse lp_{A3} in the brain during

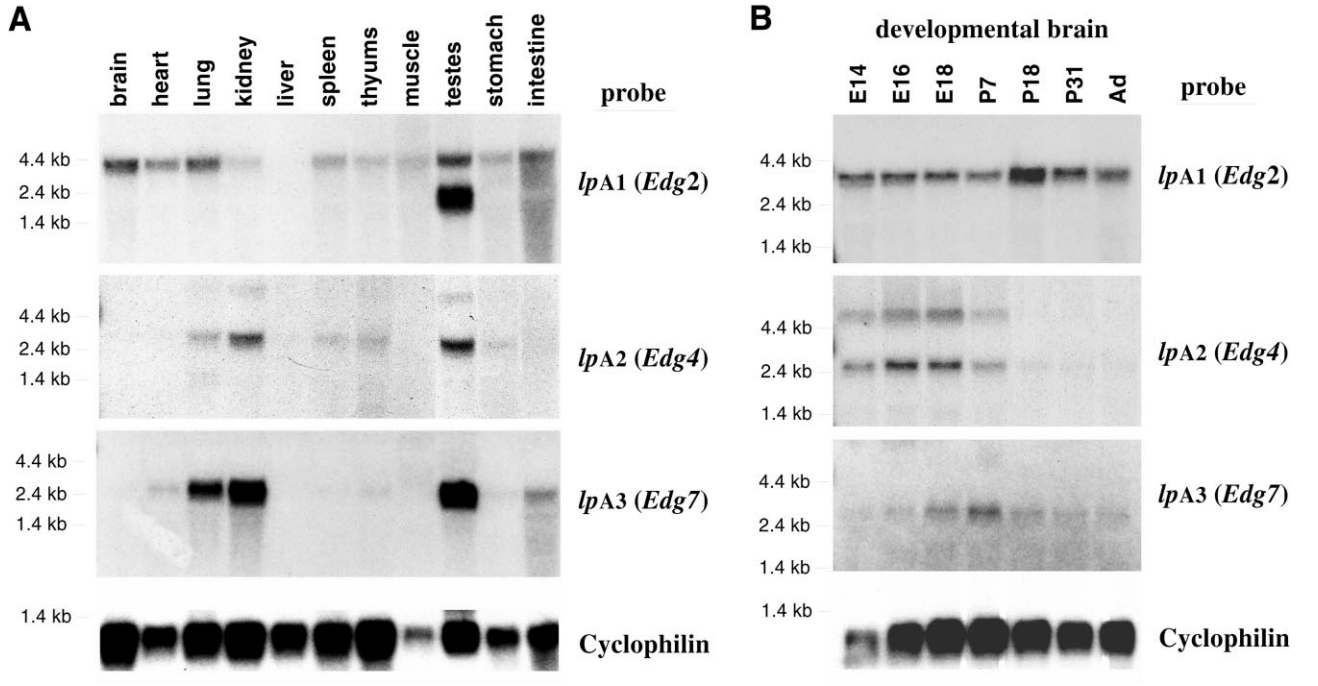

Fig. 7. Northern blot detection of lp_{A1}, lp_{A2}, and lp_{A3} transcripts. (A) Adult mouse tissue distribution. Note that the cyclophilin expression is notably lower in muscle and stomach, relative to other tissues. (B) Developmental brain expression. Total RNA (20 μg) from the indicated mouse tissues was used. Cyclophilin probe was used as a standardization control for mRNA quantity. *Edg4* refers to the non-mutant form of the gene. Partially adapted from (Contos et al., 2000b).

the perinatal/postmitotic period indicates that the receptor functions during the development of the nervous system. The finding that LP_{A3} does not mediate neurite retraction suggests that LP_{A3} likely has distinct functions compared to LP_{A1} and LP_{A2} (Ishii et al., 2000). This suggestion is particularly intriguing given that the expression of lp_{A2} appears to turn off when the expression of lp_{A3} is turned on.

We observed all three LPA receptor genes to be most abundantly expressed in adult testes. Although many genes show cryptic expression in the testes with no known function, LP_A receptors likely mediate LPA-induced stimulation of the acrosome reaction and protein kinase C activation in spermatids (Garbi et al., 1999). These responses suggest that LPA has specific roles in fertilization and that at least one cell type that expresses the transcripts is spermatogonia/spermatids. In addition to testes, we also observed expression of lp_{A2} and lp_{A3} in kidney and lung. Roles for LPA in kidney cells and the development of glomerular nephritis have been proposed, based on the observed effects of LPA on kidney cells in culture and the known production of LPA by activated platelets (Gaits et al., 1997; Inoue et al., 1999). Our results suggest redundant functions of LPA receptors in testes, kidney, and perhaps lung.

Additional conclusions can be drawn from our lp_{A3} genomic characterization data. Not surprisingly, the genomic structure of lp_{A3} is very similar to both lp_{A1} and lp_{A2}. Each gene contains introns upstream of TMD I and within TMD VI (Contos and Chun, 1998, 2000). Although the genomic structures of $lp_{A1–3}$ are similar with respect to the locations of introns adjacent to and within the coding regions, these structures do differ in the number of upstream exons. Whereas lp_{A2} has only one exon upstream of TMD I (Contos and Chun, 2000), lp_{A1} contains four such exons, one of which is present only in an alternative transcript form (Contos and Chun, 1998). This result suggests that an ancestral three-exon LPA receptor gene duplicated and diverged with the result that lp_{A1} picked up additional introns in exon 1. Because we have not mapped the 5′ end of the lp_{A3} transcript, it remains possible that additional upstream exons encode more 5′ UTR sequence.

We observed several nucleotide differences between the various mouse clones analyzed. The most likely explanation for the mouse sequence differences is simply strain polymorphism, because the cDNA was from the Swiss-Webster/NIH strain, and the two genomic sequences were from the 129/SvJ and C57BL/6J strains. The identification of these polymorphisms should be valuable for positional cloning studies in which polymorphic markers between strains are used. We also identified several polymorphisms between the human genomic and cDNA sequences (data not shown), which may be of similar value.

As we were characterizing the mouse and human lp_{A3} genomic structures, other researchers reported isolation of a human lp_{A3} cDNA clone variant (HOFNH30; accession #AF236117), with substantial sequence differences in the last part of the coding region and the 3′ UTR (Fitzgerald et al., 2000). Without knowledge of the genomic structure, it

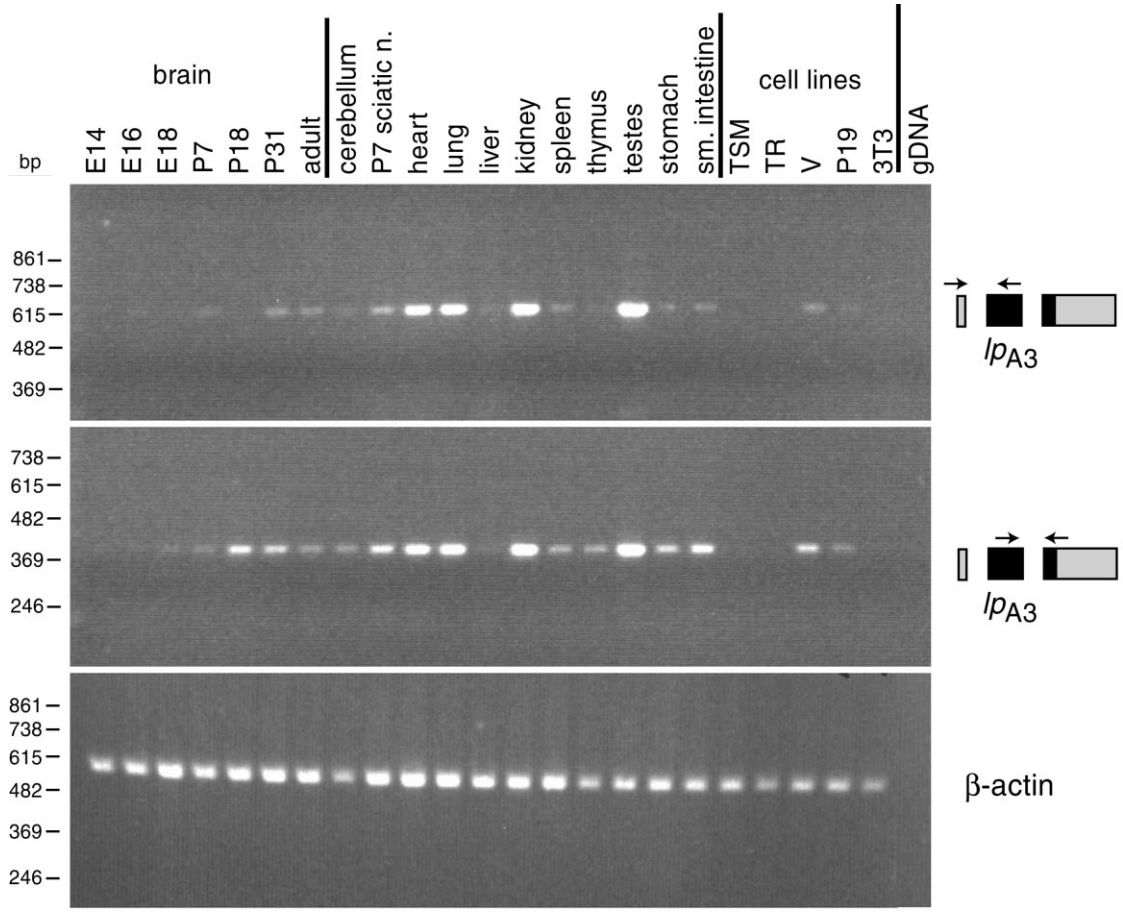

Fig. 8. RT-PCR detection of lp_{A3} in mouse. Relative locations of primers in lp_{A3} are indicated to the right (boxes indicate exons, with coding regions in black). Above each lane is the template cDNA used, including whole brain at various developmental stages (E, embryonic day; P, postnatal day), adult cerebellum, P7 sciatic nerve, nine adult organs, and five mouse cell lines. Cell lines, TR and TSM, are derived from embryonic cerebral cortical neuroblasts, V is a glia-like cell line, P19 is a teratocarcinoma, and 3T3 is a fibroblast-derived line. As a control, the last lane shows product from genomic DNA template (gDNA). The β-actin PCR demonstrates relative levels of cDNA template used in each sample.

was suggested that these sequence differences might be due to alternative splicing. We determined that all of the AF236117 sequence differences were in exon 3, and not in exons 1 or 2. This supports the hypothesis that an additional exon 3 (exon 3b) is used in the production of some transcripts. Such a phenomenon is not unreasonable because in some mouse strains, the corresponding lp_{A1} exon is duplicated (Contos and Chun, 1998). Our Southern blot results using a probe in exon 3 indicate that in mouse, there is no alternative exon 3. However, it remains possible that in human such an exon exists. This question will be resolved when sequence of the entire human genome becomes available.

We found that mouse lp_{A3} cosegregated with loci on distal Chromosome 3 at cM 70.5–72.9 (in the Mouse Genome Database). Genes in the vicinity of mouse lp_{A3} include *Rrh* (retinal pigment epithelium rhodopsin homolog; cM 67.0), *Rpe65* (retinal pigment epithelium gene, 65 kDa protein; cM 78.1), and *Va* (cM 74.8). The possible relationships between lp_{A3} and these genes remain for future studies.

Acknowledgements

We thank Joshua Weiner and Carol Akita for assistance with the northern blot, Jonathan Hecht for isolation of the initial cDNA fragment, the Jackson Laboratory for assistance in the mouse backcross analysis, and Casey Cox for copyediting the manuscript. This work was supported by the National Institute of Mental Health.

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