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The Genes Encoding the Eph-Related Receptor Tyrosine Kinase Ligands LERK-1 (*EPLG1, Epl1*), LERK-3 (*EPLG3, Epl3*), and LERK-4 (*EPLG4, Epl4*) Are Clustered on Human Chromosome 1 and Mouse Chromosome 3

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Hek and elk are members of the eph-related family of receptor tyrosine kinases. Recently, we isolated five cDNAs encoding membrane-bound ligands to hek and elk. Because of the promiscuous nature of their binding, we have termed these proteins ligands of the eph-related kinases or LERKs. The LERKs can be divided into two subgroups by virtue of their sequence identity, binding properties, and mode of cell membrane attachment. For example, LERK-2 (*EPLG2, Epl2*) and LERK-5 (*EPLG5, Epl5*) are type 1 transmembrane proteins, while LERK-1 (*EPLG1, Epl1*), LERK-3 (*EPLG3, Epl3*), and LERK-4 (*EPLG4, Epl4*) are anchored to the membrane by glycosyl-phosphatidylinositol (GPI) linkage. Using Southern hybridization analysis of human × rodent somatic cell hybrid DNAs, we have assigned the genes that encode the GPI-anchored LERKs (*EPLG1, EPLG3*, and *EPLG4*) to human chromosome 1. Fluorescence *in situ* hybridization to metaphase chromosome preparations using genomic clones from each locus refined this localization to chromosome 1, bands q21–q22. In addition, Southern blot analysis of DNA from interspecific backcross mice indicated that the mouse homologues *Epl1, Epl3*, and *Epl4* map to a homologous region on mouse chromosome 3.

INTRODUCTION

The interaction of extracellular ligands with membrane-bound receptors initiates intracellular signals required for cell proliferation, differentiation, and survival. The eph (erythropoietin-producing human hepatocellular carcinoma) family of receptor tyrosine kinases (RTKs), named after the first member to be identified (Hirai *et al.*, 1987), is the largest family of RTKs, with well over a dozen members (Scales *et al.*, 1995; van der Geer *et al.*, 1994). Its members include chicken cek4 (Sajjadi *et al.*, 1991) and cek5 (Pasquale, 1991), mouse mek4 (Sajjadi *et al.*, 1991), bsk (Zhou *et al.*, 1994), nuk (Henkemeyer *et al.*, 1994), and sek (Gilardi-Hebenstreit *et al.*, 1992), rat elk (Lhoták *et al.*, 1991), ehk-1, and ehk-2 (Maisonpierre *et al.*, 1993), and human hek (Wicks *et al.*, 1992), hek2 (Böhme *et al.*, 1993), and eck (Lindberg and Hunter, 1990). Because the eph-related RTKs are primarily expressed in the brain, it has been predicted that these receptors and their ligands may be involved in the growth, differentiation, and survival of neurons. Indeed, immunohistochemical staining and *in situ* hybridization analysis have implicated several members of the eph-related RTKs in neurogenesis in the mouse (Gilardi-Hebenstreit *et al.*, 1992; Henkemeyer *et al.*, 1994; Nieto *et al.*, 1992; Xu *et al.*, 1994; Zhou *et al.*, 1994).

We have recently isolated five cDNA clones that encode membrane-bound ligands of elk and hek (Beckmann *et al.*, 1994; Cerretti *et al.*, 1995; Kozlosky *et al.*, 1995). In addition, several groups have found that some of these proteins as well as a related protein, ELF-1 (LERK-6), bind to other eph-related receptors (Bartley *et al.*, 1994; Bennett *et al.*, 1995; Cerretti *et al.*, 1995; Cheng and Flanagan, 1994; Davis *et al.*, 1994; Shao *et al.*, 1994). Because of their promiscuous binding, we have termed these proteins LERKs (ligands for the eph-related kinases) (Beckmann *et al.*, 1994; Kozlosky *et al.*, 1995). The LERKs range in size from 201 to 345 amino acids, share an amino acid identity of 30 to 59%, have four conserved Cys residues, and all bind both elk and hek. Despite these similarities, the LERKs can be divided into two distinct subclasses. LERK-1, LERK-3, and LERK-4 are anchored to the cell membrane by

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glycosyl-phosphatidylinositol (GPI) linkage and are more homologous to each other than to LERK-2 and LERK-5, which are type 1 transmembrane proteins. In addition, the GPI-anchored LERKs have a higher binding affinity to hek than to elk, whereas LERK-2 and LERK-5 bind with higher affinity to elk.

In this report, we show that the GPI-anchored LERKs are also related genetically. While *EPLG2* (LERK-2) and *EPLG5* (LERK-5) are located on chromosomes Xq13 and 13q33, respectively (Fletcher *et al.*, 1994), *EPLG1* (LERK-1), *EPLG3* (LERK-3), and *EPLG4* (LERK-4) are clustered on human chromosome 1q21–q22 and on mouse chromosome 3.

The official HGM nomenclature committee designation for the gene loci that encode the LERKs is *EPLG* followed by their LERK number (P. J. McAlpine, Winnipeg, pers. comm., January 20, 1994). *EPLG* is derived from the gene designation for the eph tyrosine kinase receptor (EPHT) and ligand. The official MGD nomenclature committee does not accept *lg* for ligand, and thus the mouse designation for the gene loci that encode the LERKs is *Epl* followed by their LERK number (L. Maltais, Bar Harbor, pers. comm., April 24, 1995).

MATERIALS AND METHODS

Somatic cell hybrid DNA analysis. Hybrid cell lines used for the chromosomal mapping of *EPLG1, EPLG3,* and *EPLG4* were obtained from the National Institute of General Medical Sciences' Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NJ) ("GM" series) or prepared as previously described (lines A2–A6, C1, C2) (Morris *et al.,* 1991). Genomic DNAs prepared from the hybrid lines were digested to completion with *Hin*dIII and subjected to Southern blot analysis using DNA probes for LERK-1 (*EPLG1*), LERK-3 (*EPLG3*), or LERK-4 (*EPLG4*) under conditions described previously (Cerretti *et al.,* 1994).

Isolation of genomic clones. Human *EPLG1* (LERK-1), *EPLG3* (LERK-3), or *EPLG4* (LERK-4) cDNA inserts were ^{32}P-labeled and used to screen a human placenta genomic library prepared in the λ Dash II vector (Stratagene, La Jolla, CA) under the hybridization and wash conditions described above. Positive clones that hybridized uniquely with one of the three cDNA clones were plaque-purified and characterized by restriction endonuclease mapping and comparison with total human genomic DNA hybridization patterns identified with the individual cDNA clones. Three genomic phage clones representing each of the *EPLG* gene loci were used for fluorescence *in situ* hybridization studies; these clones are designated λ4 (*EPLG1*), λ22 (*EPLG3*), and λ17 (*EPLG4*) and contain inserts of 14.3, 13.8, and 13.1 kb, respectively. Each of these clones hybridized only to its corresponding cDNA clone; hybridizations of the random-primer-labeled cDNA inserts to *Hin*dIII digests of the phage clones revealed diagnostic positively hybridizing fragments of 5.5 and 1.0 kb (λ4), 12.0 kb (λ22), and 2.5 kb (λ17) that correspond in size to fragments hybridized uniquely in Southern analysis of human genomic DNA.

Fluorescence in situ hybridization (FISH). Phytohemagglutinin-stimulated peripheral blood lymphocytes of normal donors were used as a source of metaphase chromosomes. Genomic DNAs were labeled with either digoxigenin-11–UTP (*EPLG*s) or biotin-16–UTP (p58^{clk-1}) (Eipers *et al.,* 1992) and hybridized as previously described (Morris *et al.,* 1992). Signals were detected by incubation of the slides with fluorescein-conjugated sheep antidigoxigenin antibodies and Texas red-conjugated avidin followed by counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) in antifade. Analysis was performed from digitally acquired merged images that were obtained with a charge-coupled device camera and a commercially available software package. Fluorescence microscopy was performed with a Nikon Optiphot microscope.

Interspecific backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Epl1, Epl3,* and *Epl4* loci (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins *et al.,* 1982). All blots were prepared with Zetabind nylon membrane (AMF-Cuno). The probes were labeled with [α-^{32}P]dCTP using a nick-translation labeling kit (Boehringer Mannheim) or a random-primed labeling kit (Stratagene); washing was performed to a final stringency of 1.0× SSCP, 0.1% SDS, 65°C. The probe for *Epl1,* an ~640-bp fragment of human cDNA, detected fragments of 5.0, 2.4, and 1.6 kb in *Pvu*II-digested and 6.4, 5.1, and 3.4 kb in *Hin*dIII-digested C57BL/6J (B) DNA. Fragments of 5.0, 4.4, and 1.6 kb in *Pvu*II-digested and 6.4 and 3.1 kb in *Hin*dIII-digested *M. spretus* (S) DNA were detected. The presence or absence of the *M. spretus*-specific fragments was followed in backcross mice. The *Pvu*II and *Hin*dIII data were combined. The probe for *Epl3,* an ~450-bp *Sph*I/*Nco*I fragment of the human cDNA, detected fragments of 7.9 kb (B) and 8.6 kb (S) in *Kpn*I-digested DNA. The probe for *Epl4,* an ~636-bp *Bgl*II fragment of the human cDNA, detected major fragments of 7.4 kb (B) and 4.2 kb (S) in *Bgl*II-digested DNA. In both cases, the presence or absence of *M. spretus*-specific fragments was again followed in backcross mice.

A description of the probes and restriction fragment length polymorphisms (RFLPs) for loci linked to the *Epl* loci, including the trk proto-oncogene (*Ntrk1*), connexin 40 (*Gja5*), and nerve growth factor β (*Ngfb*), has been reported previously (Tessarollo *et al.,* 1993). Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

Human–rodent somatic cell hybrid analysis. Preliminary Southern analysis using full-length cDNA clones representing *EPLG1* (LERK-1), *EPLG3* (LERK-3), or *EPLG4* (LERK-4) to probe normal human genomic DNA digested with various frequently cutting restriction enzymes revealed unique hybridization patterns for each, indicating that the three genes are encoded at distinct chromosomal loci and appear to be single-copy genes (data not shown). Hybridizations of Southern blots prepared with various somatic cell hybrid cell line DNAs digested with *Hin*dIII using the individual *EPLG* cDNA probes indicated that the *EPLG* genes reside on the long arm of human chromosome 1 (reviewed but data not shown).

Fluorescence in situ hybridization analysis. To confirm and refine our mapping of the *EPLG1, EPLG3,* and *EPLG4* genes to chromosome 1, we performed FISH of metaphase chromosomes prepared from normal peripheral blood lymphocytes by cohybridizing genomic phage clones specific to each of the three loci (see Materials and Methods)—λ4 (*EPLG1*), λ22 (*EPLG3*), and λ17 (*EPLG4*)—with a genomic clone of p58^{clk-1} that maps to human chromosome 1p36 (Eipers *et al.,* 1992). Representative hybridizations with these clones to metaphase chromosomes are illustrated in Fig. 1; with each of the three locus-specific *EPLG* genomic clones,

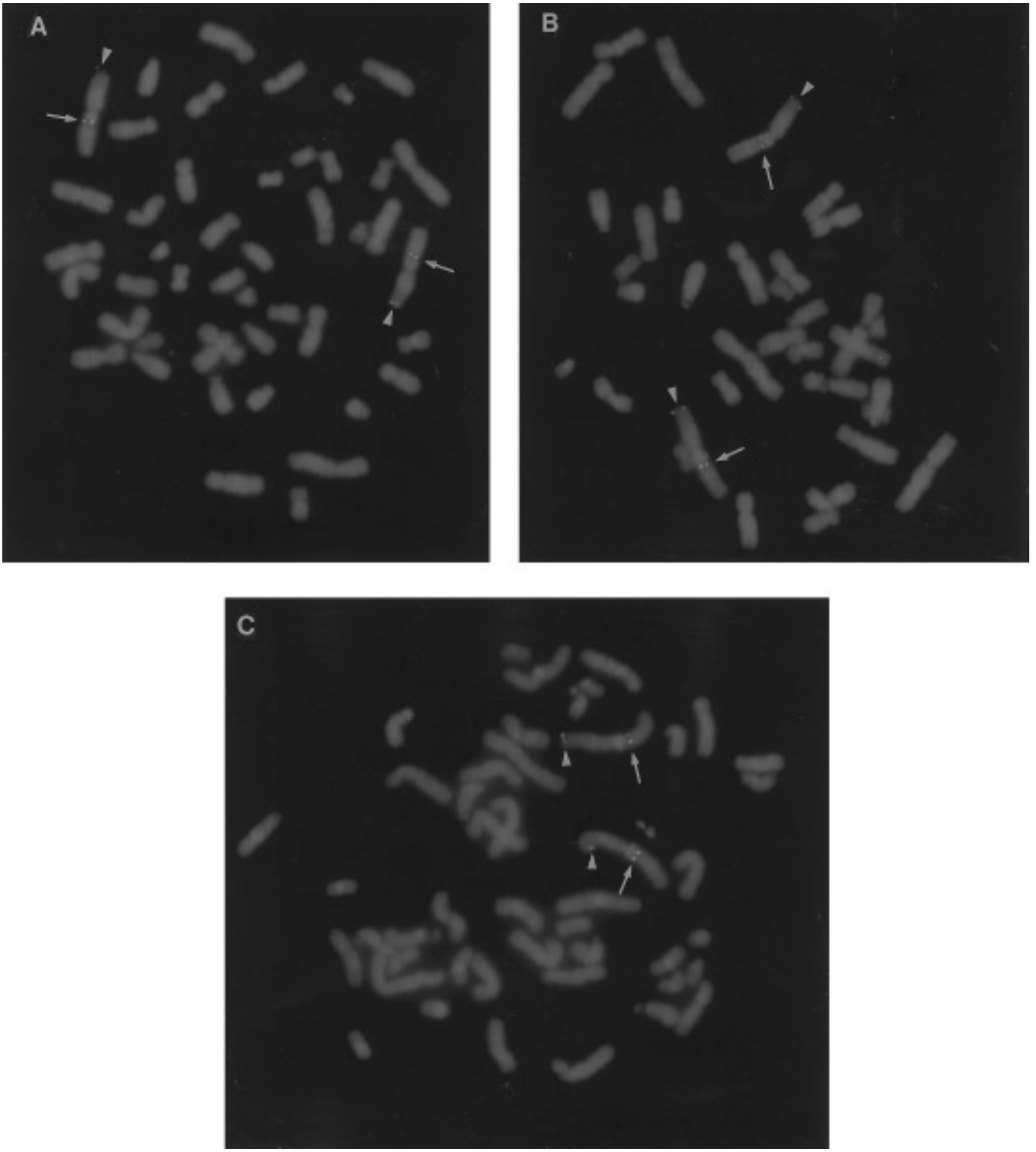

FIG. 1. Fluorescence *in situ* hybridization of human metaphase chromosomes with genomic clones specific for the (A) *EPLG1,* (B) *EPLG3,* and (C) *EPLG4* loci. Genomic λ phage clones λ4 (*EPLG1*), λ22 (*EPLG3*), and λ17 (*EPLG4)* were hybridized to normal metaphase chromosomes as described; specific hybridization of the probes (arrows) is evident in each case on chromosome 1 chromatids at identical positions just distal to the centromeric heterochromatin on the long arm in the region of bands q21–q22. A genomic clone representing the $p58^{clk-1}$ locus at 1p36 (Eipers *et al.,* 1992) was cohybridized to confirm chromosomal identity (arrowheads).

fluorescence signal was detected on the proximal long arm of chromosome 1. The positions of the fluorescent signals on chromosome 1 that were observed with each of the three *EPLG* genomic clones were cytogenetically indistinguishable (Fig. 1). Specific labeling of 64 of 66 (96%) chromatids on proximal 1q was seen in meta-

phases hybridized with clone λ4 (*EPLG1*); 61 of 68 (90%) and 69 of 70 (99%) chromosome 1 chromatids were specifically labeled at the same position when hybridized with clones λ22 (*EPLG3*) and λ17 (*EPLG4*), respectively. No nonrandom signals suggestive of alternative localization or cross-hybridization with related gene loci were observed with any of the three genomic clones. Based on the DAPI banding pattern, together with the presence of the signals just distal to the centromeric heterochromatin, we assigned the *EPLG1*, *EPLG3*, and *EPLG4* loci to the region of bands q21–q22.

The mouse chromosomal location of Epl1, Epl3, and Epl4. The mouse chromosomal location of the *Epl* loci was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 1500 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative RFLPs using human cDNA probes. *M. spretus* RFLPs (see Materials and Methods) were used to follow the segregation of each locus in backcross mice. The mapping results indicated that the *Epl1*, *Epl3*, and *Epl4* genes are tightly linked to each other and map to the central region of mouse chromosome 3 near *Ntrk1*, *Gja5*, and *Ngfb*. Although 99 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 2), up to 190 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are centromere–*Ntrk1*–2/138–*Epl1*–0/112–*Epl4*–0/147–*Epl3*–8/183–*Gja5*–8/190–*Ngfb*. The recombination frequencies (expressed as genetic distances in centimorgans ± the standard error) are *Ntrk1*–1.5 ± 1.0–(*Epl1*, *Epl4*, *Epl3*)–4.4 ± 1.5–*Gja5*–4.2 ± 1.5–*Ngfb*. That no recombination was detected between *Epl1* and *Epl4* in 112 animals typed in common and between *Epl4* and *Epl3* in 147 animals typed in common suggests that the 2 loci in each pair are within 2.6 and 2.0 cM of each other (upper 95% confidence limit), respectively.

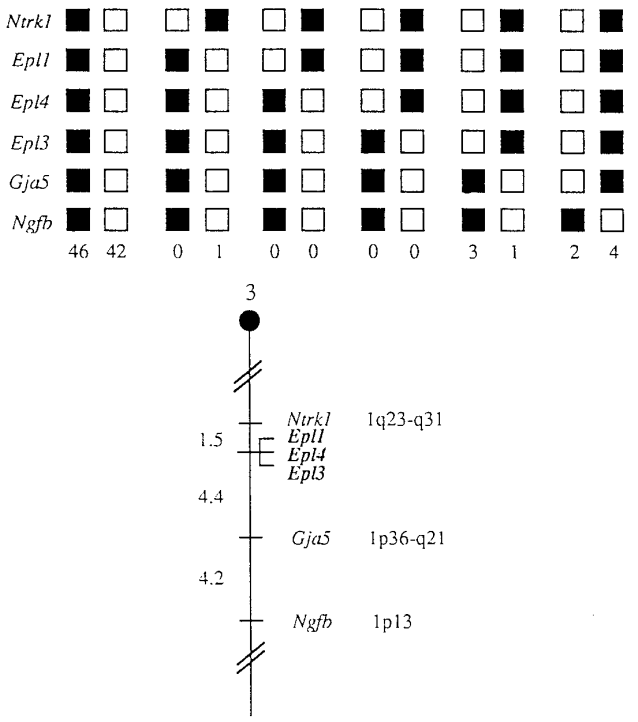

FIG. 2. *Epl1*, *Epl3*, and *Epl4* map to the central region of mouse chromosome 3. The *Epl* loci were placed on mouse chromosome 3 by interspecific backcross analysis. (Top) The segregation patterns of the *Epl* loci and flanking genes in 99 backcross animals that were typed for all loci are shown. For individual pairs of loci, more than 99 animals were typed (see text); the map distances are based on all available data for each pair of loci. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. The black boxes represent the presence of a C57BL/6J allele, and the white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. (Bottom) A partial chromosome 3 linkage map showing the location of the *Epl* loci in relation to linked genes is shown. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of the loci in human chromosomes are shown to the right. References for the human map positions of loci mapped in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

DISCUSSION

In this report, we describe the chromosomal localization of the human and mouse genes encoding three peptides, LERK-1, LERK-3, and LERK-4, recently shown to function as ligands for the receptor tyrosine kinases hek and elk (Beckmann *et al.*, 1994; Kozlosky *et al.*, 1995). These distinct gene loci, officially designated *EPLG1*, *EPLG3*, and *EPLG4*, each map to chromosome 1 in the region of bands q21–q22. Analysis of metaphase FISH results with each clone revealed essentially indistinguishable localizations, suggesting that these three genes lie in very close proximity to one another. Our mapping of these gene loci to chromosome 1q21–q22 is in agreement with their localization and tight linkage in the central portion of mouse chromosome 3. The central segment of mouse chromosome 3 shares a region of homology with human chromosome 1 (summarized in Fig. 2). In particular, *Ntrk1* has been placed on human 1q23–q31, and *Gja5* has been placed on 1p36–q12. Thus, the placement of *Epl1*, *Epl3*, and *Epl4* between *Ntrk1* and *Gja5* in the mouse is consistent with their localization to human chromosome 1. It will be of interest in future studies in both mouse and human to determine by interphase FISH measurements and long-range restriction mapping by pulsed-

field gel analysis the order of these loci and their exact physical separation on the chromosome. Recently, Shao *et al.* (1995) have also mapped B61 (*Epl1,* LERK-1) to mouse chromosome 3.

Evolutionary relatedness of *EPLG1, EPLG3,* and *EPLG4* is supported not only by their close physical localization on human chromosome 1 and mouse chromosome 3 but also by their degree of sequence homology and their functional properties. The protein products of these three genes share 38 to 44% amino acid identity, have similar predicted secondary structures, identical mechanisms of cell membrane attachment via glycosyl-phosphatidylinositol residues, and similar characteristics of binding to the hek and elk receptors (Kozlosky *et al.,* 1995). By contrast, *EPLG2* (LERK-2) and *EPLG5* (LERK-5), which are localized to human chromosome Xq13 (Fletcher *et al.,* 1994) and chromosome 13q33 (GenBank Accession No. L13819), respectively, bear significantly less homology to the other three LERKs (28 to 33% amino acid identity) than to each other (59% amino acid identity) and have divergent binding and structural characteristics (type 1 transmembrane proteins), excepting a similar secondary structure within the putative receptor binding domain.

Currently, there is no direct evidence to indicate that mutations of the *EPLG1, EPLG3,* and *EPLG4* loci result in any known human diseases. In addition, we have compared our interspecific map of chromosome 3 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (compiled by M. T. Davisson, T. H. Roderick, A. L. Hillyard, and D. P. Doolittle and provided from GBASE, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). The *Epl* loci mapped to a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in one of these genes (data not shown). However, several human abnormalities are known to colocalize to the region of chromosome 1 containing these genes. Chromosomal breakpoints in neoplastic diseases within this region occur in both hematopoietic malignancies and solid tumors with moderate frequency, but usually with varying translocation partner chromosomes (Mitelman, 1991). Nonrandom, recurrent translocations noted at 1q21 include the t(1;11)(q21;q23) found in acute nonlymphocytic leukemia and the t(1;16)(q21;q13) that has been observed in several types of solid tumors, including Ewing sarcoma and Wilm tumors (Douglass *et al.,* 1990). Nonneoplastic diseases localized to the 1q21 region include a nonprogressive congenital muscle weakness (nemaline myopathy, MIM No. 161800); a form of congenital cataracts (Coppock-type, MIM No. 116200); and a bullous skin disease (epidermolysis bullosa simplex, Koebner type, MIM No. 131900) (McKusick, 1992). A pathophysiologic role for the LERKs in any of these disorders may become evident when the biologic functions of these peptides or the eph-related receptor tyrosine kinases are more completely determined.

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