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The Mouse Prolactin Gene Family Locus

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In the mouse, there is a large family of paralogous genes closely related to PRL. The objective of this report was to investigate the organization of the mouse PRL gene family locus. PRL family genes reside on chromosome 13 of the mouse genome. The PRL gene family members were localized to a series of overlapping bacterial artificial chromosome clones and arranged based on structural relationships. Additionally, several new members of the PRL gene family were identified. Placental lactogen I (PL-I) was found to be encoded by three closely related (>98% exon sequence identity) contiguous genes (termed: PL-Iα, PL-Iβ, and PL-Iγ). Two previously unidentified mouse orthologs for members of the rat PRL family, PRL-like protein-I (PLP-I) and PLP-K were discovered, as were two new members of the PLP-C subfamily, PLP-Cγ and PLP-Cδ, and two new entirely unique members of the PRL family, PLP-N and PLP-O. Amino acid sequences predicted from the latter two genes most closely resembled proliferin-related protein. Each of the nine newly discovered genes is expressed in trophoblast cells of the mouse placenta in a gestationally specific pattern. In summary, elucidation of the mouse PRL gene family locus provides new insights into the expansion of the mouse PRL family and new tools for studying the genetics and biology of its members. (*Endocrinology* 144: 313–325, 2003)

PROLACTIN (PRL) IS a hormone/cytokine responsible for the coordination of a wide range of biological processes in vertebrates. In the mouse, rat, cow, and likely other mammalian species, there are large families of paralogous genes closely related to PRL (1). The proteins encoded by the PRL family genes have been given a variety of names, including placental lactogens (PLs), PRL-like proteins (PLPs), PRL-related proteins (PRPs), proliferin (PLF), and PLF-related protein (PLF-RP). Unfortunately, in some instances, the literature contains nomenclature that is confusing and/or incorrect. Members of the PRL family are expressed in cell- and temporal-specific patterns in the uteroplacental compartment and anterior pituitary (1). An overriding theme characteristic of the PRL family is its association with pregnancy and regulatory mechanisms controlling viviparity.

The initial identification of a substance extracted from the anterior pituitary possessing actions on the mammary gland and the initiation of lactation and its subsequent naming as PRL occurred over 70 yr ago (2–5). The notion that there may be other hormones related to PRL developed from studies with the pregnant mouse and pregnant rat. Removal of the anterior pituitary during the second half of gestation in the mouse or rat was consistent with continued development of the mammary glands and corpus luteum (another target of PRL; Refs. 6 and 7). These studies suggested an extrapituitary source of a PRL-like hormone and were followed by the discovery that extracts from the placenta possessed luteotropic (8) and mammotropic activities (9). The isolation of functional PRL mimics (PLs) from the rodent proceeded within the next few years (10–12). Their relationship with PRL was apparent following the cloning and sequence analyses of PRL cDNAs and the initial PL cDNAs (13–17). Existence of an expanded PRL family became evident during a search for mRNAs in 3T3 fibroblasts whose expression was dependent upon growth factor stimulation and as a byproduct of cloning rat placental lactogen (PL)-II. In the first instance, growth factor regulated mRNAs were identified with sequence similarity to PRL (18), and in the second instance unique cDNAs were isolated from a rat placental cDNA library with sequence similarity to the PRL sequence (19, 20). Several additional members were discovered during the characterization of known members of the PRL family at protein, cDNA, and genomic levels and through the use of degenerate probes and differential display strategies (21–32). More recent discoveries have been directly attributed to searching mouse and rat expressed sequence tag databases (33–43).

The PRL family genes in the mouse, rat, and cow appear to be clustered on chromosomes 13 (mouse: 30, 33–35, 39, 40, 44), 17 (rat: 24, 25, 27, 45–48), and 23 (cow: 49) of each respective species genome. Gene structure has been determined for a few members of the PRL family. Two conserved exon-intron arrangements are evident: 1) a prototypical five exon-four intron structure found in PRL (50–52), PL-II (48, 53), PLP-A (54), PLF (55), and two bovine PRL family members (56, 57); and 2) a six exon-five intron structure present in members of the PLP-C subfamily, including PLP-C variant (v) (27), PLP-Cα (30), and decidual (d)PRP (58). The latter exon/intron arrangement represents the addition of a short exon between exons II and III of the prototypical PRL exon/intron organization. Based on amino acid similarities, the extra exon is likely present in all members of the PLP-C subfamily, and PLF-RP. PLP-E and PLP-F may possess a nonhomologous exon situated in the same region (37).

In the present study, we have used information derived from the public mouse genome database as a tool for identifying several new members of the mouse PRL family. We

Abbreviations: BAC, Bacterial artificial chromosome; d, decidual; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PLF, proliferin; PLF-RP, PLF-related protein; PL-I or -II, placental lactogen (I or II); PLP, PRL-like protein; PRL, prolactin; PRP, PRL-related protein; SSC, standard saline citrate; v, variant.

report the identification of nine new members of the PRL family and their expression patterns, and some insights into the organization of the PRL family locus on chromosome 13.

Materials and Methods

Animals and tissue preparation

CD-1 mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). The animals were housed in an environmentally controlled facility, with lights on from 0600–2000 h, and allowed free access to food and water. Timed pregnancies were generated and tissue dissections were performed as previously described (35). The presence of a copulatory plug was designated as d 1 of pregnancy. Protocols for the care and use of animals were approved by the University of Kansas Animal Care and Use Committee.

Conceptuses with associated uteri were removed on d 11, 13, 16, and 19 of gestation. Tissues were frozen in dry ice-cooled heptane and stored at −80 C until used for *in situ* hybridization. Alternatively, placental tissues were dissected free from maternal and fetal structures, immediately frozen in liquid nitrogen, and stored at −80 C until processed for RNA analysis.

Blastocysts were isolated by flushing uterine horns on d 4 of gestation, washed, transferred to 35-mm dishes, and cultured in Roswell Park Memorial Institute 1640 medium in a humidified incubator at 37 C with an atmosphere of 95% air/5% CO2. The culture medium was supplemented with antibiotics, 1 mM glutamine, 1 mM sodium pyruvate, and 20% fetal bovine serum. Blastocysts hatched, attached, and demonstrated a significant outgrowth within a few days of the initiation of culture. Outgrowths were harvested following 6 d of culture.

Cloning and characterization of the PRL family cDNAs

Genes for new members of the PRL family were identified by BLAST analysis (59) using nucleotide sequences of previously identified members of the PRL family (Ref. 1; and Table 1) against the public mouse genome assembly (http://mouse.ensembl.org and http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html). Members of the PRL family were found on several overlapping bacterial artificial chromosome (BAC) clones localized to chromosome 13 (RP23–351I6, RP23–189A16, RP23–122C3, RP23–142M3, RP23–117C5, RP23–20A22, RP23–231P12; Children's Hospital-BACPAC Resources, Oakland, CA). Genomic sequences possessing similarity to members of the PRL family were used to search the mouse expressed sequence tag database (National Center for Biotechnology Information; Ref. 59). One cDNA (PLP-O; clone no. 5280049R, BG077704) was obtained from the American Type Tissue Collection (ATCC, Manassas, VA) and another cDNA (PLP-Cγ; Riken No. 1600019J01, AV036496) from the Riken Institute (Ibaraki, Japan; Ref. 60). The remaining cDNAs were isolated by RT-PCR from total RNA extracted with the TRIzol reagent (61) from mouse blastocyst outgrowths or placental tissues. Five micrograms of total RNA and 0.5 μg of oligo-deoxythymidine were used for reverse transcription reactions. PCRs were performed using *Pfu* polymerase with sets of primers based on genomic sequences (Table 2). PCR was performed for 30 cycles (denature, 95 C for 1 min; anneal, 55 C for 1 min; extension, 72 C for 1.5 min). Amplified products were subcloned into pCRII-TOPO vector flanked by SP6 and T7 promoters with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). cDNAs were sequenced by the Biotechnology Support Facility of the University of Kansas Medical Center. Nucleotide and amino acid sequence comparisons were performed with CLUSTAL W (version 1.8; Ref. 62). The location of signal peptides was determined by homology and the SignalP software program (version 2.0.b2; Ref. 63).

TABLE 1. Mouse PRL gene family

	PRL family	Other names	GenBank accession no.	Reference
1	PRL		X02892	14
2	PL-Iα	Midpregnancy lactogen; PL-I; PL-Im	M35662, AF525162	17; Present study
3	PL-Iβ		AF525160	Present study
4	PL-Iγ		AF525161	Present study
5	PLP-J	PLP-I, Decidualin, Prlpj	AB019118	39, 40
6	PL-II	PL	M14647	15
7	PLP-I	PLP-H	AF525154	Present study
8	PLP-B	Prlpb	AF015563	34, 36
9	DPRP	D/tPRP	AF015729	34, 35
10	PLP-K	Prlpk	AF525155	Present study
11	PLP-Cγ	Prlpc3	AF466150	Present study
12	PLP-Cβ	Prlpc2	AF158744	32
13	PLP-Cδ		AF525158	Present study
14	PLP-Cα	Prlpc1	AF090140	30
15	PLP-N		AF525156	Present study
16	PLP-E	PLP-G, Prlpe	AF020525	33, 37
17	PLP-F	Prlpf	AF020524	33, 37
18	PLP-O		AF525157	Present study
19	PLF-RP	PRP	X02594	21
20	PLF-1	MRP-1	K02245	18
21	PLF-2	MRP-2	K03235	66
22	PLF-3	MRP-3	NM_011954	55
23	PLF-4	MRP-4	AF128884	67
24	PLP-M	Prlpm	AF234636	40
25	PLP-A	Prlpa	AF015562	34, 36
26	PLP-L		AF226611	43

d/tPRP or dPRP, Decidual/trophoblast PRL-related protein; MRP, mitogen-regulated protein; PLF-RP or PRP, PLF-related protein.

Analysis of mRNA expression

RT-PCR restriction enzyme analysis of PL-I-related genes. PL-I-related cDNAs were amplified from blastocyst outgrowths or placentas from d 10 of gestation. Amplified products were digested with restriction enzymes capable of differentially cutting PL-Iα, PL-Iβ, and/or PL-Iγ. *Bsa*J1 digests each of the PL-I cDNAs at nucleotide 136 and PL-Iα uniquely at nucleotide 420. *Nla*III digests each of the PL-I cDNAs at nucleotide 140 and 740; PL-Iβ and PL-Iγ are additionally cut at nucleotide 324. The digested products were resolved by electrophoresis in 2% agarose gels and ethidium bromide staining.

Northern blot analysis. Northern blots were performed as previously described (34, 64). RNA was extracted from tissues using TRIzol (61). Total RNA (20 μg) was separated on 1% formaldehyde-agarose gels and transferred to nylon membranes. Blots were probed with [^{32}P]-labeled cDNAs for PLP-Cβ, PLP-Cγ, PLP-Cδ, PLP-I, PLP-K, PLP-L, PLP-N, or PLP-O. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was used as an internal reference to ensure integrity of the RNA samples.

In situ hybridization. PRL family mRNAs were detected in placental tissues using nonradioactive *in situ* hybridization as previously described (65). Plasmids containing cDNAs for PLP-Cγ, PLP-Cδ, PLP-I, PLP-K, PLP-L, PLP-N, and PLP-O were linearized and used as templates to synthesize sense and antisense digoxigenin-labeled riboprobes according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). Tissue sections were air dried and fixed in ice-cold 4% paraformaldehyde in PBS for 15 min. Prehybridization was carried out in a humidified chamber at 50 C in 5× SSC (standard saline citrate), 50% deionized formamide, 1× Denhardt's reagent, 10% dextran sulfate, and salmon sperm DNA (100 μg/ml). Hybridizations were performed in the same incubation conditions overnight. Slides were washed in 2× SSC at room temperature for 30 min followed by treatment with ribonuclease A (100 ng/ml) and additional washes with 2× SSC for 30 min at room temperature, followed by washes with 2× SSC for 1 h at 65 C and 0.1 × SSC for 1 h at 65 C. Tissue sections were blocked for 30 min and incubated with alkaline phosphatase-conjugated antidigoxigenin antibody (1:500) in blocking buffer (Roche Molecular Biochemicals) for 2 h at room temperature. Slides were then washed and detection was performed using nitro blue tetrazolium (250 μg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (225 μg/ml; Roche Molecular Biochemicals).

Results

Mouse PRL family gene locus

BLAST analysis of the public mouse genome assembly (http://mouse.ensembl.org and http://www.ncbi.nlm.

TABLE 2. Primer sets used for isolating PRL family cDNAs

Gene	Forward primer	Reverse primer
PL-I	5′-CTCACTTGGAGCCTACATTGT-3′	5′-GGGGAAAGCATTACAAGTC-3′
PLP-Cδ	5′-CTTAGGAAGTCCTCAAA-3′	5′-CACAGGGTAGGTGTTCC-3′
PLP-I	5′-TGTGTATTCCATGCCCATGTG-3′	5′-ATTCATTATATATGTGCATGC-3′
PLP-K	5′-TAGGTGTGACTTCTCAGC-3′	5′-GAAGCAGGATATATAAACC-3′
PLP-N	5′-AGGACACCAGTTTAGCAG-3′	5′-GGATAGCTTCAAATGGGA-3′

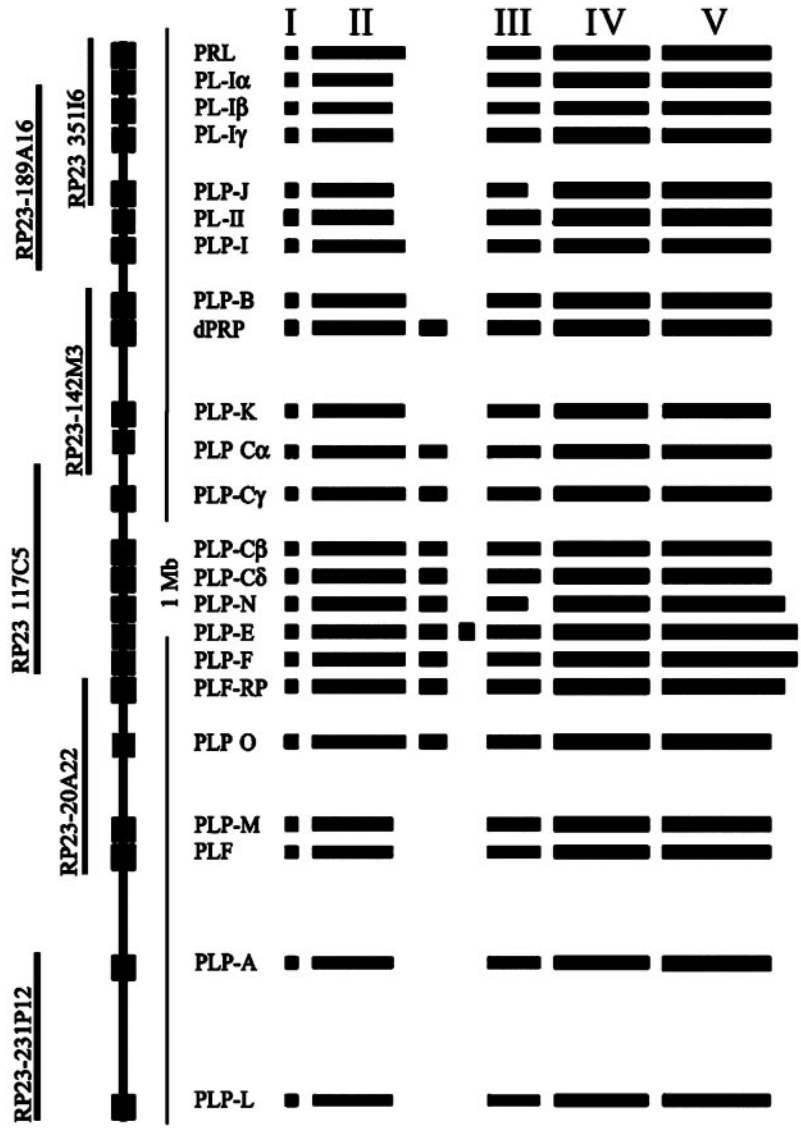

FIG. 1. Alignment of the mouse PRL family locus. The alignments of PRL family genes and BAC clones spanning the PRL family locus were determined by analysis of BAC clone sequences (RP23–351I6, AL591943; RP23–189A16, AL589679; RP23–142M3, AL592443; RP23–117C5, AL590522; RP23–20A22, AL627326; RP23–231P12, AL590616). The entire PRL gene family locus spans approximately 1 megabase. Three exon-intron organization patterns were observed: 1) five exon-four intron (PRL, PL-I subfamily, PLP-J, PL-II, PLP-I, PLP-B, PLP-K, PLP-M, PLF, PLP-A, PLP-L); 2) six exon-five intron (dPRP, PLP-C subfamily, PLP-N, PLP-F, PLP-O, PLF-RP); 3) seven exon-six intron (PLP-E). Exon lengths are only rough approximations. The beginning of exon-I was defined as the location of the translation start codon, and the end of the last exon was defined as the location of the translation stop codon.

nih.gov/genome/seq/MmBlast.html) with cDNA sequences for members of the mouse PRL family (Table 1) resulted in the identification of several overlapping BAC clones (RP23–351I6, RP23–189A16, RP23–142M3, RP23–117C5, RP23–20A22, RP23–231P12) containing PRL family genes. Each of the BACs is localized to chromosome 13. The entire locus spans approximately 1 megabase. RP23–351I6 contains PRL, three genes closely related to PL-I, and PLP-J. Two complete PL-I genes, PLP-J, PL-II, and a gene orthologous to rat PLP-I are situated on RP23–189A16. RP23–142M3 possesses PLP-B, dPRP, a gene orthologous to rat PLP-K, and PLP-Cα. Two PLP-Cα related genes, PLP-Cβ, a PLF-RP-related gene, PLP-E, and PLP-F were found on RP23–117C5. RP23–20A22 contains PLF-RP, a gene related to PLF-RP, PLP-M, and a PLF gene, whereas PLP-A and PLP-L were localized to RP23–231P12. The alignments of the BAC clones and the PRL family genes and their relationships to one another are presented in Figs. 1 and 2. The three PL-I-related genes were termed PL-Iα, PL-Iβ, and PL-Iγ. The two mouse genes similar to PLP-Cα are referred to as PLP-Cγ and PLP-Cδ and the two genes possessing similarity to PLF-RP were named PLP-N and PLP-O. Because gaps are present in the current BAC clone map, it is possible that additional members of the PRL family may exist. At least one of these

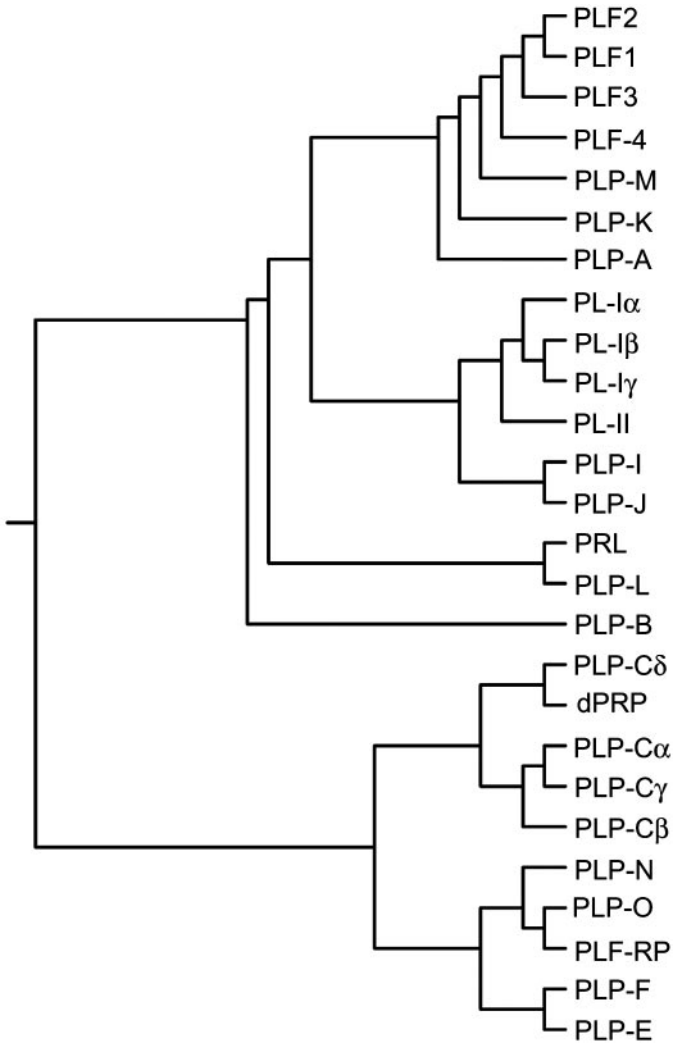

FIG. 2. The mouse PRL family tree. Comparisons of the 26 paralogous mouse PRL family cDNAs and phylogenetic tree construction were performed using CLUSTAL W (version 2.0) software program (62). See Table 1 for GenBank accession numbers and nomenclature.

gaps is situated between BAC clones, RP23–20A22, and RP23–231P12, and near the PLF gene cluster. Thus far, we have only identified a single PLF gene from a subfamily of at least four highly related PLF genes. The sequence of the identified PLF gene is very similar but different than each of the known sequences for PLF-1, PLF-2, PLF-3, and PLF-4 (18, 55, 66, 67), suggesting the PLF cluster may consist of at least five related genes.

Physical relationships of members of the PRL family within the mouse PRL family locus were best determined by sequence and structural similarities. In general, genes with greater sequence and structural similarity were located in closer proximity. This was best typified by the tandem alignment of the three PL-I related genes and the tandem alignment of the four PLP-C related genes. There are exceptions to this rule. For example, PRL and PLP-L possess considerable sequence similarities; however, they are situated at opposite ends of the locus. There was also a strong trend toward clustering of genes based on their exon-intron organization. Five exon-four intron gene clusters flanked a centrally located cluster of six/seven exon-five/six intron genes.

All PRL family genes contain five conserved exons. A subset contains an additional exon(s) situated between exons II and III of the conserved structure. dPRP, PLP-C-related genes, PLP-N, PLP-F, PLF-RP, and PLP-O contains a single extra exon, whereas PLP-E has two additional exons in this region.

PL-I subfamily: structure and expression

The mouse PL-I cDNA was cloned several years ago (17). In the original report describing the cloning of the mouse PL-I cDNA, the authors noted a complex Southern blot analysis and suggested that there may be multiple PL-I genes. As revealed from sequence analysis of two overlapping BAC clones, RP23–351I6 and RP23–189A16, there are three closely related PL-I genes that we have named PL-Iα, PL-Iβ, and PL-Iγ. Each of the PL-I cDNAs was cloned by PCR from either RNA isolated from blastocyst outgrowths or from gestation d 10 placentas and sequenced (Fig. 3). Two PL-Iα, three PL-Iβ, and four PL-Iγ cDNA clones were isolated from blastocyst outgrowths, whereas six PL-Iα and one PL-Iγ cDNA clones were isolated from gestation d 10 placenta. PL-Iα, PL-Iβ, and PL-Iγ nucleotide and predicted amino acid sequences exhibited more than 98% sequence identity when compared with one another. The predicted amino acid sequence of PL-Iα is identical to the predicted amino acid sequence for the originally cloned PL-I cDNA except for the absence of an amino acid in the signal peptide (Ala^{-20}; Ref. 17). PL-Iα differed from PL-Iβ at three amino acid residues (Thr^{+69} *vs.* Ile^{+69}; Val^{+119} *vs.* Ile^{+119}; Ser^{+147} *vs.* Thr^{+147}) and differed from PL-Iγ at five amino acid residues (Leu^{-7} *vs.* Phe^{-7}; Asp^{+10} *vs.* Glu^{+10}; Asn^{+22} *vs.* Ser^{+22}; Thr^{+69} *vs.* Ile^{+69}; Val^{+119} *vs.* Ile^{+119}). PL-Iβ and PL-Iγ differed at four amino acid residues (Leu^{-7} *vs.* Phe^{-7}; Asp^{+10} *vs.* Glu^{+10}; Asn^{+22} *vs.* Ser^{+22}; Thr^{+147} *vs.* Ser^{+147}).

The cloning results suggested that there might be differential regulation of the three PL-I genes during development. Consequently, we established an RT-PCR-restriction enzyme-based assay to distinguish the three PL-I related transcripts (Fig. 4). *Bsa*J1 and *Nla*III restriction enzymes were used to discriminate PL-Iα from PL-Iβ and PL-Iγ. PL-Iα was expressed in both blastocyst outgrowths and in d 10 mouse placentas (Fig. 4). PL-Iβ and PL-Iγ transcripts were only detectable in RNA from blastocyst outgrowths (Fig. 4). These results are consistent with the cloning data and reinforce the notion that all three PL-I genes are expressed and exhibit different patterns of regulation.

Identification of new PRL family genes

Searching the mouse genomic database resulted in the discovery of several previously unidentified members of the PRL family.

PLP-I. Perusal of mouse genomic sequences revealed the existence of a mouse ortholog for rat PLP-I on BAC clone, RP23–189A16 (38). A mouse PLP-I cDNA was cloned by RT-PCR from gestation d 19 mouse placenta, sequenced, and compared with rat PLP-I (Fig. 5). Upon careful examination,

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                  -28
PL-Iα             MQLTLTLSGSGMQLLLLVSSLLLWENVS SKPTAMVPTDDLYTRLAELSHNTFILAADVYR   32
PL-Iβ             MQLTLTLSGSGMQLLLLVSSLLLWENVS SKPTAMVPTDDLYTRLAELSHNTFILAADVYR   32
PL-Iγ             MQLTLTLSGSGMQLLLLVSSLFLWENVS SKPTAMVPTEDLYTRLAELSHSTFILAADVYR   32
                  ********************* ****** ********* *********** **********

PL-Iα             EFDLDFFDKTWITDRTLPLCHTASIHTPENREEVHETKTEDLLKAMINVSISWKEPLKHL    92
PL-Iβ             EFDLDFFDKTWITDRTLPLCHTASIHTPENREEVHEIKTEDLLKAMINVSISWKEPLKHL    92
PL-Iγ             EFDLDFFDKTWITDRTLPLCHTASIHTPENREEVHEIKTEDLLKAMINVSISWKEPLKHL    92
                  ************************************* ***********************

PL-Iα             VSALTALPGASESMGKKAADIKGRNLVILEGLQTIYNRTQANIEENENFDYPAWSGLEEL   152
PL-Iβ             VSALTALPGASESMGKKAADIKGRNLIILEGLQTIYNRTQANIEENENFDYPAWTGLEEL   152
PL-Iγ             VSALTALPGASESMGKKAADIKGRNLIILEGLQTIYNRTQANIEENENFDYPAWSGLEEL   152
                  ************************** *************************** *****

PL-Iα             QSPNEDTHLFAVYNLCRCIKRDIHKIDSYIKVLRCRVVFQNEC                    195
PL-Iβ             QSPNEDTHLFAVYNLCRCIKRDIHKIDSYIKVLRCRVVFQNEC                    195
PL-Iγ             QSPNEDTHLFAVYNLCRCIKRDIHKIDSYIKVLRCRVVFQNEC                    195
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FIG. 3. Predicted amino acid sequences for mouse PL-Iα, PL-Iβ, and PL-Iγ. An *arrow* indicates the predicted signal peptide cleavage sites. The identity of these sites as the cleavage sites is based on the SignalP software program and similarities with other members of the PRL family. Note similarities in the positioning of cysteine residues (shown as *outlined boxes*) and N-linked glycosylation sites (*gray shaded boxes*). *Asterisks below the sequences* denote identity.

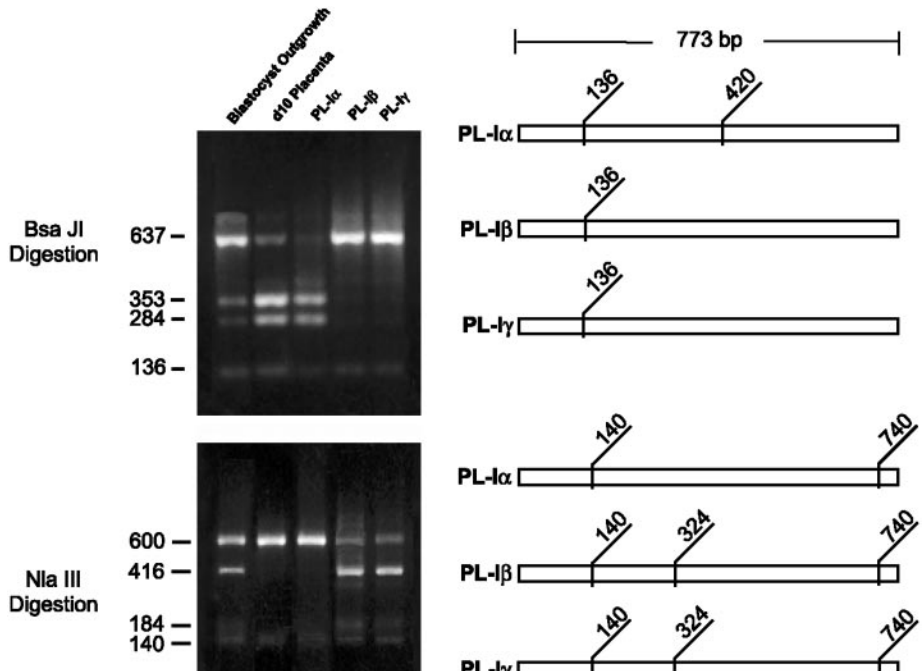

FIG. 4. Analysis of PL-Iα, PL-Iβ, and PL-Iγ expression in developing trophoblast. PL-I related cDNAs were amplified from blastocyst outgrowths or placentas from d 10 of gestation. Amplified products were digested with restriction enzymes capable of differentially cutting PL-Iα, PL-Iβ, and/or PL-Iγ. *Bsa*J1 digests each of the PL-I cDNAs at nucleotide 136 and PL-Iα uniquely at nucleotide 420. *Nla*III digests each of the PL-I cDNAs at nucleotide 140 and 740; PL-Iβ and PL-Iγ are additionally cut at nucleotide 324. The digested products were resolved by electrophoresis in 1% agarose gels and ethidium bromide staining. Blastocyst outgrowths preferentially expressed PL-Iβ and PL-Iγ and modest amounts of PL-Iα. In contrast, d 10 placenta primarily expressed PL-Iα and modest if any PL-Iβ and PL-Iγ.

we noted two errors in the original published sequence for rat PLP-I (38). Two extra nucleotides were found in the 5′-region of the cDNA resulting in two separate frame shift errors in the corresponding amino acid sequence (corrected PLP-I GenBank accession no. AF526270). Mouse PLP-I is 83% and 75% identical to rat PLP-I at nucleotide and amino acid levels. Mouse and rat PLP-I each possess a predicted 29 amino acid signal peptide; share the placement of six cysteine residues, and two conserved putative N-linked glycosylation sites. PLP-I most closely resembled PLP-J and was situated near the PL genes and PLP-J gene in the PRL gene family locus.

PLP-K. A mouse ortholog for rat PLP-K was identified based on nucleotide and predicted amino acid sequence similarities on BAC clone, RP23–142M3 (38, 40). A cDNA corresponding to mouse PLP-K was cloned by RT-PCR from gestation d 16 mouse placentas and sequenced (Fig. 6). Mouse PLP-K is 87%

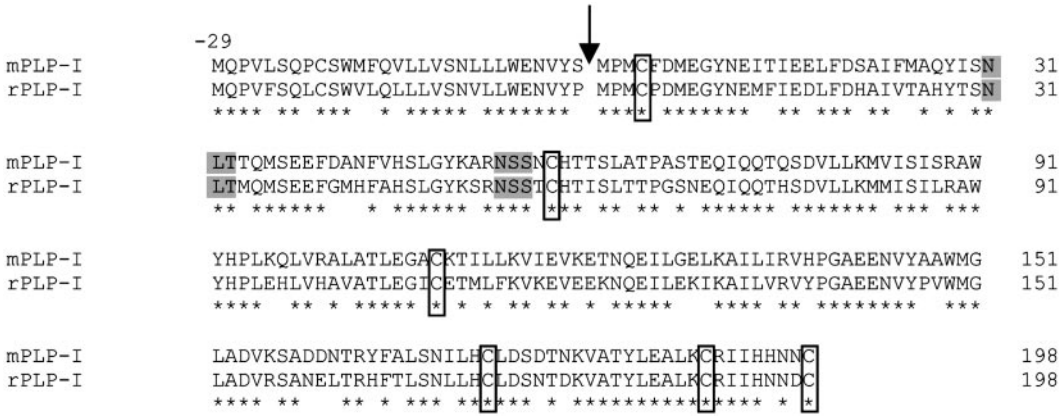

FIG. 5. Amino acid sequence comparison of mouse and rat PLP-I. The rat PLP-I sequence has been corrected from that originally published (Ref. 38; corrected rat PLP-I GenBank accession no. AF526270). An *arrow* indicates the predicted signal peptide cleavage sites. The identity of these sites as the cleavage sites is based on the SignalP software program and similarities with other members of the PRL family. Note similarities in the positioning of cysteine residues (shown as *outlined boxes*) and N-linked glycosylation sites (*gray shaded boxes*). *Asterisks below the sequences* denote identity.

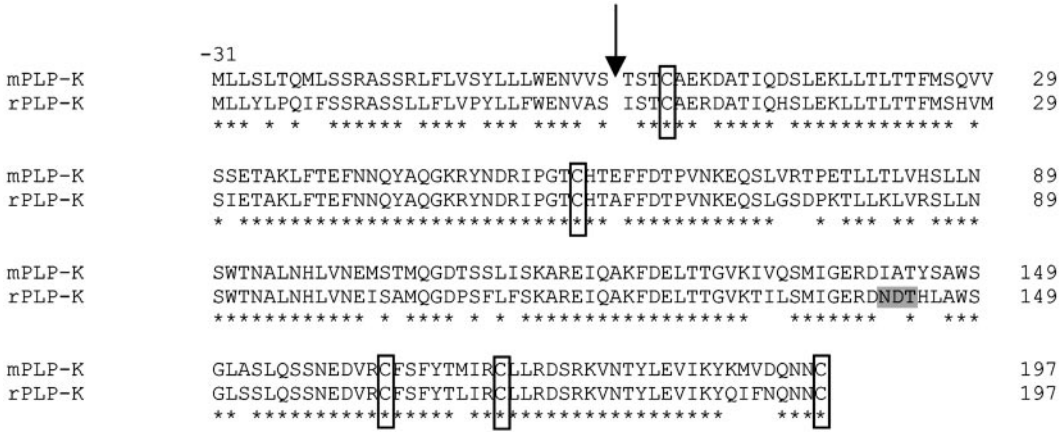

FIG. 6. Amino acid sequence comparison of mouse and rat PLP-K (Ref. 38, rat PLP-K GenBank accession no. AF234635). An *arrow* indicates the predicted signal peptide cleavage sites. The identity of these sites as the cleavage sites is based on the SignalP software program and similarities with other members of the PRL family. Note similarities in the positioning of cysteine residues (shown as *outlined boxes*). Rat PLP-K possesses a single predicted N-linked glycosylation site (denoted as a *gray shaded box*), whereas mouse PLP-K lacks putative N-linked glycosylation sites. *Asterisks below the sequences* denote identity.

and 83% identical to rat PLP-K at nucleotide and amino acid levels, respectively. Mouse and rat PLP-K each possess a predicted 31 amino acid signal peptide and five conserved cysteine residues. Rat PLP-K contains a single putative N-linked glycosylation site, whereas mouse PLP-K lacks apparent sites for the addition of N-linked carbohydrate groups. As previously reported, PLP-K bears the greatest similarity to PLP-A, PLP-M, and PLF. Unlike most other members of the PRL family, the location of the PLP-K gene within the mouse PRL gene family locus does not directly correspond with its structural similarities to its nearest neighbors.

PLP-Cγ and PLP-Cδ. Based on sequence analyses of BAC clone, RP23–117C5, two new members of the mouse PLP-C subfamily were identified and named PLP-Cγ and PLP-Cδ. The PLP-Cγ clone was obtained from the Riken Institute and the PLP-Cδ cDNA was cloned by RT-PCR from gestation d 19 mouse placentas. Both cDNAs were sequenced and their predicted amino acid sequences compared with amino acid sequences for other members of the PLP-C subfamily (Fig. 7). PLP-Cγ and PLP-Cδ genes are localized to a region of the PRL family locus near three other related genes, dPRP, PLP-Cα, and PLP-Cβ. Overall predicted amino acid sequence identities among the PLP-C subfamily range from 51–66% (Fig. 7). Each member of the subfamily contains a 28–30 amino acid signal peptide, six conserved cysteine residues, and a putative N-linked glycosylation site. PLP-Cβ, PLP-Cδ, and dPRP each possess an additional putative N-linked glycosylation site.

PLP-N and PLP-O. Two additional new members of the mouse PRL gene family were identified on two overlapping BAC clones, RP23–117C5 and RP23–20A22. The PLP-N cDNA was cloned by PCR from gestation d 19 mouse placentas, and the PLP-O cDNA was obtained from the ATCC. Both cDNAs were sequenced and relationships of their nucleotide and predicted amino acid sequences determined (Fig. 8). Based on amino acid sequence homologies PLP-N and PLP-O both showed some similarities to PLF-RP (Fig. 8).

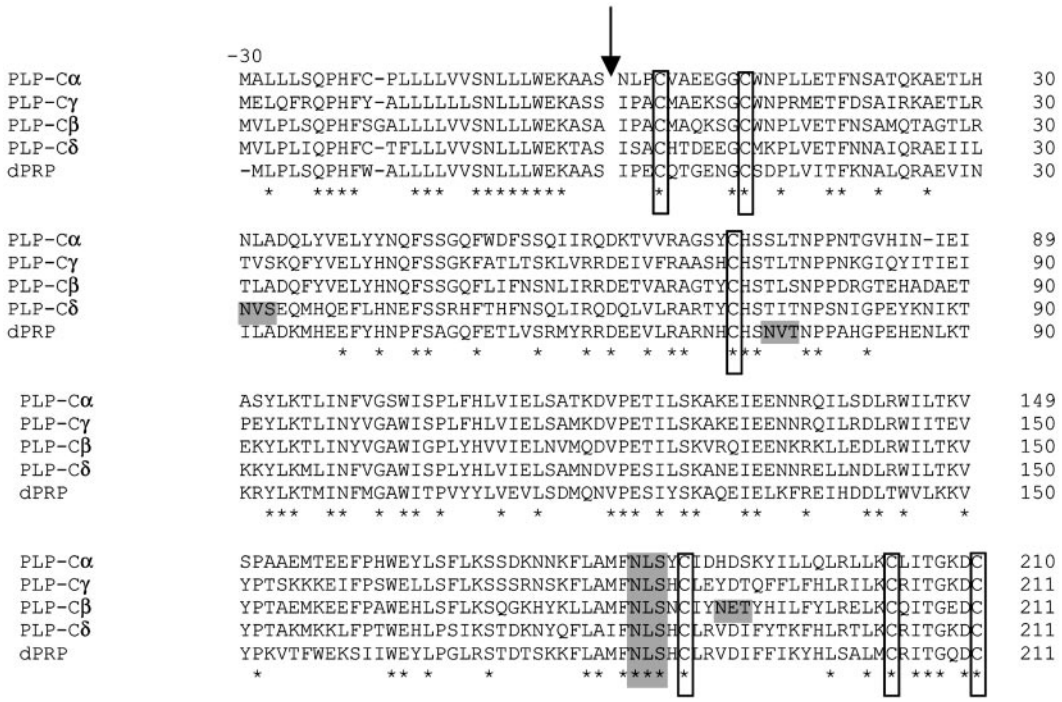

FIG. 7. Amino acid sequence comparison of members of the PLP-C subfamily. An *arrow* indicates the predicted signal peptide cleavage sites. The identity of these sites as the cleavage sites is based on the SignalP software program and similarities with other members of the PRL family. Similarities in the positioning of cysteine residues are shown as *outlined boxes*. Putative N-linked glycosylation sites are denoted as *gray shaded boxes*. *Asterisks below the sequences* denote identity.

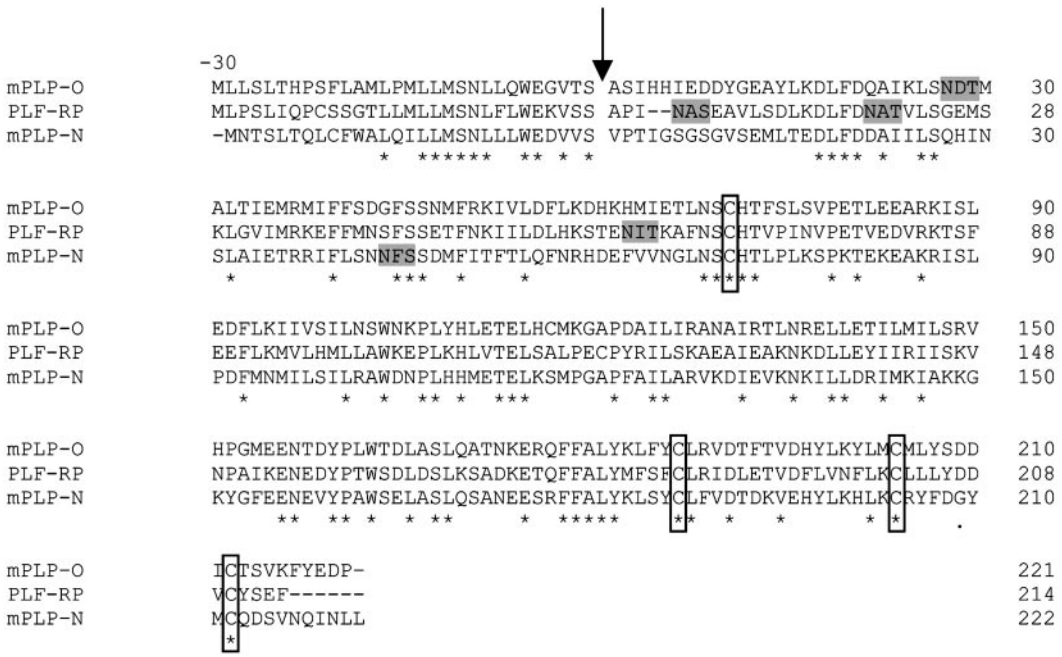

FIG. 8. Amino acid sequence comparison of PLP-N, PLP-O, and PLF-RP. An *arrow* indicates the predicted signal peptide cleavage sites. The identity of these sites as the cleavage sites is based on the SignalP software program and similarities with other members of the PRL family. Similarities in the positioning of cysteine residues are shown as *outlined boxes*. Putative N-linked glycosylation sites are denoted as *gray shaded boxes*. *Asterisks below the sequences* denote identity.

PLP-N and PLP-O predicted amino acid sequences are 38% and 46% identical to PLF-RP, respectively. The putative proteins encoded by PLP-N, PLP-O, and PLF-RP each possesses a predicted signal peptide region (29–30 amino acids) and four conserved cysteine residues. PLP-N and PLF-RP have a fifth cysteine residue situated in the middle of their predicted protein sequences. PLP-N and PLP-O each have a single putative N-linked glycosylation site within the amino-

terminal half of the proteins, whereas PLF-RP contains three putative sites for N-linked addition of carbohydrate within this region.

Expression of the new PRL family genes

The discovery of several new members of the PRL family prompted an investigation into whether they were expressed. We initiated our search with the placenta. mRNAs for the newly identified members of the PRL family were examined in placental tissues harvested from the second half of gestation (d 11, 13, 16, and 19) by Northern analysis (Figs. 9 and 10) and *in situ* hybridization (Fig. 11). PLP-Cβ and PLP-L transcripts were determined for comparative purposes. The expression pattern of mouse PLP-L has not been previously reported.

Each of the newly identified members of the PRL family was expressed in the mouse placenta as determined by Northern analysis (Fig. 9). The temporal patterns of expres-

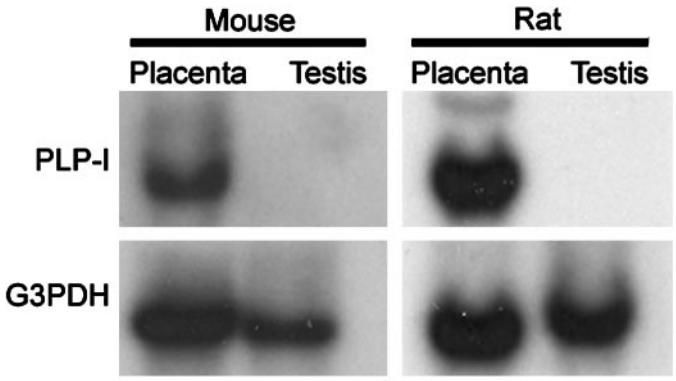

FIG. 10. Examination of PLP-I mRNA expression in adult mouse and rat testes. Total RNA was collected from placental tissues (d 19 mouse; d 20 rat) and adult mouse and rat testes. PLP-I mRNA concentrations were estimated by Northern blot analysis. G3PDH was used as a control to demonstrate loading accuracy and the integrity of the RNA.

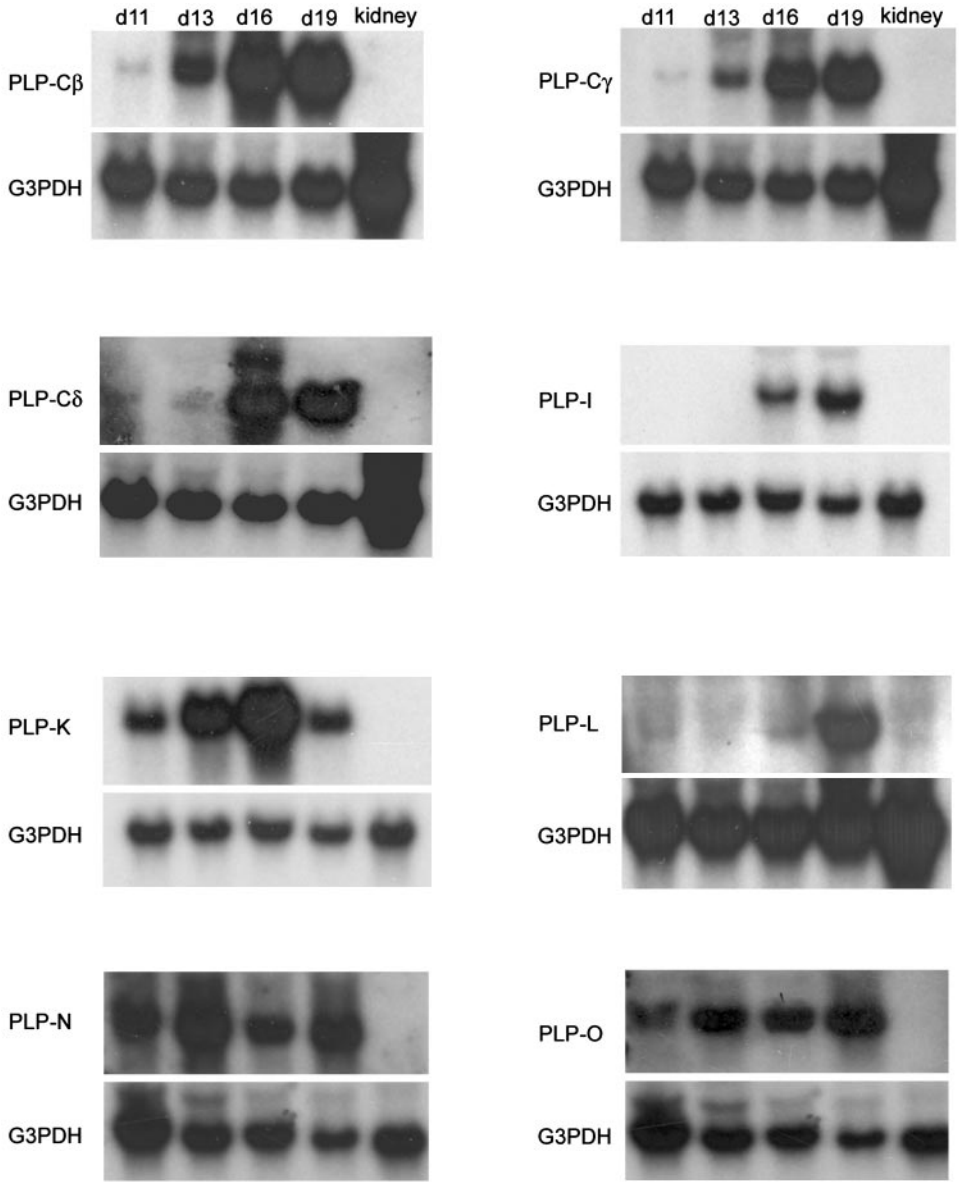

FIG. 9. Northern blot analyses of PLP-Cβ, PLP-Cγ, PLP-Cδ, PLP-I, PLP-K, PLP-L, PLP-N, and PLP-O expression during gestation in the mouse. Total RNA was collected from placental tissues (d 11, 13, 16, and 19). The relative levels of PLP-Cβ, PLP-Cγ, PLP-Cδ, PLP-I, PLP-K, PLP-L, PLP-N, and PLP-O mRNAs in mouse placental tissues were determined by Northern blot analysis. G3PDH was used as a control to demonstrate loading accuracy and the integrity of the RNA. Kidney tissues served as a negative control.

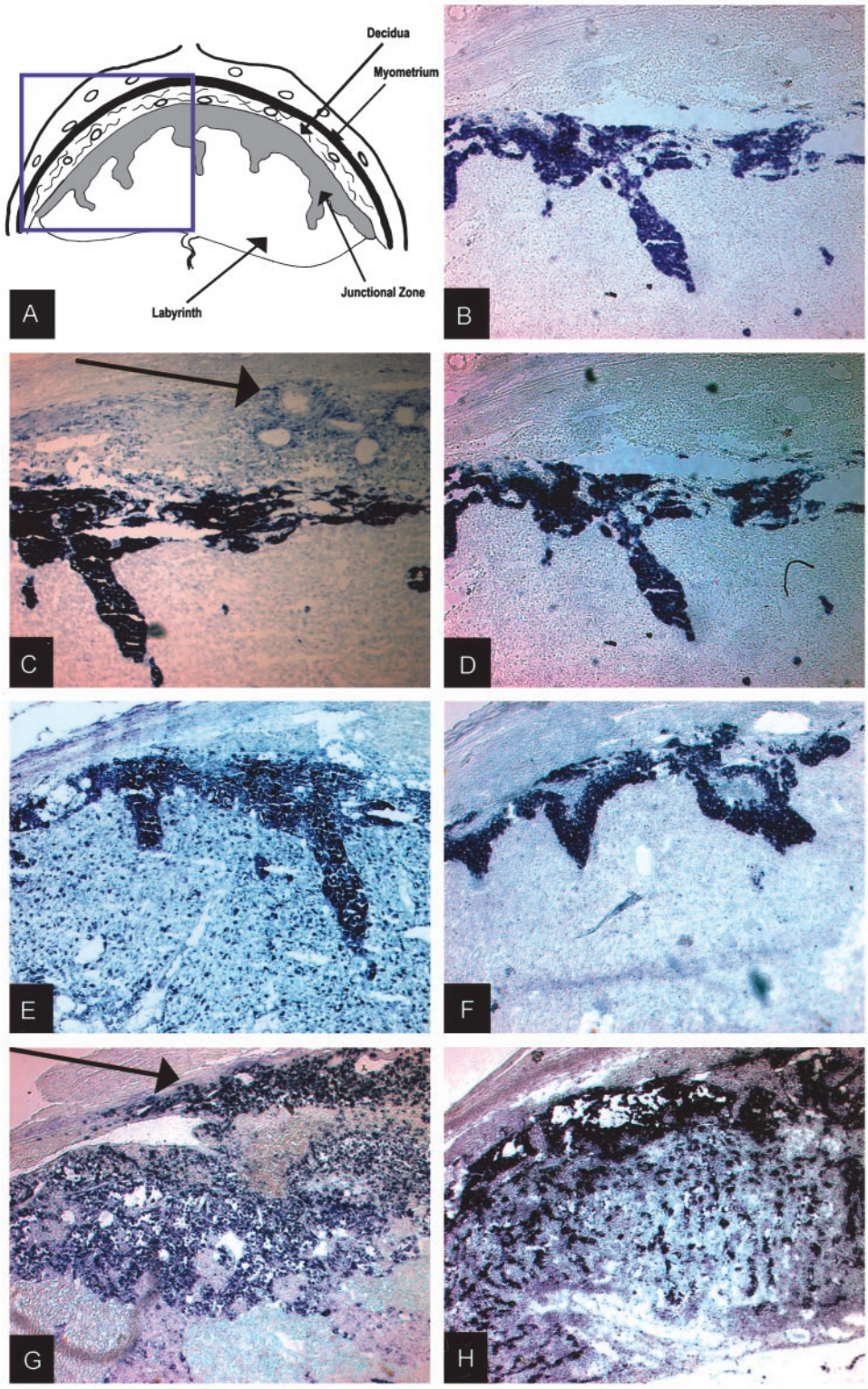

FIG. 11. Cell and tissue-specific localization of PLP-Cγ, PLP-Cδ, PLP-I, PLP-K, PLP-L, PLP-N, and PLP-O in mouse placental tissues. The *in situ* detection of mRNA expression was performed on frozen tissue sections. cDNAs were used as templates for the synthesis of digoxigenin-labeled sense and antisense RNA probes. A, Schematic diagram of the mature rodent placenta; the *boxed area* represents the histological sections presented in panels B–H. B, PLP-Cγ antisense probe on a d 19 placental section; C, PLP-Cδ antisense probe on a d 19 placental section; the *arrow* depicts the location of migratory trophoblast; D, PLP-I antisense probe on a d 19 placental section; E, PLP-K antisense probe on a d 19 placental section; F, PLP-L antisense probe on a d 19 placental section; G, PLP-N antisense probe on a d 19 placental section; the *arrow* depicts the location of migratory trophoblast located in the decidua and myometrium. H, PLP-O antisense probe on a d 13 placental section. Please note that sense probes did not provide detectable hybridization in any of the tissues investigated. Magnification, ×40.

sion differed. PLP-Cβ, PLP-Cγ, PLP-Cδ, PLP-I, and PLP-L transcripts showed increases in abundance as gestation advanced, whereas PLP-N and PLP-O mRNAs were detectable throughout the second half of gestation. None of the PRL family mRNAs tested were expressed in the kidney.

In an earlier report, PLP-I mRNA was detected in the rat testis (38). Consequently, we evaluated the expression of PLP-I in testes from adult mice and rats by Northern blot and *in situ* hybridization analyses with homologous probes (Fig. 10). We could not detect PLP-I transcripts in either the mouse or rat testes by Northern blotting (Fig. 10) or *in situ* hybridization (data not shown), and thus were not able to confirm

the earlier finding (38). Thus, based on our experimentation, PLP-I does not appear to be expressed in testes from adult mice and rats.

Each of the newly identified members of the mouse PRL family was localized to trophoblast cells. However, they exhibited some prominent differences in the trophoblast lineage responsible for their expression. Four different trophoblast lineages are involved in the expression of PRL family members: 1) trophoblast cells of the labyrinth zone; 2) spongiotrophoblast cells of the junctional zone; 3) trophoblast giant cells of the junctional zone; 4) migratory trophoblast cells invading the decidua and myometrium of the mesometrial compartment (see Fig. 11A). PLP-Cγ, PLP-Cδ, PLP-I, and PLP-L mRNAs were all localized to the junctional zone of the chorioallantoic placenta (Fig. 11, B–D, F). The expression of PLP-Cγ, PLP-I, and PLP-L was restricted to spongiotrophoblast cells, whereas PLP-Cδ was localized to spongiotrophoblast cells, trophoblast giant cells, and migratory trophoblast cells. PLP-K and PLP-O transcripts were detected in trophoblast cells of both the junctional and labyrinth zone compartments (Fig. 11, E and H). Within the junctional zone, PLP-K expression was restricted to spongiotrophoblast cells, whereas PLP-O was expressed in both spongiotrophoblast cells and trophoblast giant cells. A similar localization of PLP-K mRNA in the mouse placenta was previously demonstrated with a short mouse PLP-K probe (40). PLP-N transcripts were localized to spongiotrophoblast cells and trophoblast giant cells of the junctional zone and to migratory trophoblast cells present in the mesometrial decidua and myometrium (Fig. 11G). The PLP-N expressing cells in the decidua and myometrium were cytokeratin-positive, thus confirming their trophoblast origin (data not shown). In summary, the newly discovered PRL family genes are expressed in trophoblast cells of the mouse placenta in gestationally specific patterns.

Discussion

Progress in sequencing human and mouse genomes has led to the identification and characterization of gene families and the discovery of new genes encoding ligands and receptors (68). The PRL cytokine/hormone gene family is compelling in that it represents an example of species-specific expansion. In the mouse, the PRL gene family has undergone an extensive expansion, whereas in the human there appears to be just a single member, PRL (1). Gene families arise by gene duplication and natural selection (69). To date, at least 26 separate mouse genes encoding members of the PRL family have been reported. Core features shared among members of the mouse PRL family are their structural similarities with PRL and the localization of their genes to a 1-megabase segment of chromosome 13.

The alignment of genes along the PRL family locus primarily reflects sequence conservation. Those genes possessing greater nucleotide identities are situated in closer proximity. PRL is positioned at one end of the mouse locus, whereas PLP-L is positioned at the other end of the locus. At a nucleotide level these genes exhibit modest similarity; however, the protein encoded by the PLP-L gene exhibits marked conservation with PRLs, especially more evolutionarily ancient PRL structures (70). Commonalities in cell-specific and/or temporal-specific aspects of gene expression do not appear to be main factors governing alignment along the locus. It is likely that analogous PRL family loci exist on rat chromosome 17 and bovine chromosome 23. Although many members of the mouse and rat PRL families are orthologous, this does not seem to be the situation in the cow, which suggests the independent utilization of the ancestral PRL template (71).

Genes within the PRL family locus were also organized based on their exon-intron structure. The prototypical five exon-four intron organization characteristic of PRL was a feature of genes clustered at both ends of the locus. The central core of genes possessed an additional exon(s) situated between exons II and III of the prototypical sequence. The insertion of the additional short exon(s) is near the location of binding site 1 found in the amino acid sequences of ligands interacting with the PRL receptor (72). dPRP, PLF-RP, and PLP-E possess the extra exon(s) and do not effectively bind the PRL receptor (73–75). Whether this is also true for the remaining members of the six/seven exon subfamily is unknown. The repeated insertion of an additional exon(s) between exons II and III of the prototypical PRL gene structure in numerous members of the PRL family implies some functional significance. Whether the extra exon(s) encode for a physical spacer, separating key regulatory domains or the inclusion of a biologically relevant motif is yet to be resolved. Exon III of PLP-C and PLF-RP related genes encodes for a short exon rich in aromatic amino acids, whereas the extra exon(s) of PLP-E and PLP-F are distinct. The latter genes encode for proteins regulating hematopoiesis (75–77). The significance of the additional exon(s) in the biological actions of PLP-E and PLP-F remains to be determined.

PL-I is situated adjacent to the PRL gene and shares the same exon-intron structure, biological targets, and receptor-signaling pathway. It is now evident in the mouse that PL-I is actually encoded by three closely related genes, which encode for three closely related proteins, referred to as PL-Iα, PL-Iβ, and PL-Iγ. The PL-I cDNA was originally cloned from the midgestation mouse placenta (17) and is equivalent to PL-Iα. Each PL-I gene is expressed and exhibits distinct patterns of activation during development. PL-Iβ and PL-Iγ are activated most dramatically in trophoblast cells isolated from blastocyst outgrowths, whereas PL-Iα predominates in placenta from d 10 of gestation. These observations suggest that differences may exist in the transcriptional control of the PL-Iα gene *vs.* PL-Iβ and PL-Iγ genes. A mouse PL-I promoter, likely corresponding to PL-Iγ, has been studied in the Rcho-1 trophoblast cell model (78–80). These studies have implicated the involvement of activator protein 1 and GATA factors. Whether these regulators are restricted to a specific PL-I gene or whether they will be shared among all three PL-I genes will need to be resolved. The differential PL-I gene expression patterns suggest that there may be differences in the biological activities of each PL-I. Furthermore, the needs of an implanting blastocyst differ markedly from events at midgestation. PL-Iα is a known PRL receptor agonist (81). The abilities of PL-Iβ and PL-Iγ proteins to activate the PRL receptor-signaling pathway are not known. Amino acid sequences encoded by the three PL-I genes differ at a maximum

of only five residues in the mature proteins. These amino acid substitutions are, for the most part, conservative changes. Although the amino acid changes in PL-Iβ and PL-Iγ are not dramatically different from PL-Iα they may impact the transport, stability, or actions of the ligands.

Six additional new members of the PRL gene family were discovered via mining of the mouse genome database. Two of the new members were orthologous to previously characterized members of the rat PRL family, PLP-I and PLP-K; two were classified as part of the PLP-C subfamily and were termed PLP-Cγ and PLP-Cδ, and two exhibited structural relationships with PLF-RP and were named PLP-N and PLP-O. The amino acid sequence similarities and expression patterns may provide some clues into the biology of these new members of the PRL family.

PLP-I. PLP-I is a five exon-four intron gene situated near the PRL and PL genes. It has an ortholog in the rat. Earlier comments about peculiar structural features and expression patterns of rat PLP-I appear to be unfounded (38). Placental expression of PLP-I is prominent during the latter stages of gestation. Expression is restricted to spongiotrophoblast cells, suggesting that PLP-I may be preferentially directed toward the maternal compartment. The predicted PLP-I protein has putative N-linked sites for glycosylation and exhibits some structural relationships to classical members of the PRL family (*i.e.* those using the PRL receptor signaling pathway); however, whether it shares similar targets or modes of action are unknown.

PLP-K. An ortholog of rat PLP-K was identified and possessed a five exon-four intron structure. Mouse PLP-K is very similar to rat PLP-K with the notable exception of a putative N-linked glycosylation site present in rat but not in mouse PLP-K. Based on sequence comparisons, PLP-K shares some similarity to PLF. PLF is a known regulator of blood vessel development and uterine growth (82, 83). The actions of PLF on endothelial cells are mediated by carbohydrate structures on the PLF protein backbone (84). These carbohydrate motifs interact with the insulin-like growth factor-II/mannose-6-phosphate receptor to stimulate angiogenesis (84). The absence of N-linked glycosylation sites in mouse PLP-K may not be consistent with a PLF-like mode of action. The expression pattern of mouse PLP-K is intriguing and unique among members of the PRL family. PLP-K mRNA is expressed at high levels in trophoblast cells from both junctional and labyrinth zones of the chorioallantoic placenta during the last week of gestation. The distribution of PLP-K in both junctional and labyrinth zones indicates that the PLP-K protein may be targeted to both maternal and fetal compartments.

PLP-Cγ and PLP-Cδ. Two new members of the PLP-C subfamily possessing the characteristic six exon-five intron gene organization were found. The PLP-C subfamily appears to have been more recently evolved. Five PLP-C family members have been identified in the mouse (dPRP, PLP-Cα, PLP-Cβ, PLP-Cγ, and PLP-Cδ) and six in the rat (dPRP, PLP-C, PLP-Cv, PLP-Cβ, PLP-D, and PLP-H). dPRP and PLP-Cβ are orthologous in the mouse and rat; however, the remaining mouse and rat members are not orthologous. The biology of the PLP-C subfamily is poorly understood, other than dPRP, which binds heparin and targets eosinophils (85). Both PLP-Cγ, and PLP-Cδ are predominantly expressed in the junctional zone during the latter third of gestation. Although PLP-Cγ expression is restricted to spongiotrophoblast cells, PLP-Cδ is expressed in spongiotrophoblast and trophoblast giant cells, and to a lesser extent in a subpopulation of trophoblast cells migrating into the mesometrial decidua (see additional discussion about migratory trophoblast cells below). This pattern of gene expression indicates that PLP-Cγ and PLP-Cδ are likely targeted to maternal tissues, similar to the pattern of PLP-Cα and PLP-Cβ and the rat members of the PLP-C subfamily. The species specificity of the PLP-C subfamily of ligands may have evolved to regulate pregnancy-dependent processes that are unique to the mouse *vs.* the rat.

PLP-N and PLP-O. Two genes with six exon-five intron structures encoding proteins with sequence similarity to PLF-RP were discovered. Orthologs for PLP-N and PLP-O have not been reported for any other species, including the rat. PLF-RP orthologs exist in the mouse, rat, and likely hamster (21, 41, 86). PLP-N is abundantly expressed in trophoblast cells of the junctional zone and trophoblast migrating into the mesometrial decidua. Migratory trophoblast cells form intimate relationships with the vasculature supplying the chorioallantoic placenta and increase in numbers during the latter stages of pregnancy (87, 88). In comparison, PLP-O is expressed in spongiotrophoblast cells and trophoblast giant cells of the junctional zone and in labyrinthine trophoblast. The spongiotrophoblast and labyrinthine trophoblast cell expression overlapped with the transcript distribution for PLP-K. In the mouse, PLF-RP targets the vasculature. It is an inhibitor of angiogenesis and a potent antagonist of PLF action (82, 89). Both PLP-N and PLP-O are in close proximity to vasculature critical to the placenta and the fetus, respectively. Whether these two PLF-RP related cytokines also target blood vessels needs to be evaluated.

Overview

The mouse possesses at least 26 different genes comprising the PRL family locus. We have considerable understanding of the biology of PRL but very little knowledge of the physiology of most of the other constituents of the locus. The evolutionary survival of this expanded gene family implies functional importance and relevance to speciation. Some ligand-receptor families have undergone coordinate evolution (69). However, no evidence exists for an expanded PRL receptor family. Our current information indicates that a few members of the PRL family use the PRL receptor signaling system; however, most do not and may instead use receptor signaling pathways characteristic of other ligands. Thus, there must be something intrinsic in the structure of the ancestral PRL template that enables it to evolve into structures recognized by other receptor systems. The existence of at least 26 natural variants should facilitate understanding the significance of key structural motifs within this family of cytokines/hormones.

In summary, elucidation of the mouse PRL gene family locus provides new insights into the expansion of the mouse

PRL family and new tools for studying the genetics and biology of its members.

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Paralogous refers to the relationship of homologous genes due to a duplication event; *orthologous* refers to the relationship of homologous genes due to a speciation event (90, 91).

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