A cDNA Encoding a New Member of the Rat Placental Lactogen Family, PL-I mosaic (PL-Im)

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Abstract. We have isolated a cDNA encoding a novel placental lactogen (PL), PL-I mosaic (PL-Im), by screening the cDNA library in λ ZAP of the mid-pregnant (day 12) rat placenta. The cDNA comprised an open reading frame of 687 bp encoding 229 amino acids, in which there were two putative N-glycosylation sites. Northern blot analysis showed that PL-Im mRNA was expressed specifically during mid-pregnancy (days 10 and 12) in the rat placenta. The cDNA was highly homologous with those of other rat PL family members; in particular, the homology among PL-Im, PL-I (mid-pregnancy-specific) and PL-IV (late-pregnancy-specific) was over 90%. Interestingly, the nucleotide sequence of PL-Im cDNA was a mosaic of PL-I and PL-IV and it did not possess its own particular sequence. As the genomic pattern determined using Southern blot analysis of PL-Im was distinct from that of PL-I and PL-IV, the encoded area of PL-Im appears to be independent of those of PL-I and PL-IV on the gene. In the dendrogram of the rat PL family constructed on the basis of the nucleotide sequence homologies, PL-Im was located between PL-IV and PL-I in the process of molecular evolution. Therefore, PL-Im has a unique cDNA structure and may be a principal factor in the molecular evolution of PLs.

Key words: Rat placental lactogen, Placental lactogen I mosaic, cDNA, Cloning.

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THE PLACENTA produces a PRL- or GH-like hormone known as placental lactogen (PL), which is found in several mammals, including the rat [1], mouse [2], cow [3], sheep [4], monkey [5], and human [6]. In the rat, the PL family shows structural homology with PRL. To date, the cDNAs of 6 members of the rat PL family have been cloned: PL-I [7], PL-II [8], PL-IV [9], PRL-like protein-A (PLP-A) [10], PLP-B [11] and PLP-C [12]. Only PL-I has been found to be a mid-pregnancy-specific PL; all the others were expressed predominantly during late-pregnancy [1, 7].

In addition to PL-I, there appear to exist multiple

types of molecules with PL activity in the mid-pregnant rat placenta. Sephadex G-100 gel-filtration analysis of mid-pregnant rat serum and placentas revealed a broad elution profile of PL activity [1, 13], which we separated into two substances by high-performance liquid chromatography gel-filtration [14, 15]. Thus, the PL activity in mid-pregnant serum does not depend upon a single molecular type, but involves at least two molecular types, PL-I (molecular mass 45–50 kDa) and PL- α (55–60 kDa) [14, 15]. These PLs were demonstrated to be N-glycosylated proteins, as they were sensitive to several glycosidases [14, 15].

PRL, GH and PLs appear to have evolved from a single ancestral gene by gene-duplication and sequence divergence [16]. All the rat PLs found so far, with the exception of PL-II, have one or two putative N-glycosylation sites [7–12, 17], and the molecular evolution of the glycoresidues is also a matter of interest.

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In this paper, we describe a novel PL (PL-Im) cDNA, which is expressed specifically during mid-pregnancy in the rat placenta. The structure of PL-Im cDNA is of particular interest, as its nucleotide sequence is a mosaic of mid-pregnant and late-pregnant PLs (PL-I and PL-IV respectively) and has no particular sequence of its own.

Materials and Methods

Reagents

pBluescript and λ ZAP were purchased from Stratagene (California, USA). Oligotex dT30, AmpliTaqTM polymerase, ligase, T4 polynucleotide kinase and pUC119 were from Takara Shuzo (Kyoto, Japan). The restriction endonucleases, AMV reverse transcriptase, random primer labeling kit and hybridization blocking reagent were from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Agarose and the AutoRead sequencing kit were from Pharmacia LKB (Uppsala, Sweden). [³²P] dCTP (3000 Ci/mmol) was from New England Nuclear (Boston, USA), and all the other reagents were from Wako Pure Chemicals (Osaka, Japan).

Animal treatments and tissue preparation

Adult Wistar (Imamichi Institute for Animal Reproduction, Ibaraki, Japan) and Sprague-Dawley (Clea Japan, Inc. Tokyo, Japan) rats were kept under a lighting schedule of 14 h light (lights on 0500 h) and 10 h dark (lights off 1900 h) and allowed food and water *ad libitum*. Wistar strain rats were used, unless stated otherwise. During the evening of the proestrus day, each female was housed with a male, and the day that sperm were observed in a vaginal smear was designated as day 0 of pregnancy. The placental tissues remaining after removal of the embryos with forceps were collected every 2 days from days 10 to 20 inclusive, frozen quickly and stored at -80°C until required for RNA extraction.

Extraction of RNA

The total RNAs were isolated from the placentas obtained on days 10, 12, 14, 16, 18 and 20 of pregnancy using the CsCl-ultracentrifuge method [18], and the RNAs isolated on each day were subjected to Northern blot analysis. The poly(A) RNAs of days 10 and 12 were selected with Oligotex dT30, as described elsewhere [19], and were used in the synthesis of first-strand cDNA for Polymerase chain reaction (PCR) and construction of a λ ZAP cDNA library.

Preparation of probe for screening the cDNA library

Two pairs of PCR primers, H- and F-primers, based on the nucleotide sequences of rat PL family members, were designed (Fig. 1A) and synthesized using an Oligo 1000 DNA Synthesizer (Beckman Instruments, Inc. California, USA) [18]. The first-strand cDNA used for the PCR was transcribed from the poly(A) RNA with AMV reverse transcriptase [20], and PCR amplification was carried out in a final reaction volume of 100 μl containing the cDNA, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 6 mM $(\text{NH}_4)_2\text{SO}_4$, 1.5 mM MgCl_2 , 0.1% Triton X-100, 200 mM dNTP, 1.0 mM each PCR primer and 2.5 units of AmpliTaqTM polymerase. The reaction was carried out in a Quick Thermo (Nippon Genetics, Tokyo, Japan) and involved 30 cycles of denaturation (94°C for 1 min), annealing (45°C for 1 min) and extension (72°C for 2 min) [21]. The PCR products were analyzed by electrophoresis on 1.0% agarose gel in TAE (40 mM Tris-acetate containing 1 mM EDTA).

The amplified PCR products were ligated into a pUC119 vector cut by *Sma*I and then transformed in *Escherichia coli* strain XL1-blue. After analysis of the nucleotide sequence [18], a novel cDNA (690 bp), shown in Fig. 2, was found and designated F12.

DNA sequencing and sequence analysis

The DNA sequence was obtained on both strands of DNA by the dideoxy-chain-termination method with an AutoRead sequencing kit and an A.L.F. DNA sequencer (Pharmacia LKB, Uppsala, Sweden) [22]. Sequence analysis was carried out using MacMololyTetra computer software (Soft Gene, Berlin, Germany).

Note. The novel nucleotide sequence data published here have been deposited with the DDBJ, EMBL and GenBank Nucleotide Sequence Databases and are available under the accession number D21103.

Construction and screening of the cDNA library

The F12 was labeled with [32 P]-dCTP using random primers [23] and used as a probe to screen the cDNA library in λ ZAP, which was constructed from placental poly(A) RNA obtained on day 12 of pregnancy [18]. Approximately, 1.2×10^5 plaques of the library were plated and transferred to nylon filters, Hybond-N (Amersham, Tokyo, Japan). They were then hybridized with the probe for 24 h at 42°C in $5 \times$ SSC which contained 0.02% SDS, 0.1% lauroylsarcosine and 1% blocking reagent, washed with $1 \times$ SSC/0.1% SDS at 25°C for 10 min and then with $0.2 \times$ SSC/0.1% SDS at 60°C for 10 min, after which X-ray film was exposed to the filters at -80°C for 24 h. The phagemid-selected plaque was transformed in XL1-blue [18].

Northern blot analysis

The total RNA (40 μg), dissolved in electrophoresis buffer (20 mM MOPS (pH 7.0), 5 mM sodium acetate and 1 mM EDTA) containing 50% formamide and 6.5% formaldehyde, was heated to 65°C for 15 min, electrophoresed on 1.0% agarose/formamide gel and transferred to a nylon membrane, Hybond-N (Amersham, Tokyo, Japan) [18]. The membrane was hybridized with the [32 P]-labeled F12 in $5 \times$ SSC containing 50% formamide, 0.02% SDS, 0.1% lauroylsarcosine and 1% blocking reagent, then washed with $1 \times$ SSC/0.1% SDS at 25°C for 20 min followed by $0.2 \times$ SSC/0.1% SDS at 60°C for 10 min, after which X-ray film was exposed to the membrane at -80°C for 24 h [18].

Southern blot analysis

Genomic DNA (10 μg) isolated from the livers of Wistar and Sprague-Dawley male rats was digested with *Bam*HI, *Hind*III or *Eco*RI and electrophoresed on 0.8% agarose gel in TAE [18]. They were then transferred to nylon membranes, Hybond-N, and hybridized with the [32 P]-labeled F12 in $5 \times$ SSC containing 50% formamide, 0.02% SDS, 0.1% lauroylsarcosine and 5% blocking reagent. Then, each membrane was washed twice with $1 \times$ SSC/0.1% SDS at 25°C for 10 min each, followed by four washes with $0.2 \times$ SSC/0.1% SDS at 60°C for 30 min each, and X-ray film was exposed to the membranes at -80°C for 24 h [18].

Results

Amplification of a novel PL mRNA by the PCR

The results of the PCR using the H- and F-primers are shown in Fig. 1B. The 596 bp product in both cDNA preparations (days 10 and 12) was amplified by the H-primers, and the corresponding 690 bp bands were amplified by the F-primers. Sequence analysis showed that these bands contained several PLs including PL-II. We found that the sequence of one of the DNA bands (596 bp, H12) amplified by the H-primers was a novel one. Another DNA band amplified by the F-primers (690 bp, F12) contained H12. Interestingly, the nucleotide sequence of F12 was very similar, but not identical, to those of PL-I [7], PL-Iv [9], PL-II [8], PLP-A [10], PLP-B [11] and PLP-C [12].

Cloning of novel PL cDNA (PL-Im) by Screening the λ ZAP cDNA library

A cDNA library of 1.2×10^6 recombinants was constructed from the mRNA obtained on day 12 of pregnancy. After the second screening, a clone (pE01-a) was obtained and its nucleotide sequence was determined (Fig. 2). The sequence contained an open reading frame of 687 bp encoding 229 amino acids. There were two potential glycosylation sites, Asn-Val-Ser at position 108–110 and Asn-Arg-Thr at position 157–159. Cleavage of the signal peptide sequence would be expected to occur between Ser and Lys at position 29–30. The polyadenylation signal and poly(A) tail were contained in 130 bp of the 3'-untranslated region, although the 5'-non-coding region was truncated. The protein encoded by the insertion of pE01-a was designated PL-I mosaic (PL-Im) from its structure, as described in the Discussion.

Northern blot analysis of the rat placental PL-Im

The expression of PL-Im mRNA in the rat placenta was investigated using Northern blot analysis (Fig. 3A). Intense mRNA bands of approximately 1.0 kb were detected in the placentas obtained during mid-pregnancy (days 10 and 12), whereas this expression was not evident on the other days. The amounts of RNA obtained on days 10 to 20 inclusive subjected to analysis were almost

Fig. 1. PCR Amplification of the PL mRNAs expressed during mid-pregnancy. A) H-primers (mixed primers), based on the highly homologous parts of the nucleotide sequences of PL-I, PL-II, PLP-A and PLP-B, and F-primers containing the initiation and stop codons of PL-I were designed. The amino acids are numbered according to the PL-I. B) These primers were used to investigate mRNA expression in the placenta at mid-pregnancy by PCR. After analysis of the PCR products by 1.0% agarose electrophoresis, they were stained with ethidium bromide. The major fragments obtained, comprised of 596 bp (H-primers) and 690 bp (F-primers), were amplified (M, marker (λ -EcoT14I digest); H10, PCR product using H-primer and cDNA from day 10; H12, H-primer and cDNA from day 12; F10, F-primer and cDNA from day 10; F12, F-primer and cDNA from day 12).

equal judged by the intensity of the samples stained with ethidium bromide (Fig. 3B).

Genomic DNA restriction enzyme patterns of PL-Im

The Southern blot results are shown in Fig. 4.

When the genomic DNA from Wistar and Sprague-Dawley strain rats was pretreated with *Eco*RI, four bands were detected, including a 0.9 kb fragment. A single broad band was produced after *Hind*III and *Bam*HI treatment of both strains.

PL-Im	Met	Gln	Leu	Thr	Leu	Thr	Leu	Ser	Gly	Ser	Gly	Met	Gln	Leu	Leu	Leu	Leu	Val	
	ATG	CAG	CTG	ACT	TTG	ACT	CTT	TCG	GGC	TCT	GGT	ATG	CAA	CTG	TTG	CTG	CTG	GTG	
									27			36			45			54	
Ser	Ser	Leu	Leu	Leu	Trp	Glu	Asn	Val	Ala	Ser	Lys	Pro	Thr	Ala	Ile	Val	Ser	Thr	Asp
TCA	AGC	TTG	CTC	CTT	TGG	GAA	AAC	GTG	GCC	TCC	AAA	CCA	ACT	GCC	ATT	GTG	TCC	ACT	GAT
								81			90			99			108		
Asp	Leu	Tyr	His	Arg	Leu	Val	Glu	Gln	Ser	His	Asn	Thr	Phe	Ile	Met	Ala	Ala	Asp	Val
GAC	CTA	TAT	CAT	CGT	TTG	GTT	GAA	CAG	TCT	CAT	AAT	ACA	TTT	ATC	ATG	GCT	GCA	GAT	GTA
	123				132			141			150			159			168		
Tyr	Arg	Glu	Phe	Asp	Ile	Asn	Phe	Ala	Lys	Arg	Ser	Trp	Met	Lys	Asp	Arg	Ile	Leu	Pro
TAC	CGT	GAA	TTT	GAT	ATA	AAT	TTT	GCC	AAG	AGA	AGT	TGG	ATG	AAA	GAC	AGG	ATA	CTT	CCC
	183				192			201			210			219			228		
Leu	Cys	His	Thr	Ala	Ser	Ile	His	Thr	Pro	Glu	Asn	Leu	Glu	Glu	Val	His	Glu	Met	Lys
CTG	TGT	CAC	ACT	GCT	TCC	ATC	CAT	ACT	CCA	GAG	AAT	CTA	GAG	GAA	GTC	CAT	GAA	ATG	AAA
	243				252			261			270			279			288		
Thr	Glu	Asp	Phe	Leu	Asn	Ser	Ile	Ile	Asn	Val	Ser	Val	Ser	Trp	Lys	Glu	Pro	Leu	Lys
ACT	GAA	GAC	TTC	CTG	AAC	TCA	ATC	ATC	AAT	GTT	TCA	GTT	TCC	TGG	AAA	GAA	CCT	CTG	AAA
	303				312			321			330			339			348		
His	Leu	Val	Ser	Ala	Val	Thr	Asp	Leu	Pro	Gly	Ala	Ser	Val	Ser	Met	Gly	Lys	Lys	Ala
CAC	TTG	GTG	TCT	GCA	GTG	ACT	GAT	CTT	CCG	GGA	GCT	TCT	GTT	AGT	ATG	GGG	AAA	AAA	GCT
	363				372			381			390			399			408		
Val	Asp	Met	Lys	Asp	Lys	Asn	Leu	Ile	Ile	Leu	Glu	Gly	Leu	Gln	Thr	Leu	Tyr	Asn	Arg
GTT	GAT	ATG	AAG	GAC	AAA	AAT	CTT	ATA	ATT	CTG	GAG	GGA	CTT	CAG	ACC	TTA	TAC	AAC	AGG
	423				432			441			450			459			468		
Thr	Gln	Ala	Lys	Val	Glu	Glu	Asn	Phe	Glu	Asn	Phe	Asp	Tyr	Pro	Ala	Trp	Ser	Gly	Leu
ACT	CAG	GCT	AAA	GTT	GAA	GAA	AAT	TTT	GAA	AAT	TTT	GAC	TAC	CCT	GCC	TGG	TCT	GGA	CTC
	483				492			501			510			519			528		
Lys	Asp	Leu	Gln	Ser	Ser	Asp	Glu	Asp	Thr	His	Leu	Phe	Ala	Ile	Tyr	Asn	Leu	Cys	Arg
AAA	GAC	TTG	CAG	TCA	TCT	GAT	GAA	GAC	ACT	CAT	CTT	TTT	GCC	ATT	TAT	AAC	CTG	TGC	CGC
	543				552			561			570			579			588		
Cys	Phe	Lys	Arg	Asp	Ile	His	Lys	Ile	Asp	Thr	Tyr	Leu	Lys	Val	Leu	Arg	Cys	Arg	Val
TGC	TTT	AAA	AGG	GAC	ATC	CAT	AAG	ATT	GAC	ACT	TAT	CTC	AAA	GTC	TTG	AGG	TGC	CGA	GTT
	603				612			621			630								
Val	Phe	Lys	Asn	Glu	Cys	Gly	Val	Ser	Thr	Phe	TER								
GTC	TTT	AAG	AAT	GAG	TGT	GGA	GTG	TCC	ACC	TTT	TGA	AGT	C	T	T	G	C	A	C
	663																		
GTAATGCTTTTTTCGCCTCTCGGTGTATTTCAGAGCTGTAATGGAATCTTTTCATAAAATAAAATGGAATTATTTAGAAA																			
AAAAAAAAAAAAAAAAAAAA																			

Fig. 2. Nucleotide and deduced amino acid sequences of PL-Im cDNA. The PL-Im cDNA was cloned by screening the λ ZAP cDNA library constructed from placenta obtained during mid-pregnancy (day 12). Its nucleotide sequence was determined and the amino acid sequence of PL-Im was deduced. The PL-Im cDNA contains an open reading frame with 687 bp nucleotides encoding 229 amino acids. The nucleotides and amino acids were numbered from the initiation codon, ATG. The position of the signal cleavage site was predicted according to its homology with other members of the PL family [7, 29], and is marked with an arrow. The positions of the two potential glycosylation sites, Asn-Val-Ser at position 108–110 and Asn-Arg-Thr at position 157–159, are shown enclosed in boxes, and the nucleotides corresponding to the H- and F-primers are underlined. The nucleotide sequences of H12 and F12 correspond to these parts of PL-Im.

Fig. 3. Northern blot analysis of rat placental PL-Im. A) Total RNA preparations (40 μ g each) extracted from placentas obtained on days 10, 12, 14, 16, 18 and 20 of pregnancy were electrophoresed on 1.0% agarose/formamide gel and transferred to a nylon membrane, which was hybridized with [32 P]-labeled F12. The mobilities of 28S and 18S are shown on the left side and the arrow indicates 1.0 kb PL-Im mRNA. B) After electrophoresis, the samples were stained with ethidium bromide.

Fig. 4. Southern blot analysis of rat genomic DNA with PL-Im cDNA. Genomic DNA preparations (10 μ g each) of Wistar A) or of Sprague-Dawley B) rat strains were treated with a restriction enzyme, *EcoRI*, *HindIII* or *BamHI*, electrophoresed on 0.8% agarose gel and transferred to a nylon membrane, which was hybridized with [32 P]-labeled F12. The arrow (0.9 kb) indicates the original band detected with the PL-Im probe.

Discussion

We have successfully cloned a cDNA encoding a novel PL, PL-Im, from the rat placenta obtained during mid-pregnancy and determined its nucleotide sequence. The particularly interesting point to note is its structure and time of expression; PL-Im cDNA has a mosaic structure of PL-I (mid-pregnancy-specific) and PL-Iv (late-pregnancy-specific) and is expressed specifically during mid-pregnancy.

On the basis of its high homology with the 6 members of the rat PL family (PL-I, PL-Iv, PL-II, PLP-A, PLP-B and PLP-C), PL-Im is a new member of the PL family [7–12] (Fig. 5). In particular, PL-Im cDNA is highly homologous (over 90%) with PL-I and PL-Iv and, furthermore, detailed comparisons of these nucleotide sequences revealed that PL-Im cDNA does not possess its own sequence but is a mosaic structure of PL-I (mid-pregnancy-specific) and PL-Iv (late-pregnancy-specific) (Fig. 6).

The PL-Im contains the 20 amino acids which are conserved in these 6 other PLs. The positions of these 20 amino acids are; Cys (80, 199, 216 and 224), Leu (14, 16, 23, 103, 181, 196 and 214), Ser (19, 29 and 183), Trp (24 and 175), Glu (25), His (119), Asp (203) and Arg (217) [7–12]. Of these, the four Cys residues, which are considered to be parts of the disulfide bonds that determine the tertiary

structure of proteins [24, 25], are conserved in the rat PL family without exception. In addition, three other amino acids, Arg at 43 and 202 and Lys at 212 in PL-Im, have also been postulated to be essential for reactions with the lactogenic hormone receptor in a study of point mutation in mouse PLs [26, 27].

Northern blot analysis revealed that PL-Im mRNA was strongly expressed in the rat placenta on days 10 and 12, and this abundance of PL-Im mRNA seems to be similar to that of PL-I and PL-Iv reported by Robertson [9]. In the present experiment, the sequence of the cDNA used as a probe was more than 90% homologous with PL-I mRNA, like PL-Iv mRNA. Therefore, it is predicted that the faintly detectable band on day 16 represents cross-hybridization of PL-Iv mRNA. As PL-I and PL-Iv mRNA have much the same level of homology as the probe, it would be expected that the cross-hybridization of PL-I mRNA on days 10 and 12 would be as low as that of PL-Iv mRNA on day 16. Judging from the above, it appears that PL-Im mRNA expressed in mid-pregnant placenta can be detected with this degree of stringency.

Previously, we reported that there were at least two distinct PL-like substances in mid-pregnant rat serum, PL-I and PL- α (molecular masses of 45–50 and 55–60 kDa respectively) [14, 15], although the structure of PL- α has not been determined. PL- α was recognized preferentially by antiserum AII-86,

	PL-Im	PL-I	PL-Iv	PL-II	PRL	PLP-A	PLP-B	PLP-C
PL-Im	97.1	90.4	50.3	44.6	34.1	25.5	21.5	
PL-I	91.8	88.0	54.6	44.9	33.5	25.5	21.5	
PL-Iv	86.1	80.1	50.5	52.7	29.4	35.7	18.8	
PL-II	41.5	38.7	41.6	46.9	35.4	39.3	32.6	
PRL	29.9	26.7	30.8	35.7	46.7	27.8	24.4	
PLP-A	20.3	17.6	31.7	29.1	19.1	38.0	26.5	
PLP-B	10.9	10.2	20.4	30.2	16.5	28.8	50.0	
PLP-C	14.7	14.0	11.6	16.4	15.5	14.3	19.2	

Fig. 5. Homology of PL-Im with the rat PL family. The percentage homologies with identical amino acids and nucleotides are shown. The deduced amino acid and nucleotide sequences of PL-Im are compared with those of PL-I [7, 29], PL-II [8], PL-Iv [9], PLP-A [10], PLP-B [11] and PLP-C [12].

Fig. 6. Comparison of the amino acid structure of PL-Im with those of PL-I and PL-Iv. The nucleotide and deduced amino acid sequences of PL-Im showed it had a mosaic structure of PL-I and PL-Iv and did not possess its own sequence [7, 9].

which was specific to the hydrophilic region of PL-II, and the recognition site of the PL- α core protein appeared to be analogous to PL-II rather than to PL-I [28]. As the deduced amino acid sequence of PL-Im that corresponds to the antibody recognition site was identical of PL-I, PL-Im should be distinct from PL- α .

From the homology of PL-Im with other rat PL family members, we expected that PL-Im would have a signal peptide cleavage site between Ser at position 29 and Lys at position 30 [7, 29]. As PL-Im has two putative glycosylation sites, it may be a secretory glycoprotein, like PL-I, PL-Iv and PL- α [7, 9, 14, 30]. The amino acid sequence near the glycosylation site is believed to contain information that determines the final structure of the glycoside. Recently, the amino acid sequence motif, Pro-Leu-Arg, which is located in 6–9 residues on the amino-terminal side of the N-glycosylation sites on the hCG α and β subunits, was reported to be essential for recognition by N-

acetylglactosamine-transferase [31]. Although this motif is not included in the PL family, study of the glycoside structure(s) is of great interest in this context. The affinities of the PL-I and PL-Iv glycosides for concanavalin A differ [30]. The structure near the glycosylation sites of PL-Im is a mosaic of PL-I and PL-Iv. Therefore, structural analysis of the glycosides of these PLs will yield new information about modification of protein by glycosylation, and this would be worth studying in detail.

When subjected to Southern blot analysis, similar and simple hybridization patterns were obtained for both the genomic DNA preparations from the two strain rats, Wistar and Sprague-Dawley, which suggests there is no polymorphism in the PL-Im gene area. However, digestion of PL-Im with *EcoRI* yielded a PL-Im-specific fragment (0.9 kb), which was not present after Southern blot analysis of PL-I and PL-Iv [9]. Therefore, the restriction enzyme pattern of PL-Im differed dis-

tinctly from those of these PLs. In view of the previous report that PL-I and PL-Iv were independent of each other in the encoded area on the gene, these very similar PLs (PL-I, PL-Iv and PL-Im) appear not to be produced in the same genomic area by alternative splicing, but to be encoded in different genomic areas.

The possible molecular evolution process was deduced by constructing a dendrogram based on the nucleotide sequence homologies of PL family members (Fig. 7). The mid-pregnancy-specific PLs (PL-Im and PL-I) appear to be derived from the late-pregnancy-specific PLs, and PL-Im is located between PL-Iv and PL-I in the process of their molecular evolution. Furthermore, as shown in Table 1, the replacement to silent substitute ratio of PL-Iv to PL-Im is 1.21 (54.8%/45.2%), whereas that of PL-Im to PL-I is quite high (3.50, 77.8%/22.2%). Therefore, it is likely that positive selective influences have caused rapid fixation of replacement substitutes in the genes.

The rat has a very efficient reproductive system, which utilizes PRL as a luteotrophic factor [32]. The rat has no luteal phase during its normal ovulatory cycle and ovulates at 4–5 day intervals if the female does not have a chance to mate with a male. The PRL surges following mating, which is induced by cervical stimulation, is necessary to initiate and maintain the progesterone secretion function of the corpus luteum. PL is a primary candidate for the luteotrophic factor of gestation, in view of the observations that PRL secretion ceases at mid-pregnancy following the initiation of PL secretion [33] and PLs are able to bind to the ovarian PRL receptor [14, 15, 28]. Mid-pregnancy-specific PLs appear to be peculiar to the rat, mouse and

hamster [7, 17], while PL expression occurs predominantly during late pregnancy in other species. Therefore, acquiring mid-pregnancy PLs, especially those involved in the evolutionary process from PL-Im to PL-I, may lead to an efficient reproductive strategy for these species.

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Fig. 7. Dendrogram of the PL family. The dendrogram was constructed according to the nucleotide sequence homologies of 7 members of the PL family: PL-Im, PL-I, PL-Iv, PL-II, PLP-A, PLP-B and PLP-C.

Table 1. Codon-by-codon comparisons of PL-Im, PL-I and PL-Iv

Comparison	Identical codon	Codons differing by one base	
		Silent	Replacement
PL-Iv vs. PL-Im	84.2% (187/222)	45.2% (14/31)	54.8% (17/31)
PL-Im vs. PL-I	95.5% (210/220)	22.2% (2/9)	77.8% (7/9)

When nucleotide sequences are compared codon by codon, there can be two types of codon that differ by one base. One is the replacement substitute (one base change in a codon results in an amino acid replacement), and the other is the silent substitute (one base change does not affect an amino acid, but codes a synonymous amino acid). The percentage of identical, silent and replacement substituted codons among PL-Im, PL-I and PL-Iv are shown.

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