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Structural Organization of the 5′-End and Chromosomal Assignment of Human Placental Leucine Aminopeptidase/ Insulin-Regulated Membrane Aminopeptidase Gene

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Placental leucine aminopeptidase (P-LAP) which is identical with cystine aminopeptidase as oxytocinase was found to be a homologue of rat insulin-regulated membrane aminopeptidase (IRAP) by cDNA cloning. In this study, we confirmed 5′-end cDNA sequence of P-LAP and isolated genomic clones containing the upstream region of human P-LAP gene. The transcription initiation sites determined by primer extension located 478 and 480 bp upstream of the initiation methionine codon, 38 bp downstream of TATA box-like motif. The 5′-flanking region of human P-LAP gene contained DNA-binding motifs for several ubiquitous transcription factors such as SP1 and AP2. Chromosomal localization by fluorescence *in situ* hybridization showed that the gene was assigned to 5q14.2-q15 of the human chromosome. This study establishes the genetic basis for P-LAP gene research, thereby leading to better understanding of the molecular mechanism underlying the P-LAP gene.

Human placenta and maternal serum contain several aminopeptidases which regulate the concentrations and activity of peptide hormones such as oxytocine and angiotensine (1, 2). We have purified placental leucine aminopeptidase (P-LAP) from retroplacental serum and found that P-LAP is identical with cystine aminopeptidase (CAP) which is regarded as oxytocinase (EC 3.4.11.3) (3). The determination of the partial amino acids sequence of this purified protein enabled us to isolate human P-LAP cDNA clone from human placental cDNA library (4). By amino acid sequence comparison, human P-LAP shares 85% identity with rat insulin-regulated membrane aminopeptidase (IRAP) (5) which is present in glucose transporter isotype GLUT4 vesicles of rat adipocytes (6–8). This sequence conservation indicates that IRAP is a rat homologue of human P-LAP. Human P-LAP cDNA cloning was performed later by Laustsen also (9), but the 5′-cDNA end of P-LAP/IRAP has not been clearly determined up to now.

P-LAP tissue distribution was extensively investigated by immunohistochemistry (10) and Northern blot analysis (4, 9), which demonstrated the widespread tissue distribution besides placenta. P-LAP mRNA is expressed in placenta, brain, heart and skeletal muscle, while little or no mRNA of P-LAP can be detected in lung, liver and kidney. Gene expression in tissue specific manner is generally regulated at transcription levels. To give an example, aminopeptidase N gene expression is regulated highly in the small intestine, moderately in the liver and low in the spleen by upstream promoter region and tissue specific nuclear proteins (11, 12). Although it is quite important to elucidate the molecular mechanism underlying this tissue specific expression of P-LAP, molecular characterization of the P-LAP gene from any species has not been reported so far.

In maternal serum P-LAP activity is increased with gestational age just before onset of labor (13), which suggests that P-LAP may play an important role in suppressing labor pain through hydrolyzing oxytocin. Concerning the factors involved in the regulation of P-LAP levels during pregnancy, we have reported that several hormones such as estrogen and cortisol induced P-LAP activity in the organ culture of placenta (14).

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Sequence data from this article have been deposited with EMBL/Genbank Data Libraries under Accession No. AF145455.

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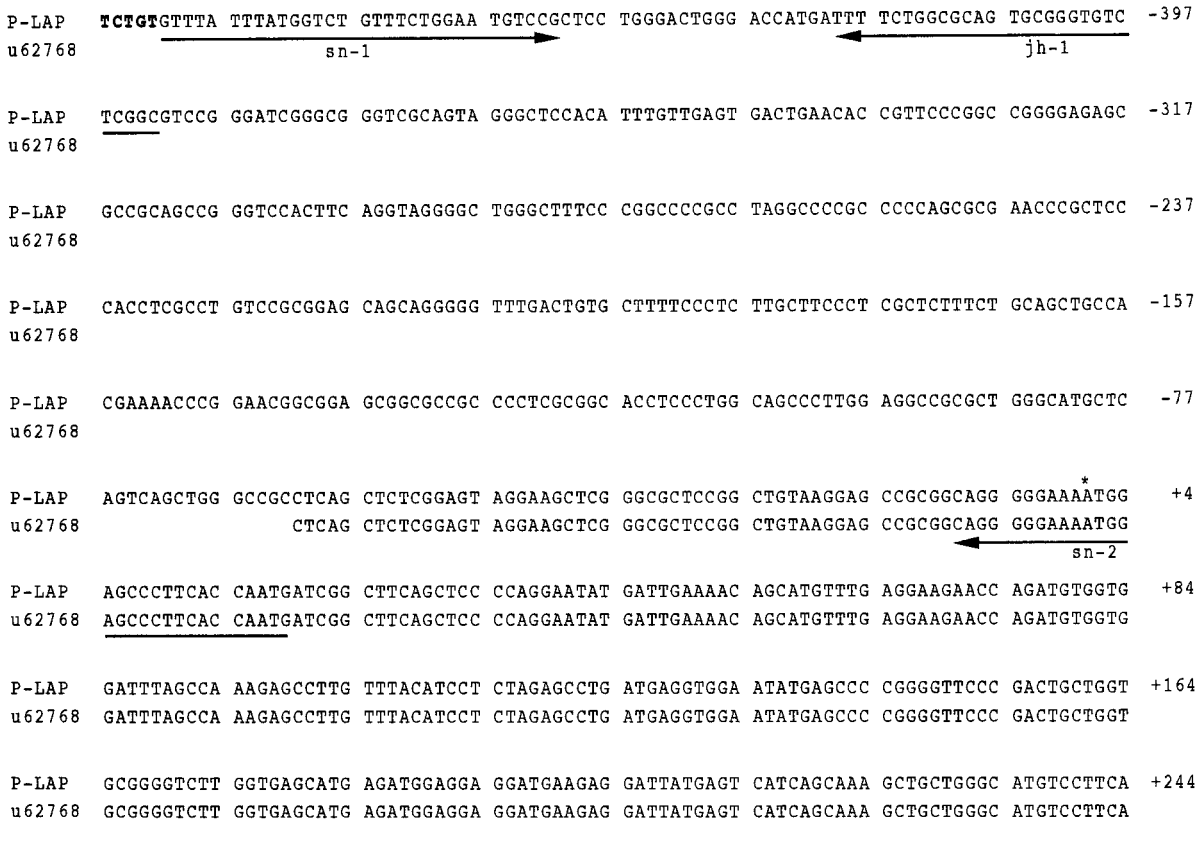

FIG. 1. Nucleotide sequence of the 5′-region of human P-LAP cDNA (upper line). Alignment of this sequence to the cDNA determined by Laustsen (Acc. No. u62768) shows a complete similarity up to −61. The additional nucleotide sequence obtained by 5′-RACE experiments is indicated in bold face. The translation start site is indicated by an asterisk and numbered as +1. The sequences of the oligomers, sn-1 and sn-2 used in PCR in Fig. 2, jh-1 and sn-2 used in primer extension analysis in Fig. 4, are underlined.

Since these hormones affect gene expression through their response element on the genome (15–18), analyzing the promoter or enhancer region of P-LAP is essential to study the hormone regulation.

These observations prompted us to investigate and analyze the P-LAP gene, which is helpful in understanding the physiological and pathophysiological roles of P-LAP in human. We report here the confirmation of cDNA sequence of P-LAP, the isolation of the promoter region of the human P-LAP gene and also chromosomal assignment of the gene using fluorescence *in situ* hybridization technique.

MATERIALS AND METHODS

Rapid amplification of 5′-cDNA ends (5′-RACE). The 5′-end of the cDNA was obtained by 5′-RACE technique with modification as previously described (19). Briefly, poly(A)$^+$ RNA purified from human placenta was used as a template to synthesize the first cDNA strand, and the second cDNA complementary to the poly-d(T)$^+$ sequence and flanked on its 5′-end by an *Eco*RI site (5′-CCAACAGAATTC(A)$_{18}$-3′). The sequence between the anchor oligomer and the human P-LAP specific oligomer complementary to residues −10 to +19 in the cDNA sequence was synthesized by PCR with a GeneAmp PCR system 2400 (Perkin Elmer). Nucleotide sequences were determined by the cycle sequencing method using BigDye terminators (ABI-Perkin Elmer).

PCR amplification. Reverse transcribed cDNA and genomic DNA from human placenta were prepared as described in our previous study (20, 21) and used for PCR. PCR was performed using sense (sn-1, −471 to −441) and antisense (sn-2, −10 to +19) oligomers shown in Fig. 1 under the thermal cycle profile consisting of 94°C for 15 s, 68°C for 30 s for 30 cycles.

Screening of human genomic library. The P-LAP cDNA sequence (−471 to +147) in Fig. 1 was labeled by random priming and used as a probe to screen three haploid genome equivalents (10^6 plaques) of a human genomic library (CLONTECH) as previously described (22).

Primer extension analysis. The primer extension was performed as previously (19) using the synthetic oligonucleotides jh-1 and sn-2 complementary to the sequence from −419 to −392 and −10 to +19, respectively in the P-LAP cDNA shown in Fig. 1. These primers were labeled with ^{32}P-ATP and hybridized to 50 μg of total RNA from human placenta and choriocarcinoma cell line NaUCC-4 (20) in 30 μl of 0.4 M NaCl, 10 mM Pipes (pH 6.5), 1 mM EDTA and 80% formamide at 42°C overnight. After phenol/chloroform extraction and ethanol precipitation, the oligonucleotides were extended and the extended fragments were analyzed on 6% polyacrylamide denaturating gel.

Fluorescence in situ hybridization (FISH). We used a genomic cosmid clone as a probe for chromosomal assignment. Direct R-banding FISH based on FISH combined with replicated prometaphase R-bands was applied (23, 24). To suppress repetitive se-

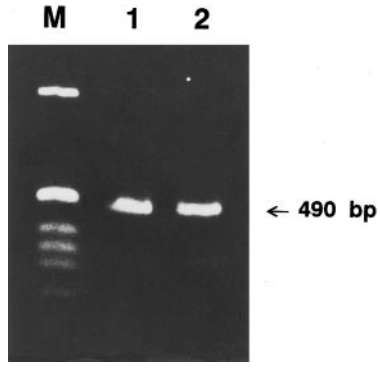

FIG. 2. PCR products from human genomic DNA (lane 1) and reverse transcribed placental RNA (lane 2). The primers using for PCR are shown in Fig. 1. M, 1 kb DNA ladder as molecular size marker.

quences contained in this clone, we used 20-fold excess of human Cot1 DNA (Gibco BRL) according to the method of Lichter *et al.* (25). Labeling, hybridization, rinsing and detection were carried out in a routine manner. Provia 400 (Fuji, ISO 400) was used for the microphotography (filter:Nikon B-2A).

RESULTS

Previously, human P-LAP cDNA cloning was performed by us (Acc. No. D50810) (4) and Laustsen *et al.* (Acc. No. u62768) (9). Alignment of our sequence to that of Laustsen's revealed a 100% similarity up to −61 calculated from the initiation methionin codon. On the other hand, our isolated cDNA clone extended further upstream to −471, although the other one ended at −61 (Fig. 1). For determining the complete 5′-sequence of P-LAP, we performed 5′-RACE experiments. To exclude the misincorporation and incomplete elongation during PCR, several clones were sequenced and analyzed. The 5′-RACE products extended to 470−476 bp upstream of the initiation methionin codon, which was compatible with the result of our cDNA cloning and suggested our clone carried nearly full length of 5′-sequence. The 5′-end of the longest 5′-RACE products located 5 bp upstream from that determined by cDNA library screening (Fig. 1).

We also performed PCR amplification to verify the presence of the sequence from −471 to −62 in human placental mRNA and human genomic DNA (Fig. 2). Both PCR experiments using reverse transcribed DNA from placenta and genomic DNA as a template yielded the same product with expected size, which confirmed the presence of the sequence in the exon region of human genome and suggested no introns in the sequence.

Using the fragment of the 5′-portion of P-LAP cDNA (−471 to +147) as a probe, we isolated three independent clones from a human placental genomic DNA library. Compared with the sequence of P-LAP cDNA, the two overlapped genomic clones contained exon 2, while the remainder was separate from those and carried an upstream sequence of cDNA and exon 1. Figure 3 shows the nucleotide sequence of P-LAP gene (Acc. No. AF145455). Since we were unable to obtain some part of the first intron by genomic library screening, we tried to isolate the missing fragment by PCR, which resulted in failure probably because the size of the fragment was too large to be amplified by PCR (data not shown). Sequence analysis of the full length of the cloned phage DNA indicated the size of the first intron was at least 15 kb. The splice donor and acceptor sequence of the first intron was gtgag and tttag, respectively. The splice donor sequence of the second intron was gtagg.

In order to determine the transcription initiation sites, we performed primer extension analysis using two different primers jh-1 and sn-2 with the total RNA extracted from human placenta and NaUCC-4 cells (Fig. 4). Primer jh-1 with either of RNA indicated the two nucleotide residues at −478 and −480 calculated from the adenosine of the initiation codon, which were 2 and 4 bp upstream from the 5′-cDNA end obtained by 5′-RACE. We concluded that the g at −480 is the main transcription initiation site for the human P-LAP gene. The numbering of the nucleotide residues in Fig. 3 has been determined starting from the transcription initiation site g numbered as +1. No specific signals were obtained with tRNA (Fig. 4, lane tRNA). Primer sn-2 yielded no significant signals (data not shown), which should be due to the long distance between the primer and the transcription start sites.

As shown in Fig. 3, upstream region from the exon 1 contained a GC rich sequence (about 63%), a TATA box-like motif (26) (ATTTAATT; −38 to −31), four GC boxes of GGGCGG or CCGCCC (27) (−789 to −794, −406 to −401, −328 to −323, −302 to −297) and an AP-2 binding site (CCCAGGGC) (28) at position −357 to −350. We could not find the estrogen responsive element (ERE) consensus sequence (15) in this region, while there were one 5′ half palindrome-like motif (AGGTCC; −334 to −329) and two 3′ half palindrome-like motif (CGACCT; −164 to −159, TGACTT; −17 to −12).

The human chromosomal localization of P-LAP gene was determined by FISH. A total of 50 metaphase cells were examined. Of these, 21 cells exhibited twin-spot signals on both homologs, 26 had incomplete signals on either or both homologs, and no specific signals were detected in 6 cells. By high-resolution banding analysis, the specific signals were localized to q14.2-q15 region of chromosome 5. No signals were observed on the other chromosomes. This accumulation of the signals indicated that human P-LAP is located on chromosomes 5q14.2-q15 (Fig. 5).

FIG. 3. Nucleotide sequence of the gene around the human P-LAP transcription initiation sites. Exon sequences are shown in upper-case letters and 5′-flanking region and intron sequences are shown in lower case letters. The arrow maps major transcriptational start site determined by primer extension (nucleotide numbered +1 in the sequence). Putative response elements are underlined with their respective identification.

DISCUSSION

The rat homologue of human P-LAP is generally referred as IRAP, which was found at first as a major component of GLUT4-containing vesicles. The protein possesses aminopeptidase activity and redistributes to plasma membrane in response to insulin (6–8). Keller isolated the cDNA clone of rat IRAP (5) almost the same time as we did that of human P-LAP (4). Alignment of P-LAP cDNA (Acc. No. D50810) from −71 to +3126 with IRAP (Acc. No. U76997) demonstrated that they are 85.4% identical at the nucleotide level. The sequence conservation exhibited they were homologous in different spieces, which was supported by the similar tissue distribution of the mRNAs (4, 5). In contrast with human P-LAP, however, 5′-end of rat IRAP cDNA locates 71 bp upstream from the ATG codon. The longer 5′-untranslated region of human P-LAP cDNA was verified by 5′-RACE experiment, PCR amplification and primer extension in this study. These results suggested that the 5′-end of rat IRAP is distinct due to the difference of the species or may be incomplete.

Then we isolated the upstream genomic DNA of the human P-LAP gene using 618 bp 5′-end fragment as the probe. Three positive clones were identified and analyzed by DNA sequence. A partial genomic map was constructed based on these three clones in sum that contained exon 1 and exon 2. We were unable to determine precisely the size of the first intron which should be too large to be contained in the single phage (average insert size, 16 kb). Within a sequence examined, splicing donor and acceptor consensus sequences were located at the putative exon/intron borders (26). The exon 1 was composed of large untranslated region and 19 bp translated region. The exon 2 spanned 844 bp translated region which included the transmembrane domain and the N-terminal sequence of soluble P-LAP in maternal serum (3). Concerning soluble

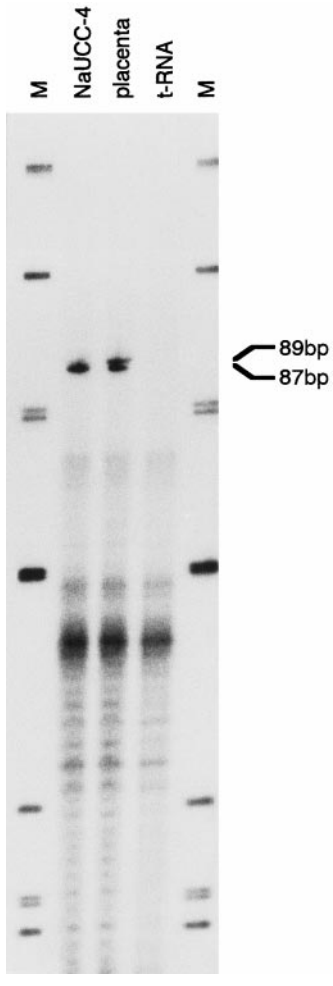

FIG. 4. Primer extension analysis to identify the transcription initiation sites. Total RNA from human placenta and choriocarcinoma cell line NaUCC-4, and tRNA control were hybridized with an end-labeled primer jh-1 and extended with reverse transcriptase. The extended products were analyzed on 7% urea/6% polyacrylamide gel. Sizes of products were determined by comparison with phaiX174 Hinf I marker (M).

P-LAP, little is known how it is produced, but genomic analysis in the current study gave a clue to resolve the question. In case of vascular endothelial growth factor (VEGF), soluble, intermediate and membrane-bound forms of VEGF are regulated by alternative splicing (29). Contrary to this, absence of the splicing site around the N-terminus of soluble P-LAP and transmembrane domain suggested that P-LAP in the serum must be produced not at transcription levels, but at post-translation levels as the cleavage by secretases.

The 5′-flanking region of the human P-LAP gene contained a TATA box-like motif and several cis-acting regulatory elements. In the placental organ culture P-LAP activity is up-regulated by estrogen (14). Within the upstream region of human P-LAP gene examined, we found three half palindromic ERE-like motif. The promoter region of cathepsin D gene which is induced by estrogen has no consensus ERE motif but functional imperfect ERE, SP1-ERE halfsite (30, 31). The sequence 5′-GCGGGGCACTGACTT-3′ (−26 to −12) located just near the transcription initiation site in P-LAP gene is quite similar to the SP1-ERE halfsite sequence. Further studies such as measuring promoter activity and gel shift assay are on the way to evaluate the estrogen effects on P-LAP gene expression.

We demonstrated by FISH that the P-LAP gene is mapped on human chromosome 5q14.2-q15. To our knowledge, this area is not associated with known genetic disorders.

In conclusion, we succeeded in the isolation and characterization of the 5′-region of human P-LAP gene. We also determined the chromosomal assignment by FISH. This study helps to establish the genetic basis of P-LAP gene research in humans, leading to better understanding of the physiological role of P-LAP in regulating labor pain and probably glucose transport.

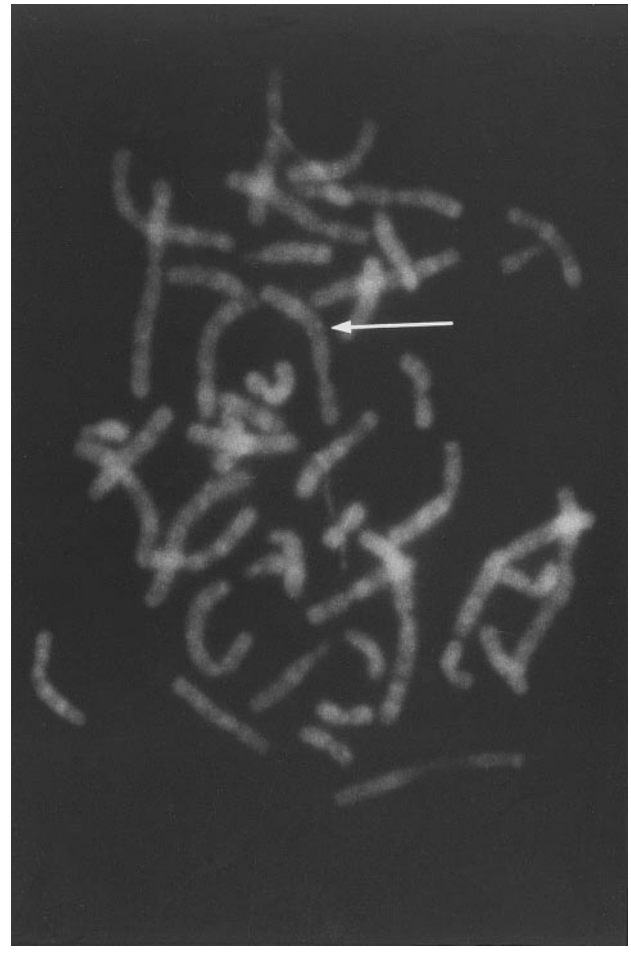

FIG. 5. R-banding metaphases after *in situ* hybridization with biotinylated human P-LAP gene. An arrow indicates signals on 5q14.2-q15.

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