Cloning, Nucleotide Sequence, and Chromosome Localization of the Human Pleiotrophin Gene†

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ABSTRACT: Pleiotrophin (PTN), midkine (MK), and retinoic acid-induced heparin-binding (RI-HB) protein are members of a recently discovered family of developmentally regulated cytokines. We report here the cloning, sequencing, chromosomal localization, and structural organization of the genomic version of the human PTN gene and its comparison to the mouse MK gene. The PTN gene was found to be arranged in five exons and four introns, in a fashion similar to that of the mouse MK gene. Exon 1, as for MK, does not appear to encode amino acid sequence. As in the case of the MK gene, exon 2 encodes the hydrophobic leader sequence of PTN, which constitutes the beginning of gene translation. The signal peptide cleavage site of both genes lies toward the 3′ end of exon 2. Exons 3 and 4 of PTN were most closely related to exons 3 and 4 of the MK gene; in particular, six of the ten cysteine residues were coded for in exon 3 and the remaining 4 in exon 4. The intron–exon splice junctions of both genes occurred through the same residues. The two genes were found to be less closely related in the fifth exon which encodes the highly basic C-terminal domains, the translation termination codon, and the polyadenylation signal of both cDNAs. We also report ~2000 bp of the 5′ untranslated sequence of the PTN gene and the site of initiation of transcription in human placenta. PTN was localized to human chromosome 7q33-34 by fluorescence in situ hybridization. These data confirm the existence of a new gene family of developmentally regulated cytokines.

The molecular events which control fetal growth and development are currently the subject of intense investigation. Pleiotrophin (PTN) (Milner et al., 1989; Li et al., 1990), midkine (MK) (Kadomatsu et al., 1988; Tomomura et al., 1990a), and retinoic acid-induced heparin-binding (RI-HB) protein (Vigny et al., 1989; Raulais et al., 1991; Urios et al., 1991) are members of a recently discovered family of developmentally regulated cytokines which display both neurotrophic and mitogenic properties. Both RI-HB (which may be the avian homologue of MK) and MK are inducible by retinoic acid at both the mRNA and protein level (Kadomatsu et al., 1988; Tomorura et al., 1990b; Raulais et al., 1991). These genes are widely expressed during development in the fetus (Li et al., 1990; Kadomatsu et al., 1990), and their protein products bind to heparin, thus remaining basement membrane and cell matrix associated. Following the period of rapid fetal growth and organogenesis, the pattern of distribution of these cytokines is dramatically reduced. By midgestation, MK mRNA was found to be expressed only in the kidney (Kadomatsu et al., 1990), hence the name midkine or *mid*gestational mouse *ki*ney g*ene*, and RI-HB protein expression was restricted to the lens capsule and iris in newly hatched chickens (Vigny et al., 1989). PTN expression, which is present at birth in the rat brain, intestine, muscle, skin, heart, lung, and kidney, appears 1 month postnatally to be limited to the intestine and brain (Li et al., 1990; Merenmies & Rauvala, 1990). In the adult, PTN mRNA expression could only be detected in the brain and uterus (Li et al., 1990), although PTN cDNA has recently been cloned from adult mouse osteoblasts (Tezuka et al., 1990), suggesting expression in bone as well.

Controversy was generated by the claim (Milner et al., 1989) that these three molecules were mitogenic (Kuo et al., 1990; Huber et al., 1990). However, each has now been independently shown to be mitogenic and PTN to be angiogenic (Milner, 1991; Courty et al., 1991). PTN purified from adult bovine brain is a potent mitogen for endothelial cells (Courty et al., 1991) with half-maximal stimulation at 0.2 ng/mL, and recombinant PTN is mitogenic for NRK cells (Li et al., 1990). Independently, RI-HB (Raulais et al., 1991) and MK (Tomomura et al., 1990b) were shown to be mitogenic for PC-12 cells with half-maximal stimulation of cell division at approximately 10 ng/mL. Recombinant MK protein has been purified from the supernatant of a transient expression system and found to be a mitogen for NIH 3T3 cells (Muramatsu & Muramatsu, 1991). The recent report of the oncogenic properties of the PTN gene, including its ability to induce tumors in nude mice (Li et al., 1991), is a clear demonstration in vivo of this molecule's biological properties as a mitogen.

High-affinity receptors (~5000/cell) for PTN with a k_d of ~0.6 nM and estimated molecular masses of ~155 and ~127 kDa have been identified on the surface of NIH 3T3 cells (Kuo et al., 1992). Receptor-mediated endocytosis occurs at this site following PTN binding, and the PTN receptor–ligand interaction can be disrupted by heparin. These data suggest that a specific cell surface receptor mediates the mitogenic and neurotrophic effects of PTN on the target cells.

The neurotrophic activity of these proteins has also been confirmed. RI-HB induced neurite outgrowth from PC-12 cells (Raulais et al., 1991), while PTN induced neurite outgrowth from rat fetal cortical neurons (Rauvala, 1989; Li et al., 1990), as did the recombinant MK protein (Muramatsu & Muramatsu, 1991).

The cDNA and genomic sequences of MK have been published (Matsubara et al., 1990) as have the mouse, rat,

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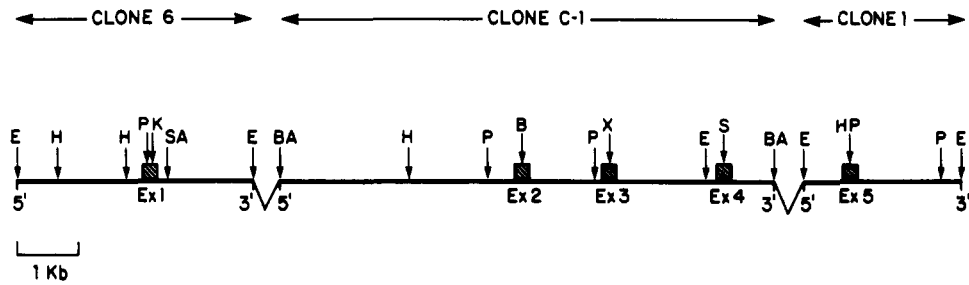

FIGURE 1: Partial restriction map of the three clones encoding exons 1–5 of the human PTN gene. Restriction sites were identified by digestion of the clones followed by Southern analysis as described in the text. Clones 1, C-1, and 6 are shown according to scale and exons 1–5 by the hatched boxes. The restriction enzyme code is as follows: BA, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; P, *Pst*I; SA, *Sac*I; S, *Stu*I; X, *Xho*I.

and bovine PTN cDNA sequences (Li et al., 1990; Merenmies & Rauvala, 1990; Tezuka et al., 1990). We report the previously unpublished sequence and organization of the human PTN gene from genomic DNA and its chromosome localization.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of PTN Genomic Clones. A PTN cDNA probe (Li et al., 1990) labeled with [^{32}P]dCTP and a random primer kit (Boehringer Mannheim) was used to screen a Charon 4A bacteriophage human genomic library (American Type Culture Collection) by plaque filter hybridization. Nitrocellulose filters were hybridized overnight at 42 °C in the presence of 6× SSC, 5× Denhardt's solution, 0.2% SDS, 200 μg/mL tRNA, and 50% formamide. The filters were then washed with 0.1× SSC containing 0.5% SDS at 68 °C for 1 h, dried, and visualized by autoradiography. Two strongly hybridizing clones were identified. *Eco*RI restriction fragments from these purified clones were fractionated by size by electrophoresis through 1% agarose gels. The gels were then dried, treated with 0.5 N NaOH, hybridized directly with the ^{32}P-labeled PTN cDNA probe, and washed using the same conditions as for plaque filter hybridization described above. One positively hybridizing fragment from each of the clones was extracted from the gel by NaI treatment using a Geneclean II kit (Bio101, Inc.) and subcloned into the *Eco*RI site of the plasmid vector pUC19. Clone 1 contained a 3-kb insert which was found upon DNA sequencing to contain exon 5. Similar DNA sequencing experiments (see below) revealed that the other clone (clone 6) contained a 4-kb insert which encodes exon 1 of the PTN gene. An additional 8-kb PTN genomic clone was obtained by colony filter screening of a human genomic pWE-15 cosmid library with a ^{32}P-labeled PTN cDNA probe. This clone, C-1, was found to encode exons 2, 3, and 4. Restriction site mapping of clones 1, 6, and C-1 was performed by Southern blot analysis (Maniatis et al., 1982) with restriction endonucleases purchased from Promega Biochem.

DNA Sequence Analysis. Microgram quantities of the pUC19 plasmids containing the three clones (1, 6, and C-1) were prepared and isolated by phenol–chloroform extraction for DNA sequencing. Double-stranded DNA sequencing of these clones was performed by Sanger's dideoxy method using a Sequenase kit (USB Corp.), 18-nucleotide sequence-specific primers, deoxyadenosine [α-^{35}S]thiotriphosphate, and 6% denaturing polyacrylamide gel electrophoresis (Sanger et al., 1977). The 5′ sequence, 2000 bp upstream of the first nucleotides of the cDNA, and the sequence of exon 1 (nucleotide −2012 to +327) were obtained from clone 6 using the universal primer M13-reverse (Pharmacia) and a series of synthetic primers derived from continuous overlapping sequences proceeding from the 5′ → 3′ direction along the 4-kb fragment. All primers were synthesized by Midland Certified Reagent Co. (Midland, Tx). The sequence of exons 2–5 and the intron–exon junction were determined from clones 1 and C-1 by sequencing in the forward and reverse directions using a series of synthetic 18-nucleotide primers. The sequence for these primers was obtained from the coding and noncoding strands of the human PTN cDNA.

Determination of Intron Size. The sizes of introns 2 and 3 were determined by PCR from clone C-1 using forward and reverse primers from exons 2 and 3 and exons 3 and 4, respectively. PCR reactions were performed using a GeneAmp Kit (Perkin-Elmer/Cetus). Because clones 1, 6, and C-1 were not overlapping, it was not possible to determine the size of introns 1 and 4. However, it was shown by restriction enzyme analysis of the three clones that introns 1 and 4 were greater than 15.0 and 6.0 kb, respectively.

Primer Extension. Oligonucleotide primers corresponding to reverse sequence from exon 1 were end labeled with [γ-^{32}P]-dATP using the dsDNA cycle sequencing system (BRL). Primer extension was performed by incubating 10 μg of total RNA, extracted from human placenta, with 30 000 cpm of labeled primer and 15 units of AMV reverse transcriptase (Promega) for 1 h at 37 °C. Samples were loaded and run simultaneously in a DNA sequencing gel with the DNA sequence from clone 6 prepared according to the method above using unlabeled primer.

Chromosome Localization by in Situ Hybridization. Fluorescence in situ hybridization was performed essentially as described by Lichter et al. (1988). Human prometaphase chromosome spreads were prepared from cultured phytohemagglutinin-stimulated peripheral blood lymphocytes from a male of normal karyotype (46,XY). Extended chromosomes were produced by standard colchicine treatment. The 8-kb insert of PTN clone C-1 was labeled with biotin-11–dUTP by nick translation (Rigby et al., 1977), and each chromosome spread was hybridized with approximately 150 ng of labeled probe (Lichter et al., 1988). For fluorochrome detection, slides were incubated with 5 μg/mL fluorescein isothiocyanate-(FITC-) conjugated avidin DCS (Vector Laboratories), amplified by incubation in 5 μg/mL FTIC-conjugated goat anti-avidin D antibodies (Vector Laboratories), and counterstained with 200 ng/mL 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and 200 ng/mL propidium iodide (PI), which were present in the final wash solution. Cytogenetic banding patterns were observed by staining the slides with giemsa following fluorescent hybridization.

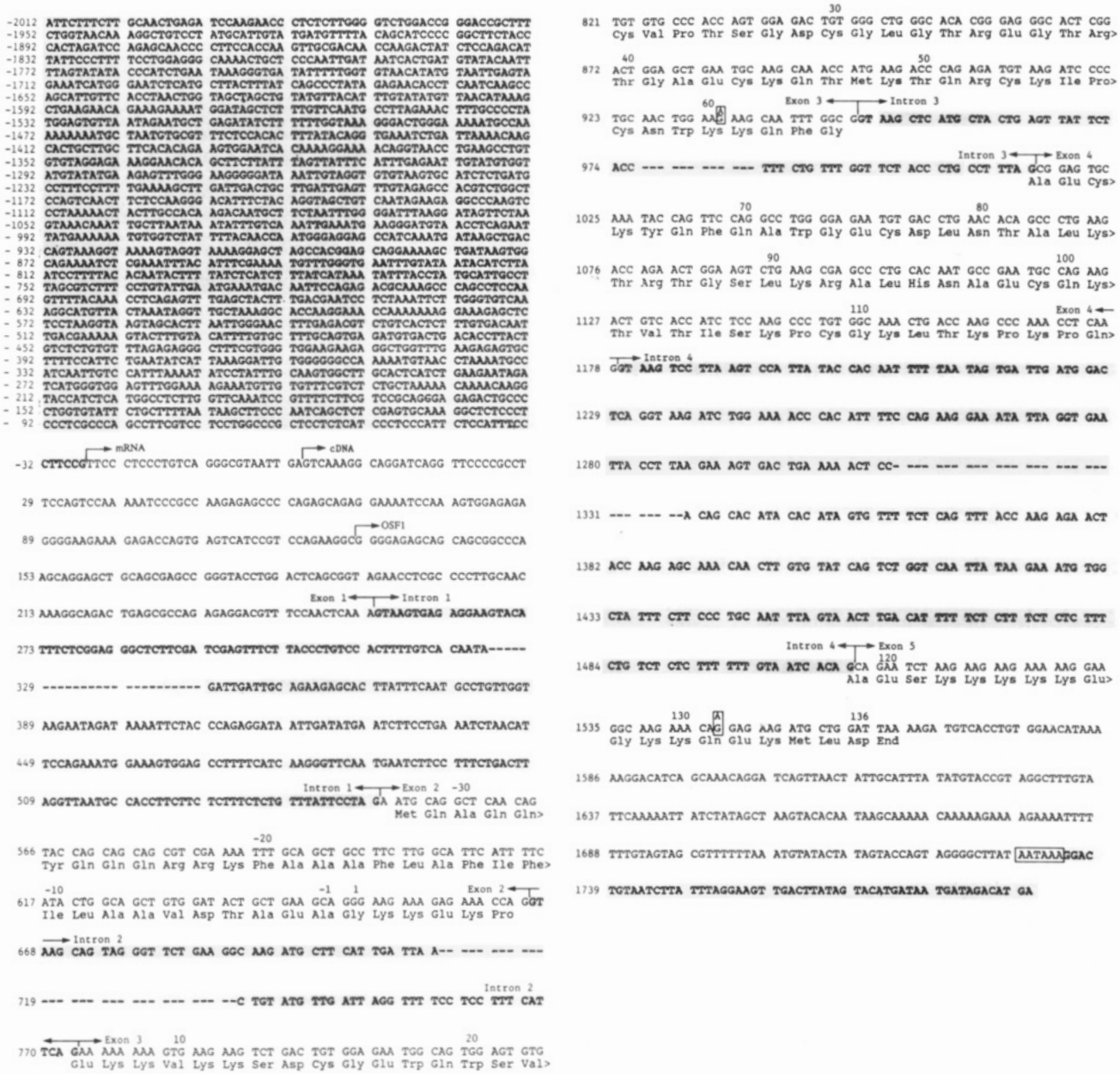

FIGURE 2: Human PTN gene sequence. Shown in the shaded type from nucleotide –2012 to –27 is the 5′ untranslated sequence of the PTN gene. Nucleotide position 1 corresponds to the beginning of the cDNA sequence as previously determined (Li et al., 1990); primer extension assays identified the transcription initiation site labeled mRNA. Limited flanking sequences from introns 1 to 4 are shown in shaded type, and each intron–exon junction is identified by arrows above the nucleotide sequence. The complete amino acid sequence of the PTN protein is shown in a three-letter code below the nucleotide sequence starting with the initiator methionine at amino acid position –32, found at the beginning of exon 2. The amino acid number of the PTN protein is shown as numbers above the nucleotide sequence. The hydrophobic leader sequence is amino acid –32 to –1, while amino acid 1 corresponds to the N-terminal glycine of the sequenced protein (Milner et al., 1989). Variations in the nucleotide sequence between the two individuals from whom the cDNA and gene sequences were derived are highlighted by boxed nucleotides at amino acids 60 and 131. The polyadenylation signal at nucleotide position 1729 is boxed, and the 3′ untranslated nucleotide sequence following this is shown in shaded type.

RESULTS

Isolation of PTN Genomic Clones. Three human genomic clones, clones 1 and 6 from the phage (Charon 4A) library and clone C-1 from a Cosmid (pWE-15) library, were isolated by virtue of their ability to hybridize under conditions of high stringency to full-length human PTN cDNA. *Eco*RI digestion of clones 1 and 6 and *Bam*HI digestion of clone C-1 yielded three strongly hybridizing fragments 4 kb (clone 6), 8 kb (clone C-1), and 3 kb (clone 1) in length, which were subcloned into plasmid vectors for further study. These three clones failed to hybridize to each other, had no DNA sequence or restriction sites in common, and so were judged not to overlap.

A partial restriction map of these three clones and the human PTN gene is shown in Figure 1, which also shows the structural organization of the PTN gene. Several unique restriction sites were found, as predicted by computer analysis of the human PTN cDNA sequence. These included the *Kpn*I site in exon 1 and *Bgl*I site in exon 2, which proved useful both in determining the organization of the human PTN gene and for comparing clones 6 to C-1 to confirm that they were not overlapping.

Sequence of the PTN Gene. The nucleotide sequence of ~2000 bp upstream of exon 1 and the complete sequence of exons 1 → 5 of the humans PTN gene, up to the polyadenylation signal AATAAA in exon 5 of the human PTN gene,

Table I: Intron–Exon Splice Junctions of the PTN Gene[a]

donor sequence	acceptor sequence	exon size (bp)	intron size (kb)
Exon 1 | Intron 1 | Exon 2		(1) 271	(1) >15.0
TTCCAACTCAAA | GTAAGTG.....ATTCTAG | A ATG CAG GCT			
*Met-Gln-Ala-			
-32 -31 -30			
Exon 2 | Intron 2 | Exon 3		(2) 115	(2) 1.2
AAA CCA G | GTAAGCA.....CATTCAG | AA AAA AAA			
-Lys-Pro-G | lu-Lys-Lys-			
+5 +6 +7 +8 +9			
Exon 3 | Intron 3 | Exon 4		(3) 175	(3) 1.9
TTT GGC G | GTAAGCT.....CCTTTAG | CG GAG TGC			
-Phe-Gly-A | la-Glu-Cys-			
+63 +64 +65 +66 +67			
Exon 4 | Intron 4 | Exon 5		(4) 162	(4) >6.0
CCT CAA G | GTAAGTC.....ATCACAG | CA GAA TCT		(5) 226	
-Pro-Gln-A | la-Glu-Ser-			
+117 +118 +119 +120 +121			

[a] Underlined sequences in the introns represent splice donor and acceptor sequences. The asterisk indicates the site of initiation of translation.

are shown in Figure 2. Only a partial sequence of the introns (1 → 4) is shown, i.e., the sequence close to and forming the intron–exon splice junction. The sequence and location of the intron–exon splice junction of the human PTN gene are shown in Table I.

Analysis of the 5′ upstream sequence failed to identify a functioning TATA or CAAT box. Primer extension identified the PTN mRNA transcription start site at 26 bp 5′ of the human PTN cDNA, and this site is labeled mRNA in Figure 2. Exon 1 is 271 nucleotides long and appears not to be translated since the site of initiation of translation, the ATG sequence of methionine 32, begins as the second nucleotide of exon 2. Exons 2, 3, 4, and 5 are 115, 175, 161, and 226 bp, respectively. The 32 amino acid hydrophobic leader sequence and the first six N-terminal amino acids of the mature protein are encoded by exon 2. The splicing of exon 2 to exon 3 occurs at glutamic acid 7, and exon 3 encodes 57 amino acids (7 → 64) including 6 of the 10 cysteine residues. The remaining four cysteines are encoded among the 53 amino acids of exon 4, which is joined to exon 3 through alanine 65. Exon 5 is joined to exon 4 through alanine 119 and encodes the C-terminal 17 amino acids of the mature protein, the TAA stop codon, and 172 nucleotides of the 3′ untranslated sequence including the polyadenylation signal AATAAA.

Two nucleotide differences were noted between the human cDNA sequence and the human PTN gene sequence, but neither was significant since they did not result in an amino acid sequence difference. These changes, an A → G switch in the third position of lysine 60 and an A → G switch in the third position of glutamine 131, most likely represent sequence variation between the two individuals from whom the cDNA and genomic libraries were prepared. The amino acid sequence of the mature protein has already been shown to be extremely highly conserved between three species, cow, man, and rat, with only one amino acid difference between rat and man and two between man and cow out of a total 136 amino acids (Li et al., 1990).

The donor and acceptor sequences for intron–exon splicing, GTAAG and AG, respectively (Table I), were identical in introns 1–4 and conformed to recognized splice donor and acceptor sequences.

Comparison to the MK Gene. Sequencing of the PTN gene confirms its homology to the MK gene and supports the claim that the genes are related. Using the published mouse MK gene sequence (Matsubara et al., 1990) and the PTN gene sequence derived here, we compared their amino acid sequence identities, functional homology, and exon structures (Figure 3). Of the 118 amino acids of the mature MK protein, 61, or 51%, are identical to PTN. In addition, 15 amino acid differences appear to be functionally conserved changes, giving an overall functional identity of 76 amino acids, or 64%.

Most striking, however, is the high degree of conservation within exons 3 and 4 of both genes, which together encode the 10 cysteine residues, and the marked similarity of intron–exon splice junctions. Exon 1 of both genes is not translated. The site of initiation of translation of both the PTN and MK proteins is at the beginning of exon 2, and in each case exon 2 encodes the hydrophobic leader sequence. The exon 2–exon 3 splice junction occurs through glutamic acid, Glu 4 for MK and Glu 7 for PTN. There are 6 cysteine residues encoded in exon 3 of both genes, and exon 3 of MK appears to have 70% functional amino acid homology with exon 3 of PTN. Exon 4 has the same number of amino acids in both proteins, encodes the remaining 4 of 10 cysteines of each protein, and is spliced to exons 3 and 5 through the same residues (Ala 65 and 119 in the case of PTN and Ala 67 and 111 in MK). The C-terminal tail of both proteins is encoded by exon 5, but this exon and the intron size of the two genes appear to be somewhat different. The human MK gene has not yet been identified and so the comparison of these two genes is incomplete.

Chromosome Localization of the Human PTN Gene. The PTN gene was cytogenetically localized using fluorescence in situ hybridization essentially as described by Lichter et al. (1988). The 8-kb insert from the PTN genomic clone C-1 was labeled with biotin and hybridized to metaphase spreads of human chromosomes. The labeled DNA was detected with fluorescein isothiocyanate- (FITC-) conjugated avidin DCS and amplified using goat anti-avidin D antibodies. As shown in Figure 4, the probe strongly hybridizes to a region on 7q. Subregional localization to 7q33-34 was achieved by analysis of the same spreads banded with giemsa. In more than 50 metaphase spreads examined, signals were observed on both chromosome 7 chromatids in 60% of the cases. No consistent secondary sites of hybridization were detected.

DISCUSSION

This is the first report of the cloning, exon sequence, genomic organization, and chromosome localization of the human PTN gene. These findings have allowed us to compare the organization of this gene to that of the mouse MK gene and thereby confirm the existence of a new gene family. The

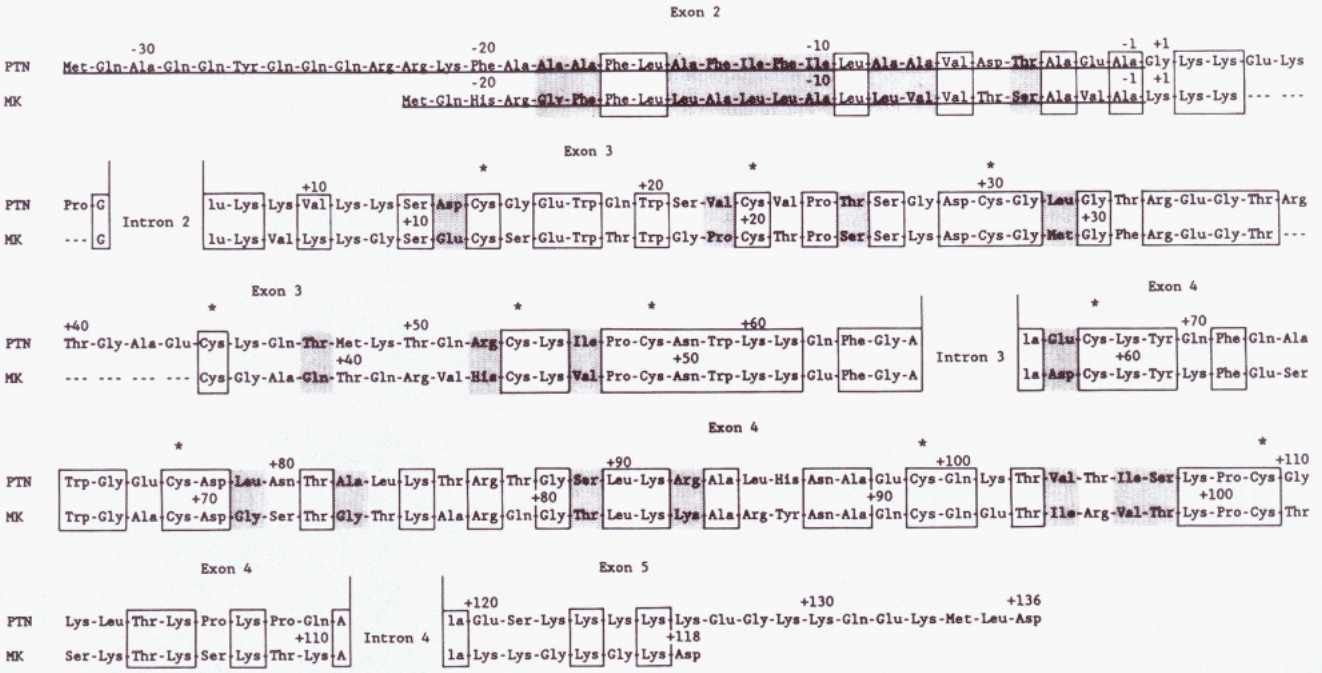

FIGURE 3: Comparison of the mouse MK and human PTN gene structures and amino acid sequences. The amino acid sequences are shown in three-letter code, and the hydrophobic leader sequence of both proteins is underlined with amino acid +1 corresponding to the N-terminus of the secreted protein. The intron–exon junctions are indicated by the tall vertical lines, and the exons are identified by numbers. Only exons 2–5 are shown. The boxed amino acids represent the regions of identity, and the shaded sequences are the regions of presumed functional conservation. The asterisks indicate the cysteine residues of both proteins, for comparison.

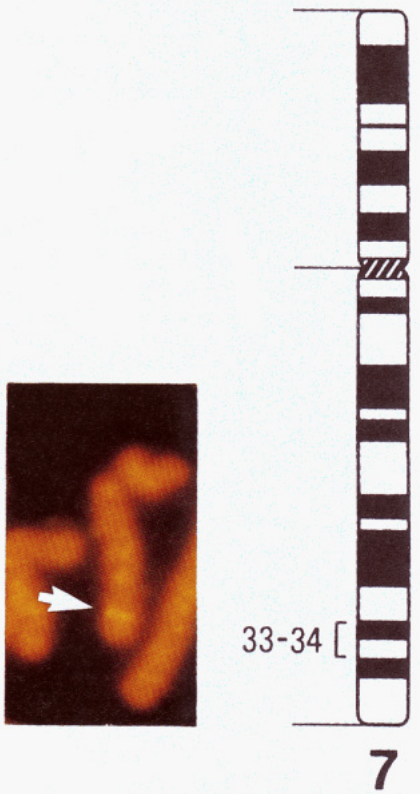

FIGURE 4: Localization of the PTN gene to 7q33-34. Fluorescence in situ hybridization using a biotin-labeled 8-kb PTN genomic clone (C-1) demonstrated a strongly hybridizing signal on 7q as the left inset panel illustrates. DAPI staining and geimsa banding confirmed subregional localization to 7q33-34. The right panel shows the standard idiogram of a giemsa-banded chromosome 7 at the 550-band resolution.

chicken RI-HB most likely represents the avian equivalent of MK.

Analysis of the 5′ upstream sequence of the human PTN gene revealed no TATA or CAAT boxes sufficiently close to the beginning of the cDNA sequence in order that they might function as promoters of initiation of transcription. GC-rich regions were found in the 5′ region of the gene. Similar findings were noted in the case of the MK gene (Matsubara et al., 1990).

The human PTN cDNA clone previously isolated from human placenta (Li et al., 1990), the sequence of which is unpublished, was found to begin at the nucleotide labeled 1 in Figure 2. The previously reported human osteoblast-specific factor or OSF-1 cDNA (Tezuka et al., 1990), which codes for a protein identical in amino acid composition to PTN, was found to be missing 125 nucleotides at the 5′ end relative to PTN cDNA and to begin at nucleotide +125 of the PTN gene with the sequence GGGAGAGC... etc. OSF-1 and PTN cDNA are most likely incomplete clones, since our primer extension studies identify the transcription initiation site at 26 bp 5′ of the beginning of the human PTN cDNA sequence. Primer extension assays in this study were done on RNA extracted from human placenta, and no transcript was detected which corresponded to OSF-1. OSF-1 may, however, represent an alternative PTN mRNA transcript expressed in osteosarcomas and brain, the two human tissues from which OSF-1 cDNA was isolated and sequenced. The MK gene has three alternative sequences for the first exon, the significance of which is not clear (Tomomura et al., 1990a).

We have compared the structure of the human PTN gene and the mouse MK gene and found many similarities in addition to the known sequence homology (Li et al., 1991). It was not possible to compare the human MK gene organization with that of PTN. However, the human MK cDNA has been characterized (Tsutsui et al., 1991) and differs from the mouse cDNA, so certain limited comparisons can be inferred between the human PTN and MK genes.

There are 15 amino acid differences between the mouse and human MK sequences, and some uncertainty exists about the correct N-terminal sequence of these proteins since neither has been purified and sequenced. In addition to the suggestion

that RI-HB is the chicken homologue of MK, Raulais et al. (1991) have suggested that the valine and alanine residues at positions −2 and −1, respectively, of MK are not in fact part of the leader sequence but rather represent the N-terminus of this protein. This would imply cleavage of the MK leader sequence between alanine −3 and valine −2 and a closer fit between the MK and PTN proteins at their N-termini than is suggested by the comparison shown in Figure 3. Of the 15 amino acid differences between mouse and human MK, only two are in the regions of identity shared between MK and PTN shown in Figure 3 (the boxed amino acids), and the changes Glu 4 to Asp and Lys 46 to Arg show functional conservation. The human MK protein appears to have a three amino acid insertion of Gly-Gly-Pro between Lys 8 and Gly 9 of the mouse.

The Glu to Asp switch at position 4 of MK is through the splice junction of exons 2 and 3; however, it does not appear to affect intron–exon splicing since only the third residue of the mouse GAG nucleotide sequence coding for Glu is altered to either GAC or GAT coding for an Asp residue in the human. The G to C or T swtich does not affect this intron–exon splice junction since it occurs between the G-A nucleotides of this triple codon.

In summary, cloning and sequencing of the PTN gene have allowed us to compare it to the MK gene and confirm the existence of a new cytokine gene family. In addition, cloning the PTN gene has allowed us to localize it to chromosome 7q33-34, a region of the human chromosome about which relatively little is known. Interestingly, the hepatocyte growth factor (HGF) gene has also recently been localized to human chromosome 7q in the region of 22-qter (Laguda et al., 1991).

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