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Structural Characterization of the Human Platelet-Derived Growth Factor A-Chain cDNA and Gene: Alternative Exon Usage Predicts Two Different Precursor Proteins

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The human platelet-derived growth factor (PDGF) A-chain locus was characterized by restriction endonuclease analysis, and the nucleotide sequence of its exons was determined. Seven exons were identified, spanning approximately 22 kilobase pairs of genomic DNA. Alternative exon usage, identified by cDNA cloning, occurs in a human glioblastoma cell line and may give rise to two types of A-chain precursors with different C termini. The exon-intron arrangement was similar to that of the PDGF B-chain/*sis* locus and seemed to divide the precursor proteins into functional domains. Southern blot analysis of genomic DNA showed that a single PDGF A-chain gene was present in the human genome.

Human platelet-derived grown factor (PDGF) is an M_r 30,000 protein composed of two related polypeptide chains, A and B, of approximately the same size (30). The B-chain gene is the normal cellular homolog to the v-*sis* oncogene of simian sarcoma virus (SSV) (7, 13, 29, 31, 53) (and therefore denoted *sis*) and is situated on the short arm of human chromosome 22 (4, 11, 48). A 4.0-kilobase (kb) PDGF-B/*sis* transcript has been detected in a variety of human tumor cell lines (14, 19, 37, 52) as well as in endothelial cells (3, 9, 25), placental cytotrophoblasts (18), and activated macrophages (35, 46). Both the B-chain/*sis* gene and v-*sis* encode a precursor molecule with a hydrophobic N-terminus characteristic of a signal peptide. The precursors are further processed by proteolytic propeptide removal at both the N and C terminals (29, 40). Structurally, the processed proteins are likely to be very similar; only three amino acid differences fall within the mature PDGF B-chain sequence, and these are thought to constitute a species variation between woolly monkey (in which SSV arose) and humans. However, the size of the fully processed monomeric v-*sis* product (12 kilodaltons [kDa] [28, 40, 41]) is smaller than that observed for the human PDGF B-chain (16 kDa [30, 53]), indicating that the proteolytic cleavages may occur at different sites in the two precursor molecules. Biologically, the v-*sis* product is very similar to PDGF (12, 16, 28, 33), and several lines of evidence suggest that acute SSV transformation is the result of autocrine PDGF receptor activation only (26, 27).

PDGF A-chain complementary DNA (cDNA) has been isolated from a human glioma cell line (6) and predicts that the A-chain is also synthesized as a prepropeptide. The A- and B-chain precursors are about 40% homologous, and in the mature chains the homology increases to 60%. All eight cysteine residues are conserved, indicating similarities in the tertiary structure. Human genomic A-chain-homologous sequences have been localized to chromosome 7 by using somatic cell hybrids (6). Three PDGF A-chain transcripts of 1.9, 2.3, and 2.8 kb have been detected in human tumor cell lines (5, 6, 54) as well as in normal human endothelial cells in culture (10) and mitogen-stimulated human fibroblasts (38).

In the present study, we have isolated and characterized genomic clones corresponding to the human PDGF A-chain locus. We show that the gene contains at least seven exons and that alternative exon usage occurs in a human glioma cell line and may give rise to two types of PDGF A-chain precursors with different C terminals.

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from either New England BioLabs or Pharmacia. DNA polymerase I (Klenow fragment), T4 DNA ligase, and *Eco*RI linkers were from Pharmacia. T4 DNA polymerase was from New England BioLabs. λEMBL3 and λEMBL4 DNAs were from Promega Biotech. Lambda phage in vitro packaging extracts were from Stratagene and Promega Biotech. A human leukocyte DNA library used for the initial screenings for genomic clones was purchased from Clontech.

Construction and screening of glioma cDNA library. The human clonal glioma cell line U-343 MGa C12:6 was the source of polyadenylated [poly(A)$^+$] RNA, which was prepared by the LiCl-urea method (2). Oligo(dT)-primed synthesis of double-stranded cDNA was performed by the method of Huynh et al. (24). The resulting cDNA was treated with T4 DNA polymerase and subcloned into *Eco*RI-cleaved λgt10 by using *Eco*RI linkers. The recombinant phage were plated on *Escherichia coli* C600 hfl. The library was screened with several double-stranded DNA probes directed against the PDGF A-chain protein sequence, synthesized as long overlapping oligonucleotides and radiolabeled with [α-^{32}P]deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I (6). Duplicate nitrocellulose filter lifts were hybridized with ^{32}P-labeled oligonucleotide probes at 42°C in 20% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM sodium phosphate (pH 7.0)–5× Denhardt–0.1% sodium dodecyl sulfate (SDS)–20 μg of sonicated salmon sperm DNA per ml and washed in 0.5× SSC–0.1% SDS at the same temperature. Approximately 10^6 independent clones were

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screened, resulting in four positive isolates which were plaque purified and grown. Lambda DNA was prepared by the method of Helms et al. (22), and the inserts were excised by *Eco*RI digestion. These were then characterized by restriction enzyme analysis and DNA sequencing.

Construction and screening of genomic libraries. Two types of human genomic libraries were constructed from human macrophage DNA, one by cloning partially *Sau*3A-digested DNA into *Bam*HI-cut λEMBL3 and the other by cloning fully *Eco*RI-digested DNA into *Eco*RI-cut λEMBL4. Both libraries were plated on *E. coli* LE392. Duplicate nitrocellulose filter lifts were hybridized with the PDGF A-chain cDNA clone D1, labeled with ^{32}P by the method of random priming (15). Hybridizations were performed at 42°C in 50% formamide–5× SSC–50 mM sodium phosphate (pH 7.0)–1× Denhardt–0.1% SDS–20 μg of sonicated salmon sperm DNA per ml and washed in 0.1× SSC–0.1% SDS at 60°C. Filters were exposed to Kodak XAR5 films overnight at −70°C, and double-positive plaques were picked, plaque purified, and grown for DNA preparation. Restriction enzyme analysis and Southern blotting were performed by standard procedures (34).

Nucleotide sequencing. The nucleotide sequence of cDNA clones and genomic restriction fragments containing sequences hybridizing to the cDNA was determined by the dideoxy nucleotide method (42) after subcloning in M13 derivatives.

RNA preparation and Northern (RNA) blotting. Total cellular RNA was prepared by the LiCl-urea method (2). Poly(A)$^+$ RNA was selected by chromatography on oligo(dT)-cellulose (Pharmacia). Electrophoresis on 0.8% agarose–formaldehyde slab gels, blotting to nitrocellulose filters (Schleicher & Schuell), and hybridization to ^{32}P-labeled DNA probes were carried out by standard procedures (34). Blots were exposed to Kodak XAR5 films overnight at −70°C.

RESULTS

Isolation and characterization of human glioma PDGF A-chain cDNA clones. Human glioma cell line U-343 MGa clone 2:6 expresses high quantities of a PDGF-like protein (36) having the characteristics of a homodimer of PDGF A-chains (M. Nistér et al., unpublished) and expresses relatively large amounts of PDGF A-chain mRNA (6). A cDNA library constructed from this cell line was used for the isolation of PDGF A-chain cDNA clones. Physical maps of the four PDGF A-chain cDNA clones isolated and sequenced are shown in Fig. 1. The sequence of one of them, clone D1, has been published previously (6). The other three clones differ from D1 in one major respect: they lack 69 base pairs (bp) (bases 968 to 1,036 in clone D1) in the 3′ protein-coding sequence. This affects the coding region, so that clone D1 will encode a 211-amino -acid PDGF A-chain precursor with an extremely basic C terminus, whereas clone 13.1 and 15.1 encode an A-chain precursor which is 15 residues smaller and lacks the basic C-terminal region (Fig. 1).

Clone 9.1 differs from the others in an additional respect: a complete sequence divergence is seen 5′ of base 64 (codon 22 in the precursor protein sequence). The presence of an in-frame stop codon in the diverging sequence suggests that the transcript from which this cDNA clone arose cannot be translated to a PDGF A-chain precursor protein.

To reveal the nature of the differences between the cDNA clones, we decided to characterize the corresponding human genomic sequences.

Isolation of human PDGF A-chain genomic clones. To isolate genomic clones corresponding to the human PDGF A-chain locus, cDNA clone D1 was used as a probe to screen a human genomic library constructed from partially *Sau*3A-digested leukocyte DNA cloned into λEMBL3. In this way, two hybridizing clones (λA.1 and λA.2) were isolated and physical maps were established by restriction enzyme analysis. The position of particular restriction sites present also in the cDNA clone suggested that λA.1 and λA.2 overlapped the 5′ part of the PDGF A-chain gene. Attempts to isolate clones covering the 3′ part of the gene from amplified genomic libraries failed; all additional positive clones isolated were fully covered by λA.1 and λA.2. As the cDNA clone recognized two bands of approximately 14 and 18 kb on human genomic Southern blots with *Eco*RI-cleaved DNA and a 17-kb *Eco*RI fragment was predicted by the consensus map of λA.1 and λA.2, we constructed a library from *Eco*RI-digested human DNA in order to isolate a clone containing the second *Eco*RI fragment. The library was screened without prior amplification, and in this way one clone containing a 12-kb *Eco*RI fragment, λB.1 (as well as numerous clones containing the 17-kb fragment, λB.2), was isolated and found to overlap the 3′ end of λA.2 and correspond to the 3′ part of the A-chain gene. Following restriction enzyme analysis of λB.1, a physical map of the PDGF A-chain locus could be established (Fig. 2). Apparently, the 3′ part of the PDGF A-chain locus contains sequences which are poorly propagated by λ phage, a conclusion based on the difficulties in isolating clones covering this region in comparison with the 5′ part of the gene. Subsequent isolation of phage DNA from λB.1 always resulted in lower yields than the other clones.

Characterization of the PDGF A-chain locus. Seven regions hybridizing to cDNA clone D1, spanning approximately 20 kb of genomic DNA, were identified (Fig. 2). The hybridizing regions were sequenced and found to constitute exons. The exon-intron borders established on the basis of sequence homology with the cDNA clone D1 and consensus splice signals (45, 49) are given in Table 1. Thus, the PDGF A-chain gene contains at least seven exons. The sequence of these, intron junctions, and flanking gene sequence is given in Fig. 3. The gene sequence confirms the previously published cDNA sequence (6), and consequently it is likely that the glioma-derived A-chain cDNA sequence encodes a normal A-chain precursor and that the three amino acid discrepancies between the deduced protein sequence from the glioma cDNA and the human platelet PDGF A-chain, determined by protein sequencing (29), are due to protein se-

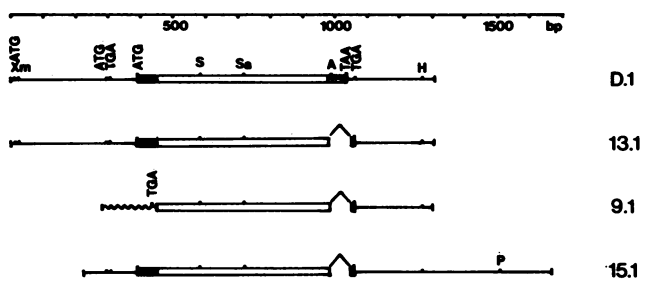

FIG. 1. Physical maps of the four characterized PDGF A-chain cDNA clones D1, 13.1, 9.1, and 15.1. Restriction sites: Xm, *Xma*III; S, *Sst*I; Sa, *Sal*I; A, *Avr*II; H, *Hin*dIII; P, *Pst*I. The hatched bar indicates the sequence coding for the signal peptide. The shaded bar indicates coding sequence unique for clone D1. Upstream in-frame termination codons as well as initiation codons are indicated.

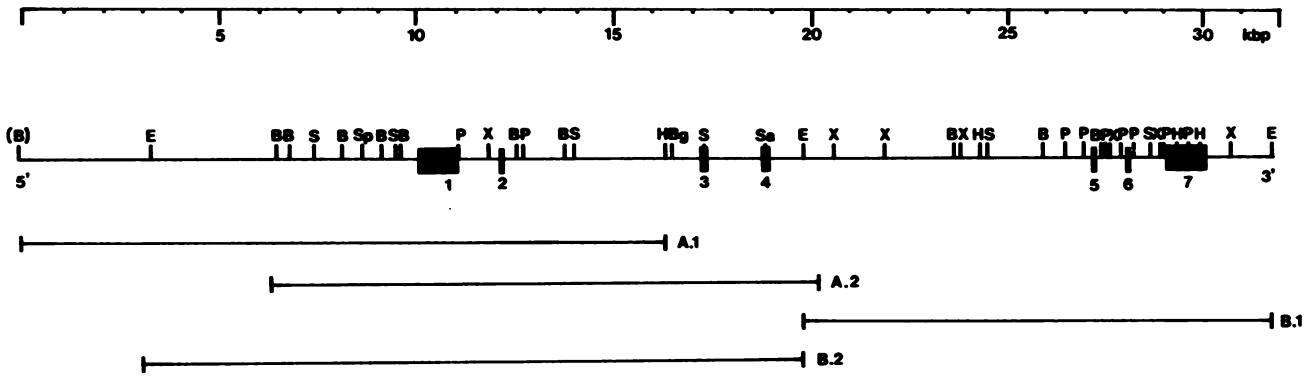

FIG. 2. Physical map of the human PDGF A-chain locus and alignment of the isolated λ clones. Restriction sites: E, *Eco*RI; B, *Bam*HI; S, *Sst*I; Sp, *Ssp*I; P, *Pst*I; X, *Xba*I; H, *Hin*dIII; Bg, *Bgl*II; Sa, *Sal*I; (B), *Sau*3A site at the 5′ end of clone λA.1. The exons are indicated as solid boxes and numbered.

quencing errors (6). It also shows that the 211-amino-acid residues present in the PDGF A-chain precursor deduced from cDNA clone D1 (+ 69 bp) are distributed throughout six of the seven exons of the PDGF A-chain gene (Fig. 3). In cDNA clones 13.1 and 15.1, in which exon 6 was not present, exon 7 contributed three codons to the C terminus (see below).

Many of the differences between the cDNA clones are explained in the gene sequence. The most intriguing difference, the 69-bp region present only in cDNA clone D1, constituted a separate exon (no. 6 in Fig. 2). Its absence in the other three cDNA clones isolated suggests alternative splicing and usage of this exon in a minority of the PDGF A-chain transcripts. This conclusion is also supported by Northern blot analysis of RNA from the glioma cell line U-343 MGa clone 2:6 as well as other tumor cell lines. When the excised exon 6 was used alone as a radiolabeled probe, the same three transcripts were detected as with the cDNA probe, although with a lower intensity and a different distribution between the cell lines (Fig. 4). The glioma cell line showed stronger hybridization to exon 6 than, for example, the melanoma cell line WM 266-4, despite the fact that they hybridized with similar intensities to the cDNA probe (Fig. 4).

Some light was also shed on the differences in the 3′ ends. The clones D1, 13.1, and 9.1 ended with a $(dGA)_6$ in D1 and 13.1, followed by a short poly(A) stretch, whereas 15.1 carried at the corresponding position a $(dGA)_{11}$ and continued for another 370 bp farther 3′, ending with a poly(T) stretch. None of these clones contained the typical AATAAA polyadenylation signal. At the corresponding position in the gene sequence, a $(dGA)_{12}$ was followed by a sequence contiguous with that in clone 15.1. Further 3′ in the genomic sequence, additional T stretches were found, the longest spanning 19 residues, and an authentic polyadenylation signal (AATAAA) was situated 182 bp downstream of the end of cDNA clone 15.1 (Fig. 3). Two AACAAA sequences were also found immediately following the dGA repeat. Whether any of these are used as polyadenylation signals in the transcripts remains to be determined.

TABLE 1. Exon-intron junctions of the human PDGF A-chain gene[a]

Sequence	Donor	Acceptor
Consensus	AG | GTRAGT..	..YYNCAG |
Intron 1	AG | GT<u>T</u><u>G</u>GT..	..TTGCAG |
Intron 2	AG | GTAA<u>A</u>T..	..CTGCAG |
Intron 3	<u>C</u><u>G</u> | GTGAGT..	..TTGCAG |
Intron 4	AG | GTGAG<u>C</u>..	..T<u>G</u>GCAG |
Intron 5	<u>G</u><u>G</u> | GTGAGT..	..T<u>A</u>ACAG |
Intron 6	AG | GTA<u>G</u><u>G</u><u>A</u>..	..CTGCAG |

[a] A, Adenine; T, thymine; G, guanine; C, cytosine; R, purine; Y, pyrimidine; N, any nucleotide; |, splice site. The consensus sequence is from references 45 and 49. Nucleotides which do not conform with the consensus are underlined.

The basis for the variations in the length of the dGA repeat is not known but could be genetic polymorphism. Since the dGA repeat was followed by an A-rich region, one might speculate on the possibility that oligo(dT) priming of the first-strand cDNA synthesis in clones D1, 9.1, and 13.1 occurred in this region.

In cDNA clone 9.1, in which exon 1 was substituted for a diverging sequence, comparison with the gene sequence revealed that this was a remaining part of the first intron. At present, we do not know whether clone 9.1 represents a partially spliced nuclear precursor mRNA or corresponds to one of the three cytoplasmic A-chain transcripts. The latter is not completely unlikely, considering that the largest of the three cytoplasmic transcripts is approximately 2.8 kb and therefore could contain intron 1, which is relatively small (0.9 kb). However, for reasons pointed out above, such a transcript could not be translated into a PDGF A-chain precursor protein.

As all our PDGF-A cDNA clones were likely to be 5′ deleted, we sequenced ca. 750 bp of the gene upstream from the most 5′-extending cDNA clones (Fig. 3). A putative promoter region, a TATA box surrounded by G+C rich sequences, was situated ca. 870 bp upstream from the translation initiation site. A GAATC sequence (an anomalous CAAT box) was situated 59 bp upstream from the TATA box. The G+C content between this and the TATA box was 97%. Between the TATA box and the translation initiation site, the G+C content was 79%. Since there is a tendency for the first base of a eucaryotic mRNA to be A flanked by pyrimidines, the mRNA start site was likely to fall within the CACAC region 25 bp downstream from the TATA box.

Comparison between the PDGF A- and B-chain loci. Comparison of the PDGF A-chain locus with the PDGF-B/c-*sis* locus revealed several similar features (Fig. 5). The exons were similar in number and size, and if, in the A-chain, exon 1 encodes all of the 5′ untranslated sequence and exon 7 contain all of the missing 3′ untranslated sequence (which

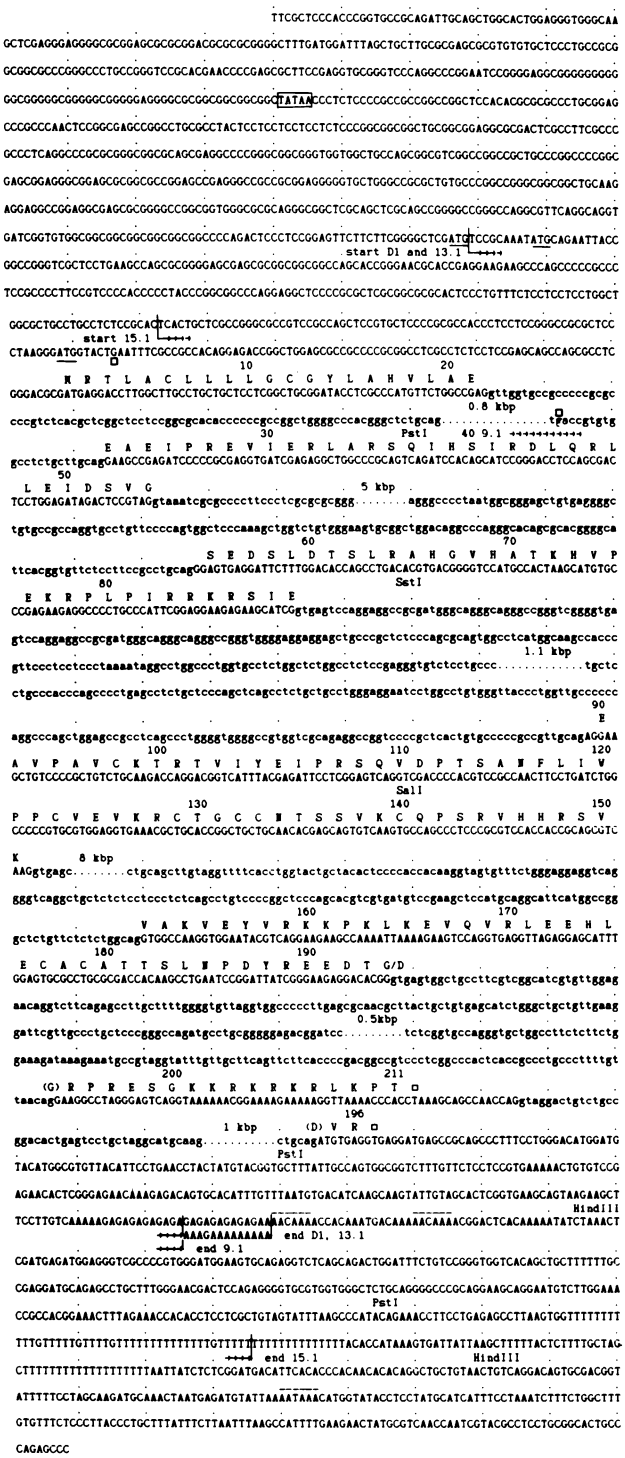

FIG. 3. Nucleotide sequence of the exons and parts of the introns of the PDGF A-chain gene. The start (|→→) and end (→→|) of the cDNA clones in relation to the gene sequence are shown. ATG codons in the 5′ untranslated sequence are underlined. In-frame stop codons in the 5′ untranslated sequence as well as in intron 1 are indicated (□). A TATAA sequence in the putative promoter region is boxed. Putative polyadenylation signals are overlined. Dotted lines indicate the part and approximate size of the intron for which the DNA sequence has not been determined.

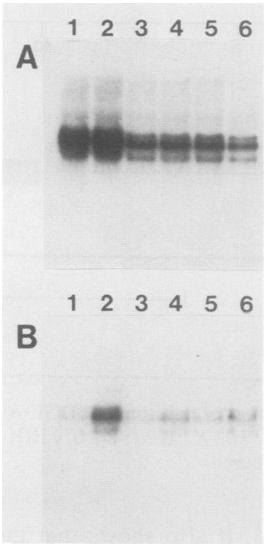

FIG. 4. Northern blot analysis of poly(A)$^+$ RNA prepared from the following cell lines: lanes 1, WM 266-4 (23) (human melanoma), 10 μg; lanes 2, U-343 MGa clone 2:6 (36) (human glioblastoma), 10 μg; lanes 3, U-2 OS (39) (human osteosarcoma), 10 μg; lanes 4, B-5 GT (50) (human osteosarcoma), 10 μg; lanes 5, A431 (17) (human squamous cell carcinoma), 10 μg; lanes 6, U-343 MGa clone 2:6, 2.5 μg. Blots were hybridized with ^{32}P-labeled DNA probes corresponding to the complete PDGF-A cDNA (A) or the excised exon 6 from the PDGF-A gene (B) under stringent hybridization conditions. The figure shows overnight exposures at −70°C to Kodak XAR5 films.

would agree with the structure of the PDGF-B/*sis* gene), the genes were also of similar size (approximately 22 kb). The introns were, however, different in size, and it is not known at present whether any nucleic acid sequence homologies between the two genes are present within the introns.

Figure 5 shows the splice junctions of the two genes in relation to the amino acid sequence of the precursor proteins. As expected, the splice junctions appeared in corresponding positions in the precursor proteins, and interestingly, they seemed to divide the precursor molecules into

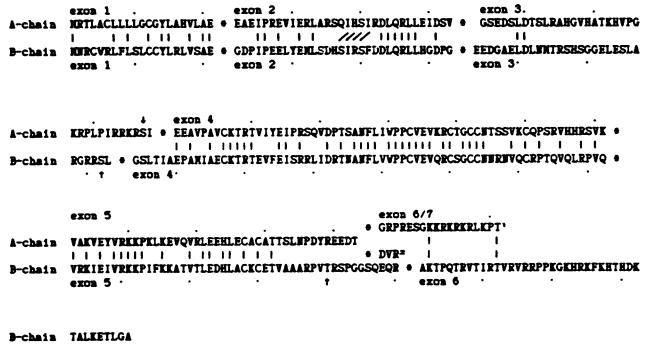

FIG. 5. Alignment and comparison of the amino acid sequences and splice junctions of the two PDGF chain precursor proteins. Straight and slanted lines indicate amino acid identity; *, approximate splice sites (see also Fig. 3); ↑ and ↓, N terminals of the mature A- and B-chains, †, C terminus of human platelet B-chains (29). The C-terminal processing site for the A-chain precursor is unknown. [1], A-chain precursor C-terminal sequence predicted by cDNA clone D1; [2], A-chain precursor C-terminal sequence predicted by cDNA clones 15.1 and 13.1.

domains with different functional properties. In both genes, the signal peptide sequence was encoded entirely by exon 1. Exons 2 and 3 encoded the entire propeptides and, in both chains, a few of the first amino acids in the mature chains. Exon 2 encoded the parts of the propeptides showing significant homology between the precursors, whereas exon 3 encoded an almost completely diverging stretch of amino acids. Exons 4 and 5 encoded the mature chains except for the first few amino acids. Whether exon 6 encoded any part of the mature chains is uncertain, since the exact C-terminal processing site for the human B-chain is not known (although amino acid sequencing of human PDGF indicates that the B-chain ends with a threonine residue at position 190 in the precursor sequence [29]). It is not known whether the A-chain is processed at the C terminus. Exon 7 was noncoding in the B-chain/*sis* gene as well as in the A-chain transcript containing exon 6 (in the transcripts lacking exon 6, exon 7 encoded the three C-terminal amino acids, as shown above). That the exons divided the PDGF B-chain into functional domains was also indicated by experiments with mutants carrying deletions of the PDGF-B/*sis* gene, which have shown that, provided the signal sequence is left intact, substantial parts of the 5′ and 3′ coding sequences can be deleted without affecting the transforming properties of the gene (32, 43). This includes the part of the N-terminal propeptide, which was homologous between the two PDGF chains. Thus, the B-chain/*sis* exons 2, 3, 6, and 7 are dispensable for the transforming activity of the gene.

A single human PDGF A-chain gene. Comparing the physical map of the characterized PDGF A-chain locus with the hybridization pattern of A-chain cDNA clones on genomic Southern blots showed that a single PDGF A-chain gene was present in the human genome. Digestion of human DNA with *Eco*RI and *Xba*I revealed hybridizing fragments only compatible with what can be predicted from the physical map shown in Fig. 2 and no additional fragments (data not shown).

DISCUSSION

The present study describes the human PDGF A-chain locus. We show that this gene contains seven exons comprising the entire A-chain protein coding region, but due to the lack of cDNA clones which are full length regarding the 5′ and 3′ untranslated sequences, we cannot completely exclude additional exons.

Exon 6 was found in only one of four cDNA clones, and its presence predicts a 211-amino-acid precursor protein containing an extremely basic C terminus instead of a 196-amino-acid precursor devoid of the basic C terminus. This is shown to be due to alternative splicing occurring to various degrees in different tumor cell lines known to express PDGF-A mRNA. Recent cloning of endothelial cell PDGF-A cDNAs revealed the version lacking exon 6 only (8, 51). Thus, it is possible that the alternative (long) PDGF-A mRNA occurs in certain tumor cells only. Using the two types of clones in expression systems, we confirmed that both encode biologically active PDGF A-chain proteins (Beckman et al., submitted; Bywater et al., unpublished), but we cannot rule out, at present, the existence of biological differences between the two types of A-chains. One would predict that the basic region encoded by exon 6 would have profound effects on the physiochemical properties of the molecule. However, such regions are also known to be susceptible to proteolytic cleavage, and it may therefore be cleaved from the A-chain precursor.

Collins and co-workers recently reported that the expression of both versions of PDGF-A cDNAs in monkey COS cells gave secretion of an immunoreactive and biologically active protein only when the longer clone (containing exon 6) was used (8). This implies differences in synthesis or processing of the two precursors. On the contrary, Tong and co-workers reported that expression of the shorter cDNA in Chinese hamster ovary cells resulted in the secretion of a biologically active protein (51). In further contrast to the data presented by Collins et al., we found that, using retroviral vectors and Rat-1 fibroblasts, the same levels of mRNA expression gave 10-fold higher amounts of immunoreactive PDGF-A protein synthesized with the shorter than with the longer of the PDGF-A cDNAs (Bywater et al., unpublished). The reason for the diverging results in the different expression systems is unknown.

It is at present unclear why the human PDGF A-chain is present as three different-sized transcripts (1.9, 2.3, and 2.8 kb) in transformed as well as normal cells (5, 6, 10, 38, 54). Exon 6 (69 bp) appears to be present in all three transcript sizes. Several other possibilities have to be considered, including additional alternative splicing (which may occur, as one of the cDNA clones appears to retain at least part of the first intron), alternative polyadenylation sites, and alternative promoters.

We present evidence for a single human PDGF A-chain locus present on chromosome 7. The PDGF B-chain/*sis* gene is also present as a single locus in the human genome and has been located to chromosome 22, in the region 22q12.3-22q13.1 (4, 11 48). The close structural relationship between the two loci proves that they stem from a common ancestral gene. After duplication and evolutionary divergence, the two daughter genes have acquired different genomic locations.

A dimer configuration is required for the biological activity of the molecule. Theoretically, three types of dimers with the configurations AA, BB, and AB can be envisioned. Several lines of evidence suggest that human PDGF is a heterodimer (Hammacher et al., unpublished), whereas porcine PDGF (47) and the v-*sis* product (40) are B-chain homodimers.

Also, A-chain homodimers exist; the first to be demonstrated was secreted by a human osteosarcoma cell line (20, 21), and more recent examples include products of both transformed (54; Nistér et al., submitted) and normal (38, 44) cells.

The biological relevance of the presence of several types of PDGF with different subunit compositions is not known, but hybridization studies indicate that both the A- and the B-chain/*sis* genes are well conserved in the mammalian and chicken genomes (Rorsman et al., unpublished), indicating that both genes probably have specific and indispensable functions. Comparing the biological properties of PDGF A-homodimers on one hand and PDGF and PDGF B-homodimers on the other hand has revealed striking differences. PDGF A-homodimers from human glioma cells are a relatively weak mitogen and lack some of the effects of PDGF on actin reorganization and cell motility (Nistér et al., submitted). PDGF-A is also severalfold less potent as a transforming gene than PDGF-B (Beckmann et al., submitted). Studies on the expression of the two PDGF genes in normal (10, 38, 44) as well as transformed cells (1, 6, 54) have revealed a number of situations where only one of the chains is expressed as well as situations in which both genes are expressed. This indicates that expression of the two PDGF genes is differentially regulated. The isolation and characterization of the human PDGF A-chain gene will now permit

investigations on the molecular-biological basis for this difference.

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