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Identification and Characterization of PF4var1, a Human Gene Variant of Platelet Factor 4

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A synthetic DNA probe designed to detect coding sequences for platelet factor 4 and connective tissue-activating peptide III (two human platelet α-granule proteins) was used to identify several similar sequences in total human DNA. Sequence analysis of a corresponding 3,201-base-pair *Eco*RI fragment isolated from a human genomic library demonstrated the existence of a variant of platelet factor 4, designated PF4var1. The gene for PF4var1 consisted of three exons and two introns. Exon 1 coded for a 34-amino-acid hydrophobic leader sequence that had 70% sequence homology with the leader sequence for PF4 but, in contrast, contained a hydrophilic amino-terminal region with four arginine residues. Exon 2 coded for a 42-amino-acid segment that was 100% identical with the corresponding segment of the mature PF4 sequence containing the amino-terminal and disulfide-bonded core regions. Exon 3 coded for the 28-residue carboxy-terminal region corresponding to a domain specifying heparin-binding and cellular chemotaxis. However, PF4var1 had amino acid differences at three positions in the lysine-rich carboxy-terminal end that were all conserved among human, bovine, and rat PF4s. These differences should significantly affect the secondary structure and heparin-binding properties of the protein based on considerations of the bovine PF4 crystal structure. By comparing the PF4var1 genomic sequence with the known human cDNA and the rat genomic PF4-coding sequences, we identified potential genetic regulatory regions for PF4var1. Rat PF4 and human PF4var1 genes had identical 18-base sequences 5′ to the promoter region. The intron positions appeared to correspond approximately to the boundaries of the protein functional domains.

Platelet factor 4 (PF4), connective tissue-activating peptide-III (CTAP-III), γ-IP-10, monocyte-derived neutrophil chemotactic factor (MDNCF), and melanoma growth stimulatory activity (MGSA) are members of a homologous multigene family of human proteins that appear to be important mediators of the inflammatory response, hemostasis, and cell growth. PF4, an α-granule protein secreted by human platelets, has several biological functions, including procoagulation, antiheparin, chemotactic, and immunoregulatory activities (9, 15, 18, 20). CTAP-III is a platelet-derived growth factor that stimulates a variety of specific metabolic and cellular activities including mitogenesis, extracellular matrix synthesis, glucose metabolism, and plasminogen activator synthesis in human fibroblast cultures (4, 5, 29). CTAP-III (also known as low-affinity PF4) may be derived from a precursor, platelet basic protein (14), that contains an additional 9 amino acids at the amino-terminal end. CTAP-III can be converted to a mitogenically inactive form, β-thromboglobulin, by proteolytic cleavage at the Lys-4–Gly-5 peptide bond (5). γ-IP-10 is a protein with unknown activity that is synthesized and secreted from several cell lines following gamma interferon stimulation (21). MDNCF is released from monocytes by an inflammatory response and is selectively chemotactic for neutrophils but not monocytes (40). This same protein also has been reported as mitogen-stimulated human leukocyte protein 3-10C (32) and neutrophil-activating factor (38). MGSA is an autocrine growth factor with potent mitogenic activity that is secreted by human melanoma cells (30). It appears to be the same as the product of a growth-regulated gene, *gro*, whose transcriptional regulation depends on the growth status of the cell and results in elevated constitutive expression levels in tumor-derived human cells (2).

All of these proteins share considerable amino acid sequence homology, including conservation of the four cysteine residues that form the two disulfide bonds in the mature protein. Although the genomic sequences for these proteins have not been reported, the cDNAs for PF4, γ-IP-10, 3-10C (MDNCF), and *gro* (MGSA) have been cloned and sequenced (2, 21, 28, 30, 40); and they exhibit considerable

```
        Val Glu Val Ile Ala Thr Leu Lys Asn Gly Arg Lys Ile Cys Leu Asp Pro Asp Ala Pro Arg Ile Lys Lys Ile Val
        *   *   *                                                   *   * *       *   **                  *
5' AGCTT GTC GAA GTG ATA GCC ACG CTG AAG AAT GGA AGG AAA ATT TGC TTG GAC CCG GAC GCC CCG CGG ATC AAG AAA ATA GTT G    3'
3'     A CAG CTT CAC TAT CGG TGC GAC TTC TTA CCT TCC TTT TAA ACG AAC CTG GGC CTG CGG GGC GCC TAG TTC TTT TAT CAA CTTAA 5'
```

FIG. 1. Sequence of the four DNA oligomers used to construct the probe that detected the PF4var1 gene. The amino acid sequence given is for a portion of CTAP-III, from positions 49 to 74 (5). The asterisks denote the bases that were changed from the human PF4 cDNA sequence (28) to match the CTAP-III amino acid sequence. The underscore indicates the position of the junction of the four DNA oligomers. This double-stranded DNA was cloned into M13mp19 by using the *Hin*dIII and *Eco*RI restriction sites.

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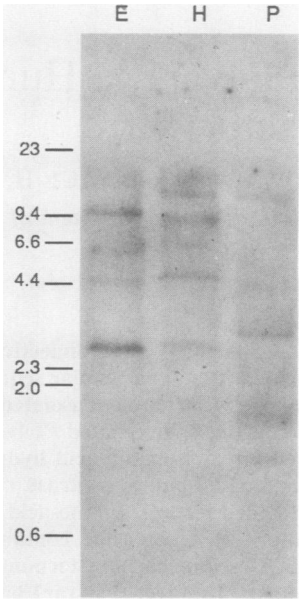

FIG. 2. Southern analysis of genomic digests of human DNA. Molecular weight markers are shown on the left (in kilobases). The enzymes used were *Eco*RI (E), *Hin*dIII (H), and *Pst*I (P). The two darkest bands in the *Eco*RI lane were approximately 3 and 10 kb. In addition, a number of lighter bands were detected.

nucleotide sequence homology. Therefore, this family of homologous proteins appears to be coded by a set of related genes that presumably have evolved by gene duplication and divergence. The genes for PF4, γ-IP-10, and MGSA map to the same region of human chromosome 4 (q12 → q21) (11, 30).

Structure-activity comparisons suggest that this family of proteins contains the following four separate domains: (i) a hydrophobic leader segment that may function in protein secretion, (ii) the amino-terminal domain of the mature protein whose integrity is necessary for the immunoregulatory properties of PF4 (18) and for the growth-stimulatory properties of CTAP-III (5), (iii) a central core region containing the two disulfide bonds, and (iv) a carboxy-terminal domain containing peptide determinants that are necessary for glycosamino-glycan binding and cellular chemotaxis (9, 13, 33).

Using a synthetic hybridization probe based on nucleotide and amino acid sequence information for both human PF4 and CTAP-III, we identified additional human sequences that are homologous to this family of proteins. One of these, a variant designated PF4var1, is very similar to PF4 but contains interesting changes in the secretory and heparin-binding domains of the protein. Comparisons of the coding and noncoding regions with human and rat PF4 genes identified potential regulatory sequences and suggested that the location of introns corresponds to the boundaries of two fo the four possible functional domains.

MATERIALS AND METHODS

Hybridization probe construction. The synthesis of oligonucleotides was performed on a DNA synthesizer (380A; Applied Biosystems) by using *O*-methylphosphoramidite chemistry. All reagents were from Beckman Instruments, Inc. (Fullerton, Calif.). The hybridization probe was constructed by ligating four oligonucleotides and cloning them into the *Hin*dIII and *Eco*RI sites of an M13mp19 vector, as described below.

Radioactive labeling. Radiolabeled probe was prepared by using the large fragment of DNA polymerase I (Klenow) to synthesize a strand that was complementary to the inserted probe sequence of the M13 single-stranded template in the presence of ^{32}P-labeled nucleoside triphosphates. A fivefold excess of the M13 primer (GGGTAACGCCAGGGTTTT) was incubated with the template (2 μg/μl) at 55°C for 5 min and was then cooled to room temperature and incubated in 10 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$–1 mM dithiothreitol–30 μM each of dGTP, dATP, and dTTP–3 μM [α-^{32}P]dCTP (800 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.)–0.1 U of Klenow polymerase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) per μl for 60 min. The partially double-stranded template was then cleaved with *Hin*dIII. The single-stranded probe was purified by electrophoresis on a 5% polyacrylamide–7 M urea gel. The radiospecific activity of the probe was approximately 10^9 cpm/μg.

Southern hybridization analysis and screening of a genomic library. The probe was hybridized to nitrocellulose blots of human genomic digests (22) in a buffer adapted from that of Amasino (1) containing 20% formamide, 10% polyethylene glycol, 0.25 M Na_2PO_4 (pH 7.2), 0.25 M NaCl, 7% sodium dodecyl sulfate, and 1 mM EDTA at 42°C overnight. The filter was washed in 0.1× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate at 40°C. Approximately 300,000 plaques from a human leukocyte genomic library in an EMBL3 lambda vector (Clonetech Laboratories, Inc.) were blotted onto nitrocellulose (22) and hybridized with the probe under the same conditions described above for Southern analysis. Positive plaques were picked and rescreened to obtain a pure clone.

DNA sequence analysis. DNA isolated from a positive clone was digested with *Eco*RI, and the 3.2-kilobase (kb) band that hybridized to the probe was subcloned into M13 vectors for sequence analysis by the dideoxy nucleotide method (24, 31). Computer analysis of nucleic acid and protein sequence data was performed by using programs that

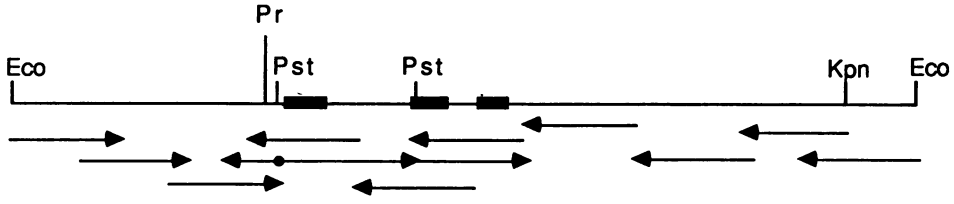

FIG. 3. Sequencing strategy used for the 3.2-kb *Eco*RI (Eco) fragment containing the PF4var1 gene. The exons are represented by the dark bars. The promoter is labeled Pr. The arrows indicate the direction of sequencing. In addition to the use of the two *Pst*I (Pst) sites and the *Kpn*I (Kpn) site for subcloning, synthetic DNA oligomers were used as sequencing primers.

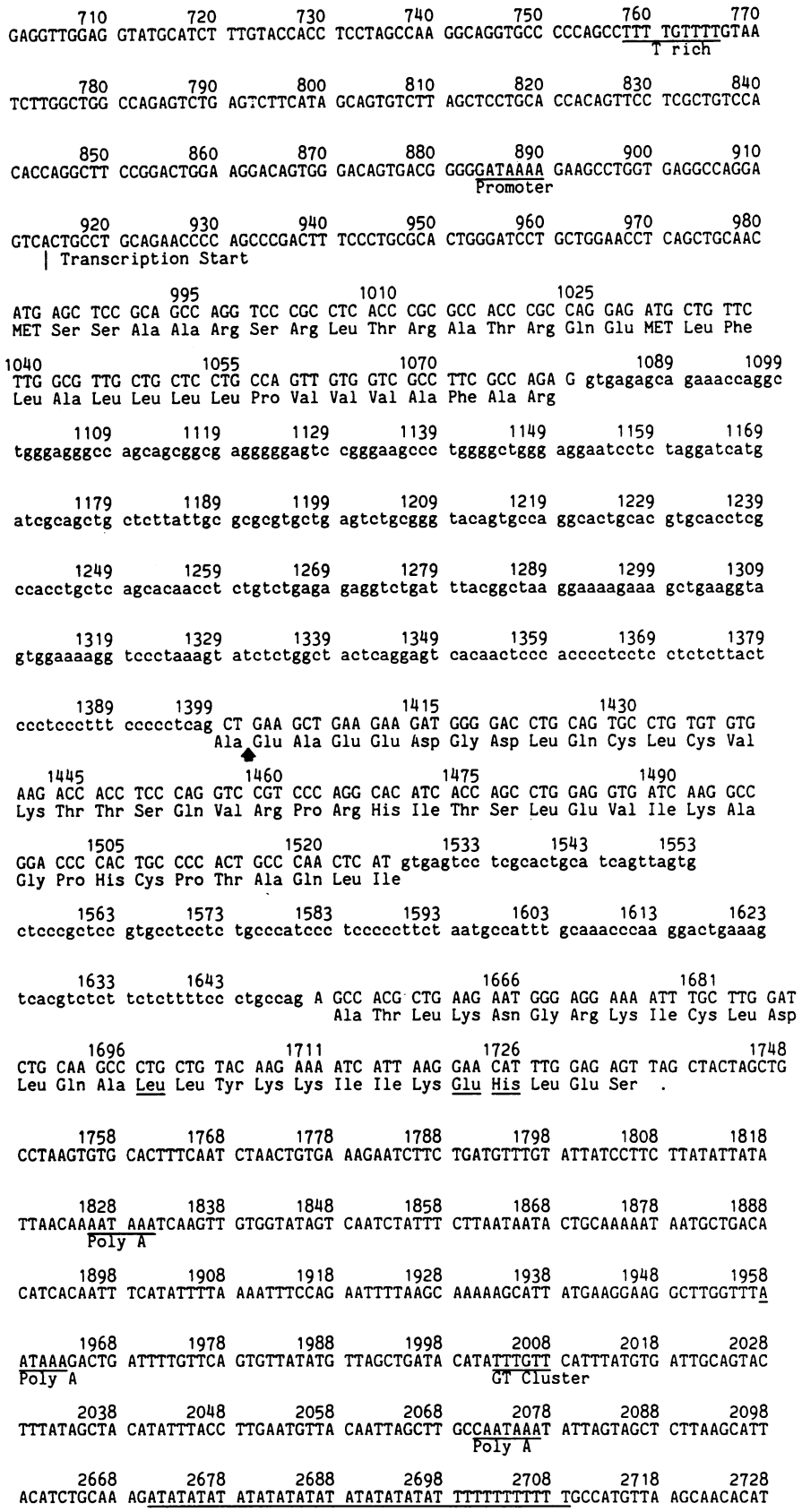

FIG. 4. Sequence of the portion of the *Eco*RI fragment containing the PF4var1 gene. The numbering is based on the entire 3,201-base-pair fragment length. Intron sequences are given in lowercase letters. The amino acid sequence is shown below the DNA sequence. The arrow denotes the expected cleavage site for the PF4var1 leader sequence. In the predicted mature protein sequence, there were only three amino acid changes from PF4. These residues are underlined.

```
        10'        20'        30'  ↓      6
MSSAARSRLT RATRQEMLFL ALLLLPVVVA FARAEAEEDG
     ***** * * ***    *     *      *
MSSAA---GF CASRPGLLFL GLLLLPLVVA FASAEAEEDG

        16         26         36      ↓ 46
DLQCLCVKTT SQVRPRHITS LEVIKAGPHC PTAQLIATLK

DLQCLCVKTT SQVRPRHITS LEVIKAGPHC PTAQLIATLK

        56         66
NGRKICLDLQ ALLYKKIIKE HLES
           *       * *
NGRKICLDLQ APLYKKIIKK LLES
```

FIG. 5. Amino acid sequence comparison between PF4var1 (top rows) and human PF4 (bottom rows). Arrows indicate the positions of the introns between residues 33' and 34' and between residues 42 and 43. Asterisks denote differences in the amino acid sequences.

are available through the BIONET National Computer Resource for Molecular Biology.

RESULTS

Hybridization analysis. Based on the known sequence of the mRNA of PF4 (28) and reverse translation analysis of the amino acid sequence of CTAP-III (5) in the region of maximum amino acid sequence homology, we designed a 78-nucleotide probe to detect the genes for both proteins (Fig. 1). This probe was similar to the PF4 gene sequence except for the minimum changes necessary to allow the codons to match the CTAP-III amino acid sequence. A contiguous sequence of 41 of the 78 nucleotides should be a perfect match for the PF4 gene, assuming the absence of an intron. The exact homology to the CTAP-III gene depended on the extent to which the two homologous amino acid sequences had the same codon usage.

When the labeled probe DNA was hybridized to a nitrocellulose blot of restriction enzyme digests of human genomic DNA, several bands were detected (Fig. 2). The *Eco*RI digest showed particularly strong hybridization signals corresponding to two fragments of approximately 3 and 10 kb. Hybridization bands corresponding to fragments with similar sizes have been reported by Guzzo et al. (12), who used a PF4 cDNA probe, and were thought to be caused by a restriction fragment length polymorphism of the PF4 gene (see below).

We used the PF4–CTAP-III probe to screen a human genomic library in the EMBL3 lambda vector. Of the several positive signals that were detected, DNA isolated from one plaque contained an *Eco*RI fragment of approximately 3 kb. This fragment was subcloned into M13mp19 and sequenced by the strategy illustrated in Fig. 3. The promoter, coding, and intron regions of the putative gene were sequenced from both strands.

DNA sequence analysis. The fragment proved to be 3,201 base pairs long. The sequence for the region (positions 701 to 2728) containing only the coding sequence and adjacent control regions is shown in Fig. 4. Sequence analysis demonstrated a gene that coded for a protein with approximately 85% homology to human PF4 (hPF4). The PF4var1 gene contained two introns. The first was 328 base pairs long and separated a sequence coding for a possible leader peptide (exon 1; amino acid residues 1' to 34') from the beginning of the mature coding sequence defined by the known structure of the PF4 protein isolated from activated platelets (8, 25, 39). The processing site for this leader peptide was predicted from its homology to the processing site for hPF4 (28) and from the consensus sequence for other mammalian leader peptides (36, 37). The second intron was 128 base pairs long and divided the coding sequence for the mature protein between exon 2 (amino acid residues 1 to 41) and exon 3 (amino acid residues 42 to 70). Exons 2 and 3 contained four silent nucleotide changes compared with a hPF4 cDNA sequence (28) and nucleotide changes that resulted in three amino acid substitutions located at the carboxy-terminal end of the protein.

The introns of the genes for PF4var1 and rat PF4 showed a high degree of homology in position and sequence, especially at the 3' ends. The 5' ends of the PF4var1 and rat introns were much less similar.

Regulatory sequences. The likely promoter for the PF4var1 gene was a GATAAAA sequence that was located 90 nucleotides upstream from the ATG codon. This sequence was identical to the putative promoter of the rat PF4 gene (10). The start site for transcription of the PF4var1 mRNA also could be predicted based on the homology with the rat PF4 transcription start site (Fig. 4). An area of 16 nucleotides on either side of the rat PF4 transcription start site was preserved in the PF4var1 gene region with only two changes.

Both genes also had a cluster of T residues approximately 120 nucleotides upstream from the promoter, although the rat PF4 gene had a much larger T cluster. The PF4var1 gene had two dyad symmetries upstream of the coding sequence. There was a 9-nucleotide inverted repeat 320 nucleotides upstream of the promoter, and there was also an 11-nucleotide inverted repeat on both sides of the promoter.

TABLE 1. Structure of the carboxy-terminal region of PF4 and related proteins

Protein	Structure[a]																	
PF4var1	L	D	L	Q	A	L	L	Y	K	K	I	I	K	E	H	L	E	S
PF4	L	D	L	Q	A	P	L	Y	K	K	I	I	K	K	L	L	E	S
CTAP-III	L	D	P	D	A	P	R	I	K	K	I	V	Q	K	K	L	A	G
Bovine PF4[b]	L	D	Q	Q	R	P	L	Y	K	K	I	L	K	K	L	L	D	G
Structure[c]	s	s	t	t	t	h	h	h	h	h	h	h	h	h	h	h	h	h
Exposure[d]	1	3	6	16	9	8	1	3	10	13	0	1	14	8	1	3	13	8
% Φ[e]	71	33	50	0	50	50	57	86	20	50	80	86	50	40	86	71	100	

[a] The sequences given are from positions 53 to 70.
[b] The three additional carboxy-terminal residues in bovine PF4 are disordered in the X-ray structure.
[c] The symbol under each residue indicates that its structure is helical (h), pleated sheet (s), or turn (t).
[d] Exposure of the bovine residue in terms of accessible water molecules (1 water/1.0 nm^2) as calculated by the program of Kabsch and Sander (17).
[e] Percent of residues within 0.4 nm of the side chain of the bovine residue that are hydrophobic. The high reading for Asp-69 reflects a single contact with the beta carbon of Leu-68.

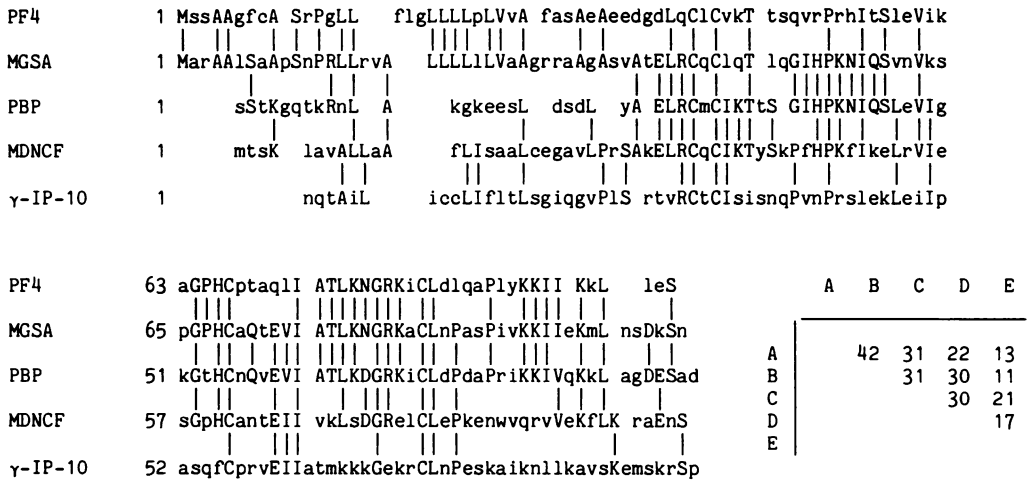

FIG. 6. Amino acid sequence alignment of human PF4 and homologous proteins. Sequences were aligned by GENALIGN, a multiple sequence alignment program based on the algorithms developed by Needleman and Wunsch (26) and Sobel and Martinez (34), and are available through the BIONET National Computer Resource. The total alignment score was 119 (compared with 13 for the randomized sequences). The insert shows the scoring matrix for PF4 (A), MGSA (B), platelet basic protein (C), MDNCF (D), and γ-IP-10 (E). The following parameters were used: amino acid residue length, 2; deletion weight, 1.0; length factor, 0; matching weight, 1.0. The score is a measure of the degree of alignment among two sequences calculated as the number of matching amino acids times the matching weight minus the number of deletions (needed to place the common regions in register) times the deletion weight. Upper- and lowercase letters indicate consensus and nonconsensus amino acids, respectively.

Immediately downstream of the T cluster in both the PF4var1 and the rat PF4 genes there was a perfectly conserved 18-nucleotide sequence (TAATCTTGGCTGGC CAGA) and an 11-nucleotide sequence (GGATCCTGCTG). However, in the rat PF4 gene region, this latter sequence appeared upstream of the promoter, overlapping an area of dyad symmetry that was not found in the PF4var1 gene region. In the PF4var1 gene region, this same sequence was found 17 nucleotides ahead of the translational start codon, ATG, which was well within the expected mRNA sequence.

The cDNA sequence of γ-IP-10, a protein with some amino acid sequence similarity to PF4 and CTAP-III (21), had an 8-nucleotide sequence (GGAACCTC) in the 5′-untranslated region that was conserved in the PF4var1 gene. Only the last 7 of the 5′-untranslated nucleotides of the hPF4 cDNA have been reported (28). The PF4var1 sequence has conserved 5 of these 7 nucleotides.

The first 17 of the 3′-untranslated nucleotides of the PF4var1 gene were perfectly conserved in the hPF4 mRNA, and this segment was very homologous to the same region of the rat PF4 gene. Immediately downstream of this region was an area that was more homologous to the rat PF4 gene that it was to the hPF4 gene. This area included a 23-nucleotide sequence (TCTTCTGATGTTTGTATTATCCT) that was conserved in the rat PF4 sequence with only a single-base deletion; this sequence was not found in the hPF4 cDNA.

There were three polyadenylation consensus sequences (AATAAA) within 350 nucleotides of the termination codon of the PF4var1 gene. There was also a fourth polyadenylation consensus sequence located approximately 1,250 nucleotides downstream (data not shown in Fig. 4). The actual polyadenylation signal could not be determined with any certainty by comparing it with that of the hPF4 mRNA because of limited homology in this region. The first three possible polyadenylation signals had GT-rich areas downstream but did not have the usual spacing that is found in most eucaryotic transcription termination regions (3, 23).

Approximately 370 nucleotides downstream from the end of the PF4var1 gene was an unusual region consisting of 39 base pairs of predominantly alternating A and T residues.

Protein structure. An amino acid sequence comparison between hPF4 and PF4var1 is shown in Fig. 5. The sequences were aligned for maximum similarity; the arrows indicate the positions of the introns. The greatest variation between the two proteins occurred in the leader sequence (residues 1′ to 34′). Whereas this region was generally hydrophobic, which is characteristic of a secretory peptide, PF4var1 showed major sequence divergence from PF4, particularly in the region between residues 6′ and 17′, where it had a much greater positive charge because of three additional Arg residues.

PF4var1 had three amino acid differences in the mature protein sequence compared with hPF4 (Fig. 5): Leu-58 (replacing Pro), Glu-66 (replacing Lys), and His-67 (replacing Leu). The nature of these changes was predicted to have a significant effect on the structure and function of PF4var1 compared with that of other PF4s.

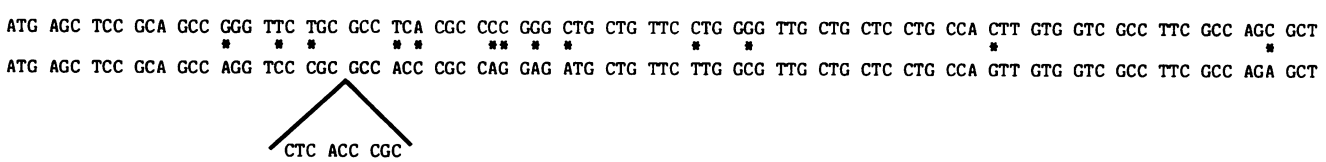

FIG. 7. Alignment of the PF4 leader peptide-coding region (top row) with the PF4var1 leader-coding region (bottom row). In addition to the base changes shown by the asterisks, the PF4var1 leader region appears to have a three-codon insert, as shown.

DISCUSSION

The crystal structure of bovine PF4, whose sequence is 73% identical to that of human PF4, has been solved to 0.3-nm resolution (35; R. St. Charles, H. Guy, and B. F. P. Edwards, Fed. Am. Soc. Exp. Biol. J., **2**:A1536, 1988; R. St. Charles, D. A. Walz, and B. F. P. Edwards, J. Biol. Chem., in press). The protein exists as a tetramer, with each identical monomer consisting of an extended loop at the amino-terminal region, three strands of β-sheet in the central core region, and an α-helix at the carboxy-terminal region. Two monomers form an extended six-stranded β-sheet with their helices antiparallel to one another on the outside. The tetramer is formed by the packing of two extended β-sheets back to back. The carboxy-terminal α-helical region of PF4 contains important peptide determinants that are necessary for heparin binding and cellular chemotaxis (7, 16, 27). The conformational and chemical requirements for effective heparin binding appear to reside in the asymmetric distribution of lysine residues on one face of an amphipathic α-helix and in the cooperative interaction of each monomer with heparin (19; St. Charles et al., Fed. Am. Soc. Exp. Biol. J., 1988; St. Charles et al., in press).

The mature form of PF4var1 differs from that of hPF4 by only three amino acids. All three residues are conserved in human (8, 25, 39), rat (10), and bovine (6) PF4s. Therefore, the differences represented by PF4var1 are not likely to be caused by insignificant variations in a duplicate PF4 gene.

The amino acid changes in PF4var1 may cause significant alterations in the secondary structure of the protein. The carboxy-terminal region of PF4var1 is less positively charged than is that of hPF4 (Lys-66 to Glu), which reduces its ability to bind heparin, as deduced by heparin-affinity chromatography studies of the recombinant protein (unpublished data). The Pro-58 to Leu change may also have a major effect on the secondary structure of the protein. Assuming that human and bovine PF4 have essentially the same three-dimensional structure, the carboxy-terminal helix in human PF4 starts at Pro-58. Proline is a helix breaker; the change to leucine could extend the helix in the amino-terminal direction and alter the turn at positions 55 to 57 that links the helix to the third strand of the β-sheet. The Leu-67 to His change could also significantly alter the characteristics of the carboxy-terminal helix. Leu-67, which is between the helix and the hydrophobic surface of the β-sheet, is buried and surrounded by hydrophobic residues (Table 1). Histidine at this position, especially if it is protonated, would weaken the packing of the helix against the sheet. To some extent, the substitutions in PF4var1 make its carboxy-terminal helix more like that of CTAP-III, in which only three of the four lysines are on one face of the helix because of an insertion of a glutamine between the corresponding positions 64 and 65 of PF4. This results in the shifting of a basic residue (lysine) into the position corresponding to Leu-67 (PF4) or His-67 (PF4var1). Both PF4var1 and CTAP-III showed significantly reduced heparin-binding properties compared with those of either human or bovine PF4 (unpublished data).

Figure 6 shows the amino acid sequence alignment for PF4 and the other known human proteins in this gene family. The sequences are arranged in order of decreasing similarity to PF4. The relative similarity is indicated by the scoring matrix shown in the insert. As with PF4, these proteins have carboxy-terminal regions that are rich in basic amino acids (lysine and arginine). γ-IP-10 and MDNCF exhibit sequence periodicities that are characteristic of amphipathic helices, while MGSA, like platelet basic protein (CTAP-III), may show a less asymmetric alignment of basic residues about an α-helix, which is indicative of weaker heparin-binding affinity compared with that of PF4.

The coding region specifying the leader sequence of the PF4var1 gene showed the greatest change from that of human PF4. The coding regions for the two leader sequences were aligned to maximize their similarities (Fig. 7). Only 15 of the 93 nucleotides were different, except for a three-codon insert in the middle of the sequence. However, these differences resulted in a significantly increased net positive charge of the PF4var1 leader sequence resulting from five arginine residues compared with only one for human PF4, three for rat PF4, and none for bovine PF4. This may imply a difference in the tissue or cell type in which PF4var1 is expressed or a difference in its mode of secretion. Although we have not yet demonstrated the natural occurrence of PF4var1 protein, work to locate its tissue(s) of origin is in progress.

The homology between the PF4var1 and hPF4 genes could easily result in the detection of both genes by the same hybridization probe. Based on a Southern analysis with a 1.4-kb *Pst*I fragment of the hPF4 gene, Guzzo et al. (12) have reported an *Eco*RI polymorphism in the hPF4 gene, with an invariant band at 10 kb and a polymorphic band at either 5 or 3.1 kb. The PF4–CTAP-III probe that we used to detect the PF4var1 gene also hybridized to the PF4 gene (unpublished data). Southern analysis of *Eco*RI digests of human DNA with this probe also detected two main bands at 10 and 3.2 kb (Fig. 2). Cloning and sequencing demonstrated that the 3.2-kb band was actually the PF4var1 gene, so it is likely that the variable band detected by Guzzo et al. (12) is actually caused by a polymorphism of the PF4var1 gene.

ACKNOWLEDGMENTS

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