

## Two different promoters direct expression of two distinct forms of mRNAs of human platelet-activating factor receptor

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The human platelet-activating factor (PAF) receptor gene exists as a single copy on chromosome 1. We identified two 5'-noncoding exons, each of which has distinct transcriptional initiation sites. These exons are alternatively spliced to a common splice acceptor site on a third exon that contains the total open reading frame to yield two different species of functional mRNA (Transcript 1 and 2). Transcript 1 has consensus sequences for transcription factor NF- $\kappa$ B and Sp-1, and the Initiator (*Inr*) sequence homologous to the murine terminal deoxynucleotidyltransferase gene. Transcript 2 also contains consensus sequences for transcription factor AP-1, AP-2, and Sp-1. Transcripts 1 and 2 were both detected in heart, lung, spleen, and kidney, whereas only Transcript 1 was found in peripheral leukocytes, a differentiated human eosinophilic cell line (EoL-1 cells), and brain. Existence of distinct promoters was thus suggested to play a role in the regulatory control of PAF receptor gene expression in different human tissues and cells.

Lipid mediator; Alternative splicing; Primer extension; Initiator (*Inr*); Human leukocyte

### 1. INTRODUCTION

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a lipid mediator that possesses diverse and potent biological effects on a variety of cells and tissues [1–4]. PAF has a role in activation of neutrophils and platelets, microvascular leakage, smooth muscle contraction, and activation of early oncogenes. Specific PAF receptors on the plasma membrane of these target cells mediate these versatile processes.

Recently, we isolated cDNAs for a PAF receptor from guinea pig lung and human leukocytes [5,6]. Northern blot analysis showed that the PAF receptor mRNA was extremely abundant in human peripheral leukocytes, a human eosinophilic cell line (EoL-1 cells) differentiated with GM-CSF, IL-5, and *n*-butyrate, and a human promyelocytic leukemia cell line (HL-60 cells)

differentiated with dibutyryl cAMP or 1 $\alpha$ ,25(OH)<sub>2</sub>vitamin D<sub>3</sub> [6–8]. The transcript was also detected in the spleen, lung, kidney, and placenta, but scarcely present in undifferentiated EoL-1 cells and undifferentiated HL-60 cells, as determined by Northern blot analysis [5–7,9,10].

Human PAF receptor cDNAs have also been cloned from human heart and cell lines (U937 myeloid cell line and differentiated HL-60 cells) [7,11,12]. All these human cDNAs contained an identical coding region sequence. Isolation of a cDNA from a human heart cDNA library [11] revealed a different sequence beyond 39 base pairs upstream of the ATG initiation codon (–186 to –39). It suggested to us the possibility that human PAF receptor cDNA may have at least two different 5'-termini. We report here cloning of the human PAF receptor gene, which possesses two alternatively spliced 5'-noncoding exons that might be under control of two distinct promoter sequences.

### 2. MATERIALS AND METHODS

#### 2.1. Screening of a genomic library

A human placental genomic library constructed in a  $\lambda$  phage vector EMBL3/SP6/T7 (Clontech) was screened with the human PAF receptor cDNAs isolated from human peripheral leukocytes [6] and from the human heart [11] as a probe, under high-stringency hybridization conditions. Various restriction fragments from positive clones were subcloned into the plasmid vector pBluescript SK(–) (Stratagene) for further analysis and sequencing by dideoxy nucleotide chain-termina-

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Abbreviations: PAF, platelet activating factor; *Inr*, initiator; EoL-1 cells, human eosinophilic cell line; GM-CSF, granulocyte macrophage colony stimulating factor; IL-5, interleukin-5; HL-60 cells, human promyelocytic leukemia cell line; SSC, standard saline citrate; BSA, bovine serum albumin; M-MuLV, Moloney murine leukemia virus; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-polymerase chain reaction; TdT, terminal deoxynucleotidyltransferase.

tion method [13] using Sequenase Ver. 2.0 (United States Biochemical).

## 2.2. Southern blot analysis of genomic DNA

Human placental DNA digested with *Xho*I, *Hind*III, *Bgl*II, and *Eco*RI were electrophoresed in a 0.7% agarose gel, and blotted onto a nylon membrane (Hybond-N, Amersham) [14]. The membrane was hybridized with a <sup>32</sup>P-labeled *Bgl*II/*Hind*III fragment of human PAF receptor cDNA (nucleotides 161–1,219) [6]. Hybridization conditions were as follows: 6 × SSC, 5 × Denhardt's solution (0.1% BSA, 0.1% polyvinylpyrrolidone and 0.1% Ficoll 400), 0.5% SDS, 400 µg/ml heat-denatured salmon sperm DNA, and the radiolabeled probe (> 5 × 10<sup>6</sup> cpm/ml) at 65°C for 20 h. The membrane was washed with 0.1 × SSC/0.5% SDS at 68°C for 30 min, and exposed to X-ray films at –70°C for three days, using intensifying screens.

## 2.3. Preparation of poly(A)<sup>+</sup> RNA

EoL-1 cells were grown and differentiated as described [6]. RNAs were extracted from EoL-1 cells and human leukocytes by the acid-phenol method [15], and poly(A)<sup>+</sup> RNA were purified using Oligotex-dT30 (Takara Shuzo, Kyoto, Japan). Poly(A)<sup>+</sup> RNAs for human lung, spleen, and kidney were obtained from Clontech. Human heart poly(A)<sup>+</sup> RNA was obtained as described [11].

## 2.4. Primer extension analysis

Primer extension analysis was performed with specific oligonucleotides for each form of the transcript: L2 (5'-CTGTAG-CAGGGGAGCGGCTTC-3', complementary to nucleotides –60 to –39 of human leukocyte PAF receptor cDNA [6]), H2 (5'-TGACTTCTCGGGAGCTCAGG-3', complementary to nucleotides –149 to –169 of human heart PAF receptor cDNA [11]). Both primers were <sup>32</sup>P-labeled at their 5'-termini by T4 polynucleotide kinase (Takara Shuzo). Poly(A)<sup>+</sup> RNA (3 µg) from the heart or differentiated EoL-1 cells was used as a template for analysis of transcription start site of each form of the transcript. Each <sup>32</sup>P-labeled primer (2 × 10<sup>5</sup> cpm) was annealed to either 3 µg of heart or EoL-1 cell poly(A)<sup>+</sup> RNA at 65°C for 3 h in a 30 µl solution of hybridization buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA, 0.25 M KCl). The annealed RNA/primer mixture was extended with 5 units of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (BRL) at 37°C for 60 min. The product was ethanol-precipitated and redissolved in 5 mM Tris-HCl pH 7.5 containing 48% formamide. After electrophoresis in 6% polyacrylamide gel containing 8 M urea [14], the length of each transcript was compared with a DNA sequence ladder obtained using the same primer.

## 2.5. Isolation of human PAF receptor 5'-cDNA ends

Rapid amplification of cDNA ends (RACE) was carried out essentially as described [16]. First strand cDNA was synthesized from EoL-1 cell poly(A)<sup>+</sup> RNA using a specific primer (C3: 5'-TGATGAA-GAAAAGGCAGCCAGC-3'), which is complementary to nucleotides +277 to +298. Excess primer was removed using a Centricon-100 spin filter (Amicon), and the single-stranded cDNA was polyadenylated at its 3'-terminus using terminal deoxynucleotidyltransferase in excess presence of dATP. First-stage polymerase chain reaction (PCR) was then performed on a small aliquot of the polyadenylated first strand cDNA, with three primers: C2 (5'-CCATGGT-GAGGTTCACC-3', complementary to nucleotides +168 to +184); adaptor (GACTCGAGTCGACATCG); and adaptor-(dT)<sub>17</sub>, using AmpliTaq DNA polymerase (Perkin-Elmer Cetus). Finally, second-stage PCR was performed using 1 µl of the first-stage PCR products as template with the adaptor and L2 as primers. RACE products were separated by electrophoresis, and specific products, determined by Southern blot analysis, were gel-excised, cloned in a pBluescript SK(–) vector, and sequenced for analysis of the 5'-cDNA ends.

## 2.6. Chromosome localization of the PAF receptor gene

A panel of 25 human-hamster somatic hybrid DNAs (BIOS Corp., New Haven, CT) was analyzed by PCR to determine the chromosomal

localization of the human PAF receptor gene. PCR was carried out using specific pairs of primers for exon 1 (L1 and L2), exon 2 (H1 and H3), and exon 3 (C3 and C4). H3 (5'-CTGAGAAACAG-CAGGGACAA-3') is complementary to nucleotide –39 to –58 of exon 2. C4 (5'-GCATCATCTTTGTGCTCGGG-3') is nucleotide +68 to +87 of exon 3. Amplified fragments were electrophoresed and visualized by ethidium bromide staining. Aliquots were analyzed by restriction enzyme digestion to verify authenticity of the amplified product.

## 2.7. Detection of expressed PAF receptor mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR)

Poly(A)<sup>+</sup> RNA (1 µg) was transcribed with M-MuLV reverse transcriptase into cDNA, using random hexanucleotides (0.2 µg) as primers and the cDNA was then used for PCR. PCR primers used were C1 (5'-CCCCGAGCACAAGATGATGC-3', complementary to nucleotide +87 to +68 of exon 3) [6], in combination of either L1 (5'-GGCTGGGGCCAGGACCCAGA-3', nucleotide –104 to –85 of exon 1) [6] or H1 (5'-CCTGAGCTCCCCGAGAAGTCA-3', nucleotide –165 to –145 of exon 2) [11]. Amplified fragments were analyzed by agarose gel electrophoresis. Authentic bands were determined by selective enzyme digestion.

# 3. RESULTS AND DISCUSSION

## 3.1. Isolation of genomic clones for the PAF receptor

Upon screening of approximately 1 × 10<sup>6</sup> plaques of the human genomic library with the cDNA probe for the human leukocyte PAF receptor, we obtained three positive clones. Partial sequence analysis of these clones revealed that each contained the entire PAF receptor open reading frame and the entire 3'-untranslated region reported by Nakamura et al. [6]. All three clones, however, contained only –1 to –38 of the 5'-untranslated region, which is common to both leukocyte and heart PAF receptor cDNAs [6,11]. They all lacked either the sequence from –39 to –112 of leukocyte or from –39 to –186 of heart cDNAs. A human genomic library was thus rescreened using DNA probes corresponding to these specific cDNA sequences: –39 to –104 (66 bases) of the leukocyte PAF receptor cDNA and –39 to –180 (142 bases) of the heart PAF receptor cDNA. The screening of about 1 × 10<sup>6</sup> plaques yielded 2 independent clones each for the specific probe. Sequences around exon-intron junctions were determined by comparison of cDNA and genomic sequences. As shown in Fig. 1, we identified two distinct exons for the 5'-flanking noncoding regions (exons 1 and 2). The coding region is situated as an intronless exon designated as exon 3. The gene spreads out over more than 20 kilobase pairs in length. Sequences around splice junctions are shown in Fig. 2. The intron/exon boundary sequences conform to the consensus GT/AG sequence [17,18].

## 3.2. Southern blot analysis

For Southern blot analysis, the human placental DNA was digested with several restriction enzymes. As shown in Fig. 3, the digestion with *Xho*I, *Hind*III, *Bgl*II, or *Eco*RI gave a single positive fragment of 17, 5.4, 6.0,

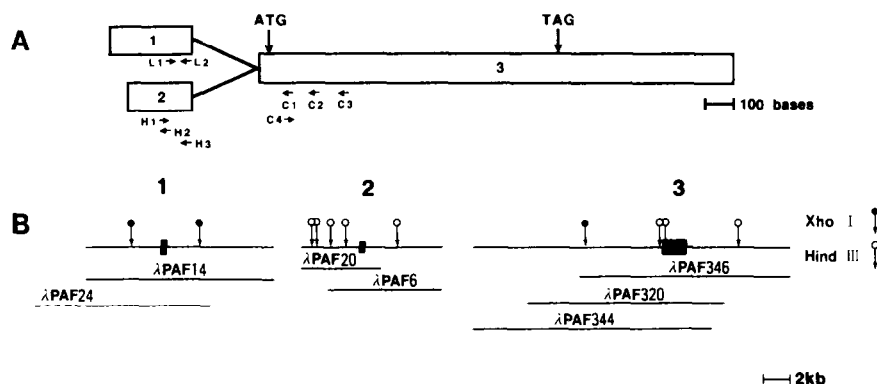


Fig. 1. Map of the human PAF receptor gene. (A) A schematic representation of the human PAF receptor mRNA. Small arrows indicate synthetic oligonucleotides for primer extension, RACE and PCR (see the text). (B) Genomic organization of the three exons of the human PAF receptor gene. Seven phages were isolated with the PAF receptor cDNA. Exons are indicated by closed boxes. Restriction sites for *Xho*I and *Hind*III are indicated by vertical arrows.

or 12 kilobase pairs (kb), respectively. The lengths of positive bands were in agreement with expected DNA fragments, as presented in the restriction map shown in Fig. 3. Southern blot analysis showed that the coding region of the PAF receptor gene exists as a single copy in a haploid genome.

### 3.3. Chromosomal localization of PAF receptor gene

There was no mutual overlap between genomic clones coding for three exons, hence large introns must separate exons 1, 2, and 3. The chromosomal localization of three exons for the PAF receptor gene was determined, using a panel of somatic cell human-hamster hybrid DNA samples. PAF receptor genomic DNAs specific to exons 1, 2, or 3 were amplified by PCR only in a normal human genomic DNA sample and hybrid clones 867, 937, and 1099. Specific PCR products were undetectable in the remaining 20 hybrid and hamster DNA samples (data not shown). Thus, we could assign the PAF receptor gene (exons 1, 2, and 3) unequivocally to human chromosome 1 [19].

### 3.4. Determination of the transcription start sites of the human PAF receptor gene

The transcription initiation site was determined by either primer extension and/or 5'-RACE. Two oligonucleotides, L2 and H2 were synthesized, based on sequences from the 5'-termini of the published sequences for human leukocyte and heart PAF receptor cDNAs, respectively [6,11]. Since the EoL-1 PAF receptor cDNA shared a common sequence with the leukocyte cDNA at the 5'-terminus beyond -39 base pairs (bp) from initial ATG (Izumi, T., unpublished observation), poly(A)<sup>+</sup> RNA from either EoL-1 cells or heart was used to determine the transcriptional start site for the leukocyte-type transcript (Transcript 1) or the heart-type transcript (Transcript 2), respectively. By primer extension analyses, a single start site was identified for

Transcript 1, and two sites for Transcript 2. The major bands were observed at position -327 in EoL-1 cells (Fig. 4A) and at positions -259 and -249 in the heart (Fig. 4B).

With the 5'-RACE method, three different products were identified using EoL-1 cell poly(A)<sup>+</sup> RNA, subcloned and sequenced. The starting site for each product was assigned at position -400, -327, and -137, respectively. Thus, the initiation site at -327 was completely identical to that determined by primer extension. The other two starting positions (-400 and -137) by 5'-RACE were not detected by primer extension under our reaction conditions. Thus, a cytidine residue at position -327 was considered as the major transcription initiation site for the leukocyte-type transcript (Transcript 1).

Sequence analysis of the region upstream of the transcription initiation site of Transcript 1 revealed that the putative promoter region of this gene has neither TATA nor CCAAT box, but does have consensus sequences for transcription factors NF- $\kappa$ B and Sp-1. Interestingly, the nucleotide sequence surrounding the putative transcriptional start site of Transcript 1 (TCCTCTTTCTCAC, -4 to +9 relative to the initiation site) was repeated 73 bp upstream, where a 5'-RACE product was indeed found to start. Thus, this initiation sequence was identical in two (-400 and -327) of the three putative initiation sites of Transcript 1 suggested by 5'-RACE. This sequence is highly homologous to the Initiator (*Inr*) (CCTCATTCT), which was found in the murine terminal deoxynucleotidyltransferase gene (TdT) [20,21]. Though the murine TdT gene also lacks TATA box and CCAAT box, the sequence (*Inr*) is reported to represent the functional promoter related to cellular differentiation and to development [20,21]. It remains to be clarified, however, whether these consensus motifs are related to gene activation during cellular differentiation of HL-60 cells or EoL-1 cells [6-8].

The 5'-upstream region for the two initiation sites of



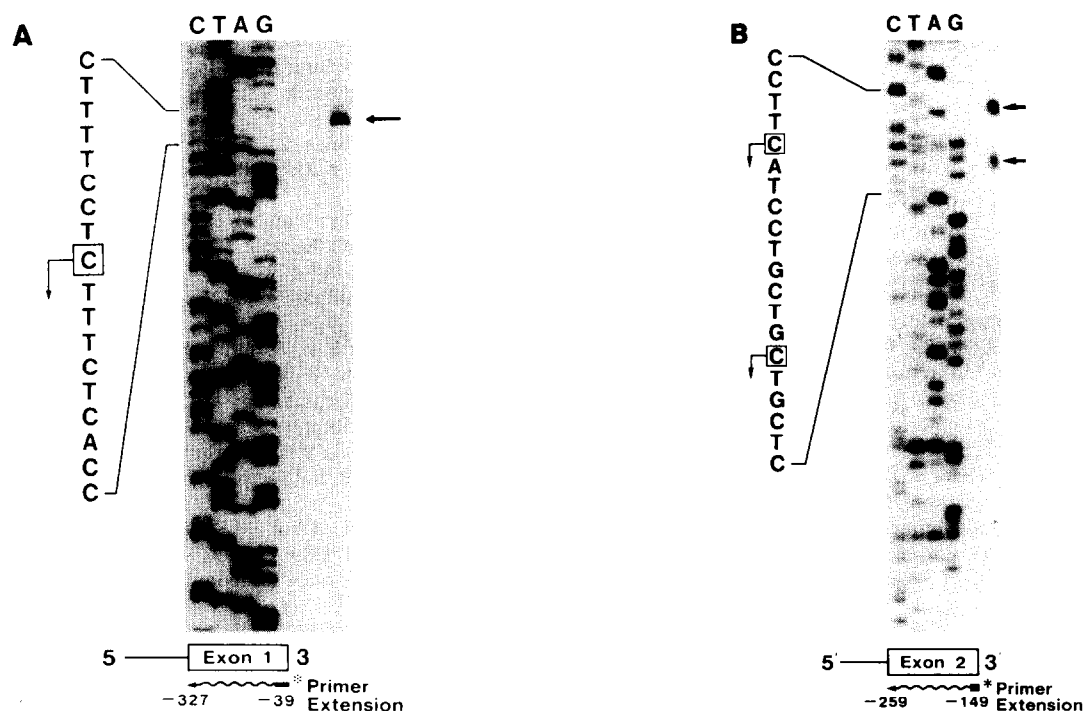


Fig. 4. Mapping of the transcriptional start site of human PAF receptor gene by primer extension analysis. (A) As depicted in the diagram at the bottom of the figure, a 22-mer synthetic oligonucleotide complementary to nucleotides -60 to -39 in the cDNA sequence for exon 1 of human PAF receptor was 5' end-labeled with  $^{32}\text{P}$ , annealed to 3  $\mu\text{g}$  poly(A) $^{+}$  RNA from EoL-1 cells, and extended as described in section 2. (B) Similarly, a 20-mer synthetic oligonucleotide complementary to nucleotide -149 to -169 in the cDNA sequence for exon 2 was 5' end-labeled with  $^{32}\text{P}$ , annealed to 3  $\mu\text{g}$  poly(A) $^{+}$  RNA from heart, and extended. The products were analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea. Marker lanes C, T, A, and G indicate sequencing ladders of exon 1 or 2, using the same primer. The arrows indicate the major start sites of transcription.

on different exons separated by introns, PCR fragments of expected sizes were derived from mRNAs. Amplification products by PCR from Transcript 1 were found in all tissues and the cell line examined (Fig. 5A). However, Transcript 2 was not detected in leukocytes, EoL-1 cells or brain (Fig. 5B). The different distribution of two transcripts suggests that their expression is differentially

regulated by use of a dual promoter system. In addition, two mRNAs may differ in the stability or transcriptional efficiency. More quantitative analysis of the expression level of two transcripts in various tissues and their regulation require further investigation.

Similar cases of alternative 5'-termini and promoters found in distinct exons have been reported for the

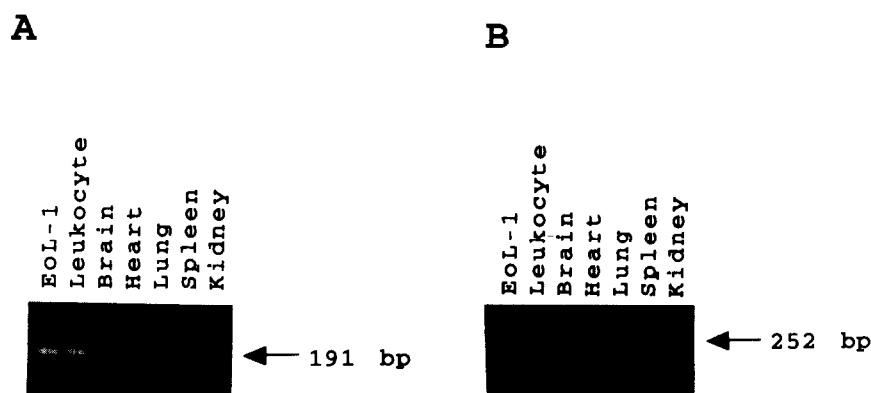


Fig. 5. PCR analysis for expression of two forms of transcripts. Amplification products from EoL-1 cells, leukocytes, brain, heart, lung, spleen, kidney using the primer-pairs L1/C1 (191-bp fragment) and H1/C1 (252-bp fragment) were examined by separation in agarose gels. Primer C1 is complementary to the genomic sequences between +68 and +87 to the ATG in the exon 3. Primer L1 is the sequence between -104 and -85 in the exon 1. Primer H1 is the sequence between -165 and -145 in the exon 2. Identical PCR reaction mixtures were used for all amplifications except for the addition of either one of primer L1 or H1.

mouse glucocorticoid receptor gene [22] and the human dystrophin gene [23,24]. As discussed by Schibler and Sierra [25] and Kozak [26], transcription of a single gene from multiple promoters provides additional flexibility in the control of gene expression. Such promoters could have different cell type- and/or development-specific activities. Elucidation of the existence of two distinct promoters is thus an important step to decipher expressional regulation of the PAF receptor gene.

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