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Isolation and Characterization of the Human Chromosomal Gene for Prostacyclin-Stimulating Factor

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Prostacyclin-stimulating factor (PSF) is a protein which acts on vascular endothelial cells and stimulates the production of prostacyclin. Recently, we were able to purify PSF from the conditioned medium of cultured human diploid fibroblasts and clone PSF cDNA. In this study, we screened a human genomic library and isolated genomic clones to determine the structure of the human chromosomal PSF gene. By determining the nucleotide sequence and transcription initiation site of this gene, we found that it comprises 5 exons and 4 introns. Southern hybridization analysis indicated the presence of a single copy of the PSF gene per haploid set of chromosomes. The 300 bp upstream of the transcription initiation site had a very high GC content, and 7 binding sites for the transcription regulating factor Sp1 were present.

Key words: exon-intron structure, genomic DNA, promoter structure, prostacyclin (PGI_2)-stimulating factor, transcription initiation site.

Prostacyclin (PGI_2) is a prostaglandin synthesized by vascular endothelial cells; it inhibits platelet aggregation and regulates vascular tonus. It plays an important role in maintaining biological homeostasis (*1, 2*). MacIntyre *et al.* reported the presence of a factor that stimulates prostacyclin production in human plasma (*3*). Further, we have demonstrated the presence in rats and in human plasma-derived serum (PDS) of a stimulatory activity on prostacyclin production (prostacyclin stimulating activity [PSA]) by cultured aortic endothelial cells, and we showed that PSA was significantly decreased in PDS from patients with diabetes (*4, 5*). In other words, it is possible that in diabetic patients the incidence and progress of vascular damage are associated with a decrease of PSA.

Recently, we discovered a similar PSA in the conditioned medium (CM) of cultured human diploid fibroblasts and successfully purified a prostacyclin stimulating factor (PSF) and cloned its cDNA (*6*). PSF is a protein of about 31 kDa and its cDNA codes for 282 amino acids. From the results of northern hybridization analysis using human PSF cDNA as a probe, we demonstrated that PSF mRNA is expressed in various tissues including the brain, lungs, liver, kidneys, skeletal muscles, and fatty tissue of Wistar rats (especially in the lungs and kidneys), and in human cultured fibroblasts, vascular endothelial cells and smooth muscle cells (*7*).

Thus, it would be of interest to investigate whether there is a relationship between abnormal expression of the PSF gene and diseases associated with vascular abnormalities, including diabetes. In the present study, we isolated genomic clones including the gene of human PSF and analyzed the structure of the PSF gene.

MATERIALS AND METHODS

Isolation of Genomic Clones—The *Nco*I-*Pvu*II (397 bp) fragment of PSF cDNA (*6*) was labeled with an enzyme using an ECL direct nucleic acid labeling and detection system (Amersham International plc, Amersham, Bucks, UK) according to the instructions provided by the manufacturer. Then, 4×10^5 clones of a human lung fibroblast genomic library cloned into λFIX II vector (Clontech, Palo Alto, CA) were screened by plaque hybridization analysis. Two positive clones (λPG5, λPG7) were isolated, and each restriction enzyme fragment was subcloned into plasmids pUC118 or pBluescript II KS(+) (Stratagene, La Jolla, CA). Furthermore, the *Sma*I-*Sph*I fragment (391 bp) of PSF cDNA and the *Xba*I-*Sac*I fragment (about 1.2 kb) of λPG5 subclone A5-7 were labeled with ^{32}P using a random primer DNA labeling kit (TaKaRa, Shiga), and 1.6×10^6 clones of the human lung fibroblast genomic library were screened by plaque hybridization analysis. λPG46 and λPG56 were isolated and each restriction enzyme fragment was then subcloned into plasmid pUC118. The nucleotide sequence was determined using a PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Southern Hybridization Analysis—A genomic DNA blot

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Abbreviations: PSF, prostacyclin-stimulating factor; IGFBP, insulin-like growth factor binding protein; FGF, fibroblast growth factor; hRPB140, human RNA polymerase II second largest subunit; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

(Human GENO-BLOT) was purchased from Clontech. The blot is a nylon membrane to which 8 μg of human DNA digested with either *Eco*RI, *Hin*dIII, *Bam*HI, *Pst*I, or *Bgl*II was transferred by Southern blotting. The probe was prepared by labeling PSF cDNA fragment *Sma*I-*Sph*I with ^{32}P as described above. After prehybridization according to the standard procedure, human digested DNA was hybridized with the probe in 5×SSPE, 10×Denhardt's solution, 2% SDS and 100 μg/ml heat-denatured salmon sperm DNA at 65°C overnight. The filter membrane was then washed with 0.1×SSC and 0.1% SDS at 68°C and autoradiographed.

S1 Mapping—S1 mapping was carried out essentially as described by Berk and Sharp (*8*). Fragment *Dra*I-*Mlu*I (533 bp) of the PSF gene was subcloned into plasmid pBluescript II KS(+) to construct plasmid pDMKS. After cleaving plasmid pDMKS with *Sma*I, asymmetric PCR was done using a T3 primer to obtain the ^{32}P-labeled single strand probe for S1 mapping. RNA from human diploid fibroblasts was extracted by the AGPC method (*9*). Then, 30 μg of RNA was hybridized with the ^{32}P-labeled single strand probe for S1 mapping at 50°C for 17 h, and subsequently digested with 200 U of S1 nuclease. The resulting fragments were electrophoresed in 6% denaturing polyacrylamide gel and then detected by autoradiography.

5′-Rapid Amplification of cDNA Ends (5′-RACE)—5′-RACE of PSF mRNA was done using human lung 5′-RACE-Ready™ cDNA (Clontech) according to the instructions provided by the manufacturer. First, PCR was conducted using an anchor primer (Clontech) and the antisense primer P210R (5′-CACATAGGGCAGCAGCCGCA-3′, complementary to nucleotides 191-210 in Ref. *6*). Using this PCR product as the template, PCR was conducted again employing the same anchor primer and the antisense primer P99R (5′-GAAGAGGAGAGGGGCAGGAG-3′, complementary to nucleotides 80-99 in Ref. *6*). The condition for PCR included a total of 30 cycles, with a melting temperature of 94°C for 30 s, an annealing temperature of 55°C for 30 s, and an extension temperature of 72°C for 60 s. The PCR product obtained was electrophoresed in a 2% agarose gel, then transferred to a nylon membrane and hybridized with the ^{32}P-labeled PSF cDNA specific oligonucleotide probe (5′-GGGCGCGCAGCGACGGCCGCTCCAT-3′, complementary to nucleotides 23-47 in Ref. *6*). The PCR product was subcloned into plasmid pUC18, and the nucleotide sequence was determined by the method mentioned under "*Isolation of Genomic Clones.*"

RESULTS AND DISCUSSION

Structure of the Human PSF Gene—Using the 5′ region probe of PSF cDNA (fragment *Nco*I-*Pvu*II) (*6*), the human lung fibroblast genomic library was screened and two positive clones, λPG5 and λPG7, were obtained (Fig. 1). According to the PCR analysis conducted using PCR primers prepared based on the nucleotide sequences of PSF cDNA, these clones did not contain the 3′ region of the PSF gene (data not shown). Therefore, we screened the human lung fibroblast genomic library again using the *Xba*I-*Sac*I fragment of λPG5 insert 3′ subclone A5-7 and the 3′ region of the PSF cDNA (PSF cDNA fragment *Sma*I-*Sph*I) (*6*) as probes and obtained 29 positive clones. Among these clones, the 3′ region exon of the PSF gene was apparently covered by λPG46 and λPG56 (Fig. 1). However, a clone that cross-hybridized both λPG5 and λPG46 was not obtained from the remaining clones of the PSF gene (data not shown). As a result, analysis of intron 1 was not conducted. Thereafter, we analyzed λPG5, λPG7, λPG46,

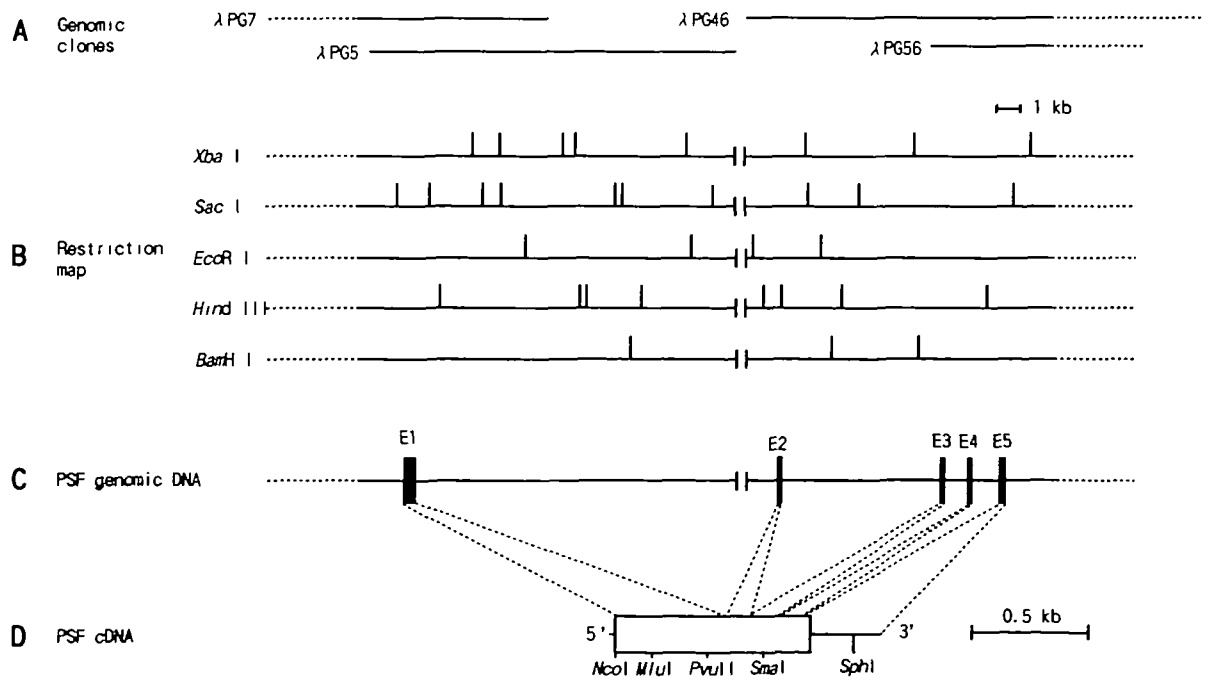

Fig. 1. **Genomic structure of the human PSF gene.** (A) Relative positions of the four genomic clones. (B) Partial restriction enzyme mapping of the gene. The sites recognized by restriction enzymes *Xba*I, *Sac*I, *Eco*RI, *Hin*dIII, and *Bam*HI are shown. (C) Schematic diagram of the human PSF gene. Exons are indicated by closed boxes. (D) Relationship of genomic DNA to cDNA. The hatched box indicates the protein coding region.

and λPG56.

Restriction enzyme fragments of 4 positive clones were subcloned, and the subclones possessing a DNA fragment that included the PSF exon region were selected by PCR screening using PCR primers prepared according to the nucleotide sequences of the PSF cDNA. Based on these subclones, the nucleotide sequences of the exon region and the exon-intron junctions were determined. Comparison of these nucleotide sequences with the PSF cDNA nucleotide sequence indicated that the PSF gene was fragmented by 4 introns and all the spliced sites satisfied the GT-AG rule (Fig. 2) (*10*). Furthermore, the nucleotide sequence of the exon region was in perfect correspondence with that of PSF cDNA.

Exon 1 consisted of 509 bp, had a high G/C content (75%) and coded a region similar to IGFBP-4 (*11*). The amino acid sequence coded in this region shared 36.2% identity with IGFBP-4. It was noteworthy that 10 of the 11 Cys were conserved by both proteins. The region was conserved not only by IGFBP-4, but by the whole IGFBP family, and since the Cys cluster plays an important role in various physiological activities (*12*) of IGFBP, this Cys cluster is assumed to be important for the physiological activity of PSF. Exon 4 coded a region similar to FGF receptor family (*13, 14*). To determine the number of genes encoding PSF, restriction enzyme digests of human genomic DNA were probed with the *Sma*I-*Sph*I cDNA fragment. After hybridization, the following bands were observed (Fig. 3): one of about 10 kb in *Eco*RI-digested DNA, one of about 6 kb and another of 20 kb (very faint) in *Hin*dIII-digested DNA, and one band of 23 kb or more (very faint) in *Bam*HI-digested DNA. The results were in agreement with those predicted based on the restriction enzyme mapping. These results demonstrated the presence of a single copy of PSF gene per haploid set of chromosomes. Bands of about 7 kb and about 1.2 kb were observed in *Pst*I- and *Bgl*II-digested DNA, respectively.

Determination of the Transcription Initiation Site of the PSF Gene—The transcription initiation site of the PSF gene was determined by S1 mapping and 5′-RACE. The ^{32}P-labeled probe which is considered to contain the transcription initiation site and RNA from human diploid fibroblasts were hybridized and subsequently digested with S1 nuclease. The product was then analyzed by electrophoresis on a denaturing polyacrylamide gel. Some bands of about 200 b were detected by S1 mapping (Fig. 4A). Then 5′-RACE was done to confirm the result of S1 mapping. Using human lung 5′-RACE-Ready cDNA as the template, 5′-RACE was conducted and the PCR product was analyzed by agarose gel electrophoresis. Two bands were obtained, a clear one of about 150 bp and a wide, smeared band ranging between 600 and 180 bp. Hybridization using the PSF cDNA-specific oligonucleotide probe demonstrated that the amplified product of about 150 bp was the PSF-specific 5′-RACE product (Fig. 4B). This was then subcloned to determine its nucleotide sequence. The PSF cDNA 5′-ends of the clones obtained were all at 34 b upstream of the PSF translation initiation site, and this result was similar to that estimated by S1 mapping. The site was 12 b upstream from the 5′-end of the PSF cDNA already reported, which was not full length (*6*). According to the results of S1 mapping and 5′-RACE, it was considered that the PSF gene has plural transcription initiation sites and the transcription of PSF gene is initiated most frequently at 34 b upstream of the PSF translation initiation site (Fig. 5).

Analysis of the 5′-Flanking Sequence and the 3′-Flanking Sequence—When we determined the nucleotide sequence about 400 bp upstream of the transcription initiation site, we found that although no typical TATA box and CAAT box were observed in this region, the area from the

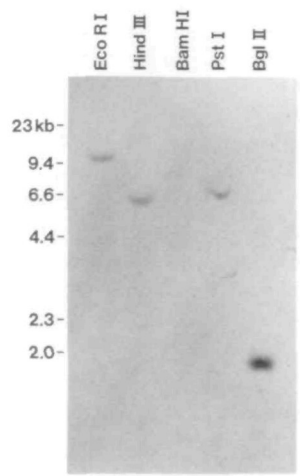

Fig. 3. **Southern hybridization analysis of human genomic DNA digested with various restriction endonucleases.** Human Geno-Blot containing five lanes of human genomic DNA that has been digested with different restriction endonucleases. The positions of DNA molecular weight markers are shown on the left.

GAG	CAA	G	gtgggcacga...(Intron 1 20kb <)...tctcctgcag	GT	CCT	TCC
Glu	Gln	Gly			Pro	Ser
157	158	159			160	161
TGG	AAC	AAG	gtcagtggca...(Intron 2 7.3kb)...gtttttgcag	GTA	AAA	AGG
Trp	Asn	Lys		Val	Lys	Arg
193	194	195		196	197	198
TGG	GTG	CTG	gtgagtacca...(Intron 3 0.7kb)...tctttcacag	GTA	TCT	CCT
Trp	Val	Leu		Val	Ser	Pro
232	233	234		235	236	237
AAA	AAA	G	gtacacaata...(Intron 4 1.2kb)...ttttaaacag	GT	GAA	GGT
Lys	Lys	Gly			Glu	Gly
275	276	277			278	279

Fig. 2. **Sequences of the exon-intron junctions of the human PSF gene.** Exon sequences are shown in uppercase letters, and intron sequences in lowercase letters. Each encoded amino acid and its position are indicated below the exon sequences. The length of each intron is shown in parentheses. The amino acid residues are numbered from the initiation methionine (*6*).

transcription initiation site to 300 bp upstream had a high G/C content (79%) and a large number of CpG sequences were observed (Fig. 5). Seven binding sequences of transcription regulating factors Sp1 (*15*) were present in the region from position −227 to position −31. Regarding promoters characterized by having no TATA sequence and a high G/C content, housekeeping genes including those for human hypoxanthine guanine phosphoribosyltransferase (*16*) and human 3-phosphoglycerate kinase (*17*), cancer genes such as human c-Ha-*ras* (*18*) and mouse *c*-Ki-*ras* (*19*) and growth factor receptor genes such as human insulin-like growth factor receptor (*20*) and human epidermal growth factor receptor (*21*) have been reported. The features of these genes reportedly include their expression in a comparatively large range of tissues and cells (*22*), as is the case for the PSF gene (*7*), though at a relatively low expression level (*22*). There were also a binding sequence of the transcription regulating factor GATA, which is involved in the growth and differentiation of erythroblasts and megakaryoblasts, from 407 b upstream of the major transcription initiation site (*23*), and two repeats each of 5′-AGCAGGAGC-3′ (positions −237 to −229 and −144 to −136) and 5′-CGGGCGGGGC-3′ (positions −100 to −91 and −46 to −37). Whether these binding sequences and repeat sequences are actually functioning or not is

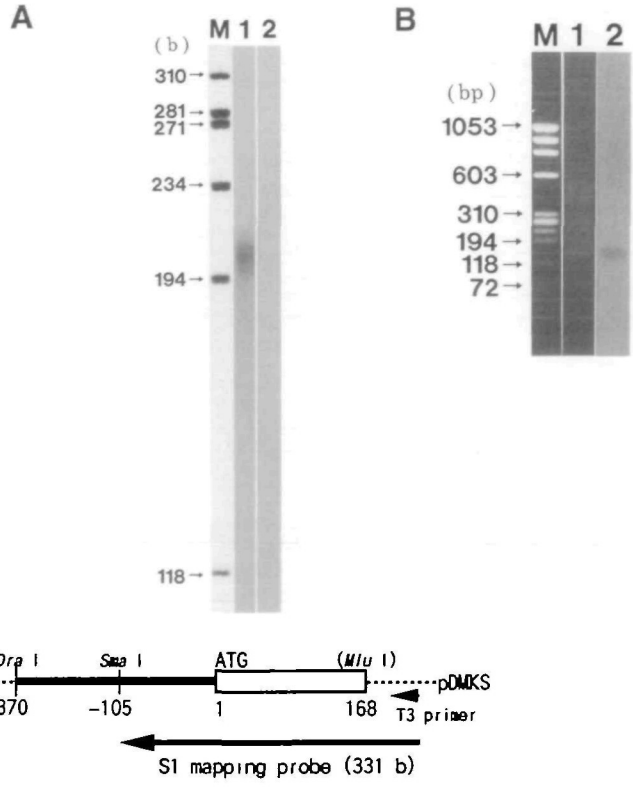

Fig. 4. **Determination of the transcription initiation site for the human PSF gene.** (A) S1 mapping analysis. The radiolabeled S1 mapping probe was hybridized to RNA from human diploid fibroblasts (lane 1) and yeast RNA (lane 2), digested with S1 nuclease, analyzed by electrophoresis on a denaturing polyacrylamide gel, and autoradiographed. The size of labeled S1-products was determined by comparison with a radiolabeled ϕX174 *Hin*dIII DNA marker (lane M). Adenosine, which is the translation start site, is numbered +1 in the diagram of pDMKS. (B) 5′-RACE analysis. A portion of the 5′-RACE products was electrophoresed on an agarose gel (lane 1), transferred to a nylon membrane, hybridized to a labeled oligonucleotide probe, and detected by autoradiography (lane 2). Lane M: ϕX174 *Hin*dIII DNA marker.

Fig. 5. **Nucleotide sequence of the 5′-flanking region of the human PSF gene.** Adenosine, which is the major transcription start site, is marked by an arrow and is numbered +1. Putative Sp1 and GATA sites are boxed. Repeated sequences are underlined.

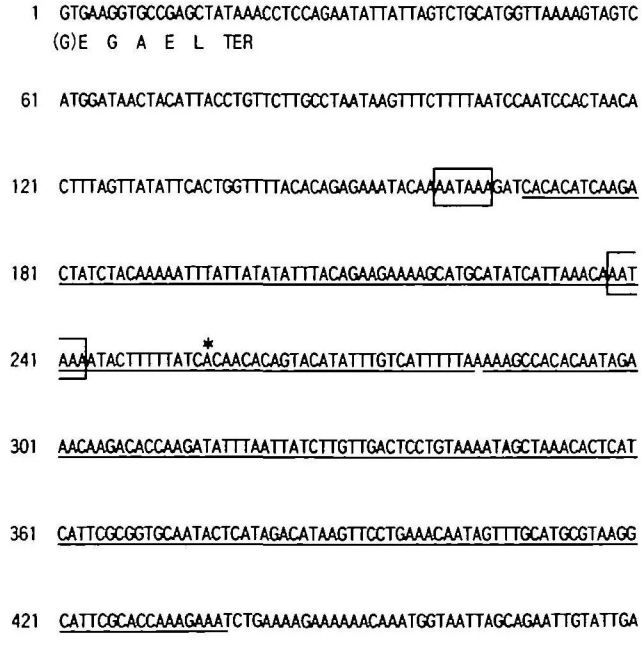

Fig. 6. **Nucleotide sequence of exon 5 and the 3′-flanking region of the human PSF gene.** Potential polyadenylation signals are enclosed by boxes. The putative poly-adenylation site is marked by an asterisk. The region identical with hRPB140 cDNA is underlined. Guanosine, which is the 5′-end of exon 5, is numbered +1.

unknown, but we assume it may be possible to elucidate the control mechanism of PSF expression by closely analyzing this 5′-flanking region.

We determined the nucleotide sequence for about 200 bp downstream of the PSF gene 3′-end (Fig. 6). There were two typical polyadenylation signals in exon 5 (positions 160-165 and 238-243). It is of interest to note that the nucleotide sequence in the region from 169 bp downstream of the 5′-end of exon 5 to 182 bp downstream of the PSF gene 3′-end was identical with the antisense nucleotide sequence in part of the coding region and 3′-flanking region of human RNA polymerase II second largest subunit (hRPB140) cDNA (*24*) (nucleotide position from 3479 to 3748 in EMBL-GDB AC; X63563), except for 1 base (Fig. 6).

In the present study, we isolated the PSF chromosomal gene and analyzed its structure. It would be very interesting to elucidate the relationship, if any, between the control of PSF expression and the presence of structural abnormalities in systemic diseases associated with vascular damage, including diabetes. The results of the present study should be helpful in this regard.

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