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Nucleotide Sequence of Gene PBII Encoding Salivary Proline-Rich Protein P-B[1]

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The nucleotide sequence of gene PBII encoding salivary proline-rich protein P-B was determined. PBII is 7.1 kb long and contains 3 exons. PBII exhibits considerable nucleotide sequence homology not only in exons but also in introns with PBI (accession number D89501), the gene whose nucleotide sequence was determined previously [Isemura and Saitoh (1997) *J. Biochem.* 121, 1025–1030]. PBI and II constitute a gene family distinct from that to which the majority of salivary proline-rich protein ones belong. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB031740.

Key words: P-B, PBI, PBII, proline-rich protein, saliva.

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Human saliva is known to contain acidic, basic, or glycosylated forms of proline-rich proteins (PRPs). These proteins are also detected in the saliva or salivary glands of animals including monkeys, rats, mice, and rabbits. Several investigators have studied their chemical, physicochemical and genetic features extensively (*1*).

There have also been some reports concerning their physiological roles in the oral cavity. Moreno *et al.* described the roles of acidic PRPs in the maintenance of teeth through inhibition of hydroxyapatite crystal growth on their surface (*2*). Mehansho *et al.* reported that PRPs detoxify dietary tannin by inhibiting its absorption from the digestive organs (*3*). Gu *et al.* (*4*), and Robinovitch *et al.* (*5*) showed that basic PRPs had anti-virus activity.

I and coworkers have elucidated the amino acid sequences of several basic PRPs of low molecular weight (<10,000) in saliva (*6–11*). All these proteins have high contents of proline (>20%) and repetitive sequences, but only P-B is different from the others in the structure of the repeating unit, that is GPGXX′PPPP (X and X′ are any amino acid) in P-B and PPGKPQGPP in the others. Basic PRPs other than P-B exhibit sequence homology with acidic PRPs and glycosylated PRPs (*12*).

The nucleotide sequences of the cDNAs (*12*) and genes (*13*) of PRPs other than P-B have revealed that the polymorphism of PRPs is due to the presence of multiple genes belonging to the same family, alternative splicing of mRNA precursors, and post-translational modification.

The nucleotide sequence of P-B cDNA (*14*) that we determined did not exhibit significant homology with other PRP cDNAs or genes, suggesting that it may belong to a distinct PRP gene family, and showed that P-B was not a degradation product of a larger protein, but a mature protein itself.

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Abbreviations: dNTP, deoxy nucleotide triphosphate; PRP, proline-rich protein.

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During the course of cloning of the P-B gene through the screening of a human genomic library using the P-B cDNA fragment as a probe, we characterized one gene (*15*). However, the gene isolated was not the P-B gene, and we designated it as PBI because putative translational product P-B1 was homologous to P-B (*6, 15*). In the course of this line of experiments, I have successfully isolated a candidate gene encoding P-B. In this paper, I report the cloning and nucleotide sequence of this P-B gene termed PBII.

MATERIALS AND METHODS

The following materials were purchased from the sources indicated: PCR kit, LAPCR kit, LAPCR *in vitro* cloning kit, ligation kit, Dr. GenTLE and *Escherichia coli* JM109 competent cells, Takara Shuzo; dye terminator sequencing kit, Perkin-Elmer; ECL direct nucleic acid labeling system, ECL 3′-oligonucleotide labeling system and ECL detection system, Amersham; M13 forward and reverse primers used for sequencing, and enzymes and reagents used for molecular cloning, Toyobo and Takara Shuzo; custom-made oligonucleotides used as probes and primers, Asahi Emerse; DNA extraction kit, Wako Pure Chemicals; and QIAEXII gel extraction kit, QIAprep spin miniprep kit and QIAquick PCR purification kit, QUIAGEN.

Human Genomic DNA Preparation—Human genomic DNA was prepared from the author's blood by following the instructions for a DNA extraction kit or Dr. GenTLE. LAPCR, *in vitro* cloning, nucleotide sequencing reaction, hybridization, and detection by ECL, transformation and ligation were performed by following the instructions recommended by the respective manufacturers.

DNA Sequencing—The nucleotide sequences were determined with a DNA sequencer (Perkin Elmer 373S) using the dye terminator dideoxy method according to the protocol of the manufacturer. M13 forward and reverse primers and/or custom-made primers were used for sequencing inserts in plasmids or PCR products. The determined sequences were each confirmed by reanalyzing a different clone or batch of PCR products using primers designed so

as to be hybridizable with the determined sequence. The sequencing strategies are summarized in Fig. 1. The probes and primers used in this study are listed in Table I. The reaction conditions and programs for PCR are listed in Table II.

PCR Cloning of the P-B Gene—P-B genes were cloned by using LAPCR and *in vitro* cloning kits. The template for PCR and LAPCR was human genomic DNA, and that for *in vitro* cloning was a mixture obtained by ligation of restriction enzyme digests of human genomic DNA into the cassette provided in the kit.

Cloning of A10—Primers 3 [corresponding to P-BcDNA (–39)-(–20) (*14*), sense primer] and 4 [corresponding to P-BcDNA (34)-(16) (*14*), with an additional 5′AAGGAT introduced to create a *Bam*HI site, antisense primer] were used. *Bam*HI digests of PCR products were inserted into PUC18. A plasmid containing a 1.3 kbp insert hybridizable with probe 9 [corresponding to P-BcDNA (13)-(30) (*14*)] was selected and sequenced.

Cloning of A5—Primers 9 [corresponding to P-BcDNA (13)-(30) (*14*), sense primer] and 10 [corresponding to P-BcDNA (516)-(496) (*14*), antisense primer] were used. *Eco*RI digests of PCR products were inserted into PUC18. A plasmid containing a 2.7 kbp insert hybridizable with probe 11 [corresponding to P-BcDNA (378)-(397) (*14*)] was selected and sequenced.

Cloning of D9—Primers 5 [corresponding to P-BcDNA (–8)-(14) (*14*), with an additional 5′TTAGGC introduced to create a *Stu*I site, sense primer] and 6 (corresponding to a partial sequence of A5, with a *Stu*I site inside, antisense primer) were used. *Stu*I digests of PCR products were inserted into a *Hinc*II site of PUC18. The plasmid containing 3.3 kbp insert hybridizable to probe 21 (corresponding to the partial sequence of A5) was selected and sequenced.

In Vitro Cloning of E3—Human genomic DNA was digested extensively with *Eco*RI and then the products were ligated to an *Eco*RI cassette supplied with the *in vitro* cloning kit. The first PCR was performed using cassette primer C1 in the kit (sense primer) and primer 2 (corresponding to a partial sequence of A10, antisense primer). The template was the ligation product. The second PCR was performed using the first PCR products as templates. The primers used were cassette primer C2 (sense primer) and primer 1 (antisense primer corresponding to the upstream region of primer 2). After purification using the QIAquick PCR purification kit, the PCR product was digested with *Eco*RI and *Hin*dIII. The digests were ligated to an *Eco*RI/*Hin*dIII site of PUC18. The plasmid containing a 1.5 kbp insert hybridizable with the direct-labeled A10 fragment was selected and sequenced.

In Vitro Cloning of A2—Human genomic DNA was digested extensively with *Bam*HI and then the products were ligated to a *Sau*3AI cassette provided with the *in vitro* cloning kit. The first PCR was performed using primer 12 (corresponding to the partial sequence of A5, sense primer) and cassette primer C1 in the kit (antisense primer). The template was the ligation product. The second PCR was performed using the first PCR products as templates. The primers used were primer 11 [sense primer corresponding to the downstream region of primer 12 and corresponding to P-BcDNA (378)-(397) (*14*)] and cassette primer C2 (antisense primer). After purification using the QIAquick PCR purification kit, the PCR product was digested with *Sau*3AI and *Eco*RI. The digests were ligated to an *Eco*RI/*Bam*HI site of PUC18. The plasmid containing a 0.25 kbp insert hybridizable with probe 10 [corresponding to the partial sequence of A5 and to P-BcDNA (516)-(496) (*14*)] was selected and sequenced.

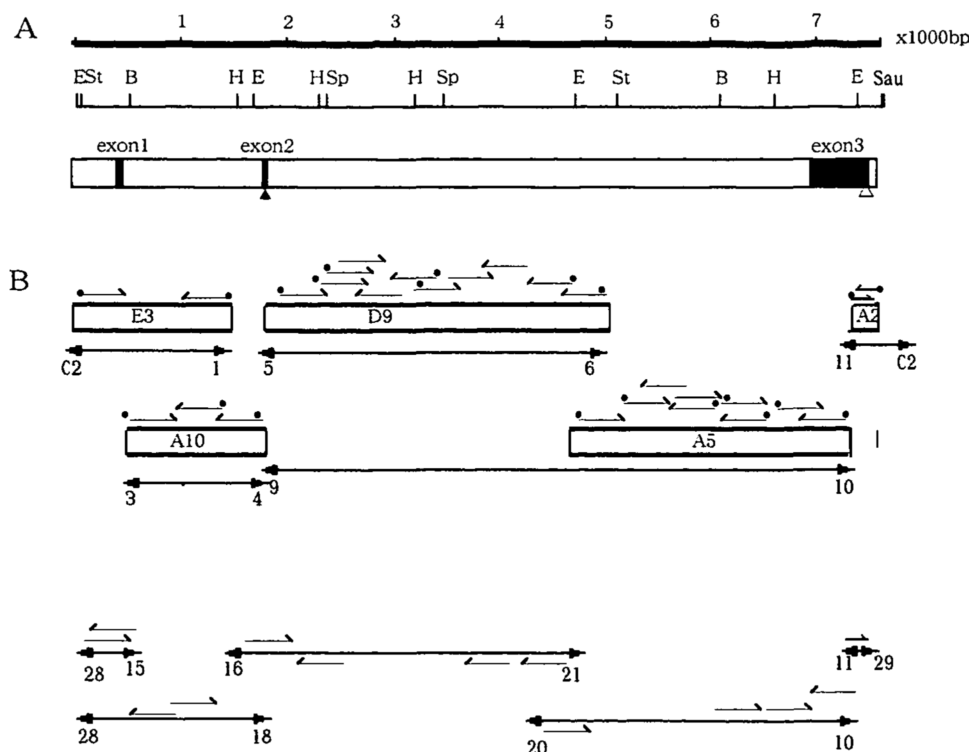

Fig. 1. **Restriction map of the PBII gene and sequencing strategy.** (A) Restriction map and gene organization. Exons are shown as black boxes. Solid and open triangles indicate the positions of an initiation codon and a termination codon, respectively. Restriction enzymes: E, *Eco*RI; St, *Stu*I; B, *Bam*HI; H, *Hin*dIII; SP, *Sph*I; Sau, *Sau*3AI. *Sau*3AI sites other than the 3′ end are not shown in this figure. (B) Sequencing strategy. Double-headed arrows indicate PCR products. The numbers below arrowheads refer to the primer numbers (see Table I). Boxes above double-headed arrows indicate the plasmid inserts derived from enzyme digests of PCR products. Small arrows indicate the sequenced range and the direction of sequencing. A dot at the origin of a small arrow shows that this part was sequenced using a M13 forward or reverse primer after subcloning into PUC18. A part with a small arrow without a dot was sequenced using a custom-made primer.

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TABLE I. **Nucleotide sequences of primers and probes.**

No.	Sequence 5′ → 3′	Base No. in PBII[a] (5′ → 3′)	Base No. in PBcDNA[b] (5′ → 3′)
1	caaagagttg ttcatacatt gctctgagca	1125 → 1096	
2	taaaatttga cttcaaaata gtggaagtag	1195 → 1166	
3	ctgtaagacg gatcctcaca	63 → 82	(−39) → (−20)
4	(aaggat)ccca aaggcccaag atcca	1419 → 1401	34 → 16
5	(ttaggc)ctga aaggatgaaa tcactgac	1378 → 1399	(−8) → 14
6	agaggcctgt aattttccaa ga	4659 → 4638	
7	cccagcatgt agtttatgga	1764 → 1783	
8	tttaatggga ctaatagtag	3368 → 3349	
9	acttggatct tgggcctt	1398 → 1415	13 → 30
10	attctcatcc aaaaatggaa g	6999 → 6979	516 → 496
11	aacaactgca gcaggtgcca	6860 → 6879	378 → 397
12	tctctctgat ttcttctgtc tctg	6339 → 6362	
13	gaatcccacc tcctcctccc gcaccctatg	6655 → 6684	
14	gtacctccac atgaccctcc ccacaacatg	6262 → 6291	
15	gttgaaaatg ttatctttga	162 → 143	
16	tatctcaaac ccatccatag a	963 → 983	
17	aatcggtcca tccaagtaca	314 → 333	
18	ttgcctctga aagtaagcac	1377 → 1358	
19	gcctgggtga cagagtgaga	3117 → 3136	
20	catttgttgt attagtgatc cta	3864 → 3886	
21	ggcaggaatt ataaactgga	4364 → 4345	
22	tctagaatac cctttcaaag ttt	3964 → 3986	
23	tcaacctggc ttctgccttt	3928 → 3909	
24	gggggagaag ggtgagtgaa	4292 → 4311	
25	aaagaggccc caggggacca tat	6550 → 6572	68 → 90
26	ttgaggaggg ggtggtggaa atat	6716 → 6693	234 → 211
27	gtctttcaac tggcaagagt cattttgacc	19 → 48	
28	gaattcagtt taaaaatatt tgta	−424 → −401	
29	gatctatgtt cactcattta	7194 → 7175	
30	gccacaccca catatatata	513 → 532	
31	gtgtggtctg agcaatgcaa	596 → 577	
32	tggaattaat ctaacactac	2094 → 2075	
33	taatattctc tttctgtcac	2114 → 2095	
34	ttcttttgta ttctgaggga	2782 → 2763	
35	cttatgttat cagaaaacta	3196 → 3177	
36	accatgccca gcctcagcta	5274 → 5293	
37	atggtttaca taaagcacaa	5329 → 5310	
38	atacaggaca caaaactaca	4910 → 4891	
39	caattgactt aaatgacaga	5785 → 5804	

[a]See Fig. 2. [b] See Fig. 2 in Ref. 7.

Homology Search—A computerized homology search in the database was performed using the FASTA search service. Regional comparison of genes PBI and PBII was performed with a search homology program of Gentyx Mac (Software Development).

RESULTS

By overlapping the sequences of E3, A10, D9, A5, and A2, the sequence of the P-B gene was determined and has been registered to DDBJ. The accession number is AB031740. The gene was named PBII because this is the candidate gene for salivary proline-rich protein P-B and because it is the second member of the gene family to which it belongs. PBII contained the entire sequence of P-BcDNA (*14*) divided into 3 parts. PBII had 3 exons for coding P-B, the sizes being 87, 68, and 582 bp for the first, second, and third exons, respectively. The size of the first exon is approximate, since the transcriptional start point has not been precisely determined experimentally. The putative transcriptional start point was assigned by considering that transcription usually starts at about 20 to 30 bases downstream of a TATA box (*16*). The first exon contained a 5′-untranslated region. The 2nd exon was composed of 14 bp of the 5′ non-coding sequence and 54 bp of a coding sequence corresponding to 18 amino acid residues of the signal sequence of P-B. The third exon contained 12 bp coding for 4 amino acid residues of the signal sequence, 171 bp coding for the entire mature protein (57 amino acid residues), and 399 bp of the 3′ non-coding sequence from the stop codon TAA through the poly A addition signal AATAAA to the poly A addition site. The positions of exons are shown in Figs. 1 and 2. The sizes of the 1st and 2nd introns were 1,284 and 5,097 bp, respectively. Both introns satisfied the GT–AG rule (*17*), and had sequences homologous to the consensus sequence, YNYRAY (Y = pyrimidine, R = purine, N = any base), required for branching for splicing (*18*) about 30 bp upstream of the 3′ end. The promoter sequence TATATAA (*16*) was found at 21–27 bp upstream of the putative transcriptional site. Another promoter sequence, CCAAT (*19*), was present at 123–127 bp upstream of the transcriptional site as an inverse complementary form. In Fig. 2, the nucleotide sequences of the 5′ upstream region of exon 1, the three exons, and regions adjacent to the exons are shown. The regulatory elements for transcription and splicing are also shown in Fig. 2.

A computerized homology search of PBII in databases revealed that PBI has the highest score. The percent homology between the corresponding regions of the two genes is shown in Table III. PBI and PBII have the same gene organization. They have the same promoter sequences (TATATAA and CCAAT) at similar positions. The two genes exhibit homology over the entire molecules. Exons 2 of the two genes are completely identical. The homology in the middle parts of the molecules was relatively low and the length of intron 2 of PBII was longer than that of PBI by 600 bp. In spite of that exon 3 of PBII was longer than that of PBI, the gene product of PBII (salivary proline-rich protein P-B) is shorter than the putative gene product (P-B1) of PBI by 55 residues, mainly because of the shift of the stop codon of PBI in the 3′ direction.

Rat PR-Vβ1 (*20*) is a protein homologous to human P-B. The gene organizations of PBI, PBII, and VCSβ1 (the gene for PR-Vβ1) (*15, 20*) are compared schematically in Fig. 3. The three genes fundamentally have the same gene organization. The corresponding exons in the three genes are similar in size. In all three genes, exons 1 are for 5′ non-coding sequences, exons 2 for the rest of the 5′ non-coding sequences plus signal sequences, and exons 3 for a few residues of the C-terminal signal sequences and the mature PRPs plus 3′ non-coding sequences.

P-B is expressed in the human submaxillary gland, since P-BcDNA has been PCR-cloned from human submaxillary gland cDNAs (*14*). P-B is also considered to be expressed in the human parotid gland, since this protein has been isolated from human parotid duct saliva (*8*). The expression of P-B seems to be tissue-specific because no mRNAs from various tissues including heart, brain, lung, liver, skeletal muscle, kidney, and pancreas gave positive signals on Northern blotting, for which a P-B cDNA fragment (corresponding to residue numbers [(72–78)+(1372–1439)+(6537–6870) of PBII, see Fig. 2] was used as a probe (*14*). In this Northern blot analysis, placenta mRNA gave a positive signal but the size was larger (5–8 kb) than in the case of P-B cDNA (*14*).

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TABLE II. **Conditions and programs for PCR reactions.** The kits used were: PCR amplification kit, for reaction 1; LAPCR kit, for reactions 2, 3, and 6–10; and LAPCR *in vitro* cloning kit, for reactions 4, 4′, 5, and 5′. The reaction volume was 50 µl, and the compositions, in 50 µl, were: template, 0.2 µg; primers, 1 µM each; dNTP, 200 µM each; *Taq* polymerase, 1.25 U; buffer concentration, 1 × PCR buffer for reaction 1. Template, 0.2 µg; primers, 0.2 µM each; dNTP, 400 µM each; LA *Taq* polymerase, 2.5 U; buffer concentration, 1 × LAPCR buffer for reactions 2, 3, and 6–10. Heat-treated (94°C 10 min) template, 0.5 µg; primers, 0.2 µM each; dNTP, 400 µM each; LA *Taq* polymerase 2.5 U; buffer concentration, 1 × LAPCR buffer for reactions 4 and 5. Template, 1 µl; primers, 0.2 µM each; dNTP, 400 µM each; LA *Taq* polymerase 2.5 U; buffer concentration, 1 × LAPCR for reactions 4′ and 5′.

No.	Product	Template	Primer		Program
			Sense	Antisense	
1	Precursor of A10	Human genomic DNA	3	4	94°C 1 min, 55°C 1 min, 72°C 2 min, 30 cycles
2	Precursor of A5	Human genomic DNA	9	10	94°C 1 min, 55°C 1 min, 72°C 5 min, 30 cycles
3	Precursor of D9	Human genomic DNA	5	6	94°C 1 min, 56°C 1 min, 72°C 5 min, 35 cycles
4	Precursor of E3, 1st PCR	Ligation products of *Eco*RI digests of human genomic DNA and *Eco*RI casette	C1	2	94°C 30 s, 55°C 2 min, 72°C 1 min, 30 cycles
4′	Precusor of E3, 2nd PCR	1 µl of 1st PCR product	C2	1	94°C 30 s, 55°C 2 min, 72°C 1 min, 30 cycles
5	Precursor of A2, 1st PCR	Ligation products of *Bam*HI digests of human genomic DNA and *Sau*3AI casette	12	C1	98°C 20 s, 60°C 20 s, 68°C 15 min, 40 cycles
5′	Precursor of A2, 2nd PCR	1 µl of 1st PCR product	11	C2	94°C 30 s, 55°C 2 min, 72°C 1 min, 40 cycles
6	(–424)–1377	Human genomic DNA	28	18	98°C 15 s, 47°C 10 s, 72°C 3.5 min, 35cycles
7	(–424)–162	Human genomic DNA	28	15	98°C 15 s, 48°C 10 s, 72°C 3.5 min, 35 cycles
8	963–4364	Human genomic DNA	16	21	98°C 15 s, 58°C 10 s, 68°C 6 min, 35 cycles
9	3864–6999	Human genomic DNA	20	10	98°C 15 s, 55°C 10 s, 68°C 6 min, 35 cycles
10	6860–7194	Human genomic DNA	11	29	98°C 15 s, 48°C 10 s, 72°C 3.5 min, 35 cycles

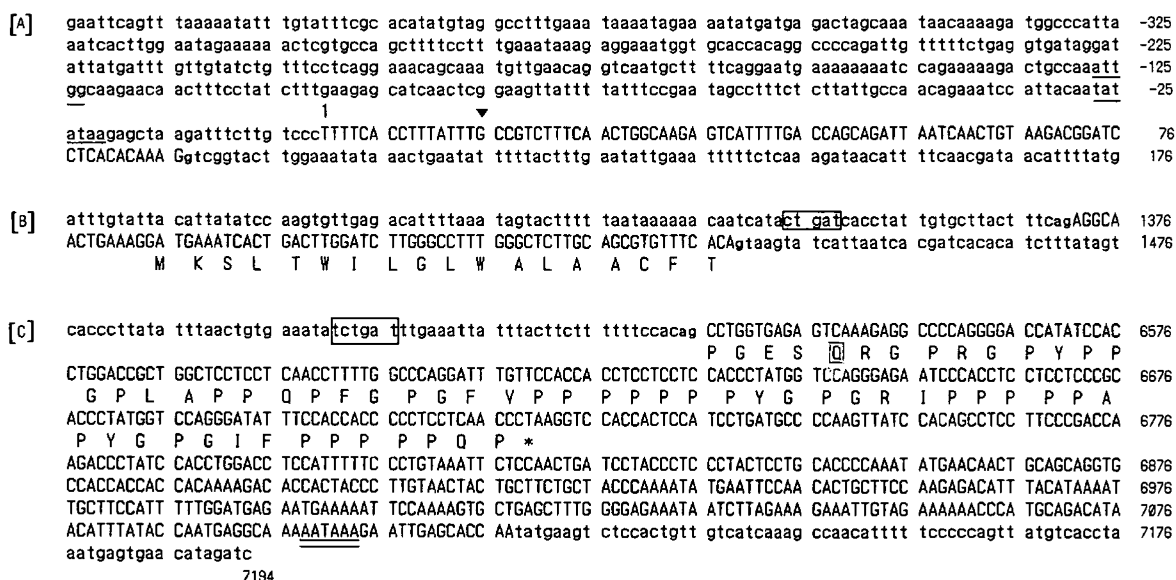

Fig. 2. **Nucleotide sequences of the 5′-upstream region, the 3′-downstream region, exons 1–3, and the regions adjacent to the exons of PBII.** The putative transcriptional site is numbered 1. The exon sequences are given in capital letters while the intron sequences are given in lower case letters. Encoded amino acid residues are given as one-letter abbreviations. [A] Exon 1 and the regions adjacent to it. The TATA box and CCAAT box are underlined. The 5′ end of the sequenced P-B cDNA is indicated by ▾. [B] Exon 2 and the regions adjacent to it. The sequence required for branching for splicing is boxed. [C] Exon 3 and the regions adjacent to it. The sequence required for branching for splicing is boxed. Polyadenylation signals are double-underlined. The amino-terminal amino acid of salivary proline-rich protein P-B is boxed. For the rest of the PBII sequence, refer to AB037140 in the DDBJ database.

DISCUSSION

In the present work, the nucleotide sequence of a candidate gene for basic proline-rich protein P-B of human saliva was determined. The amino acid sequence deduced from the determined sequence was completely in accordance with that of P-B (*6*). We have named this gene PBII, because it is the second cloned member of a gene family. The first member of this gene family is PBI and its putative translational product is similar to but distinct from P-B (*15*).

The present data show that the two genes PBII and PBI have the same gene organization and promoter sequences. Both are AT-rich DNAs (each with an AT content of about 63%). They exhibit homology over their entire molecules. However, P-B1 (the gene product of PBI) appeared not to be expressed in salivary glands, since only protein P-B (the gene product of PBII) was detected when human whole and parotid saliva was analyzed (*6, 8*). In fact, in our previous study on PBI, the restriction enzyme digestion pattern of human submaxillary gland cDNA implied the absence of cDNA derived from gene PBI (*15*). Although TATA box and

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TABLE III. **Comparison of PBII and PBI, percent of homology between their corresponding regions.**

PBI region base No.[b]	size[c]	PBII region base No.[a] size[c]: Upstream (−424)–(−1) [424]	Exon 1 1–87 [87]	Intron 1 88–1371 [1284]	Exon 2 1372–1439 [68]	Intron 2 1440–6536 [5097]	Exon 3 6537–7118 [582]	Downstream 7119–7194 [76]
Upstream (−1)–(−435)	[435]	79.2%/427 bp						
Exon 1 1–88	[88]		93.1%/87 bp					
Intron 1 89–1347	[1259]			83.5%/1275 bp				
Exon 2 1348–1415	[68]				100%/68 bp			
Intron 2 1416–5888	[4473]					66.0%/4579 bp		
Exon 3 5889–6396	[508]						83.5%/510 bp	
Downstream 6397–6472	[170]							84.8%/66 bp

[a]See Fig. 2. [b]See Fig. 2 in Ref. (*15*). [c]Expressed in bp.

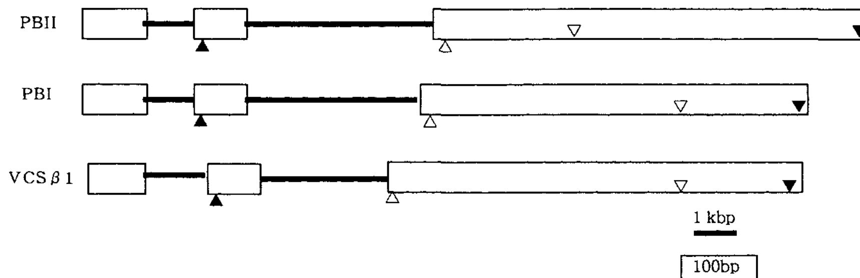

Fig. 3. **Comparison of the gene organizations of PBII, PBI, and rat VCSβ1.** ▲, △, ▽, and ▼ indicate the initiation codon, codon for the N-terminal amino acid, stop codon, and poly A addition signal, respectively.

CCAAT boxes are present almost at identical positions, the homology upstream of the transcriptional sites of the two genes is somewhat low, as shown in Table III. This fact may explain the difference in the expression of the two genes. There is a possibility of the presence of other members of this gene family for the following reasons. Firstly, Southern blotting of restriction enzyme digests of human genomic DNAs gave multiple signals (*14*), and secondly, on Northern blot analysis with P-B cDNA as a probe, the human placenta gave a positive signal, the size being larger than that in the case of salivary gland (*14*).

This gene family seems to be ubiquitous, since a proline-rich protein having N-terminal 57 residues identical to P-B and an apparent molecular weight larger than that of P-B has been detected in developing bovine enamel (*21*), and various species of animals including man, monkey, rat, mouse, dog, cow, rabbit, and chicken, and yeast gave positive signals on Southern blot analysis (*14*). In the rat, two members of this gene family, VCSα1 and VCSβ1, have been characterized (*20, 22*), and in the mouse, three cDNAs, MSG1-, MSG2-, and MSG3 cDNA, transcriptional products of the genes belonging to this gene family, have been cloned (*23*). They clearly exhibit homology at the nucleotide sequences level, but only the gene product PR-Vβ1 of rat VCS β1 and mouse MSG1 are PRPs homologous to human P-B and P-B1. The protein sequences of these four PRPs were compared schematically in our previous paper (*15*). SMR1, the gene product of VCSα1 (*22*), MSG2, and MSG3 (*23*) are not so-called PRPs, because of the high frequency of nucleotide substitutions in coding sequences or deletion of the region coding for proline-rich repeating units from its cDNA. These findings suggest that some human genes belonging to the PB gene family may code for non-PRPs.

Studies on other members of this gene family, the efficiency of their expression and their tissue expression sites may help reveal the physiological significance of P-B and related proteins.

Gu *et al.* (*4*) have shown the ability of proline-rich proteins and salivary cystatins to inhibit viral replication, with basic proline-rich peptides being more effective. Their results also suggested that basic proline-rich peptides reduced the virus titer by interfering with penetration and/or cellular processing of the virus within the target cell. More recently, Robinovitch *et al.* (*5*) reported that certain basic proline-rich proteins exhibit inhibitory activity toward HIV-I infection. Collectively, these results suggest that salivary PRPs play an important role in the host defense mechanism against virus infection.

Since certain differences were found between the published nucleotide sequence of P-B cDNA (*14*) and the exon 3 sequence of PBII, the nucleotide sequence of the corresponding part of P-B cDNA used in our previous study was reexamined. The results indicated that in the previous sequence G (464) should be changed to A and C (473) to CC. These changes resulted in the exon 3 sequence and the corresponding one in P-B cDNA being completely identical.

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