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ON THE cDNA'S FOR TWO TYPES OF RAT PANCREATIC SECRETORY TRYPSIN INHIBITOR

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Two types of cDNA, which code for the two types of rat pancreatic secretory trypsin inhibitors (PSTIs), were cloned and sequenced. Both predicted amino acid sequences consisting of 79 amino acids, with the secretion signal peptide consisting of 18 and 23 amino acids for PSTI-I and PSTI-II, respectively. The nucleotide sequences were 91% homologous between the two cDNAs, but 68% and 65% homologous, respectively, when compared with human *PSTI* cDNA. Northern blot analyses showed that *PSTI-I* is expressed in the pancreas, whereas *PSTI-II* is expressed in the pancreas and the liver using the same promotor. Southern blot analyses suggested that both *PSTI-I* and *PSTI-II* genes are single copy genes per haploid genome. Duplication of rat *PSTI* gene seems to have occurred recently, after the divergene of humans and rats.

The pancreatic secretory trypsin inhibitor (PSTI) is produced in mammalian pancreatic acinar cells and secreted into pancreatic juice. Its physiological role has been thought of as the prevention of premature activation of zymogens while they are in the pancreas and pancreatic duct. Recently, this polypeptide has been widely detected in various normal and malignant tissues (1) and the same mRNA as that in the pancreas has been found in certain cancerous tissues (2). These findings suggest that PSTI may have other roles. In addition, PSTI is detected in sera (3), particularly in patients who have undergone major surgery or have suffered from injuries or inflammations. In these patients, the serum level of PSTI

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is closely correlated with that of acute phase reactants (4, 5), of which PSTI seems to be a member. To clarify the physiological effects of PSTI, we started to analyze rat PSTI cDNAs and found two different molecules, unlike in humans for which only a single type of molecule is known (18, 21). Here we describe the structures of these two PSTI cDNAs and show that one of them is identical with a CCK-releasing peptide which Iwai et al. (6) reported recently.

MATERIALS AND METHODS

Preparation of poly(A) RNA and construction of the cDNA library. Rat pancreas and liver were removed from male Wistar rat, immediately frozen in liquid nitrogen, and stored at −70°C. Total cellular RNA was isolated essentially as described previously (7). Poly(A) RNA was purified from the total RNA by repeated passages through oligo dT cellulose (Type 7, Pharmacia Co., Sweden) column. Double-stranded cDNAs were prepared from rat pancreas poly(A) RNA and then ligated to λgt10 with *Eco*RI linkers as described previously (8).

Library screening. The 17-mer oligodeoxynucleotide probes (Fig. 1) were synthesized on an Applied Biosystems Model 381A instrument. These probes were mixtures of 48 and 64 possible oligodeoxynucleotides corresponding to amino acid sequences for rat PSTI-I and PSTI-II (9), respectively. Plaque transfer to nitrocellulose filter was performed as described elsewhere (10). The filters were hybridized at 31°C and washed at 4°C as described previously (11). Filters were exposed at −70°C for 40 hr. to Kodak XAR-5 x-ray film with a Du Pont Lightning-Plus intensifying screen.

Sequencing analysis. cDNA clones of λgt10, subcloned in pUC18, M13 vectors mp18 and mp19 (12), were subjected to DNA sequencing analyses by the dideoxy method (13, 14).

Southern and Northern blot hybridizations. Ten micrograms of high molecular weight DNA prepared from rat liver was digested with restriction enzymes (Takara Shuzo, Kyoto, Japan and Toyobo, Osaka, Japan), electrophoresed in 0.7% agarose gel (15) and transferred to a nitrocellulose or a nylon filter (18). mRNA was denatured by heating at 65°C for 15 min in 50% (vol/vol) formamide, electrophoresed in a 1% agarose / 2.2M formaldehyde gel as described (19), and then transferred to a nitrocellulose filter (15). The filters were hybridized for 15 hours with total cDNA probe or regional probes (see Fig. 2A) labeled with [^{32}P] in 6×SSC 1%SDS (1×SSC is 150 mM NaCl, 15 mM Na citrate) at 65°C or with 5'-end-labeled synthetic oligodeoxynucleotide probes (Fig. 2B) in 6×NET / 1×Denhardt's (1×NET is 150 mM NaCl, 1 mM EDTA, 30 mM TrisCl pH 8.0) at 50°C. The filters were washed at 65°C with 2×SSC, except that washing was employed at room temperature with 6×SSC for synthetic oligodeoxynucleotide probes, and then exposed at −70°C to Kodak XAR-5 x-ray film with a Du Pont Lightning-Plus intensifying screen.

Primer extension analysis. Primer extension analyses were carried out using poly(A) RNAs as templates and 5'-end-labeled synthetic oligodeoxynucleotide primers (Fig. 2B) as described previously (2).

RESULTS AND DISCUSSION

Recently, Uda et al. (9) showed that the rat has two different types of PSTI, PSTI-I and PSTI-II, and described their total amino acid sequences. Out of 61 and

56 amino acids, two regions, amino acid numbers 10 through 15 in PSTI-I and amino acid numbers 31 through 36 in PSTI-II, show significant sequence differences from the counterpart of the other PSTI molecule. We synthesized two oligodeoxynucleotide probes, probe I and probe II, representing these two regions (Fig. 1), and used them as probes for screening a cDNA library constructed from rat pancreas poly(A) RNA using λgt10. Out of 1×10^5 plaques screened, 15 clones gave weak signals with a mixture of probes I and II, and among them, a clone giving a strong signal with probe II was selected (λRTIB10). The insert of λRTIB10 was subcloned in pUC18 (pRTIB10) and was used as a probe for rescreening the cDNA library. Altogether 22 clones were obtained, and 6 and 16 clones, respectively, hybridized specifically to probes I and II. The insert DNAs were purified and subcloned in pUC18, M13mp18 and 19 for sequencing studies. The λRTIB10 was found to be a member of *PSTI-II* cDNAs.

The restriction maps and the nucleotide sequences are shown in Fig. 2A and 2B. Assignment of *PSTI-I* and *PSTI-II* corresponding to the nomenclature by Uda et al. (9) was done by sequencing (see below). Both *PSTI-I* and *-II* cDNAs contain 237 nucleotide regions that can code for 79 amino acids, along with 80 nucleotide 3'-noncoding regions. The 5'-noncoding regions for *PSTI-I* and *-II* cDNAs were 42 and 62 nucleotides, respectively, although neither 5' end may represent the initiation site for transcription. The predicted amino acid sequences completely agreed with those reported by Uda et al. (9), who showed that the PSTI-I and -II in

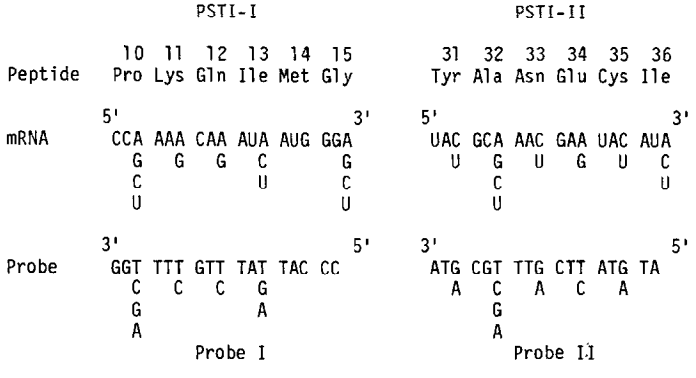
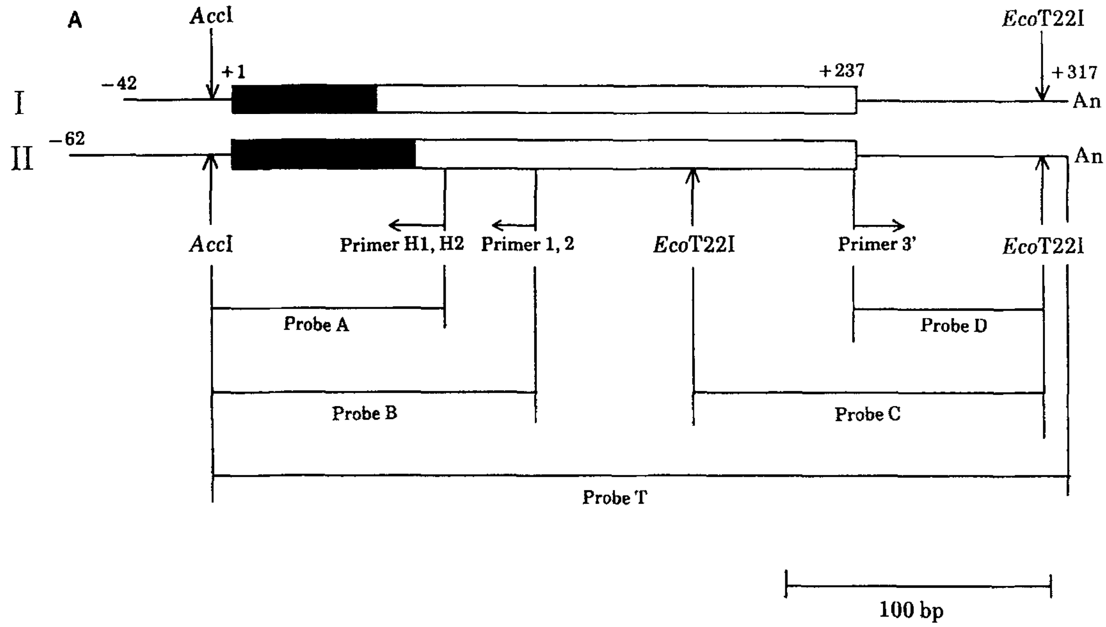


Fig. 1 Sequences of the synthetic oligodeoxynucleotide probes used for screening the rat pancreatic cDNA library. Probe I and II are mixtures of 48 and 64 oligodeoxynucleotides, respectively.



B

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                                                    -62                   -42                                         -1
PSTI I                                                                     CCCTGCACAGTTCTTCTGAGTTTTGGACCTAGGTCTACAACC
PSTI II                                             TCAGTTTCTGAAGAGAAGCA

      +1                                                                        +60     Primer H1, H2         +81
PSTI I    ATG AAG GTA GCA ATT ATC TTT CTT CTC AGT GCT TTG GCC CTG CTC AGT TTA GCA GGT AAC CCT CCA GCT GAG GTG AAT GGA
PSTI II                                                                A                   A   A       A       T   G
          Met Lys Val Ala Ile Ile Phe Leu Leu Ser Ala Leu Ala Leu Leu Ser Leu Ala Gly Asn Pro Pro Ala Glu Val Asn Gly
                                                                      Asn                 Thr Thr     Lys     Ile
          -23                                                                     -5                  +1          +4

     +82                     +99     Primer 1, 2  +115                                                             +162
PSTI I    AAA ACG CCT AAT TGC CCT AAG CAA ATT ATG GGA TGT CCC AGG ATT TAT GAC CCT GTG TGT GGG ACT AAC GGA ATT ACT TAC
PSTI II        A  G                    T AC  C   G T    C          GA       T               T   G       AA
          Lys Thr Pro Asn Cys Pro Lys Gln Ile Met Gly Cys Pro Arg Ile Tyr Asp Pro Val Cys Gly Thr Asn Gly Ile Thr Tyr
              Lys Ala                 Asn Thr Leu Val                 Asp                         Asp     Lys
          +5                  +10     Probe I     +15                                                             +31

                                                                                                Primer 3 -> +237   +243
     +163
PSTI I    CCC AGT GAA TGC AGT CTG TGC TTT GAA AAC AGG AAA TTC GGA ACA TCT ATC CAC ATT CAG AGG AGA GGG ACT TGC TGA ATG
PSTI II   G    A           T    A                        T                   G                       CT
          Pro Ser Glu Cys Ser Leu Cys Phe Glu Asn Arg Lys Phe Gly Thr Ser Ile His Ile Gln Arg Arg Gly Thr Cys ***
          Ala Asn         Ile                                             Arg                         Leu     ***
          +32  Probe II   +36                                                                             +56

     +244    +252                                                 +317
PSTI I    TCCTGATTTTGAAATCTTTTAGGGCTACCATAATGTTTAGCAAGAAGGTTTGCTGAATAAATGCATCTGAACATAn
PSTI II            C                                                           An
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Fig. 2 (A) Restriction maps of two types of rat *PSTI* cDNAs. The boxes represent amino acid coding regions. The solid and open boxes are coding regions for the signal peptides and peptides in secreted form, respectively. Total cDNA probe (probe T), regional probes (probe A~D) along with five primers used for extension analyses and for making probes (A, B and D) are also shown. (B) The nucleotide sequences and deduced amino acid sequences of rat *PSTI*s. The numbers start from the first base of the ATG translation initiation codon. The amino acid numbers start from the N-terminal of PSTI-II in the secreted form. The primers used for extension analyses and for making regional probes are boxed. The asterisks indicate the termination codons. The poly(A) additional signals and the locations of probes I and II which were used for the screening of a cDNA library are underlined. The processing sites for the signals of PSTI-I and -II are indicated by downward and upward arrows, respectively. (C) Comparison of human and rat *PSTI* cDNAs. The numbers start from the first base of the ATG translation initiation codon. The asterisks indicate the same nucleotides between the human and rat *PSTI* cDNAs.

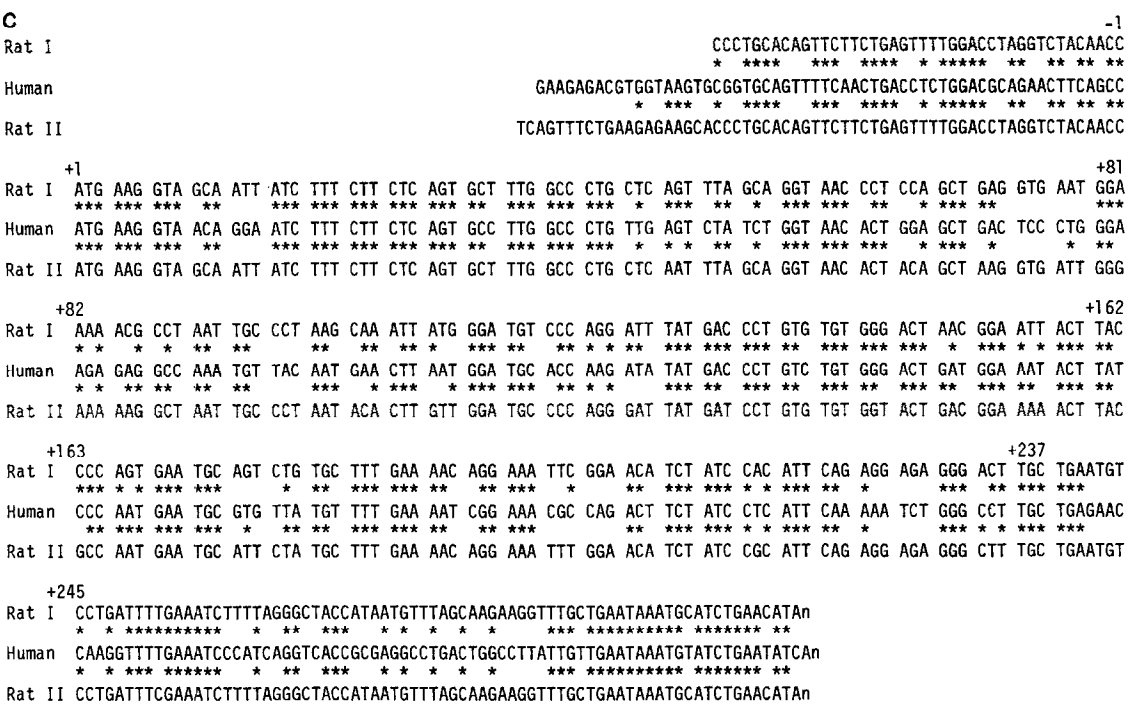

Fig. 2 - Continued

the secreted form consisted of 61 and 56 amino acids, respectively, and determined their N termini. Taking their results into consideration, the signal peptides for PSTI-I and -II must be 18 and 23 amino acids, respectively, with sequences that are almost identical except for the region around the cleavage sites of the signal peptides.

The homology between human (2, 18) and the rat *PSTI* cDNAs was 68% and 65% as can be seen in Fig. 2C. The homology was higher in the coding regions (both 72% homologous) and lower in the noncoding regions (61% and 55% homologous, respectively), suggesting that the nucleotide sequences in the coding regions had been conserved in the course of evolution.

Northern blotting analyses of poly(A) RNA from rat pancreas demonstrated that *PSTI-I* and *-II* mRNAs are about 550 nucleotides (Fig. 3A), in contrast to 359 and 379 nucleotides for the two cDNAs. If we assume that the size of poly(A) tail is roughly 200 nucleotides, then the discrepancy disappears, but this must be subjected to more stringent examination. To determine the border of the 5'-noncoding regions, we performed primer extension analyses. The results shown in Fig. 3C demonstrate that the major transcription of *PSTI-I* and *PSTI-II* starts,

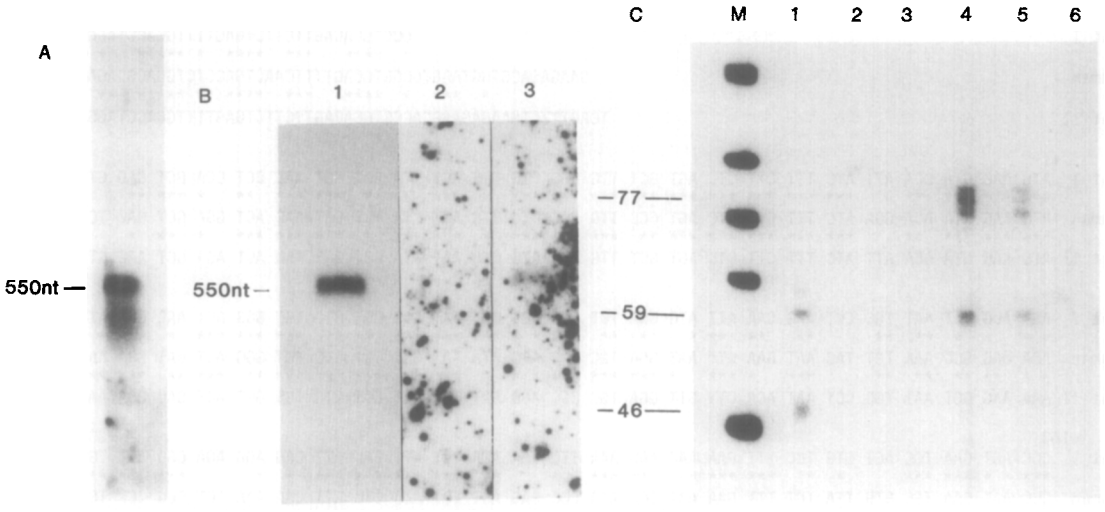

Fig. 3 (A) Northern blotting profile of poly(A) RNA (2 μg) from rat pancreas using ^{32}P-randomly-labeled probe T (see Fig. 2A). (B) Northern blotting profiles of poly(A)RNA (10 μg) from rat liver. Probes are ^{32}P-randomly labeled probe T (lane 1), 5'end-labeled 22-mer synthetic oligodeoxynucleotide for *PSTI-I* (lane 2), 5' end-labeled primers H1 (lane 2) and H2 (lane 3) which are specific for *PSTI-I* and *-II*, respectively (see Fig 2A, 2B). Autoradiography was performed for two hours (lane 1) or 3 days (lane 2, 3). (C) Primer extension analyses to determine 5' ends of *PSTI-I* and *-II*. Lane M: 5'-end-labeled, *Hap*II digested pBR322 as size marker. [^{32}P]-end-labeled synthetic oligodeoxynucleotide primer H1 for *PSTI-I* (lane 1, 2, 3) and primer H2 for *PSTI-II* (lanes 4, 5, 6) were used for primer extension studies (2). Poly(A) RNA(5μg) from rat pancreas (lanes 1, 4), rat liver (lanes 2, 5), and a human colon as a negative control (lanes 3, 6) were subjected to the same tests. Numbers indicate the nucleotide positions from the first base of the initiation codon.

respectively, at 46 nucleotides and 77 nucleotides upstream from the first base of the initiation codon. In both cases, there were signs of minor transcription initiation sites (both at the same position, −59).

To elucidate the genomic organization of rat PSTI genes, Southern blotting analyses were done using whole or regional cDNA probes (see Fig. 2A). Four bands, 10kb, 9kb, 2.5kb, 2.0kb, each with almost identical intensity, were detected when probe T was used (Fig. 4, lane 1). The 5'-regional probes (probes A and B) hybridized only with 10kb and 9kb bands, whereas the 3'-regional probes (probes C and D) hybridized only with 2.5kb and 2.0kb bands. DNA cleaved with *Bam*HI, *Hin*dIII, *Bgl*II or *Xba*I gave similar results (data not shown). Therefore, it is highly likely that each PSTI is coded for by the respective copy of the gene. We have cloned the *PSTI-II* gene (our unpublished observation) and shown that in *PSTI-II* gene, exons 1 through 3 belonged to the 9kb band and exon 4 to the 2.5kb band.

The overall nucleotide homology between these two cDNAs is 91% (Table 1). Within the molecules, the region for the secreted form is 84% homologous, whereas the 5'- and 3'-noncoding regions are 100% and 99% homologous, respectively. In many genes, the most rapidly evolving bases are those in synonymous positions in codons or 3'-noncoding regions (19). A different case has been reported by Hill and Hastie (20) who analyzed a group of serine protease inhibitors, and found accelerated evolution of the reactive center regions in these proteins. They proposed positive darwinian selection to account for this observation. Our results seem to coincide with their case. Humans have only one *PSTI* gene (18, 21), whereas rats have two. This may be due to a duplication that occurred after divergence of rats and humans; the two genes then may have evolved into two related, but different genes.

In rat, both PSTI-I and -II have the same trypsin inhibitory activity (9). Besides these activities, PSTI-I has been shown to be identical to CCK-releasing peptide, which is also known as a monitor peptide (6, 9). PSTI-I also stimulates

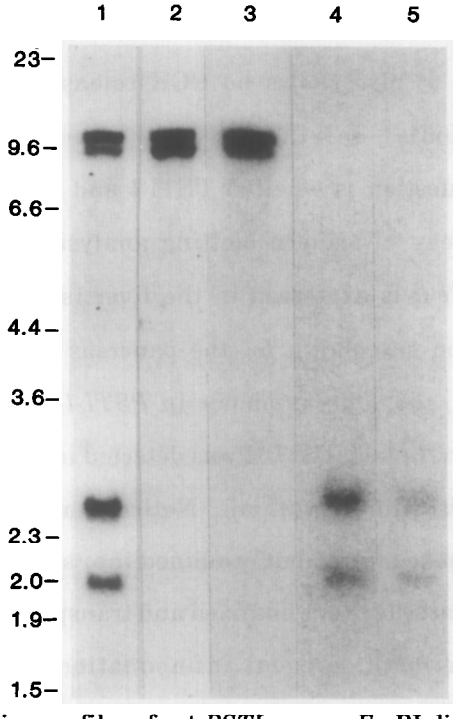

Fig. 4 Southern blotting profiles of rat *PSTI* genes. *Eco*RI digested rat genomic DNA (10μg) was hybridized with probe T (lane 1), probe A (lane 2), probe B (lane 3), probe C (lane 4) and probe D (lane 5) (see Fig. 2A).

Table 1. Summary of the nucleotide sequence homology

Region	Homology	
Total cDNA (-42 - +317)	328/359	91%
5'-noncoding region (-42 - -1)	42/42	100%
Coding region (+1 - +237)	207/237	87%
Region for signal peptide (+1 - +54)	53/54	98%
Region for secreted peptides (+70 - +237)	141/168	84%
3'-noncoding region (+238 - +317)	79/80	99%

thymidine uptake by 3T3 fibroblasts (22) and binds to 3T3 fibroblasts competitively with EGF (23). Human PSTI also can stimulate DNA syntheses in human fibroblasts (24) and is identical to one of the human endothelial cell growth factors derived from hepatoma cells (25), but no CCK-releasing activity has been reported for it. As for PSTI-II, there have been no reports as to its stimulatory activity for growth. It also shows no CCK-releasing activity (K. Miyasaka personal communication).

An interesting question is whether PSTI-I and -II are expressed in organs other than the pancreas. Northern blotting analysis has shown that the gene *PSTI-II*, but not *PSTI-I*, is expressed in the liver (see Fig. 3B), with the same transcription initiation site shown for the pancreas (see Fig. 3C). It is likely, therefore, that in rat, the same promotor in *PSTI-II* gene is used in both the pancreas and the liver. In fact, PSTI-II was detected immunologically in bile juice of rat (K. U. unpublished observation). Notice that in rat a bile duct and a pancreatic duct are not separated but combined into a long hepatopancreatic duct where bile and pancreatic juice are admixed and transported. The PSTI-II which is produced in rat liver might prevent inflammation caused by the reflux of pancreatic juice in the bile duct. Further studies are needed to clarify the functions of these PSTIs.

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