Identification of Novel Pancreas-specific Regulatory Sequences in the Promoter Region of Human Pancreatic Secretory Trypsin Inhibitor Gene*

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The human pancreatic secretory trypsin inhibitor (PSTI) genes introduced into mice are specifically expressed in pancreas. The 1.0 kilobase pairs of PSTI 5'-flanking sequence directed preferential expression of a linked reporter chromomembril acetyltransferase, which was active in a PSTI-expressing pancreatic cell line (AR42j) but not in a PSTI-nonexpressing fibroblast cell line (XC). Two positively acting elements were found, Region I (−161/−116) and Region II (−103/−74), as defined by transfection and binding assays with AR42j cells. Region II is sufficient for the pancreas-specific expression, but the presence of both Regions I and II is needed for the maximum activity. Sequence studies also revealed that these two elements differ from the previously identified recognition sequence for pancreas transcription factor 1 (PTF1). When the same set of experiments was done with XC cells, one negatively acting element was identified, Region IV (−154/−137). Interestingly, Regions I and IV share a core sequence (−149/−139), CAATCAATAAC. These results suggest that this novel element regulates the human PSTI gene expression positively in pancreatic cells but negatively in non-pancreatic cells.

The differentiation and maintenance of phenotype in eukaryotic cells depends on the regulated expression of selected genes, most of which relies on interaction of specific regulatory protein(s) (transcription factor) and its cognate cis-acting DNA elements including a promoter and enhancer(s).

A cis-acting element has been shown to be involved in the control of the expression of pancreas-specific genes, such as mouse α-amylase 2, elastase 2, trypsin, rat elastase I, and chymotrypsin B. This element sequence lies 300 bp upstream from the cap site in the 5'-flanking region of these genes (1–4), has a consensus sequence GXXX(A/C)TGGGAAA, CTX-CAG/G/C/TGTTG/C/A/CTX, and consists of bipartite binding regions (Box A and Box B) separated by one or two DNA turns.

This sequence interacts with a pancreas-specific transcription factor 1 (PTF1) (5), which consists of a 64-kDa protein subunit that binds to Box A and a 48-kDa protein subunit that binds to Box B (6). This dimer complex is further associated with a 75-kDa protein that does not directly bind to DNA (7) but acts in transporting the complex into the nucleus. Besides PTF1, several other trans-acting factors have been discovered, such as exocrine pancreas transcription factor 1 (8), which interacts with the (CACACTG,TTTCCC) motif and Pan/E12, E47, and AP4, which interact with the CAGCTG motif in Box B (9–11). Thus, the expression of pancreas-specific secretory proteins in acinar cells has been thought to employ essentially the same cis-acting regulatory elements.

Pancreatic secretory trypsin inhibitor (PSTI), consisting of 56 amino acids (molecular mass 6.2 kDa) (12), was first isolated from bovine pancreas in 1948 by Kazal et al. (13) and has been thought to be the cognate inactivation factor for preventing intra-pancreatic trypsin activity. In mammals, it is secreted from pancreatic acinar cells into the pancreatic juice, and naturally, this gene expression control was expected to involve the pancreas-specific common cis-acting regulatory sequence. However, the structure of the genomic PSTI gene, as determined by Horii et al. (14), has neither the consensus sequence nor the typical TATA nor CAAT promoter sequence. Therefore, we initiated an analysis of the 5'-sequences flanking the human PSTI gene for identifying the cis-acting elements involved in this pancreas-specific expression through deletion studies and protein binding studies. We show here that the PSTI gene expression control involves two novel 5' cis-acting elements defined by their effect on transcription and their interaction with a protein(s) from pancreatic cells. In addition, competition binding assays showed that one of the core sequences of the two elements is CAATCAATAAC, which is unique and can interact with another protein(s) from nonpancreatic cells; this interaction is likely to repress the expression of PSTI gene in non-pancreatic cells. These results suggest that this unique sequence (CAATCAATAAC) functions as a positively acting element in pancreatic cells and a negatively acting element in nonpancreatic cells in the control of the expression of the human PSTI gene.

EXPERIMENTAL PROCEDURES

Identification of the Integrated PSTI Gene.—A 27-kbp human PSTI genomic DNA that contains 8.3 kbp of the 5'-flanking sequence has been cloned into pWE16 by Horii et al. (14). The DNA for transgenic mice was extracted from this cosmid clone, named cosT4, by NotI and Kpn1 digestion and followed by agarose gel electrophoresis.

Production and Identification of Transgenic Mice.—Human PSTI genomic DNA in cosT4 (a NotI-Kpn1 fragment, see Fig. 1) was microinjected into the male pronucleus of fertilized BDF1 mouse eggs that
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were allowed to develop to term. Mouse pups were screened for the presence of the injected gene by genomic Southern blotting using 5 μg of tail DNA digested with BamHI and hybridization with a 32P-labeled human PSTI cDNA probe (15, 16).

Identification of and Poly(A) RNA and Northern Blot Analysis—Total cellular RNA was extracted from source cells using acid guanidinium thiocyanate/phenol/chloroform (17). Poly(A) + RNA was purified from total RNA by repeated passage through an oligo(dT) cellulose (type 7, Amersham Pharmacia Biotech) column. Total RNA or poly(A) + RNA was denatured by heating at 65 °C for 15 min in 50% (v/v) formamide and resolved by electrophoresis in a 1% agarose gel in 1× TBE buffer.

Cell Lines and Tissue Culture—AR42j is a rat pancreatic exocrine cell line that expresses PSTI (18), and XC (19) and RAT2 (20) are rat fibroblast cell lines that do not express PSTI. These were obtained from Dainippon Pharmaceutical Co. (Suita, Japan). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 500 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37 °C under a 5% CO2 atmosphere. The XC cells were prepared essentially as described by Dignam et al. (26). All steps were performed at 4 °C. The cells (3 to 5 × 106) were collected from monolayer cultures, washed with phosphate-buffered saline, and pelleted by centrifugation for 5 min at 700 × g. The cell pellets were resuspended in washing buffer (10 mM Tris-HCl, pH 7.5, 130 mM NaCl, 5 mM KCl, and 8 mM MgCl2) to a final volume of 5 times that of the packed cells. The cells were precipitated by centrifugation for 5 min at 700 × g and resuspended in hypotonic buffer (20 mM HEPES-KOH, pH 7.9, 5 mM KCl, 0.5 mM MgCl2, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), to a final volume of 3 times that of the original packed cells and were allowed to swell for 10 min on ice. The cells were swelled by hypertonic magerin and disrupted slowly by 20 strokes. The nuclei were collected by centrifugation for 10 min at 700 × g. The nuclear pellets were resuspended in an equal volume of extraction buffer (20 mM HEPES-KOH, pH 7.9, 5% glycerol, 500 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 0.5 μM/ml pepstatin A, and 1.3 μM/ml spermidine), stirred gently for 1 h, then precipitated by centrifugation at 45,000 × g for 30 min in a swing-out rotor. The supernatant was collected as the nuclear extract and dialyzed against binding buffer (20 mM HEPES-KOH, pH 7.9, 0.5 mM EDTA, 100 mM KCl, 10% glycerol, 0.5 mM DTT, and 0.5 mM PMSF) for 12 h. After the pellets were removed by centrifugation at 11,000 × g for 30 min, the supernatant was collected as the nuclear extract, concentrated with polyethylene glycol 20000 to 5 mg/ml, and stored at −80 °C.

Preparation of DNA Probes for Gel Mobility Shift Assay—A probe containing the nucleotides 179 to 1 of the human PSTI gene was excised from PSTI/159/CAT by digestion with HindIII (for a coding strand) or BssHII (a noncoding strand), end-labeled with [α-32P]dCTP by the Klenow fragment, and then digested again with BssHII or HindIII. The single end-labeled DNA probe was run in a 5% polyacrylamide gel, and the band was eluted by ammonium acetate, ethanol precipitated by Maxam and Gilbert (27). The end-labeled fragment (3 fmol; specific activity, 3 × 107 cpm) was incubated with 20 ng of nuclear protein for 30 min at room temperature in a volume of 50 μl in the presence of 12 μM HEPES-KOH, pH 7.9, 60 μM KCl, 0.12 mM EDTA, 1 mM MgCl2, 12% glycerol, 1 mM DTT, and 1 mM PMSF with 1 or 2 μM of poly(dI-dC). The MgCl2 concentration was raised to 3 mM, DNase I was added to a final concentration of 5 μg/ml, and the incubation was stopped at 0 °C with 10 mM HEPES-KOH, pH 7.9, 5 mM NaCl, 1% SDS, 10 mM EDTA, and 25 μg/ml calf thymus DNA. Samples were digested with 50 μg/ml proteinase K for 30 min at 37 °C and extracted with phenol/chloroform; nucleic acids were then precipitated with ethanol.

DNase I-resistant material was dissolved in 6 μl of sequencing dye and analyzed on 6% sequencing gels after heating at 90 °C for 5 min. The dried gel was autoradiographed at −70 °C. Nucleotide sequence markers (G + A) were prepared by the Maxam-Gilbert reaction (27).

Preparation of DNA Probes for Gel Mobility Shift Assay—Wild-type and individual 10-bp substitution mutant probes (161/116) were prepared as follows. Oligonucleotides (33-mer) were synthesized from the 5′-end of coding and noncoding strands, using a DNA synthesizer (Applied Biosystems, Foster, CA). Labeled double-stranded DNA probes were formed by annealing these synthesized oligonucleotides in 10 mM Tris-Cl, pH 7.9, 0.5 mM MgCl2, then filling in the 3′-recessed termini using Sequenase (Sequenase® Ver 2.0, U. S. Biochemical Corp.) in the presence of 1 mM dNTP and [α-32P]dCTP (3000 Ci/mmol). The products were purified by electrophoresis on an 8% polyacrylamide gel. Wild-type and individual 10-bp substitution mutant probes (161/116) were denatured by boiling and annealed on 6% sequencing gels after heating at 90 °C for 5 min. The dried gel was autoradiographed at −70 °C. Nucleotide sequence markers (G + A) were prepared by the Maxam-Gilbert reaction (27).

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This line, we obtained lines 12 and 24 that had integrated truncated sequences, 4.5 and 1.0 kbp respectively, in the 5'-flanking region. The DNA integrated in these animals are shown in Fig. 1B. Aside from the truncation in the distal portion of the 5' region, the other regions of the gene were intact. The human PSTI gene, as examined by Northern blots, was expressed in the pancreas of the three transgenic mouse lines despite the differences in the 5'-flanking region. The 11–4L mice, which had integrated the intact DNA, expressed the PSTI gene at an extremely high level in the pancreas (Fig. 1C). The low level expression detected in the stomach, kidney, and lung reflected that seen in human organs (28) (data not shown). With the mouse lines 12 and 24, the expression of this gene, whose levels were lower than that of 11–4L, was detected only in the pancreas (Fig. 1C and 1D). These data show that the human PSTI gene expression is regulated in the mouse pancreas at the transcriptional level and that its control element(s) is conserved even in the line 24 mouse, which contains only 1.0 kbp of the 5'-flanking sequence hooked to the 7.5 kbp of the coding sequence followed by 1.2 kbp of the 3'-flanking sequence.

The 5'-Flanking Sequence of the Human PSTI Gene Directs the Preferential Expression in the Pancreatic Tumor Cell Line—
The genes of pancreas secretory proteins such as amylase, trypsin, elastase I, and chymotrypsin have a similar cis-acting DNA element in the 5'-flanking region, and this sequence has been known to be responsible for their pancreas-specific expressions (1–4). To see whether the 1.0-kbp 5' sequence of human PSTI gene were able to drive expression of a reporter gene, as shown in the transgenic mouse line 24 (Fig. 1D), a construct of PSTI(1.0)CAT was made in promoterless plasmid pBS0CAT vector with 1.0 kbp of the 5' sequence from genomic fragment of the PSTI gene. The AR42j cell line (a rat pancreatic acinar cell carcinoma) expressing amylase, trypsin, and chymotrypsin genes (29) was confirmed to be able to express RNAs for this line, we observed lines 12 and 24 that had integrated truncated sequences, 4.5 and 1.0 kbp respectively, in the 5'-flanking region. The DNA integrated in these animals are shown in Fig. 1B. Aside from the truncation in the distal portion of the 5' region, the other regions of the gene were intact. The human PSTI gene, as examined by Northern blots, was expressed in the pancreas of the three transgenic mouse lines despite the differences in the 5'-flanking region. The 11–4L mice, which had integrated the intact DNA, expressed the PSTI gene at an extremely high level in the pancreas (Fig. 1C). The low level expression detected in the stomach, kidney, and lung reflected that seen in human organs (28) (data not shown). With the mouse lines 12 and 24, the expression of this gene, whose levels were lower than that of 11–4L, was detected only in the pancreas (Fig. 1C and 1D). These data show that the human PSTI gene expression is regulated in the mouse pancreas at the transcriptional level and that its control element(s) is conserved even in the line 24 mouse, which contains only 1.0 kbp of the 5'-flanking sequence hooked to the 7.5 kbp of the coding sequence followed by 1.2 kbp of the 3'-flanking sequence.

The Human PSTI Gene Is Specifically Expressed in the Pancreas of Transgenic Mice—Transgenic mice were prepared by introducing a human PSTI gene containing the 8.3 kbp of the 5'-flanking sequence (Fig. 1A). One line that contains the full-length of the introduced DNA was named 11–4L. Along with this line, we obtained lines 12 and 24 that had integrated truncated sequences, 4.5 and 1.0 kbp respectively, in the 5'-flanking region. The DNA integrated in these animals are shown in Fig. 1B. Aside from the truncation in the distal portion of the 5' region, the other regions of the gene were intact. The human PSTI gene, as examined by Northern blots, was expressed in the pancreas of the three transgenic mouse lines despite the differences in the 5'-flanking region. The 11–4L mice, which had integrated the intact DNA, expressed the PSTI gene at an extremely high level in the pancreas (Fig. 1C). The low level expression detected in the stomach, kidney, and lung reflected that seen in human organs (28) (data not shown). With the mouse lines 12 and 24, the expression of this gene, whose levels were lower than that of 11–4L, was detected only in the pancreas (Fig. 1C and 1D). These data show that the human PSTI gene expression is regulated in the mouse pancreas at the transcriptional level and that its control element(s) is conserved even in the line 24 mouse, which contains only 1.0 kbp of the 5'-flanking sequence hooked to the 7.5 kbp of the coding sequence followed by 1.2 kbp of the 3'-flanking sequence.

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FIG. 2. CAT assays with deletion mutants with progressive loss of the 5' border. Serial deletion mutants containing various 5'-flanking sequences of PSTI gene with the 5' border deletion ends at −320, −245, −179, −159, −141, −134, −115, −98, −77, −34, and 0 bp were transfected into the PSTI-expressing AR42j cells and the nonexpressing XC cells by lipofection (24), and CAT activities were measured. Each activity was normalized by β-galactosidase activity (25) and is shown as the fold increase against that of the vector.

The 5'-Sequence Contains the Cell Specific Cis-acting Elements—To examine the functionally significant sequences in the 5'-flanking region of the human PSTI gene, we constructed a set of deletion derivatives from PSTI(1.0)CAT by stepwise elimination of the 5' border and transfected them into AR42j cells. The results are shown in Fig. 2. PSTI(320)CAT, which possesses 320 bp of the 5'-flanking sequence, showed activity identical to that of the starting plasmid (data not shown), demonstrating that the specific regulatory element(s) must be located downstream of the −320 site. Deletions extending to −159 showed no measurable effect (activity of around 80-fold that of the vector plasmid), whereas deletions that extended to −141 and −134 resulted in a significant reduction of activity. Thus, the 5' border of the positively acting cis-element lies between −159 and −141. Upon further deletion to −115, the activity was restored (activity of about 70-fold), but this augmenting activity was negated when a region covering between −134 and −115 was added or a deletion extended further to −98. However, some weak activities (around 10-fold), which seemed to be basal level activities, remained. These observations suggest that there is a negatively acting element within −134 and −115 and another positively acting element downstream of −115, the effect of which is eliminated with the −98 construct. Deletion to −77 resulted in a further loss of activity from 10- to 3-fold, and with the deletion extended to −34, the activity was completely eliminated. Thus, the loss of activities with −77 and −34 constructs can be regarded as the loss of an element regulating basal level activity and the promoter for transcription initiation, respectively, because the human PSTI gene lacks a CAAT box or TATA box and has multiple transcription start points (14), but the major site for transcription is at −60 (16). Altogether, these results suggest that the 5’ sequence downstream of −179 is crucial for human PSTI gene expression in AR42j cells and contains an upstream positively acting element followed (or overlapped) by a negatively acting element and then a downstream positively acting element and a basal activity-regulating element just in front of the promoter for transcription initiation.

Multiple Sites for Protein Interaction in the 5'-Flanking Region of the Human PSTI Gene—To examine trans-acting factors in the 5’-region of the human PSTI CAT genes, we carried out in vitro DNase I footprinting assays using nuclear extracts from the PSTI-expressing AR42j cells or nonexpressing XC cells. The assays were done with both coding and noncoding strands. With the coding strand, three protected regions appeared in a dose-dependent fashion in AR42j nuclear extracts (Fig. 3A) and one protected region appeared in XC nuclear extracts (Fig. 3B): Region I (−161/−116), Region II (−103/−74), Region III (−65/−42) in AR42j, and Region IV (−154/−137) in XC. With the noncoding strand, the binding profile was similar with Regions I and IV but weak with Region II and none with Region III (Fig. 3B). Comparing the protection profiles of AR42j and XC, both Regions I and II are AR42j-specific, and Region IV is XC-specific, because they show the cell-type-specific protection. Region III is also AR42j-specific but is likely to be the promoter region, judging from the results of the transfection assays and the transcription start point of the PSTI gene.

Region I is divided into two subregions, Region Ia (−161/−136) and Region In (−131/−116), separated by a 5-bp nonprotective gap sequence. The left end of Region Ia (−161) is almost identical to the 5’ border of the upstream positively acting element (−159/−141) in Fig. 2, and the left end of Region In (−131) lies within the −134 to −115 region, where the negatively acting element is located. The left end of Region II (−103) lies within the −115 to −98 region, where the 5’ border of the downstream positively acting element exists.

To examine the roles of Regions I and II, we checked the influences of the presence or absence of Regions I and/or II on CAT activities in AR42j cells (Fig. 4). PSTI(159)CAT that contains both Regions I and II was used as a reference; although its 5’ region is 2 bp inside Region I, this did not affect the results. PSTI(115)CAT without Region I showed about half of the activity. PSTIΔel(104-78)CAT without Region II showed only marginal activity, although it retained Region I. PSTI(77)CAT without Regions I and II showed no activity at all. Thus, Region II is sufficient for the pancreas-specific expression of the PSTI gene, but for the maximum activity, the presence of the Region I is needed. Region I in combination with the promoter is inactive.

To check the interaction of Regions I and II, the deletion set was then used for DNase I footprinting assays (Fig. 5). Deletions from −179 to −134 did not affect the protections. However, deletion extending to −115, completely eliminating Region I, weakened the protection of the 5’ portion (−103/−95) of Region II. These results suggest, although they do not prove, that the binding proteins for Regions I and II are likely to have some mutual influence. But they also indicate that Region II seems to consist of two subregions, Region IIa (−103/−95) and Region IIb (−94/−74). Thus, the 5’ border of the downstream positively acting element is found to be involved in the Region IIa, and also the left end of Region IIb lies within the −98 to −77 region, where there is the 5’ border of the basal activity-regulating element.

Region IV protection (−154/−137) was observed with coding and noncoding strands and was found to be specific to the PSTI-nonexpressing XC cell nuclear extract. Region IV seemed to be overlapping with the negatively acting element. In accordance with this idea, the transfection assays in XC cells using the same set of deletion mutants (Fig. 2) showed essen-
ments were labeled with $^{32}$P at the 3’-end of coding (A) and at the 5’-end of noncoding (B) strands, respectively, and were incubated in the presence of increasing concentrations (0, 5, 10, 20 μg) of AR42j nuclear extracts or 20 μg of XC nuclear extract. Protein-DNA complexes were partially digested with DNase I, and the digestion products were separated by electrophoresis in a 6% sequencing gel. Maxam and Gilbert (27) G + A chemical cleavage reactions served as size markers. DNA regions that were protected by nuclear protein(s) are indicated by open boxes on the right side of each autoradiograph with nucleotide positions representing the footprint boundaries and have been termed Regions I, II, III, and IV. Region I can be divided into two subregions (termed Ia and Ib) as described in the text.

Fig. 3. DNase I footprinting analysis of the 5’-flanking region of the human PSTI gene. The −179/−1 fragments were labeled with $^{32}$P at the 3’-end of coding (A) and at the 5’-end of noncoding (B) strands, respectively, and were incubated in the presence of increasing concentrations (0, 5, 10, 20 μg) of AR42j nuclear extracts or 20 μg of XC nuclear extract. Protein-DNA complexes were partially digested with DNase I, and the digestion products were separated by electrophoresis in a 6% sequencing gel. Maxam and Gilbert (27) G + A chemical cleavage reactions served as size markers. DNA regions that were protected by nuclear protein(s) are indicated by open boxes on the right side of each autoradiograph with nucleotide positions representing the footprint boundaries and have been termed Regions I, II, III, and IV. Region I can be divided into two subregions (termed Ia and Ib) as described in the text.

Fig. 4. Effects of deleting Region I and/or Region II on CAT activity. PSTII(159)CAT contains both Regions I and II. PSTII(115)CAT and PSTIdel(104–78)CAT contain Region II and Region I, respectively. PSTII(77)CAT contains neither. These plasmids were transfected into AR42j cells, and the CAT activities were measured as in Fig. 2.

The Binding Sequences Are Different from That for PTF1—To further examine the nature of the footprint sequences, we performed gel mobility shift and competition assays using $^{32}$P end-labeled synthetic oligonucleotides that correspond to Region I (−161/−116) or Region II (−103/−74). The Regions I and II oligonucleotides yielded two and three complexes in the binding reaction with and AR42j nuclear extracts, respectively (A1 and A2 for Region I and A3, A4, and A5 for Region II) (Fig. 6). Among these, the A1, A3, and A4 complexes were affected in the presence of excess unlabeled homologous oligonucleotides, resulting in a disappearance of A3 complexes and a dose-dependent inhibition of the formation of the A1 and A4 complexes, whereas the A2 and A5 complexes showed no significant change. A similar set of binding reactions with XC nuclear extracts and Region IV oligonucleotides was performed. Four complexes were yielded, X1-X4 (right panel of Fig. 6), none of which matched A1-A3 complexes, and which are likely to involve different protein factors from those of AR42j nuclear extracts. The X1, X2, and X3 complexes were competed with excess unlabeled homologous oligonucleotides, whereas X4 complex did not. Furthermore, XC nuclear extracts formed three complexes in the binding reaction with Region II oligonucleotides, although protein binding in Region II was not detected in footprinting assay (Fig. 5). These are indistinguishable from A2-A5 complexes with AR42j nuclear extracts, the data are omitted from Fig. 6 for clarity. The binding proteins for Region II are likely to be cell-type-nonspecific proteins and to be repressed in the footprinting assay with the XC nuclear extracts. Thus, A4 for Region I, A5, and A6 for Region II, and X4, X5, and X6 for Region IV are the sequence-specific protein-binding complexes, whereas A2, A3, and X3 are not.

To analyze whether these sequence-specific-binding proteins involve the previously identified PTF1 (5), which is the major pancreas-specific transcription factor, gel mobility shift assays were performed in the presence of the PTF1 binding sequences for mouse α-amylase (−158/−122) and trypsin (−128/−96) genes. The PTF1 oligonucleotides formed two complexes in AR42j nuclear extracts as reported by Cockell et al. (5) (Fig. 7A). However, the A1, A2, A3, and X1-X3 complexes were different in mobilities from those PTF1 complexes and did not compete with a 200-fold molar excess of the PTF1 oligonucleotides (Fig. 7B). These results demonstrate that PTF1 is not the factor involved in the binding with Regions I, II, and IV of the PSTI gene.

The Core Binding Sequence CAATCAATAAC Plays the Most Important Role in the Cell-specific Expression of Human PSTI Gene—To further analyze the recognition sequences in Regions I and II, we synthesized a set of mutant competitor oligonucleotides (mut probes) in which a stretch of 10-bp sequence was substituted by a target sequence for XbaI or EcoRV (Fig. 8A). The results are demonstrated in Fig. 8B. The binding assays with AR42j nuclear extracts showed that the formation of A1 complex was inhibited with three of the mutant competitors and not with mut(148−139). The formation of A2 and A4 complexes was competed with one of the mutant competitors, but...
double-stranded oligonucleotides are shown. separated by electrophoresis in 4% polyacrylamide gels and autoradiographed. In each or XC. In the assays for Region IV, we used Region I oligonucleotide, within which Region IV is present. The DNA-protein complexes were incubated in the presence of 10
strands were end-labeled probe is very close to the binding site. These probes, whose coding
9
5
script II plasmid sequences linked to the 5
and PSTI(115)CAT, respectively. Each excised probe has 41 bp of Blue-
Sac excising the corresponding regions using the
The probes were prepared by
* the human PSTI 5
protection regions are shown to the right of the autoradiograph; open
and shaded boxes
Nucleotide positions at footprint boundaries are noted. Bold bars
subregions I A (2
136), I B (2
115) contains no Region I. Lane (2
2
131/2)
and is likely to function as a positively and negatively acting
importance in both PSTI-expressing and nonexpressing cells
sequence CAATCAATAAC (Fig. 8
2
139 containing this core sequence has the characteristic se-
sequence in Region I in the AR42j nuclear extract, although the
binding proteins are different. The region between −149 and −139 containing this core sequence has the characteristic se-
CAATCAATAAC (Fig. 8C). This unique sequence is important in both PSTI-expressing and nonexpressing cells and is likely to function as a positively and negatively acting element in AR42j and XC cells, respectively.

FIG. 5. DNase I footprinting analysis of a set of 5′ deletions in the human PSTI 5′-flanking regions. The probes were prepared by excising the corresponding regions using the SacI and HindIII sites from PSTI(179)CAT, PSTI(159)CAT, PSTI(141)CAT, PSTI(134)CAT, and PSTI(115)CAT, respectively. Each excised probe has 41 bp of Blue-
HindIII site by the Klenow fragment, were
partial digestion with DNase I and electrophoresed on 6% sequencing
gels. The probes (−179) and (−159) contain Region I, (−141) and (−134) contain only Region Ia, and (−115) contains no Region I. Lane (−
shows the (−179) probe DNA without AR42j nuclear extract. DNase I protection regions are shown to the right of the autoradiograph; open and shaded boxes indicate strong and weak protections, respectively. Nucleotide positions at footprint boundaries are noted. Bold bars indicate the common 41 bp of Bluescript II plasmid vector sequences that all of the probes have in common at the 5′-end. Regions I and II and their subregions Ia (−161/−136), Ib (−131/−116), Ia (−103/−95), and Ib (−94/−74) described in the text are shown to the right.

FIG. 6. Competition analyses for sequence-specific binding of proteins in AR42j or XC nuclear extract to oligonucleotides corresponding to Region I or Region II. Samples (0.1 ng; 15,000 cpm) of a 32P end-labeled double-stranded oligonucleotide corresponding to Region I or II were mixed with various amounts of cold competitor oligonucleotides and incubated with 10 μg of crude nuclear extracts from AR42j or XC. In the assays for Region IV, we used Region I oligonucleotide, within which Region IV is present. The DNA-protein complexes were separated by electrophoresis in 4% polyacrylamide gels and autoradiographed. In each panel, the amount (in fold molar excess) of cold homologous double-stranded oligonucleotides are shown. Lane N.E. (−) indicates no nuclear extract reaction, and lane N.S. shows the reaction in the presence of a 200-fold molar excess of cold nonspecific double-stranded oligonucleotides corresponding to Region III (−65/−42). Positions of protein-DNA complexes are indicated as A1, A2, etc. For explanations, see text. Open triangles indicate unbound DNA.

The present study revealed that the PSTI gene, which is expressed selectively in the pancreas, involves a novel recognition sequence, CAATCAATAAC, and possibly novel proteins in the regulation of its expression. The role of the PSTI gene is to inhibit the activity of trypsin when the activation of trypsinogen occurs in the pancreatic duct. Thus, to protect the pancreas from the risk of autodigestion, it may be important to have a system in which excess PSTI is expressed and secreted to the pancreatic duct, especially because the PSTI is not a very potent inhibitor of trypsin (30). It is not surprising, therefore, that the regulation of PSTI gene expression is different from that of pancreatic exocrine enzymes involving trypsin.

Analyses of transgenic mice that have integrated the human PSTI gene showed that the expression of human PSTI gene is maintained in the pancreas of transgenic mice that contains the 5′-truncated flanking sequence. The relevant cis-acting control element was shown to reside in the 1.0-kbp genomic

the mut(93–84) and mut(83–74) did not show the competition. Thus, the most important recognition sequences, as reflected by these noncompeting oligonucleotides, lie in the region between −148 and −139 in Region I, which is involved in Region Ia (−161/−136), and the region between −93 and −74 in Region II, which is identical to the Region IIA (−93/−74) (Fig. 8C). In these studies, a recognition sequence was not detected in Regions Ib and IIA, because these protections were weaker than those of Regions Ia and IIB in the DNase I footprinting assay.

In the binding studies using XC nuclear extracts, the formation of X1, X2, and X3 complexes were competed weakly with mut(158–149) and completely with mut(138–129) and mut(128–119), but the mut(148–139) did not show competing ability. The competition of complex formation in Region II is the same as that in AR42j. Thus, the sequence between −148 and −139 is likely the most important target in Region IV. Interestingly, this core sequence overlaps the crucial binding sequence in Region I in the AR42j nuclear extract, although the binding proteins are different. The region between −149 and −139 containing this core sequence has the characteristic sequence CAATCAATAAC (Fig. 8C). This unique sequence is important in both PSTI-expressing and nonexpressing cells and is likely to function as a positively and negatively acting element in AR42j and XC cells, respectively.

DISCUSSION

The present study revealed that the PSTI gene, which is expressed selectively in the pancreas, involves a novel recognition sequence, CAATCAATAAC, and possibly novel proteins in the regulation of its expression. The role of the PSTI gene is to inhibit the activity of trypsin when the activation of trypsinogen occurs in the pancreatic duct. Thus, to protect the pancreas from the risk of autodigestion, it may be important to have a system in which excess PSTI is expressed and secreted to the pancreatic duct, especially because the PSTI is not a very potent inhibitor of trypsin (30). It is not surprising, therefore, that the regulation of PSTI gene expression is different from that of pancreatic exocrine enzymes involving trypsin.

Analyses of transgenic mice that have integrated the human PSTI gene showed that the expression of human PSTI gene is maintained in the pancreas of transgenic mice that contains the 5′-truncated flanking sequence. The relevant cis-acting control element was shown to reside in the 1.0-kbp genomic
region 5’ to the transcription initiation site. The conclusion obtained with transgenic animals has been supported by an independent system using CAT assay system with a rat pancreas-derived cell line (AR42j) but not with fibroblast-derived cell lines. The cis-acting control elements are located in a relatively short region immediately upstream of the PSTI gene, consistent with the other known pancreas-specific genes (5, 8–11).

The present detailed analyses demonstrated that the control region consists of Region I (−161/−116), Region II (−103/−74), Region III (−65/−42), and Region IV (−154/−137) and further-
more, that Regions I and II are composed of two subregions, a positively active Region IA (−161/−136) and a negatively active Region IB (−131/−116), and a positively active Region II A (−103/−95) and a basal activity-regulating Region II B (−93/−74), respectively, as summarized in Fig. 9. Region III is the promoter region, because the major transcription start point of the human PSTI gene is at −60 bp. Transfection assays revealed that Region II is an indispensable element responsible for the pancreatic cell-specific expression of the human PSTI gene, whereas Region I, especially Region IA, is an element augmenting the promoter activity only in combination with Region II. So both Regions I and II are needed to show the maximal activation. A similar additive effect has been observed with immunoglobulin heavy chain enhancers that contain four motifs (31) as well as with the simian virus 40 enhancer that contains three segments (32). This functional additivity may involve an interaction of the multiple elements with a cognate protein(s), culminating in the production of an active enhancer, as discussed by Schaffner et al. (33).

The gel mobility shift competition assays using AR42j nuclear extracts showed that several nuclear factors bind to these elements. In Region I, the A4 complex, which binds with positively active Region IA, was revealed, but a binding protein for negatively active Region IB could not be detected (Figs. 8 and 9). The A4 complex was expected to involve the positive transcription factor, for example PTF1, similar to other pancreatic genes. However, this complex was revealed not to involve PTF1 by following three points. 1) The mobility of this protein is different from that of PTF1, 2) this protein does not compete with PTF1 for the recognition sequence, and 3) the core binding sequence is CAATCAATAAC (−149/−74), which is different from PTF1 binding sequence (Figs. 7 and 8). The A4 complex was revealed not to involve PTF1 by following three points. 1) The mobility of this protein is different from that of PTF1, 2) this protein does not compete with PTF1 for the recognition sequence, and 3) the core binding sequence is CAATCAATAAC (−149/−74), which is different from PTF1 binding sequence (Figs. 7 and 8). We do not know at present the nature of the protein involved in the formation of A4 complex. There is a possibility that a novel transcription factor may participate. However, because the core recognition sequence is related to CCAAT, which is recognized by C/EBP, NF1, CP1, and CP2 or eCBF, the possibility of one or several of these factors being involved cannot be ruled out.

Region II is the element that is sufficient for pancreas-specific expression of the human PSTI gene. The gel mobility shift competition assays revealed two complexes, A3 and A4, which bind with Region III B but could not show that there was a binding factor for positively acting Region IA (Figs. 8 and 9). The A3 and A4 complexes do not involve PTF1 (Fig. 7) and may be the same as two sequence-specific complexes formed by the binding with Region II and XC nuclear extract, judging from the mobility, affinity, and core binding sequence of the complexes. Therefore, these two complexes are likely to contain constitutive factors that are needed for basal level activation, for example Sp1 protein, because the core sequence in Region II carries the GACCC motif (−83/−79), which is related to the CACCC motif for Sp1 binding (34, 35). However, the question of whether the Sp1 protein is involved awaits further analyses.

With the extract of PSTI nonexpressing XC cells, we noted a protein binding sequence lying between −154 bp and −137 bp (Region IV). The transfection assays in XC cells showed no activity with deletion mutants containing intact Region IV, but deletion extended from −159 bp to −141 bp, impairing Region IV, resulted in the appearance of low level activity (around 10-fold), which was similar to the basal activity that PSTI(98)CAT in AR42j cells showed (Fig. 2). This finding raises the possibility that Region IV may play a role in a negatively regulating process of the expression of PSTI gene in nonpancreatic cells. The gel mobility shift assays using XC nuclear extracts showed that Region IV yields complexes X1, X2, and X3 (Fig. 6), which are likely to involve the negative factors, although such factors have never been detected so far in other pancreatic genes. The core sequence of Region IV binding complexes is the sequence CAATCAATAAC (−149/−139), which is superimposable to that of A1 complex in a positively active Region IA (Figs. 8 and 9). Positive and negative regulations for tissue-specific gene expression have been reported in several cases, including albumin (36), β-globin of chick (37), pancreatitis-associated protein I (PAP I) of rat (38), p12 of mouse (39), and MUC1 of humans (40); however, none of these carries a shared recognition sequence(s). This sequence CAATCAATAAC that we revealed in the promoter region of human PSTI gene is likely to function to activate PSTI gene expression in pancreatic cells by the interaction of the binding factors for Regions I and II and to repress the same expression in nonpancreatic fibroblast cells by the interaction of binding factors for Region IV, refining the pattern of the pancreas-specific expression. The sequence CAATCAATAAC is a novel element, whose function may alter positively and negatively depending on cell type. The analysis of the putative positively acting and negatively acting factor(s) participating in the human PSTI gene expression is of utmost importance for future study.
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