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Isolation of the murine ribonuclease gene *Rib-1*: structure and tissue specific expression in pancreas and parotid gland

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ABSTRACT

The mouse pancreatic ribonuclease gene *Rib-1* was isolated from a library of mouse genomic DNA and sequenced. This small gene contains a nontranslated exon of 52 base pairs, an intron of 791 base pairs, and a coding exon of 741 base pairs. *Rib-1* transcripts were detected in parotid gland as well as in pancreas. The abundance of the transcripts were approximately 200-fold greater in pancreatic RNA than in parotid RNA. The sites of transcription initiation were mapped by primer extension and ribonuclease protection assays. One major initiation site and several minor initiation sites were identified in pancreatic RNA. Transcription in parotid appears to be initiated from the same sites. Parotid-specific transcripts were not detected. The data suggest that *Rib-1* is transcribed in pancreas and parotid from the same promoter. This is in contrast with the mechanism for production of amylase in pancreas and parotid, which is accomplished by tissue specific expression of different gene copies.

INTRODUCTION

Ribonuclease is one of the digestive enzymes produced and secreted by the exocrine pancreas. Because of its abundance, small size, and stability, ribonuclease has been the subject of intensive investigation. The amino acid sequence of the protein from a variety of species has been determined (1, 2). Large quantities of pancreatic ribonuclease are produced by rodents and by species with ruminant-like digestion.

Ribonuclease and several other pancreatic digestive enzymes have a second site of expression in the parotid salivary gland. Ribonuclease activity has been identified in parotid of several species including cow (3), rat (4), and human (5). Other digestive enzymes expressed in both tissues include amylase (6), deoxyribonuclease (7, 8), phospholipase A_2 (9, 10), and kallikrein (11). An evolutionary relationship between parotid and pancreas is suggested by their structural and functional similarities, in addition to their production of related gene products. From an evolutionary perspective, the origin of salivary glands in amphibian species occurred subsequent to the development of the acinar pancreas in fish (12, 13). One approach to understanding the mechanism of this apparent 'organ duplication' is molecular characterization of tissue specific expression of enzymes which are produced in both organs.

The production of amylase in pancreas and parotid gland has been well characterized. Distinct members of the amylase multigene family have diverged with regard to promoter structure and tissue specific regulation (14–16). The basis for dual expression of other digestive enzymes in pancreas and parotid has not been determined. The present study was undertaken to determine the molecular basis for ribonuclease expression in the two tissues.

We previously reported the cloning of a mouse ribonuclease cDNA and assignment of the *Rib-1* gene to mouse chromosome 14 (17). The cDNA hybridized with a single restriction fragment from mouse genomic DNA, indicating that *Rib-1* is not a member of a multigene family. In this report we describe the cloning and sequencing of the *Rib-1* gene and characterize its expression in pancreas and parotid gland.

MATERIALS AND METHODS

Isolation of Ribonuclease Clones

Genomic DNA from inbred strain C3H/HeJ was cloned in the lambda vector EMBL3 (18). This library was screened with the insert from the mouse ribonuclease cDNA clone pMPR1 (17). Two recombinant phage with overlapping restriction maps, λ17 and λ18, were isolated.

Sequence Analysis

Restriction fragments were isolated from λ17 and subcloned into pGEM vectors (Promega). Double-stranded plasmid DNA was sequenced with the Sequenase kit (U.S. Biochemicals, version 2.0) or the Gem Seq K/RT system (Promega). Identification of

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DNA consensus elements was carried out with Pustell Sequence Analysis software (International Biotechnology Inc., version 2.02). The *Rib-1* genomic sequence has been submitted to the EMBL data library with accession number X60103.

Primer Extension Analysis

A synthetic 17 nucleotide antisense primer (12.5 pmol) (Figure 2, nucleotides +850 to +866) was annealed to total pancreatic RNA (25 μg) by heating to 80°C for 5 minutes followed by slowly cooling to 50°C in a volume of 12.5 μl containing 20 mM Tris (pH 8.0), 300 mM KCl, and 0.2 mM EDTA. Sequencing primer extension reactions were carried out with AMV reverse transcriptase (0.3 units/μl) in extension buffer (10 mM Tris-HCl pH 8.0, 150 mM KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 10 mM DTT) containing dATP (500 μM), dTTP (500 μM), dCTP (500 μM), [α-^{32}P]dGTP (0.5 μM; 3000 Ci/mM, Amersham), and either dideoxy-ATP (500 μM), dideoxy-TTP (500 μM), dideoxy-CTP (500 μM) or dideoxy-GTP (1 μM). After incubation at 45°C for 10 min, dGTP was added to a final concentration of 500 μM, and the samples were incubated an additional 10 min. Reactions were stopped with sequencing load buffer, heated to 95°C, and loaded directly onto a denaturing gel containing 6% acrylamide and 8 M urea.

The method described by Sambrook *et al.* (19) was used to compare full length primer extension products in pancreatic and parotid. The 17 nucleotide primer described above was end-labelled with T4 DNA kinase in the presence of [γ-^{32}P]ATP. Labelled primer (2×10^5 cpm) was annealed overnight at room temperature with 1 to 5 μg of total RNA from pancreas or 10 μg of poly A$^+$ RNA from parotid. Extension reactions were performed with AMV reverse transcriptase at 37°C for 2 h.

RNA Isolation and RNase Protection Assays

Total cellular RNA was isolated from tissues using a modification of the guanidine thiocyanate homogenization-cesium chloride centrifugation method (20,21) as previously described (22). RNA was quantitated by absorbance at 260 nm. The integrity of RNA samples was evaluated by ethidium bromide staining of ribosomal RNA after agarose gel electrophoresis. Parotid polyA$^+$ RNA was isolated by a single round of purification over an oligo(dT)-cellulose column as described in Sambrook *et al.* (19).

For RNase protection assays, a 1.1 kb *Pst* I fragment containing the first exon of *Rib-1* (Figure 1) was subcloned into pGEM1. An antisense RNA probe was generated with [α-^{32}P]UTP (800 Ci/mM, Amersham) and T7 polymerase, and assays were carried out as previously described (22). Protected fragments were analyzed by electrophoresis through a denaturing gel containing 6% acrylamide and 8 M urea.

Northern Analysis

Total pancreatic RNA (0.25 μg) and poly A$^+$ parotid RNA (5 μg) were fractionated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose filters (19). The *Rib-1* cDNA clone pMPR-1 (17) was labelled by the random oligonucleotide primed method (23) and hybridized to the filter at 50°C in a 50% formamide buffer (24) without bovine serum albumin.

Affinity Purification of PTF1 and Gel Mobility Shift Experiments

Nuclei were isolated from the pancreatic acinar cell line AR42J (25) and protein was extracted by the method of Dignam *et al.* (26). An affinity column was prepared using the method of Chodosh *et al.* (27) for the isolation of the pancreatic nuclear protein PTF1. A 31 bp double-stranded oligomer containing the PTF1 binding site of the rat elastase 1 gene (nucleotides −122 to −92 from reference 28) was biotinylated at one end by filling in a three nucleotide single stranded terminus with the Klenow fragment of DNA polymerase in the presence of 20 μM biotin-7 dATP (Bethesda Research Laboratories). Streptavidin agarose was saturated with the biotinylated oligomer and equilibrated with binding buffer containing 10 mM Hepes, pH 7.9, 1 mM EGTA, 20% glycerol, 1 mM DTT, 1 mM PMSF, 100 mM KCl, 250 μg/ml bovine serum albumin, and 50 μg/ml poly(dI:dC). Nuclear proteins were incubated with the matrix at 4°C for 30 minutes in binding buffer. PTF1 was eluted with 1.0 M KCl. PTF1 activity was defined by its binding activity in a gel shift assay with labelled probes containing amylase and elastase consensus sequences, as previously described (29).

Fragments containing the *Rib-1* consensus elements PTF1-A and PTF1-B were amplified by polymerase chain reaction using 22 nucleotide primers under standard conditions. The sequence −221 to −121, containing PTF1-A, was amplified with the coding strand primer (5′)CATTGTCCCTGTGGCTAGCTCT and the noncoding strand primer (5′)TGGAAAGGAAATCCCC-AGAAGG. The sequence +196 to +288, containing PTF1-B, was amplified with the coding strand primer (5′)TAAAGAA-CGAGTGGGTCGGGGA and the noncoding strand primer (5′)CTGGCAGTATACAGGGTTTGGG. Full length DNA fragments were gel purified and radiolabelled with [γ-^{32}P]ATP using T4 DNA kinase. The gel purified fragments were quantitated by ethidium bromide staining and by absorbance at 260 nm.

RESULTS

Rib-1 clones were isolated by screening a mouse genomic library with the pancreatic ribonuclease cDNA probe pMPR1, as described in Methods. Southern blot analysis of DNA from clone λ17 with the cDNA probe identified two hybridizing *Bam* HI fragments of 2.9 kb and 3.7 kb. Restriction fragments containing the gene were subcloned and sequenced by the strategy indicated in Figure 1. The resulting sequence (Figure 2) contains one long open reading frame whose deduced amino acid sequence is in agreement with the published sequence of the mouse pancreatic ribonuclease protein (30). The gene includes a 25 residue N-terminal putative signal peptide not found in the mature protein.

The coding region is contained in a single exon. The presence of a consensus splice acceptor sequence 24 base pairs upstream

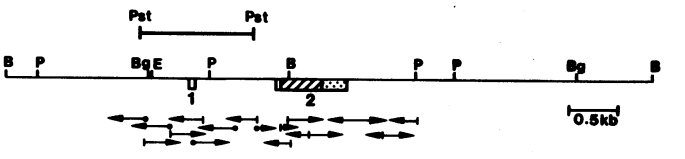

Figure 1. Mouse ribonuclease gene structure. The restriction map of the region surrounding the ribonuclease gene in clone λ17 and the sequencing strategy are shown. Arrows indicate the direction and extent of sequencing. Sequencing primers corresponding to ribonuclease sequences are represented by closed circles. The 1.1 kb *Pst* I fragment used as a probe in RNase protection assays is indicated. Open boxes, 5′ untranslated sequence; striped box, coding sequence; stippled box, 3′ untranslated sequence. B, *Bam* HI; P, *Pvu* II; Bg, *Bgl* II; E, *Eco* RI.

from the ATG start codon suggested that an intron may interrupt the 5′ untranslated portion of the gene.

Identification of Transcription Start Sites by Primer Extension

Primer extension analysis of pancreatic RNA was carried out with a 17 nucleotide primer complementary to the sequence adjacent to the proposed splice acceptor site (Figure 2, overlined). Several primer extension products were observed, ranging from 71 to 77 nucleotides in length, with a predominant product of 75 nucleotides (Figure 3, lane 1). Chain terminating dideoxynucleotides were added to the reverse transcription reactions to determine the sequence of the 5′ end of the *Rib-1* pancreatic transcript (Figure 3, lanes 2–5). The sequence matched a genomic region 791 base pairs further upstream, identifying the location of the first exon. Thirty-nine of 51 nucleotides correspond to the sequence of the genomic clone, which is shown at the right in Figure 3; the other eleven nucleotides could not be assigned. The major primer extension product of 75 nucleotides is marked on the genomic sequence by the arrow in Figure 3.

The sequences of the proposed intron/exon borders are in good agreement with consensus splice site sequences (31). Comparison of the *Rib-1* sequence with the sequence of a recently reported mouse pancreatic ribonuclease cDNA clone (32) confirms the intron placement and identification of the first exon. The *Rib-1* exonic sequence is identical to the available cDNA sequence (32).

The length of the gene is 1589 bp from the major pancreatic start site to the polyadenylation site. It includes a nontranslated exon of 52 bp, an intron of 791 bp, and a coding exon of 741 bp. The length of the predicted mRNA (excluding polyA tail) is 793 nucleotides, with 76 nucleotides of 5′ untranslated

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-803  TTTTTTTTTT TAATTTTTTT CTTTTTCAGA TTTATTTATT TATTTTATGT ATATGAGTAC
-743  ACTGTCACTG TCCTTAGACA CACACCAGAA GCCACCATGA GGTTGCTGGG AATTGAACTC
-683  GGGACCTCTG GAAGAGTAGT CTGTGCTCTT AACTACTGAG CCATCTCTCC AGCCCCTTCA
-623  CTGGACTCTC TTTACTCCCT TTCCCAATTA GCCACATTGA ATGCACTGTA TTTTTCCACT
-563  TTTTTGATAT TATTTGTCGA TAATTGGTCT ATTGAGGATG AGTGGCTGAG TTTGGCTTGG
-503  CAAGTCTGCC ACAACCCAGA CTTTCACCCT ACCAACTCCA CTAGTAGGGA ACCCTTCAAG
-443  CTGCAGACAG GACGGCCTCT TATTTTCTCC CCAAGAGAAG CACGGCTGGG AGATCTGGAA
-383  TTCTGACTAC CATCAATTTG ATTCAAATAG AGAAGGAGGG CTTCCCTCTT CCATGATTCC
-323  CAGGATCTCT GTGTGTCCTT TGTTAACCTC CAGGTTGTAT ATGATGCAAA CAGCATGCTC
-263  ATAAATTACA AGGCTTTGTG AAAGACTGTG AAGTGTATGT GCCATTGTCC CTGTGGCTAG
-203  CTCTGCTGCC CCTCTGGGAAT CAGCACAGGA GGCACAAAAG GTGTTTTCTC TTTCCTGGCA
-143  GCCTTCTGGG GATTTCCTTT CCAAGGTTTC AATGCCCTTG TTTTTATTAT TCAGATAAGA
 -83  TTGACCCAGA TTTACTCCGC CCATTTTTTC TTAGAAGCCA ACCCCTTGGA ATCCTTTTAA
 -23  ACTCCACATC ACAGAGACTG TGTCACTGAA GAAACTGACT TCCAATTCGT CTTGGAGTTC
 +38  AAAGTAAAAT TTTAGGTAAG TGGCCTCAGT AACTATTTCT GCTTGAGAGT TAGAACTGAG
 +98  AGAGTCCAGG CCAGGCACTC TTGACTGACA CCAACTCCAG AAAAGCTGAG CCCAGCAAAC
+158  AGCTCGAGGC AGGGGTGGAG GCATGGCACA GTCAGATATA AACAACGAGT GGGTCGGGGA
+218  GGATGGAGCT CCCAAGCCTGG GGAAGGGAGC ACGTGTCTTT AGACTAAAAC CCAAACCCTG
+278  TATACTGCCA GAGTCAGGCA CTAGACCAAG TTCCCCTATG TCATAAGATA ATGAGACTAC
+338  AGCCACAGTC CTGGATGTCA GTAAAGACCC AGAAATACAA CTTAGCTTAA GGCGTCTTCC
+398  AAATGACCTT CTGACGAGGT ATTTGGACAC TATTTACAAA TGTGCTCTAG TTTTCAAATG
+458  TATTTTATAT GAGGAAAAAA AAACCCAACC GTATTGAGCA TTTTGCCAAG AGTTTAGGAG
+518  ACACAGCCCA CCACCTAGAG AAAAGAATGG CTTCTCTTCA GTAGGTAATG AAGAAAAACT
+578  GTCTCACTTG CAGGATCTCT CAGCAGAGGA AATCTTTCTG GCTCACCTAA AGAATGGACA
+638  GTCTTGAGAT TGGGGCGTAT GCTGGTGGCT GCAGGGACTA GGGTAGTGGT GGAGGGGGTG
+698  CTGGGGAGTC TGGGGAGATT GTGCTGGGGA GGCTGGGGGT GTTAATGTGT AAGGAAGCTG
+758  AGGTGGGAGA GTCTTCTGTG GAGACAGAGG TGGGTGCTGC AGCTCTTCTG AGCATTTGTC
                                          primer                  Met Gly
+818  CTTTTTTTCT CTTCCTTTCC TTTCAGGCTT TTCCGAGCAG CGAAGCCACC  ATG GGT
     Leu Glu Lys Ser Ler Ile Leu Phe Pro Leu Phe Phe Leu Leu Leu Gly
+874  CTG GAG AAG TCC CTC ATT CTG TTT CCA TTG TTT TTC CTG CTG CTT GGA
     Trp Val Gln Pro Ser Leu Gly Arg Glu Ser Ala Ala Gln Lys Phe Gln
+922  TGG GTC CAG CCT TCC CTG GGC AGG GAA TCT GCA GCA CAG AAG TTT CAG
     Arg Gln His Met Asp Pro Asp Gly Ser Ser Ile Asn Ser Pro Thr Tyr
+970  CGG CAG CAC ATG GAT CCA GAT GGT TCC TCC ATC AAC AGC CCC ACC TAC
     Cys Asn Gln Met Met Lys Arg Arg Asp Met Thr Asn Gly Ser Cys Lys
+1018 TGC AAC CAA ATG ATG AAA CGC CGG GAT ATG ACA AAT GGG TCA TGC AAG
     Pro Val Asn Thr Phe Val His Glu Pro Leu Ala Asp Val Gln Ala Val
+1066 CCC GTG AAC ACC TTC GTG CAT GAG CCC TTG GCA GAT GTC CAG GCC GTC
     Cys Ser Gln Glu Asn Val Thr Cys Lys Asn Arg Lys Ser Asn Cys Tyr
+1114 TGC TCC CAG GAA AAT GTC ACC TGC AAG AAC AGG AAG AGC AAC TGC TAC
     Lys Ser Ser Ser Ala Leu His Ile Thr Asp Cys His Leu Lys Gly Asn
+1162 AAG AGC AGC TCT GCC CTG CAC ATC ACT GAC TGC CAC CTG AAG GGC AAC
     Ser Lys Tyr Pro Asn Cys Asp Tyr Lys Thr Thr Gln Tyr Gln Lys His
+1210 TCC AAG TAT CCC AAC TGT GAC TAC AAG ACC ACT CAA TAC CAG AAG CAC
     Ile Ile Val Ala Cys Glu Gly Asn Pro Tyr Val Pro Val His Phe Asp
+1258 ATC ATT GTG GCC TGT GAA GGG AAC CCG TAC GTA CCA GTC CAC TTT GAT
     Ala Thr Val stop
+1306 GCT ACT GTG TAG GGGTC CACATAGGCC AAACCAGTGA GATGTCTATG TCTCCCAACC
+1363 TCATCACCTG TCTCCCCTCT GTCTACCATT CTTTCTCTGA AAGAAGTAAG TCATGTTAGG
+1423 ACTTCCATAT GACAAAACAT GCGTTCCTGT CCTGTGTCAT GCTCCAGAAT CCCTCTCTTG
+1483 CTGTAGACAA ATGAAGCAAG ATGGCCCTGA CGAGAACATG TCCGTTCAAC GTTAGACTTT
+1543 CTTGACTTTA GAAAACAACC AAATAAAATT CTGTTCATCA TAAATTGTGC ATTTAGGGCA
+1603 GTTCGAAGTG GAGTAAGGAC AATGGGCAAG TCACCTCATT CATTGGTCCA GAATTTCCTC
+1663 ACTCCTGACT ACTTTAACTA CACTATTGAA ACTAGACTTT TCTGCCTCTC TGCTGATCAT
+1723 TCATTCTTCG CATATACAAT AAAGTCTAAC ATCTATTTCA GCACTCAGTA GCCATAGCTC
+1783 CCAAGTCCAT CTGCTCCTCA TACCAATACA GATCAGTCAC ATCTACTGAA GAGAGTTCTT
+1843 CCATTAAGAG AGTAGAAGAT GGTTACCTTT GCCTCTGCCT TTCAGCTATC TTCTTCCATC
+1903 CATGTTTCAC TTTGTACTTT CTTTCTCATT GTAAGTGAAA AGCAGCATCC AGTCTTGCCT
+1963 TCCAGGGGCT GCTTCCTACT CTCCTGCCTC CTTACCTTGA CTCAGACCTC ATGGAACAAA
+2023 TAGGAATGTG GAGGGTAGGG GGTGGGGAGG CTTCAGTTTT AACAACCACA GTGGAAATCC
+2083 AATTTCGATG ATGCTTTTGG GAAACAGTCA CATGCACAGT CTTG
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Figure 2. Complete sequence of the *Rib-1* gene and flanking regions. The major start site in pancreas (+1) is marked with an arrowhead. Exonic sequences are underlined. TATA-like sequences are boxed. The potential CCAAT element at position −78, Sp1 binding site (48) at −62, and AP-4 binding site (49) at +161 are double underlined. The two PTF1 consensus binding sites at −180 and +245 are bracketed. The sequence of the 17 nucleotide primer extension oligomer is overlined.

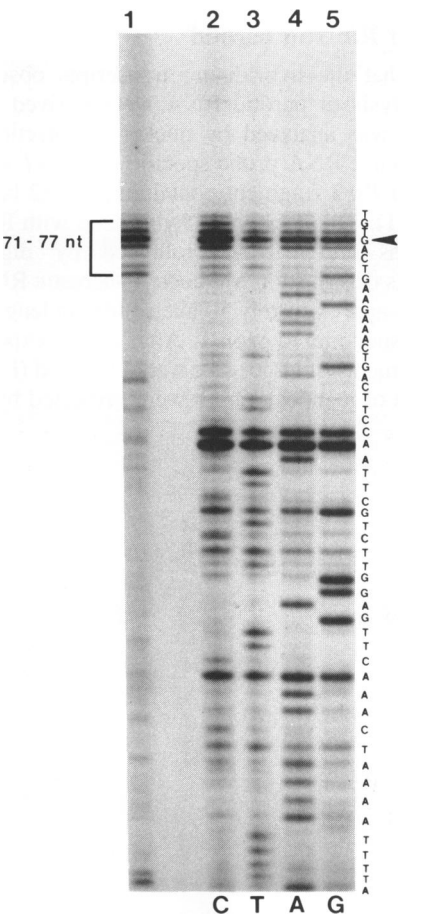

Figure 3. Sequence of the 5′ terminus of the pancreatic ribonuclease transcript. Primer extension reactions were performed with pancreatic RNA in the absence (lane 1) or presence of chain terminating dideoxynucleotides (lanes 2–5) as described in Materials and Methods. Genomic sequence is displayed at the right for comparison. The arrow marks the residue corresponding to the predominant primer extension product of 75 nucleotides. Lane 1, primer extension without the addition of dideoxy-NTPs; lane 2, dideoxy-GTP added; lane 3, dideoxy-ATP added; lane 4, dideoxy-TTP added; lane 5, dideoxy-CTP added.

sequence, 450 nucleotides of protein coding sequence, and 267 nucleotides of 3′ untranslated sequence. The gene organization is represented in Figure 1.

Rib-1 transcripts in pancreas and parotid

Northern analysis was used to analyze ribonuclease transcripts in RNA isolated from pancreas and parotid gland. A similar sized transcript was observed in both tissues using a mouse pancreatic ribonuclease cDNA probe (Figure 4). Ribonuclease mRNA is much more abundant in pancreatic RNA than in parotid RNA. A comparable signal was observed with 0.25 μg of total pancreatic RNA and 5 μg of parotid poly A^+ RNA (Figure 4). When total RNA from both tissues was compared directly, transcripts were 200-fold more abundant in pancreas (not shown). The slight mobility difference between pancreatic and parotid transcripts observed by Northern analysis (Figure 4) is probably due to the 20-fold difference in the amounts of RNA loaded on the gel, although we can not rule out minor differences between the two transcripts. By comparison with the mobility of 28s and 18s ribosomal RNAs, the length of the ribonuclease transcript was estimated to be 1 kb. Since the total exon length is 793 nucleotides, the length of the polyA$^+$ tail in the mature transcript appears to be approximately 200 nucleotides.

Expression of *Rib-1* in parotid

To confirm that the hybridizing transcripts observed in the Northern analysis of parotid RNA were derived from *Rib-1*, parotid RNA was analyzed by nuclease protection. A single stranded antisense RNA probe specific for *Rib-1* was prepared from a 1.1 kb *Pst* I fragment containing the 52 base pair first exon (Figure 1). This probe was hybridized with RNA isolated from pancreas and parotid, followed by digestion with ribonuclease as described in Methods. Pancreatic RNA protected two fragments approximately 50 nucleotides in length (Figure 5, lane 6), corresponding to exon 1. After longer exposure, minor protected fragments of larger size were observed (Figure 5, lane 2). Fragments of identical length were protected by RNA from parotid, with a difference in relative abundance (Figure 5, lanes 3 and 4). The similarity in the lengths of protected fragments indicates that the same gene is expressed in pancreas and parotid gland, and that the same transcription initiation sites are utilized in the two tissues. The abundance of *Rib-1* transcripts was estimated to be 200-fold higher in pancreas, since a comparable signal was observed with 0.25 μg of total pancreatic RNA and 50 μg of total parotid RNA (not shown). This is in agreement with the Northern results, indicating that the ribonuclease mRNA detected in parotid RNA is derived from *Rib-1*.

The activity of the *Rib-1* promoter in eight additional tissues was examined. Transcripts were not detected in 50 μg of total RNA from liver (Figure 5, lane 5), submaxillary gland, mammary gland, lung, kidney, ovary, testes, and brain (not shown). We conclude from these experiments that expression of this *Rib-1* promoter is restricted to pancreas and parotid.

Identical Transcription Initiation Sites in Pancreas and Parotid

The initiation sites for pancreatic and parotid *Rib-1* transcripts were also compared in a primer extension assay, using the 17 nucleotide *Rib-1* specific oligonucleotide primer described above. The predominant 75 nucleotide extension product observed with pancreatic RNA is of the length predicted for transcripts initiating at +1 (Figure 6). A minor product of 92 nucleotides is also visible (Figure 6, right lane). Because of the lower concentration of ribonuclease transcripts in parotid, we purified polyA$^+$ RNA for this analysis (Figure 6, left lane). The primer extension products for parotid polyA$^+$ RNA corresponded in length to those observed with pancreatic RNA, indicating that transcription

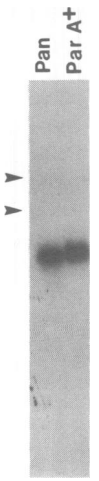

Figure 4. Ribonuclease transcripts in pancreas and parotid gland. Total RNA from pancreas and poly A^+ RNA from parotid were analyzed by Northern blotting and hybridization with the mouse pancreatic ribonuclease cDNA clone pMPR1. The positions of 28s and 18s ribosomal RNA are indicated. Pan, pancreatic RNA (0.25 μg); Par A^+, parotid poly A^+ RNA (5 μg).

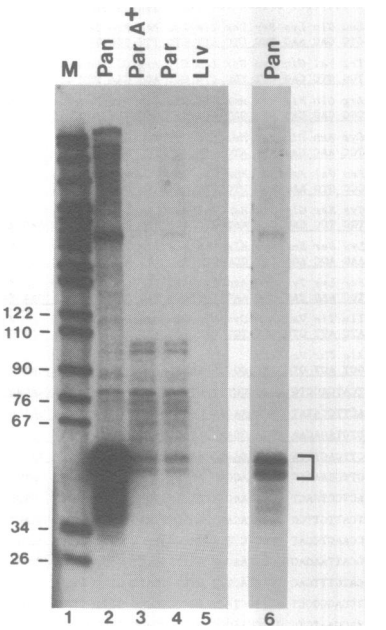

Figure 5. *Rib-1* is expressed in both pancreas and parotid gland. RNA was analyzed by RNase protection assay. The riboprobe corresponds to a 1.1 kb *Pst* I fragment which includes the 52 nucleotide first exon (Figure 1). The bracket indicates the approximately 50 nucleotide protected fragments observed with pancreatic RNA. Lane 6 is a shorter exposure of lane 2. M, molecular size standard pBR322 X *HpaII*; Pan, total RNA from pancreas (5 μg); Par A^+, polyA$^+$ RNA from parotid (5 μg); Par, total RNA from parotid (50 μg); Liv, total RNA from liver (50 μg).

in parotid is initiated at the same sites as in pancreas, and providing additional evidence for the expression of the same gene in both tissues. In parotid, transcripts corresponding to the 75 and 92 nucleotide extension product are approximately equal in abundance. The excess of transcripts initiating from +1 in pancreas accounts for the 200-fold difference in the steady state levels of *Rib-1* transcripts in pancreas and parotid.

Potential Transcription Regulatory Motifs

Several potential transcriptional control elements were identified in the promoter region of the *Rib-1* gene by similarity to consensus binding sites. These regions are marked on the *Rib-1* sequence in Figure 2. An AT-rich region 28 nucleotides upstream of the major pancreatic start has similarity to the TATA consensus. An SP1 consensus binding site is located at position −62 and a CCAAT box homology at -78. Within the intron there is an AP-4 site at +161 and a TATA consensus at +194. The sites in the intron are of interest because the intron of the bovine ribonuclease gene also contains potential regulatory sequences (33). We did not detect transcripts initiating from this region of the gene (data not shown).

PTF1 is a pancreatic nuclear protein which binds an enhancer element common to genes expressed in the exocrine pancreas (29, 34). The *Rib-1* gene contains two regions related to the PTF1 binding consensus, one in the 5′ flanking region (PTF1-A) and the other in the intron (PTF1-B) (Figure 7A). PTF1 binding sites of other pancreatic genes have been localized to the 5′ flanking region. To determine whether the *Rib-1* consensus sites are recognized by PTF1, they were amplified by polymerase chain reaction and tested in a gel mobility shift assay. PTF1 was purified from the rat pancreatic cell line AR42J by retention on an affinity column containing the PTF1 binding site of the rat elastase 1 gene. Incubation of PTF1-B with the affinity-purified protein generated a complex with reduced mobility (Figure 7B, lane 2). Binding specificity of the probe was demonstrated by competition in the presence of a 350 molar excess of unlabelled probe (lane 3), and the lack of competition in the presence of a 750 molar excess of an unrelated 119 bp fragment from pBR322 (29) (data not shown). A 63 bp fragment containing the mouse amylase PTF1 binding site (29) was also an effective competitor (lane 5). PTF1-A competed weakly (lane 4), suggesting that it contains a low affinity binding site.

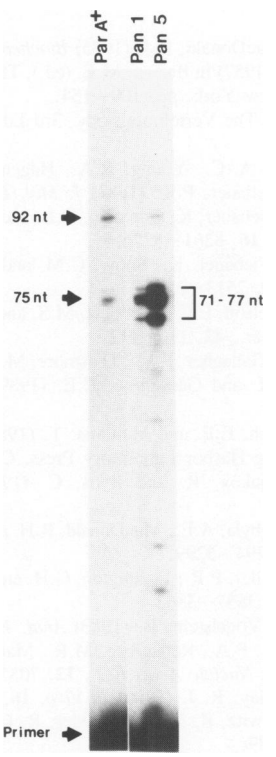

Figure 6. Parotid and pancreatic ribonuclease transcripts are initiated from the same start sites. Primer extension reactions were performed with poly A^+ RNA from parotid (5 μg), or total pancreatic RNA (1 and 5 μg). The position of the 17 nucleotide primer and the extension products are indicated. The sizes of the primer extension products were determined from a DNA sequencing ladder on the same gel.

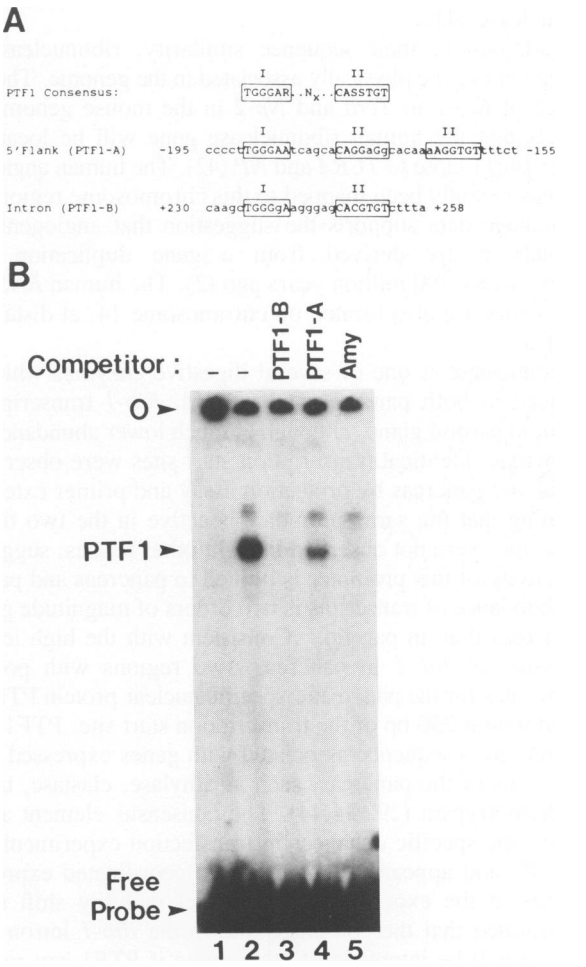

Figure 7. PTF1 binding sites in *Rib-1*. A) Two regions with homology to the PTF1 consensus binding site. The PTF1 consensus sequence is comprised of two elements separated by a variable spacer (Nx), which ranges from 5 to 15 bp in the previously characterized pancreatic genes (34). Two alternatives for consensus element II of PTF1-A are indicated. Nucleotides which match the consensus sequence are in capitals, spacer sequence and mismatches are in lower case. R = A or G; S = G or C. B) Binding of purified PTF1 to the intron consensus site PTF1-B. A DNA fragment corresponding to nucleotides +196 to +288 was amplified, and used as a probe in a gel mobility shift assay. PTF1 was purified by affinity chromatography as described in Methods. Lanes 1 and 2 contain the PTF1-B probe in the absense and presence of purified PTF1, respectively. Competitor DNAs were present at a 350-fold molar excess. PTF1-B, intron fragment +196 to +228; PTF1-A, 5′ flanking fragment −221 to −121; Amy, amylase PTF1 fragment −172 to −110 (29); O, origin.

DISCUSSION

We have cloned the mouse pancreatic ribonuclease gene, determined its complete nucleotide sequence, and characterized its transcription in pancreas and parotid gland. The structure of *Rib-1* is similar to that of the bovine ribonuclease gene (33) and other members of the ribonuclease superfamily: eosinophil cationic protein (*RNS2*) (35), eosinophil neurotoxin (*RNS3*) (35), and angiogenin (36). All of these genes contain one uninterrupted coding exon. A single noncoding exon is present in *Rib-1*, *RNS2*, and *RNS3*. The amino acid sequences of eosinophil cationic protein, eosinophil neurotoxin, and angiogenin are approximately 30% identical to pancreatic ribonuclease (36–38), and eosinophil cationic protein and eosinophil neurotoxin contain some ribonuclease activity (39, 40). The bovine genome also contains a second ribonuclease gene which is expressed in seminal vesicle and is 83% identical in amino acid sequence to bovine pancreatic ribonuclease (41).

In addition to their sequence similarity, ribonuclease and angiogenin may be physically associated in the genome. The close linkage of *Rib-1* to *Tcra* and *Np-2* in the mouse genome (17) predicts that the human ribonuclease gene will be located on human 14q11 close to *TCRA* and *NP* (42). The human angiogenin gene has recently been mapped to this chromosome region (43). The linkage data supports the suggestion that angiogenin and ribonuclease are derived from a gene duplication event approximately 300 million years ago (2). The human *RNS2* and *RNS3* genes are also located on chromosome 14, at distal band 14q24-q31.

Ribonuclease is one of several digestive enzymes which are produced in both pancreas and parotid. *Rib-1* transcripts are present in parotid gland, although at much lower abundance than in pancreas. Identical transcription start sites were observed in parotid and pancreas by protection assay and primer extension, indicating that the same promoter is active in the two tissues. Transcripts were not observed in eight other tissues, suggesting that activity of this promoter is limited to pancreas and parotid. The abundance of transcripts is two orders of magnitude greater in pancreas than in parotid. Consistent with the high level of expression of *Rib-1* in pancreas, two regions with potential binding sites for the pancreatic-specific nuclear protein PTF1 are located within 250 bp of the transcription start site. PTF1 binds to a consensus sequence associated with genes expressed in the acinar cells of the pancreas, such as amylase, elastase, trypsin and chymotrypsin (29, 34,44). The consensus element acts as a pancreatic-specific enhancer in transfection experiments (29, 34,44,45) and appears to contribute to coordinated expression of genes in the exocrine pancreas. Gel mobility shift assays demonstrated that the consensus site in the *Rib-1* intron binds PTF1. It will be interesting to determine if PTF1 can regulate transcription from a site in the intron.

In contrast to the amylase multigene family, mouse ribonuclease appears to be encoded by a single gene which is expressed in pancreas and parotid. The expression of the same *Rib-1* promoter in both tissues was unanticipated. *Rib-1* is unlike the mouse salivary amylase gene which contains two functional promoters with different tissue specificity (46,47). In view of their evolutionary relationship, it is possible that pancreas and parotid produce related transcription factors. The expression of the pancreatic *Rib-1* promoter at a lower level in parotid may represent a primitive mechanism for gene expression in parotid. Similar studies of other enzymes with dual expression in pancreas and parotid, such as deoxyribonuclease, phospholipase A_2, and kallikrein, would contribute to a deeper understanding of the evolution of parotid specificity.

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