Volume 16 Number 12 1988 Nucleic Acids Research

Structure of the bovine pancreatic ribonuclease gene: the unique intervening sequence in the 5' untranslated region contains a promoter-like element

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Received February 5, 1988; Revised and Accepted April 13, 1988 Accession no.X07283

ABSTRACT

Although pancreatic ribonucleases are extensively studied proteins, little information is available on nucleic acids coding for these enzymes. Here, for the first time, the structure of a gene coding for such an enzyme, the well known bovine pancreatic ribonuclease, is reported. The coding region of this gene is devoid of introns, whereas the 5' untranslated sequence of the pancreatic transcript contains an intron of 735 nucleotides. This intervening sequence is endowed with signals (CAAT and TATA boxes) which might act as regulatory elements. The structural organization of this gene suggests that the sequence coding for the bovine pancreatic ribonuclease might be expressed under the control of two different promoters.

INTRODUCTION

The exocrine pancreas of mammals secretes, among other lytic activities, a ribonuclease enzyme. Pancreatic ribonucleases isolated from different species are homologous proteins whose primary structure was conserved during evolution. The amino acid sequence of as many as 41 "pancreatic-type" RNAases was determined and their enzyme activity was characterized (1). Among them two bovine enzymes, the pancreatic and the seminal ribonucleases, are the most extensively studied. The pancreatic enzyme is a monomer of 124 amino acid residues (2) while the bovine seminal ribonuclease is a dimer made up of two identical 124 amino acid subunits linked through two interchain disulfide bridges (3,4). The primary structure of the subunits is strictly homologous (80%) to that of bovine pancreatic RNAase (5). A structurally related enzyme was purified from bovine brain (6), and other distantly related proteins (the so-called non secretory ribonucleases)

have been found in bovine liver (7) and kidney (8). The occurence of many homologous proteins in different organs of the same animal suggests the existence of a family of homologous genes regulated in a tissue-specific fashion. Whereas the enzyme molecules were extensively studied, scarce information is available on the nucleic acid sequences coding for these ribonucleases. Rat pancreatic (9) and bovine seminal (10) cDNAs were cloned and the 5'flanking sequence of the rat gene has been reported (11). Moreover, the gene coding for human angiogenin, a ribonuclease structurally related to the non secretory RNAases, has been cloned (12).

The aim of our work is the study of gene organization of ox ribonucleases in order to elucidate the mechanism involved in their tissue specific expression. In this paper we report the structure of the pancreatic ribonuclease gene which suggests that the same coding unit may be expressed under the control of two different promoter sequences.

MATERIALS AND METHODS

Isolation and characterization of the genomic clone

The genomic library was generated by partial MboI digestion of high molecular weight bovine DNA. Digestion products were cloned into the BamHI site of the EMBL3 phage. This library was a kind gift of Dr. Ken Reed, the University of Canberra, Australia. Screening of the library was performed with a nick-translated probe, as described (13). The probe was a 569 bp PstI fragment of the plasmid 17G3 (10) which contains the sequence coding for the bovine seminal ribonuclease. For the construction of subclones, restriction fragments were isolated from agarose gels and ligated into convenient sites of plasmids pUC18 or pUC19. DNA was sequenced by the procedure of Maxam and Gilbert (14). Five sequencing reactions (G, dimethyl sulfate; G+A, formic acid; C+T, hydrazine; C, hydrazine+salt; A> C, sodium hydroxide) were carried out to enhance sequence accuracy.

Sequence of the extended primer

The PstI fragment of 0.7 Kb of the bovine pancreatic RNAase gene (see Figure 1b) was subcloned in the pUC19 vector.

The recombinant plasmid was linearized and kinased in the presence of $\gamma^{32}P$ ATP at the HindIII site of the vector and restricted with the AvaII enzyme. The resulting 30 bp fragment was then elongated in the presence of poly A+ RNA from bovine pancreas. The AvaII-PstI double stranded fragment (8 pmoles) were hybridized to 30 μg of polyadenylated RNA from ox pancreas in the presence of 80% formamide. The resulting heteroduplex was incubated with avian myeloblastosis virus reverse transcriptase in the presence of deoxynucleoside triphosphates, $\alpha^{32}P$ dATP and chain terminator dideoxynucleoside triphosphates. The products were analyzed in an urea-5% polyacrylamide gel. In a parallel experiment the primer was elongated in the presence of dNTP precursors, the longest product was purified and sequenced with the Maxam-Gilbert technique.

Southern blot analysis

High molecular weight bovine DNA from peripheral blood of individual local strain animals was isolated as described (13). The DNA was digested to completion with restriction endonucleases (Boehringer) and digestion monitored by the extent of cleavage of an internal pBR322 DNA marker in a parallel reaction. Samples were electrophoresed on 0.8% agarose gel, transferred to nitrocellulose paper and hybridized to a ^{32}P nick-translated probe in a medium consisting of 0.3XSSC, 5X Denhardt's solution, 0.5% SDS, 10mM Na_2EDTA, 150 μg/ml denatured salmon sperm DNA, at 65 °C. Filters were washed in 0.1XSSC, 0.5% SDS, 10 mM Na_2EDTA, at 65 °C, and exposed to Kodak films at -70 °C with intensifying screens.

Preparation and analysis of RNA from bovine pancreas

Polyadenylated RNA from bull pancreas was isolated as described (15). RNA samples were denatured by incubation at 65 °C for 5 minutes in a solution containing 50% formamide, 6% formaldheyde and running buffer (20 mM 4-morpholine propanesulphonate buffer, 5mM Na acetate, 1mM Na_2EDTA, pH 7). Electrophoresis was carried out at 100 V in 1.7% agarose gel containing 6% formaldheyde and running buffer. Hybridization of the RNA transferred to nitrocellulose paper (Schleicher and Schuell, FRG) was performed according to Thomas (16).DNA probes were labeled by nick-translation with $\alpha^{32}P$ dCTP (400 Ci/mMol,

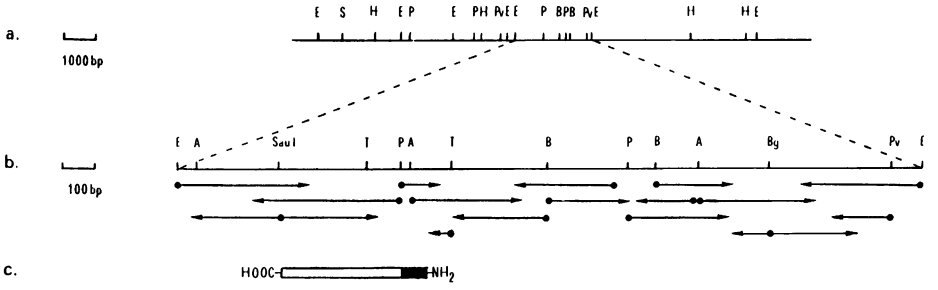


Figure 1
(a) Restriction map of the genomic clone containing the sequence coding for bovine pancreatic ribonuclease. Only relevant restriction sites are shown. (b) Sequencing strategy of the 2.3 kb EcoRI fragment of the recombinant phage. (c) Orientation of the coding sequence relative to the 2.3 kb EcoRI fragment. The black box corresponds to the signal peptide. A: AvaII; B: BamHI; Bg: BglII; E: EcoRI; H: HindIII; P: PstI; Pv: PvII; S: SalI; T: TaqI.

Amersham U.K.). Blots were washed four times (5 min each) with 2xSSC in 0.1% SDS at room temperature, twice (60 min each) with 0.1xSSC in 0.1% SDS at 45 °C and exposed to Kodak film.

RESULTS

Isolation of the gene coding for pancreatic ribonuclease

A bovine genomic library was screened with the seminal RNAase cDNA as a probe (10), and five recombinant phages out of 7.5×10^5 were isolated; four exihibit identical restriction pattern and one of them (called FB) was chosen for further analysis. Fig. 1a shows the restriction map of the 16 Kb FB insert which contains a unique 2.3 Kb EcoRI fragment, able to hybrydize to the above mentioned probe. The 5th clone contains a sequence coding for a protein molecule highly homologous to both pancreatic and seminal ribonucleases (17, and unpublished results).

Nucleotide sequence of the bovine pancreatic ribonuclease gene

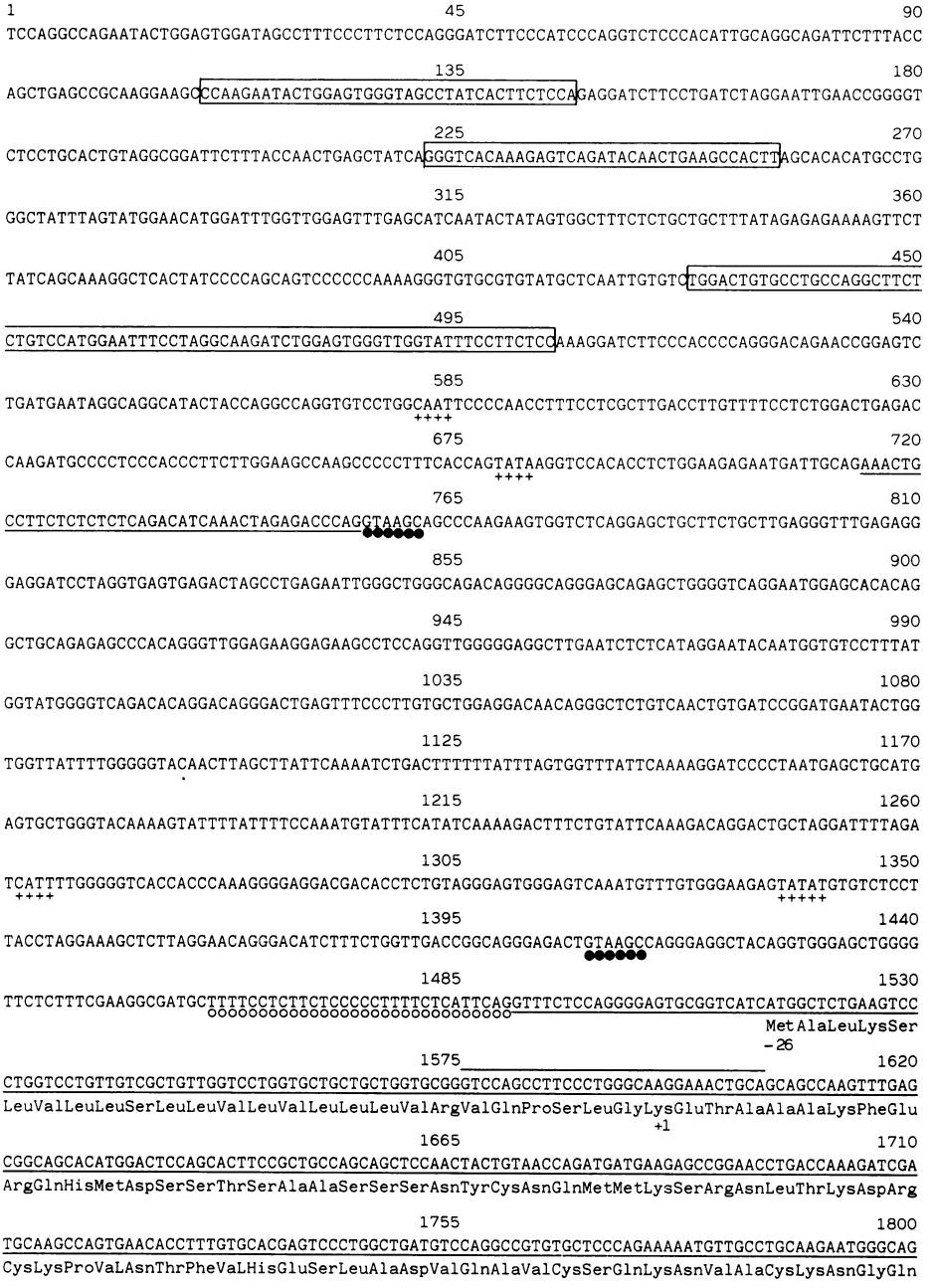
The primary structure of the 2.3 Kb EcoRI genomic fragment was determined with the Maxam-Gilbert technique for about 75% in both directions. The sequencing strategy is reported in Figure 1b. As shown in Figure 2, the amino acid sequence

derived from nt 1516 to nt 1965 codes for pancreatic ribonuclease. The open reading frame is interrupted by a TAG triplet which corresponds to a stop codon. No discrepancies between the known primary structure and that deduced from the nucleotide sequence was found. The sequence coding for the native enzyme is preceded by a sequence specifying an extra peptide of 26 amino acids which is highly hydrophobic (77% of non-polar amino acid residues), bears a positive charge close to the NH_2-terminus (lys -23), and terminates with a glycine residue. These features are distinctive of the known structure of signal peptides of other secreted proteins (18). No intervening sequences were found within the translated region of the gene. However, 25 nts upstream to the first in frame methionine codon, a 3' splice signal was found.

The polypyrimidine stretch (from nt 1460 to nt 1487) contains a single adenine residue and is followed by the sequence CAGG. An exanucleotide GTAAGC is located 79 nts upstream. These sequences match, respectively, with the 3' and the 5' consensus usually found at the exon-intron boundaries of protein coding genes (19).

The 5' end of the bovine pancreatic ribonuclease mRNA

In order to identify the transcription initiation site of the RNAase message, a primer extended on bovine pancreatic polyA+ RNA was sequenced. This analysis could also ascertain if the putative exon borders were actually joined in the ribonuclease message. The primer used was the AvaII-PstI fragment coding from amino acids -6 to +4, located between nucleotides 1576-1605 (Figure 2). This 30 bp fragment was elongated in the presence of polyadenylated RNA and the nucleotide sequence was determined according to the Sanger procedure. Some ambiguities in the sequence ladders, due to pause sites of reverse transcriptase, were clarified by sequencing the fully elongated primer, which consists of 156 nts, with the Maxam-Gilbert procedure (data not shown). The sequence determined corresponded to the genomic region from the primer to nt 1491 and from nt 755 to nt 713. The result of this experiment demonstrated that: 1) the actual transcription initiation site is located about 800 nts upstream to the


1
45
90
TCCAGGCCAGAATACTGGAGTGGATAGCCTTTCCCTTCTCCAGGGATCTTCCCATCCCAGGTCTCCCACATTGCAGGCAGATTCTTTACC
135
180
AGCTGAGCCGCAAGGAAGCCCAAGAATACTGGAGTGGGTAGCCTATCACTTCTCCAGAGGATCTTCCTGATCTAGGAATTGAACCGGGGT
225
270
CTCCTGCACTGTAGGCGGATTCTTTACCAACTGAGCTATCAGGGTCACAAAGAGTCAGATACAACTGAAGCCACTTAGCACACATGCCTG
315
360
GGCTATTTAGTATGGAACATGGATTTGGTTGGAGTTTGAGCATCAATACTATAGTGGCTTTCTCTGCTGCTTTATAGAGAGAAAAGTTCT
405
450
TATCAGCAAAGGCTCACTATCCCCAGCAGTCCCCCCAAAAGGGTGTGCGTGTATGCTCAATTGTGTCTGGACTGTGCCTGCCAGGCTTCT
495
540
CTGTCCATGGAATTTCCTAGGCAAGATCTGGAGTGGGTTGGTATTTCCTTCTCCAAAGGATCTTCCCACCCCAGGGACAGAACCGGAGTC
585
630
TGATGAATAGGCAGGCATACTACCAGGCCAGGTGTCCTGGCAATTCCCCAACCTTTCCTCGCTTGACCTTGTTTTCCTCTGGACTGAGAC
675
720
CAAGATGCCCCTCCCACCCTTCTTGGAAGCCAAGCCCCCTTTCACCAGTATAAGGTCCACACCTCTGGAAGAGAATGATTGCAGAAACTG
765
810
CCTTCTCTCTCTCAGACATCAAACTAGAGACCCAGGTAAGCAGCCCAAGAAGTGGTCTCAGGAGCTGCTTCTGCTTGAGGGTTTGAGAGG
855
900
GAGGATCCTAGGTGAGTGAGACTAGCCTGAGAATTGGGCTGGGCAGACAGGGGCAGGGAGCAGAGCTGGGGTCAGGAATGGAGCACACAG
945
990
GCTGCAGAGAGCCCACAGGGTTGGAGAAGGAGAAGCCTCCAGGTTGGGGGAGGCTTGAATCTCTCATAGGAATACAATGGTGTCCTTTAT
1035
1080
GGTATGGGGTCAGACACAGGACAGGGACTGAGTTTCCCTTGTGCTGGAGGACAACAGGGCTCTGTCAACTGTGATCCGGATGAATACTGG
1125
1170
TGGTTATTTTGGGGGTACAACTTAGCTTATTCAAAATCTGACTTTTTTATTTAGTGGTTTATTCAAAAGGATCCCCTAATGAGCTGCATG
1215
1260
AGTGCTGGGTACAAAAGTATTTTATTTTCCAAATGTATTTCATATCAAAAGACTTTCTGTATTCAAAGACAGGACTGCTAGGATTTTAGA
1305
1350
TCATTTTGGGGGTCACCACCCAAAGGGGAGGACGACACCTCTGTAGGGAGTGGGAGTCAAATGTTTGTGGGAAGAGTATATGTGTCTCCT
1395
1440
TACCTAGGAAAGCTCTTAGGAACAGGGACATCTTTCTGGTTGACCGGCAGGGAGACTGTAAGCCAGGGAGGCTACAGGTGGGAGCTGGGG
1485
1530
TTCTCTTTCGAAGGCGATGCTTTTCCTCTTCTCCCCCTTTTCTCATTCAGGTTTCTCCAGGGGAGTGCGGTCATCATGGCTCTGAAGTCC
Met AlaLeuLysSer
-26
1575
1620
CTGGTCCTGTTGTCGCTGTTGGTCCTGGTGCTGCTGCTGGTGCGGGTCCAGCCTTCCCTGGGCAAGGAAACTGCAGCAGCCAAGTTTGAG
LeuValLeuLeuSerLeuLeuValLeuValLeuLeuValArgValGlnProSerLeuGlyLysGluThrAlaAlaAlaLysPheGlu
+1
1665
1710
CGGCAGCACATGGACTCCAGCACTTCCGCTGCCAGCAGCTCCAACTACTGTAACCAGATGATGAAGAGCCGGAACCTGACCAAAGATCGA
ArgGlnHisMetAspSerSerThrSerAlaAlaSerSerSerAsnTyrCysAsnGlnMetMetLysSerArgAsnLeuThrLysAspArg
1755
1800
TGCAAGCCAGTGAACACCTTTGTGCACGAGTCCCTGGCTGATGTCCAGGCCGTGTGCTCCCAGAAAAATGTTGCCTGCAAGAATGGGCAG
CysLysProValAsnThrPheValHisGluSerLeuAlaAspValGlnAlaValCysSerGlnLysAsnValAlaCysLysAsnGlyGln

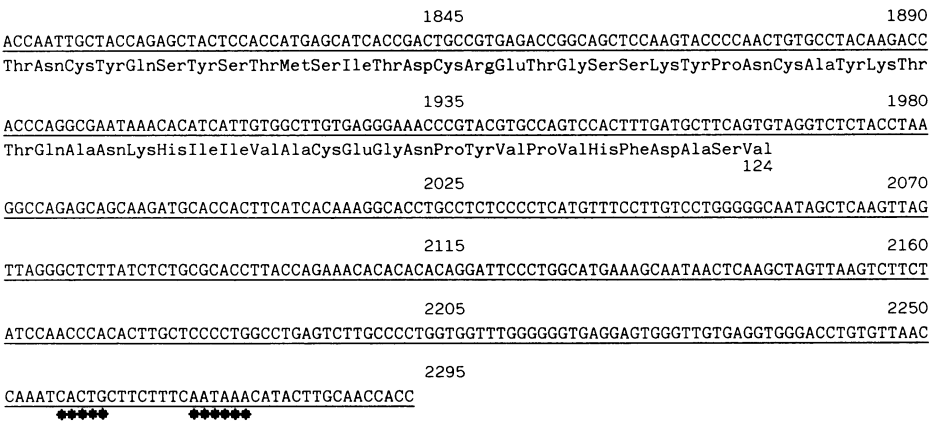


Figure 2.
Nucleotide sequence of the bovine pancreatic ribonuclease gene. Translation of the open reading frame is shown; amino acids are numbered relative to the first residue of the native protein. CAAT and TATA boxes are marked by crosses. The sequence corresponding to the pancreatic ribonuclease message is underlined. 5' splice signals are marked by filled circles (●). The polypyrimidine stretch and the 3' splice site are marked by open circles (o). Polyadenylation signals are marked by asterisks (✱). Repetitive sequences are boxed. The AvaII-PstI fragment used in the primer elongation experiment is upperlined.

translated region of the gene, near the A residue at position 713 and, 2) a relatively large intervening sequence (from nt 756 to nt 1490) is present in the 5' untranslated region of the bovine pancreatic RNAase gene. The 3' border of the untranslated exon lies at nt 755, where the sequence CAG:GTAAGC (a typical 5' splice signal), is present. In the mature message the 3' exon border is joined to the splice site located at position 1490.

The 3' end of the message

The coding sequence of the pancreatic RNAase gene is followed by an untranslated region of about 320 bp. The so-called polyadenylation signal (AATAAA) is located at nt 2269. A second recognition element CACTG, which is frequently found close to the site of polyA addition (20), is present at position 2256 (Figure 2). No splice signals within this region were found. Moreover, this sequence is highly homologous (about

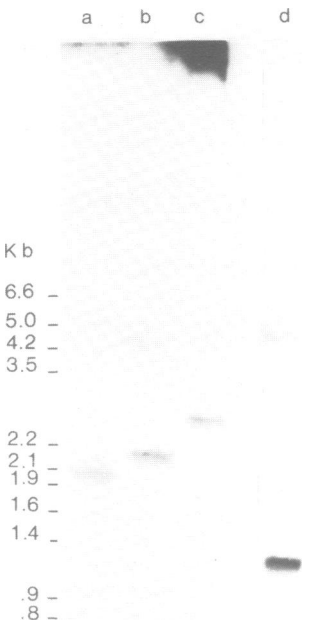


Figure 3..
Southern blot analysis of bovine genomic DNA. DNA digested with PstI (lane a), EcoRI (lane b), PvuII (lane c) or EcoRI and BamHI (lane d) restriction endonucleases was hybridized to the 0.6 Kb EcoRI-PstI fragment of the pancreatic RNAase gene, labeled by nick-translation.

90%) to the 3' untranslated region (consisting of about 330 nts) of the bovine seminal cDNA. From these observations and the experimental results described above it can be deduced that only one intervening sequence is present in the pancreatic RNAase gene.

Regulatory signals in the bovine pancreatic ribonuclease gene

The region upstream to the transcription initiation site contains elements which are known to control transcription of protein coding genes (21, 22). CAAT and TATAA boxes were found, at nts 581 and 679, respectively, that is about 130 and 30 residues upstream to the cap site (Figure 2). Interestingly, the intervening sequence located within the untranslated region of the gene also contains regulatory elements. A TATAT sequence

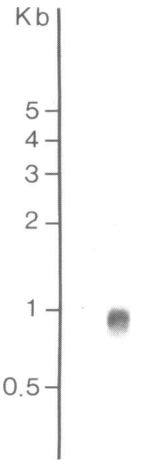


Figure4.
Northern blot analysis of pancreatic poly A+ RNA. The probe used was the the same as in Southern blotting experiment.

can be found at position 1337 and a variant CAAT box lies 76 nts upstream. The CATT variant of the canonical CAAT box has already been found within the promoter structure of the glyceraldheyde-3-phosphate dehydrogenase gene (23).

Repetitive sequences

Repetitive DNA sequences were found in the region upstream to the pancreatic ribonuclease promoter (see Figure 2). The region from nt 222 to nt 256 is homologous to the 3' end of the consensus derived from repetitive sequences found in the bovine ACTH-βLPH precursor gene (24). Sequences from nt 112 to nt 145 and from nt 429 to nt 504 are homologous to the 5' end of the consensus mentioned above, in the inverted orientation.

Southern blot analysis

Figure 3 shows the restriction patterns of bovine genomic DNA hybridized to the EcoRI-PstI fragment which contains the coding sequence and the 3' untranslated region of the pancreatic RNAase gene (see Figure 1b). Lanes a), b) and c) show the analysis of the genomic DNA digested with PstI, EcoRI and PvuII restriction enzymes, respectively. Lane d) shows the hybridization pattern of the genomic DNA subjected to double digestion with EcoRI and BamHI endonucleases. A major band

appears in each case (2.0 Kb in lane a), 2.2 Kb in lane b), 2.6 Kb in lane c), and 1.1 Kb in lane d). These results agree with the restriction pattern determined for the recombinant phage (Figure 1). Fainter bands represent cross-hybridization to the seminal RNAase gene and to another homologous gene (17, and data not shown). These data indicate that the cloned ribonuclease gene probably represents a unique sequence in the bovine genome.

Northern blot analysis of pancreatic polyadenylated RNA

Northern blot analysis of bovine pancreatic poly A+ RNA shows that a single RNA species of about 950 nts hybridizes to the pancreatic ribonuclease probe (Figure 4). The length of this message is similar to that of mRNAs coding for bovine seminal (10), pig (25) and rat (9) pancreatic ribonucleases, and agrees with the transcription unit characterized in the experiments described above. The mRNA coding for the ribonuclease is a very abundant species in the bovine exocrine pancreas; in fact, 2.5 μg of polyadenylated pancreatic RNA, hybridized with a ^{32}P nick-translated specific probe (see Materials and methods), give a strong signal after 15 minutes of film exposure (Figure 4).

DISCUSSION

In this work we report the sequence of the gene coding for bovine RNAase and analysis of its expression in the pancreas. The coding region of this gene is devoid of intervening sequences, but a relatively large one was found within the 5' untranslated region. This intervening sequence is endowed with signals which could, in principle, allow transcription of a downstream gene. The structural organization of the pancreatic RNAase gene suggests that the same protein coding unit might be expressed under the control of two different promoters. The expression of a coding sequence depending on two different promoters was reported for some viral and eukaryotic genes. In the genes of mouse amylase ($Amy1^A$) (26), insulin-like growth factor II (27,28), Drosophila alcohol dehydrogenase (29) and myosin light chain (30,31,32), a promoter structure was identified in the 5' flanking region and a second one within an

intervening sequence. In each case the presence of different transcripts is related to tissue or developmental stage specificity. As for the mouse amylase gene ($Amy1^A$), the mature transcripts synthesized under the control of the two different promoters bear the identical coding unit and differ only in the 5' untranslated region. This regulatory mechanism may also work for the bovine pancreatic RNAase gene. Ribonuclease molecules undistinguishable from the pancreatic enzyme have been found in bovine kidney (8), milk (33), serum (34), seminal vesicles (35) and salivary glands (36). RNA analysis from these tissues could ascertain whether indeed the regulatory elements within the intervening sequence promote transcription of the RNAase message.

ACKNOWLEDGEMENTS

This work was carried out with the financial support of the project Ingegneria genetica e basi molecolari delle malattie ereditarie of CNR, and by a grant from the Ministero della Pubblica Istruzione, Italy.

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