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Molecular cloning of the gene encoding the bovine brain ribonuclease and its expression in different regions of the brain

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ABSTRACT

In this paper we report the molecular cloning of the gene encoding the bovine brain ribonuclease. The nucleotide sequence determined in this work shows a high degree of identity to the homologous gene encoding the bovine pancreatic ribonuclease. Processing of the primary transcripts of these genes also follows a similar pathway, splicing of the unique intron in the 5′ untranslated region occurs at corresponding positions. Expression of the bovine brain ribonuclease gene can be detected both at the transcriptional and translational levels in all the regions of the brain examined.

INTRODUCTION

The major ribonucleolytic activity in the bovine brain is associated with an enzyme whose primary structure has been elucidated by Irie and coworkers (1). The brain ribonuclease (BRb RNAase) shares a high degree of identity in its primary structure with pancreatic ribonucleases of various species (2). The cerebral enzyme contains, however, a unique hydrophobic carboxy-terminal extension, in which the two polar amino acid residues, are covalentely bound to oligosaccharide chains. In the ox in addition to the pancreatic and cerebral RNAases, a homologous enzyme is synthesized in seminal vesicles (3). These three closely related but structurally different secretory ribonucleases, are expressed in the bovine species according to a tissue specific pattern. To date the physiological significance of extracellular ribonucleolytic activities in tissues other than the pancreas remains unclear.

We have recently cloned the gene of the pancreatic RNAase (4) and a large portion of the cDNA encoding the seminal ribonuclease (5). More recently a cDNA clone containing the complete coding sequence of the seminal enzyme was characterized by Preuβ et al. (6). In this paper we report the molecular cloning of the gene encoding the BRb RNAase and an analysis of its expression in different regions of the bovine brain.

MATERIALS AND METHODS

Isolation and characterization of the genomic clone

The genomic library of bovine DNA, cloned in the EMBL3 phage vector, was a kind gift of Dr. Ken Reed, the University of Canberra, Australia. Screening of the library was performed as described (4), using as a probe the cDNA coding for the bovine seminal ribonuclease. Subclones of the phage insert were generated by ligating the restriction fragments of interest into pUC18 or pUC19 plasmids. DNA was sequenced either by the procedure of Maxam and Gilbert (7) or by the dideoxynucleotide enzymatic method (8) using the Sequenase kit (USB).

Purification and analysis of RNA

RNA was purified by phenol extraction, according to Parish (9). Polyadenylated RNA of bovine brain was isolated by chromatography on oligo dT cellulose, as described previously (10). The primer extension analysis was carried out using the *Ava*II-*Hpa*II fragment (nt 1410 to nt 1450) of the BRb RNAase gene, 5′ labelled with ^{32}P at nucleotide 1450. 0.1 picomoles of the fragment were hybridized to 150 μg of total RNA for 16 hr at 40°C under standard conditions (12). The sample was precipitated with ethanol, elongated with the avian myeloblastosis virus reverse transcriptase and the products were analyzed on 5% denaturing polyacrylamide gel. Oligonucleotide 1 (5′AT-$C^A/_G{}^A/_T$AGTGGACTGGCACG$^G/_T$A3′), which is complementary to the coding region of the gene from nt 1767 to nt 1787, was used to prime reverse transcription of polyadenylated RNA purified from ox brain, using the murine leukemia virus reverse transcriptase (Boehringer). The BRb RNAase specific cDNA was amplified with Taq polymerase (Perkin Elmer Kit) using a Perkin

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Elmer Cetus DNA Thermal Cycler; Taq polymerase activity was primed by hybridization to oligonucleotide 1 and oligonucleotide 2 (5'CTGCCTTCTTTCTCAGAGACC3'), which corresponds to the region from nt 500 to nt 520 of the BRb RNAase gene. The amplified products were purified by 2% agarose gel electrophoresis and sequenced with the Sequenase kit, with minor modifications, using as a primer the oligonucleotide 3 (5'AGGGACTTCAGAGCCAT3') which is complementary to the sequence (nt 1347 to nt 1363) encoding the $-NH_2$ terminus of the BRb RNAase. Northern blot analysis was carried out using total RNA purified as described (11), electrophoresed on 1% agarose gel containing 6% formaldehyde and running buffer (20 mM MOPS, 5 mM Na acetate and 1 mM Na_2EDTA, pH 7) and transferred to Hybond N membranes (Amersham). Filters were hybridized as described (12). The probes used were: 1) a 54mer oligonucleotide complementary to the sequence (nt 1797 to nt 1850) coding for the carboxy terminal segment of the brain ribonuclease labelled with ^{32}P at the 5' end; 2) a fragment encoding the brain enzyme (nt 1280 to nt 1698); 3) the human actin cDNA. The latter two probes were labelled with ^{32}P using the Multiprime system (Amersham). Filters were washed at high stringency and exposed to Kodak films. Slot blot analysis was carried out following the same hybridization conditions used for the Northern blot analysis. The autoradiograms were scanned with a laser densitometer (LKB).

Preparation of brain cells and extracts

Fractions enriched in astrocytes and neurons were prepared from bovine brain according to the procedure of Farooq and Norton (13).

Tissue samples (3 g wet weight) were homogenized at 4°C in 20 ml of 40 mM MOPS, pH 7.5, containing 0.1 M NaCl. The homogenates were centrifuged at 17,000×g for 20 min at 4°C and the supernatants were used for the determination of RNAase activity. Protein concentration was determined according to Bradford (14) using the bovine serum albumin as a standard.

Enzyme assays

Ribonuclease activity was measured by the formation of acid-soluble nucleotides according to the method of Anfinsen *et al.* (15) with some modifications. The incubation mixtures (final volume, 1 ml) contained 0.1−0.2 ml of the sample to be assayed and 5 mg yeast RNA (Sigma, further purified as described by Blackburn *et al.* (16)) or 1 mg poly(C) in 40 mM MOPS, pH 7.5, containing 0.1 M NaCl and 0.5 mM p-hydroxymercuribenzoic acid (PHMB). One unit of RNAase activity was defined as the amount of enzyme producing an absorbance increase of 1.0 at 260 nm in 30 min. Bovine pancreatic RNAase A (Sigma, type XII-A) was used as a Tstandard.

RESULTS

Isolation of the gene encoding the bovine brain ribonuclease

A bovine genomic library was screened using the seminal RNAase cDNA as a probe, and five recombinant phages out of 7.5×10^5 were isolated. Four clones exhibited an identical restriction pattern and were shown to contain the pancreatic ribonuclease gene (4); the fifth clone was further analyzed in order to characterize the hybridizing sequence. Fig. 1 shows the restriction map and the sequencing strategy for the 13 kb insert containing a unique PstI-EcoRI fragment of about 1400 bp, which hybridizes to the seminal RNAase cDNA probe.

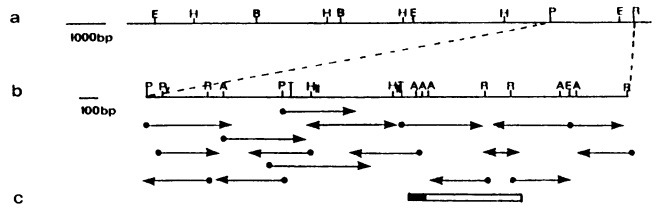

Figure 1. (a) Restriction map of the genomic clone containing the sequence coding for bovine brain ribonuclease. Only relevant restriction sites are shown. (b) Sequencing strategy for a 2.4 kb fragment of the recombinant phage containing the ORF. (c) Orientation of the coding sequence. The black box corresponds to the signal peptide. A: *Ava*II; B: *Bam*HI; E: *Eco*RI; H: *Hind*III; HII: *Hind*II; P: *Pst*I; Pv: *Pvu*II; R: *Rsa*I; T: *Taq*I.

Nucleotide sequence of the bovine brain ribonuclease gene

The nucleotide sequence of a genomic DNA fragment containing the bovine brain ribonuclease gene is shown in Fig. 2 (line 2); the homology to the gene encoding the bovine pancreatic enzyme (line 1) is also indicated. The region from nt 1347 to nt 1847 contains an open reading frame (ORF) which is not interrupted by intervening sequences. The first 78 nts of the ORF code for a peptide of 26 amino acid residues, which is absent in the native protein, and shows a high degree of identity to the signal peptides of pancreatic and seminal RNAases (fig. 3). The DNA sequence from nt 1425 to nt 1847 codes for the native brain ribonuclease and its deduced protein sequence is in good agreement with the protein sequence determined by Watanabe *et al.* (1). Only two discrepancies were detected. Firstly a Thr instead of a Ser residue was found at position 129. Like Ser 133, a serine at position 129 was not determined directly from the protein sequence, but its existence was inferred by the transformation to Ala following deglycosylation and reduction; a threonine will form amino butyric acid upon this treatment. Secondly an additional Leu residue was found at the C-terminal end of the ORF. This finding is in agreement with the amino acid composition of C-terminal peptides presented by Watanabe *et al.* (1). The carboxy-terminal extension of the brain enzyme is encoded by a DNA region which diverges considerably from the corresponding sequences of the pancreatic and seminal ribonuclease (4−6) messages. The homology between the two genes is not restricted to the open reading frames but it extends to the upstream and downstream flanking regions. CAAT and TATA elements, as well as the splice signals located at the boundaries of the unique intervening sequence present in the 5' untranslated region of the homologous pancreatic gene, are conserved. A relatively large insertion (nt 877 to nt 948) and a deletion (nt 352) were found in the BRb RNAase gene, relative to the pancreatic RNAase gene. The 3' untranslated region of the gene is characterized by a stretch (23 repeats) of the CA dinucleotide. This sequence is also present, but less extended, in the pancreatic ribonuclease gene and in the mRNA of the seminal enzyme. The polyadenylation signal is located at corresponding sites in the transcription units of the brain and pancreatic ribonuclease coding genes.

Repetitive elements were found within the sequence shown in fig 2. Regions from nt 15 to nt 43 and from nt 886 to nt 916 are homologous to the 3' end of the consensus sequence derived from repetitive sequences of the bovine ACTH-βLPH precursor gene (17). These elements were also found in the gene coding for the pancreatic enzyme (4), although their relative locations are different.

Figure 2. Nucleotide sequence of the bovine brain ribonuclease gene (line 2). The homology to the bovine pancreatic ribonuclease gene (line 1) is shown. CAAT and TATA consensus sequences conserved in both genes are underlined. 5′ and 3′ splice sites are marked by arrows. The polyadenylation signal is marked by dots. Translation of ORF coding for the bovine brain ribonuclease is shown.

BRb RNAase gene is present as a single copy in the bovine genome as suggested by Southern blot analysis performed under stringent conditions (4). A single band was detected with *EcoR*I, *Pvu*II or *Pst*I restriction enzymes using a fragment encoding the brain enzyme (nt 1280 to nt 1698) as a probe (data not shown).

The transcription unit of the BRb RNAase gene

The elongation of a fragment corresponding to the coding region of the BRb RNAase gene (see Methods), labelled with ^{32}P at its 5′ end, yielded an extended product of 168 nts (not shown). Assuming that excision of an intervening sequence occurs at positions corresponding to the splice sites of the pancreatic RNAase gene (4), the results of this experiment locate the transcription initiation site at nt 497. In order to demonstrate that the processing of primary transcripts is similar in the two homologous genes, we sequenced a PCR amplified fragment of a cDNA obtained by priming the reverse transcriptase with oligonucleotide 1, complementary to the coding sequence of the BRb RNAase gene. Oligonucleotide 1 and oligonucleotide 2 were used to prime the Taq DNA polymerase activity, while the nested

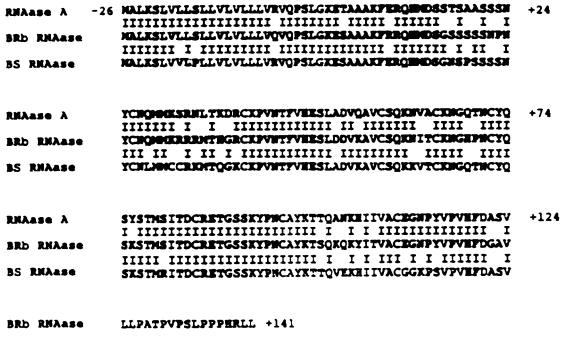

Figure 3. Comparative analysis of the amino acid sequences of the bovine pancreatic (RNAase A), cerebral (BRb RNAase) and seminal (BS RNAase) ribonuclease preproteins.

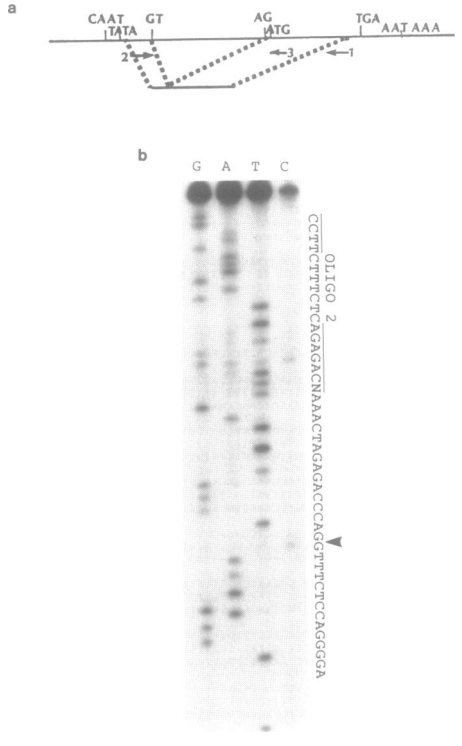

Figure 4. (a) Diagram of the BRb RNAase gene structure and the PCR amplified fragment; not in scale. 1, 2, 3, indicate respectively the position and orientation of the oligonucleotide 1, oligonucleotide 2, oligonucleotide 3 (see Methods). (b) Sequence analysis of the PCR amplified products. The complementary nucleotide sequence is shown. The position corresponding to the splice junction is marked by an arrow.

oligonucleotide 3 was used as a primer to sequence the amplified fragment (see Fig. 4a and Methods). The size of this fragment (about 500 bp, not shown) and its nucleotide sequence indicate (Fig. 4b) that splicing of the BRb RNAase gene primary transcript occurs at positions corresponding to the splice sites of the bovine pancreatic RNAase message. The nucleotide region sequenced in this experiment is identical in the two genes. However, the restriction pattern of the amplified fragment, as well as the sequence of a clone obtained from the amplified products, demonstrate that they are derived from the BRb RNAase mRNA (data not shown).

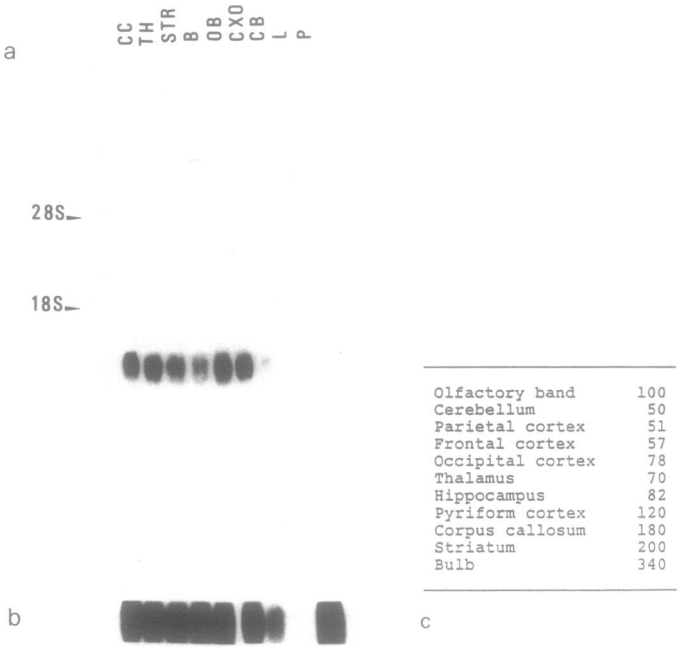

Figure 5. Northern blot analysis of the RNA isolated from different regions of the brain and hybridized to (a) an oligonucleotide encoding the carboxy-terminal peptide of the BRb RNAase or (b) a fragment encoding the majority of the BRb RNAase. CC: corpus callosum; TH: thalamus; STR: striatum; B: bulb; OB: olfactory band; CX: cortex; CB: cerebellum; L: liver; P: pancreas. (c) Levels of BRb RNAase mRNA. Values are expressed as a percentage of mRNA determined in olfactory band.

Table I. Ribonuclease levels in extracts of bovine brain.

Extract	Specific activity Units/mg of protein		Protein mg/g of tissue
	yeast RNA	Poly(C)	
Grey matter	1.48	8.94	11.5
White matter	3.27	19.78	5.5
Cerebellum	0.87	n.d.	9.2
Temporal lobe	1.53	n.d.	8.5
Occipital lobe	1.48	n.d.	8.1
Parietal lobe	1.64	n.d.	7.9
Frontal lobe	1.13	n.d.	7.6
Astrocytes	n.d.	7.59	n.d.
Neurons	n.d.	2.90	n.d.

Each value is the average of duplicate determinations obtained with three different specimens of brain tissue; n.d.: not determined.

Distribution of the BRb RNAase mRNA in the brain

Total RNA purified from the corpus callosum, thalamus, striatum, bulb, olfactory band, occipital cortex and cerebellum was hybridized to a ^{32}P labelled 54mer oligonucleotide complementary to the region encoding the carboxy terminal segment of the brain ribonuclease (nt 1797 to 1850). This probe does not hybridize to the transcripts of the pancreatic and seminal enzymes. The BRb RNAase mRNA was found to be present in all the region of the brain tested, although it was not expressed at a detectable level in the liver and pancreas (Fig. 5a). This mRNA is about 900 nucleotides in length which is similar to that of mRNAs of bovine seminal (5) and ox (4), rat (18), mouse (19) and pig (20) pancreatic ribonucleases. A band of the same

size was also detected when a fragment encoding the brain enzyme, which also hybridizes to the pancreatic transcript, was used as a probe (Fig. 5b). The size of the brain RNAase mRNA is in agreement with the transcription unit defined in the experiments described above. The level of the specific message detected in various regions of the ox brain was determined by slot blot hybridization of total RNA isolated from different cerebral areas. All the results were normalized relative to the level of actin mRNA and expressed as a percentage of the brain RNAase mRNA present in the olfactory band (Fig. 5c).

Distribution of the BRb RNAase in the brain

Ribonuclease activity was assayed in homogenates of regional brain sections in order to study the distribution of the enzyme in different areas of the brain (Table I). Ribonuclease activity can be measured reliably only in the presence of PHMB, because the extracts contain a high amount of an inhibitory protein (21) which is sensitive to the -SH titrating agent. The ribonucleolytic activity measured in extracts of grey and white matter is inhibited by antibodies raised against BRb RNAase (data not shown). Ribonuclease specific activity in extracts of white matter is twofold higher than in extracts of grey matter, and is lower in the cerebellum than in the temporal, occipital, parietal and frontal lobes. In order to investigate whether a specific cell type is responsible for the ribonucleolytic activity, extracts derived from fractions enriched in neurons or astrocytes were analyzed. As shown in Table I, ribonuclease activity appears to be present both in neurons and glial cells.

DISCUSSION

Although the genes encoding the bovine brain and pancreatic ribonucleases are very similar, both the coding and flanking regions sharing a high degree of identity, there are nevertheless some interesting differences. Thus an insertion of 168 nucleotides, located 11 nts upstream to the conserved CAAT element is present in the pancreatic ribonuclease gene. The region upstream to the CAAT and TATA promoter elements is known to be involved in the transcriptional regulation of downstream sequences and thus lack of homology in this region may be related to the differential expression of these genes. The transcription unit of the brain ribonuclease gene, defined in this study, is similar to that determined for the pancreatic ribonuclease gene. The unique intervening sequence in the 5′ untranslated region of the message is a structural feature conserved in the genes of the mammalian pancreatic ribonuclease superfamily characterized to date (22). A comparative analysis of the genes coding for bovine ribonucleases (4, 5) clearly shows that the difference of the carboxy-terminal segment in ox ribonucleases is not generated by a shift of the stop codon as a consequence of a point mutation, but by the occurrence of substitutions and deletions in this region. This observation may be of some interest with respect to the low rate of evolutionary divergence reported for ribonucleases of the pancreatic type in Ruminants (2). Phylogenetic analysis based on the primary structure of 41 mammalian ribonucleases, indicates that gene duplication events, leading to the presence of three closely related genes in the bovine species, took place during the evolution of Ruminants. In agreement with this observation Southern analysis indicates that three sequences related to the coding region of the ox pancreatic ribonuclease gene are present in the genome of Ruminants, whereas only one seems to exist in other mammals (23).

The presence of ribonucleases active at alkaline pH and sensitive to the protein inhibitor in the central nervous system of various mammalian species both in the grey and white matter has been already reported (24–30). We have shown herein that the synthesis of the BRb RNAase is not restricted to a specific area of the brain or to a specific cell type, both the mRNA and the ribonuclease activity were in fact detected in all the regions examined, suggesting a role for the BRb RNAase in brain physiology.

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