An Alu-linked Repetitive Sequence Corresponding to 280 Amino Acids Is Expressed in a Novel Bovine Protein, but Not in Its Human Homologue*

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A novel protein harboring a 280-amino acid region from an Alu-linked repetitive sequence (bovine Alu-like dimer-driven family) was isolated from a bovine brain S-100 fraction using monoclonal antibodies against a rat GTPase-activating protein that shares the same epitope. The protein has an apparent molecular mass of 97 kDa (p97). Western blot analysis using extracts prepared from various tissues showed p97 to be predominantly detected in brain and moderately in liver and lung. From sequence analysis of the cDNA encoding p97, it was found that the 840-base pair sequence homologous to a part of the bovine Alu-like dimer-driven family, which has never been shown to be expressed, occurs in the middle of the protein coding region. The protein also contains a pair of intramolecular repeats composed of 40 highly hydrophilic amino acids at the C terminus. Human cDNA homologous to p97 was cloned, and its nucleotide sequence demonstrates that the 840-base pair repetitive sequence and one of the intramolecular repeats are missing. We named p97 bovine BCNT after Bucentaur. These results show that bovine BCNT is a unique molecule and suggest that an analysis of the relationship between bovine bent and its human homologue may help further the understanding of gene organization and evolution.

Short and long interspersed repetitive DNA elements, SINE and LINE, are widely distributed in mammals, and a few copies move within the genome as transposable elements. The Alu family is a representative of SINE and exists in nearly 10^6 copies/molecule and suggest that an analysis of the biological meaning of repetitive sequences. In bovine, in addition to the Alu-like elements, there is a family of 3.1-kbp repetitive sequences, called the bovine Alu-like dimer-driven family (BDDF), whose 5′- and 3′-ends are flanked by sequences homologous to the bovine Alu-like sequence (7). During the preparation of monoclonal antibodies (mAbs) against a ras GTPase-activating protein (GAP), GAP1m (a GAP with two C2 domains and Bruton’s tyrosine kinase homology domain) (8), we identified a protein from bovine brain extract whose biochemical characteristics are similar to rasGAP1m such as molecular mass and affinity for a heparin. Since we observed that ras GTPase activating activity other than that attributable to known rasGAPs might be present in the heparin column chromatography fractions (9), we suspected that this might be a novel protein with a GAP-like structure. To see if this was true, we purified the protein and cloned its cDNA. The cDNA sequence analysis showed that this is a novel protein containing a region homologous to a part of BDDF, but without GAP-like structure. Since a BDDF region has never been shown to exist as a protein, we further characterized p97, including its tissue expression, and compared it with its human homologue. The repetitive sequence was not detected in the human homologue. From the processes involved in the isolation of the new molecule, we named p97 bovine BCNT after Bucentaur.

EXPERIMENTAL PROCEDURES

Preparation of Antigen—Escherichia coli cells containing a plasmid expressing the rat gene, gap1m, covering 90% of the ORF (from Ile^55 to Ser^94) (8) fused to glutathione S-transferase (GST) were grown in LB medium. The fusion protein was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside at 25°C for 14 h. The cells were pelleted from 5 liters of culture and lysed with a French press in the presence of protease inhibitors (100 μM Pefabloc and 20 μg/ml each antipain, leupeptin, peptatin A, and aprotinin); the lysates were cleared by centrifugation at 15 k rpm for 30 min (Beckman JA-20 rotor) at 4°C. The supernatant was applied directly to a column of glutathione-Sepharose (Pharmacia Biotech Inc.), and the adsorbed proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 9.6) after washing with 50 mM glutathione.

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‡ The abbreviations used are: kbp, kilobase pair(s); bp, base pair(s); BDDF, bovine Alu-like dimer-driven family; mAb, monoclonal antibody; GAP, Ras GTPase-activating protein; ORF, open reading frame; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; IR, intramolecular repeat.


**Alu-linked Repetitive Sequence-containing Novel Bovine Protein**

A (9) containing 0.2M NaCl and applied to a heparin column (1 ml of dialyzed against phosphate-buffered saline and then against buffer A containing 0.2M NaCl and 10 mM Tris-HCl (pH 8.0), without dithiothreitol, and then eluted with 0.1 M glycine HCl (pH 2.5). Purified IgGs were concentrated by AmSO4 precipitating 0.5M NaCl. The preparation (2.5 mg) was then coupled to HiTrap/Pharmacia Biotech Inc.) by incubation for 4 h at 4 °C on a rotary shaker. The coupling yield was estimated to be 88% by determination of protein concentration.

**Isolation of a Anti-p97 (BCNT) IgG Column—Supernatants from serum-free cultures of four hybridoma clones (GIT medium, Nissui, antibody isotyping kit (Amersham Corp.). Four of the clones were used overallyield was estimated to be 88% by determination of protein concentration.

**Isolation of p97 (Bovine BCNT)—Detection of p97 (bovine BCNT) was carried out by Western blotting as described previously (12). A bovine brain S-100 fraction (4.4 mg/ml, 300 ml) prepared as described previously (9) was fractionated with AmSO4, (30–55%). The precipitate was dialyzed against phosphate-buffered saline and then against buffer A (9) containing 0.2 M NaCl and applied to a heparin column (1 ml of HiTrap, Pharmacia Biotech Inc.). The column was sequentially washed with buffer A containing 0.2 or 0.3 M NaCl, and the antigen was eluted with buffer A containing 0.7 M NaCl but without dithiothreitol. The eluates were applied to an anti-p97 IgG-HiTrap column, washed with buffer A containing 0.2 M NaCl and 10 mM Tris-HCl (pH 8.0) but without dithiothreitol, and then eluted with 0.1 M glycine HCl (pH 2.5). The eluate was immediately neutralized with Tris-HCl (pH 8.8). The samples were concentrated 10-fold with Ultrafree (30,000-Da cutoff; Millipore Corp.). Purity was estimated by subjecting the preparations to two-dimensional gel electrophoresis as described previously (13). The overall yield was ~10%, and the degree of purification was ~20,000-fold compared with the S-100 fraction, as estimated by densitometric analysis.

**Determination of the Partial Amino Acid Sequence of p97 (Bovine BCNT)—** Partially purified p97 (bovine BCNT) was separated by 7.5% SDS-PAGE, blotted onto a polyvinylidene difluoride membrane (Immo-B-P, Millipore Corp.), and stained with 0.1% Ponceau S in 1% acetic acid.

**Isolation of p97 (Bovine BCNT)—** Detection of p97 (bovine BCNT) was carried out by Western blotting as described previously (12). A bovine brain S-100 fraction (4.4 mg/ml, 300 ml) prepared as described previously (9) was fractionated with AmSO4, (30–55%). The precipitate was dialyzed against phosphate-buffered saline and then against buffer A (9) containing 0.2 M NaCl and applied to a heparin column (1 ml of HiTrap, Pharmacia Biotech Inc.). The column was sequentially washed with buffer A containing 0.2 or 0.3 M NaCl, and the antigen was eluted with buffer A containing 0.7 M NaCl but without dithiothreitol. The eluates were applied to an anti-p97 IgG-HiTrap column, washed with buffer A containing 0.2 M NaCl and 10 mM Tris-HCl (pH 8.0) but without dithiothreitol, and then eluted with 0.1 M glycine HCl (pH 2.5).

**Expression of p97 (Bovine BCNT) Antibodies—** Monoclonal antibodies against p97 (bovine BCNT) were then used for epitope mapping. A plasmid containing the full-length cDNA of bovine BCNT was fragmented to an average size of 300 bp and used for screening with anti-p97 (BCNT) mAbs. Seven positive clones were obtained among ~9 × 10^6 bacterial colonies. The sequence common to all clones was determined as the possible epitope for anti-p97 antibodies.

**Northern Blotting of the Bovine Genome—** Bovine genomic DNA was prepared from liver and digested with various restriction enzymes. Ten micrograms of DNA lane was electrophoresed on a 0.7% Tris borate-EDTA-agarose gel and blotted onto a nylon membrane. The membrane was prehybridized at 68 °C in hybridization buffer (6 × SSC, 5 × Denhardt’s solution, 0.1% SDS, 0.1 mg/ml salmon sperm DNA) and probed with a 32P-labeled 317-bp HindIII-AIII (1480–1796) fragment encompassing most of the IR sequences. Final washing was performed at 66 °C with 0.1 × SSC, 0.1% SDS.

**Expression of p97 (Bovine BCNT) Gene Clones—** Five micrograms of bovine brain poly(A)+ mRNA prepared as described above were separated and blotted onto nitrocellulose as described previously (18). The filter was probed with the 5′-region of p97 (bovine BCNT) DNA (nucleotides 1–697 of the cDNA) labeled with [32P]-dCTP (3000 Ci/mmol) by a random priming method at 42 °C for 14 h and then washed under stringent conditions (0.1 × SSC, 0.1% SDS at 68 °C for 80 min).

**Expression of p97 (Bovine BCNT) Expression in Various Tissues—** Organs were freshly isolated, cut into small pieces, and frozen immediately. The tissue samples were then subjected to protein extraction buffer (1% SDS, 1 mM EDTA, 10 mM Hepes/NaOH (pH 7.4)) supplemented with protease inhibitors (100 μM Pefabloc and 20 μg/ml each antipain, leupeptin, pepstatin A, and aprotinin) with a Dounce homogenizer. The extracts were then boiled immediately for 5 min and subjected to sonication and centrifugation at 10,000 × g for 10 min. The protein concentrations in the supernatants were estimated using a BCA kit (Pierce). Constant amounts of protein were separated by 12.5% SDS-PAGE and subjected to Western blotting.

**Miscellaneous—** Protein was detected by Coomassie Brilliant Blue or silver (Bio-Rad) staining, depending on the amount of protein. Bovine brain was obtained from a local slaughterhouse.

**RESULTS**

**Purification of Bovine BCNT—** Although a GST fusion protein of rat GAP1 was used for immunization, the five isolated mAbs recognized a 97-kDa protein in bovine brain (bovine BCNT) more strongly than the purified rat GAP1 (data not shown). Both proteins share similar molecular characteristics such as molecular mass on SDS-PAGE and heparin affinity. To examine the relationship between BCNT and GAP1, we isolated the BCNT protein. As shown in Fig. 1, the isolated preparations showed homogeneity with respect to both isoelectric point and molecular mass on two-dimensional gels, the
silver staining patterns and Western blots were quite similar to each other, indicating high purity of the protein. Therefore, these bands were blotted onto polyvinylidene difluoride filters and excised for lysyl endopeptidase digestion followed by HPLC. Amino acid sequences of the separated peptides were determined and found to represent a novel protein. Thus, we went on to clone the cDNA of bovine BCNT.

Cloning of the cDNA Encoding Bovine BCNT—We performed PCR on bovine brain cDNA using degenerate primers corresponding to the determined amino acid sequence and obtained a 1-kbp fragment. Then, the rest of the gene was obtained using the 5'- and 3'-RACE method. The ORF deduced from the DNA sequence consists of 592 amino acids and includes all the determined amino acid sequences of the peptides derived from the digests of purified BCNT (Fig. 2). To eliminate PCR artifacts, we also isolated five cDNA clones from a bovine brain phage library by screening with 53-mer oligonucleotides corresponding to a partial peptide sequence. Nucleotide sequence analysis revealed that the RACE product and all phage clones share the same part of the ORF, but one clone resulted in a shorter ORF (Fig. 3). We used the longest ORF for further analysis.

Sequence Analysis—Contrary to our expectations, the BCNT cDNA and gap1m genes from rat (8) and human (20) showed no significant homology except for a small amino acid sequence as shown below. A data base search of the obtained DNA sequence revealed that BCNT is a novel protein with a calculated molecular mass on SDS-PAGE (97 kDa), the cloned cDNA was expressed in COS-7 cells. As shown in Fig. 5, bovine BCNT mRNA included a major band at 3.1 kilobases, which is consistent with the size of the 2.8-kbp cDNA obtained by RACE. Western blot analysis of the total proteins from untreated COS-7 cells or their transfectants with vector alone or plasmid expressing the antisense cDNA showed a 47-kDa band, but not the 97-kDa band (Fig. 5b). On the other hand, the extracts from cells with plasmids expressing the sense cDNA showed the presence of a protein with the same molecular mass as BCNT (97 kDa) from bovine brain extracts on SDS-PAGE (Fig. 5b).

Expression of BCNT in Various Tissues—To gain insight into the biological function of BCNT, we examined the expression of BCNT in various bovine tissues by Western blotting. BCNT was found to be expressed predominantly in brain, moderately in liver and lung, and in small amounts in heart (Fig. 6).
a

FIG. 2. Nucleotide sequence of BCNT and its deduced amino acid sequence. a, the full-length cDNA of BCNT was constructed by 3’- and 5’-RACE or was isolated from a bovine brain cDNA library. Numbers start with the ATG start codon (GenBankTM accession number D84513). The peptide sequences determined are underlined. The BDDF region is shaded. The postulated poly(A) addition signal sequence is in italics.

b

amino acids while human BCNT does not, to understand its function, it is essential to clarify whether there is another copy of the bcnt gene without the repetitive sequence present in bovine. As described above, anti-bovine BCNT mAbs that recognize the molecule in a region outside the repetitive sequence detect one band in the bovine extract. To obtain data at the
genomic level, we performed Southern blotting of the bovine genome cleaved with different restriction enzymes using the 3'-region of bovine \textit{bcnt} excluding the 840-bp repetitive sequence as a probe. The result revealed only one band with \textit{Bam}HI, \textit{Eco}RI, and \textit{Hind}III and two bands with \textit{Bgl}II, strongly suggesting that there is only one gene (Fig. 7), consistent with the result that PCR carried out on bovine brain cDNA using a set of primers flanking the repetitive region gave no shorter fragment (data not shown).

\section*{DISCUSSION}

In this paper, we describe the structure and characteristics of a novel protein, BCNT. The BCNT cDNA sequence from bovine brain shows the existence of a repetitive sequence of BDDF in its ORF, which has never been revealed as a protein. However, this cannot be discounted as a simple PCR artifact because 1) a peptide sequence was found to match this region; 2) library screening resulted in no clone without this region; and 3) PCR carried on bovine brain cDNA using a set of primers flanking this region gave no shorter fragment. Although several proteins are known to possess a part of the repetitive sequence (4), a sequence as long as that in BCNT is very rare. Recently, we found that the 840-bp repetitive sequence also occurs in sheep and giraffe, but not in human and pig.\textsuperscript{3} Therefore, the repetitive sequence associated with the \textit{bcnt} gene was specific to \textit{Ruminantia}, and the cDNA difference between human and bovine might be caused at the level of genomic organization. Two 7-nucleotide sequences (TAATACC) and three hexamer repeats (GTCAGG) at both boundaries of the 840-bp and IR sequences might be involved in the integration of the repetitive sequence.

The molecular mass deduced from the ORF of the bovine BCNT cDNA is only 66,000 Da, which is less than the apparent molecular mass on SDS-PAGE. However, the apparent size is consistent with that of the protein expressed in COS-7 cells.

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Bovine extracts containing 40 μg of protein from the various tissues indicated were probed with anti-BCNT mAbs.

transfected with plasmids expressing the cDNA. The results of Northern blotting show a major band at 3.1 kilobases. The cDNA sequence around the potential initiation Met codon matches Kozak’s rule well (21). Sequence comparison with human BCNT cDNA shows that the 5′-end of this region is not conserved between the two despite high homology in the postulated ORF regions. Therefore, although post-modification such as phosphorylation may also contribute to the apparent molecular size difference, we conclude that the cDNA codes the full-length ORF. The GST fusion protein of human EST expressed in E. coli migrates around 68 kDa on SDS-PAGE, although it was calculated to be 48 kDa. This result implies that the BDDF homologous region is not the sole reason for the anomalous behavior of bovine BCNT on SDS-PAGE. This tendency has been reported in some hydrophilic proteins (22, 23) such as neuramidin, tau, and calpastatin. Since bovine BCNT is rich in charged amino acids (Glu, 11.2 mol %; and Lys, 8.8 mol %) and is especially rich in Glu in its N-terminal region, it provides another example of abnormal electrophoretic mobility on SDS-PAGE.

It is essential to clarify the function of BCNT and how the repetitive region is related to this function. Therefore, it is critical to evaluate whether there is only one bcnt gene in bovine and whether there is an isomer(s) of BCNT without the repetitive sequence region. Southern blotting of the bovine genome probed with the 5′-region of bcnt excluding the repetitive sequence showed that there is one gene. Consistent with this result, all phage clones obtained with the 53-mer nucleotide corresponding to the region other than the repetitive portion included the same 280-amino acid repetitive region, although one clone had a shorter C terminus (Fig. 3). Furthermore, we immunoblotted bovine tissue extracts with anti-bovine BCNT mAbs whose epitopes are located in regions other than the repetitive region. These mAbs detected only one major band, although a more detailed study is required.

The expected molecular mass of human BCNT was calculated to be 24 kDa. It is intriguing that anti-BCNT mAbs recognized a 47-kDa protein, but not a 97-kDa protein, in extracts from COS-7 cells (see Fig. 5b) or human neuroblastoma SH-SY cells (24). A 47-kDa protein was also found in rat brain and rat fibroblast 3Y1 cells. Its identification is now under investigation.

To gain insight into the function of BCNT, we examined BCNT expression levels in various tissues and found that bovine BCNT is expressed predominantly in brain. Furthermore, we observed that BCNT is localized in Purkinje cells in bovine cerebellum. Both bovine and human BCNT proteins have many consensus phosphorylation sites for casein kinase II (25), which is localized in nuclei. In fact, both human and bovine BCNT proteins are phosphorylated by casein kinase II.

A mouse EST (GenBank™ accession number W83351) shows high homology to human bcnt. This may indicate that BCNT is highly conserved in mammals, but that a repetitive sequence is inserted in the bovine protein. No sequence homologous to bcnt is found in the genome sequence of Saccharomyces cerevisiae. Mammalian conservation suggests a biologically important role for this family. Although the function of BCNT is not yet clear, an analysis of the relationship between bcnt and its human homologue may shed light on gene development and organization.

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