Predicted structure of the bovine calcitonin gene-related peptide and the carboxy-terminal flanking peptide of bovine calcitonin precursor

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ABSTRACT

We have isolated from a bovine genomic library a clone which contains the calcitonin (CT) and CT gene-related peptide (CGRP) sequences, using probes representing the human CT and CGRP sequences. Sequence analysis has identified the nucleotide sequence coding for bovine CT, its C-terminal flanking peptide and bovine CGRP. The deduced amino acid sequence of bovine CGRP revealed a significant homology with other CGRPs so far reported. It differs by only one amino acid from rat CGRPζ and porcine CGRP, and by three and four amino acids from human CGRPβ and α respectively. Bovine CT has, however, only 14 out of 32 residues in common with human CT. As in the human CT precursor, the C-terminal flanking peptide of bovine CT precursor is a 21 amino acid peptide. It shares only 11 residues in common with its human counterpart. This study thus provides further evidence that CGRP, in contrast to CT and its C-terminal flanking peptide, is a highly conserved molecule.

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INTRODUCTION

The organization and sequence of the calcitonin (CT) gene in two species, rat (Amara, Jonas & Rosenfeld, 19826) and man (Edbrooke, Parker, McVey et al. 1985; Jonas, Lin, Kawashima et al. 1985), have been established. In these two species the CT gene codes for the precursor of two peptides: CT' and CT gene-related peptide (CGRP). The two mRNAs arise by alternate splicing of a common precursor RNA. The gene stretches over approximately 6.5 kb and consists of six exons; the first three exons are present in both CT and CGRP mRNA, although exon one is not translated. Exon four contains the CT-coding sequence and a sequence encoding its C-terminal flanking peptide and this is followed by an untranslated sequence. Exon five encodes the CGRP sequence. Exon six, which is also part of the CGRP transcript, is not translated (for review see Breimer, MacIntyre & Zaidi, 1988).

Calcitonin is a hypocalcaemic hypophosphataemic peptide which is implicated in bone conservation during times of calcium stress (Stevenson, Hillyard & MacIntyre, 1979). It is produced by C cells of the thyroid gland in mammals (Foster, Baghdiantz, Kumar et al. 1964) and by ultimobranchial glands in submammalian vertebrates (Moseley, Matthews, Breed et al. 1968). Based on structure, three groups of CTs can be distinguished: (1) the primate and rodentian group: man (Neher, Riniker, Maier et al. 1968) and rat (Raulais, Hagaman, Ontjes et al. 1976); (2) the artiodactyl group: cattle (Brewer & Ronan, 1969), sheep (Potts, Niall, Keutmann et al. 1970) and pig (Potts, Niall, Keutmann et al. 1968); and (3) the teleostean and avian group: salmon (Niall, Keutmann, Copp & Potts, 1969), eel (Noda & Narita, 1976) and chicken (Lasmoles, Jullienne, Desplan et al. 1985). The degree of homology between CTs within each of the three groups is high, e.g. man and rat differ by only two amino acids. However, the homology between the groups is much lower, e.g. man and cattle share only 14 out of the 32 amino acids.

Calcitonin gene-related peptide is a neuropeptide which is widely distributed in the central and peripheral nervous system (Rosenfeld, Mermod, Amara et al. 1983; Tschopp, Henke, Petermann et al. 1985; Inagaki, Kito, Kubota et al. 1986) where it plays the role of a neuromodulator. The peptide is also a most potent vasodilator (Brain, Williams,
Tippins *et al.* 1985) and is likely to modulate the vascular tone. In contrast to CT, CGRP appears to be a highly conserved molecule. Thus the CT gene is an interesting model for evolutionary studies as it expresses, by alternate splicing, the precursor of two peptides, CT and CGRP, the rate of divergence of which varies considerably. Until now, nucleotide sequence information has not been available for the artiodactyl type of CT. Here we provide this information and also report the deduced amino acid sequence of the bovine CGRP molecule.

**MATERIALS AND METHODS**

**Screening of the bovine genomic library and Southern blot analysis**

A bovine genomic library in λEMBL3 was kindly provided by Dr S. Ruppert (Ruppert, Scherer & Schutz, 1984). Recombinant phages (0·6 x 10⁶) were screened by plaque hybridization (Maniatis, Fritsch & Sambrook, 1982; Alevizaki, Shiraishi, Rassool *et al.* 1986), under low stringency conditions, with human DNA probes derived from Ph TB58 and Ph TB3 (Edbrooke *et al.* 1985) representing exons 5 and 4 of the human CT gene (Alevizaki *et al.* 1986). Probes were labelled by nick translation using a kit from Amersham International plc (Amersham, Bucks, U.K.) and hybridized for 18 h at 34 °C in 5 x SSPE (1 x SSPE is 180 mM NaCl, 10 mM Na phosphate buffer, pH 7·7, and 1 mM EDTA), 50% formamide, 0·1% dried milk, 0·2% sodium dodecyl sulphate (SDS) and 10% dextran sulphate. Filters were washed in 2 x SSC (1 x SSC is 150 mM NaCl and 15 mM Na citrate)/0·2% SDS at 50 °C. λDNA was prepared from positive clones (Maniatis *et al.* 1982), and a 7·5 kb EcoRI fragment containing the sequence hybridizing to the human CGRP probe was subcloned into a plasmid vector (p-Bluescript; Stratagene, San Diego, CA, U.S.A.).

DNA from this clone was subjected to restriction analysis using KpnI and PstI, electrophoresed, transferred to nitrocellulose and then probed with 32P-labelled DNA specific for human CGRP. The KpnI/Pst fragments (six) were further subcloned into Bluescript vector.

**Sequence strategy**

DNA from the KpnI/Pst clones containing sequences hybridizing with DNA probes for exons 4 and 5 of the human CT/CGRP gene were further subjected to restriction enzyme analysis using AluI and HaeIII. Restriction fragments were subcloned into p-Bluescripts, and the DNA from these clones was sequenced. Single-stranded DNA (4 μg for each primer T3 and T7) was prepared from these clones by an alkali denaturation method as follows. DNA (10 μl) was incubated with an equal volume of 2 M NaOH/5 mM EDTA at 37 °C for 10 min. The DNA was precipitated by the addition of 2 μl 3 M sodium acetate (pH 5·2) and 2–3 volumes absolute ethanol. The DNA pellet was washed in 70% ethanol and the dry pellet resuspended in 20 μl sterile double-distilled water. Amounts (10 μl) of this DNA were then mixed with 2 μg primer, heated at 70 °C for 5 min and allowed to anneal at 37 °C for 30 min. The DNA was sequenced by the dideoxy chain-termination method (Sanger, Nicklen & Coulson, 1977) using the protocol provided by the United States Biochemical Corporation (Cleveland, OH, U.S.A.), using a Sequenase kit, version 2.

**RESULTS**

When the bovine genomic library was screened under low stringency conditions with a human CGRP gene probe, five independently derived clones were identified and one was selected for detailed study, as it contained sequences hybridizing with probes for exons 4 and 5 of the human CT/CGRP gene. The restriction map of the 7·5 kb EcoRI fragment isolated from the λ clone is shown in Fig. 1.

The nucleotide sequence of a 532 bp AluI fragment revealed the presence of an open reading frame coding for bovine CGRP, a 37 amino acid peptide.

![Figure 1](https://via.placeholder.com/150)

**FIGURE 1.** Restriction site map of the isolated bovine genomic DNA fragment which contains the calcitonin and calcitonin gene-related peptide sequences.

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with a C-terminal glycine residue which is presumed to give rise to an amidated peptide (Fig. 2).

Sequence analysis of DNA from a cloned 705 bp HaeIII fragment revealed the presence of an open reading frame coding for bovine CT and its C-terminal flanking peptide. As in human, murine and chicken procalcitonin, bovine CT is preceded by a Lys-Arg cleavage site and followed by a Gly-Lys-Lys-Arg sequence, characteristic of an amidation and proteolytic cleavage site (Fig. 3).

**DISCUSSION**

We have isolated from a bovine genomic library a clone which contains both CRGPs and CT sequences within a 7.5 kb fragment which presumably represents the entire gene. Determination of the bovine CRGP sequence brings the total number of CRGPs now characterized to seven (Fig. 4). The amino acid sequence is remarkably conserved, with 26 of the residues absolutely conserved. The capture ring structure (residues 2–7) appears to be particularly important and is also conserved in the distantly related pancreatic peptide amylin (Cooper, Willis, Clark et al. 1987; Westmark, Wernstedt, Wilander et al. 1987). There is strong homology with other CRGPs; bovine CRGP differs by only one amino acid from porcine CRGP (Kimura, Sugita, Kanazawa et al. 1987) (aspartic acid at position 31 in porcine CRGP instead of asparagine in bovine and other CRGPs which is the result of a single nucleotide base substitution) and from rat CRGP\(\beta\) (Amara et al. 1982b), by two residues from rat CRGP\(\beta\) (Amara, Arriza, Leff et al. 1985), by just four amino acids from human CRGP\(\alpha\) (Morris, Pánico, Etienne et al. 1984) and chicken CRGP (Minvielle, Cressent, Lasmoles et al. 1986) and by only three residues from human CRGP\(\beta\) (Steenbergh, Höppener, Zandberg et al. 1985).

The deduced amino acid sequence of bovine CT is in complete agreement with the published amino acid sequence of the isolated peptide (Brewer & Ronan, 1969). Bovine CT is highly diverged from its human counterpart, only 14 of its residues corresponding with those of human CT.

```plaintext
GGCCTTTTTCCTTTTCATCTCTGGAAACCACG
ATCTACTGGCAGAAGG

AGG TCC TGC AAC ACT GCC ACC TGT GTG ACC CAT CGG CTP
R S C N T A T C V T H R L

GCA GCC TGG CTC AGG AGA TCT GGG GGT GTG GTA AAG AGC
A G L L S R S C G G V V K S

AAC TTT GTG CCC ACC AAC GTG GCC TCT GAA GCC TTT GCC
N P V T P T N V G S E A F G

CGG CGC CGC AGG ACC CTT CAG GAC TGA gcagagaagatgactctgg
R R R R T L Q D -

aggtgatttgccctttgtactgtgacaagggagaagggattttggagattagggactcgatatttt

gaaaccactgatgtttcagatctcctcatcataaattttcttgatgtagttagtgacctctcatagctgt

tggcatagccttattgcaactaccagatctctggttcagacgaccagcgatgtgcaccccctcctacaatcccgtgc

tagctcaacactttctactcgggtggttcacagaccagctgactgctggtccacggttaaaggtgacctggcaac

aggtcatgggcttacacatcttcctactcgggtgttcagacgaccagcgatgtgcaccccctcctacaatcccgtgc

acgcgcggtgtacaccgcttgattgcaactaccagatctctggttcagacgaccagcgatgtgcaccccctcctacaatcccgtgc

ggttgg

**FIGURE 2.** Nucleotide sequence of the 532 bp AluI fragment showing the predicted amino acid sequence of bovine calcitonin gene-related peptide (underlined). Upper-case letters represent the exon sequence and lower-case letters the intron sequence.

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Like many peptides, CT is synthesized as a large precursor molecule, where it is flanked by amino- and carboxy-terminal peptides. The C-terminal flanking peptide of human CT precursor (Craig, Hall, Edbrooke et al. 1982) is a 21 amino acid non-amidated peptide, while that of rat is a 16 amino acid peptide (Jacobs, Goodman, Chin et al. 1981; Amara, Jonas, O’Neil et al. 1982a). Chicken and human C-terminal peptides share only four amino acids (Lasmoles et al. 1985). In cattle (the present study) it is predicted to be 21 amino acids long. Furthermore, salmon CT precursor C-terminal flanking peptide is
only 18 amino acids long (Pöschl, Lindley, Hofer et al. 1987). The bovine C-terminal flanking peptide shares 11 out of 21 amino acids with its human counterpart (52% homology). Therefore, C-terminal flanking peptide appears to be the least conserved of all the CT/CGRP peptides (Fig. 5).

In conclusion, this study is the first report of the predicted sequence of bovine CGRP and of bovine CT precursor C-terminal peptide. It provides further evidence that CGRP, in contrast to CT and its C-terminal flanking peptide, is a highly conserved molecule.

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