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# Nucleotide sequence of cDNA of bone-mineralizing hormone calcitonin in medaka (Teleostei)

**Hidenori Sakamoto and Yuichi Sasayama***

*Noto Marine Laboratory, Division of Biodiversity, Institute of Nature and Environmental Technology, Kanazawa University, Uchiura, Ishikawa 927-0553, Japan.*

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**Abstract** Although we succeeded in amplifying cDNA fragments of calcitonin in 16 species of teleosts using reverse transcription-polymerase chain reaction (RT-PCR) with several primer sets, we could not amplify cDNA fragments of medaka calcitonin using the same primer sets. By combining the RT-PCR and rapid amplification of cDNA ends (RACE)-PCR methods, however, we were able to determine the full-length nucleotide sequence of medaka calcitonin. It was determined that the nucleotide sequence of medaka calcitonin was dissimilar to that of calcitonins from salmon, angler fish and puffer fish, but resembled that of surf fish or sole.

## Introduction

Calcitonin is a peptide hormone composed of 32 amino acid residues. This hormone is produced by cleaving procalcitonin, which consists of an N-terminal peptide, calcitonin and a C-terminal peptide (Amara *et al.*, 1982). In mammals, calcitonin lowers blood Ca levels by depressing the activity of osteoclasts to suppress mobilization of Ca from bones (Copp *et al.*, 1970). In lower vertebrates, calcitonin is secreted from the ultimobranchial gland. We recently reported that, in freshwater eels fed a high Ca consommé solution, plasma Ca and calcitonin levels increased rapidly (Suzuki *et al.*, 1999a). This suggests that calcitonin also plays an important role in Ca homeostasis in teleosts.

The teleost medaka (*Oryzias latipes*) has many advantageous characteristics as an experimental animal: it is easy to keep, it is salt-tolerant, and its reproductive system is controllable by changing the photoperiod. Therefore, we believed that elucidation of the nucleotide sequence of medaka calcitonin might contribute to increasing our knowledge of Ca homeostasis in teleosts.

In a previous study, we determined the nucleotide sequences of calcitonin from 16 species of teleosts by amplifying cDNA fragments using reverse transcription-polymerase chain reaction (RT-PCR) (Suzuki *et al.*, 1999b). As a result of that study, it became clear that the primary structure of this hormone is highly conserved among teleosts. Nevertheless, we were not able to amplify medaka calcitonin cDNA fragments, despite using the same primer set as in the RT-PCR method. In this study, therefore, we attempted to clarify the nucleotide sequence of medaka calcitonin by combining the RT-PCR method with the 5'-rapid amplification of cDNA ends (RACE)-PCR and 3'-RACE-PCR methods.

## Materials and Methods

Medaka individuals were purchased through a commercial source. Tissues containing ultimobranchial glands were dissected, immediately frozen in liquid nitrogen, and stored at –80°C until analysis. Total RNA was obtained using an isolation kit (Nippon Gene, Tokyo).

The RT-PCR method was performed as described by Iwami *et al.* (1996). Figure 1 (A) shows the locations of the nucleotide sequences of the calcitonin region and the primer regions used in RT-PCR. The N-terminal sense primer (N1), which was a 24-mer, was synthesized based on the nucleotide sequences from the 5'-region, excluding 1 bp prior to the consensus portions of the human, salmon and chicken calcitonin genes. Two types of C-terminal antisense primer were synthesized. The first C-primer (C1) was a 30-mer whose sequence included the nucleotides from the 3'-region of the salmon calcitonin gene. The second C-primer (C2) was a mixed 26-mer primer

* Corresponding to: Dr. Yuichi Sasayama, Division of Biodiversity, Institute of Nature and Environmental Technology, Kanazawa University, Kanazawa, Ishikawa 920-1192, Japan. Tel.: 81-76-264-6307, Fax: 81-768-74-1644, Email: sasayama@kenroku.kanazawa-u.ac.jp

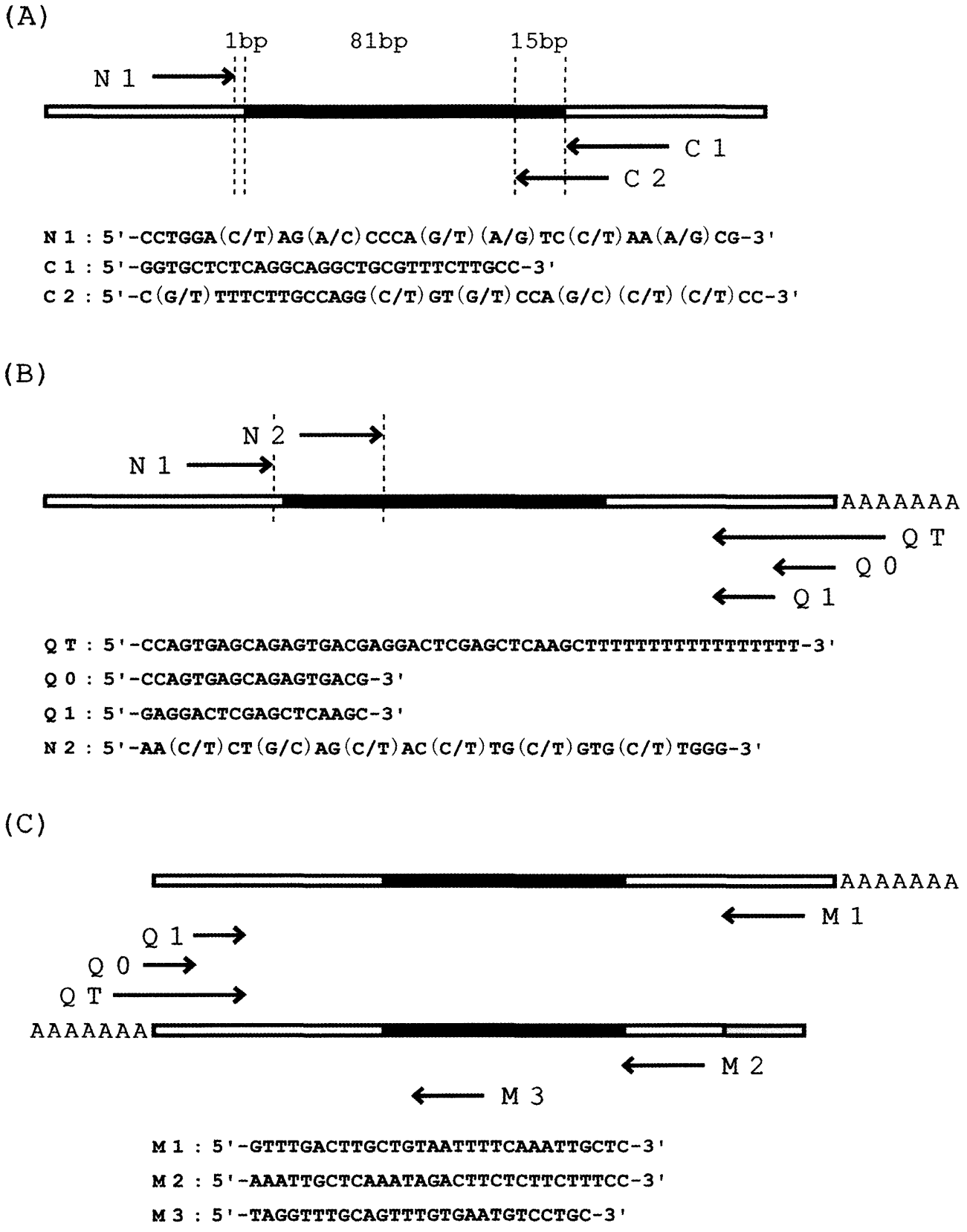


**Fig. 1.** Primer locations for RT-PCR (A), 3'-RACE-PCR (B) and 5'-RACE-PCR (C). In the figure, the black bar is the coding region for the calcitonin molecule (96 bp).

based on the consensus portions of the salmon and human calcitonin genes, and whose sequence was 15 bp inside the 96 bp of the calcitonin region. The compositions of the reverse-transcription and PCR solutions, and PCR conditions were as described previously (Suzuki *et al.*, 1997).

The RACE-PCR method was performed as described by Frohman *et al.* (1988). In Figure

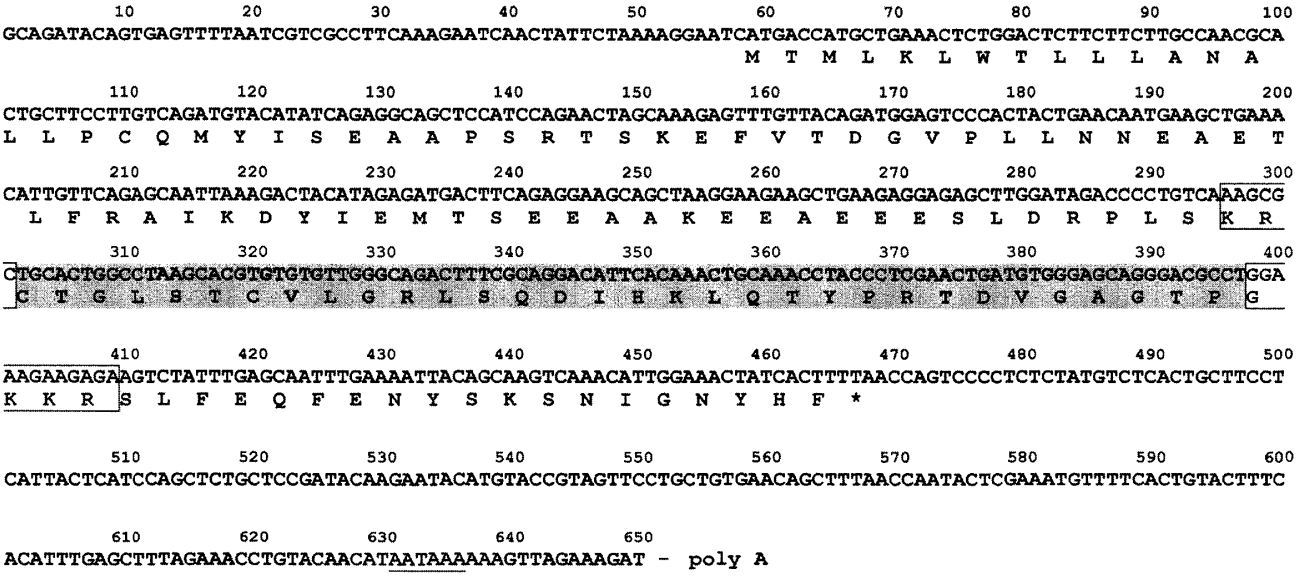


**Fig. 2.** Nucleotide sequence and deduced amino acid sequence of cDNA of medaka procalcitonin. The region shaded is the calcitonin molecule. The putative cleavage sites of the precursor are boxed. The polyadenylation signal (AATAAA) in the 3'-untranslated region is underlined.

1 (B), the primers used for 3'-RACE-PCR are shown. In the first PCR, after extension with the QT primer, the Q0 primer and the N1 primer were used to amplify cDNA fragments. In the second PCR, the Q1 primer and a new N-terminal sense primer (N2) were used. The N2 primer was a mixed 23-mer based on the consensus nucleotides of several teleost calcitonins.

In Figure 1 (C), the primers used for 5'-RACE-PCR are shown. Three types of C-terminal antisense primer (M 1,2,3) were synthesized based on the 3'-region of cDNA fragments clarified by RT-PCR and 3'-RACE-PCR. The M1 and M2 primers were 30-mers, and the M3 primer was a 27-mer. In the first PCR, after extension with the M1 primer, the Q0 primer and the M2 primer were used to amplify cDNA fragments. In the second PCR, the Q1 primer and the M3 primer were used. The conditions of RACE-PCR were as described previously (Sakamoto *et al.*, 2000).

Five micrograms of total RNA was reverse-transcribed to synthesize cDNA fragments. These cDNA fragments were separated from a reaction mixture containing the M1-primer using a PCR purification kit (Boehringer Manheim, Tokyo). Poly(A) was appended to the purified cDNA fragments. The first PCR and the second PCR were performed in the same way as 3'RACE-PCR. In the first RACE-PCR process, the QT primer was bound to the poly(A) tail portion, and double-stranded DNA was obtained. After the second reaction, the Q0 primer and the M2 primer were used to amplify the double-stranded DNA.

The PCR products were electrophoresed on 3% agarose gels and visualized by ethidium bromide staining. Only cDNA fragments located at the same position as the calcitonin gene were extracted from the agarose gel, purified, and ligated into the pT7 Blue T-Vector (Takara, Tokyo). After screening, the nucleotide sequence was determined by a DNA sequencer (Model 373S, Applied Biosystems) using the dideoxynucleotide chain terminal method.

## Results

In RT-PCR with the C1 primer, cDNA fragments coding medaka calcitonin were not amplified. Only the C2 primer was compatible. As a result, the cDNA fragments lacked the last 15 bp of the 96 bp sequence.

In order to elucidate the full-length sequence of the calcitonin gene, 3'-RACE-PCR and 5'-RACE-PCR were used. Using the 3'-RACE-PCR method, the region lacking the 15 bp and the 3'-region up to the poly(A) tail were identified. Using the 5'-RACE-PCR method, the 5'-region was clarified.

The-full-length medaka calcitonin cDNA consisted of 650 nucleotides, excluding poly(A) tail (Fig. 2). The beginning of the sequence was composed of a 58-bp 5'-untranslated region. The N-terminal peptide (237 bp) and a 6-bp putative cleavage site followed. Calcitonin (96 bp) was located in the next region. Furthermore, a 12-bp putative cleavage site, a 57-bp coding C-terminal peptide, and a 184-bp 3'-untranslated region, which includes the hexanucleotide AATAAA,

**Table 1.** Percent similarity of nucleotide sequence (A) and amino acid sequence (B) of calcitonins from various teleosts.

(A)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| MEDAKA | | | | | | | | |
| 82 | SURFFISH | | | | | | | |
| 80 | 88 | SOLE | | | | | | |
| 76 | 72 | 78 | WRASSE | | | | | |
| 75 | 72 | 76 | 81 | EEL | | | | |
| 75 | 75 | 78 | 94 | 84 | FILEFISH | | | |
| 74 | 79 | 83 | 83 | 80 | 85 | GOLDFISH | | |
| 74 | 76 | 79 | 91 | 83 | 94 | 84 | PUFFER | |
| 71 | 73 | 72 | 91 | 82 | 93 | 80 | 92 | ANGLERFISH |
| 70 | 69 | 77 | 83 | 79 | 84 | 80 | 84 | 81 SALMON |

(B)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| MEDAKA | | | | | | | | |
| 91 | SURFFISH | | | | | | | |
| 94 | 97 | SOLE | | | | | | |
| 78 | 81 | 84 | WRASSE | | | | | |
| 84 | 81 | 84 | 94 | EEL | | | | |
| 78 | 81 | 84 | 100 | 94 | FILEFISH | | | |
| 81 | 84 | 88 | 94 | 94 | 94 | GOLDFISH | | |
| 78 | 81 | 84 | 100 | 94 | 100 | 94 | PUFFER | |
| 75 | 84 | 81 | 97 | 91 | 97 | 91 | 97 | ANGLERFISH |
| 75 | 78 | 81 | 91 | 91 | 91 | 91 | 91 | 88 SALMON |

were identified. The AATAAA region is a typical polyadenylation site that located 14 nucleotides upstream of poly(A). Thus, the open reading frame of medaka calcitonin cDNA encoded an N-terminal peptide of 79 amino acid residues (aa), a putative cleavage site of 2 aa, the 32 aa calcitonin protein, a putative cleavage site of 4 aa and a C-terminal peptide of 19 aa.

## Discussion

Among teleosts, the full-length cDNA of calcitonin has only been clarified in salmon (Pöschl *et al.*, 1987). The similarity of the nucleotide sequence of the N-terminal peptide of medaka to that of salmon was 65% (154/237 bp). On the other hand, in the C-terminal peptide, the nucleotide sequence had only 30% similarity (17/57 bp). Thus, the C-terminal part of medaka calcitonin is very different from that of salmon. This would explain why RT-PCR was not able to amplify cDNA fragments coding the calcitonin region in medaka.

The nucleotide sequence of medaka calcitonin was similar to that of surf fish or sole. Furthermore, the deduced amino acid sequence of medaka calcitonin was very similar to that of surf fish or sole, as only 2 aa and 3 aa differed, respectively. However, medaka calcitonin was different from that of other fish, e.g. salmon, angler fish and puffer fish (Table 1). At present, however, the reasons for the discrepancy between these 2 teleost groups are unclear.

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