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Anuran Calcitonins are Diverse in Lower Vertebrates

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ABSTRACT—We investigated the nucleotide sequences of cDNA fragments coding calcitonin from ultimobranchial glands in 2 species of urodelans (1 salamander and 1 newt) and 4 species of anurans (1 toad and 3 frogs) by the reverse transcription polymerase chain reaction (RT-PCR) method and rapid amplification of cDNA ends (RACE)-PCR method. The salamander and newt calcitonins were each 97% and 94% similar to the lungfish and caiman calcitonins that we have already reported, in the amino acid sequences. However, anuran calcitonins were not only dissimilar (63–81%) to the lungfish and caiman calcitonins but also diversified (59–91%) even among anurans. The sequence identity of toad calcitonin was always low (59−66%) among anurans.

INTRODUCTION

Calcitonin, a peptide hormone composed of 32 amino acid residues, prevents excessive rises of blood Ca levels (Azria, 1986). As all jawed vertebrates produce calcitonin, this hormone seems to play an important role on Ca homeostasis. The primary structure of calcitonin has so far been determined in some vertebrate groups: mammals, birds, teleosts and stingrays (see Sasayama and Oguro, 1993). Recently, we reported the nucleotide sequences of cDNA fragments coding calcitonin in 4 species of reptiles (Suzuki *et al*., 1997) and in 4 primitive bony fishes such as lungfish (Suzuki *et al*., 1999). Reptile calcitonins were extremely similar (88–100% in the amino acid sequences) to both chicken calcitonin and those of the primitive bony fishes. These facts suggest that calcitonin is a very conservative hormone, at least among these animal groups. Similarly, also in amphibians, we clarified the primary structure of bullfrog calcitonin (Yoshida *et al*., 1997), which was a unique molecule because of a chimera including some amino acid residues common to mammalian and fish calcitonins. However, it is not clear whether or not this characteristic of bullfrog calcitonin is common to all amphibian calcitonins.

In this study, we tried to amplify cDNA fragments coding calcitonin molecule of 2 species of urodelans and 4 species of anurans. Furthermore, we compared the sequences of the amplified nucleotides and of the deduced amino acid sequences to those of the reptiles and fishes (Suzuki *et al*., 1997; 1999) and among amphibians as well.

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MATERIALS AND METHODS

We collected 2 species of urodelans (Tohoku black salamander: *Hynobius nigrescens*; Japanese newt: *Cynops pyrrhogaster*) and 4 species of anurans (Japanese toad: *Bufo japonicus*; Japanese tree frog: *Hyla japonica*; black-spotted pond frog: *Rana nigromaculata*; and forest green tree frog: *Rhacophorus arboreus*) near our laboratory in Uchiura Town, Ishikawa Prefecture. These amphibians were anesthetized by neutralized tricaine methanesulfonate.

Calcitonin-producing ultimobranchial glands were dissected under a binocular microscope and immediately frozen in liquid nitrogen. Total RNA was extracted using an RNA isolation kit (Nippon gene, Toyama), and subjected to a RT-PCR kit (Takara, Kyoto) according to the method of Iwami *et al*. (1996). Figure 1A shows locations of primers used in the RT-PCR method. As an N-terminal primer (N-1 primer), a mixture of 24 mer was synthesized from the 5'region excluding 1 bp prior to the portion coding calcitonin molecule, on the basis of the consensus of the nucleotide sequences of the human, salmon and chicken calcitonins. On the other hand, three kinds of C-terminal primers were synthesized. The first C-primer (C-1) was composed of 30 mer from the 3'region of the portion coding salmon calcitonin. The second C-primer (C-2) was 30 mer as well but was based on the chicken calcitonin. The third C-primer (C-3) was a mix primer of 26 mer based on the consensus of the portions coding salmon and human calcitonins. The compositions of the reverse-transcription solution, the PCR solution, and the conditions of PCR reaction have been previously described (Suzuki *et al*., 1997).

Furthermore, 3'-RACE-PCR method was also applied. Oligo(dT) primers were synthesized as Frohman *et al* (1988) suggested. In Fig.1B and C, primers adopted in this method are shown. After the extension with QT primer, in the first PCR, cDNA fragments were amplified by the Q0 primer and N-1 primer used in the RT-PCR method. In the nested PCR, the Q1 primer and each sense primer (gene specific primer, of which nucleotide sequence was obtained in the RT-PCR) of 22 mer in the salamander and 23 mer in the toad were used (Fig. 1B). In the case of newt, we synthesized another N-terminal primer (N-2 primer) for the 3'-RACE-PCR method (Fig. 1C), of which region was at the up-stream of the N-1 primer. The primer was composed of a mixture of 24 mer synthesized on the basis of the

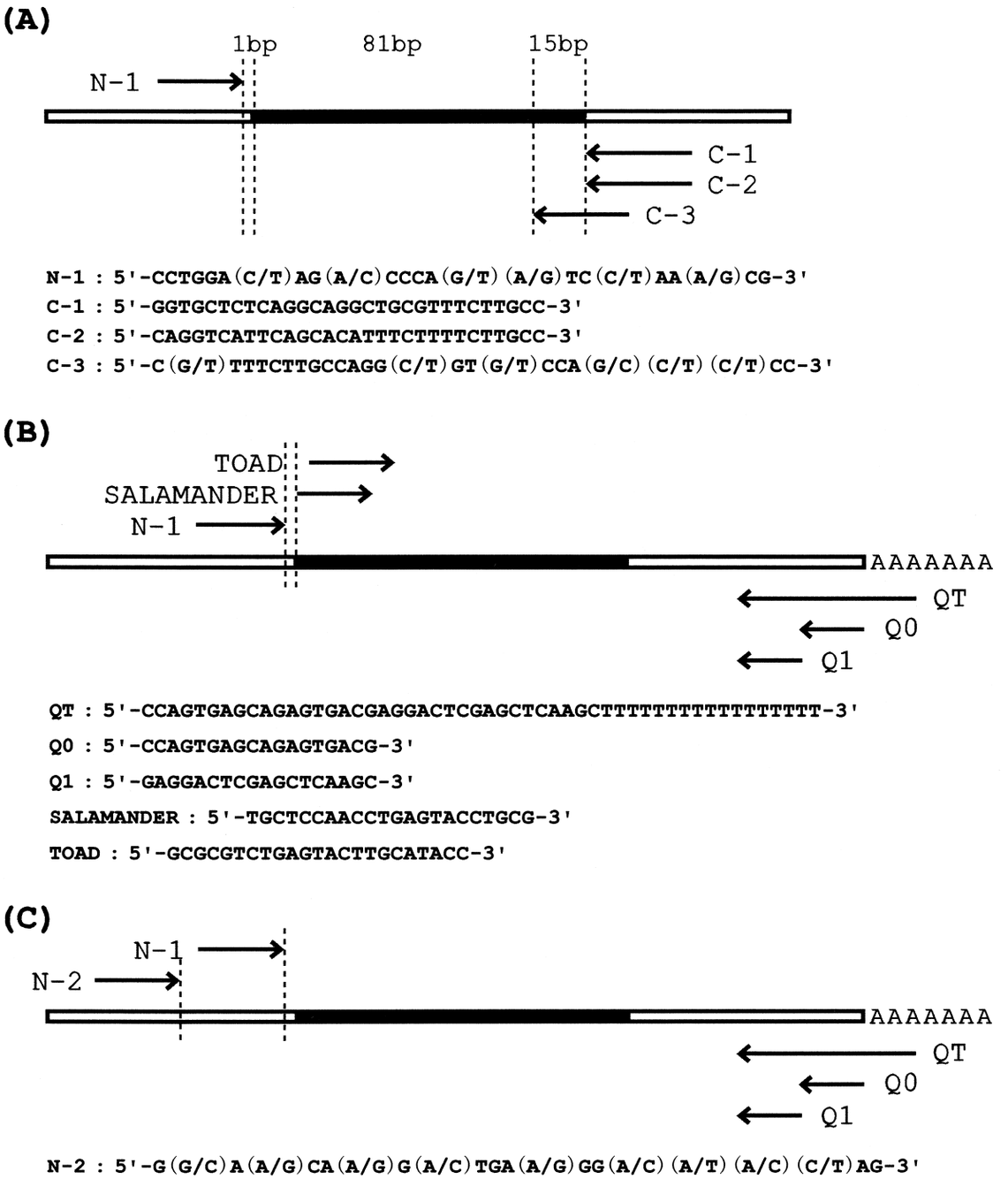

Fig. 1. (A), (B) and (C) Primers' location in the RT-PCR method and 3'-RACE-PCR method. Calcitonin gene is composed of six exons. Exon 4 encodes calcitonin. Figure 1 shows only one part of exon 4. Black portion on the line is a coding region of calcitonin molecule (96 bp). Nucleotide sequences of every primer are also cited. (A) shows the RT-PCR method for all amphibians. (B) and (C) exhibits the 3'-RACE-PCR method for the salamander and the toad, and the newt, respectively.

consensus of the nucleotide sequences of mammal, chicken and salmon calcitonins. In the first PCR for the newt, this N-2 primer and Q0 primer set was used. In the nested PCR, the N-1 primer and Q1 primer set was used.

The conditions for the RACE-PCR are followings. Five μg of total RNA was reverse-transcribed. The solution was incubated at 55°C for 1 hr. Complementary DNA fragments were subjected to the first PCR. The procedures were done as follows: for 5 min at 97°C, 3 min at 75°C, 3 min at 55°C, 40 min at 72°C, and next 30 cycles of 1 min at 94°C, 1 min at 55°C, 3 min at 72°C, and finally 15 min at 72°C. Furthermore, nested PCR was performed for 2 min at 95°C, 30 cycles of 45 sec at 95°C, 30 sec at 55°C, 1 min at 72°C, and 30 min at 72°C.

The purified cDNA fragments were ligated into pT7 Blue vector (Takara, Kyoto). After screening, nucleotide sequence was determined by a DNA sequencer (Model 373S, Applied Biosystems) using the dideoxynucleotide chain terminal procedure with T7 primer for the vector. At least 4 clones were analyzed in each species.

RESULTS

In the Tohoku black salamander and the Japanese toad, neither the C-1 nor the C-2 primer could amplify the cDNA fragments coding calcitonin molecule. Only the C-3 primer was compatible. Therefore, cDNA fragments of 132 bp lacking the information of the last 15 of the 96 bp were amplified. However, those lacking regions were covered by the 3'-RACE-PCR method. In the Japanese newt, only the 3'-RACE-PCR method with the N-2 primer was successful to amplify the full length of the cDNA fragments coding calcitonin molecule. In the 3 species of frogs (Japanese tree frogs, black-spotted pond frogs and forest green frogs), cDNA fragments covering calcitonin molecules were amplified by the RT-PCR method.

Figure 2 shows the nucleotide sequences and the deduced amino acid sequences of amphibian calcitonins. Bullfrog calcitonin and some other calcitonins are also cited for reference.

In these amphibian calcitonins, the amino acid residues located in the 15th and 19th positions, which are common to those of mammalian calcitonins in bullfrog calcitonin, were not always common even among the amphibians examined. On the other hand, the nucleotide sequence of the salamander calcitonin was 86% similar to that of the lungfish, and 85% to the caiman, 76% to those of the goldfish and sole (Table 1). The sequence identities of the amino acid residues deduced from the nucleotides in these animals were higher than those of the nucleotides, as those values range from 84% to 97% (Table 2). The amino acid sequence of the newt calcitonin coincided completely with that of the salamander. In contrast,

Fig. 2. Nucleotide sequences and amino acid sequences of amphibian calcitonins. Horizontal bars indicate identical bases and amino acid residues to those of the lungfish calcitonin. Data other than amphibians are also cited for reference (Suzuki *et al*. 1997; Yoshida *et al*. 1997; Suzuki *et al*. 1999).

Table 1. Sequence identity of nucleotides of cDNA coding calcitonin molecules shown in Fig. 1.

LUNGFISH									
91	GOLDFISH								
81	88	SOLE							
97	94	84	TOHOKU BLACK SALAMANDER						
97	94	84	100	JAPANESE NEWT					
63	59	63	63	63	JAPANESE TOAD				
81	78	78	81	81	66	JAPANESE TREE FROG			
72	75	75	72	72	59	84	BULLFROG		
75	72	72	75	75	66	91	78	BLACK-SPOTTED POND FROG	
69	69	75	69	69	63	72	75	72	FOREST GREEN TREE FROG
91	94	84	94	94	66	78	69	72	66	CAIMAN

Table 2. Sequence identity of amino acid residues of calcitonin molecules shown in Fig. 1.

LUNGFISH									
72	GOLDFISH								
72	83	SOLE							
86	76	76	TOHOKU BALCK SALAMANDER						
78	78	76	91	JAPANESE NEWT					
66	65	64	72	67	JAPANESE TOAD				
71	74	74	77	77	77	JAPANESE TREE FROG			
68	72	71	73	72	73	82	BLACK-SPOTTED POND FROG		
61	68	67	67	69	69	74	80	FOREST GREEN TREE FROG	
89	72	75	85	78	73	71	69	63	CAIMAN

the nucleotide sequences of the 4 species of anurans were 67–77% similar to those of the salamander and newt, 64–74% to those of the goldfish and sole, 63–73% to that of the caiman, 61–71% to that of the lungfish (Table 1). The sequence identities of the deduced amino acid residues in those animals ranged from 59% to 81% (Table 2). Among anurans as well, the sequence identities of the deduced amino acid residues ranged widely from 59% to 91%. The calcitonin of the Japanese toad was clearly distinctive, since the sequence identities of the amino acid residues were only 59–66%. However, the calcitonin of the Japanese tree frog was relatively similar to those of other frogs. The sequence identities of the amino acid residues were 66 - 91%, of which the highest value was obtained with the black-spotted pond frog.

DISCUSSION

The present study did not support the hypothesis that the chimera-like characteristic of bullfrog calcitonin is general among amphibian calcitonins. Therefore, its characteristic is limited so far to the bullfrog calcitonin alone. On the other hand, the salamander and newt calcitonins were very similar to those of the lungfish and the caiman. This fact suggests that urodele calcitonin may have originated from calcitonin of the ancestor common to the primitive bony fishes and that the calcitonin has been transported to reptiles as well. In anurans, however, the sequence identities of the nucleotides coding calcitonin were rather low in comparison to those of other lower vertebrates. Especially, calcitonin of the Japanese toad was distinctive even among amphibians. Recently, Conlon *et al*. (1998) reported the diversity of the amino acid sequences of pancreatic polypeptide (PP) among amphibians. This peptide is composed of 36 amino acid residues whose molecular size is similar to that of calcitonin. The amino acid sequence of the PP of *Rana sylvatica* was the same as that of the bullfrog PP. The PP from the green frog (*Rana ridibunda*) was different only in one amino acid residue from that of the bullfrog. However, the PP of the cane toad (*Bufo marinus*) was different in 6 amino acid residues from that of the bullfrog PP. As paleontological evidence shows the early diversity of toads from other frogs (Duellman and Trueb, 1985), comparative aspects of calcitonin and PP may contribute our understanding of the phylogenetic location of toads in anurans. In the frog phylogeny, Hylidae (Japanese tree frog) is closer to Bufonidae (Japanese toad) than to Ranidae (black-spotted pond frog) (Duellman and Trueb, 1985). However, the sequence identity of the tree frog calcitonin to the pond frog calcitonin was higher in the amino acid residues than to the toad calcitonin. Therefore, the relationship based on the sequence identity of frog calcitonins did not coincide with the phylogenetic relationship of the frogs.

As above-mentioned, anuran calcitonins were distinctive and diversified from those of lower vertebrates. Amphibians are the first vertebrates that advanced on land. In general, it has been accepted that new environments relax various restrictions to suppress gene changes (Kimura, 1983). Recently, Arai *et al*. (1998) reported that the alpha subunit of the glycoprotein hormone in the pituitary diversifies more largely in amphibians than in mammals, birds and lungfish. Therefore, results from calcitonin and the study of the subunit of glycoprotein seem to coincide with the theory established by Kimura (1983). Salamanders and newts changed their morphology and physiology moderately from their ancestor on the way of evolution. Anurans, on the other hand, altered drastically. On the path to adaptation, frogs acquired a characteristic physiology known as metamorphosis. In the metamorphic processes, frogs form their leg bones in a manner different from that of other vertebrates. At first, tadpoles develop endolymphatic sacs in their vertebral bones and accumulate Ca in those sacs preceding metamorphosis, since they can not obtain Ca through food on account of the reconstruction of their digestive tracts. Ca deposited in the sacs is utilized to make four leg bones during metamorphosis. Calcitonin accelerates the accumulation of Ca into the sacs (Sasayama and Oguro, 1985). Such function of calcitonin has not been known in other vertebrates. Furthermore, Stiffler *et al*. (1998) recently reported that, in frogs, calcitonin stimulates Ca influx through the skin into the body. In other vertebrates, such a role of

calcitonin has not been known, because this hormone accelerates primarily to decrease blood Ca levels. On the other hand, frogs changed their moving style from crawling to jumping, which deformed their skeletal structure to a large extent. Some of their vertebral bones became united to fix the waist for jumping, and some parts of the skull regressed to lighten the weight. These various characteristics of phenomena related with Ca homeostasis in anurans might have been brought about by the relaxation on the suppression of changes in the calcitonin molecule, or they might have caused the relaxation on the molecule.

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