Nucleotide sequences of reptile calcitonins: Their high homology to chicken calcitonin

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Nucleotide Sequences of Reptile Calcitonins: Their High Homology to Chicken Calcitonin

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ABSTRACT—The calcitonin genes of four species of reptiles (Reeve's turtle, rat snake, grass lizard, and spectacled caiman) were amplified from the genomic DNA, as well as from the mRNA of the ultimobranchial glands of the former three species, by the polymerase chain reaction (PCR) method, and were sequenced. Among several primer sets, only one primer set synthesized from the chicken calcitonin gene was compatible with those of the reptiles. The nucleotide sequences of the reptile calcitonin genes were highly homologous with that of chicken calcitonin (100% for turtle, 99% for caiman, 96% for lizard and 93% for snake). The products amplified from mRNA by the RT-PCR method matched completely those from genomic DNA in the turtle, snake and lizard.

INTRODUCTION

Calcitonin, a hypocalcemic hormone composed of 32 amino acid residues, is secreted from the C-cells of the thyroid gland in mammals and from the ultimobranchial glands in non-mammals (Copp *et al.*, 1970). The primary structure of calcitonin has been determined in 13 species of animals (Sasayama *et al.*, 1993). Those calcitonins have been classified into four lineages according to the similarity of amino acid sequences: human lineage (human, rabbit and rat); pig lineage (pig, cattle, dog and sheep); bony fish lineage (salmon, eel, goldfish, sardine and chicken); cartilaginous fish lineage (stingray). Recently, however, we clarified the sequence of amino acid residues of bullfrog calcitonin (Yoshida *et al.*, 1997), which belongs to a fifth lineage. Therefore, only the sequence of calcitonin of reptiles, phylogenetically located between birds and amphibians, has not been reported.

Reptiles are the first vertebrates to be completely independent of the water environment by developing an eggshell and an amnion. This fact shows that reptiles can perform calcium metabolism of the terrestrial type, different from that of the amphibians. In reptiles, however, the role of calcitonin in calcium metabolism has not been clarified. Thus, information on the primary structure of reptile calcitonin is of interest also from the viewpoint of biological function. This study may contribute to our knowledge of the molecular evolution of calcitonin.

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MATERIALS AND METHODS

Preparation of genomic DNA and RNA

Spectacled caiman (*Caiman crocodilus*) was purchased through a commercial source. Experimental use of this species is not contrary to the Washington treaty. Rat snake (*Elaphe climacophora*), grass lizard (*Takydromus tachydromoides*) and Reeves's turtle (*Geoclemys reevesii*) were caught around our laboratory in Ogi district of Uchiura Town, Ishikawa Prefecture. Genomic DNA was isolated from the liver using a genomic DNA isolation kit (Funakoshi, Tokyo).

Tissue (20–100 mg) containing ultimobranchial glands was dissected out from lizard, turtle and snake using a binocular dissecting microscope, and was immediately frozen in liquid nitrogen. These samples were stored at –80°C until analysis. The total RNA was obtained using an isolation kit (Nippon Gene, Toyama).

Polymerase chain reaction (PCR)

Figure 1A shows the genomic structure of the human calcitonin gene and the location of the primers. Three primers (C-1, C-2, C-3 primers) from 3' region of 30 mer were synthesized according to the nucleotide sequences of human (Steenbergh *et al.*, 1986), salmon (Pöschl *et al.*, 1987) and chicken (Lasmoles *et al.*, 1985) calcitonins (Fig. 1B). As a 5' primer, a mixture of 24 mer containing 64 sequence combinations from the consensus of the three species was synthesized (N-1 primer) (Fig. 1B). Therefore, the region which should be amplified is 96 bp of the calcitonin gene and 1 bp just prior to the gene (Fig. 1A).

One μg each of genomic DNA was subjected to PCR. The PCR was performed for 30–45 cycles of 30–60 sec at 95°C, 30 sec at 47–55°C, and 30–60 sec at 72°C in 20 μl of a solution containing 50 mM

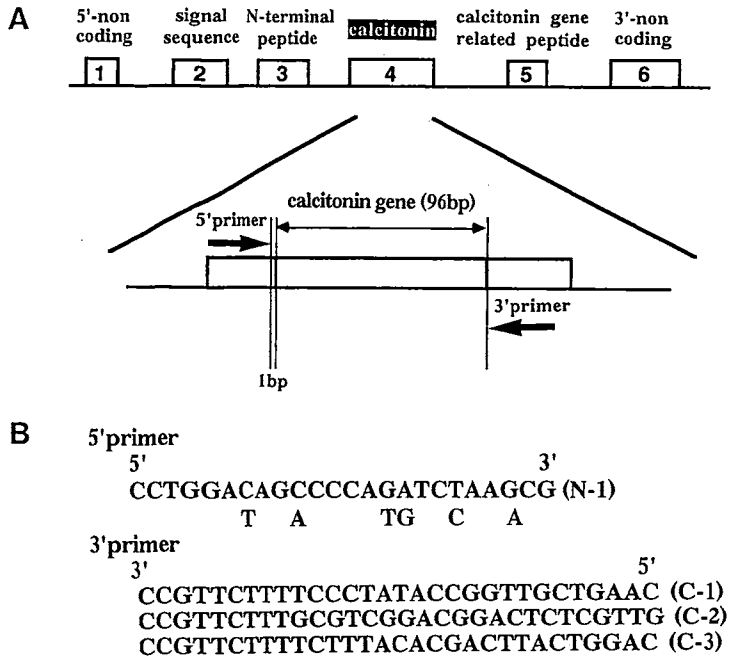

Fig. 1. (**A**) Genomic gene structure of human calcitonin gene. Calcitonin gene is composed of six exons. Exon 4 encodes mature calcitonin (96 bp). 5' primer and 3' primer for PCR were synthesized up- and down-stream of mature calcitonin, respectively. (**B**) Primers designed for PCR method. Three primers (C-1, C-2, C-3 primers) from 3' region of 30 mer were synthesized according to the nucleotide sequences of human, salmon and chicken calcitonins. As a 5' primer, a mixture of 24 mer (N-1 primer) containing 64 sequence combinations from human, salmon and chicken calcitonin gene was synthesized.

KCl, 10 mM Tris-HCl (pH 8.8), 2.5–8.5 mM $MgCl_2$, 1% Triton X-100, 250–500 μM of dNTPs, 1 unit of *Taq* polymerase (Nippon Gene, Toyama) and 0.5 μM of each of the primers. An aliquot of the amplified product containing 8 μl of the solution was analyzed on 3% NuSieve GTG agarose gel (FMC Bioproducts, Rockland).

Reverse-transcriptase PCR (RT-PCR)

An RT-PCR was made by the method of Iwami *et al.* (1996). This method was useful to prevent contamination of genomic DNA. One μg of total RNA was reverse-transcribed in 20 μl of solution composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM of dNTPs, 0.5 unit/μl of RAV-2 reverse transcriptase (Takara, Kyoto) and 1 μl of Oligotex-dT 30 Super (Takara, Kyoto). This solution was incubated for 60 min at 42°C, boiled for 5 min and immediately chilled on ice. By this treatment, cDNA was synthesized and bonded to the latex. This cDNA bound to the latex was separated from the reaction mixture by centrifugation at 15,000 rpm for 5 min at 4°C, and was washed twice with 200 μl of TE solution (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). The washed latex was suspended in 100 μl of solution composed of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2.5 mM $MgCl_2$, 75 μM of each dNTP in the presence of 0.5 μM of N-1 primer and the C-3 primer. One μg of cDNA supported by the latex was subjected to PCR as described in the previous section.

Cloning and sequencing of the PCR product

As the positive control of PCR, salmon calcitonin gene was amplified with the primers of N-1 and C-2 under the same condition. The DNA fragment amplified was composed of 151 bp, since the calcitonin region is 96 bp, primer regions 54 bp and 1 bp just prior to the calcitonin. After electrophoresis, a band of the same size as the 151 bp was eluted in 300 μl of TE solution at 70°C, followed by two times of phenol extraction. The purified DNA fragments were ligated into pT7 Blue vector (Takara, Kyoto). After screening, the nucleotide sequence was determined by a DNA sequencer (Model 373S, Applied Biosystems) using the dideoxynucleotide chain terminal procedure with T7 primer for the vector.

RESULTS

Among primers applied, the ones synthesized on the bases of nucleotide sequences of human calcitonin (C-1) and salmon calcitonin (C-2) could not amplify the reptile calcitonin gene. However, when chicken primer (C-3) was used, in all four species of reptiles, the portion of 96 bp out of the probable 151 bp, which codes a mature hormone composed of 32 amino acid residues, was amplified from genomic DNA. The nucleotide sequences of those calcitonins were highly homologous to that of chicken calcitonin. Furthermore, the nucleotide sequence obtained from RT-PCR performed in snake, lizard and turtle coincided completely with that of genomic DNA in each species. Figure 2 summarizes the results. In this study, in one PCR performance at least four clones were sequenced and we confirmed that their nucleotide sequences coincide completely. In caiman (sequence identity to chicken, 99%), only the 24th nucleotide for the 8th amino acid residue differs, although the substitution is silent. In snake (sequence identity, 93%), seven nucleotides differ at positions 8, 9, 15, 54, 63, 90 and 93. Although the substitution at positions 8 and 9 resulted in different amino acid residue in the 3rd amino acid residue, that is, Ser in chicken and Asn in snake, other substitutions are silent. In lizard (sequence identity, 96%), although

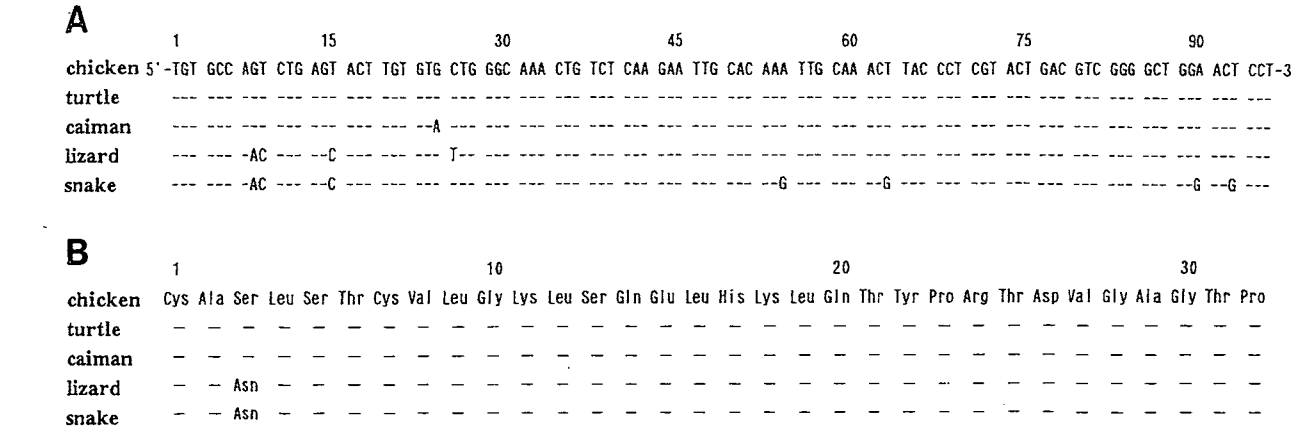

Fig. 2. Nucleotide sequences (**A**) and amino acid sequences (**B**) predictable from reptile PCR products. The nucleotide sequence and the amino acid sequence of chicken calcitonin are also shown for comparison. Horizontal bars indicate identical bases and amino acid residues to the chicken calcitonin.

four nucleotides differ at positions 8, 9, 15 and 25, only the 3rd amino acid residue differs from chicken. In turtle (sequence identity, 100%), the nucleotide sequences obtained from the PCR and RT-PCR methods completely coincided with that of the chicken calcitonin.

DISCUSSION

Table 1 shows the sequence identity of the amino acid residues of reptile calcitonins to those of other vertebrates. The similarity of reptile calcitonins is 77% to the stingray of cartilaginous fish, 84–97% to bony fish, 69% to the bullfrog of anuran amphibians, 97–100% to the chicken of birds, and 34–53% to mammals. Thus, it is clear that reptile calcitonins are highly homologous to chicken calcitonin, and are never intermediate phylogenetically between amphibians and birds. The

Table 1. Sequence identity of amino acid residues of calcitonins among reptiles, ray, bony fishes, frog, chicken and mammals.

	turtle	caiman	lizard	snake
ray	78	78	75	75
salmon	84	84	88	88
eel	94	94	97	97
goldfish	94	94	91	91
sardine	84	84	88	88
frog	69	69	69	69
chicken	100	100	97	97
rat	50	50	53	53
rabbit	47	47	50	50
human	47	47	50	50
canine	47	47	50	50
porcine	34	34	38	38
bovine	34	34	38	38
ovine	38	38	41	41

Data from Sasayama *et al.* (1993), Yoshida *et al.* (1997) and this study.

number of reptile hormones of which the primary structure has been clarified, is not so many at present. However, the amino acid sequence of the α-subunit of insulin of the red ear turtle and alligator coincides completely with that of the chicken (Conlon and Hicks, 1990). Recently, amino acid sequences of growth hormone and prolactin of the sea turtle were determined (Yasuda *et al.*, 1989, 1990). The sequence identity of the amino acid residues of those hormones between sea turtle and other vertebrates is 32–35% to bony fish, 66–75% to amphibians, 85–88% to birds, and 58–88% to mammals (Rand-Weaver *et al.*, 1993). Furthermore, the amino acid sequence of luteinizing hormone (LH) of sea turtle was reported (Koide *et al.*, 1995). The sequence identity of α-subunit of the turtle LH to that of chicken was high, 95–98%, in contrast to the 70–80% homology to other vertebrate groups. Therefore, also in these hormones, a high homology is recognized between reptiles and birds. Although studies of the morphology of the temporal fossa of the skull (Frazzetta, 1968; Radinsky, 1987) and of the 12S and 16S ribosomal RNA of mitochondria (Hedges, 1994) show that birds are closely related to reptiles, this relationship may be supported also from hormonal aspects.

Furthermore, in this study, sequence identity of the amino acid residues of the turtle calcitonin was 100% the same to chicken calcitonin. According to the usual taxonomy, turtles are primitive reptiles and belong to a different lineage from other reptiles because of the peculiar morphology of the skull (Colbert and Morales, 1991). Recently, however, it was reported that turtles are affinities to other reptiles, and not so different, on the bases of cladistic analysis using 168 characters (Rieppel and deBraga, 1996). Therefore, the results obtained in this study seem not to contradict to the reptile phylogeny.

On the other hand, in both reptiles and birds, the physiological role of calcitonin in adults is not clear (Dacke, 1979). In the chick, however, calcitonin has been suggested to function to prevent hypercalcemia when calcium moves from the

eggshell for the rapidly growing skeleton in the egg (Baimbridge and Taylor, 1980), and to regulate the acid-base balance of the blood by calcium carbonate (Taylor *et al.*, 1975). In fact, the blood calcitonin levels and calcitonin contents of the ultimobranchial gland of chicks are higher in individuals just before and just after hatching, compared to the adult levels (Dacke, 1979). Also in snakes, the calcitonin contents of the ultimobranchial gland are higher in hatchlings than those of the adults (Uchiyama *et al.*, 1981). Only the ultimobranchial glands at the stages around the hatching in snakes react intensely with calcitonin antiserum in immunohistochemistry (Sasayamsa *et al.*, 1990). These facts suggest that the physiological role of calcitonin in reptiles is similar to that of birds.

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