CNP is the only Natriuretic Peptide in an Elasmobranch Fish, Triakis scyllia

Authors: Kawakoshi, Akatsuki, Hyodo, Susumu, and Takei, Yoshio

Source: Zoological Science, 18(6) : 861-868

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.18.861

ZOOLOGICAL SCIENCE **18**: 861–868 (2001)

CNP is the only Natriuretic Peptide in an Elasmobranch Fish, *Triakis scyllia*

Akatsuki Kawakoshi*, Susumu Hyodo and Yoshio Takei

Ocean Research Institute, The University of Tokyo, Nakano, Tokyo 164-8639, Japan

ABSTRACT—The natriuretic peptide (NP) family consists of endocrine ANP/BNP/VNP in the heart and paracrine CNP in the brain in vertebrates ranging from teleosts to mammals. In elasmobranchs, however, only CNP has been identified thus far in the heart and brain. To delineate the molecular evolution of this hormone family, it is essential to determine whether CNP is the only NP in this primitive fish group. In the present study, PCR cloning of all types of piscine NP was performed from the heart and brain of a dogfish, *Triakis scyllia,* using degenerate primers that amplified eel ANP, VNP and CNP. However, only CNP cDNA with an identical sequence was cloned from the heart and brain. Southern blot analysis showed that the CNP gene is a single copy gene, showing that endocrine CNP from the heart and paracrine CNP in the brain originate from the same gene. Since expression of the CNP gene was so high as demonstrated by Northern blot analysis, the abundantly expressed CNP mRNA could have interfered the amplification of other NP mRNAs expressed in small amounts. Therefore, a method was developed to cleave the cloned CNP mRNA specifically at the 3'-untranslated region with RNase H. After removal of the cloned CNP mRNA by this technique, no other NP cDNAs could be cloned, but small amounts of CNP cDNAs with shorter 3' sequence were amplified. These results strongly suggest that only CNP is present in elasmobranchs. Thus, it is likely that CNP is an ancestral form of the NP family and endocrine ANP/BNP/VNP have appeared later in the vertebrate evolution.

INTRODUCTION

Natriuretic peptides (NP) comprise hormone family that plays a pivotal role in cardiovascular and body fluid homeostasis in most vertebrate species (Brenner *et al*., 1990; Evans, 1990; Takei, 2000; Farrell and Olson, 2000; Loretz and Pollina, 2000). NPs have been isolated throughout vertebrate classes except for cyclostomes. In mammals, amphibians and teleost fish, three types of NPs (ANP, BNP/VNP and CNP) have been identified; ANP, BNP and VNP are produced mainly in the heart and act as circulating hormones, while CNP functions as a paracrine factor in the brain and peripheral tissues such as the endothelium (Chen and Burnett, 1998). ANP and BNP genes are localized in tandem on the same chromosome in the human and mouse (Tamura *et al*., 1996). However, the CNP gene is present on a different chromosome (Ogawa *et al*., 1994). These results suggest that NPs have functionally and evolutionarily diverged into two distinct groups, endocrine NP from the heart and paracrine NP principally in the brain, in teleost fishes and tetrapods.

In elasmobranchs, however, only CNP has been isolated from the heart and brain despite several attempts to isolate ANP and other cardiac NPs from the heart (Schofield *et al*., 1991; Suzuki *et al*., 1991, 1992, 1994; Takano *et al*., 1994). The mature CNP-22 is a brain form in elasmobranchs as in mammals, while proCNP is a cardiac form stored in the granules of cardiocytes in two species of dogfish, *Triakis scyllia* and *Scyliorhinus canicula* (Suzuki *et al*., 1991, 1992). Thus, proCNP secreted from the heart circulates in the blood of *Triakis* at concentrations even higher than ANP in the blood of teleosts and mammals (Suzuki *et al*., 1994). Circulating CNP stimulates Na^+ and Cl^- secretion from the rectal gland of spiny dogfish, *Squalus acanthias* (Solomon *et al*., 1992; Silva *et al*., 1999). These results suggest that, in elasmobranchs, CNP functions as both a circulating hormone and a paracrine factor. However, there is still a possibility that other NPs are also present in the elasmobranch heart. It is known that CNP is much more potent than other NPs for the relaxant activity in the chick rectum that is used for assay during isolation of NPs (Suzuki *et al*., 1992). Consequently, it may be difficult to isolate other forms of NP from the heart in the presence of CNP. There is also a possibility that paracrine CNP-22 in the brain and circulating proCNP from the heart are produced from different genes since it is known that different CNP genes are expressed in the brain and periphery of the bullfrog (Kojima *et al*., 1994). For these reasons and in order to trace the evolu-

* Corresponding author: Tel. +81-3-5351-6467;
FAX. +81-3-5351-6463.
E-mail: kawakosi@ori.u-tokyo.ac.jp

tion of the NP family, it is imperative to determine whether CNP is the only NP in elasmobranchs and whether cardiac and brain CNP originate from a single gene.

In the present study, we attempted to isolate NP cDNAs from the brain and heart of a dogfish, *Triakis scyllia*, with a PCR-based method using degenerate primers that amplify all NP types. Using these primers, it was preliminarily shown that CNP and ANP/VNP were amplified from the eel brain and heart, respectively. Since the CNP gene was so abundantly expressed in the brain and heart of the dogfish, CNP mRNA was specifically removed before PCR so that other NPs that may be expressed in small quantities could be effectively amplified. We report here that CNP is the sole NP in the dogfish and is expressed from a single gene in the heart and brain.

MATERIALS AND METHODS

Animals and sampling

Japanese dogfish, *Triakis scyllia*, of either sex and weighing from 800 to 1800 g, were collected from the Koajiro Bay, near Misaki Marine Biological Station, the University of Tokyo. They were kept without feeding in a 4 kl round tank with running seawater (ca. 13°C), usually for one week before use. For tissue sampling, fish were anaesthetized in 1% (w/v) 3-aminobenzoic acid ethyl ester (Sigma). The heart and brain were dissected out and frozen quickly in liquid nitrogen.

3'-end amplification of cDNA (3'-RACE)

A two-step PCR with RACE method was applied for isolation of NP cDNA using 3'- and 5'-Full RACE Core Sets (Takara). Total RNA was extracted from the heart and brains of dogfish with ISOGEN (Wako). Poly-A^+ RNA was purified with Oligotex-dT30 (Takara). For 3'-RACE, 1 μg of poly-A^+ RNA was incubated in reaction mixture containing dT-adaptor primer and AMV reverse transcriptase at 50°C for 30 min. One-fortieth of the first strand cDNA was then subjected to PCR amplification with a degenerate sense primer and the universal antisense adaptor primer. PCR reaction was performed using high-fidelity Ex-Taq DNA polymerase (Takara). Two degenerate sense primers corresponding to the conserved region of known NP peptides were designed as follows: NP-1; TG(C/T)TT(C/T)GGGGT(A/C/G/T)AA(A/G)CTGGA(C/T)(A/C)G(A/C/G/T)AT(A/C/T)GG, and NP-2; TC(A/C/G/T)TGGAG(C/T)GG(A/C/G/T)(C/T)T(A/C/G/T)GG(A/C/G/T)TG. The efficiency of these primers had been confirmed using cDNAs from the eel brain and heart. Although eel ANP, CNP and VNP were all amplified with each primer, ANP and CNP were major products with the NP-1 primer, and VNP with the NP-2 primer (data not shown). After an initial denaturation at 94°C for 3 min, 40 cycles of PCR were performed, each consisting of 50 sec denaturation at 94°C, 30 sec annealing, and 1.5 min extension at 72°C. The annealing temperature varied from 50 to 60°C. The resulting products were electrophoresed through 1.2% (w/v) agarose gel, and purified using the GENECLEAN kit (BIO 101). The purified product was ligated into pT7Blue T-Vector (Novagen), and transformed into XLI-Blue competent cells. The nucleotide sequence was determined in an automated DNA sequencer (PRISM 310, Perkin-Elmer/Applied Biosystems).

5'-RACE

A circular or concatemeric first-strand cDNA-mediated RACE method was applied to amplify the 5'-end of CNP mRNA (Maruyama *et al.*, 1995). One-half μg of poly-A^+ RNA was reverse-transcribed as described above except for the substitution of a phosphorylated gene

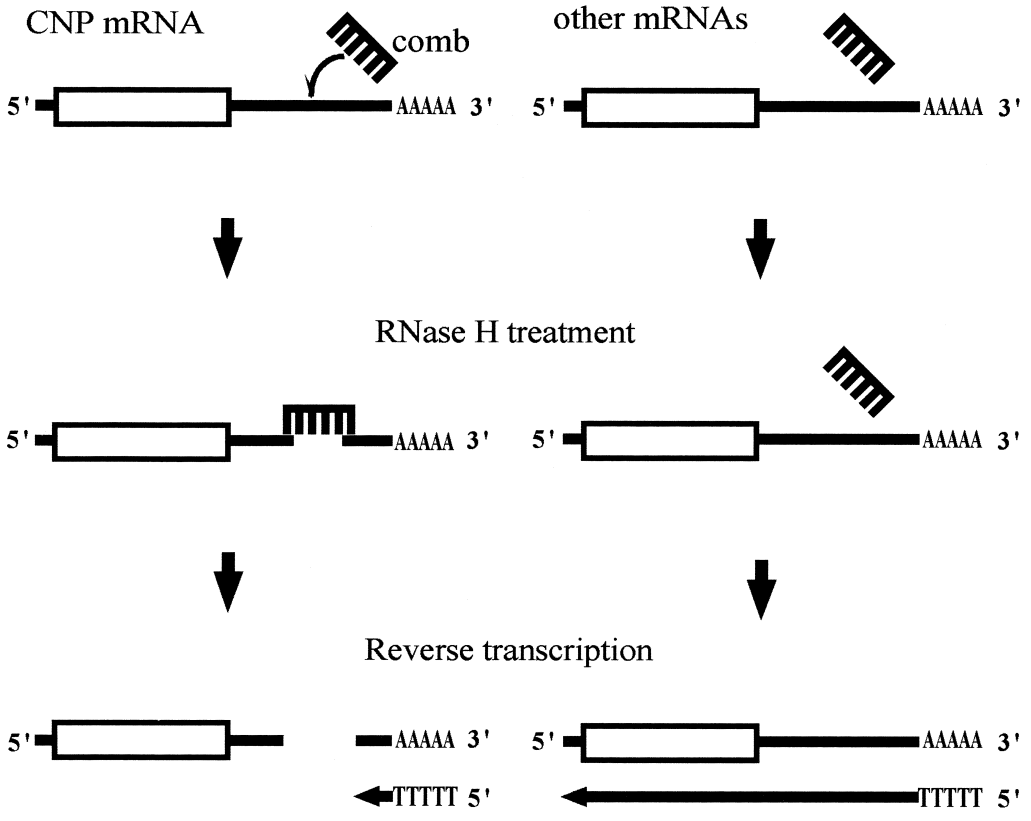

Fig. 1. Schematic drawing showing specific removal of CNP mRNA by RNase H treatment. CNP mRNA is cleaved at 3'-untranslated region after hybridization with an oligonucleotide "comb" complementary to the CNP mRNA, while other cDNAs remain intact after this treatment.

```
    5'
  1 gaaataaaagacccagagtctgagcacagaggcagcagggaaagagggagcaagtgaagaaactaattcgccgtgaagagtagtccaata  90

 91 caaactcaaacagagaag ATGAGCGGACAAACGAGTTTCTACTGCGGACTCTTGCTTGTGCTTCTGATCCAAGCGCAAGCTAGACCAAG 179
             Pst I      M  S  G  Q  T  S  F  Y  C  G  L  L  L  V  L  L  I  Q  A  Q  A  R  P  R

180 ATCAGATGATAGCCTGCAGACCTTGTCCAGGCTTTTGGAAGATGAATATGGGCACTACTTGCCCTCAGACGAGCTGAACAATGAGGCACA 269
     S  D  D  S  L  Q  T  L  S  R  L  L  E  D  E  Y  G  H  Y  L  P  S  D  E  L  N  N  E  A  Q

270 AGAGATGTCGCCAGCCGCTTCCCTTCCAGAATTTAACGCTGACCAGTCCGATTTGGAGCTTCCCTGGGATCGCGAATCCCGAGAGATTGG 359
     E  M  S  P  A  A  S  L  P  E  F  N  A  D  Q  S  D  L  E  L  P  W  D  R  E  S  R  E  I  G

360 TGGCCGGCCTTTCCGTCAGGAAGCGGTCCTGGCCCGTCTCCTGAAAGACCTCAGCAACAATCCTCTGCGGTTTAGAGGCAGGAGCAAAAA 449
     G  R  P  F  R  Q  E  A  V  L  A  R  L  L  K  D  L  S  N  N  P  L  R  F  R  G  R  S  K  K

450 GGGGCCATCACGGGGCTGCTTCGGAGTGAAACTGGACCGAATCGGTGCCATGAGCGGCCTGGGCTGTTAG cggctcgtttaaaggtctg 538
     G  P  S  R  G  C  F  G  V  K  L  D  R  I  G  A  M  S  G  L  G  C  end

539 actccgggagcaacactgccggcaccgccttttcatcgacctacccacatttaattcactcctctgatttttgctccacaaactctgact 628

629 gatgacaagtagtgtcccacctgtagactgtttacaagctcgactcttggattaaatccttaatgtacatacagtgccagctgctattt 717

718 aaacaatatttaattctatttatattaattatgaaactatttatttgtattgtatttgtttttgttgctgtgacactgactgtcagtca 807

808 cttgctgtgagctctggtgcagattaaacaaagccatcagtggagttggtattttaccttcagtacatgtagacacagtgtatggaacc 896

897 tactgcagaataaaatttaaagt 3' (919 bp)
```

Fig. 2. Nucleotide sequence of cloned cDNA encoding *Triakis* CNP precursor. Amino acid sequence of preproCNP deduced from the nucleotide sequence is also shown. Mature CNP-22 is in reversed font. White arrowhead indicates cleavage site of signal peptide. ATTTA motifs are shown in boxes. Polyadenylation signals are underlined. Arrows indicate polyadenylation sites of two shorter cDNAs. NP-1 primer corresponds to nucleotide sequence from 466 to 494. NP-2 primer corresponds to nucleotide sequence from 496 to 515. This cDNA sequence has been deposited in the DDBJ database with accession number of AB047081.

specific primer for the dT-adaptor primer. The RNA strand of the reverse-transcribed cDNA was digested with RNase H. First strand cDNA was then circularized and/or joined into a concatemeric form by T4 RNA ligase. The resulting single-stranded DNA was used as a template for amplification of 5'-end by PCR with gene specific primers. After determination of the full-length CNP cDNA by two-step RACE method using at least three clones, full length CNP cDNA was also amplified with newly designed specific primers to confirm the sequence using three clones.

Southern and Northern blot analyses

For Southern blot analysis, genomic DNA was extracted from a single dogfish heart as described by Sambrook *et al*. (1989). Restriction enzymes, *Pst* I and *Hin*d III, were used to digest extracted DNA (10 μg). The digests were then electrophoresed in a 0.6% agarose gel and finally transferred to a NYTRAN membrane (Schleicher and Schuell) according to the manufacturer's instructions. For Northern blot analysis, poly-A^+ RNA (4 μg) extracted from the heart and brain was electrophoresed in a 1.2% agarose/formaldehyde gel and transferred to a NYTRAN membrane. The cloned dogfish CNP cDNA was labeled by a random priming method using a megaprime DNA labeling system and [α-^{32}P] dCTP (Amersham). Hybridization was performed in a sealed plastic bag containing 6×SSC, 0.1% SDS, 1×Denhardt's reagent and 10 μg/ml sonicated salmon sperm DNA at 50°C. After hybridization, the filters were washed twice for 1 h in 0.2×SSC/0.1% SDS at 60°C for Southern blot analysis, or 0.1×SSC/0.1% SDS at 65°C for Northern blot analysis. Hybridization signals were detected with an auto image analyzer (FLA 2000, Fuji Film) after exposure to an imaging plate.

Removal of CNP mRNA with RNase H

Since consensus sequences of all NP mRNAs are well conserved, it was not possible to design a specific primer for each NP. In fact, NP-1 and NP-2 primers used in the current study were not specific for a single NP but cross-hybridized with multiple NPs. Therefore, if large amounts of CNP mRNA are expressed in the heart and brain, this

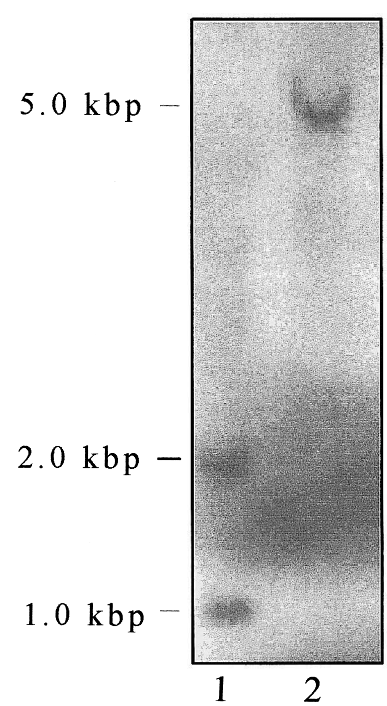

Fig. 3. Southern blot analysis of CNP gene from *Triakis*. Ten μg of DNA was digested with *Pst* I (lane 1) or *Hin*d III (lane 2) and electrophoresed. Single signal was detected at ca. 5 kbp with *Hin*d III digest. Digestion with *Pst* I resulted in two signals due to the cleavage site in the CNP cDNA (see Fig. 2).

may prevent amplification of other NP mRNAs that might be expressed at much lower levels than CNP mRNA. To avoid this, CNP mRNA was specifically removed by RNase H after hybridization with oligonucleotide complementary to its 3'-untranslated region (Fig. 1). One μg of poly-A^+ RNA was hybridized with 0.5 μg of oligonucleotide corresponding to dogfish CNP cDNA (885–904) in 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM DTT and 2.5 mM $MgCl_2$. In order to avoid non-specific hybridization, the reaction mixture was initially heated to 70°C for 10 min then cooled gradually until it reached 37°C. RNase H (2 units) was then added and maintained at 37°C for 30 min. Subsequently, RNase H was removed by phenol/chloroform treatment, and then reverse transcription and the 3'-RACE were performed as described above. To examine the specificity of this method, both CNP cDNA and vasotocin cDNA were amplified with a gene specific primer and universal antisense adaptor primer after RNase H treatment and confirmed that only vasotocin cDNA was amplified.

RESULTS

Cloning of cDNAs encoding dogfish CNP

A full length cDNA coding for dogfish CNP was cloned from the heart and brain by the described RACE method only with the NP-1 primer (Fig. 2). The CNP cDNA consisted of 919 nucleotides, and was a size consistent with that shown by the Northern blot analysis (data not shown). The CNP precursor deduced from the cDNA was composed of a signal peptide of 21 amino acid residues and proCNP carrying mature CNP-22 at its C-terminal end. A Lys-Lys residue pair prior to the mature CNP may serve as a signal for proteolytic processing. The sequence of CNP cDNA, even in the untranslated regions, was identical in the brain and heart.

Southern blot analysis

Genomic DNA digested with *Hin*d III hybridized as a single band with radiolabelled dogfish CNP probe at approximately 5 kbp (Fig. 3). The *Pst* I digestion resulted in two bands at ca. 2 kbp and 1 kbp with similar signal intensity as expected from the cleavage site within the CNP sequence (Fig. 2).

PCR cloning after RNase H treatment

The PCR amplification after RNase H treatment abolished the band of CNP cDNA at ca. 900 bp that was apparent in the

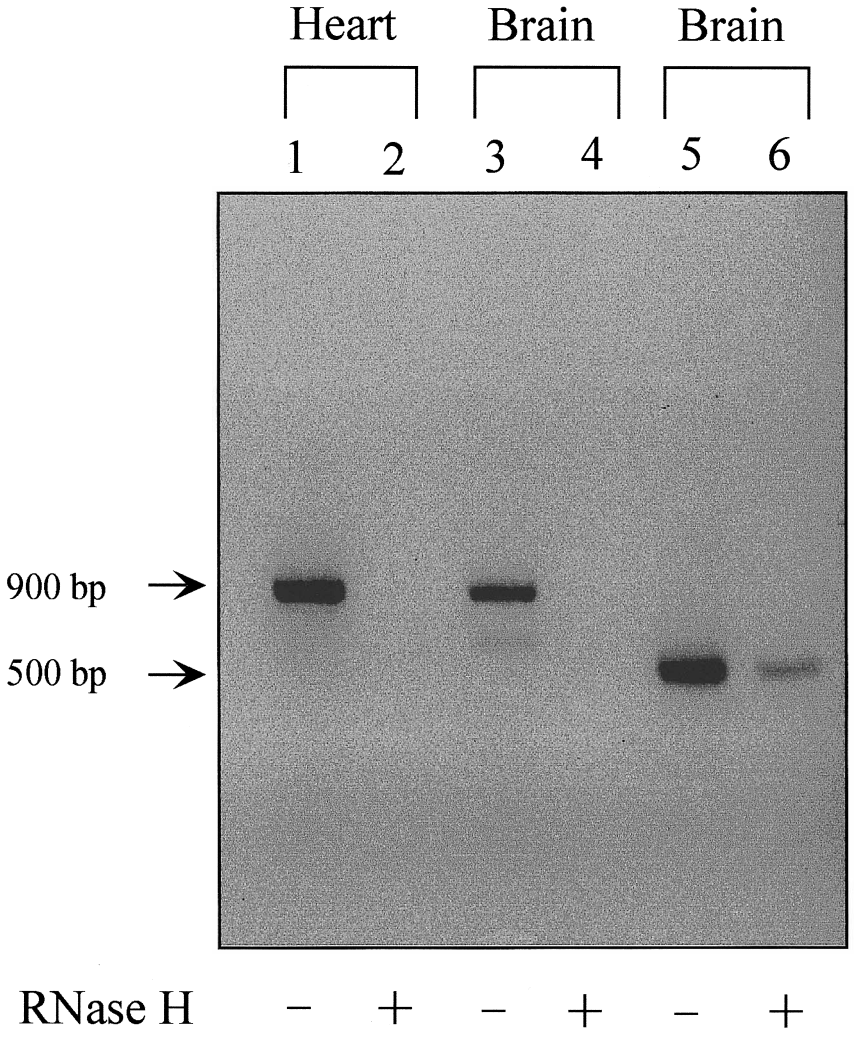

Fig. 4. RT-PCR of RNase H-treated poly-A^+ RNA after hybridization with a specific sequence corresponding to CNP cDNA-(885-904) (see Fig. 2). CNP cDNA is not amplified in the heart and brain (lanes 1–4), while vasotocin cDNA is amplified even after RNase H treatment (lane 5 and 6).

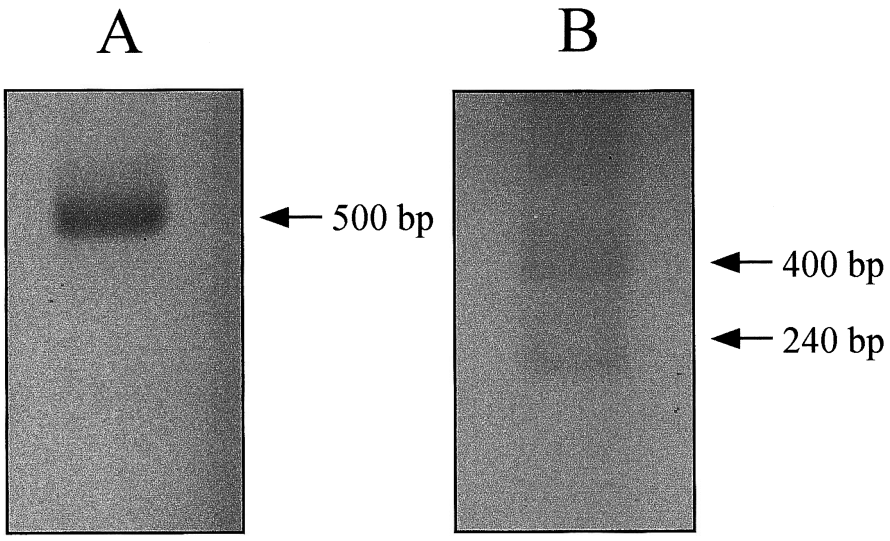

Fig. 5. 3'-RACE using intact (A) and RNase H-treated (B) poly-A$^+$ RNA. Cloned CNP cDNA amplified at 500 bp without RNase H treatment disappeared and two bands appeared at ca. 400 bp and 240 bp.

heart and brain samples without RNase H treatment (Fig. 4, lanes 1-4). However, vasotocin cDNA was amplified from the brain mRNA even after RNase H treatment (Fig. 4, lanes 5 and 6), showing specific removal of the CNP mRNA. The 3'-RACE using RNase H-treated heart mRNA gave two faint bands at ca. 400 bp and 240 bp (Fig. 5). Sequence analysis of these cDNAs revealed that both encode CNP precursor with shorter 3'-untranslated sequences (Fig. 2). The shorter CNP cDNAs were also cloned from RNase H-treated brain mRNA.

DISCUSSION

In the present study, we cloned only CNP cDNA from the dogfish, *Triakis scyllia*, using primers that can amplify all piscine NPs (Fig. 2). The deduced amino acid sequence was identical to that of previously isolated proCNP from the dogfish heart (Suzuki *et al.*, 1992). The cloned CNP cDNAs from the heart and brain of the dogfish were identical even at untranslated regions. Furthermore, even after the specific removal of cloned CNP cDNA that was expressed in large amounts, no other NP cDNAs were amplified by the subsequent PCR. Therefore, it is highly probable that only a CNP gene is expressed in the heart and brain of the dogfish.

We have developed a method to remove cloned CNP mRNA specifically before reverse transcription (Fig. 1). The specificity of this method was confirmed by amplification of vasotocin cDNA after treatment of RNase H (Fig. 4). Owing to the method, we were able to clone different CNP cDNA forms with shorter 3'-untranslated sequences that could not have been cloned otherwise because of their small amounts. Therefore, it is likely that other NPs, e.g. ANP and VNP, may have been cloned in the dogfish if they are in fact present even in small amounts. This method can be applied to any family of hormones with similar structures to clone all members of the family. Furthermore, it can be applied more universally to any family of proteins that consist of many subtypes such as water/ion channels, cell adhesion molecules (Tsukita and Furuse, 1999; Uchida, 2000).

After removal of the cloned CNP mRNA, two CNP cDNAs with shorter 3'-untranslated sequences were successfully cloned (Fig. 2). Since all CNP cDNAs have identical sequences except for the length of the 3'-untranslated sequence, they most probably derive from the same gene. The physiological significance for the presence of shorter mRNA is unknown; however, since the number of AUUUA motifs in the 3'-untranslated region, the repetition of which is thought to facilitate mRNA degradation (Wilson and Treisman, 1988), differs among the three cDNAs (Fig. 2), one possible explanation for the presence of multiple mRNA forms may relate to the regulation of CNP synthesis by changing the ratio of the three mRNAs. The importance of the 3'-untranslated region of dogfish CNP mRNA is supported by the high sequence identity between *Triakis* and *Squalus acanthias* (Schofield *et al.*, 1991), which is even higher (93.1%) than that of translated region corresponding to preproCNP (89.3%).

Southern blot analysis demonstrated that the CNP mRNA originates from a single-copy gene (Fig. 3). Therefore, endocrine-type NP from the heart and paracrine-type NP in the brain have not diverged genetically in elasmobranchs. However, processing of CNP differs between the heart and brain; the main form that is stored in the heart and that circulates in plasma is proCNP, whereas mature CNP-22 is stored in the brain (Suzuki *et al.*, 1994). Mature CNP-22 stimulates NaCl secretion from the *Squalus* rectal gland (Silva *et al.*, 1999). However, it is not known whether circulating proCNP is cleaved to CNP-22 before acting on target tissues, since dogfish guanylyl cyclase-coupled receptor, probably NPR-B (Aller *et al.*, 1999), has low ligand selectivity in *Triakis* (Sakaguchi and Takei, 1998).

CNP is highly conserved in elasmobranchs; this high degree of conservation extends not only to mature CNP-22 that is located at the C-terminus, but also to the whole proCNP sequence as shown in Fig. 6 (Schofield *et al.*, 1991; Suzuki *et al.*, 1991). This is also true in mammals where the entire proCNP sequence is well conserved (Kojima *et al.*, 1990; Tawaragi *et al.*, 1991). However, the homology of proCNP is very low among different vertebrate classes (e.g., between elasmobranchs and mammals, except for the mature CNP-22 sequence; Fig. 6). With regard to other proNPs, proBNP is variable including the mature sequence while proANP is conserved even at the prosegment (Maki *et al.*, 1984; Oikawa *et al.*, 1984; Seidman *et al.*, 1984; Yamanaka *et al.*, 1984; Kojima *et al.*, 1989; Sudoh *et al.*, 1989). Thus, biological actions were assigned to each fragment of the prosegments although further studies are required to establish their physiological functions (Denker *et al.*, 1990). Since proCNP is even more highly conserved than proANP (Fig. 6), it is possible that prosegments of CNP may also possess some biological functions.

The current series of studies demonstrate that CNP appears to be the only NP in the dogfish. There are several hypotheses with respect to the phylogenetic position of Chondrichthyes (Rasmussen and Arnason, 1999). Among them, it is generally accepted that Chondrichthyes are basal to other gnathostome fishes in the cladogram (Janvier, 1996). Since CNP is the only NP in the dogfish, we suggest here that CNP is the ancestral molecule of the NP family. Thus, it seems that endocrine ANP, BNP and VNP may have appeared later during the vertebrate evolution. With respect to more phylogenetically ancient cyclostomes, immunoreactive ANP and its binding sites are present (Reinecke *et al.*, 1987; Donald *et al.*, 1992; Toop *et al.*, 1998; Callahan *et al.* 2000); however, molecular identity of hagfish NP has not been determined yet. Identification of the cyclostome NP and its receptors by molecular cloning is awaited to delineate more convincingly the evolutionary history of the NP family in vertebrates.

Accumulating evidence indicates that the NP family is a key endocrine system for body fluid regulation in vertebrates. ANP is a primary hormone for regulation of plasma Na^+ concentration in teleost fishes, whereas in mammals ANP is important for blood volume regulation (Evans, 1990; Takei, 2000). Since patterns of body fluid regulation in vertebrates are diverse, ranging from osmo-conforming cyclostomes to osmo-regulating teleosts and tetrapods, it is of interest to

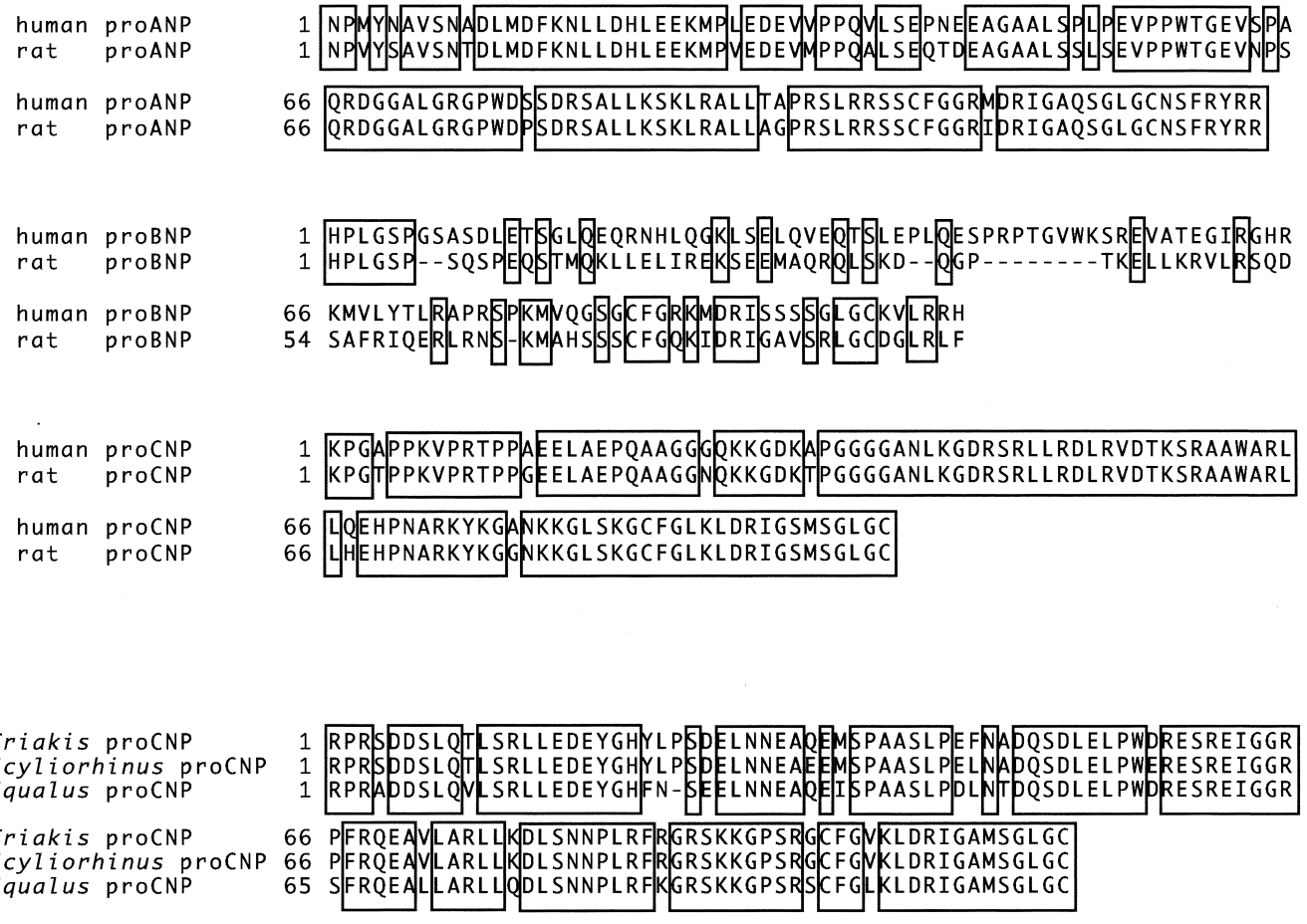

Fig. 6. Comparison of amino acid sequences of elasmobranch proCNP and mammalian (human and rat) proANP, proBNP and proCNP. The same amino acid residues in each group are shown in boxes.

examine the molecular evolution of the NP family in relation to the evolution of body fluid regulation. Marine elasmobranchs are unique in that they are partial osmo-regulators by maintaining plasma ion concentrations lower than seawater while accumulating urea to increase plasma osmolality to the seawater level. It is possible, therefore, that the sole presence of CNP in elasmobranchs might be related to their special pattern of osmoregulation.

ACKNOWLEDGEMENTS

We thank Dr. Hajime Sakaguchi of Ocean Research Institute for advice and Prof. Christopher A. Loretz of State University of New York at Buffalo for his critical reading of the manuscript. We are also grateful to Misaki Marine Biological Station, the University of Tokyo, for maintenance of dogfish. This work was supported by Grant-in-Aid for Specially Promoted Grant (01902008) and for Creative Basic Research (12NP0201) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

Aller SG, Lombardo ID, Bhanot S, Forrest Jr JN (1999) Cloning, characterization, and functional expression of a CNP receptor regulating CFTR in the shark rectal gland. Am J Physiol 276: C442–C449

Brenner BM, Ballermann BJ, Gunning ME, Zeidel ML (1990) Diverse biological actions of atrial natriuretic peptide. Physiol Rev 70: 665–699

Callahan W, Forster M, Toop T (2000) Evidence of a guanylyl cyclase natriuretic peptide receptor in the gills of the New Zealand hagfish *Eptatretus cirrhatus* (Class Agnatha). J Exp Biol 203: 2519–2528

Chen HH, Burnett Jr JC (1998) C-type natriuretic peptide: The endothelial component of the natriuretic peptide system. J Cardiovasc Pharmacol 32: S22–S28

Denker PS, Vesely DL, Gomez-Sanchez CE (1990) Effect of proatrial natriuretic peptides 1–30, 31–67 and 99–126 on angiotensin II-stimulated aldosterone production in calf adrenal cells. J Ster Biochem Molec Biol 37: 617–619

Donald JA, Vomachka AJ, Evans DH (1992) Immunohistochemical localisation of natriuretic peptides in the brains and hearts of the spiny dogfish *Squalus acanthias* and the Atlantic hagfish *Myxine glutinosa*. Cell Tissue Res 270: 535–545

Evans DH (1990) An emerging role for a cardiac peptide hormone in fish osmoregulation. Annu Rev Physiol 52: 43–60

Farrell AP, Olson KR (2000) Cardiac natriuretic peptides: A physiological lineage of cardioprotective hormone? Physiol Biochem Zool 73: 1–11

Janvier P (1996) Early vertebrates. Oxford University Press, Oxford

Kojima M, Minamino N, Kangawa K, Matsuo H (1989) Cloning and sequence analysis of cDNA encoding a precursor for rat brain natriuretic peptide. Biochem Biophys Res Commun 159: 1420–1426

Kojima M, Minamino N, Kangawa K, Matsuo H (1990) Cloning and sequence analysis of a cDNA encoding a precursor for rat C-type natriuretic peptide (CNP). FEBS Lett 276: 209–213

Kojima M, Ohyama Y, Miyamoto K, Minamino N, Kangawa K, Matsuo H (1994) Cloning and characterization of a novel natriuretic peptide in frog (*Rana catesbeiana*). J Biol Chem 269: 13136–13140

Loretz CA, Pollina C (2000) Natriuretic peptides in fish physiology. Comp Biochem Physiol 125: 169–187

Maki M, Takayanagi R, Misono KS, Pandey KN, Tibbetts C, Inagami T (1984) Structure of rat atrial natriuretic factor precursor deduced from cDNA sequence. Nature 309: 722–724

Maruyama IN, Rakow TL, Maruyama HI (1995) cRACE: A simple method for identification of the 5' end of mRNAs. Nucl Acids Res 23: 3796–3797

Ogawa Y, Itoh H, Yoshitake Y, Inoue M, Yoshimasa T, Serikawa T, Nakao K (1994) Molecular cloning and chromosomal assignment of the mouse C-type natriuretic peptide (CNP) gene (Nppc): Comparison with the human CNP gene (NPPC). Genomics 24: 383–387

Oikawa S, Imai M, Ueno A, Tanaka S, Noguchi T, Nakazato H, Kangawa K, Fukuda A, Matsuo H (1984) Cloning and sequence analysis of cDNA encoding a precursor for human atrial natriuretic polypeptide. Nature 309: 724–726

Rasmussen A-S, Arnason U (1999) Molecular studies suggest that cartilaginous fishes have a terminal position in the piscine tree. Proc Natl Acad Sci USA 96: 2177–2182

Reinecke M, Betzler D, Forssmann WG (1987) Immunocytochemistry of cardiac polypeptide hormones (cardiodilatin/atrial natriuretic polypeptide) in brain and hearts of *Myxine glutinosa* (Cyclostomata). Histochemistry 86: 233–239

Sakaguchi H, Takei Y (1998) Characterization of C-type natriuretic receptors in the gill of dogfish *Triakis scyllia*. J Endocrinol 156: 127–134

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. Cold Spring Harbor laboratory, Cold Spring Harbor, New York

Schofield JP, Jones DSC, Forrest Jr JN (1991) Identification of C-type natriuretic peptide in heart of spiny dogfish shark (*Squalus acanthias*). Am J Physiol 261: F734–F739

Seidman CE, Duby AD, Choi E, Graham RM, Haber E, Homcy C, Smith JA, Seidman JG (1984) The structure of rat preproatrial natriuretic factor as defined by a complementary DNA clone. Science 225: 324–326

Silva P, Solomon RJ, Epstein FH (1999) Mode of activation of salt secretion by C-type natriuretic peptide in the shark rectal gland. Am J Physiol 277: R1725–R1732

Solomon R, Protter A, Mcenroe G, Porter G, Silva P (1992) C-type natriuretic peptides stimulate chloride secretion in the rectal gland of *Squalus acanthias*. Am J Physiol 262: R707–R711

Sudoh T, Maekawa K, Kojima M, Minamino N, Kangawa K, Matsuo H (1989) Cloning and sequence analysis of cDNA encoding a precursor for human brain natriuretic peptide. Biochem Biophys Res Commun 159: 1427–1434

Suzuki R, Takahashi A, Hazon N, Takei Y (1991) Isolation of high-molecular-weight C-type natriuretic peptide from the heart of a cartilaginous fish (European dogfish, *Scyliorhinus canicula*). FEBS Lett 282: 321–325

Suzuki R, Takahashi A, Takei Y (1992) Different molecular forms of C-type natriuretic peptide isolated from the brain and heart of an elasmobranch, *Triakis scyllia*. J Endocrinol 135: 317–323

Suzuki R, Togashi K, Ando K, Takei Y (1994) Distribution and molecular forms of C-type natriuretic peptide in plasma and tissue of a dogfish, *Triakis scyllia*. Gen Comp Endocrinol 96: 378–384

Takano M, Sasayama Y, Takei Y (1994) Molecular evolution of shark C-type natriuretic peptides. Zool Sci 11: 451–454

Takei Y (2000) Structural and functional evolution of the natriuretic peptide system in vertebrates. Int Rev Cytol 194: 1–66

Tamura N, Ogawa Y, Yasoda A, Itoh H, Saito Y, Nakao K (1996) Two cardiac peptide genes (atrial natriuretic peptide and brain natriuretic peptide) are organized in tandem in the mouse and human genomes. J Mol Cell Cardiol 28: 1811–1815

Tawaragi Y, Fuchimura K, Tanaka S, Minamino N, Kangawa K, Matsuo H (1991) Gene and precursor structures of human C-type natriuretic peptide. Biochem Biophys Res Commun 175: 645–651

Toop T, Grozdanovski D, Potter IC (1998) Natriuretic peptide binding sites in the gill of the pouched lamprey *Geotria australis*. J Exp Biol 201: 1799–1808

Tsukita S, Furuse M (1999) Occludin and claudins in tight-junction strands: Leading or supporting players? Trends Cell Biol 9: 268–

273
Uchida S (2000) In vivo role of CLC chloride channels in the kidney. Am J Physiol 279: F802–F808
Wilson T, Treisman R (1988) Removal of poly (A) and consequent degradation of c-fos mRNA facilitated by 3' AU-rich sequences. Nature 336: 396–399
Yamanaka M, Greenberg B, Johnson L, Seilhamer JJ, Brewer M, Friedemann T, Miller J, Atlas SA, Laragh J, Lewicki J, Fiddes JC (1984) Cloning and sequence analysis of the cDNA for the rat atrial natriuretic factor precursor. Nature 309: 719–722

(Received March 12, 2001 / Accepted June 6, 2001)