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Cloning and sequence analysis of complementary DNA encoding a precursor for chicken natriuretic peptide

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Chicken α-natriuretic peptide (α-chNP) has been identified in chicken heart, which showed higher homology to brain natriuretic peptide (BNP) than to atrial natriuretic peptide (ANP) [1]. Complementary DNA (cDNA) clone encoding a chNP precursor (pre-chNP) was isolated from cardiac cDNA library and sequenced. Pre-chNP was 140-residue peptide carrying a 24-residue signal peptide at the N-terminus and α-chNP at the C-terminus, and did not exhibit high homology to porcine BNP except for the C-terminal region. However, a characteristic AT-rich nucleotide sequence commonly found in mammalian BNPs was also present in the 3'-untranslated region. Thus, chNP is concluded to be classified into the BNP-type.

Chicken natriuretic peptide; Atrial natriuretic peptide; Brain natriuretic peptide; cDNA cloning; Chicken heart

1. INTRODUCTION

We have recently isolated from chicken heart a novel natriuretic peptide of 29 amino acid residues, which elicits a pharmacological spectrum, such as diuretic, natriuretic and vasorelaxant activity, very similar to that of mammalian α-ANP [1]. Thus, the peptide was designated chicken α-natriuretic peptide (α-chNP). The complete amino acid sequence determined for α-chNP was, however, more highly homologous to porcine BNP than to mammalian α-ANP, although α-chNP was isolated from heart tissue [1,2]. Based on these facts, it is essential to determine the cDNA sequence of chNP precursor in order to obtain further structural information for chNP. The present paper describes cloning and sequence analysis of a cDNA for chicken cardiac mRNA encoding a precursor for chNP.

2. MATERIALS AND METHODS

2.1. *cDNA library construction*

Since α-chNP was found both in cardiac atrium and ventricle [1], total RNA was extracted from whole chicken heart tissue according to the method of Okayama et al. [3], and subjected to oligo(dT)-cellulose chromatography. Five μg of purified poly(A)$^+$ RNA thus obtained was subjected to synthesis of double-stranded cDNA by the method of Gubler and Hoffman [4] using a cDNA synthesis kit (Pharmacia). After the addition of *Eco*RI adaptors, a portion, corresponding to 70 ng of poly(A)$^+$ RNA, was ligated to 1 μg of *Eco*RI-digested and dephosphorylated λgt10 DNA (Promega Biotec). Half of the resulting ligation reaction mixture (10 μl) was used for in vitro packaging using Gigapak Plus (Stratagene).

2.2. *cDNA library screening*

Oligodeoxyribonucleotide probes were synthesized by the phosphoramidite method as three pools (probe Ia, Ib and II in Fig. 1A) and labeled at 3' end ($2–5 \times 10^7$ cpm/pmol) by the tailing method with a modification using [α-^{32}P]dCTP. The cDNA library was screened by plaque hybridization with in situ amplification of phage plaques [5]. The filters were hybridized with probe(s) in a hybridization solution ($3–6 \times 10^6$ cpm/ml) for 16 h at 37°C, and washed in a solution containing 3 M tetramethylammonium chloride at 53°C for probe II or 44°C for probes Ia and Ib [5,6].

2.3. *DNA sequencing*

cDNA fragments subcloned into M13 vectors (mp18 and mp19) were sequenced by the chain-termination method using 2'-deoxy-7-deaza-GTP (7-DEAZA sequencing kit; Takara Shuzo) [7].

2.4. *Primer extension*

Approximately 0.02 pmol of the *Hha*I-*Dde*I fragment (nucleotide −2 to 89) ^{32}P-labeled at 5' ends (5×10^7 cpm/pmol; preferentially labeled at *Dde*I-protruding-5' end) was denatured in 40 μl of 80% formamide containing 40 mM Pipes (pH 6.4) and 1 mM EDTA at 75°C for 10 min and then hybridized to 2 μg of chicken cardiac poly(A)$^+$ RNA in the same solution at 42°C for 3 h. Following ethanol precipitation, the primer/template complex was incubated with 0.1 μmol of dNTP and 8 units of murine reverse transcriptase (Pharmacia) for 1 h at 37°C. One-fifth of the primer-extension product was denatured in 50% formamide at 95°C for 3 min, and was electrophoresed through 6% acrylamide/7 M urea gel. *Hin*cII fragments of φX174 labeled with [α-^{32}P] dCTP by T4 DNA polymerase were used as a standard [5].

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Abbreviations: chNP, chicken natriuretic peptide; cDNA, complementary DNA; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; p.f.u., plaque forming unit; Pipes, piperazine *N,N'*-bis(2-ethanesulfonic acid); bp, base pairs; EDTA, ethylenediaminetetraacetic acid; dNTP, each of dGTP, dATP, dCTP, and dTTP

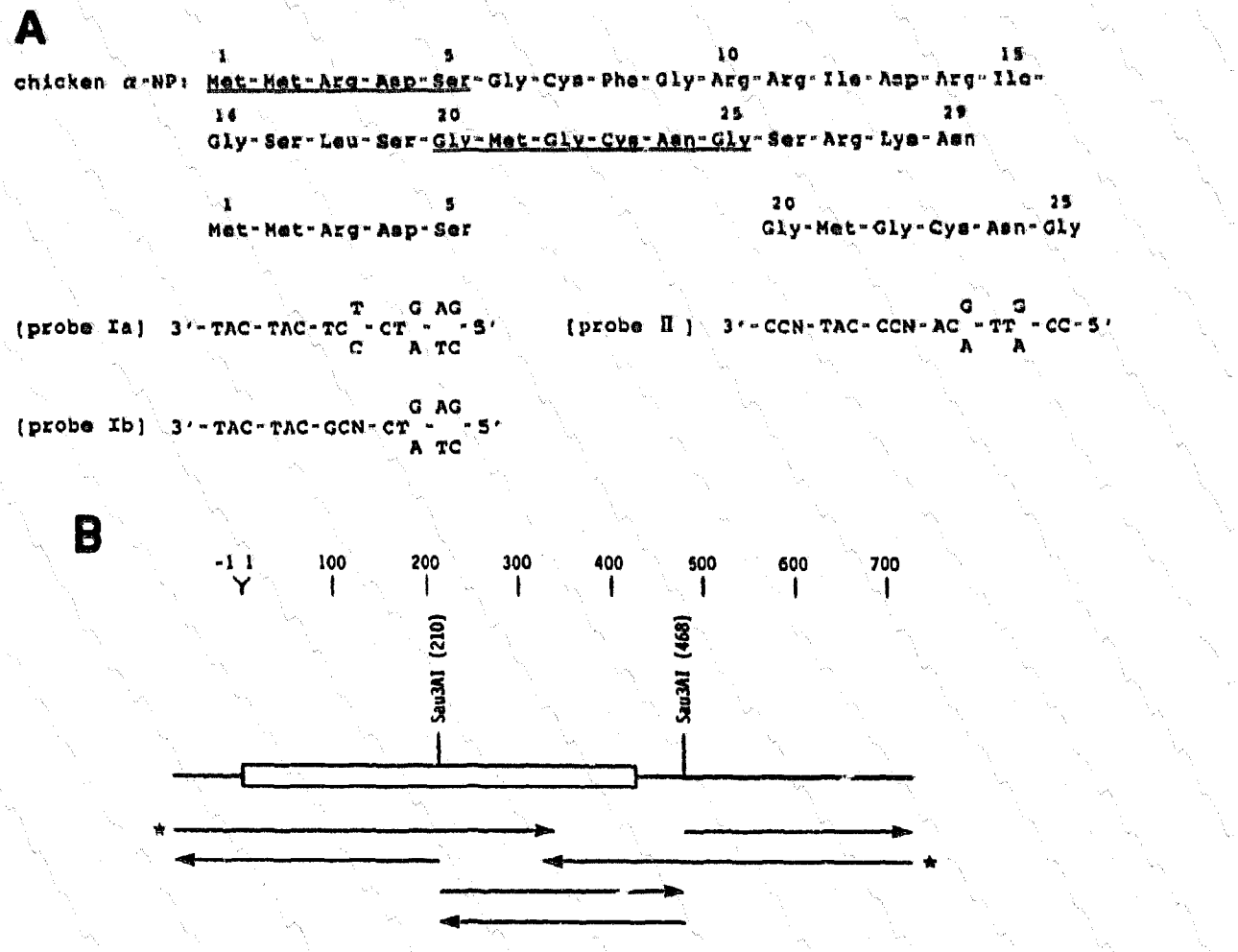

Fig. 1. (A) Amino acid sequence of chicken α-NP and synthetic oligodeoxyribonucleotide probes. The regions utilized for the syntheses of probes are underlined. (B) Strategy of sequencing an insert in λ chNP-E. Relevant *Sau*3AI sites in the insert were obtained by direct sequencing from each end (shown by asterisked arrows). Each arrow shows the direction and extent of sequence determination.

3. RESULTS AND DISCUSSION

According to the established amino acid sequence of α-chNP, oligodeoxyribonucleotides which represented all possible complementary sequence corresponding to two subsequenes, (Met-Met-Arg-Asp-Ser:probe Ia and probe Ib, Gly-Met-Gly-Cys-Asn-Gly: probe II) were synthesized (Fig. 1A) and used for screening cDNA clones for chNP precursor. A cDNA library representing chicken cardiac poly(A)$^+$ RNA was constructed (2.2×10^6 p.f.u. on C600hfl$^-$). A portion of the library (approximately 1×10^5 p.f.u.) was subjected to a first screening with probe II to isolate 29 positive clones. By a second screening of 9 of these clones using a mixture of probes Ia and Ib (molar ratio 1:2), 8 positive clones were isolated. A clone designated λchNP-E, which harbored the longest insert of approximately 800 base pairs (bp), was sequenced according to the strategy indicated in Fig. 1B. The complete nucleotide sequence of 755 bp long (excluding poly(A) tract and *Eco*RI adaptors) and predicted amino acid sequence are shown in Fig. 2. The sequence of nucleotides 334–420 corresponded precisely to the amino acid sequence of 29-residue α-chNP and was followed directly by a TAG termination codon. In the region preceding nucleotide 334, 111 amino acid residues were encoded from the first ATG codon (nucleotides 1–3). The nucleotide 1 was preceded by two TGA termination codons (nucleotides -60 to -58 and -33 to -31). Characteristic features of signal peptides were observed in the sequence of nucleotides 1–72, especially in nucleotides 31–51 encoding consecutive hydrophobic amino acid residues. These findings indicated that the first ATG (nucleotide 1) was the translation initiation site. The initiation site was also confirmed by the primer extension method (Fig. 3). The primer was extended, starting at nucleotide 91, to approximately 180 nucleotides. This meant that the mRNA for chNP precursor started around nucleotide -90, which was acceptable length as a 5'-untranslated region. Thus, pre-chNP was concluded to be composed

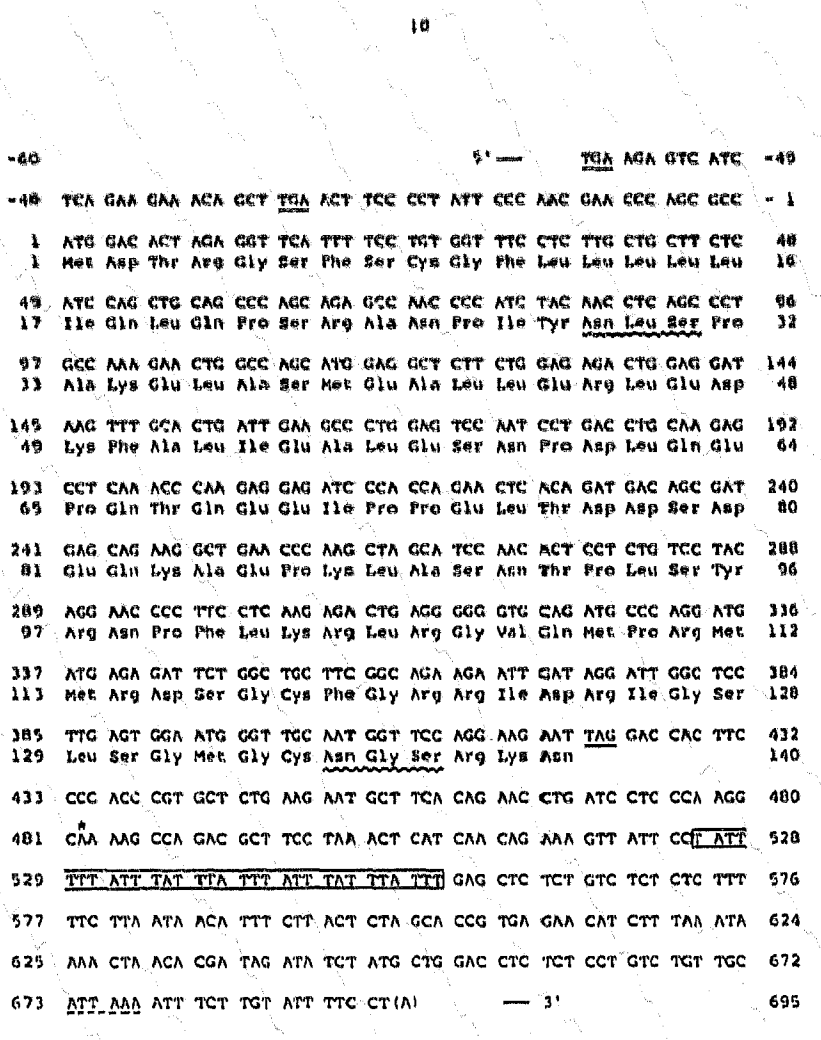

Fig. 2. Nucleotide sequence of the cDNA in λchNP-E with predicted amino acid residues. Nucleotides are numbered starting from the first nucleotide of putative ATG initiation codon. Nucleotides located on the 5′ side of nucleotide 1 are indicated by negative numbers. Predicted amino acid residues are shown under the nucleotide sequence and numbered from the initiating methionine. Wavy lines indicate potential glycosylation signals. Termination codons and the sequence ATTAAA are shown by solid and broken lines. AT-rich sequence in the 3′-untranslated region is boxed. Asterisked A at nucleotide 482 is substituted by G in another clone.

of 140 amino acid residues (nucleotide 1–420). Nucleotide 482 (asterisked A in Fig. 2) in the 3′-untranslated region was substituted by G in another clone. This might be due to the fact that the cDNA library was prepared from poly(A)$^+$ RNA of 3 chickens. The sequence ATTAAA (23–18 nucleotides before poly(A) tract) is probably a variant of the sequence AATAAA which is known to be a polyadenylation signal [8].

Concurrent with the present study, we have succeeded in isolating chicken γ-NP(γ-chNP) of 12000 Da from chicken cardiac ventricular extracts as a major storage form of chNP [9]. The 116-residue sequence determined for the isolated γ-chNP was found to be identical to subsequence 25–140 of pre-chNP deduced from the cDNA sequence analysis. Therefore, γ-chNP of the major molecular form of chNP in heart, is concluded to be generated from pre-chNP by removal of a signal peptide in a manner similar to mammalian ANP and BNP [10–12]. Although there are two potential glycosylation sites in the molecule of pre-chNP (see Fig. 2), no glycosylation has been observed on either position in the amino acid sequence analyses of γ-chNP and α-chNP [1,9].

During the course of the present study, a cDNA encoding porcine BNP precursor has been identified and sequenced [12]. Furthermore, amino acid sequences of human and rat BNP precursors have also been deduced by the cDNA cloning method [13,14]. In this paper, the nucleotide and amino acid sequences of pre-chNP are compared with those of human pre-ANP and porcine pre-BNP, each as a representative of mammalian ANP-

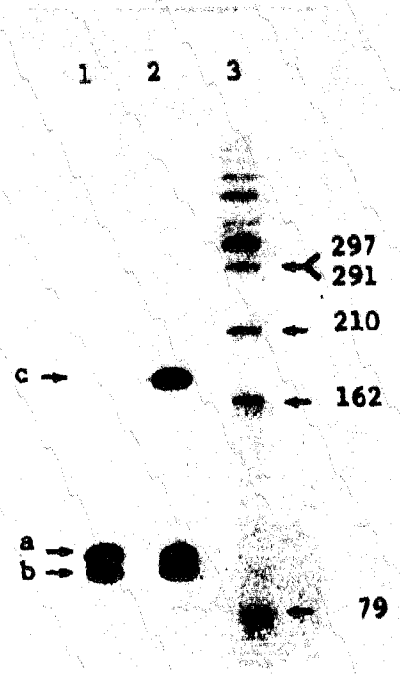

Fig. 3. Identification of the 5′-terminus of chNP precursor mRNA by primer extension. (Lane 1) *Hhal-Ddel* fragments (nucleotides −2 to 89) before primer extension. (Lane 2) Product after primer extension. (Lane 3) *Hin*cII fragments of ϕX174. Numbers on the right side indicate nucleotide length. (Arrow a) Primer fragment (95 nucleotides). (Arrow b) Primer-complement fragment (90 nucleotides) labeled at *Hha*I-recessed-5′ end. (Arrow c) Primer-extended fragment (about 180 nucleotides).

type or BNP-type (Fig. 4A). When the 420-nucleotide sequence encoding pre-chNP is aligned to give maximum matching, it shows 59.7% and 58.7% homology to porcine pre-BNP and human pre-ANP, respectively. At the amino acid sequence level, only 41.0% and 38.9% of the residues of pre-chNP are identical to those of porcine pre-BNP and human pre-ANP, respectively (Fig. 4A). In the C-terminal 32-residue peptide, however, sequence homology between chNP and porcine BNP increases up to 71.9% and 70.7% at the amino acid and nucleotide levels, respectively. Moreover, it should be noted that chNP cDNA carries several repetitive ATTTA sequences in the 3′-untranslated region, as is also observed in mammalian BNP cDNAs (Fig. 4B) [12–14]. Especially, nucleotides 525–555 of chNP cDNA completely consist of A and T, and show 90.3% homology to the corresponding region of porcine BNP cDNA. Since recent studies have verified that repetition of ATTTA sequence destabilizes mRNA in the cell, the mRNA encoding chNP may be transcribed in response to stimuli and degraded in a short period [15,16]. Such AT-rich nucleotide sequences should now be recognized as one of structural features of the BNP-type, since cDNAs for mammalian ANPs never carry such an AT-rich sequence in the 3′-untranslated region [10]. Although nucleotide and amino acid sequences of chNP do not exhibit extremely high homology to porcine BNP, this peptide shares with mammalian BNPs the highly

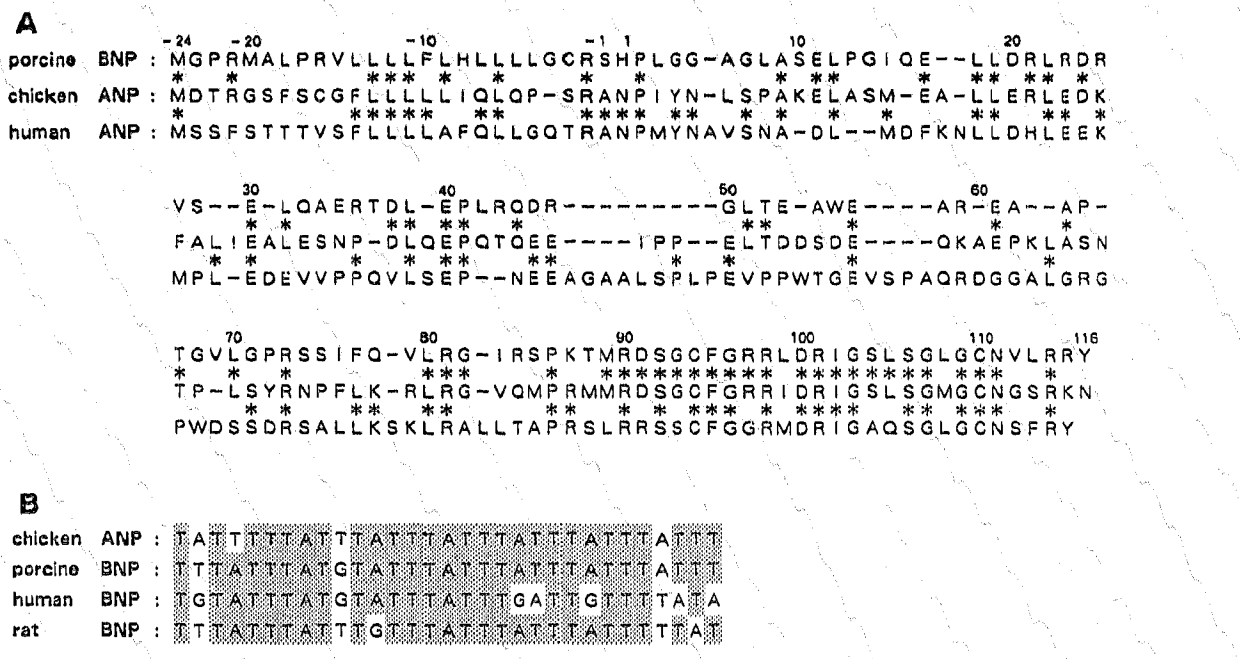

Fig. 4. (A) Amino acid sequence of chicken pre-NP compared with those of porcine pre-BNP and human pre-ANP. Amino acids are written in one letter and numbered from the N-terminus of γ-form (pro-form) according to chicken NP numbering system. Signal peptides are indicated by negative number. Amino acid residues are aligned to give maximum matching and the matched one is indicated by an asterisk. (B) Alignment of AT-rich sequences in the 3′-untranslated regions of cDNAs for chicken NP, and porcine, human and rat BNPs. Columns, in which more than two nucleotides are identical, are shaded.

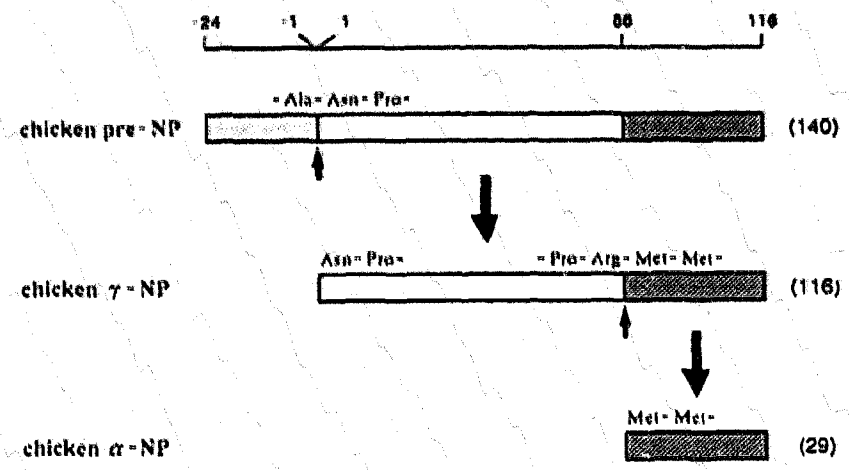

Fig. 5. Putative proteolytic processing steps of chicken pre-NP. The scale is numbered in the manner of Fig. 4A. Proteolytic processing sites (shown by arrows) and neighboring amino acid residues are added. Numbers on the right side indicate amino acid residue numbers. (▭) signal peptide; (▬) α-chNP.

homologous C-terminal sequence essential for exerting bioactivity and a characterisic AT-rich sequence in the 3'-untranslated region. Thus, chNP is concluded to be a member of the BNP-type.

Identification of α-chNP and γ-chNP in chicken heart indicates that proteolytic processing of pre-chNP takes place by two steps after the translation event [9]. Amino acid sequence of -Arg-$\overset{-1}{\underline{\text{Ala}}}$-$\overset{1}{\underline{\text{Asn}}}$-Pro-Ile-Tyr-Asn- in pre-chNP, in which Ala-Asn bond is first cleaved by a signal peptidase, is more highly homologous to that in human pre-ANP(-Arg-$\overset{-1}{\underline{\text{Ala}}}$-$\overset{1}{\underline{\text{Asn}}}$-Pro-Met-Tyr-Asn-) than that in porcine pre-BNP(-Arg-$\overset{-1}{\underline{\text{Ser}}}$ -$\overset{1}{\underline{\text{His}}}$-Pro-Leu-Gly-Gly-). This processing removes the signal peptide and yields 116-residue γ-chNP as a major storage form in heart. The location of an α-chNP structure at the C-terminus of γ-chNP indicates that further processing takes place after Pro-Arg sequence (residues 86–87) to afford α-chNP. It is notable that an identical sequence is known as a processing signal for converting mammalian γ-ANP into α-ANP [10]. Thus, proteolytic processing signals of pre-chNP to generate mature α-chNP are found to be identical to those of mammalian ANPs. Accordingly, processing of pre-chNP in heart probably takes place in a manner similar to that of mammalian ANP, as summarized in Fig. 5.

We have first demonstrated the existence of the two distinct types, ANP-type and BNP-type, in the mammalian natriuretic peptide family by identification of porcine BNP [2]. By the present study, chNP is shown to be classified into the BNP-type based not only on the amino acid sequence of a bioactive unit but also on the mRNA sequence. These facts indicate that BNP ancestor gene was already present in lower vertebrates and preserved in mammals and avians. On the other hand, chNP of the BNP-type is predominantly expressed in chicken heart and is present as a major natriuretic peptide [1,9]. In contrast to chNP, porcine BNP is also present in heart but only as a minor component as compared to ANP [17]. In should be clarified why chNP of the BNP-type is predominantly expressed in chicken heart instead of the ANP-type.

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