

## Molecular cloning of hamster brain and atrial natriuretic peptide cDNAs. Cardiomyopathic hamsters are useful models for brain and atrial natriuretic peptides.

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### Research Article

Brain and atrial natriuretic peptides (BNP and ANP) are cardiac hormones with diuretic, natriuretic, and vasodilatory activities. Cardiomyopathic hamsters are widely used animal models of heart failure. Due to the structural divergence of BNP among species, examination on pathophysiological roles of BNP using cardiomyopathic hamsters is so far impossible. We therefore isolated hamster BNP and ANP cDNAs, and investigated synthesis and secretion of these peptides in normal and cardiomyopathic hamsters. The COOH-terminal 32-residue peptide of cloned hamster preproBNP with 122 amino acids, preceded by a single arginine residue, supposedly represents hamster BNP showing < 50% homology to rat BNP. Alpha-hamster ANP, 28-residue peptide, is identical to alpha-rat ANP. In hamsters, BNP and ANP occur mainly in the ventricle and the atrium, respectively. The 32-wk-old hypertrophic cardiomyopathic BIO14.6 strain exhibited ventricular hypertrophy. The 32-wk-old dilated cardiomyopathic BIO53.58 strain remained at the stage without apparent heart failure. In BIO14.6 and BIO53.58 strains at this age, ventricular BNP and ANP gene expressions are augmented, and the plasma BNP concentration is elevated to 136 and 108 fmol/ml, respectively, three times greater than the elevated plasma ANP concentration, which well mimics changes of the plasma BNP and ANP concentrations in human heart failure. Cardiomyopathic hamsters, therefore, are useful models to investigate the implication of BNP in human cardiovascular diseases.

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# Molecular Cloning of Hamster Brain and Atrial Natriuretic Peptide cDNAs

## Cardiomyopathic Hamsters are Useful Models for Brain and Atrial Natriuretic Peptides

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### Abstract

Brain and atrial natriuretic peptides (BNP and ANP) are cardiac hormones with diuretic, natriuretic, and vasodilatory activities. Cardiomyopathic hamsters are widely used animal models of heart failure. Due to the structural divergence of BNP among species, examination on pathophysiological roles of BNP using cardiomyopathic hamsters is so far impossible. We therefore isolated hamster BNP and ANP cDNAs, and investigated synthesis and secretion of these peptides in normal and cardiomyopathic hamsters. The COOH-terminal 32-residue peptide of cloned hamster pre-proBNP with 122 amino acids, preceded by a single arginine residue, supposedly represents hamster BNP showing < 50% homology to rat BNP. Alpha-hamster ANP, 28-residue peptide, is identical to  $\alpha$ -rat ANP. In hamsters, BNP and ANP occur mainly in the ventricle and the atrium, respectively. The 32-wk-old hypertrophic cardiomyopathic BIO14.6 strain exhibited ventricular hypertrophy. The 32-wk-old dilated cardiomyopathic BIO53.58 strain remained at the stage without apparent heart failure. In BIO14.6 and BIO53.58 strains at this age, ventricular BNP and ANP gene expressions are augmented, and the plasma BNP concentration is elevated to 136 and 108 fmol/ml, respectively, three times greater than the elevated plasma ANP concentration, which well mimics changes of the plasma BNP and ANP concentrations in human heart failure. Cardiomyopathic hamsters, therefore, are useful models to investigate the implication of BNP in human cardiovascular diseases. (J. Clin. Invest. 1994; 94:1059–1068.) Key words: cardiac hypertrophy • congestive heart failure • gene expression • radioimmunoassay • tissue distribution

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1. Abbreviations used in this paper: AMI, acute myocardial infarction; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CHF, congestive heart failure; CNP, C-type natriuretic peptide; DOCA, deoxycorticosterone acetate; HOCM, hypertrophic obstructive cardiomyopathy; HP-GPC, high-performance gel permeation chromatography; LI, like immunoreactivity; SHR, spontaneously hypertensive rats.

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### Introduction

Since the discovery of atrial natriuretic peptide (ANP)<sup>1</sup> in the heart (1), it has been demonstrated that ANP is synthesized in and secreted from the heart, implicated in body fluid homeostasis and blood pressure control as a cardiac hormone (2). In normal hearts, ANP is produced mainly in the atrium and only slightly occurs in the ventricle, whereas in patients with congestive heart failure (CHF) and animal models of ventricular hypertrophy, ANP synthesis and secretion are greatly augmented in the ventricle (3–6).

Brain natriuretic peptide (BNP), a second natriuretic peptide, was originally isolated from the porcine brain (7). This peptide shows a sequence homology to ANP (7) and has peripheral and central actions similar to those of ANP (8, 9). We previously demonstrated that BNP is present at the highest concentration in the heart rather than in the brain, and also that BNP is synthesized in and secreted from the heart and acts as a cardiac hormone (10, 11). Furthermore, we disclosed that BNP occurs mainly in the ventricle, which contrasts with ANP, a cardiac hormone from the atrium (12, 13).

BNP synthesis and secretion are augmented in diseased ventricles (12–19). We previously reported that the plasma BNP concentration (0.9 fmol/ml) is only one sixth of the plasma ANP concentration in normal humans, whereas it is elevated several hundredfold ( $\sim$  300 fmol/ml) and exceeds the plasma ANP concentration ( $\sim$  200 fmol/ml) in patients with severe CHF (12). In addition, in the early phase of acute myocardial infarction (AMI), the plasma BNP concentration is rapidly and prominently increased, while the plasma ANP concentration is slightly altered (20). These findings suggest that BNP has pathophysiological roles distinct from those of ANP.

The pathophysiological implication of BNP has so far been studied using spontaneously hypertensive rats (SHR), deoxycorticosterone acetate (DOCA)-salt rats, and experimental AMI rats (13, 15).<sup>2</sup> In these experimental rat models, however, the plasma BNP concentration remains much lower than the elevated plasma ANP concentration simultaneously determined, which is quite different from our observations on human heart diseases (12). It is therefore difficult to assess the significance of BNP in human heart diseases using these rat models. In addition, these animals are not suitable models for CHF.

Cardiomyopathic hamsters have been widely used in studies on pathogenesis, therapy, and prevention of heart failure (21–23). Structures of hamster ANP and BNP, however, have never been disclosed. Mammalian ANPs so far identified are 28-resi-

2. Hama, N., H. Itoh, G. Shirakami, O. Nakagawa, S. Suga, Y. Ogawa, T. Yoshimasa, Y. Hashimoto, M. Yamaguchi, R. Hori, and K. Nakao, manuscript submitted for publication.

due peptides, and their amino acid sequences are identical except for a single residue substitution at the 12th position (Ile in rodents, Met in humans) (1, 24), which enabled investigators to detect ANP-like immunoreactivities (—LIs) in hamster tissues using the RIA for rat ANP (5, 25). In cardiomyopathic hamsters, it has been demonstrated that ANP synthesis and secretion are augmented in the ventricle and that the plasma ANP-LI concentration is increased (5, 25). In contrast, the structure of BNP is divergent among species. The major circulating forms of BNP are 26, 45, and 32-residue peptides in pigs, rats, and humans (9), respectively. We could not detect any BNP-LIs in hamster tissues and plasma samples using RIAs for rat and human BNPs (11, 12), suggesting that the structure of hamster BNP is also quite different from those of BNPs of other species. Therefore, to investigate the pathophysiological significance of BNP using cardiomyopathic hamsters, it is essential to clarify the structure of hamster BNP.

In this context, in the present study, we isolated the full-length hamster BNP cDNA and examined the synthesis and secretion of BNP in normal golden Syrian hamsters. We also isolated the full-length hamster ANP cDNA. Then we compared the synthesis and secretion of both natriuretic peptides in hearts of hypertrophic (BIO14.6) and dilated (BIO53.58) cardiomyopathic hamsters with those in the heart of the control hamster (F1B). The present study indicates that cardiomyopathic hamsters are useful animal models to investigate the pathophysiological significance of BNP and ANP in human cardiovascular diseases.

## Methods

**Preparation of hamster BNP-specific cDNA probe.** To obtain a hamster BNP-specific cDNA probe, we performed the PCR. First strand cDNA was generated from ventricular total RNA of golden Syrian hamsters (*Mesocricetus auratus*) by the oligo(dT)-primed reverse transcription (26). The PCR was carried out under the standard conditions (27). The reaction mixture was incubated at 94°C for 0.5 min at first, then it was run through 31 cycles of denaturing (0.5 min at 94°C), annealing (0.5 min at 60°C), and extension (1 min at 72°C). The reaction was finished with a 4-min incubation at 72°C. The sense and antisense primers were 5'-TCGGTCGACGGAGGTCACTCCATCCTCTGG-3' and 5'-AGAGTCGACTGCAGCTGCAGCCAGGAGGTCTTC-3' (SalI and PstI sites are underlined), corresponding to regions around the signal-sequence cleavage sites and just 3' to the stop codons of rat, porcine, and human BNP cDNAs (28–30), respectively. Nucleotide sequences of these regions are highly conserved in BNP cDNAs from these species (28–30). The PCR resulted in a 0.3-kb hamster BNP cDNA fragment (Fig. 1A).

**Cloning of hamster BNP and ANP cDNAs.** A hamster heart cDNA library was constructed from 5 µg of poly(A)<sup>+</sup> RNA of golden Syrian hamster hearts. Double-stranded cDNA, synthesized by the method of Gubler and Hoffman (26), was cloned into the phage vector λZAPII (Stratagene, La Jolla, CA), which resulted in ~2 × 10<sup>6</sup> primary recombinants.

To isolate the full-length hamster BNP and ANP cDNAs, we screened approximately 4 × 10<sup>5</sup> plaques each from the library using the obtained hamster BNP cDNA fragment and HincII-StuI fragment of rat ANP cDNA (4) as probes, respectively. Screening was carried out as previously reported (27). Positive phage clones were excised in vivo as recommended by the supplier, allowing the cDNA to be analyzed in the pBluescript vector (Stratagene).

**DNA sequencing.** DNA sequencing was carried out by the dideoxy chain-termination method (26). The final sequence was determined from both DNA strands.

**Table I. Profiles of Cardiomyopathic (BIO14.6 and BIO53.58) and Control (F1B) Hamsters**

Strains	F1B	BIO14.6	BIO53.58
Body wt (g)	168±12	126±5*	121±5*
Vt wt (mg)	370±29	447±15	325±25
Vt/BW (×10 <sup>-3</sup> )	2.37±0.29	3.56±0.05*	2.69±0.12

All values are expressed as the mean±SEM from three individual animals. \*P < 0.05, compared with values in the F1B strain. Vt wt, ventricular wt; Vt/BW, ventricular wt to body wt ratio.

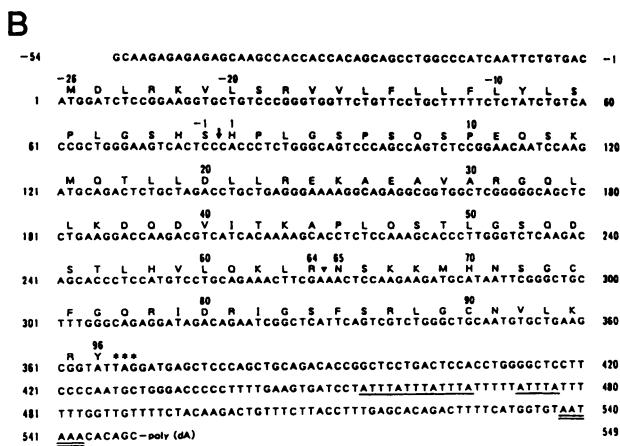
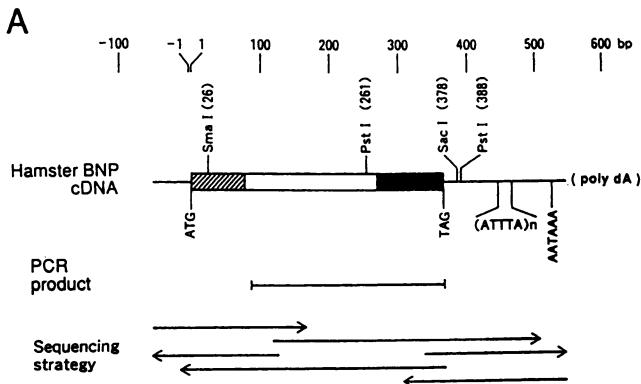
**Peptides.** Hamster BNP[83–96], hamster BNP[65–96], rat BNP, human BNP, porcine BNP, and C-type natriuretic peptide (CNP) were synthesized by the solid phase method. α-rat ANP and α-human ANP were purchased from Peptide Institute, Inc. (Minoh, Japan).

**Development of RIA for hamster BNP.** Hamster BNP[83–96] was conjugated to bovine thyroglobulin (Sigma Chemical Co., St. Louis, MO) using the carbodiimide coupling procedure (31). Three adult female Japanese white rabbits were immunized with the conjugate as previously reported (31). Elevation of the antibody titer was observed in one rabbit, and its serum was used as the antiserum against hamster BNP (HR-1). Hamster BNP[65–96] was radioiodinated by the chloramine T method (31). The specific activity of <sup>125</sup>I-hamster BNP[65–96] ranged from 50 to 100 µCi/µg. The RIA for hamster BNP was performed following the method of the RIA for the COOH-terminal fragment of ANP (31). The antiserum HR-1 was used at a final 1:5 × 10<sup>4</sup> dilution.

**Animals.** 28-wk-old male golden Syrian hamsters (Shimizu Experimental Supplies, Kyoto, Japan) were used to examine BNP and ANP gene expressions and BNP and ANP concentrations in hamster tissues. Furthermore, to examine the synthesis and secretion of these peptides in cardiomyopathic hamsters, 32-wk-old male hypertrophic (BIO14.6) and dilated (BIO53.58) cardiomyopathic strains, and their age-matched control (F1B) strain were obtained from Bio Breeders Inc. (Watertown, MA). They were housed in a temperature-, humidity-, and light-controlled room with free access to water and standard food (CE-2, Japan CLEA, Tokyo, Japan; NaCl 0.85/100 g). Table I shows brief profiles of cardiomyopathic and control hamsters used in the present study. In the present study, the BIO14.6 strain showed ventricular hypertrophy, as judged by the ventricular wt/body wt ratio (Table I), without any apparent signs of CHF. The BIO53.58 strain exhibited no signs of ventricular hypertrophy and CHF.

**Plasma samplings and tissue preparations.** After hamsters were anesthetized by intraperitoneal injections of pentobarbital (60 mg/kg), blood was sampled from the jugular vein and immediately transferred to chilled polypropylene tubes, containing Na<sub>2</sub>EDTA (1 mg/ml) and aprotinin (Ohkura Pharmaceutical Inc., Kyoto, Japan; 1,000 kallikrein inactivator U/ml), and centrifuged at 4°C. Hearts, brains, and other tissues were immediately removed from hamsters as previously described (13). To prevent atrial contamination, the apical half of the ventricle was used as the ventricular sample. Plasma and tissue samples were frozen in liquid nitrogen, and stored at -70°C until use.

**Northern blot analysis.** Total RNA was extracted by the guanidinium thiocyanate method (26), and Northern blot analysis was carried out with the <sup>32</sup>P-labeled full-length hamster BNP cDNA (Fig. 1A) and 217-bp BglII fragment of hamster ANP cDNA (Fig. 2A) as probes, as previously described (13). A human β-actin genomic probe (Wako Pure Chemical Industries Ltd., Osaka, Japan) was used to monitor the amount of total RNA in each sample. Relative amounts of mRNA species were determined by laser densitometric scanning, and the BNP mRNA and ANP mRNA concentrations were expressed relative to those of the right atrium of the F1B strain (amounts of BNP mRNA and ANP



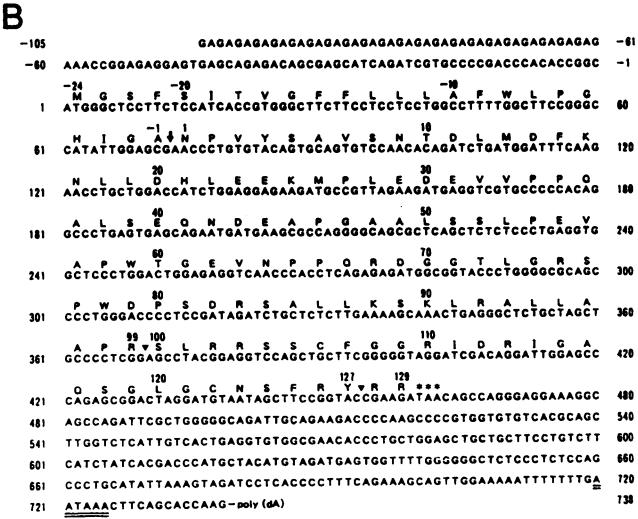
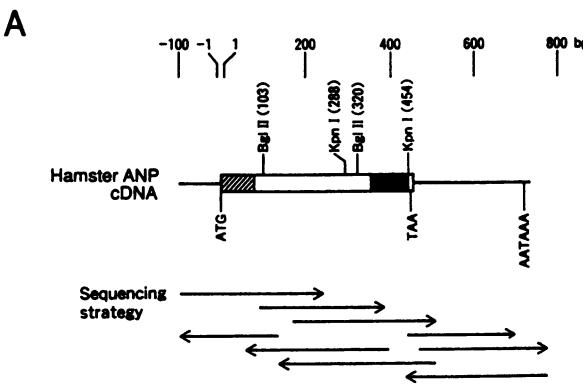
**Figure 1.** Structure of hamster BNP cDNA. (A) The restriction enzyme map of hamster BNP cDNA (clone phamBNP15), location of hamster BNP-specific cDNA probe obtained by PCR, and sequencing strategy. The coding region is boxed, and regions encoding the signal peptide (■) and BNP (■) are indicated. Positions of the translation start and stop codons, "ATTTA" motifs, and a polyadenylation signal (AA-TAAA) are indicated. The nucleotide number of the first nucleotide of the ATG translation start codon is designated as +1. Selected restriction enzyme sites are shown with nucleotide numbers in round brackets. (B) Nucleotide and deduced amino acid sequences of hamster BNP cDNA. Amino acids are expressed in one letter code. The translation stop codon is indicated by \*\*\*. "ATTTA" motifs and a polyadenylation signal are underlined with solid and double lines, respectively. The signal-sequence cleavage site (arrow) and putative processing site for BNP (arrowhead) are indicated. These sequence data are available from DDBJ/EMBL/GenBank under accession number D17314.

mRNA in 1.0  $\mu$ g of total RNA from the right atrium of the F1B strain were defined as 1.0 unit).

**Measurements of BNP-LI and ANP-LI concentrations.** Tissue and plasma extractions were carried out as previously reported (13). The BNP-LI and ANP-LI concentrations were measured by the RIA for hamster BNP and the RIA for the COOH-terminal fragment of ANP (31), respectively. The cross-reactivity of hamster BNP[65-96] in the RIA for ANP was < 0.01%. Recoveries of 100 fmol of hamster BNP[65-96] and  $\alpha$ -rat ANP added to 1-ml plasma were 80 and 70%, respectively.

*High-performance gel permeation chromatography (HP-GPC).* HP-GPC was performed on a TSK-GEL G2000 SW column (7.5 × 600 mm, Toyo Soda, Tokyo, Japan), as previously reported (31). The flow rate was 0.3 ml/min, and the fraction volume was 0.36 ml.

*Statistical analysis.* All data were expressed as the mean  $\pm$  SEM



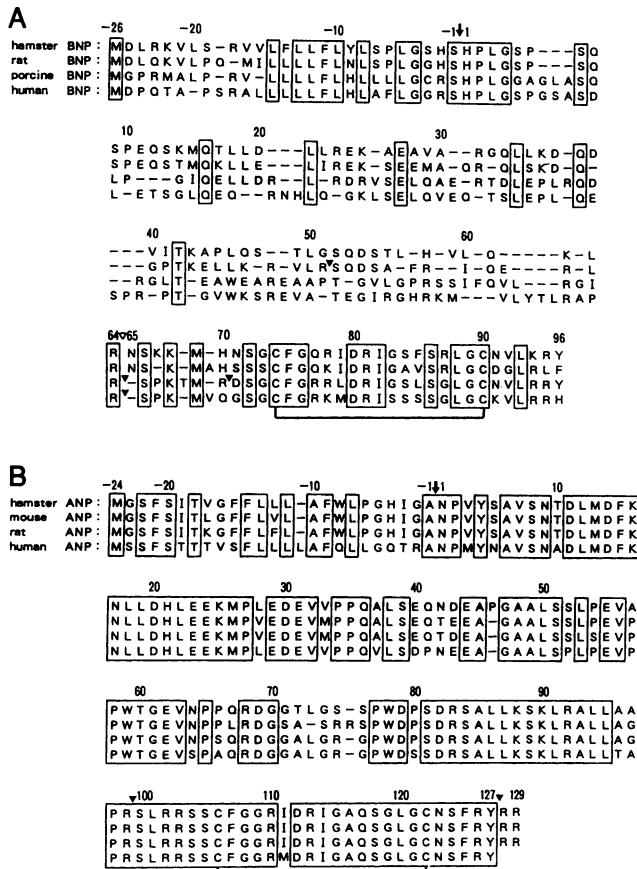
**Figure 2.** Structure of hamster ANP cDNA. (A) The restriction enzyme map of hamster ANP cDNA (clone phamANP4) and sequencing strategy. (B) Nucleotide and deduced amino acid sequences of hamster ANP cDNA. Symbols are the same as in Fig. 1. These sequence data are available from DDBJ/EMBL/GenBank under accession number D17313.

from three individual animals. The statistical significance of differences in mean values was assessed by unpaired Student's *t* test.

## Results

*Isolation and characterization of hamster BNP cDNA.* Approximately  $4 \times 10^5$  plaques were screened and 76 primary positives were obtained. The longest clone, phamBNP15, contained a 603-bp insert with an open reading frame of 366 bp (Fig. 1). The coding region of hamster BNP cDNA was 78, 64, and 63% homologous to those of rat, porcine, and human BNP cDNAs (28–30), respectively. The nucleotide sequence from + 89 to + 369 was identical to that of the hamster BNP cDNA fragment obtained by the PCR (Fig. 1 A). The 3'-untranslated region contained a typical polyadenylation signal, “AATAAA” (32), and 4 copies of “ATTTA” motif implicated in mRNA instability (33) (Fig. 1).

Analysis of the deduced amino acid sequence revealed that hamster preproBNP consists of 122 amino acids (Fig. 1 B). The NH<sub>2</sub>-terminal hydrophobic 26-residue peptide represented



**Figure 3.** Alignment of amino acid sequences of preproBNPs and preproANPs. (A) Alignment of amino acid sequences of hamster, rat, porcine, and human preproBNPs. Conserved amino acids are boxed, and two cysteine residues to form an intramolecular disulfide-linkage are connected by the solid line. A dash indicates a space introduced to maximize homology. Amino acids of hamster preproBNP are numbered above the sequence (the NH<sub>2</sub> terminus of proBNP is designated as +1). The signal-sequence cleavage site (arrow), processing sites for rat BNP, porcine BNP-32, porcine BNP, human BNP (▼), and putative processing site for hamster BNP (▽) are indicated. (B) Alignment of amino acid sequences of hamster, mouse, rat, and human preproANPs.

a signal peptide (Fig. 1 B). The COOH-terminal sequence of preproBNP contained two cysteine residues (Cys<sup>74</sup> and Cys<sup>90</sup>) to form a 17-residue ring structure, which commonly occurs in members of the natriuretic peptide family (24, 28–30, 34, 35) (Fig. 3). The COOH-terminal 32-residue peptide of hamster preproBNP (hamster BNP[65–96]) was preceded by a single arginine residue, which is the proteolytic processing site for porcine, rat, and human BNPs (28–30) (Fig. 3 A). The amino acid sequence of hamster preproBNP was 63, 46, and 43% homologous to rat, porcine, and human preproBNPs (28–30), respectively.

**Isolation and characterization of hamster ANP cDNA.** From approximately  $4 \times 10^5$  plaques, 255 primary positives were obtained. The longest clone, phamANP4, contained a 843-bp insert with an open reading frame of 459 bp (Fig. 2). The coding region of hamster ANP cDNA was 89, 90, and 81% homologous to those of rat, mouse, and human ANP cDNAs

(24, 34, 35), respectively. The 3'-untranslated region contained a typical polyadenylation signal, “AATAAA” (32).

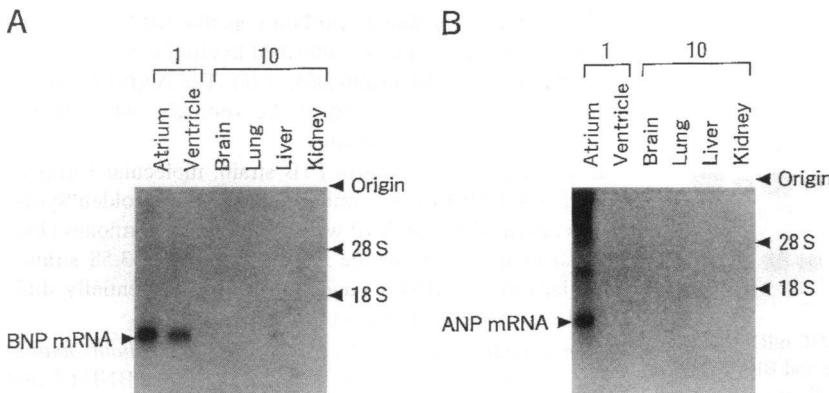
Hamster preproANP consisted of 153 amino acids (Fig. 2 B). The NH<sub>2</sub>-terminal 24-residue peptide was a signal peptide (Fig. 2 B).  $\alpha$ -Hamster ANP sequence of 28 amino acids was located at the COOH-terminal region of preproANP, and was flanked by Pro<sup>98</sup>-Arg<sup>99</sup> and Arg<sup>128</sup>-Arg<sup>129</sup> dipeptides which are proteolytic processing sites for rat and mouse  $\alpha$ -ANPs (Fig. 3 B) (34, 35). Alpha-hamster ANP was identical to  $\alpha$ -rat ANP (Fig. 3 B) (34). Hamster preproANP was 90, 90, and 81% homologous to rat, mouse, and human preproANPs (24, 34, 35), respectively.

**BNP and ANP gene expressions in hamster tissues.** Northern blot analysis identified a single 0.8-kb BNP mRNA species both in the atrium and ventricle (Fig. 4 A). The BNP mRNA concentration in the ventricle was about half of that in the atrium. Taking account of tissue weight, ~80% of the total content of BNP mRNA in the whole heart was of ventricular origin. In contrast, 0.9-kb ANP mRNA was detected mainly in the atrium (Fig. 4 B). The concentration and total content of ANP mRNA in the ventricle were only 2% and <20% of those in the atrium, respectively. No significant amounts of BNP and ANP transcripts were detected in other tissues including the brain (Fig. 4).

**Development of RIA for hamster BNP.** A typical standard curve of hamster BNP[65–96] and dilution curves of cross-reactivities of related peptides in the RIA for hamster BNP are shown in Fig. 5 A. The minimal detectable quantity in the RIA was 1 fmol/tube and the 50% binding intercept was 7 fmol/tube. In the RIA for hamster BNP, cross-reactivities of rat BNP, human BNP,  $\alpha$ -ANP, and CNP were <0.01%, while porcine BNP showed a cross-reactivity of 35% on a molar basis. The intra- and inter-assay coefficients of variation were 7.5% ( $n = 10$ ) and 4.8% ( $n = 6$ ), respectively.

**Distributions and molecular forms of BNP-LI and ANP-LI in hamster tissues.** Serial dilution curves of extracts of the atrium, ventricle, lung, and plasma were parallel to the standard curve (Fig. 5 B). In 28-wk-old golden Syrian hamsters, the BNP-LI concentration in the ventricle ( $112 \pm 9$  pmol/g,  $n = 3$ ) was 4% of that in the atrium ( $3.11 \pm 0.52$  nmol/g), while the ANP-LI concentration in the ventricle ( $9.78 \pm 1.61$  pmol/g) was only 0.1% of that in the atrium ( $8.34 \pm 0.64$  nmol/g). In the brain, ANP-LI was detected ( $0.55 \pm 0.14$  pmol/g), while BNP-LI was not detected (<0.2 pmol/g). In the lung, BNP-LI was detected ( $4.35 \pm 0.56$  pmol/g), while ANP-LI was not detected (<0.07 pmol/g). In the liver and kidney, BNP-LI and ANP-LI were not detectable. The plasma BNP-LI and ANP-LI concentrations were below the detection limits of the respective RIAs (BNP-LI, <15 fmol/ml; ANP-LI, <10 fmol/ml).

The HP-GPC revealed that BNP-LI in the atrial and ventricular extracts of 28-wk-old golden Syrian hamsters comprised a single component with an approximate mol wt of 14 kD (corresponding to proBNP). ANP-LI in the atrium was eluted as a single peak at the elution position of the precursor form of rat ANP ( $\gamma$ -rat ANP, 14 kD). In the ventricle, the major component of ANP-LI was eluted at the elution position of  $\alpha$ -rat ANP, and there was also a minor component of ANP-LI eluted at the position of  $\alpha$ -hamster ANP. These HP-GPC profiles of BNP-LI and ANP-LI in the heart of the golden Syrian hamster were essentially similar to those in the heart of the F1B strain illustrated below in Fig. 8.



**Figure 4.** Northern blot analysis of BNP mRNA (A) and ANP mRNA (B) in tissues of 28-wk-old golden Syrian hamsters. One  $\mu$ g each of total RNAs from the atrium and ventricle, and 10  $\mu$ g each of total RNAs from the brain, lung, liver, and kidney were used.

**BNP and ANP gene expressions in cardiomyopathic hamster hearts.** Representative data of Northern blot analyses of BNP and ANP mRNAs in hearts of 32-wk-old cardiomyopathic hamsters (BIO14.6 and BIO53.58 strains) and age-matched control hamsters (F1B strain) are depicted in Fig. 6. Actin transcript levels were equivalent among different RNA samples (data not shown). In the heart of the control F1B strain, the rank order of the BNP mRNA concentration was right atrium = left atrium > left ventricle  $\geq$  right ventricle, and that of the ANP mRNA concentration was right atrium = left atrium  $\geq$  left ventricle > right ventricle (Fig. 6). The BNP mRNA and ANP mRNA concentrations in the left ventricle were one order of magnitude higher than those in the right ventricle, respectively (Fig. 6).

The BNP mRNA and ANP mRNA concentrations in the three strains are summarized in Fig. 7 A. The BNP mRNA concentration in the ventricle was  $\sim$  50% of that in the atrium, and the total content of BNP mRNA in the ventricle was 87% of that in the whole heart. By contrast, the ANP mRNA concentration in the ventricle was only 2% of that in the atrium, and the total content of ANP mRNA in the ventricle was 20% of that in the whole heart. These observations were similar to those on the golden Syrian hamster.

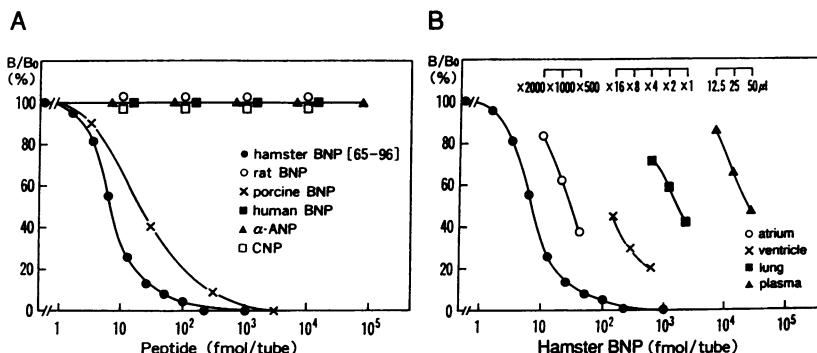
In the present study, the 32-wk-old hypertrophic cardiomyopathic BIO14.6 strain exhibited apparent ventricular hypertrophy as judged from the ventricular wt/body wt ratio (Table I) and no signs of CHF. In this strain, as compared with the F1B strain, the BNP mRNA concentration was elevated 1.5-fold in the atrium and fourfold in the ventricle (Fig. 7 A). As a result, the BNP mRNA concentration in the ventricle exceeded that in the atrium, and the total content of BNP mRNA in the ventricle reached to 95% of that in the whole heart. On the other hand,

although unchanged in the atrium, the ANP mRNA concentration was increased more than 10-fold in the ventricle (Fig. 7 A). The ANP mRNA concentration in the ventricle was one forth of that in the atrium, and the total content of ANP mRNA in the ventricle reached to 80% of that in the whole heart.

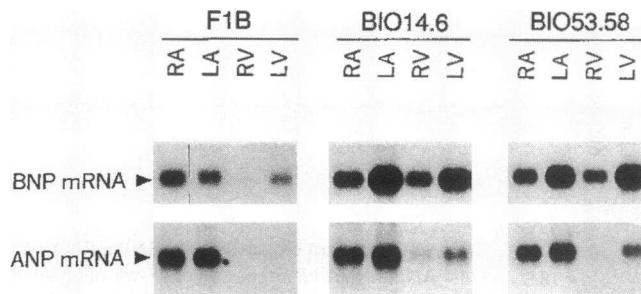
The 32-wk-old dilated cardiomyopathic BIO53.58 strain used in the present study did not show ventricular hypertrophy (Table I) and CHF. In this strain, the BNP mRNA concentration was elevated 3–4-fold in the atrium and ventricle (Fig. 7 A). The BNP mRNA concentration in the ventricle was two thirds of that in the atrium, and 85% of the total BNP mRNA content in the whole heart was expressed in the ventricle. The pattern of increase in ANP mRNA concentration in the BIO53.58 strain was similar to that in the BIO14.6 strain.

**Concentrations and molecular forms of BNP-LI and ANP-LI in cardiomyopathic hamster hearts.** In the heart of the F1B strain, the BNP-LI concentration in the ventricle ( $35 \pm 8$  pmol/g) was 5% of that in the atrium ( $720 \pm 30$  pmol/g), and the ANP-LI concentration in the ventricle ( $2.1 \pm 0.5$  pmol/g) was only 0.1% of that in the atrium ( $2.53 \pm 0.20$  nmol/g) (Fig. 7 B). The distribution pattern of BNP-LI and ANP-LI in the heart of the F1B strain was similar to that in the golden Syrian hamster heart. The BNP-LI and ANP-LI concentrations in the left ventricle were 3 and 10 times as high as those in the right ventricle, respectively.

In the heart of the hypertrophic cardiomyopathic BIO14.6 strain, as compared with the F1B strain, the BNP-LI concentration was elevated fourfold in the ventricle, while unchanged in the atrium (Fig. 7 B). The ANP-LI concentration was increased more than 11-fold in the ventricle, while it tended to be decreased in the atrium.



**Figure 5.** A typical standard curve of hamster BNP[65–96] and cross-reactivity profiles of its related peptides (A) and dilution curves of extracts of various samples (B) in the RIA for hamster BNP with the antisera HR-1.

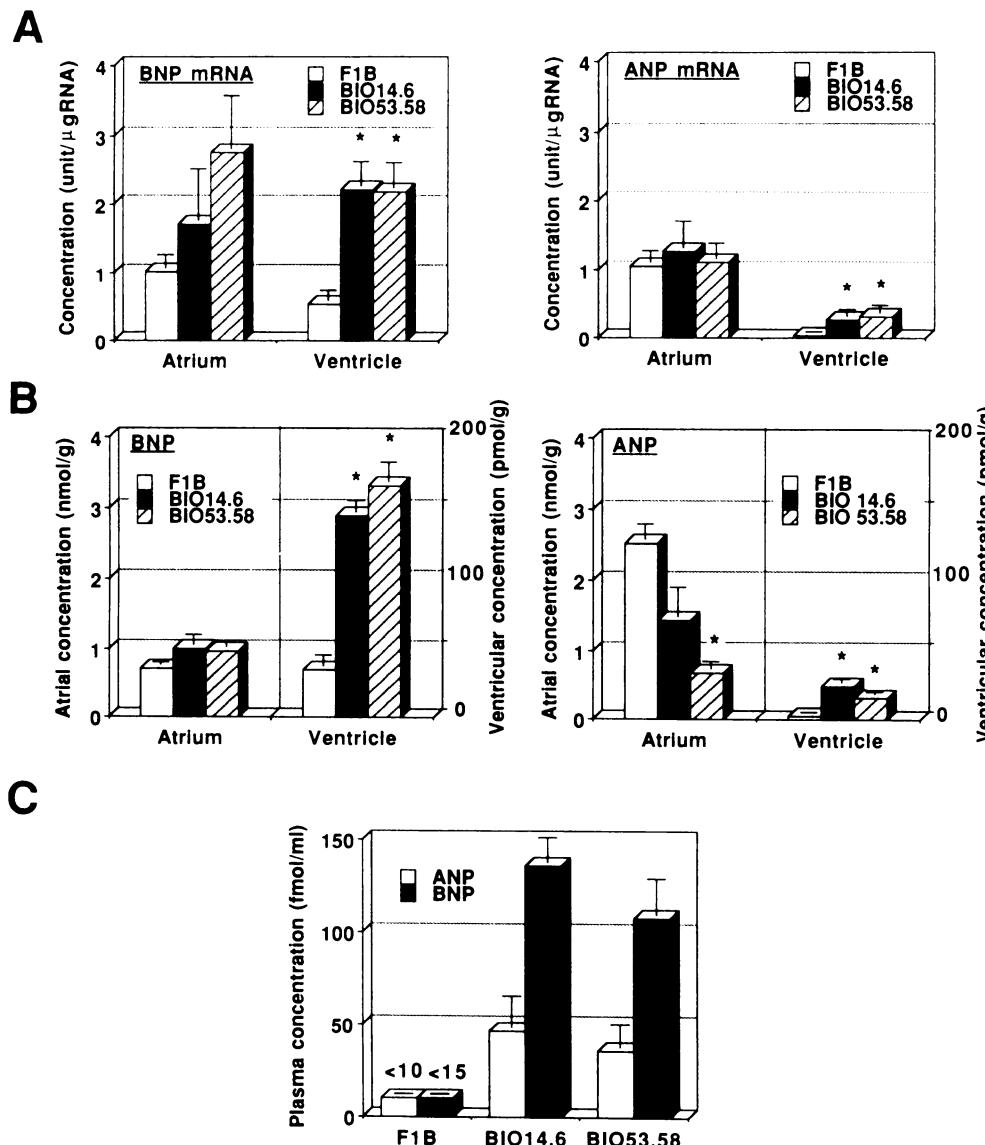


**Figure 6.** Northern blot analysis of BNP mRNA and ANP mRNA in hearts of 32-wk-old cardiomyopathic hamsters (BIO14.6 and BIO53.58 strains) and age-matched control hamsters (F1B strain). Representative cases are shown. One  $\mu$ g of total RNA is applied to each lane, and actin transcript levels are equivalent among different RNA samples (data not shown). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.

In the heart of the dilated cardiomyopathic BIO53.58 strain, the BNP-LI concentration was elevated fivefold in the ventricle, while unchanged in the atrium (Fig. 7B). The ANP-LI concentration was elevated eightfold in the ventricle, while it was decreased by 68% in the atrium.

In the heart of the control F1B strain, molecular forms of BNP-LI and ANP-LI were similar to those in the golden Syrian hamster; both BNP and ANP were stored as prohormones (Fig. 8). Also in the hearts of the BIO14.6 and BIO53.58 strains, molecular forms of BNP-LI and ANP-LI were essentially similar to those in the F1B strain (Fig. 8).

**Concentrations and molecular forms in cardiomyopathic hamster plasma.** In the F1B strain, the plasma BNP-LI and ANP-LI concentrations were below the detection limits of the respective RIAs (BNP-LI, < 15 fmol/ml; ANP-LI, < 10 fmol/ml; Fig. 7C). In the BIO14.6 strain, as compared with the F1B strain, the plasma ANP-LI concentration was increased at least fourfold to  $47 \pm 17$  fmol/ml, whereas the plasma BNP-LI concentration was remarkably elevated more than ninefold to



**Figure 7.** Summary of mRNA and peptide concentrations of BNP and ANP in atria and ventricles and the plasma BNP-like immunoreactivity (BNP-LI) and ANP-LI concentrations in 32-wk-old cardiomyopathic hamsters (BIO14.6 and BIO53.58 strains) and age-matched control hamsters (F1B strain). (A) The BNP mRNA and ANP mRNA concentrations in the atrium and ventricle, expressed relative to those in the right atrium of the F1B strain (amounts of BNP mRNA and ANP mRNA in 1.0  $\mu$ g total RNA from the right atrium of the F1B strain are defined as 1.0 U). The data are expressed as the mean  $\pm$  SEM from three individual animals. (B) The BNP-LI and ANP-LI concentrations in the atrium and ventricle. (C) The BNP-LI and ANP-LI concentrations in the plasma. Significantly different from the control F1B strain, \* $P < 0.05$ .

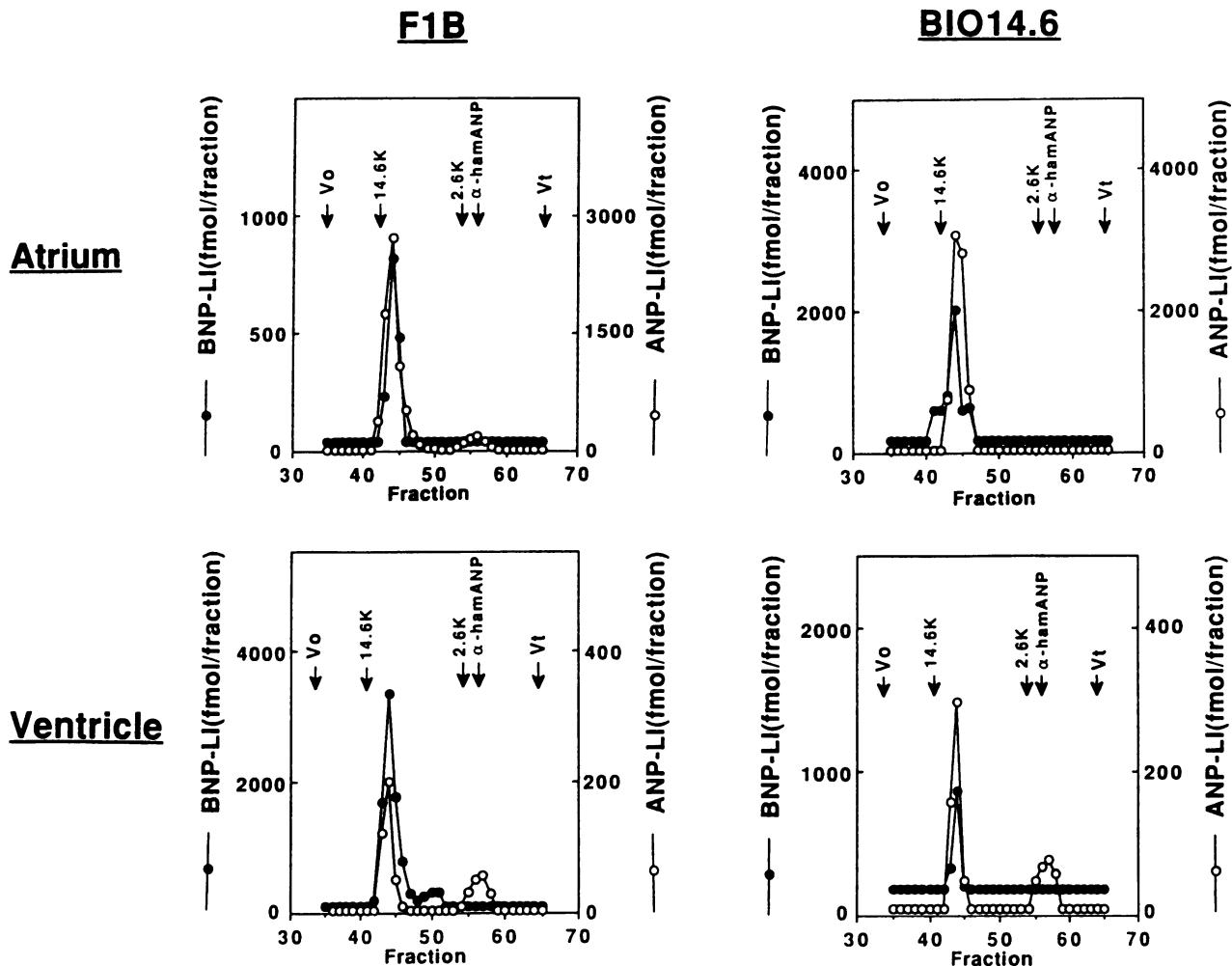


Figure 8. HP-GPC profiles of BNP-like immunoreactivity (–LI) and ANP-LI in atria and ventricles of the control F1B strain and the hypertrophic cardiomyopathic BIO14.6 strain. Arrows denote selected positions of a series of myoglobins of a polypeptide molecular weight calibration kit (Pharmacia, Uppsala, Sweden), void volume ( $V_0$ ), and total volume ( $V_1$ ), and the elution position of  $\alpha$ -hamster ANP ( $\alpha$ -hamANP).

$136 \pm 13$  fmol/ml, three times higher than the elevated plasma ANP-LI concentration (Fig. 7 C). In the BIO53.58 strain, the plasma BNP-LI concentration was elevated more than sevenfold to  $108 \pm 19$  fmol/ml, as compared with the F1B strain, which is three times higher than the elevated plasma ANP-LI concentration ( $36 \pm 13$  fmol/ml, Fig. 7 C). Furthermore, we also measured plasma BNP and ANP concentrations in a hamster from the BIO53.58 strain with signs of overt CHF (generalized edema, pleural effusion, and ascites), which were 285 and 105 fmol/ml, respectively.

In the plasma of the BIO14.6 strain, BNP-LI and ANP-LI were composed of small mol-wt forms corresponding to hamster BNP[65–96] and  $\alpha$ -hamster ANP, respectively (Fig. 9). In the plasma of the BIO53.58 strain, BNP-LI and ANP-LI also consisted of hamster BNP[65–96] and  $\alpha$ -hamster ANP, respectively.

## Discussion

BNP is a cardiac hormone which occurs mainly in the ventricle (12, 13). BNP synthesis and secretion are extremely augmented

in human failing ventricles (12, 14, 18, 19). Cardiomyopathic hamsters are widely used animal models of heart failure (21–23). However, the structure of hamster BNP has never been disclosed. Due to the extraordinary structural divergence of BNP among species, it was impossible to examine the pathophysiological significance of BNP using cardiomyopathic hamsters. In this context, we first isolated the full-length hamster BNP cDNA and elucidated the structure of hamster BNP.

We could not clone hamster BNP cDNA by the cross-hybridization method using rat, porcine, and human BNP cDNA fragments as probes (not shown). By the PCR technique as described in Methods, we successfully obtained a hamster BNP-specific cDNA probe. Using the obtained probe, we isolated the full-length hamster BNP cDNA. We determined that the obtained clone is a hamster BNP cDNA clone, based on the following reasons. First, in the COOH-terminal region of the deduced amino acid sequence, there are two cysteine residues to form a 17-residue ring structure which commonly occurs in the natriuretic peptide family (Fig. 3). Second, in the 3'-untranslated region of the obtained cDNA, there are four copies of “ATTTA” motif implicated in mRNA instability (33) (Fig.

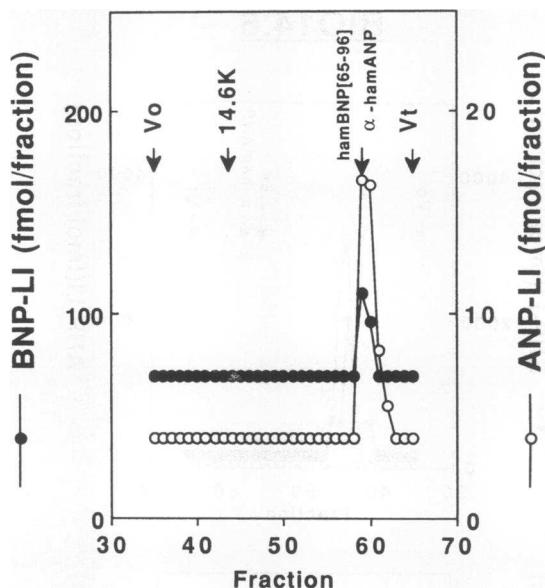


Figure 9. HP-GPC profiles of BNP-like immunoreactivity (LI) and ANP-LI in the plasma of the hypertrophic cardiomyopathic BIO14.6 strain. Arrows denote selected positions of a series of myoglobins of a polypeptide molecular weight calibration kit (Pharmacia), void volume ( $V_0$ ), and total volume ( $V_t$ ), and elution positions of  $\alpha$ -hamster ANP ( $\alpha$ -hamANP) and hamster BNP[65–96] (hamBNP[65–96]).

1). This “ATTTA” motif is also present in the 3'-untranslated region of rat, porcine, and human BNP cDNAs (28–30), while it is absent in ANP cDNAs (24, 34, 35). Third, in the coding region, the nucleotide sequence of the obtained cDNA is 78% homologous to that of rat BNP cDNA (28). Fourth, by Northern blot analysis using the cloned cDNA as a probe, the gene expression is detected in the ventricle as well as in the atrium, and not detected in extracardiac tissues, which is consistent with the tissue-specific BNP gene expression in rats and humans (9).

It is highly likely that the COOH-terminal 32-residue peptide of hamster preproBNP (hamster BNP[65–96], Fig. 1B) is hamster BNP. Hamster BNP[65–96] is preceded by a single arginine residue, which is the proteolytic processing site for porcine, rat, and human BNPs (28–30) (Fig. 3A). Furthermore, our HP-GPC study indicates that BNP-LI in cardiomyopathic hamster plasma is eluted as a single peak at the elution position of hamster BNP[65–96] (Fig. 9). Isolation and sequence determination of hamster BNP are ongoing in our laboratory.

In the present study, we also isolated the full-length hamster ANP cDNA, and elucidated that  $\alpha$ -hamster ANP of 28 amino acids is identical to  $\alpha$ -rat ANP. The 28-amino acid sequence of hamster preproANP was flanked by Pro<sup>98</sup>-Arg<sup>99</sup> and Arg<sup>128</sup>-Arg<sup>129</sup> dipeptides which are proteolytic processing sites for rat and mouse  $\alpha$ -ANPs (Fig. 3B) (34, 35). Moreover, a previous HPLC study showed that the major peak of ANP-LI in hamster plasma corresponds to the elution position of  $\alpha$ -rat ANP (25).

The nucleotide and deduced amino acid sequences of the coding region of hamster ANP cDNA are ~90% homologous to those of rat and mouse ANP cDNAs. By contrast, the nucleotide and deduced amino acid sequences of the coding region of hamster BNP cDNA was 78 and 63% homologous to those of

rat BNP cDNA, respectively. Moreover, the amino acid sequence of hamster preproBNP is <50% homologous to those of porcine and human preproBNPs. These observations further support the notion that the structure of BNP is considerably divergent among species. Thus, the cloning of hamster BNP cDNA is essential to investigate the significance of BNP using cardiomyopathic hamsters.

In the golden Syrian hamster, the BNP mRNA concentration in the ventricle is about half of that in the atrium (Fig. 4A). Taking account of tissue weight, however, the total content of BNP mRNA in the ventricle reaches to ~80–85% of that in the whole heart, while 80% of the total ANP mRNA content in the whole heart is expressed in the atrium. Because the ventricle secretes BNP by the constitutive pathway with much smaller storage pool as compared with the atrium as we have previously reported (13), the BNP-LI concentration should be lower in the ventricle than in the atrium even if the ventricle is the major source of BNP. Taken together, the ventricle is the major site of the BNP synthesis, while ANP occurs mainly in the atrium. These results are consistent with those in rats and humans (12, 13). To determine conclusively whether BNP is secreted mainly from the ventricle in hamsters or not, further studies such as perfusion of the hamster heart with or without the atrium (13) will be required.

In the present study, HP-GPC demonstrated that BNP-LI in the cardiac extract comprises a single 14-kD component in hamsters (Fig. 8) as in pigs (10), whereas the major component of BNP-LI in cardiac extracts of rats and humans is BNP (3 kD) (11, 12). These observations indicate that the processing pattern in the cardiac tissue, as well as the structure, of BNP is divergent among species. On the other hand, the processing pattern of ANP in the hamster heart is the same as those in hearts of rats and humans; ANP-LI in the cardiac extract comprises a single component corresponding to  $\gamma$ -ANP (11, 12).

Although BNP was originally isolated from the porcine brain, neither BNP mRNA (Fig. 4A) nor BNP-LI is detected in the hamster brain. These observations are consistent with previous findings showing that the tissue distribution of BNP is also divergent among species; BNP-LI is detected in the porcine brain, but not in the rat brain (10, 11). In extracardiac tissues of the hamster, BNP-LI is detected in the lung. Because ANP-LI is not detected in the hamster lung, we can exclude the possibility of atrial contamination. While we could not detect BNP transcript in the hamster lung by Northern blot analysis in the present study (Fig. 4A), it is reported that BNP transcript can be detected in the rat lung by the PCR technique (36). Taken together, it is possible that the BNP gene is expressed in the hamster lung at a low level not detectable by Northern blot analysis.

To assess the pathophysiological significance of BNP and ANP, we examined the synthesis and secretion of these natriuretic peptides in the hypertrophic (BIO14.6) and dilated (BIO53.58) cardiomyopathic strains. In the present study, we used the F1B strain as the control, because the F1B strain is a non-myopathic strain widely used as the control in studies on cardiomyopathic hamsters (5, 22).

We used the BIO14.6 strain to assess implications of BNP and ANP in ventricular hypertrophy. In the BIO14.6 strain, spotty cellular myolysis in the heart occurs at 6 wk of age. Ventricular hypertrophy ensues in a progressive manner after 14–17 wk, and becomes most prominent at 30–40 wk of age.

CHF occurs in a substantial proportion of animals after 21–29 wk, and becomes prominent from 40 wk of age (23). In the present study, the 32-wk-old BIO14.6 strain exhibits ventricular hypertrophy, and shows no signs of CHF. In our BIO14.6 hamsters, the plasma ANP-LI concentration was elevated as compared with the F1B strain, but remained < 50 fmol/ml (Fig. 7 C). It is reported that the plasma ANP-LI concentration is elevated to 100 fmol/ml in cardiomyopathic hamsters with moderate to severe CHF (5, 25). The low plasma ANP-LI concentration is, therefore, consistent with the observation that the 32-wk-old BIO14.6 strain used in the present study does not manifest CHF. In contrast, the BNP gene expression is augmented fourfold in the ventricle (Fig. 7 A), and the plasma BNP-LI concentration is elevated at least ninefold to more than 100 fmol/ml (Fig. 7 C). Elevations in the plasma BNP-LI and ANP-LI concentrations can be good markers of ventricular hypertrophy without overt CHF. The fold increase of the plasma BNP-LI concentration was greater than that of the plasma ANP-LI concentration (Fig. 7 C). The elevation in the plasma BNP-LI is, therefore, a more sensitive marker of ventricular hypertrophy than the plasma ANP-LI concentration. The observation on the BIO14.6 strain in the present study well accords with that on patients with hypertrophic obstructive cardiomyopathy (HOCM) (16). In patients with HOCM, although there are no signs of apparent CHF and the plasma ANP concentration remains at 30 fmol/ml, the plasma BNP concentration is increased to 200 fmol/ml.

We also examined the BIO53.58 strain to assess the significance of both natriuretic peptides in ventricular overload. In the BIO53.58 strain, significant cardiac hypertrophy does not occur. The ventricular dilatation and CHF appear from 30–36-wk of age, earlier than in the BIO14.6 strain (22). As the disease progresses, myolytic lesions are increased in number and size, and those in healing stage resolve into collagenous fibrillar scar (21). The ventricular filling pressure is increased, and the peak ventricular pressure and peak rate of ventricular pressure development are depressed (23). In the present study, the 32-wk-old BIO53.58 strain does not show overt CHF. The plasma BNP-LI concentration reaches to 100 fmol/ml (Fig. 7 C), although CHF is still not overt and the plasma ANP-LI concentration remains at < 50 fmol/ml (Fig. 7 C). Our results, therefore, suggest that the elevated plasma BNP-LI concentration sensitively reflects ventricular overload preceding impairment of systemic circulation. The profile of elevations of the plasma BNP-LI and ANP-LI concentrations in the BIO53.58 strain used in the present study well mimics that of the plasma BNP and ANP concentrations in patients with ventricular overload. In the early phase of AMI, the plasma BNP concentration is extremely elevated to 100 fmol/ml even in patients without overt CHF, while the plasma ANP concentration is only slightly elevated (~ 30 fmol/ml) (20).

Among the F1B, BIO14.6, and BIO53.58 strains, the pattern of the plasma BNP-LI concentration is well parallel to that of the total content of BNP mRNA in the heart. In the F1B, BIO14.6, and BIO53.58 strains, plasma BNP-LI concentrations are < 15, 136, and 108 fmol/ml, respectively, and total contents of BNP mRNA in hearts are 50, 300, and 210 U, respectively. Among the three strains, the pattern of the total content of BNP-LI in the heart is also parallel to that of the plasma BNP-LI concentration. The plasma BNP-LI concentration, therefore, directly reflects the BNP synthesis in the heart.

In the present study, in both cardiomyopathic strains, the ANP-LI concentration in the atrium was significantly decreased, while the ANP mRNA concentration in the atrium was unchanged (Fig. 7). This observation indicates the accelerated secretion of ANP from atrial storage pool, which is consistent with the previous reports on DOCA-salt rats and cardiomyopathic hamsters (15, 25). By contrast, both of the BNP-LI and BNP mRNA concentrations in the atrium were 1.5-fold increased in both cardiomyopathic strains (Fig. 7), which suggests that the synthesis and secretion of BNP are regulated differently from those of ANP in the atrium.

There are some reports showing that the plasma ANP concentration is higher than the plasma BNP concentration in patients with CHF. In these studies, the plasma BNP concentration is measured with extraction by commercially available antiserum against BNP (17–19). We established the sensitive RIA for human BNP with the monoclonal antibody which does not require plasma extraction and reported previously that the plasma BNP concentration is extremely elevated and exceeds the plasma ANP concentration in patients with severe CHF (12).

To assess the pathophysiological significance of BNP, SHR, DOCA-salt rats, and experimental AMI rats are so far used as animal models (13, 15).<sup>2</sup> In these rat models, the plasma BNP concentration remains at a relatively low level as compared with the elevated plasma ANP concentration, which is quite different from our observations on patients with CHF (12). Moreover, these experimental rat models are not animal models of CHF. It was, therefore, difficult to assess the significance of BNP in human heart failure using these rat models. By contrast, in the present study, in both cardiomyopathic hamsters, the plasma BNP-LI concentration is elevated to more than 100 fmol/ml and much higher than the elevated plasma ANP-LI concentration, which strongly suggests that cardiomyopathic hamsters are useful experimental animal models to investigate the pathophysiological significance of BNP in cardiovascular diseases. Further examinations on BNP synthesis and secretion in cardiomyopathic hamsters at various clinical stages are required in the future.

In conclusion, we isolated the full-length hamster BNP and ANP cDNA clones and studied BNP and ANP gene expressions in normal and cardiomyopathic hamsters. The present study elucidated that cardiomyopathic hamsters are good animal models to investigate the pathophysiological significance of BNP.

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