Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 410-416

CLONING AND SEQUENCE ANALYSIS OF cDNA ENCODING A PRECURSOR FOR PORCINE BRAIN NATRIURETIC PEPTIDE

Keiji MAEKAWA[1], Tetsuji, SUDOH[2], Mitsuru FURUSAWA[1], Naoto MINAMINO[3], Kenji KANGAWA[3], Hiroaki OHKUBO[4], Shigetada NAKANISHI[4] & Hisayuki MATSUO*[3]

[1]Daiichi Seiyaku Co. Ltd., Edogawa, Tokyo 134,

[2]Daiichi Pure Chemicals Co. Ltd., Sumida, Tokyo 130,

[3]Department of Biochemistry, Miyazaki Medical College, Kiyotake, Miyazaki 889-16, and

[4]Institute for Immunology, Kyoto University, Sakyo, Kyoto 606, Japan

Received October 11, 1988

SUMMARY: Brain natriuretic peptide (BNP) is a new type of natriuretic peptide recently identified in porcine brain. Since the highest concentration of BNP was found in the cardiac atrium, the cDNA library of porcine cardiac atrium was constructed, and the cDNA clone encoding a BNP precursor was isolated and sequenced. The precursor for porcine BNP (porcine prepro-BNP) is 131 amino acids in length, including a 25 residue putative signal peptide at the N-terminus. Porcine BNP structure is located at the C-terminus of the precursor and is directly followed by a termination codon. Based on structural data recently obtained for γ-BNP (a main storage form of BNP in the heart), prepro-BNP is processed to 106-residue γ-BNP by removal of the signal peptide in the heart, and to low molecular weight forms, such as BNP-26 and BNP-32, in the brain.

Recent identification of a new type of natriuretic peptide, designated BNP (brain natriuretic peptide), in porcine brain and heart disclosed the possibility that BNP may function in concert with ANP (atrial natriuretic peptide) to regulate the homeostatic balance of body fluid and blood pressure (1). BNP-26 (26 amino acid long) and its N-terminally elongated version (BNP-32), which are definitely distinct from ANP but elicit a pharmacological spectrum very similar to that of ANP, have already been identified in porcine brain (1-3). Furthermore, BNP is also found to exist in porcine cardiac atrium as a high molecular weight form of about 12K daltons, at a concentration about 100 times higher than that in brain (4). The presence of multiforms of BNP suggests that they may all derive from the same precursor. The established amino acid sequences of BNP-26 and BNP-32 have provided us the means to elucidate the structure of the BNP precursor. The present paper describes the cloning and sequence analysis of the cDNA of porcine atrial mRNA

*To whom correspondence should be addressed.

0006-291X/88 $1.50

encoding a precursor for BNP. The biosynthetic pattern of BNP will also be discussed in comparison with that of ANP.

MATERIALS AND METHODS

cDNA library construction: Total RNA was extracted from cardiac atria of one pig by the guanidine thiocyanate method (5). Poly(A)$^+$ RNA was purified from crude RNA by oligo(dT)-cellulose column chromatography (Pharmacia) (6). Using 3 μg of poly(A)$^+$ RNA, 1 μg of double-stranded cDNA was obtained by the method of Gubler and Hoffman (7). At the first strand synthesis, oligodeoxyribonucleotide, the 31mer, which had 15dT followed by 16 nucleotides including Not I site was added into the reaction mixture as a primer. The cDNA thus prepared had Not I site at the 3'-end. After the blunt end formation, the cDNA was ligated with Xho I linker and then was digested with Xho I and Not I restriction nucleases. The cDNA library was then constructed by ligation of linker-attached double-stranded cDNA to Xho I and Not I treated Bluescript KS(+) plasmid (Stratagene).

cDNA library screening: To screen cloned cDNA for the precursor of BNP, oligodeoxyribonucleotide probes were chemically synthesized by the phosphoamidite method using a DNA synthesizer (Model 380B, Applied Biosystems). Tetradecamers were synthesized as six pools (probe Ia, Ib and IIa-IId) as shown in Fig. 1. Escherichia coli HB 101 was transformed with Bluescript KS(+) plasmid and ampicillin-resistant clones were selected (8). Approximately 80,000 transformants were screened by hybridization for 16 hr at 37°C with a mixture of 5'-^{32}P-labeled probe Ia and Ib. After autoradiography, probes Ia and Ib were thoroughly removed from the filters, and then rehybridization was carried out with a mixture of labeled probes IIa and IIb. The third hybridization was performed with a mixture of labeled probes IIc and IId after washing the filters. By comparison with the results of serial hybridization, 20 hybridization-positive clones were identified. These clones were subjected to a second screening using each pool of probes independently (8). The second screening finally yielded 6 positive clones.

DNA sequencing: Clone pBNP84 carrying the largest cDNA insert among the 6 clones obtained was subjected to nucleotide sequence analysis by the chain-termination method using 2'-deoxy-7-deaza GTP (7-DEAZA sequence kit, Takara Shuzo)(9,10). In addition to pBNP84, subsequences around position 76 of three other clones carrying cDNA inserts comparable to or shorter than pBNP84 were also analyzed in order to confirm the nucleotide sequence of pBNP84.

Northern blot analysis: Porcine atrial poly(A)$^+$ RNA (5 μg) was denatured with 1M glyoxal and 50% dimethylsulfoxide, subjected to electrophoresis on a 1.5% agarose gel and transfered to Hybond-N nylon membrane (Amersham). The mambrane was hybrdized with labeled cDNA probe derived from pBNP84 (11).

RESULTS AND DISCUSSION

In a systematic examination of the central nervous system, gastrointestinal tract and other peripheral tissue, surveying for BNP by using RIA specific to BNP, we found the highest concentration of immunoreactive (ir-) BNP in heart (4). In heart, atria contained far more abundant ir-BNP than ventricles, as was true of ANP. Therefore, cDNA library for isolation of clones for a BNP precursor was constructed with poly(A)$^+$ RNA of porcine cardiac atrium, in which mRNA of BNP was thought to be most frequently transcribed.

According to the established amino acid sequences of BNP-26 and BNP-32 (1,2), oligodeoxyribonucleotide mixtures representing all possible complementary sequences were synthesized as probes for screening a cDNA clone of a porcine BNP precursor (Fig. 1). Probes Ia and Ib corresponded to the

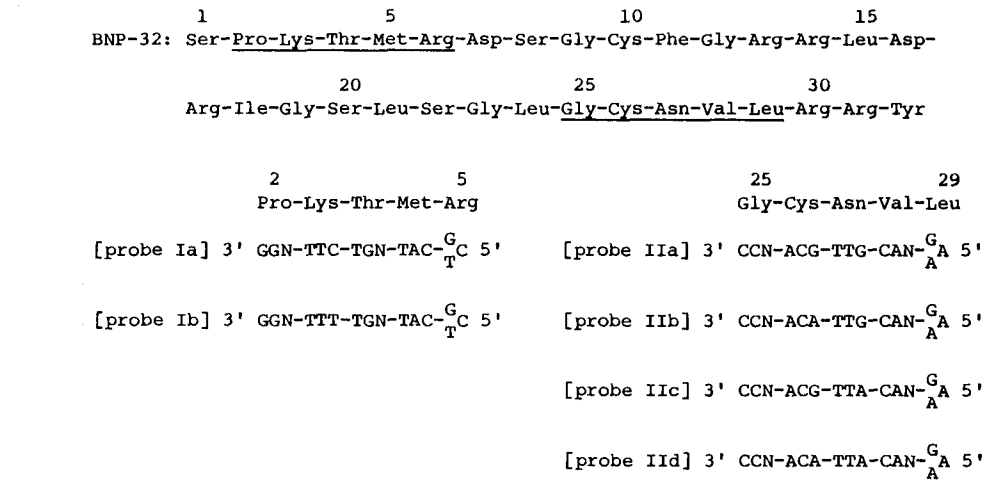

Figure 1. Amino acid sequence of porcine BNP-32 and synthetic oligodeoxyribonucleotides used for probes. Tetradecamer oligodeoxyribonucleotides representing all possible complementary sequences corresponding to the two pentapeptide sequences underlined (excluding the third nucleotides of the last amino acids) were synthesized as six pools. N = G, A, T and C.

subsequence Pro-Lys-Thr-Met-Arg (excluding the third nucleotide residue of the Arg codon) and probes IIa-IId corresponded to the subsequence Gly-Cys-Asn-Val-Leu (excluding the third nucleotide residue of the Leu codon). These probes were used for screening after 5'-^{32}P-labeling. A cDNA library, constructed by ligation of double strand DNA prepared from porcine poly(A)$^+$RNA to Bluescript plasmid, gave 2,000 ampicillin resistant transformants per nanogram of DNA. This highly efficient transformation was partly derived from ligation with heterogenous ends generated by Xho I and Not I nuclease treatment. A portion of the library was used for transformation of Escherichia coli and the resulting transformed colonies were submitted to the first screening by three successive and independent hybridizations with probes (a) Ia+Ib , (b) IIa+IIb and (c) IIc+IId. Among the 20 clones obtained by the first screening, 6 positive clones were finally isolated by the second screening. A clone designated pBNP84 harboring the longest DNA insert of approximately 750 base pairs (bp) was sequenced according to the strategy shown in Fig. 2.

The complete nucleotide sequence is 670 bp long (excluding poly(A) tract) and deduced amino acid sequence are shown in Fig. 3. The initiator ATG codon, nucleotide 1-3, was deduced from the fact that a termination codon TGA preceded this ATG codon. The sequence of nucleotide 316-393 precisely corresponded to the amino acid sequence of BNP-32 (2), which was followed directly by a TGA termination codon. In the total putative BNP precursor, 131 amino acid residues were encoded from the first ATG codon. The first 25 amino acid residues, starting with the initiator methionine and preceding the

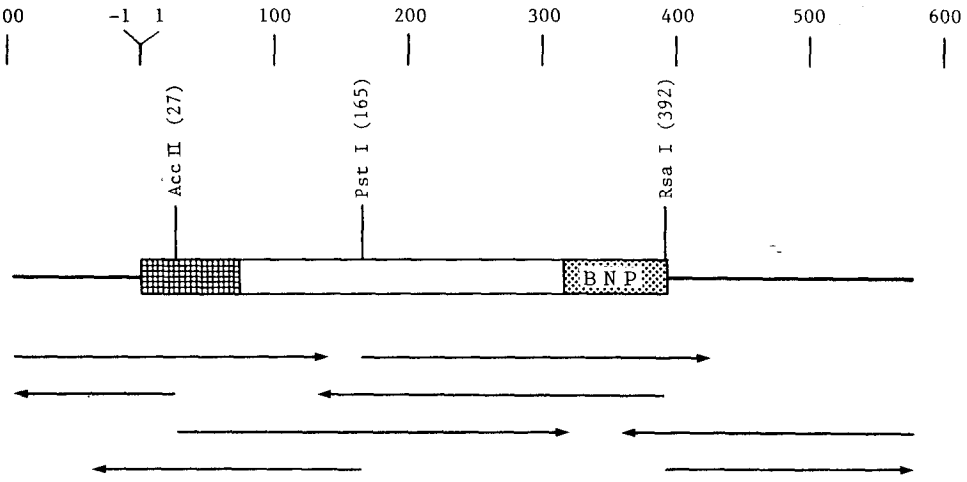

Figure 2. Strategy of sequencing the cDNA insert in clone pBNP84. The restriction map displays only relevant restriction sites, which are identified by numbers indicating the 5'-terminal nucleotide generated by cleavages of the enzymes. Each arrow shows the direction and extent of sequence determination.

putative pro-BNP, consisted of a large number of hydrophobic amino acids, including two repeated sequences of four consecutive leucine residues. These results indicate that the first 25-residue peptide is a signal peptide and its removal may afford pro-BNP of 106 residues.

Concurrent with the present study, we have recently succeeded in isolating γ-BNP of 12,000 daltons from pig atrium as a major storage form of the BNP family (to be reported elsewhere). The amino acid sequence determined for the isolated γ-BNP was found to be exactly identical to that deduced from cDNA sequence analysis of pBNP84, except that only one amino acid replacement of Tyr to His was observed at the N-terminus. In order to examine this difference, three other clones were submitted to sequence analysis. Two clones including pBNP84 had T at position 76, while two others had C at this position. No other difference in the nucleotide sequence was observed among the clones examined. This nucleotide difference at position 76 (TAT to CAT), the first nucleotide of Tyr^{26} codon, results in amino acid substitution from Tyr to His. The observed nucleotide difference may be due to polymorphism in the porcine BNP gene, since the template RNA used for in vitro cDNA synthesis was derived from somatic cells of only one pig, which had chromosomes originated from two haploids. So far as we screened two different batches of porcine atrial extracts, only the γ-BNP having His at the N-terminus was isolated. Whether another pro-BNP carrying Tyr at the first residue exists or not remains to be examined.

Northern blot analysis of porcine atrial poly(A)$^+$RNA indicated that a BNP precursor was probably encoded in a single mRNA of about 1,000 bp, which

```
     5'··· CAG GCT GCT AGG AAG TGA AAA GTG AAC CTG GAC CCA GCT CAG CGG  -49

-48  CAG CAG CAG CGG CAG CAG GCA GCA GCC TCT ATC CTC TCC TCC AGC CAC   -1

  1  ATG GGC CCC CGG ATG GCG CTT CCC CGC GTG CTC CTG CTC CTG TTC TTG   48
  1  Met Gly Pro Arg Met Ala Leu Pro Arg Val Leu Leu Leu Leu Phe Leu   16

                                          *
                                         (C)
 49  CAC CTG TTG CTG CTA GGA TGC CGT TCC TAT CCA CTG GGT GGC GCT GGC   96
 17  His Leu Leu Leu Leu Gly Cys Arg Ser Tyr Pro Leu Gly Gly Ala Gly   32
                                         (His)

 97  CTG GCC TCA GAA CTG CCA GGG ATA CAG GAG CTG CTG GAC CGC CTG CGA  144
 33  Leu Ala Ser Glu Leu Pro Gly Ile Gln Glu Leu Leu Asp Arg Leu Arg   48

145  GAC AGG GTC TCC GAG CTG CAG GCG GAG CGG ACG GAC CTG GAG CCC CTC  192
 49  Asp Arg Val Ser Glu Leu Gln Ala Glu Arg Thr Asp Leu Glu Pro Leu   64

193  CGG CAG GAC CGT GGC CTC ACA GAA GCC TGG GAG GCG AGG GAA GCA GCC  240
 65  Arg Gln Asp Arg Gly Leu Thr Glu Ala Trp Glu Ala Arg Glu Ala Ala   80

241  CCC ACG GGG GTT CTT GGG CCC CGC AGT AGC ATC TTC CAA GTC CTC CGG  288
 81  Pro Thr Gly Val Leu Gly Pro Arg Ser Ser Ile Phe Gln Val Leu Arg   96

289  GGA ATA CGC AGC CCC AAG ACG ATG CGT GAC TCT GGC TGC TTT GGG CGG  336
 97  Gly Ile Arg Ser Pro Lys Thr Met Arg Asp Ser Gly Cys Phe Gly Arg  112

337  AGG CTG GAC CGG ATC GGC TCC CTC AGC GGC CTG GGC TGC AAT GTG CTC  384
113  Arg Leu Asp Arg Ile Gly Ser Leu Ser Gly Leu Gly Cys Asn Val Leu  128

385  AGG AGG TAC TGA GAA GTC CTG GCT GAC AAC CTC TGT GTC CGC TTC TCC  432
129  Arg Arg Tyr ***

433  AAC GCC CCT CCC CTG CTC CCC TTC AAA GCA ACT CCT GTT TTT ATT TAT  480

481  GTA TTT ATT TAT TTA TTT ATT TGG TGG TTG TAT ATA AGA CGG TTC TTA  528

529  TTT GTG AGC ACA TTT TTT CCA TGG TGA AAT AAA GTC AAC ATT AGA GCT  576

577  C(Poly A)·· ··3'
```

Figure 3. Nucleotide sequence of the cDNA in pBNP84 with predicted amino acid residues. Nucleotide residues are numbered beginning with the first residue of ATG triplet encoding a putative initiator methionine, and nucleotides on the 5' side of nucleotide 1 are indicated by negative numbers. Termination codon after BNP is marked with three consecutive asterisks. The dashed line on the first row indicates a termination codon before a putative initiator methionine. The asterisk at nucleotide 76 indicates the position where C is observed in other clones, which results in an amino acid substitution from Tyr to His.

corresponded to the size of porcine prepro-BNP mRNA thus determined. Heterogeneity observed at position 76 was at least not reflected as differences in the molecular size of mRNA (Fig. 4).

The nucleotide sequence of porcine BNP precursor thus determined was compared with that of human ANP precursor, since the cDNA sequence of porcine ANP has not been elucidated yet. Even when the 393 nucleotide sequence encoding the BNP precursor was aligned to give maximum matching, it showed 50.6% homology to human ANP. Nucleotide homology was 65.7% in the regions

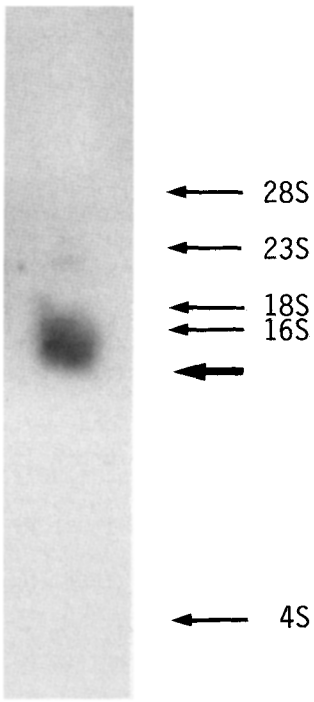

Figure 4. Northern blot analysis of mRNA of porcine atrium. Positions of rat ribosomal RNAs (28s and 18s), bacterial ribosomal RNAs (23s and 16s) and bacterial transfer RNA (4s) are indicated by thin arrows. Positive band hybridizing labeled cDNA probe derived from pBNP84 is shown by the thick arrow.

corresponding to BNP-32 and α-ANP (nucleotide 298-393), but 45.6% in other region of the 5'-side. Thus, the BNP precursor gene probably diverged from the ANP precursor gene in the early stage of evolution. Our recent identification of chicken ANP, which resembles porcine BNP more closely than mammalian ANPs, also supports this speculation (12).

It should be noted that the cDNA encoding a BNP precursor has four sets of ATTTA sequence in the 3'-untranslated region. The repetition of this specific nucleotide sequence is known to destabilize mRNA in the cell, as is observed in the case of granulocyte-monocyte colony stimulating factor (13). Lymphokines, such as α-γ interferons and interleukins 1-3, and proto-oncogenes, such as c-fos and c-myc, also include an AT-rich repetitive sequence in the 3'-untranslated region of their cDNA sequences. These mRNAs are transiently transcribed in response to stimuli, and are degraded and removed from the cell in a short period (13). So far nothing is known about stimulation of production and release of BNP. Messenger RNA encoding an ANP precursor does not contain these destabilizing sequences in its structure (14). These facts suggest the possibility that BNP and ANP have different regulation systems for biosyntheses at the level of transcription.

ACKNOWLEDGMENTS: The authors are grateful to Dr. Yoshinari Satoh and Mrs. Mitsuko Murakami for syntheses of oligodeoxyribonucleotide probes, and Dr. Yasumasa Marumoto for useful advice.

REFERENCES

1. Sudoh, T., Kangawa, K., Minamino, N. & Matsuo, H. (1988) Nature, 332, 78-81.
2. Sudoh, T., Minamino, N., Kangawa, K. & Matsuo H. Biochem. Biophys. Res. Commun. in press.
3. Ueda, S., Minamino, N., Sudoh, T., Kangawa, K. & Matsuo, H. Biochem. Biophys. Res. Commun. in press.
4. Minamino, N., Aburaya, M., Ueda, S., Kangawa, K. & Matsuo H. Biochem. Biophys. Res. Commun. in press.
5. Chirgwin, R.M., Przybyla, A.E., MacDonald, R.J. & Rutter, W.J. (1979) Biochemistry, 18, 5294-5299.
6. Avib, H. & Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A., 69, 1408-1412.
7. Gubler, U. & Hoffman, B.J. (1983) Gene, 25, 263-269.
8. Maniatis, T., Fritsch, E.F. & Sambrook, J. (1987) Molecular Cloning: A laboratory manual, Cold Spring Harvor Laboratory, New York.
9. Sanger, F., Nicklen, S. & Coulson, A.R. (1977) Proc. Natl. Acad. Sci. U.S.A., 74, 5463-5469.
10. Mizusawa, S., Nishimura, S. & Seela, F. (1986) Nucleic Acids Res., 14, 1319-1324.
11. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. U.S.A., 77, 5201-5205.
12. Miyata, A., Minamino, N., Kangawa, K. & Matsuo H. Biochem. Biophys. Res. Commun., in press.
13. Shaw, G. & Kamen, R. (1986) Cell, 46, 659-667.
14. Oikawa, S., Imai, M., Ueno, A., Tanaka, S., Noguchi, T., Nakazato, H., Kangawa, K., Fukuda, A. & Matsuo H. (1984) Nature, 309, 724-726.