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ORGANIZATION OF THE GENE FOR iso-rANP, a RAT B-TYPE NATRIURETIC PEPTIDE

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Using the polymerase chain reaction and oligonucleotide primers constructed from knowledge of the cDNA sequence we have sequenced the gene for iso-rANP, a peptide of the B-type of atrial natriuretic peptides. The overall organization of the rat iso-ANP gene is the same as that of ANP and BNP consisting of three exons and two introns at relatively similar positions. Iso-rANP and it's gene are more closely related to BNPs than ANP and yet there are significant differences at both the protein and DNA levels. Our results suggest that iso-rANP and BNP are distinct members of the same sub-family (B-type natriuretic peptides) within the family of natriuretic peptide genes.

The natriuretic, diuretic and hypotensive actions of atrial natriuretic peptide (ANP) are now well established (For review see Inagami, 1989 (1)). Over the past two years new natriuretic peptides have been discovered. These include pig brain natriuretic peptide (pBNP) (2) and peptides from rat atria which have been termed iso-rANP$_{[1-45]}$ in our laboratory (3,4) rat BNP-45 by Aburaya et al. (5) and 5K cardiac peptide by Kambayashi et al. (6). In addition, another peptide from pig brain, termed pCNP, has recently been reported (7). The rat peptides are identical in sequence (except for a gln for leu at position 44 in iso-rANP which is probably an isomorphism) and like ANP and pBNP (8) form the C-terminal portion of a pro-protein precursor (9).

Iso-rANP is isolated from rat atria as a 45-amino acid peptide (3) and this also appears to be the circulating form (G. Hira and T.G. Flynn, unpublished results). The disulfide-bonded ring area of iso-rANP$_{[1-45]}$ has significant homology with the same area of ANP and with BNPs (Fig. 1). However, the amino acid identity in this region (from residue 21 of iso-rANP to the C-terminus) is much greater among pig, dog and human BNPs (pig vs dog = 96%; human vs pig or dog = 76%) than between iso-rANP and these BNPs (iso-rANP vs pBNP, hBNP or dBNP = 56%).

The complete amino acid sequence of prepro iso-rANP/BNP-45/5K cardiac peptide was deduced from the cDNA sequence by Kojima et al. (9) who termed it preproBNP from rat, presumably to reflect the fact that a portion of pBNP cDNA was used as a probe to isolate a clone from a rat atrial cDNA library. It would appear, therefore, that iso-rANP may be the rat form of BNP. However, differences in amino acid sequence between iso-rANP and BNP from other species have led us to investigate this relationship at the gene level.

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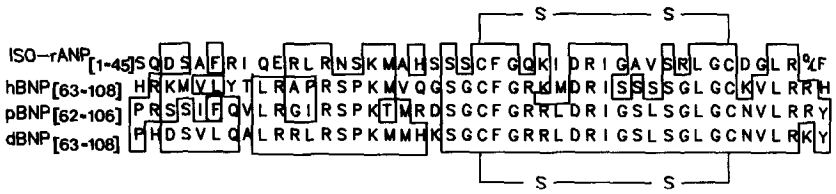

Figure 1. Comparison of the C terminal sequences of rat iso-ANP and porcine, dog and human BNPs.

We have elucidated the organization of this gene by determining the nucleotide sequence of a DNA fragment synthesized in vitro by the polymerase chain reaction (PCR) using oligonucleotide primers based on the cDNA sequence. The DNA sequence thus derived has been compared with those of ANPs and BNPs.

MATERIALS AND METHODS

Synthesis of rat atrial cDNA: Total RNA from (Sprague Dawley) rat atria, purified by guanidiniumthiocyanate phenolchloroform procedure (10) was used for synthesizing single-stranded cDNA by oligo $d(T)_{18}$ primed reverse transcription (11). The second strand synthesis of the cDNA pool was accomplished according to the method of Gubler and Hoffman (12). An aliquot of the double-stranded cDNA, in pg amount, was used for amplifying iso-ANP cDNA by polymerase chain reaction.

Rat genomic DNA: DNA from Sprague Dawley rat liver was isolated according to a standard procedure (11).

Oligonucleotide primers: Oligonucleotide primers, used in PCR synthesis and sequencing, were synthesized in an automated DNA synthesizer (Biosearch 8600) at the Oligonucleotide Synthesis Laboratory at Queen's University. Their nomenclature, designated as sense (S) or antisense (AS), and the sequences are as follows: 5'S/30 and 3'AS/30: 5'GCGAGACAAGAGAGAGCAGGACACCATCGG3' and 5'CCAAAAGCAAGAAATAGGCTATGTTTATTA3', corresponding to 5' and 3' ends respectively of the rBNP cDNA sequence (9); 30S and 39AS: 5'TCTCAGGACTCTGCCTTCCGCATCCAGGAG3' and 5'GCTATGCGCCATCTTGCTGTTTCTCAGTCTCTC CTGAAT3', corresponding to amino acid residues 1-10 and 8-20, respectively, of the iso-ANP peptide sequence (3); 19AS: 5'GAGAGCTGTCTCTGAGCCA3', 18S:5'GCACATAGTTCAAGCTGC3' and 22AS: 5'GCCGGAGTCTGCAGCCAGGAGG3', corresponding to nucleotides 164 to 182, 280 to 297 and 372 to 393, respectively of the rBNP cDNA sequence (9).

Polymerase chain reactions (PCR): The amplifications of DNA fragments were accomplished according to conditions described by Saiki et al. (13), using Taq DNA polymerase (Cetus) in 30 cycles. The denaturation, annealing and extension steps were performed at 94°, 65° and 72 °C respectively, in all amplifications.

DNA sequencing and analysis: The sequencing of 5'-end labelled PCR fragments was done according to the procedure described by Seif et al. (14). The deduced sequence was confirmed by resequencing PCR fragments, using sequenase kit (US Biochemical) and internal ^{32}P-kinased primers, (18S, 19AS and 22AS) as described by Owerbach et al. (15).

The derived sequence was analyzed using DNA sequence analysis programs - Version 2.4 written and compiled by Dr. Alan Goldin (Cal. Inst. Tech.). Comparisons of sequences were made by coalignments, using the University of Wisconsin Best fit program (16) and the ESEE program devised by Dr. Eric Cabot of Simon Fraser University, B.C.

RESULTS AND DISCUSSION

The complete prepro cDNA sequence elucidated by Kozima et al. (9) provided us with the opportunity to synthesize in vitro the DNA fragment containing the iso-ANP gene by polymerase chain reaction (PCR). Two 30-mer oligonucleotide primers, termed 5'S/30 and 3'AS/30, were initially utilized to amplify the 628 bp cDNA fragment, using the total atrial cDNA as template.

The identity of this fragment was established by partial sequencing. Subsequently, the same primers were used to synthesize a genomic PCR fragment approximately 1.3 Kb in size, which, by partial sequencing, was confirmed to harbor the iso-ANP gene.

To facilitate localization and sequencing of intronic segments, truncated products of the 1.3 Kb fragment were also PCR synthesized. Primers 5'S/30 and 39AS generated a 566 bp fragment harboring the 1st intron and 30S and 3'AS/30 primers produced a 795 bp fragment containing the 2nd intron, as suggested from difference in size with corresponding segments of the cDNA. Sequencing across introns was performed by direct sequencing of PCR products as detailed in the Methods section and precise assignment of exon/intron junctions was ensured by running sequencing reactions of cDNA segments in the same gel. The elucidated sequence is shown in Figure 2.

It is apparent from the DNA sequence (Fig. 2) that the overall organization of the rat iso-ANP gene is the same as that of ANPs (17,18,19) and BNPs (20,21), consisting of three exons and two introns at relatively similar positions. Also, like ANPs and BNPs, the mature peptide, with the exception of the last few amino acid residues, is located in exon 2, including the biologically important disulfide-bonded ring region. In addition, the coding sequence is interrupted after the 16th amino acid from the anticipated Ser/His cleavage site of the signal peptidase to accommodate the first intron, like ANPs and porcine BNP. All three peptides, therefore, belong to the same gene family of natriuretic peptides. The location of the second intron in iso-ANP is, however, like that of BNPs (21) and not ANPs, interrupting the codon for the fifth amino acid from the carboxy terminal. It is relevant in this regard to reiterate that heterogeneity exists among BNPs with regard to the location of the first intron. Compared to porcine BNP and ANPs, the location of the first intron of the human BNP is shifted by two additional amino acids (21). The coding regions of dog BNP have been assigned previously on a genomic clone by co-alignment with porcine and human genomic sequences (21). Possible uncertainty exists with regard to the exact location of the first intron in canine BNP because co-alignment was difficult in this region due to diversity within the DNA sequence. Therefore, at the present moment, the organizational identity of the rat iso-ANP gene could be established with certainty only with reference to porcine BNP.

In order to ascertain the degree of homology as well as differences, the DNA sequence of the rat iso-ANP gene was co-aligned with pig, dog and human BNPs (Fig. 2) and separately with rat ANP (not shown). At the nucleotide level, the extent of homology of iso-ANP with BNPs is exceedingly more than with ANP, but is not as extensive as it is among porcine, canine and

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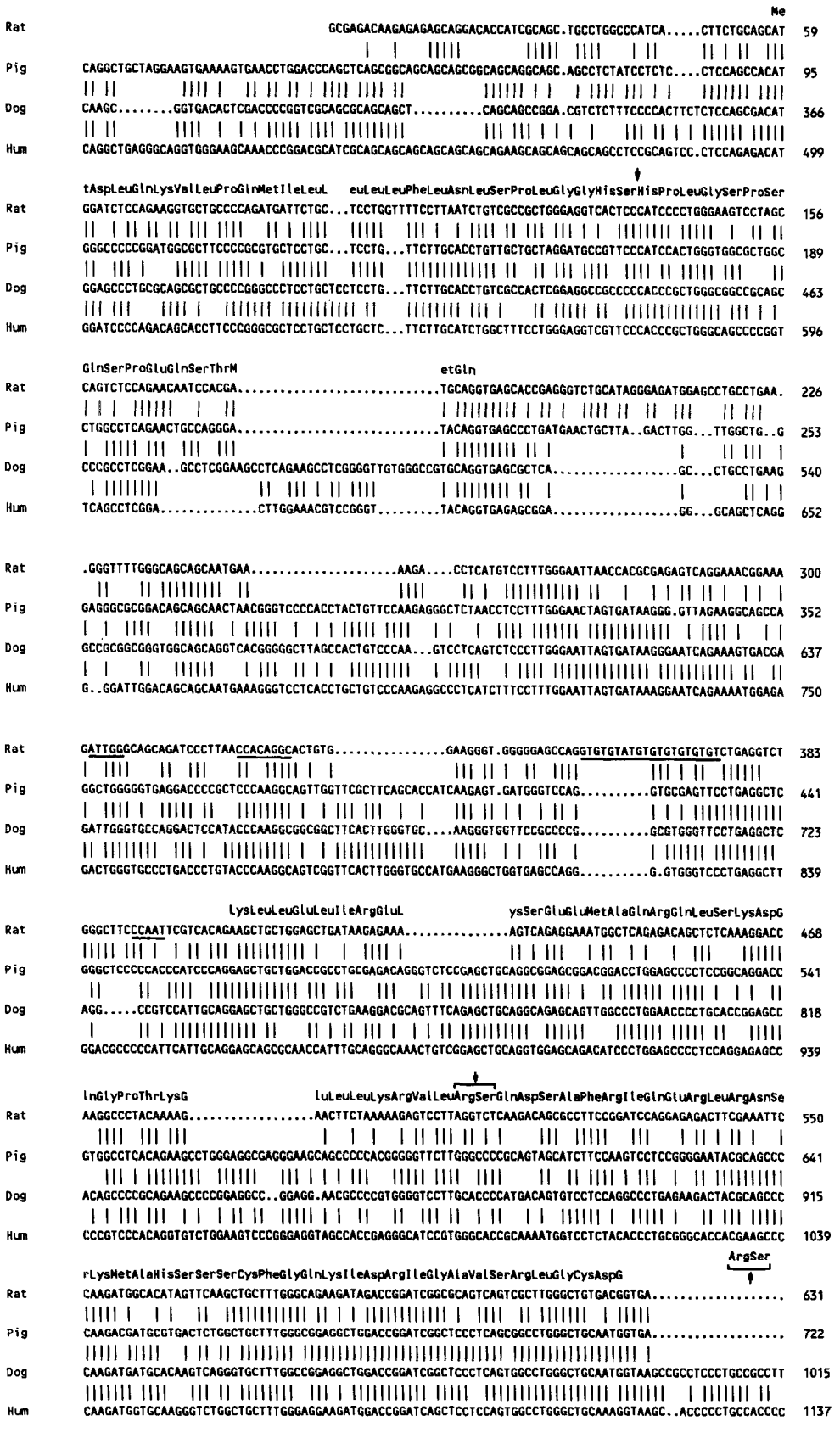
Figure 2. The nucleotide sequence of the rat Iso-ANP genomic PCR DNA was aligned with sequences from porcine, dog and human BNP genomic DNA (21). Only relevant portions of BNP genes are shown. The exon sequences of the iso-ANP are same as rBNP cDNA sequence reported previously (9). The amino acid sequence is shown above the nucleotide sequence of the iso-ANP. The anticipated processing sites are indicated by arrows and Arg/Ser cleavage sites are shown in brackets (underneath human sequence for BNPs). Potential regulatory DNA motifs within introns, as discussed in the text, are underlined.



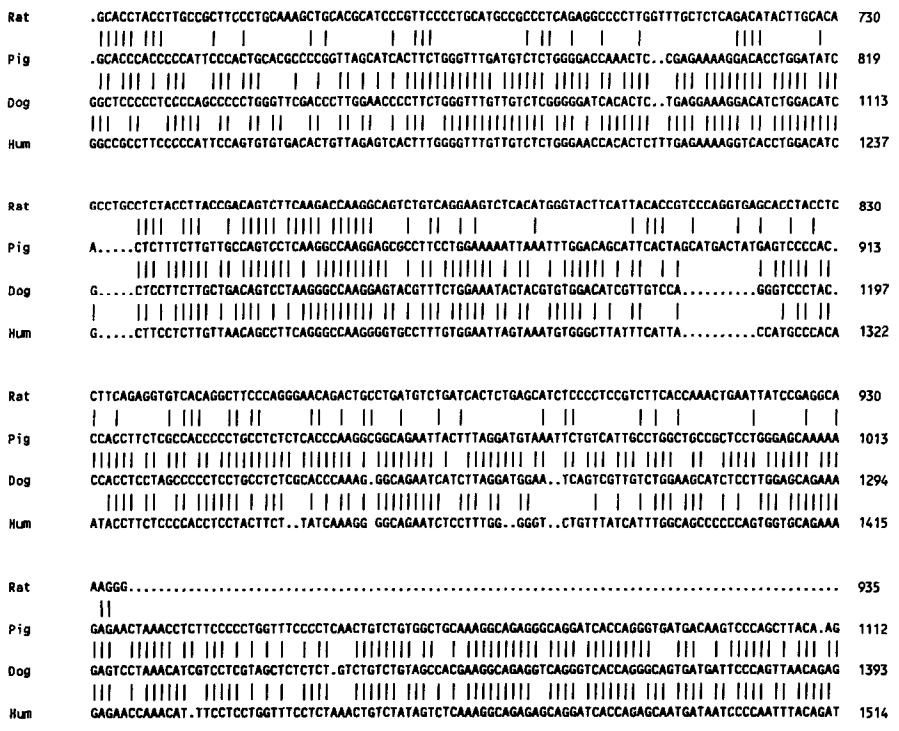


Fig. 2-Continued

human BNPs. The sequence comparison of the constituent segments of the iso-ANP gene with corresponding segments of BNPs of other mammalian species is summarized in Table 1. The extent of sequence homology of iso-ANP with BNPs lies outside the range of homology observed among BNPs (with the possible exception of the 3rd exon). The divergence of the genes for the two peptides is especially noticeable in the 2nd exon and 2nd intron. Although less conspicuous than the divergence observed within the 2nd intron, the difference within the 2nd exon is of considerable functional importance. Firstly, compared to BNPs, the 2nd exon of iso-rANP is smaller in size and consequently has reduced coding capacity, reflected in the two gaps in this segment shown in Fig. 2, introduced to maximize homology with BNPs. Located upstream from the anticipated boundary of the mature peptide, these gaps together account for a total of 30 to 33 nucleotides of reduced coding sequence or 10/11 less amino acid residues than BNP propeptides of other mammalian species. Secondly, the divergence in nucleotide sequence within the 2nd exon has strikingly affected the processing of the iso-rANP propeptide. Whereas the location of the anticipated Arg/Ser peptidase cleavage site is unaffected among various BNP species generating primarily a 32 residue peptide, in iso-rANP, due to changes in the 2nd exon, the amino acid residue at this site has changed from Ser to Asn, rendering it non-functional as a cleavage site. Instead, the sequence divergence has created a new Arg/Ser cleavage site 13 amino acid residues upstream in iso-rANP, consequently producing a mature peptide of 45 amino acid residues, which is apparently not processed any further. The 32-residue BNP may, however, be further processed in different species.

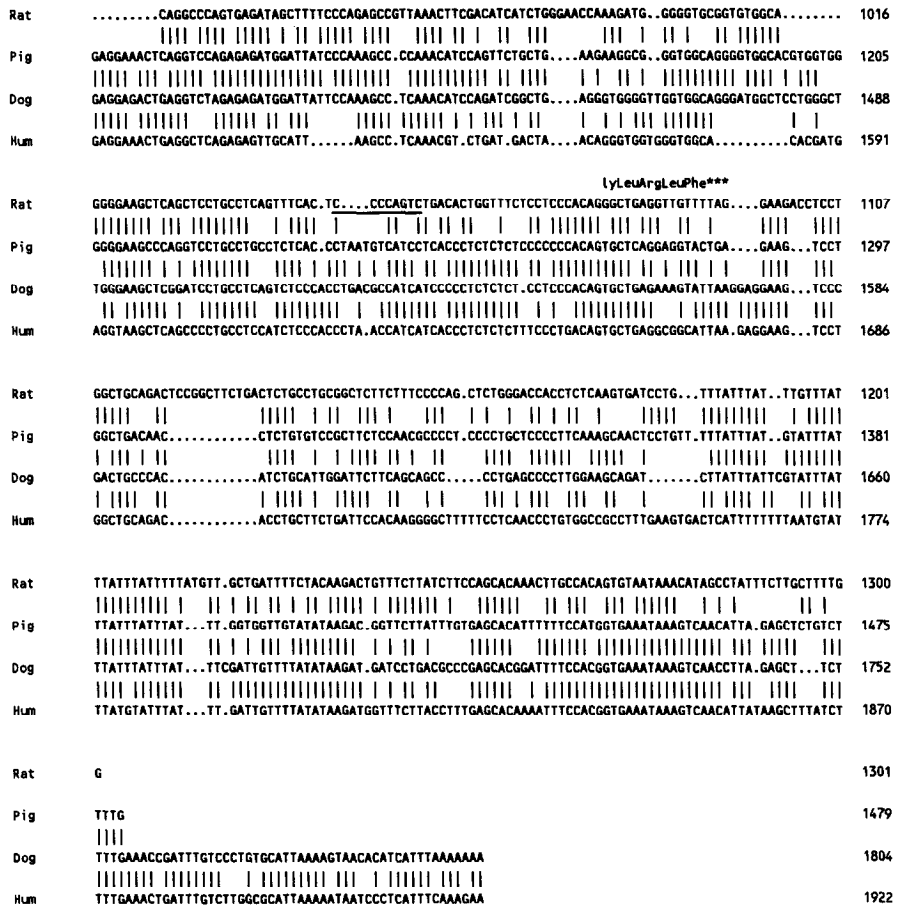


Fig. 2-Continued

Table 1

Extent of homology between iso-rANP and BNPs at the nucleotide level

	Iso-ANP sequence versus			Extent of homology among BNPs
	pBNP	dBNP	hBNP	
1st exon	59.0%	61.2%	59.0%	63.0% - 72.5%
1st intron	58.8%	56.6%	60.6%	60.8% - 71.2%
2nd exon	60.6%	56.5%	60.6%	68.7% - 75.0%
2nd intron	42.9%	53.1%	42.7%	69.3% - 74.4%
3rd exon	58.1%	58.6%	66.7%	65.7% - 76.9%

The homology has been determined by coaligning sequences with computer programs mentioned in Methods section. The extent of homology in 1st and 3rd exons has been calculated only within the area spanned by the Iso-rANP gene sequence (Figure 2).

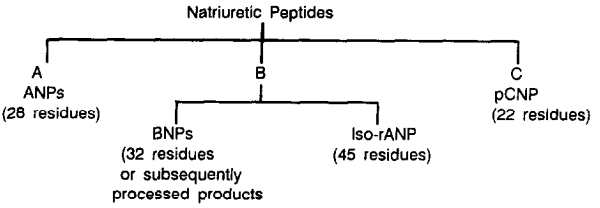


Figure 3. The relationship of rat Iso-ANP with other natriuretic peptides. The classification into three types of peptides is according to Sudoh et al. (7).

We have noticed potential regulatory DNA motifs within intronic sequences of iso-ANP (underlined in Fig. 2). Most conspicuous is a 20 nucleotide long alternating purine pyrimidine stretch in the first intron. Such an element has the potential of adopting a Z-DNA conformation and is often associated with transcriptional enhancers (22). Putative Z-DNA motifs have previously been reported at the 3' flanking regions of the rat and mouse ANP genes (17). A potential CCAAT element and its complementary form (ATTGG) (23) are also present 16 nucleotides downstream and 48 nucleotides upstream, respectively, from the putative Z-DNA element. In addition, DNA elements with high degree of homology (87.5%) with the consensus AP2 sequence (CCCCAGGC) (24) are also present in both introns. At the present moment we do not know the functional significance, if any, of these motifs with regard to expression of the rat iso-ANP gene. However, it is worth mentioning in this regard that a potential cis-acting regulatory element, namely, a glucocorticoid responsive element (GRE) has previously been reported within the second intron of the human ANP gene (19).

In reporting the cDNA sequence of rat BNP (iso-ANP) Kozima et al. (9) have observed nucleotide substitutions of C-to-A at three positions, without involving concomitant amino acid changes. Two of these sites are located in the 2nd exon, 14 nucleotides (involving the codon CGC for arginine) and 29 nucleotides (involving the ATC codon for isoleucine) upstream from the exon/intron junction (Fig. 2). The third site is located 24 nucleotides downstream from the stop codon, in the 3rd exon (nucleotide 1120, Fig. 2). We have noticed a T nucleotide in all three polymorphic sites of the rat iso-ANP, again without accompanying amino acid changes. Since two of the involved amino acid residues are located within the biologically important disulfide-bonded ring region, the selection pressure against their change is not surprising. What is surprising, however, is to find that the change at the third site, involving the 8th triplet (TCC) downstream from the stop codon, is also constrained as if to conserve a particular amino acid (serine) coded for by this triplet. Since there is an adjacent TGA triplet, 6 nucleotides downstream from this site, in phase with the reading frame, it is tempting to speculate that this triplet was perhaps functional previously as a stop codon of a longer peptide and that the presently functional stop codon resulted due to a C-to-A change of a previously functional serine (TCG) codon. In addition to these neutral polymorphisms, we have previously reported a Gln as well as a Leu residue at the penultimate position of iso-ANP (3,4). At the nucleotide level, we have observed the codon (TTG) for a leu residue, confirming the report of Kojima et al. (9). The discrepancy could be due to genetic variation among rats used for the atrial source of iso-rANP.

As discussed above, rat iso-ANP has a close structural relationship with BNPs from other mammalian species and yet has significant differences. We, therefore, view these two peptides as

distinct members of the same sub-family, within the family of genes for natriuretic peptides. In terms of the recently proposed nomenclature of A-, B- and C-type natriuretic peptides (7), the relationship of iso-rANP with other functionally related peptides can be depicted as shown in Fig. 3.

Whether iso-ANP is the functional equivalent of BNPs in rat or whether there is yet another peptide in rat which is more homologous to BNPs of other mammalian species, remains to be determined.

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