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# Molecular cloning and characterization of DNA sequences encoding rat and human atrial natriuretic factors

**(atrial pronatriodilatin/cardiodilatin/cardionatrin/cDNA cloning/genomic cloning)**

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ABSTRACT **A cDNA copy of the message encoding rat atrial natriuretic factor (ANF) has been cloned in *Escherichia coli*, and its nucleotide sequence was determined. ANF appears to be synthesized as a larger precursor, atrial pronatriodilatin. The cDNA has an open reading frame potentially encoding a protein of 152 amino acids, of which the first 24 amino acids strongly resemble a signal sequence. This is followed by a sequence with 80% homology to a second vasoactive protein, porcine cardiodilatin. The ANF peptide is contained in the COOH-terminal portion of the protein. The DNA sequence corresponding to human ANF is also presented and displays a high degree of homology to its rat counterpart. These data provide further evidence for the expression in cardiac atria of a multifactor system that may contribute to the regulation of blood pressure and extracellular fluid volume.**

It has been postulated for many years that cardiac atria possess sensors that are important in detecting changes in extracellular fluid volume (1). Such a system could respond and compensate for these changes through release of a humoral substance.

The existence of such an atrially produced substance, which enhances urinary sodium excretion and, therefore, might be involved in regulation of extracellular fluid volume, was recently demonstrated by de Bold *et al.* (2). When a partially purified extract of rat cardiac atria was injected into anesthetized rats, a large increase of urine flow and urinary sodium excretion was observed. This relatively crude extract, which possessed the appropriate characteristics for an endogenous natriuretic substance, was named atrial natriuretic factor (ANF).

In addition to its potent and natriuretic effects, it also was discovered that these extracts have potent smooth muscle relaxant activity. This property implies that they might have a potential role in regulating blood pressure as well as extracellular fluid volume.

Several laboratories have reported the purification of peptides possessing the above activities (3–5). Seidah *et al.* (6) identified a sequence common to all of the active peptides, which when synthesized, show activity in animals similar to endogenous ANF.

In this paper, we describe the isolation of a cDNA clone containing the sequence encoding this active ANF peptide. The clone spans nearly the entire rat ANF mRNA. Sequence analysis revealed that ANF was contained in a large precursor polypeptide, atrial pronatriodilatin (7), which also demonstrated homology to a second vasoactive peptide, cardiodilatin (8). Thus, the products of the *ANF* gene might act at several levels to control blood pressure and fluid volume.

In addition, we have used the rat cDNA to isolate clones of the corresponding human gene and have found a high degree of sequence homology between rat and human *ANF*.

## MATERIALS AND METHODS

**Materials.** Avian myeloblastosis virus reverse transcriptase was obtained from Seikagaku America (St. Petersburg, FL). Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, and DNA polymerase I Klenow fragment were obtained from New England Biolabs, Boehringer Mannheim, or Pharmacia P-L Biochemicals. Oligo(dT)-cellulose (type 7), mung bean nuclease, and terminal deoxynucleotidyltransferase were from Pharmacia P-L Biochemicals. Except as noted, all enzymes were used as recommended by the supplier. Radioisotopes were purchased from Amersham. (dG)-tailed pBR322 and reagents for Maxam–Gilbert sequencing were obtained from New England Nuclear. The human genomic Charon 4A library was a generous gift from T. Maniatis (Harvard University). pUC12 and pUC13 were obtained from Pharmacia P-L Biochemicals.

**Isolation of Polyadenylylated RNA.** Sixteen grams each of Sprague–Dawley rat cardiac atrial and ventricular tissue was collected and immediately frozen in liquid nitrogen. Three grams of human atrial appendage tissue was obtained by biopsy and immediately frozen. Total cellular RNA was isolated using guanidine thiocyanate (9) and the polyadenylylated poly(A)$^+$ RNA fraction was obtained using oligo(dT)-cellulose (10).

**Synthesis of Oligonucleotide.** All oligonucleotides used were made by the solid-phase triester method (11). The following sequences were made and will be designated as ANF primer pool, d(C-C-N-C-C-Pu-A-A-Pu-C-A); rat creatine kinase primer, d(A-G-G-T-C-G-G-T-C-T-T); proANF pool 1, d(A-C-T-T-C-N-C-C-N-G-T-C-C-A); proANF pool 2, d(A-C-C-T-C-N-C-C-N-G-T-C-C-A); and 5′ mRNA primer, d(G-G-G-G-C-A-C-G-A-T-C-T-C-T-G-A-T-C).

**Construction of Atrial cDNA Libraries.** First- and second-strand cDNA syntheses were carried out by the sequential method (12). Rat cDNA synthesis was initiated by using the ANF primer pool and the rat creatine kinase (13) primer, each at a poly(A)$^+$ RNA/primer weight ratio of 15:1. Human cDNA was primed as described (12). The cDNAs were phenol-extracted, ethanol-precipitated, and dried under vacuum. They then were resuspended in 250 μl of 30 mM NaOAc, pH 4.7/50 mM NaCl/1 mM $ZnCl_2$/5% (vol/vol) glycerol. Mung bean nuclease (125 units) was added and the mixture was incubated for 30 min at 38°C. The reaction was terminated by the addition of 1/10 vol of 800 mM Tris·HCl, pH 8.5/50 mM $Na_2EDTA$.

Homopolymeric tailing with dCTP was carried out, and this material was annealed to pBR322, which was (dG)-tailed

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Abbreviations. bp, base pair(s); ANF, atrial natriuretic factor.

at the *Pst* I site (14). An aliquot was used to transform *Escherichia coli* strain MM294 (15) to tetracycline resistance.

**Screening of Rat cDNA Clones.** Approximately 5000 colonies were transferred to nitrocellulose filters (14). The ANF-specific tetradecamers (proANF pool 1 and proANF pool 2) were labeled at their 5′ ends with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase (16), and hybridized to the above colonies as described (17). Sixty-six potentially positive colonies were transferred to duplicate filters and separately hybridized to either proANF pool 1 or proANF pool 2 probes. Four colonies hybridized strongly to proANF pool 1 only. The cDNA insert from the plasmid contained in the most intensely hybridizing colony, pANF3A, was subcloned into the *Pst* I site of pUC8 (18) and was subjected to DNA sequence analysis.

**Screening of Human Clones.** A 650-base-pair (bp) *Pst* I fragment derived from pANF3A was labeled with $^{32}$P by nick-translation (19) and used as a probe to identify human ANF clones. Approximately 50,000 tetracycline-resistant colonies containing human cDNA were transferred to nitrocellulose (14) for screening. Filters were prehybridized in 50 mM $NaPO_4$, pH 6.8/0.5% $NaDodSO_4$/1 M NaCl/2 mM EDTA/10× Denhardt's solution (1× Denhardt's solution = 0.2% bovine serum albumin/0.2% Ficoll/0.2% polyvinylpyrollidone) for 2 hr at 65°C; 1–5 × $10^6$ cpm of the above probe was added and the hybridization continued for 24–48 hr at 65°C. The filters were washed at 65°C with two changes of 50 mM Tris·HCl, pH 8.6/1 M NaCl/0.5% $NaDodSO_4$/2 mM EDTA, one change of 50 mM $NaPO_4$, pH 6.8/0.5% $NaDodSO_4$/0.5 M NaCl/2 M EDTA, and one change of 50 mM Tris·HCl, pH 8.6/0.5 M NaCl/0.5% $NaDodSO_4$/2 mM EDTA, for 1 hr each, followed by several rinses of 0.3 M NaCl/0.03 M Na citrate, pH 7.2, at room temperature. The filters were air dried and autoradiographed. One hundred five positive colonies were picked and rescreened as described above. Of these, 3 gave consistently strong hybridization. The clone containing the longest insert was subcloned into the *Pst* I site of pUC8 for subsequent analysis.

Human genomic ANF clones were isolated from the Charon 4A λ library of Lawn *et al.* (20) by plaque filter hybridization (21), using the rat ANF cDNA clone as a probe as described above. Twenty potentially positive plaques were purified and rescreened from an initial 6 × $10^5$ plaques. Two positive plaques were identified after the second screen. A liquid phage lysate of one clone was prepared and DNA was isolated (14). DNA was digested with *Eco*RI and subcloned into *Eco*RI-digested pUC13. Subclones hybridizing to the rat ANF cDNA were identified by colony hybridization as described above. The *ANF* gene was localized using restriction mapping and Southern blot analysis (22), and subfragments were recloned into pUC13 for DNA sequencing.

**Nucleic Acid Sequencing.** DNA for sequencing was labeled at the 5′ ends with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase (16) or at the 3′ ends with [$\alpha$-$^{32}$P]dCTP and Klenow fragment of DNA polymerase T (14) or [$\alpha$-$^{32}$P]ddATP and terminal deoxynucleotidyltransferase (23). Maxam–Gilbert sequencing reactions and gel electrophoresis were carried out as described (16).

Sequencing at the 5′ end of the RNA was carried out by reverse transcriptase-mediated primer extension in the presence of dideoxynucleotides (24). The 5′ mRNA primer was used to initiate the synthesis.

The DNA sequence presented for the rat ANF clone was a composite of at least two sequencing reactions along the entire length of the clone and, except for the region between nucleotides 392 and 445, is derived from information off both strands. The human DNA sequence presented was determined from both strands. The primer extension sequence was determined twice.

**RNA Blot Analysis.** To examine the abundance of ANF mRNA in atrial and ventricular tissues, 10 $\mu$g of poly(A)$^+$ RNA was electrophoresed on a 1.2% agarose gel containing formaldehyde (14). The RNA was transferred to nitrocellulose by the method of Thomas (25) and then baked at 80°C for 2 hr. Filters were hybridized with pANF3A and washed as described above.

## RESULTS

**Cloning of Rat ANF cDNA.** Rat atrial cDNA was synthesized using as primer two pools of undecamers complementary to the ANF peptide sequence, amino acids 129–132 (Cys-Phe-Gly-Gly) (Fig. 1; ref. 6), and to rat muscle creatine kinase (13). Upon transformation of *E. coli* MM294 (15), ≈5000 colonies were obtained. These colonies were hybridized to 14-base oligonucleotide probes complementary to all possible codons of a segment of the pronatrodilatin region, amino acids 82–86 (Trp-Thr-Gly-Glu-Val) (7). The most strongly hybridizing colony was isolated and found to contain an insert of ≈850 bp. An ≈650-bp *Pst* I fragment of this insert was subcloned into the *Pst* I site of pUC8, and the DNA sequence was determined (Fig. 1).

Based on the size of the mRNA (see below) and the size of the cloned cDNA, it was estimated that ≈50 nucleotides at the 5′ end of the mRNA were not contained in the insert. To obtain the remaining sequence, primer extension was used to sequence the mRNA directly (Fig. 1). As predicted, 42 nucleotides not present in the cDNA clone were identified (the 5′-terminal 6 nucleotides could not be read). The resulting DNA sequence revealed an open reading frame 456 bp (152 amino acids) long, beginning at the first ATG, which, when translated into amino acid sequence, displayed perfect homology to the published 106 amino acids of pronatrodilatin (7). The additional encoded amino acids obtained by DNA sequencing, all but two of which are $NH_2$-terminal to the reported protein sequence, are probably all included in the primary translational product. The possible significance of domains within the precursor protein will be discussed below.

**Analysis of ANF-Specific Transcripts.** To determine the size and relative tissue abundance of rat ANF-specific transcripts, poly(A)$^+$ RNA isolated from atrial or ventricular tissue was fractionated on agarose/formaldehyde gels and transferred to nitrocellulose filters for blot analysis.

When nick-translated pANF3A was used as a probe, a single major band of ≈900 nucleotides was observed in the atrial RNA lane (Fig. 2). Two high molecular weight bands also were seen but in much less abundance. These bands might represent precursor forms of the message or perhaps distantly related transcripts that cross-hybridize to rat ANF cDNA. The ventricular RNA lane showed little homology to the probe, although a small amount of hybridization was observed routinely at the same position as the atrial transcript. These data indicate that ANF-specific RNA is far more abundant in the cardiac atria than in the ventricles, demonstrating tissue-specific expression of the *ANF* gene. Probing of the same material with a labeled creatine kinase cDNA gave nearly equivalent signals with both tissues (data not shown), indicating equivalent expression of this common gene product in both areas of the heart.

**Comparison of Rat and Human ANF.** Human cDNA and genomic clones were obtained using the rat ANF plasmid as a probe. Sequence analysis of the human cDNA clone (pHANF4) revealed a high degree of homology between one end of the human clone and the rat cDNA. The homologous region spans the sequences encoding amino acids 12–35 (Fig. 3), the TAA terminal codon, and 6 bp of the 3′ untranslated

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nnnnngtgacggacaaaggctgagagagaaacccagagagtGAGCCGAGACAGCAAACATCAGATCGTGCCCCGACCCACGCCAGC    87

ATG GGC TCC TTC TCC ATC ACC AAG GGC TTC TTC CTC TTC CTG GCC TTT TGG CTC CCA GGC CAT ATT  153
Met Gly Ser Phe Ser Ile Thr Lys Gly Phe Phe Leu Phe Leu Ala Phe Trp Leu Pro Gly His Ile
·1·····

GGA GCA AAT CCC GTA TAC AGT GCG GTG TCC AAC ACA GAT CTG ATG GAT TTC AAG AAC CTG CTA GAC  219
Gly Ala Asn Pro Val Tyr Ser Ala Val Ser Asn Thr Asp Leu Met Asp Phe Lys Asn Leu Leu Asp
·23····

CAC CTG GAG GAG AAG ATG CCG GTA GAA GAT GAG GTC ATG CCT CCG CAG GCC CTG AGC GAG CAG ACC  285
His Leu Glu Glu Lys Met Pro Val Glu Asp Glu Val Met Pro Pro Gln Ala Leu Ser Glu Gln Thr
 45

GAT GAA GCG GGG GCG GCA CTT AGC TCC CTC TCT GAG GTG CCT CCC TGG ACT GGG GAA GTC AAC CCG  351
Asp Glu Ala Gly Ala Ala Leu Ser Ser Leu Ser Glu Val Pro Pro Trp Thr Gly Glu Val Asn Pro
 67

TCT CAG AGA GAT GGA GGT GCT CTC GGG CGC GGC CCC TGG GAC CCC TCC GAT AGA TCT GCC CTC TTG  417
Ser Gln Arg Asp Gly Gly Ala Leu Gly Arg Gly Pro Trp Asp Pro Ser Asp Arg Ser Ala Leu Leu
 89

AAA AGC AAA CTG AGG GCT CTG CTC GCT GGC CCT CGG AGC CTG CGA AGG TCA AGC TGC TTC GGG GGT  483
Lys Ser Lys Leu Arg Ala Leu Leu Ala Gly Pro Arg Ser Leu Arg Arg Ser Ser Cys Phe Gly Gly
111                         118

AGG ATT GAC AGG ATT GGA GCC CAG AGC GGA CTA GGC TGC AAC AGC TTC CGG TAC CGA AGA TAA CAG  549
Arg Ile Asp Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr Arg Arg
133                                                                          152

CCAAATCTGCTCGAGCAGATCGCAAAAGATCCCAAGCCCTTGCGGTGTGTCACACAGCTTGGTCGCATTGCCACTGAGAGGTGGTGA  636

ATACCCTCCTGGAGCTGCAG                                                                    656
```

FIG. 1. Nucleotide sequence of the cDNA encoding rat atrial pronatriodilatin. The deduced amino acid sequence is shown. Numbers on the right denote nucleotide length. Numbers below the sequence indicate amino acid number. Leucine residue 118 was previously described as the first amino acid of rat ANF (6). The 5′ nucleotides denoted in lower case letters were obtained from primer extension RNA sequencing. The putative signal sequence (·····), the cardiodilatin-related peptide (------), and the ANF sequences (——) are shown.

portion of the rat ANF clone. These data suggest that the COOH termini of rat and human ANF are very similar in amino acid composition and that they terminate at the same position. Beyond the coding regions of the messages, their nucleotide sequences rapidly diverge and the human and rat sequences show no homology.

Genomic clones were analyzed to obtain the remainder of the human ANF DNA sequence. The portion of the human cDNA clone encoding rat amino acids 12–32 (Fig. 3) was colinear with the genomic clone but diverged at amino acid 33. The divergence between the human cDNA and genomic clones beyond amino acid 32 implies a splice junction at this point. There also was good agreement between the genomic clone and the remainder of the rat ANF sequence. Fig. 3 shows a composite of the human genomic and cDNA sequences that demonstrate homology with rat ANF peptide 1–35. There is a striking amount of identity between the two species. Only seven single base changes and three amino acid differences are evident with no additions or deletions between the two sequences. A more detailed description of the human gene structure will be presented elsewhere (unpublished observations).

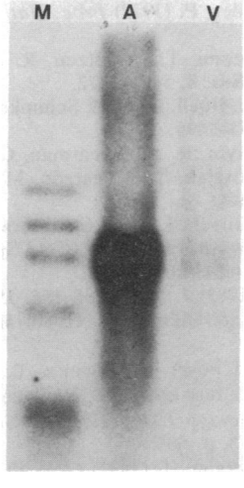


FIG. 2. Expression of pronatriodilatin mRNA in the rat myocardium. Poly(A)$^+$ RNA (10 $\mu$g) was isolated from rat atria (lane A) or rat ventricles (lane V), run on a 1.2% agarose/formaldehyde gel, blotted, and hybridized to $^{32}$P-labeled pANF3A. Lane M contains $^{32}$P-labeled *Hae* III fragments of $\phi$X174.

## DISCUSSION

We have isolated a cDNA clone containing the sequences encoding the precursor to rat ANF pronatriodilatin. Of the 152 amino acids derived from our DNA sequence, 106 have been confirmed by direct protein sequence analysis (7). The ATG at nucleotide positions 88–90 (Fig. 1) is likely to be the initiation codon for the primary translation product. It is the first methionine codon in the message sequence, and a protein product in the proper reading frame for pronatriodilatin is produced by starting at this point.

The first 24 amino acids of this protein bear a striking resemblance to a consensus signal sequence (26). After the putative signal sequence, the next 30 amino acids display strong homology to those reported for the $NH_2$-terminal portion of porcine cardiodilatin (7, 8). The ANF peptide resides in the COOH-terminal domain of the precursor protein.

```
                1                                   9                                  18
Rat AA    Leu Ala Gly Pro Arg Ser Leu Arg Arg Ser Ser Cys Phe Gly Gly Arg Ile Asp
Rat Nt    CTC GCT GGC CCT CGG AGC CTG CGA AGG TCA AGC TGC TTC GGG GGT AGG ATT GAC
Hum Nt       A   C                    G   A   C                   C       G
Hum AA       THR ALA                                                      MET

               19                                  27                  33
Rat AA    Arg Ile Gly Ala Gln Ser Gly Leu Gly Cys Asn Ser Phe Arg Tyr Arg Arg  .
Rat Nt    AGG ATT GGA GCC CAG AGC GGA CTA GGC TGC AAC AGC TTC CGG TAC CGA AGA TAA
Hum Nt                                 G       T
Hum AA
```

FIG. 3. Comparison of nucleotide and deduced amino acid sequences of rat and human ANF. Nucleotide and amino acid differences in human ANF sequence are underlined. Numbering system corresponds to that in ref. 6.

In addition to the $NH_2$-terminal processing event discussed elsewhere (6–8), the DNA sequence implies the existence of two arginine residues at the COOH terminus. These residues have never been described in any of the numerous peptides reported to display ANF activity. This suggests COOH-terminal processing of the peptide.

The sequence results reported above are not consistent with the method used to clone the rat ANF cDNA. Use of an oligonucleotide primer in a reverse transcriptase reaction should result in the production of a cDNA, which, at best, has the 5′ sequence of the primer as its terminal sequence. The rat cDNA clone described above extends beyond the putative priming site. It is possible that some oligo(dT) was stripped off the oligo(dT) cellulose column during the elution of the poly(A)$^+$ RNA and subsequently served as primer for the cDNA clone obtained. It also could be the result of aberrant priming by one of the oligonucleotides, as has been reported previously.

The demonstration of tissue-specific expression of the ANF message in cardiac atria is consistent with the previously reported distribution of the ANF peptide (4, 5). This suggests that ANF is made and stored in the atria.

In contrast, we have detected only trace amounts of ANF-specific mRNA in ventricular tissue. This is in agreement with the reported lack of ANF activity in ventricular extracts (2).

The DNA sequence encoding human ANF shows a high degree of homology with the rat sequence. Only seven base changes between the two are evident. These translate into only three amino acid differences in the ANF peptide, all of which are conservative. The inferred peptide sequence agrees with that reported for human ANF (27).

The human DNA sequence presented is a composite of data obtained from cDNA and genomic clones. Colinearity is lost between the two types of clones at the sequence corresponding to amino acid 33 (Fig. 3). Thereafter, the human cDNA sequence agrees with the rat sequence through the termination codon, while the human genomic sequence diverges completely. We conclude that this defines the location of a splice point.

Preliminary sequence data from another human cDNA clone suggests the existence of two expressed alleles of *ANF* (data not shown). This second allele possesses several base changes from that reported above, one of which introduces a termination codon at amino acid position 34, resulting in the loss of the two COOH-terminal arginines. Such a truncated protein would yield a product identical to the mature form of the original allele.

Lastly, the presence of at least two peptides with similar but distinct functions on the same gene product, as well as the high level of interspecific conservation (refs. 7 and 27; this paper), suggest pronatriodilatin is an important regulatory component of the cardiovascular system. Post-translational processing and selective secretion could allow for simultaneous vasodilation, naturesis, and fluid volume control.

**Note Added in Proof.** While this manuscript was under review, several other groups reported on the cloning and sequencing of ANF. Our results are in general agreement with their reports (28–31).

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