# The Structure of Rat Preproatrial Natriuretic Factor as Defined by a Complementary DNA Clone

Abstract. *The structure of rat preproatrial natriuretic factor (preproANF) was determined by nucleotide sequence analysis of an ANF complementary DNA clone. PreproANF is composed of a hydrophobic leader segment (20 amino acids), a precursor containing one glycosylation site (106 amino acids), and ANF (24 amino acids). Atrial natriuretic factor is located at the carboxyl terminus of the precursor molecule. The human, mouse, and rat genomes each contain a single ANF gene which is highly conserved.*

Atrial natriuretic factor (ANF) is a potent vasoactive substance that is synthesized in mammalian atria and is thought to play a key role in cardiovascular homeostasis (*1–5*). Purification and amino acid sequence analysis of rat ANF has delineated two peptides that have sequences differing only in length yet elicit different physiologic responses (*6*). The smaller peptide (21 amino acids) exhibits natriuretic and diuretic activity and selectively relaxes intestinal but not vascular smooth muscle strips. The larger peptide (23 amino acids) has more potent natriuretic and diuretic activity and relaxes both vascular and intestinal smooth muscle strips. The larger peptide can be converted to the smaller by proteolytic digestion. To study further the source, processing, and regulation of this cardiac factor, we have cloned DNA sequences complementary to rat messenger RNA (mRNA) coding for ANF. Nucleotide sequence analysis of this nearly full-length complementary DNA (cDNA) predicts the amino acid sequence of the ANF precursor. This molecule has features typical of hormone precursors, suggesting that ANF is a cardiac hormone. A new atrial function—production of a secreted hormone—is therefore postulated.

To clone DNA sequences complementary to ANF mRNA, we constructed a library of 180,000 cDNA clones from rat atria polyadenylated [poly(A)$^+$] mRNA and screened them by hybridization with a synthetic oligonucleotide probe (*7, 8*).

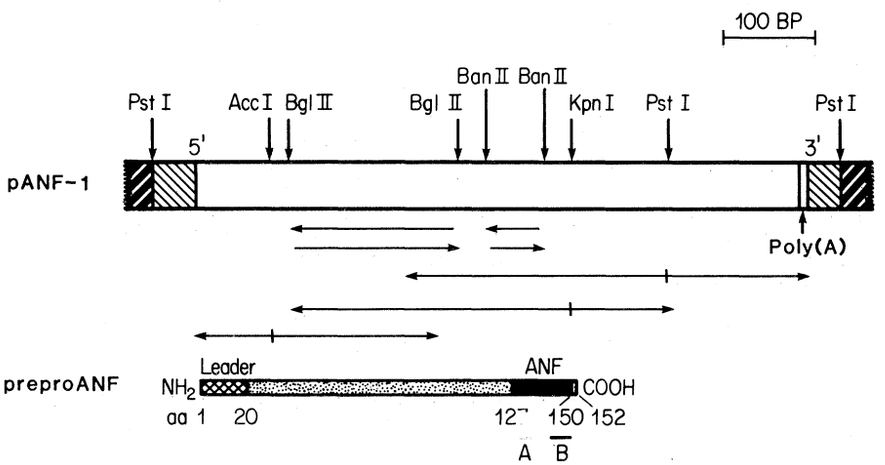


Fig. 1. Strategy for determining the nucleotide sequence of the ANF cDNA clone pANF-1. Two mixed oligonucleotide probes derived from the amino acid sequence of ANF (*6*) were synthesized (solid lines A and B). These correspond to peptides Ser-Cys-Phe-Gly-Gly (14-nucleotide probe; A) from the amino terminus and Gly-Cys-Asn-Ser-Phe-Arg (17-nucleotide probe; B) from the carboxyl terminus of ANF. Poly(A)$^+$ mRNA was isolated from atria from Sprague-Dawley rats by the guanidine isothiocyanate procedure (*19*) and oligodeoxythymine cellulose column chromatography. A cDNA library was constructed from atrial poly(A)$^+$ mRNA by cloning polydeoxycytosine-tailed, double-stranded cDNA into polydeoxyguanine-tailed, Pst I–cleaved pBR322 vector (*7*). The location of vector sequences is indicated by dark diagonal shading; the poly(G-C) linker sequence (G, guanine; C, cytosine) is indicated by light diagonal shading. The plasmid pANF-1, containing the largest cDNA insert (750 bp) among the positively hybridizing plasmids, was chosen for subsequent analysis. The restriction enzyme sites in the pANF-1 insert that were used for nucleotide sequencing are indicated above pANF-1. Restriction fragments were cloned into M13 vector mp9 and sequenced by the dideoxy chain termination method (*12*). Arrows represent the direction and extent of reading from a restriction site. There is no ambiguity of sequence in the short regions analyzed only in one direction. The nucleotide sequence predicted the structure of the 152–amino acid preproANF molecule. The first 20 amino acids correspond to a hydrophobic leader segment. The 24 amino acids corresponding to ANF are located at the carboxyl terminus of the peptide (amino acids 127 to 150).

A mixed 14-nucleotide probe was synthesized (*9–11*), corresponding to the sequence Ser-Cys-Phe-Gly-Gly from the amino terminus of ANF. This probe contained 96 individual oligomers, all of the possible sequences that could encode these five amino acids. Fifteen bacterial colonies in the atrial cDNA library hybridized to the mixed 14-nucleotide probe. Because of the complexity of the mixed probe, some of these positive colonies were likely to contain nonANF cDNA's. To identify more precisely only the ANF cDNA clones, these 15 colonies were screened with a second, 17-nucleotide probe corresponding to amino acids Gly-Cys-Asn-Ser-Phe-Arg from the carboxyl terminus of ANF. This 17-nucleotide probe contained 384 individual oligonucleotides. Of the 15 cDNA clones, 11 were again positive when hybridized to the second probe. All 11 of these plasmids have been shown to contain some portion of ANF nucleotide sequences derived from mRNA.

Plasmid pANF-1 was chosen for further analysis because it contained the largest insert [750 base pairs (bp)]. The nucleotide sequence of this insert was determined by the dideoxy chain termination procedure (*12*) (Fig. 1). The sequence of pANF-1 confirms that this cDNA clone was derived from ANF mRNA, since the plasmid insert encodes the entire 24–amino acid ANF peptide (Fig. 2). The sequence of the pANF-1 insert suggests that ANF mRNA contains an open reading frame which could encode a 152–amino acid polypeptide (preproANF) that begins with a methionine residue and is in the same reading frame as the ANF peptide (Fig. 2).

The nucleotide sequence of pANF-1 predicts many of the characteristics of preproANF mRNA, which appears to be a typical eukaryotic mRNA. The nucleotides surrounding the initiating site maintain the highly conserved adenosine, three bases before, and guanosine, one base beyond, the initiating methionine found in all eukaryotic mRNA's (*13*). Furthermore, ANF mRNA contains the normal AAUAAA polyadenylation signal (A, adenine; U, uracil) (*14*) near its 3′ end (Fig 2). PreproANF defined by the nucleotide sequence of the ANF mRNA has all of the features expected of a prepropeptide hormone. First, the 19 amino acids following the initial methionine are hydrophobic. A hydrophobic stretch of amino acids near the amino terminus of a protein is typical of secreted proteins (*15*), including preprohormones (*16*). This hydrophobic leader segment is probably cleaved to generate proANF from preproANF at the glycine

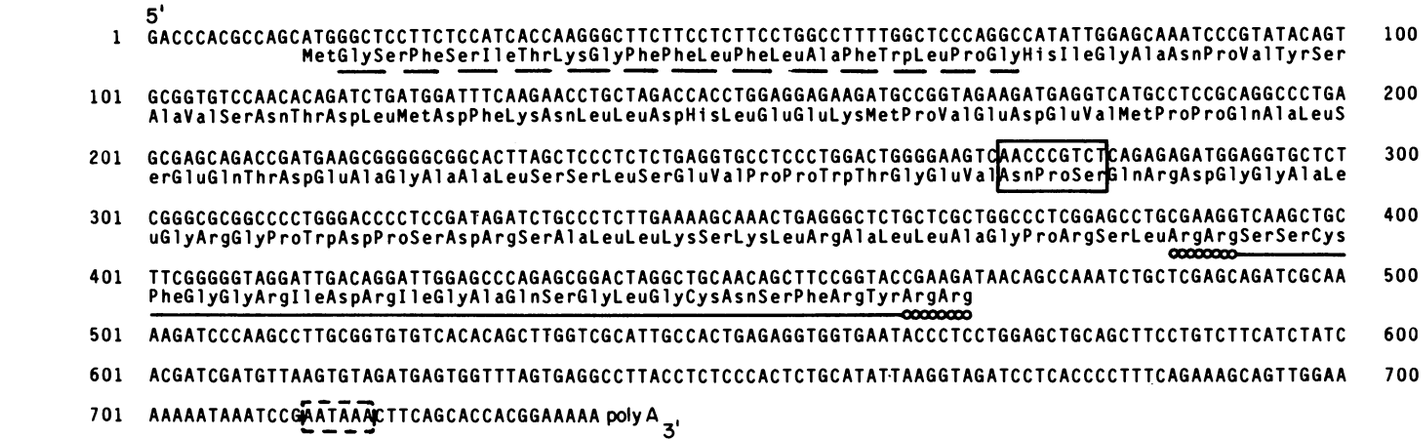


Fig. 2. The nucleotide and translated protein sequences of the pANF-1 insert. The initiating methionine is followed by a hydrophobic leader segment (dashed underline) of 20 amino acids. The subsequent 106 amino acids contain one potential site for glycosylation (Asn-Pro-Ser; boxed area). Two peptide cleavage sites (circles under sequence) occur immediately before and after the ANF peptide (underlined). The nucleotide sequence was deduced by the strategy shown in Fig. 1. The location of the poly(A) sequence and AATAAA (A, adenine; T, thymine) found at the 3′ end of all eukaryotic mRNA's is indicated (dashed box).

residue (amino acid 20), since leader segments generally end at a residue with a small neutral side chain (*17*). Second, the putative proANF consists of 106 amino acids separated from the ANF peptide by flanking basic amino acids Arg-Arg. The ANF peptide, nearly at the carboxyl terminus of the proANF molecule, is presumably cleaved from its precursor by the action of processing proteases, as has been suggested for other peptide hormone precursors (*16*). Third, the proANF sequence Asn-Pro-Ser (Fig. 2) suggests that this protein is glycosylated.

The ANF sequence is bounded on both its amino and carboxyl terminal ends by paired basic residues (Fig. 2). This is seemingly analogous to other preprohormone molecules (*16*) in which small peptide hormones are bounded by paired basic residues, indicating cleavage sites for proteolytic processing enzymes. However, a termination codon immediately follows the Arg-Arg cleavage signal in preproANF. Why peptide synthesis terminates directly after a cleavage site is unclear. Three products related to ANF that have different carboxyl terminal residues have been identified in rat atria (*6, 18*). The 24–amino acid ANF peptide (terminating in Phe-Arg-Tyr) exhibits properties like those of the 23–amino acid peptide (terminating in Phe-Arg) but differs from the 21–amino acid peptide (terminating with Ser) in selectivity for smooth muscle relaxation and diuretic and natriuretic potency. Perhaps the carboxyl dibasic peptide plays a role in this differential proteolytic processing.

To correlate the length of rat ANF mRNA with the size of the cDNA insert, we subjected rat atria and liver poly(A)$^+$

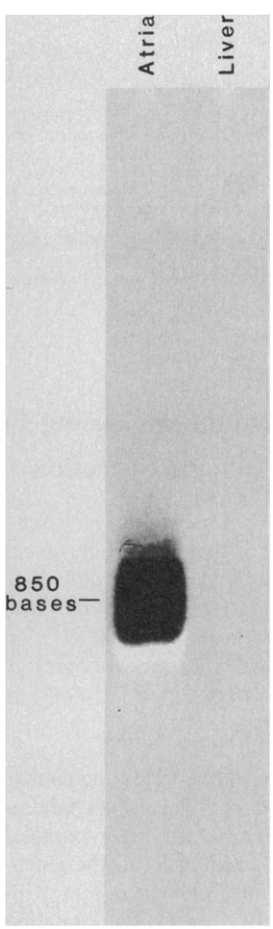


Fig. 3 (left). Identification of ANF mRNA by Northern blot analysis. Poly(A)$^+$ mRNA (1 μg) isolated from atria and liver from Sprague-Dawley rats was fractionated on a 1 percent agarose gel, transferred to nitrocellulose filter (*7*), and hybridized to a nick-translated probe derived from Pst I–restricted pANF-1 (nucleotides 1 to 580). ANF mRNA was not detected in liver mRNA. Rat atria ANF mRNA migrated as an 850-base RNA species relative to 28*S* and 18*S* ribosomal RNA's. The autoradiogram was exposed for 40 minutes.

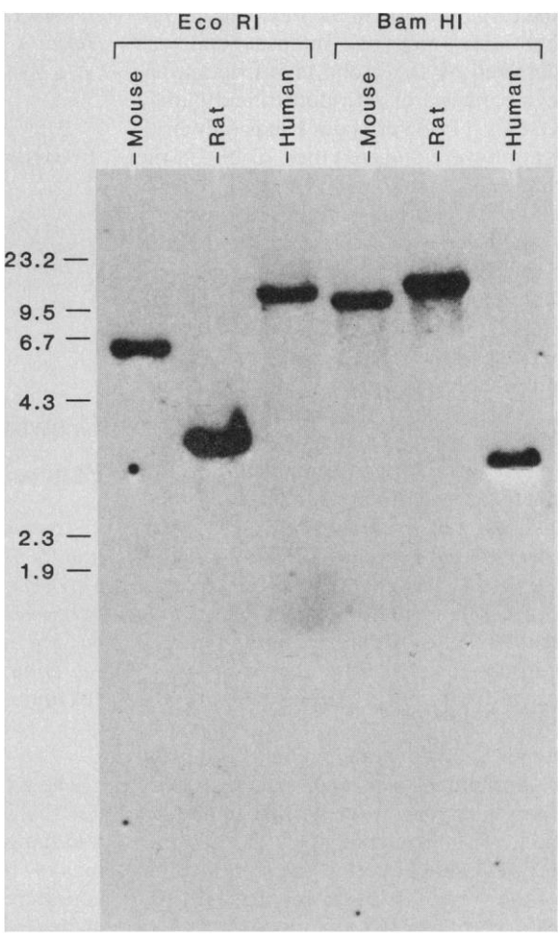


Fig. 4 (right). Southern blot hybridization of rodent and human DNA's to rat ANF probe. DNA from liver from Sprague-Dawley rats, liver from C57BL/6 mice, and human T cell lymphoma (10 μg each) was digested with Bam HI and Eco RI enzymes and fractionated on a 0.9 percent agarose gel (*7*). The DNA was transferred to a nitrocellulose filter and hybridized to an ANF nick-translated probe (see Fig. 3). Variations in fragment sizes represent differences in the location of restriction enzyme sites, not differences in gene identity or length. The size of known fragments derived from DNA digested with stained λ Hind III are indicated.

RNA to Northern blot analysis using the $^{32}$P-labeled pANF-1 insert as a hybridization probe (Fig. 3). With a 40-minute exposure, a single band was observed from atrial mRNA, suggesting that the ANF message is plentiful. No hybridizing mRNA's were detected in rat liver mRNA even after 3 days of exposure (results not shown). The length of ANF mRNA is 850 bases. Assuming that the poly(A)$^+$ tail is approximately 100 bases in length, the estimated size of ANF mRNA indicates that pANF-1 contains a nearly full-length cDNA insert.

To determine how many genes similar to ANF occur in the rat genome and whether these genes occur in other species, we subjected rat, mouse, and human DNA to Southern blot analysis and hybridization to $^{32}$P-labeled pANF-1 insert DNA (Fig. 4). Under relatively stringent conditions, where sequences of less than 70 percent homology should not hybridize, only one intense band was detected by the probe in restriction enzyme digests of rat, mouse, and human DNA's. [Two very faint bands of hybridization were detected (not visible on the autoradiogram shown in Fig. 4). Whether these faint bands represent distantly related genes or artifacts of the hybridization procedure remains uncertain.] These observations allow two conclusions. First, there are no other closely related (more than 70 percent identical) preprohormone genes encoded in the rat genome. Second, the preproANF gene is highly conserved among human, rat, and mouse, which suggests an important role for this preproANF structure.

These results show that ANF is synthesized as a preprohormone in atrial tissue that has characteristics typical of processed secreted peptides, suggesting that ANF is a cardiac hormone. The conserved similarity between ANF genes from distant species supports the hypothesis that this peptide plays an important role in maintaining cardiovascular homeostasis and that its prepro form is important for its subsequent action. The availability of the plasmid pANF-1 should permit the identification of the various roles for preproANF. For example, this cDNA clone should allow the synthesis of large amounts of the protein, enabling the physiological role of preproANF to be tested. Furthermore, the physiological changes that lead to the synthesis of the protein can be studied at the transcriptional level. Finally, mutations in the transcriptional and regulatory portions of the gene for ANF may account for some pathophysiologic states. With the use of the cDNA clone and isolated genomic clones, we can now investigate the potential role of ANF in animal models of hypertension and congestive heart failure.

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## Growth Hormone–Releasing Factor Stimulates Pancreatic Enzyme Secretion

Abstract. *Growth hormone–releasing factors (GRF's) from two human pancreatic tumors (hpGRF's) that caused acromegaly and from the rat hypothalamus (rhGRF) were recently isolated and characterized. Although these peptides are potent growth hormone secretagogues, they have not until now been described to have actions outside the pituitary. These GRF's were shown to stimulate digestive enzyme secretion from an exocrine pancreatic preparation in vitro, rhGRF being more than 100 times as potent as hpGRF. Adenosine 3',5'-monophosphate mediates this action of the GRF's.*

The growth hormone–releasing factors (GRF's) isolated from the human pancreatic tumors (*1–3*) and the rat hypothalamus (*4*) belong structurally to the glucagon-secretin family of peptides that include vasoactive intestinal peptide (VIP), glucagon, secretin, gastric inhibitory peptide (GIP), and PHI, a peptide with histidine as its $NH_2$-terminus and isoleucine amide as its COOH-terminus. Because members of the glucagon-secretin family of peptides have various physiological effects in the gastrointestinal tract and because the tumor GRF's were of pancreatic origin, we pursued the possibility that GRF has an effect in the exocrine pancreas.

In our preparation of dispersed acini from the guinea pig pancreas, VIP, PHI, and secretin—but not glucagon or GIP—act to stimulate digestive enzyme secretion by increasing cellular adenosine 3',5'-monophosphate (cyclic AMP) (*5, 6*). In contrast to VIP, PHI, and secretin, secretagogues of enzyme secretion such as cholecystokinin, bombesin, and carbachol cause mobilization of cellular calcium and enzyme secretion with no change in cyclic AMP (*7*). Another characteristic of this preparation is that the enzyme secretion from acini incubated with a combination of an agent that acts by increasing cyclic AMP and an agent that causes mobilization of cellular calci-