Extra-atrial expression of the gene for atrial natriuretic factor
(heart/tissue-specific expression/peptide hormone)

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ABSTRACT Atrial natriuretic factor (ANF) is a group of peptides, originally isolated from the cardiac atria, that have a number of important effects on blood pressure, renal function, and salt balance. In the current study, expression of the ANF gene in certain extra-atrial tissues of the rat has been examined by radioimmunoassay of extracted ANF protein and by blot-hybridization, nuclease S1 analysis, and primer-extension analysis of the ANF mRNA. ANF peptides and mRNA were detected in cardiac ventricles, lung, and pituitary gland at levels generally ≤1% those of cardiac atria. The ANF transcripts in extra-atrial tissue appear to be very similar to those synthesized in the atria. They are polyadenylated, are equivalent in overall length (950–1050 nucleotides), and have identical 5' termini. A secondary transcription start site mapping approximately 80 base pairs upstream from the primary start site is employed in atria and to a lesser extent in other tissues. The ANF transcript is present throughout the cardiac ventricles from apex to base and in the septum as well as the ventricular free walls. The transcript is more prevalent in the left ventricle and interventricular septum than in the right ventricle. Immunocytochemistry using various anti-rat ANF antibodies localized ANF immunoreactivity to the atrial myocytes; the ventricular myocytes, particularly along the endothelial surface of the ventricular chamber; perilveolar cells in the lung; and the gonadotropin-producing cells of the pituitary. The data indicate that the capacity for ANF gene expression extends beyond atrial tissue, albeit at much reduced levels, and may suggest alternative, perhaps paraendocrine, functions for the peptide in these tissues.

The atrial natriuretic factor (ANF) consists of a group of peptides with potent diuretic, natriuretic, and vasorelaxant activity (1, 2). As such, they have implied importance as naturally occurring "antihypertensive" regulators of blood pressure, volume status, and cardiovascular homeostasis.

Following the description of ANF bioactivity by deBold et al. (1), it was thought that synthesis and secretion of these peptides were confined to the cardiac atria and absent from ventricular as well as other tissues. The atria were found to be rich in secretory granules, while the ventricles were not (3), and atrial extracts had significant natriuretic activity, while no activity was found in ventricular extracts (1, 4, 5).

A number of more recent studies (6–8) also failed to identify either the ANF peptide or its mRNA in the ventricle, as determined by immunocytochemistry and blot hybridization, respectively.

Nevertheless, there have been several lines of evidence suggesting that expression of the ANF gene does take place in extra-atrial tissue. First, ANF immunoreactivity has been detected by radioimmunoassay in extracts of rat hypothalamus (9). Second, immunocytochemical studies have revealed several areas in the kidney, adrenal medulla, pituitary (10), and central nervous system, including the hypothalamus (6, 11), that appear to harbor the ANF peptides. Third, at least one other group (12) has identified putative ANF transcripts in a ventricular RNA preparation.

The present study was initiated in an attempt to define better the nature and distribution of the atrial and ventricular transcripts and to assess potential expression of the ANF gene in a number of other tissues.

MATERIALS AND METHODS

Materials. Restriction enzymes, DNA-modifying enzymes, and placental RNase inhibitor (RNasin) were obtained from standard commercial suppliers. Deoxyribonucleoside 5’-α-32P-3P-triphosphates were obtained from ICN, and [γ-32P]ATP was from Amersham.

A 4.2-kilobase (kb) EcoRI fragment of the rat ANF (rANF) gene was subcloned in pBR322 and provided to us by B. Greenberg of California Biotechnology, Inc. Restriction mapping and limited DNA sequencing confirmed that it represented a clone similar to that described by Argentin et al. (13).

RNA Blot Hybridization. Tissues were dissected from freshly decapitated 250-g male Sprague–Dawley rats, quick frozen in liquid nitrogen, and stored at −70°C until use. RNA was prepared from individual tissues, usually pooled samples from 3–5 animals, by the technique of Cathala et al. (14). Where appropriate, poly(A) RNAs were selected using oligo(dT)-cellulose (15). After quantitation by absorption at 260 mm, a predetermined amount of RNA was denatured in glyoxal and dimethyl sulfoxide, loaded on an agarose gel, fractionated electrophoretically, and blotted to nitrocellulose paper according to the technique of Thomas et al. (16).

Hybridization was carried out using a 32P-labeled strand-specific rANF cDNA (7) probe (specific activity = 1–5 × 106 cpm/μg), synthesized by the replacement-synthesis technique (17).

Primer-Extension Analysis. A synthetic single-stranded primer (≈500 pg) corresponding exactly to a 24-nucleotide segment of the ANF cDNA in the 5’ untranslated region of the gene (nucleotides 33–56, ref. 7), was 32P-labeled at its 5’ terminus and hybridized with 10–100 μg of total RNA in 10 mM Tris-HCl, pH 7.5/1 mM EDTA at 50°C for 2 h. Then the temperature was decreased to 45°C, 15 units of reverse transcriptase in 10 mM Tris-HCl, pH 8.0/10 mM MgCl2 was added together with all four deoxyribonucleoside triphosphates (250 μM), 5 mM dithiothreitol, 40 units of RNasin, and the reaction was allowed to proceed for 30 min. The reaction was terminated by adding EDTA (10 mM final) and heating at 65°C for 5 min. Radiolabeled products were then size-fractionated in a 6% polyacrylamide gel containing 8 M urea.

Abbreviations: ANF, atrial natriuretic factor; rANF, rat ANF; bp, base pair(s); kb, kilobase(s).

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Nuclease S1 Analysis. Nuclease S1 protection studies were carried out employing an 840-base-pair (bp) EcoRI-Bgl II rANF genomic fragment that spans the putative 3' end of the mature rANF transcript (15). Total RNA (10–100 μg) was added to $10^6$ cpm (≈100 ng) of 5'-radiolabeled probe in 30 μl of hybridization buffer [40 mM Pipes, pH 6.4/1 mM EDTA, pH 8.0/0.4 M NaCl/80% (vol/vol) formamide] and heated at 85°C for 5 min. The tube was then immediately transferred to 55°C and allowed to hybridize for 24 hr. At that point the volume was increased to 300 μl in S1 buffer (300 mM NaCl/300 mM sodium acetate, pH 4.5/3 mM ZnSO₄ containing salmon sperm DNA at 25 μg/ml), nuclease S1 (10 units/μg of RNA) was added, and incubation was continued at 25°C for 90 min. The reaction was terminated by addition of EDTA (10 mM final), phenol/chloroform (1:1, vol/vol) extraction, and ethanol precipitation. Reaction products were separated in a 6% polyacrylamide gel containing 8 M urea.

Protein Analysis. Tissue ANF was extracted employing a variation of the method described by Lang et al. (18). In brief, 0.5–1.0 g of frozen tissue was pulverized and added to 1 ml of 0.1 M HCl at 100°C for 5 min. The tissue was then homogenized with a Polytron (Brinkmann) and 5 ml of 2 M Tris-HCl (pH 7.5) was added to restore neutrality. The mixture was centrifuged at 10,000 × g for 30 min. The supernatant was decanted and stored frozen at −20°C. ANF was further purified by extracting the supernatant with Carboxymethyl cellulose powder (140 mesh) for 10 min. Immunoreactive peptides were recovered (65–75% recovery) by stripping the glass with aceton/0.25 M HCl (1:1, vol/vol) and evaporating the volatile solvent under a nitrogen stream. After resuspension in phosphate-buffered saline (0.15 M NaCl/20 mM phosphate, pH 7.4) containing 50 mM EDTA and 0.1% bovine serum albumin, radiounoimmunoassay was carried out using rabbit antisera directed against a 25 amino acid carboxyl-terminal fragment of the rANF molecule (residues 4–28), an identical rANF-(4–28)-peptide standard, and chloramine-T-iodinated rANF tracer (≈300 μCi/μg of peptide; 1 Ci = 37 GBq); dextran-coated charcoal treatment was used to separate bound and free ligand. Tissue ANF was displaced radiolabeled tracer from the antibody in parallel with the standard curve. Sensitivity of the assay and interassay coefficients of variance were 11% and 4%, respectively. Neither corticotropin, [Arg⁸]vasopressin, nor angiotensin II at concentrations as high as 1 μg per tube displaced 125I-labeled rANF from the antibody. Protein determinations were made according to the method of Bradford (19).

Immunocytochemistry. Anesthetized male Sprague–Dawley rats were perfused with 50 ml of 0.9% NaCl followed by 250 ml of Bouin–Holland sublimate (20) through a right ventricular cannula. Tissues were dissected and postfixed in the same fixative overnight. The tissues were then rinsed in water, dehydrated in graded alcohols, cleared in xylene, and embedded in Paraplast (Sherwood Medical Industries, St. Louis, MO). Sections (5 μm) were cut and collected on gelatin-coated slides.

Immunocytochemical staining was done as described (20), according to the avidin–biotin method. The major antisera employed in this study was generated against the human ANF-(4–28) molecule. This antisera was fully reactive with rANF-(4–28) but failed to react with ANF peptides lacking the phenylalanine-arginine-tyrosine-carboxyl-terminal tripeptide. A second antisera, employed in the examination of pituitary tissue, was purchased from Research and Diagnostics (Berkeley, CA). This rabbit antibody was generated against the human ANF (1–28) molecule and was fully reactive with both human and rat ANF. The anti-ANF antisera were used at 1:1000 dilution. Method specificity was tested by confirming the decrease in staining when the primary antibody was used at increasing dilutions. Antibody specificity was tested by preincubating the antiserum (at 1:1000 dilution) with the ANF-(4-28)-peptide (final concentration 1 μg/ml).

RESULTS

Fig. 1 shows blot-hybridization analyses of RNA from several tissues. Analysis of both the left and the right atrium revealed a transcript 950–1050 nucleotides long that hybridized to the radiolabeled probe (Fig. 1A). A similar-sized transcript was also present in the ventricular preparation (Fig. 1B), albeit at 1–2% the concentration found in the atria. Small amounts of the transcript were also found in lung and pituitary gland. Transcripts were not detected in liver, kidney, or whole brain (data not shown).

Immunoreactive ANF was detected in tissue extracts in rough proportion to the levels of ANF mRNA (Table 1). Again, the levels found in atrium were at least 100-fold higher than those found in ventricle, lung, and pituitary gland. Very little ANF immunoreactivity was detected in liver.

The comparable sizes of the ANF transcripts in different tissues suggested that they might arise from a common RNA polymerase II promoter in the ANF gene. To examine this possibility more closely, RNA from a number of these tissues was examined by the primer-extension technique (Fig. 2). The atrial RNA preparation generated the most primer-extended product, as expected. A total of 3–5 fragments were found that mapped predominantly to positions 70–76 bp upstream from the 5' end of the cDNA fragment and 20–26 bp downstream from the TATAAAA sequence. This lies very close to the presumptive transcriptional start site of the rANF gene, as described by Argentin et al. (13). Ventricular, pulmonary, and pituitary RNA produced the same cluster of primer-extended fragments, albeit at reduced levels, suggesting that these tissues share a common start site for transcription. In addition, a second terminus, which maps further upstream (=80 bp) from the primary start, is moderately prevalent in atrial RNA though not obvious in RNA from other tissues. This secondary start site lies =30 bp downstream from an A+T-rich sequence (ATAACTT-TAAA) that may function as a weak promoter for this gene.

Nuclease S1 analysis (Fig. 3) confirmed the findings described above. Atrial, ventricular, pulmonary, and pituitary RNA all protected a fragment of =185 nucleotides, mapping in a region identical to that defined by primer-extension analysis. The second upstream start site for the atrial ANF mRNA was also confirmed with a protected fragment of 265 nucleotides (see Fig. 2B). By nuclease S1 analysis, the upstream start is also apparent at low levels in RNA extracted from ventricular samples. Several additional protected fragments of intermediate size (i.e., 185–265 nucleotides) were also found, suggesting that there is consid-
erable heterogeneity in the 5' termini of the ANF transcripts in both atria and ventricles.

Immunocytochemistry was used to identify those regions within these various tissues which might be enriched in the ANF protein. As mentioned above, previous studies (6, 10) employing this technique have not provided evidence for ANF immunoreactivity in the ventricle. As shown in Fig. 4A, ANF immunoreactivity was detected in a homogenous distribution throughout the atrial section. Activity was also found in solitary ventricular cardiocytes, usually in close proximity to small blood vessels, at a very low frequency within the myocardium (Fig. 4D) and at a much higher frequency in the ventricular subendocardium (Fig. 4B and C). This activity represented specific binding of the antibody to the ANF peptide in that it could be effectively inhibited by preincubating the antibody (1:1000 dilution) with ANF peptide (1 μg/ml) prior to exposing it to the tissue sections. In the lung (Fig. 4E), ANF peptide appeared to be enriched in perivascular cells that clearly were not vascular in nature. Pituitary ANF was confined to large polygonal cells with anatomic features typical of gonadotropin-producing cells in that gland (Fig. 4F), confirming an earlier report of McKenzie et al. (10).

### Table 1. Tissue levels of ANF protein

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ANF, pg/mg of soluble protein</th>
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<tbody>
<tr>
<td>Atria*</td>
<td>207.800 ± 70.300</td>
</tr>
<tr>
<td>Ventricle†</td>
<td>2.100 ± 700</td>
</tr>
<tr>
<td>Lung</td>
<td>1.500 ± 260</td>
</tr>
<tr>
<td>Liver</td>
<td>314 ± 130</td>
</tr>
<tr>
<td>Pituitary</td>
<td>4,300 ± 900</td>
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ANF was measured by radioimmunoassay. Each value is the mean ± SD for three samples of pooled tissue.

*Represents combined left and right atria.†Ventricles were sectioned transversely at mid-chamber, and only the distal tissue was analyzed.

**DISCUSSION**

The results show that expression of the rANF gene takes place in a number of extra-atrial tissues. In addition to the cardiac atria, the cardiac ventricles, lung, and pituitary appear to make detectable quantities of ANF mRNA and protein. Additional data, not presented here, indicate that this gene is also expressed in hypothalamus and the aortic arch. Nevertheless, the relative level of ANF expression in extra-atrial tissues is much less than that in the cardiac atria, reflecting the fact that ANF is synthesized by a minority of cells within these tissues. These findings argue that some tissue-specific (or, more appropriately, cell-specific) factor, present predominantly in atrial and selected extra-atrial cells, is required for efficient expression of this gene.

Within the heart, the levels in the right and left atria were roughly equivalent. Earlier work (21) has noted an enrichment of ANF peptide in the right vs. the left atrium, raising the possibility that other factors in addition to ANF biosynthesis may regulate tissue ANF content. ANF proteins in the ventricles, lung, and pituitary were detected at levels 1–2% those found in the atria, a ratio similar to the relative ANF mRNA levels found in those tissues vs. the atria.

The finding of rANF transcripts in ventricle was of particular interest, in that only a few groups have reported the presence of ANF bioactivity, ANF immunoreactivity, or ANF mRNA in this tissue. Earlier studies of cardiocyte granularity (3), natriuretic activity (4, 5), ANF immunoreactivity (6, 10), and ANF mRNA levels (7, 8) all indicated that ANF and its associated activity were confined to the atrium and absent from the ventricle. However, ANF peptide has been identified in ventricles of amphibians (22), and Zivin et al. (12) have provided evidence for a ventricular ANF transcript.

It is not likely that our findings reflect contamination of the ventricular preparation with atrial RNA, since ANF transcripts can be detected throughout the ventricle from base to apex (D.G.G., unpublished observations). It is probable that the differences between the present findings and those reported earlier are explained in part by the difficulties...
attached to the detection of very low levels of ANF mRNA and protein. The cDNA probe we employed in these studies was labeled exclusively on the strand complementary to the mRNA, at a high specific activity. This, together with the appropriate selection of antisera, is probably responsible for providing the requisite sensitivity to demonstrate very low levels of ANF gene expression.

The demonstration of ANF immunoreactivity within the ventricular cardiocyte, as well as along the ventricular endothelial surface, is of interest. Although the presence of the ANF mRNA within ventricular tissue makes it tempting to speculate that this immunoreactivity results from synthesis of the protein within the identified cells, we cannot exclude a coexistent cellular extraction of circulating ANF from plasma (e.g., on surface or subsequently internalized ANF receptors). This is of particular importance in the case of subendothelial localization of ANF immunoreactivity and precludes quantitative assessments of ANF synthetic capacity in subendothelial and intramural cardiocytes. In situ hybridization (23) using the ANF cDNA to identify which ventricular cells contain ANF mRNA may help resolve this question.

The role of ANF in extra-atrial tissues remains unknown. In the atria, this protein is thought to be regulated predominantly by pressure receptors that sense intravascular volume (i.e., filling pressures). It is conceivable that ventricular...
and/or pulmonary ANF could be released into the circulation by increased filling pressures in these organs. Although the concentration of ANF mRNA in ventricles and lung is much lower than that in atria, it is possible, given the large mass of these tissues, that the ventricles and lungs could contribute a significant quantity of ANF to the circulating pool under certain conditions. Alternatively, it is possible that ANF in these organs is synthesized not for circulation as a hormone but to serve some local paraendocrine, autocrine, or neurotransmitter function, perhaps linked to some pressure-sensitive phenomenon and responsive to local or central nervous system modulation. Cardiopulmonary baroreceptors, with vagal afferents, have been described in lung and ventricular (particularly left ventricular) tissue (24). Stimulation of these receptors results in a decrease in blood pressure and bradycardia, effects that are suppressed by prior vagotomy. Ackermann et al. (25) have reported that infusion of atrial extracts into rats led to a decrease in blood pressure which, in large part, resulted from a relative bradycardia and diminished cardiac output. This effect also was reversed by vagotomy. The authors concluded that some of the cardiovascular effects of the atrial extract might be explained by a direct effect of some constituent in the extract on the chemosensitive cardiopulmonary receptors with vagal afferents. Other studies have shown that stimulation of similar left ventricular pressure receptors with veratridine results in vagal-dependent diuresis (26). Taken together, these data suggest a potential role for ventricular and pulmonary ANF in these reflexes. If true, this model might suggest that ANF evolved originally not as an atrial hormone but as a more general participant in baroreceptive phenomena, perhaps as a neurotransmitter, and that only later did the system acquire systemic hormonal activity.

The presence of ANF in gonadotropin-secreting cells is more difficult to explain but could well involve some paraendocrine regulatory function governing secretion of one or more of the pituitary hormones. Both angiotensin II and renin have been localized to similar pituitary cells (27). The significance of these findings is as yet undefined.

In conclusion, the ANF gene is expressed in several extra-atrial sites. Some of these (e.g., ventricle and lung), by virtue of their large mass, could conceivably contribute to the circulating ANF pool. On the other hand, the low levels of expression in these as well as other tissues (e.g., pituitary, hypothalamus, and aortic arch) suggest that ANF could be acting at a local level in an autocrine, paraendocrine, or neurotransmitter role to modulate functions relevant to fluid and electrolyte balance and maintenance of systemic blood pressure. If true, this would suggest that this protein plays a much broader role in physiology than previously recognized.

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