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Localization of atrial natriuretic peptide mRNA and immunoreactivity in the rat heart and human atrial appendage

(*in situ* hybridization/immunocytochemistry/cell culture)

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ABSTRACT **The localization of mRNA encoding prepro-atrial natriuretic peptide was investigated in tissue sections and cultures of rat heart and in sections of human right atrial appendage using the technique of *in situ* hybridization with ^{32}P- and ^{35}S-labeled RNA probes. Rat atrial natriuretic peptide (ANP) transcripts were demonstrated in numerous atrial myocytes and, to a lesser extent, in ventricular myocytes in both tissue sections and newborn rat heart cultures. These findings are consistent with those obtained by RNA blot analysis of rat heart total RNA, indicating that a single prepro-ANP transcript of ≈900 nucleotides was present in the ventricles as well as the atria. Using a ^{35}S-labeled RNA probe for human ANP mRNA, ANP transcripts were also localized to the majority of myocytes in the human right atrial appendage. Only background levels of autoradiographic labeling were obtained when RNA probes identical to the coding sequence of rat or human ANP mRNA were used. A close correlation was found between the distribution of ANP immunoreactivity and prepro-ANP mRNA in these preparations. These results provide unequivocal evidence for the expression of the ANP gene in the rat ventricles, as well as the atria, because myocytes in these tissues have been established as the sites of both ANP localization and precursor biosynthesis. The combined use of cardiac cultures and *in situ* hybridization may be of value in future studies investigating the regulation of ANP synthesis in cardiac myocytes.**

Cloning and sequence analysis of the complementary DNA (cDNA) encoding rat and human atrial natriuretic peptide (ANP) mRNA has revealed the structure of the prepro-ANP molecule, which consists of 151 and 152 amino acids in the human and rat, respectively (1–5). The nucleotide sequence of the genomic DNA encoding prepro-ANP has also been determined (6–11) and in man the gene is known to consist of 2 kilobase pairs, comprising three exons and two introns, located on the distal short arm of chromosome 1 (12). The ANP gene is expressed mainly in atrial tissues that contain both ANP transcripts (6, 13–16) and ANP immunoreactivity, the latter being localized to secretory granules in the atrial myocytes (17–19).

However, in nonmammalian species ANP-like immunoreactivity occurs in the ventricles as well as the atria (18), and the results of mRNA blot analysis (6, 16) and radioimmunoassay (15, 19) together indicate at least some expression of the ANP gene in the ventricles of the rat, although normally at a lower level than in the atria. While the presence of atrial natriuretic peptides in ventricular cells might result from their synthesis at these sites, the possible uptake of circulating ANP cannot be excluded (15). Furthermore, immunocytochemical techniques alone are not very useful for examining the regulation of prepro-ANP production because it is not possible to distinguish between changes in synthesis and secretion. *In situ* hybridization provides a method for demonstrating gene expression at the cellular level and may be of value for investigating the regulation of ANP synthesis in the heart.

In this study we have employed techniques of *in situ* hybridization, RNA blot analysis, and immunocytochemistry on tissue sections and cultures of rat heart to establish the sites of both ANP localization and precursor biosynthesis.

MATERIALS AND METHODS

Materials. Two plasmid clones containing cDNA fragments encoding the precursor of rat and human atrial natriuretic peptides, prANP10 (3) and phANP1 (2), respectively, were used as sources of cDNA for the preparation of complementary RNA probes. SP6 RNA polymerase and the plasmids pSP64 and pSP65 were obtained from both Promega Biotec (Madison, WI) and Amersham. Labeled nucleotide triphosphates were provided by Amersham.

SP6 Plasmid Construction. The SP6 plasmid used for the synthesis of probes complementary to the coding sequence (cRNA probe) of rat ANP mRNA was constructed by inserting the *Pst* I fragment (620 nucleotides) of prANP10 into the polylinker region of pSP64. This recombinant plasmid, pSP-rANP-PP, (Fig. 1) was linearized with *Eco*RI and transcribed *in vitro* as previously described (20). A plasmid that generates an RNA transcript identical to the coding strand (mRNA probe) of the rat ANP mRNA was prepared in an identical manner except the *Pst* I fragment was cloned into the polylinker region of pSP65. This recombinant plasmid, pα-rANP/SP, was linearized with *Hin*dIII before transcription.

Another plasmid was constructed for the production of a cRNA probe for human ANP mRNA by inserting the *Pst* I–*Sst* I fragment (586 nucleotides) of the human cDNA plasmid (phANP1) into the polylinker region of pSP64. This recombinant plasmid, phANP/SP64, was linearized with *Sst* I or *Eco*RI. A similar human cDNA fragment was inserted into the *Pst* I–*Sst* I site of a pSP65 plasmid. The pα-hANP/SP65 plasmid was linearized with *Hin*dIII and produced transcripts identical to the human ANP mRNA.

Preparation of SP6 Transcript. Labeled rat and human cRNA or mRNA transcripts of ANP cDNA were synthesized by incubating 0.2–1.0 μg of linearized plasmid template in a 10-μl reaction mixture containing transcription buffer, 100

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Abbreviation: ANP, atrial natriuretic peptide.

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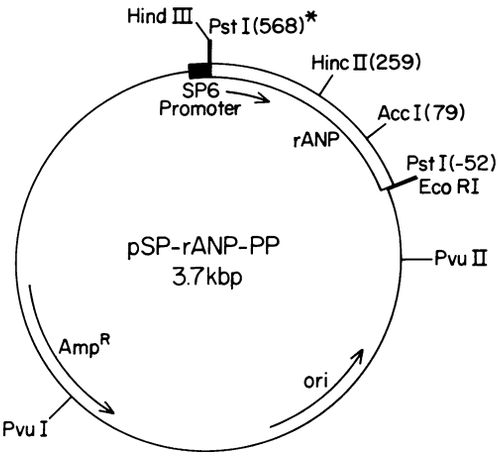

FIG. 1. Schematic diagram of the SP6 plasmid (pSP-rANP-PP) used for *in vitro* transcription. The *Eco*RI-linearized plasmid generates a transcript complementary to the rat ANP mRNA. Open-boxed portions indicate the coding region of rat ANP; numbers refer to rat ANP cDNA sequences. The location of the phage SP6 transcription initiation site and direction of transcription are shown. *, Numbering refers to rat ANP cDNA sequence.

nmol of dithiothreitol, 1 unit of RNase inhibitor (RNasin) per μl, 5 nmol each of ATP, GTP, and UTP, 120 pmol of CTP, 31.3 pmol of [α-^{32}P]CTP (800 Ci/mmol, 1 Ci = 37 GBq; Amersham) or 29.4 pmol of [α-^{35}S]CTP (850 Ci/mmol; Amersham), and 5 units of SP6 RNA polymerase. The reagents were added in the above order at room temperature, and the mixture was incubated for 60 min at 37°C. The cDNA template was removed by the addition of 1 μl of RNase-free DNase (1 μg/μl) for 10 min at 37°C, and the RNA probe was purified by extraction with phenol/chloroform and precipitated in ethanol overnight at −20°C. The pellet was dissolved in 10 μl of sterile distilled water before dilution in hybridization buffer. The total counts per minute were $\approx 1.1 \times 10^7$ cpm for ^{32}P-labeled and 2.5×10^7 cpm for ^{35}S-labeled probes with 19% and 36% incorporation, respectively.

Isolation of Total RNA and RNA Blot Analysis. Total RNA was purified from separately pooled atria and ventricles by the guanidinium isothiocyanate method of Chirgwin *et al.* (21). RNA was analyzed as follows: Total RNA isolated from rat atrium and ventricle was electrophoresed on a 1.0% agarose gel with 2.2 M formaldehyde and 50% formamide (22). The RNA was transferred to a nitrocellulose filter by the method of Thomas (23). The filter was prehybridized for 5 hr at 37°C in buffer that contains 50% (vol/vol) formamide, 10% (wt/vol) dextran sulphate, 5 μg of sodium pyrophosphate per ml, 0.01 M Tris·HCl at pH 7.5, 2× standard saline citrate (SSC; 1× = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), modified Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidine/0.02% gelatin), and 250 μg of sonicated calf thymus DNA per ml. Hybridization was done in the above solution at 37°C for 24 hr with ^{32}P-labeled rat cRNA probe (1×10^6 cpm/filter). A similar experiment was done using a probe generated from the SP6 promoter, which yields a transcript identical to the rat ANP mRNA. The filters were washed three times for 20 min each with 4× SSC, once for 30 min with a solution containing RNase A at 20 μg per ml/0.05 M NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA, once with 2× SSC for 30 min, and once with 0.1× SSC for 30 min. All washes were done at 37°C. The filters were then blotted dry and subjected to autoradiography for 56 hr at −80°C with an intensifying screen.

Tissue Preparation. The investigated tissues included atrium and ventricle from adult rat heart, cultured rat atrial and ventricular myocytes, and samples of human right atrial appendage. The human samples were obtained at surgery from patients ($n = 5$) undergoing coronary artery bypass graft operations and adult rat hearts ($n = 6$, Wistar) were dissected immediately after death. Tissues for hybridization were fixed by immersion in a freshly prepared solution of 4% (wt/vol) paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 4–6 hr at 4°C. They were then transferred to PBS containing 15% sucrose, and after washing, cryostat blocks were prepared. Sections (15-μm thick) were cut, mounted on poly-L-lysine-coated glass slides (24) and allowed to dry overnight at 37°C before processing for hybridization. Samples of rat and human heart tissues were also fixed by overnight immersion in Bouins' fluid, dehydrated, and processed to wax sections for immunostaining.

Rat atrial and ventricular cultures were prepared from the hearts of newborn rats by a technique used previously for guinea pig atrial cultures (25). Hearts were obtained from 7- to 8-day-old rats immediately after death; the atria were removed and pooled, and a section was cut transversely from the apical part of the heart to provide distinct ventricular tissue. The two tissues were kept separately but were treated in the same way: they were chopped into fragments before enzymatic and mechanical dissociation in 0.125% trypsin and 0.2% collagenase solutions at 37°C. Dissociated cells were centrifuged at 600 rpm for 5 min and resuspended in medium 199 containing 10% fetal calf serum, 5 mg of glucose per ml, and 100 units of penicillin per ml. The cultures were seeded at $\approx 5 \times 10^5$ cells per ml in modified Rose chambers (26) and kept in a humid environment at 37°C in 2% CO_2. The medium was replaced after 24 hr and every 2–3 days thereafter. After 5–10 days in culture the Rose chambers were dismantled, and the coverslips with attached cultures were rinsed in cold PBS. They were then fixed by immersion in a solution of 4% paraformaldehyde/PBS for 60 min at 4°C and rinsed several times in PBS and distilled water. The cultures were dried for ≈6 hr at 37°C and stored in a desiccator at −70°C until required for hybridization or immunocytochemistry.

***In Situ* Hybridization.** Hybridization was done under conditions similar to those used in RNA blot analysis. Tissue sections and culture preparations were permeabilized first with a 0.3% solution of Triton X-100 in PBS for 15 min and then with a solution of proteinase K (1 μg/ml) in 0.1 M Tris, pH 8, containing 50 mM EDTA for 20–30 min at 27°C. The reaction was stopped by immersing the preparations in PBS containing 4% paraformaldehyde for 5 min. Preparations were then immersed in a solution containing 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8, for 10 min to reduce background binding and prehybridized in 50% formamide and 2× SSC for 15 min at 37°C. Hybridization was done with 2–3 ng of cRNA probe ($\approx 5 \times 10^5$ cpm/section; 0.2–0.3 ng/μg) diluted in buffer containing 50% formamide/2× SSC/10% dextran sulfate/0.25% bovine serum albumin/0.25% Ficoll 400/0.25% polyvinyl pyrolidone 360/250 mM Tris, pH 7.5/0.5% sodium pyrophosphate/250 μg of denatured salmon sperm DNA per ml. The preparations were covered with dimethyl-dichlorosilane-coated coverslips to prevent evaporation, and hybridization was done in a moist chamber for 16–20 hr at 37°–42°C. After hybridization, the coverslips were removed, and the preparations were washed three times for 20 min each in 4×, 2×, and 0.1× SSC at 37°C. Unhybridized single-stranded cRNA probe was removed by treating the preparations with a solution containing 20 μg of RNase A per ml, 0.5 M NaCl, 10 mM Tris (pH 8.0), and 1 mM EDTA for 30 min at 37°C.

The preparations were dehydrated through graded concentrations of alcohol containing 0.3 M ammonium acetate, dried and dipped in Ilford K-5 emulsion, and stored in light-tight boxes for 2–4 days at 4°C. The autoradiographs were developed in Kodak D-19 developer, and the preparations were

lightly counterstained with hematoxylin, dehydrated, cleared, and mounted in synthetic medium (DPX).

Control Experiments. Separate sets of sections and tissue culture preparations were hybridized with probes identical to the coding strand of the mRNA of either rat or human ANP mRNA (mRNA probes). A further control procedure was done by treating a separate set of preparations with an RNase A solution (20 μg/ml at 37°C for 50 min) before the prehybridization step. Hybridization was then done with labeled cRNA probes, as already described.

The absence of hybridization after these procedures, as indicated by a lack of silver grain deposits over the cells, was taken as confirmation of the specificity of the results obtained during hybridization with cRNA probes.

Immunohistochemistry. Immunostaining of 4-μm-thick tissue sections was done using the peroxidase–antiperoxidase method of Sternberger (27) and counterstained with hematoxylin. Primary rabbit antisera were raised against synthetic α human ANP (Peninsula Laboratories, San Carlos, CA), as previously described (19), and used at dilutions of 1:800–1600.

Rat atrial and ventricular cultures were immunostained using an indirect immunofluorescence technique (28). The cultures were first rinsed in PBS containing 0.1% Triton X-100 for 1 hr at room temperature and then washed in PBS before applying the primary antiserum to ANP at a dilution of 1:200. After incubation overnight at 4°C the preparations were washed and incubated with a fluorescein isothiocyanate-labeled sheep anti-rabbit IgG (Miles; 1:100 dilution) for 1 hr at room temperature. After further washing the immunostained cultures were examined using a fluorescence microscope equipped for epi-illumination.

Controls included the use of nonimmune serum as first layer, the applications of second and third layers alone, and incubating preparations with ANP antiserum preabsorbed with synthetic α human (1–28) ANP (10 nmol/ml of diluted antiserum). The antisera showed no cross reactivity with other non-ANP peptides (19), and all specific immunostaining for ANP immunoreactivity was abolished by the control procedures.

RESULTS

RNA blot analysis of the rat atria revealed a single transcript of ≈900 nucleotides that hybridized specifically with the labeled cRNA probe. A similar transcript was also detectable in the rat ventricle, but at a lower concentration (Fig. 2). An SP6 transcript generated from *Hin*dIII-cleaved pα-rANP/SP was also used to probe the RNA blot, but no bands at all were detected upon hybridization with this probe, which was identical to the coding strand of the rat ANP mRNA. In addition, no hybridization to ribosomal 18S or 28S RNA was seen. The ventricular ANP mRNA was more readily demonstrated with poly(A)$^+$ RNA preparations, but the amount of message was still relatively low compared with that found in the atria (data not shown).

Hybridization between labeled cRNA probes and mRNA encoding prepro-ANP, was demonstrated by specific and discrete deposits of silver grains in the emulsion overlying atrial and ventricular myocytes. Using ^{32}P- and ^{35}S-labeled cRNA probes for rat prepro-ANP mRNA, an intense selective labeling was seen over a large number of myocytes in cultures of rat atrium (Fig. 3*A*). When the same probe and hybridization conditions were used on cultures of rat ventricle, labeled myocytes were also detected (Fig. 3*B*), but the frequency of labeled cells and density of silver grains were less than in atrial cultures. In both types of culture silver grains were localized mainly over the cytoplasm of myocytes, with fewer grains over the nuclei. The distribution and number of hybridized cells were comparable when using

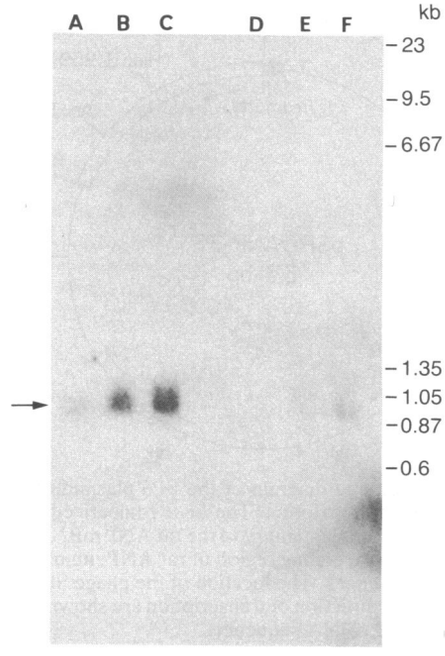

FIG. 2. RNA blot analysis of total RNA from rat atria and ventricles. Total RNA from rat atria (lanes A, B, and C) and rat ventricles (lanes D, E, and F) were subjected to electrophoresis on a 1.0% agarose gel containing 2.2 M formaldehyde/50% formamide, transferred to nitrocellulose, and hybridized with 1×10^6 cpm of the complementary mRNA generated by *in vitro* transcription of pSP-rANP-PP. Total RNA was 5 μg (lanes A and D); 10 μg (lanes B and E); and 20 μg (lanes C and F). Arrow, position of ANP mRNA transcript (900 base pairs). Molecular size markers are in kilobases.

either ^{32}P- or ^{35}S-labeled cRNA probes. However, the ^{35}S-labeled probes generally gave lower background and better resolution. Other cell types present in the cultures, including fibroblasts, Schwann cells, and neurons (atrial cultures), displayed only background labeling with scattered randomly distributed silver grains.

In situ hybridization on sections of rat heart revealed ANP transcripts in both atrial and ventricular myocytes (Fig. 3*C*). Significant numbers of myocytes were labeled throughout both atria, whereas in the ventricles only scattered myocytes possessed ANP transcripts; these cells occurred mainly in the subendocardium of the interventricular septum (Fig. 3*D*). Sections of human right atrial appendage also displayed significant labeling of myocytes when hybridized with ^{35}S-labeled cRNA probe for human ANP mRNA (Fig. 3*E*). No specific labeling occurred over other cell types in the myocardium, endocardium, or epicardium. Only background levels of silver grains were detected in cultures and tissue sections that were hybridized with probes identical to the coding sequence of rat (Fig. 3*F* and *G*) or human ANP mRNA. In addition, the autoradiographic labeling was significantly reduced by RNase treatment of cultures and sections before *in situ* hybridization. These control findings and the RNA blot analysis results together emphasize the specificity of the cRNA probes—i.e., no extraneous RNA was detected.

ANP immunoreactivity was localized in both cultures and tissue sections to myocytes, and the distribution of immunoreactivity in these cells was similar to the distribution of ANP transcripts in like cells. In rat atrial cultures numerous mono- and binuclear myocytes displayed an intense perinuclear ring of ANP immunoreactivity (Fig. 4*A*). Immunoreactive myocytes were also detected in ventricular cultures, but they occurred less frequently than in the atrial cultures, and the perinuclear ring of immunofluorescence was both weaker

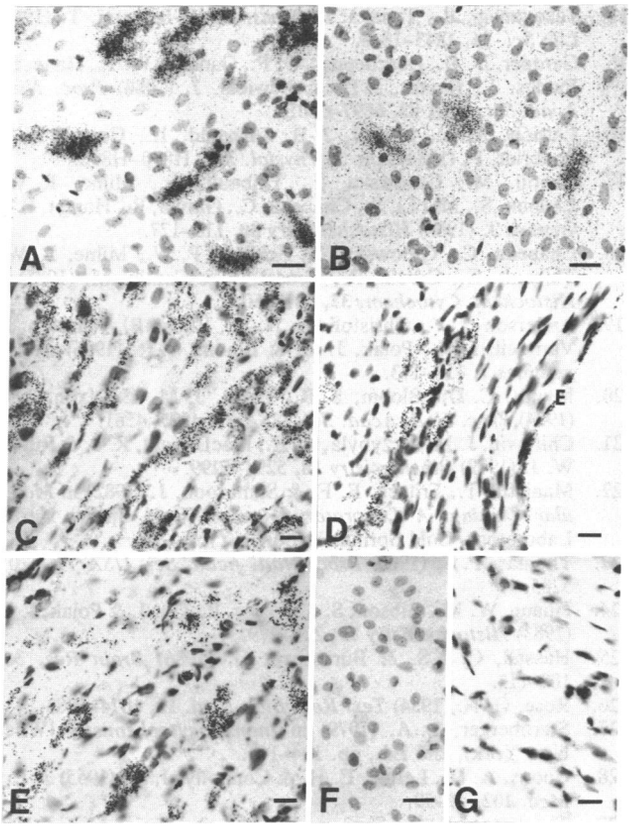

FIG. 3. *In situ* hybridization of ANP mRNA in cultured myocytes of rat atrium (*A*) and ventricle (*B*), in sections of rat atrium (*C*) and interventricular septum (*D*), and in a section of human atrial appendage (*E*). ^{35}S-labeled (*A*, *B*, and *E*) and ^{32}P-labeled cRNA probes (*C* and *D*). Autoradiographic preparations using cultures and sections (10-μm thick) fixed in 4% paraformaldehyde and counterstained with hematoxylin. E, endocardium. *In situ* hybridization using ^{35}S-labeled mRNA probes identical to the coding strand of ANP mRNA in cultured rat atrial myocytes (F) and a section of the rat interventricular septum (G). Scale bars = 20 μm.

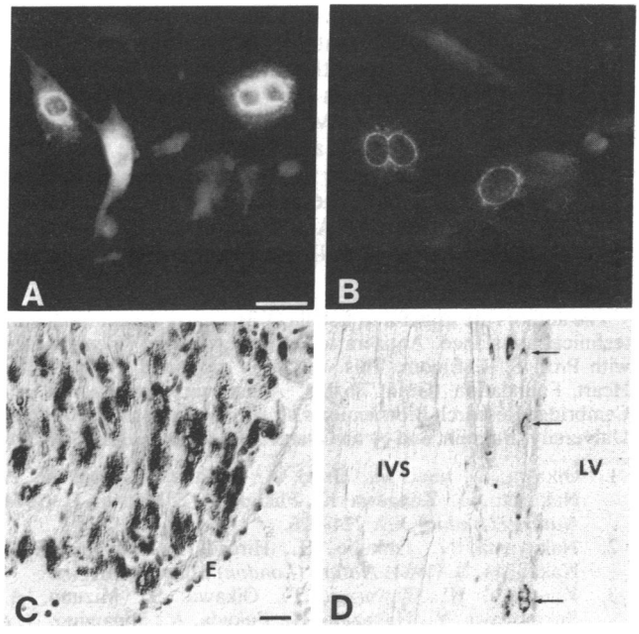

FIG. 4. Paraformaldehyde-fixed cultures of neonatal rat atria (*A*) and ventricles (*B*) immunostained for ANP using the indirect immunofluorescent technique. Mono- and binucleate immunoreactive myocytes occur in both atrial and ventricular cultures but the latter display much less immunoreactivity. ANP-immunoreactivity localized to myocytes in sections of the rat right atrium (*C*) and interventricular septum (*D*). Bouins'-fixed wax sections immunostained with the peroxidase-antiperoxidase technique and photographed using Nomarski optics. Scattered ANP-immunoreactive myocytes (arrows) occur in the subendocardium of the interventricular septum (IVS). LV, left ventricle; E, endocardium; scale bars = 20 μm.

and finer (Fig. 4*B*). Sections of rat atria contained numerous positive myocytes with ANP immunoreactivity occurring around the nuclei and concentrated at the nuclear poles (Fig. 4*C*). In contrast to the atria, where most myocytes contained ANP-immunoreactive material, only scattered immunostained myocytes were detected in sections of rat ventricle; these cells occurred mainly in the subendocardium of the interventricular septum (Fig. 4*D*). Although myocytes in sections of human atrial appendage also displayed ANP immunoreactivity, this staining generally appeared less dense than that seen in rat atrial sections.

DISCUSSION

We have used RNA probes complementary to rat and human ANP mRNA to demonstrate ANP gene expression in the heart. The results provide evidence of the cellular localization of ANP gene transcripts in both atrial and ventricular myocytes, as demonstrated by *in situ* hybridization in tissue sections and cultures of the rat heart. RNA blot analysis revealed the presence of a single transcript in atrial and ventricular tissues that corresponded in size to that previously reported for the full-length transcript of ANP mRNA (5). The demonstration of ventricular myocytes expressing the ANP gene also indicated that ANP immunoreactivity can be synthesized in the ventricles, as well as the atria, rather than merely being taken up from the circulation. However, the level of ventricular ANP transcription is generally lower than that found in the atria, a finding in agreement with other recent studies using RNA blot analysis (6, 15, 16) and also consistent with the occurrence of only scattered ANP-immunoreactive cells in the ventricular walls and relatively small amounts of immunoreactivity in extracts of the ventricles (15, 19). ANP transcripts in cultures of the neonatal rat ventricle support the claim that ANP immunoreactivity occurs in the ventricular myocardium of the developing rat heart (29), and the occurrence of ANP transcripts and immunoreactivity in the interventricular septum of the adult rat also suggests that this is not just a developmental phenomenon.

The precise nature of ventricular myocytes producing ANP *in vivo* is not clear, but in rat ventricle cultures we have found ANP immunoreactivity to be localized to secretory granules similar to those found in atrial myocytes (unpublished observation). ANP immunoreactivity was detected in subendocardial myocytes in the interventricular septum, and these cells may represent ventricular conduction tissue (30, 31), but we have been unable to demonstrate ANP immunoreactivity in the rat atrioventricular node. The role of the ANP produced by the ventricles is uncertain, but, as in the atria, ANP gene expression increases in acute and chronically volume-loaded rats (16). This increased transcription could contribute to raised circulating levels of ANP immunoreactivity, particularly when accompanied by ventricular hypertrophy. However, it is not known whether all ventricular myocytes can be induced to express the ANP gene.

Although the relative sensitivity of *in situ* hybridization and immunocytochemical techniques remain to be established, the present results showed similar numbers and distribution patterns of myocytes in both cultures and sections containing

ANP mRNA and immunoreactivity. The value of *in situ* hybridization for localizing sites of peptide biosynthesis has been described by various authors (32–34). Furthermore, the use of cRNA probes has been shown to give hybridization of higher specificity and sensitivity (35, 36) than that obtained with other probe types. Rat atrial cultures have previously been used to investigate the biosynthesis and secretion of prepro-ANP (13, 37, 38). The present findings indicate the potential value of using cRNA probes and cardiac cultures to examine the regulation of ANP gene expression in atrial and nonatrial tissues.

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