Upstream Sequences Confer Atrial-specific Expression on the Human Atrial Natriuretic Factor Gene*

(Received for publication, January 27, 1988)

Margot C. LePointe, Jiangping Wu, Barry Greenberg*, and David G. Gardner
From the Department of Medicine and Metabolic Research Unit, University of California, San Francisco, San Francisco, California 94143 and California Biotechnology, Inc., Mountain View, California 94043

5'-Flanking sequences from the human atrial natriuretic factor (hANF) gene were subcloned into a reporter plasmid (pSVOCAT) and transfected into primary cultures of neonatal rat atrial cardiocytes. Hybrid hANFCAT genes containing either 2500 or 409 base pairs of 5'-flanking sequence DNA were expressed at similar levels. When sequences between -409 and -332 were deleted, reporter gene (CAT) activity decreased significantly. Expression of the hANFCAT constructs was specific for atrial cells, as no expression was detected in primary cultures of ventricular cardiocytes or nonmyocardial cells derived from the neonatal hearts. Correct transcription start sites for the transfected hANF genes were confirmed by S1 nuclease mapping and RNase protection analysis. A “gel shift” assay was used to identify a specific cardiac nuclear protein which bound to the 5'-flanking sequences of the hANF gene. A 192-base pair PvuII fragment (−400 to −208) associated with a protein in these extracts in a tissue- and sequence-specific fashion. These findings indicate that the DNA sequence between −409 and −332 in the hANF gene harbors a tissue-specific element whose activity may involve association with a cardiac-specific nuclear protein.

The atrial natriuretic factor (ANF) is a cardiac hormone with potent natriuretic, diuretic, and vasorelaxant activities (1). Recent studies indicate that the adult cardiac atria expresses the ANF gene at a level of 50–100 times higher than that of nonatrial tissues (2). The highly selective nature of this expression prompted us to examine the 5'-flanking sequences of the human ANF (hANF) gene for DNA elements which might confer atrial specific expression upon the gene. We also investigated the possible involvement of specific cardiac nuclear proteins in this expression.

EXPERIMENTAL PROCEDURES

Cell Culture—Hearts were removed from 1-day-old rat pups, and atria were separated from ventricles by a transverse dissection slightly below the atrioventricular groove. The upper 30% of the heart mass was used to generate the atrial cardiocyte-enriched preparation, while the lower segment was used for the ventricular cardiocyte preparation. Nonmyocardial cell cultures were generated by passaging cells collected at the “preplating” step (3) for 3–4 weeks. Tissues were digested according to a scheme described previously (3) except that bromodeoxyuridine was eliminated from the culture medium.

Plasmid Constructions—To ensure that sufficient 5'-regulatory sequence could be examined, a 4.6-kilobase EcoRI fragment from the 10-kilobase hANF genomic clone (4) was subcloned into pBR322. This clone contained an additional 2000 bp of 5'-flanking sequences upstream from those sequences previously described (4). The 2500 bp of 5'FS (PstI to the HaelIII site at +18) was subcloned into pBR322 and subjected to restriction analysis using conventional techniques. To facilitate subcloning of hANF 5'-flanking sequences (82-2500 bp) were subcloned into this polylinker upstream from the CAT coding sequences. pRSVCAT (6) was used as a positive control for expression of transfected CAT sequences. As a negative control we placed a 444-bp promoterless HindIII fragment from the human lamin cDNA (position 766–1210, Ref. 7) into the HindIII site of pSVOCAT-L.

Transfection and CAT Assays—DNA (10 μg) of the hANFCAT constructions and 5 μg of pRSV-β-galactosidase (8) was transfected into cells using the CaPO4, precipitation method (9). Cells were cultured in DME-H21 containing 10% fetal calf serum and 1 mM sodium butyrate (10). Sixty-six hours later, cells were harvested and lysed in 0.25 M Tris-HCl, pH 7.5, and 0.1% Triton X-100 for 10 min. Cellular debris was sedimented, and 100 μg of protein from the supernatant was assayed for either CAT (5) or β-galactosidase (11) activity. Quantitation of CAT assays was accomplished by scraping and pooling acetylated products from the TLC plates and counting them by liquid scintillation. To control for variability in transfection efficiency, CAT activity (percent of total radioactivity in acetylated product(s)) was normalized to an arbitrary unit of β-galactosidase activity for each sample.

RNA Extraction—Total RNA was extracted from at least eight 10-cm dishes of cardiocytes (~7 × 106 cells/plate) using the guanidinium monothiocyanate/cesium chloride method (12). After isolation, RNA was treated with DNase I (Cooper Biomedical) in the presence of RNasin (Promega Biotec) (20 μg of DNase I and 60 units of RNasin per pg of RNA for 2 h at 23 °C. Reaction products were $^32$P- labeled at the Sac1 site, the 3'overhang was eliminated with T4 RNA ligase, and 32P-labeled RNA was hybridized to total RNA (20–40 μg) in 30 μl of hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide) at 55 °C overnight. S1 nuclease digestion was carried out with 20 units of S1 nuclease (Bethesda Research Laboratories) per μg of RNA for 2 h at 37 °C. Reaction products were separated on 10% polyacrylamide gels under denaturing conditions.

For mapping the start site of the hybrid hANFCAT transcripts, a 583-bp PstI-EcoRI fragment isolated from −409 hANFCAT was cloned into Gemi 4 (Promega Biotec) in an orientation appropriate
for the generation of a radiolabeled cRNA probe from the SP6 polymerase promoter. 1 µg of KpnI cut vector was labeled with 40–50 µCi of [α-32P]UTP (Amersham Corp., 800 Ci/mmol) using SP6 polymerase (Promega Biotec) under conditions recommended by the vendor. The labeled probe was isolated from a 6.5% denaturing polyacrylamide gel. ~400,000 cpm were hybridized to 20–40 pg of total RNA overnight at 60 °C in 0.1% sodium dodecyl sulfate, 20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.4 M NaCl, and 50% formamide. After digestion (37 °C for 60 min) of the hybrids with RNase A (10 µg/ml) and RNase TI (0.1 µg/ml), protected fragments were separated on a 6.5% denaturing polyacrylamide gel.

**Gel Shift Assay—**Neonatal cardiocytes or fibroblast monolayers were harvested after 5 days in culture. Nuclei were isolated and extracts were prepared as described by West et al. (14).

A 192-bp Psull fragment from the hANF gene 5'-flanking sequences (position −400 to −208 relative to the transcription start site) was end-labeled with [γ-32P]ATP and T4 polynucleotide kinase and employed as a probe for the gel shift assay. Eight micrograms of nuclear protein was added to ~20,000 cpm (~1–3 ng) of probe, 2 µl of 10 × binding buffer (0.1 M Tris-HCl, pH 7.5, 10 mM dithiothreitol, 10 mM EDTA (all final concentrations)), and 6 µl of H2O. After a 5-min incubation at room temperature, 0.1–2.0 µg of poly(dI:dC) (Pharmacia LKB Biotechnology Inc.) in 4 µl was added as a nonspecific competitor. Other competitors, i.e. unlabeled 192-bp Psull fragment, a 229-bp SalI fragment from hANF intron B, and a 700-bp fragment from the human growth hormone cDNA (100 bp at the 5'-end deleted from the full-length cDNA) were added in amounts which varied between 60 and 400 ng in 2-µl volume. Reaction products were separated by electrophoresis through a 4% polyacrylamide gel in 0.2 × TEA buffer (1 × 40 mM Tris acetate and 1 mM EDTA) at room temperature.

**RESULTS AND DISCUSSION**

To determine whether putative regulatory elements from the hANF gene were capable of functioning in primary cultures of rat cardiocytes, we transfected a 4.6-kilobase hANF genomic clone (~2500 bp of 5'-FS plus the structural sequences described in Ref. 4) into atrial cardiocytes and quantitated hANF gene transcripts by S1 nuclease analysis. Hybridization conditions were chosen to preclude binding of the hANF S1 probe to endogenous rANF mRNA. Fig. 1A shows that RNA from transfected atrial cells protected a fragment of approximately 100 bp whereas no protected fragments were seen in untransfected atrial cardiocytes. RNA from human fetal atrial tissue protected a fragment identical in size, indicating that transcription is correctly initiated on the transfected gene.

Having shown that the transfected hANF gene could be transcribed in atrial cells, we employed this system to identify elements governing the tissue-specific expression of the gene. Variable lengths of 5'-flanking sequences from the hANF gene were subcloned into pSVOCAT-L in the orientation appropriate for directing CAT gene transcription from the ANF promoter. DNA was transfected into atrial cardiocytes, and 66 h later either RNA or protein was extracted from cells for analysis. Fig. 2 shows that CAT expression in atrial cardiocytes was highest in constructions containing sequences between −2500 and −409 bp (numbered relative to the start site of transcription). When sequences between −409 and −332 were deleted, expression fell 60%. Further deletion to position −82 did not cause any further diminution of CAT activity. Background levels of CAT expression were seen using a “promoterless” construct containing 450 bp of human lamin cDNA cloned upstream of the CAT sequences. When the −409 fragment of 5'-flanking sequences was cloned in the inverse orientation upstream of CAT and subsequently transfected into cells, no CAT expression was seen, indicating that the hANF promoter is functional only in the proper orientation (data not shown).

To test the tissue specificity of this response, the hybrid gene constructions were transfected into ventricular cardiocytes, primary cultures of nonmyocardial cells derived from neonatal rat hearts, and GC cells, a rat pituitary tumor cell line. As shown in Fig. 2, CAT expression in ventricular cardiocytes was present at relatively low levels which were not statistically different from the “promoterless” construction. Similarly, no expression was evident either in the nonmyocardial cells or in the GC cells (data not shown).

The levels of expression of the transfected hANFCAT constructions were low even in the atrial cardiocytes. Transfected pRSVCAT routinely provided levels of CAT expression which were 10–20-fold higher than that provided by ~2500 hANFCAT suggesting that the low level of hANFCAT expression resulted from low intrinsic activity of the hANF promoter element rather than a generalized inefficiency of transfection and/or expression of the genes introduced into these cultured cells.

**RiNase protection analysis was used to map the start site of hANFCAT expression in atrial and ventricular cardiocytes.** Sixty-six hours after transfection, protein extracts were made from transfected atrial and ventricular cardiocyte cultures. Extracts were assayed for both CAT and β-galactosidase activity. CAT activity has been normalized to β-galactosidase activity to correct for differences in transfection efficiency. Numbers represent means ± standard deviations. For atria, numbers represent data from three separate experiments. For ventricles, the numbers are from a representative experiment done in triplicate.

---

**Fig. 1. Mapping of transcription start sites for transfected hANF genes.** A. Nuclease S1 analysis. Lane 1 contains 40 µg of RNA from untransfected atrial cardiocytes. Lane 2 shows the protected fragment (arrow) generated with 40 µg of RNA from atrial cells transfected with the hANF gene. Lane 3 contains 3 µg of RNA from human fetal atrial tissue. Numbers to the left of the figure represent positions of molecular weight markers run in parallel. B. RNase protection analysis. Lane 1 contains 40 µg of RNA from control untransfected atrial cardiocytes, and lane 2 contains 40 µg of RNA from atrial cardiocytes transfected with −2500 hANFCAT. M represents molecular weight markers (size noted on the right). The arrow points to the protected fragment. No protected fragments were seen in the absence of exogenous RNA.

**Fig. 2. hANFCAT expression in atrial and ventricular cardiocytes.** Sixty-six hours after transfection, protein extracts were made from transfected atrial and ventricular cardiocyte cultures. Extracts were assayed for both CAT and β-galactosidase activity. CAT activity has been normalized to β-galactosidase activity to correct for differences in transfection efficiency. Numbers represent means ± standard deviations. For atria, numbers represent data from three separate experiments. For ventricles, the numbers are from a representative experiment done in triplicate.
hANFCAT hybrid mRNA. The results (Fig. 1B) indicate that mRNA from transfected atrial cells, but not from untransfected cells, protected a fragment of about the size predicted (261 nucleotides) for a transcript originating from the hANF promoter.

To determine whether tissue-specific expression of the hANFCAT gene involved interaction of sequences between -409 and -332 with specific cardiac nuclear proteins, fragments of 5'-flanking sequences were tested for their ability to bind protein extracted from myocardial or nonmyocardial cells using a "gel shift" assay. As shown in Fig. 3, a small fraction of a radiolabeled 192-bp PvuII fragment (-400 to -208) was retarded in the gel by protein extracts obtained from cultured cardiocytes (containing both atrial and ventricular cells). In contrast, the labeled 192-bp PvuII fragment did not bind to nuclear proteins extracted from nonmyocardial cells (lanes 3-6) or from GC cells (data not shown).

Competition experiments were done to determine the sequence specificity of binding to the cardiac nuclear protein. In these experiments labeled 192-bp PvuII fragment was incubated with nuclear protein extract in the presence of varying amounts of unlabeled competitor DNA. Fig. 4 (lanes 1-4) shows that only the unlabeled 192-bp PvuII fragment was able to displace binding totally. As shown in lanes 5-12, neither a 229-bp Rsal fragment from hANF intron B nor a 700-bp fragment from human growth hormone cDNA displaced the labeled PvuII fragment from the nuclear protein extract. These data indicate that the -400 to -208-bp PvuII fragment of hANF binds to a nuclear protein extract from cardiocytes in a tissue- and sequence-specific manner. This same region has functional importance as defined in the transfection studies described above. Thus, the data are compatible with the hypothesis that the DNA region from -400 to -332 harbors a tissue-specific element whose function involves association with a cardiac-specific nuclear protein.

None of the hANFCAT constructions were expressed to a significant degree in ventricular cardiocytes. The adult cardiac ventricle in the rat is known to express the ANF gene at a very low levels.

In summary, we have defined a region of the 5'-flanking sequences of the hANF gene (-409 to -332) which confers atrial-specific expression to the gene. Moreover, a PvuII fragment from the 5'-flanking sequences (-400 to -208), which includes the regulatory region defined by the CAT assays, was shown to bind a protein factor present in the nuclei of cultured myocardial cells. These data suggest that the 5'-flanking sequences of the hANF gene contains a tissue-specific element whose function may involve association with a cardiac-specific nuclear protein.

Acknowledgments—We thank LaMont Basham for preparing the manuscript. We wish to acknowledge the assistance of Dr. Norman E. Eberhardt in carrying out the computer search and Dr. John Baxter for his support and review of the manuscript.

Addendum—While this manuscript was under review, Field (19) reported that 472 bp of hANF 5'-flanking sequences was sufficient to confer atrial-specific expression upon the SV40 large T antigen (Tag) following the introduction of the chimeric gene (hANF-Tag) into transgenic mice.

REFERENCES

\(^2\) J. Wu, C. Deschepper, and D. G. Gardner, unpublished data.
Atrial-specific hANF Expression