Proximal Regulatory Domains of Rat Atrial Natriuretic Factor Gene

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Background. At least three cis-acting regulatory elements are required for expression of the rat atrial natriuretic factor (ANF) gene. One distal cis-acting regulatory element lies more than 640 base pairs from the transcription initiation site.

Methods and Results. In this report, we identify two other proximal regulatory elements that lie within 609 base pairs of the transcription initiation site. One proximal regulatory element contains an activator protein-1 (AP-1)–like binding site and is recognized by the AP-1 protein, the c-fos/c-jun proto-oncogene heterodimer in vitro. The second regulatory element contains a cyclic AMP-responsive element (CRE)–like recognition site.

Conclusions. In vitro binding of the c-fos/c-jun heterodimer to ANF gene sequences suggests that the heterodimer may play a role in the regulation of gene transcription in vivo. This observation may also explain the correlation between c-fos/c-jun expression and ventricular ANF gene expression found in hypertrophic states. Nuclear extracts from normal cardiocytes contain proteins that bind these regulatory elements but do not appear to bind at the AP-1 site, suggesting that the levels of fos/jun heterodimer in nonhypertrophied cardiocytes are quite low. (Circulation 1991;84:1256–1265)

Atrial natriuretic factor (ANF) is a cardiac hormone that exerts powerful natriuretic, diuretic, and vasodilatory effects when administered exogenously (for recent reviews, see References 1 and 2). In the healthy adult, the ANF gene is expressed predominantly in cardiac atria where the level of ANF messenger RNA (mRNA) is several hundredfold greater than that found in the ventricles.3 However, ventricular ANF gene expression is significant during prenatal development4 and is increased during pathological states such as cardiac hypertrophy.5,6 Extracardiac expression of ANF is even less than ventricular expression, although it has been reported in a few tissues, including brain and vascular tissue.3,7,8 Therefore, the ANF gene serves as a useful model of cardiac-specific gene expression. The present study examines control of ANF expression in atrial cardiocytes. It is likely that other cis- and trans-acting elements are important in extra-atrial ANF expression to allow differential regulation.

Defining the features of the ANF gene that direct its expression in the atria is critical to understanding the mechanisms that control molecular cardiac function. Identification of cis-acting sequences that direct ANF gene expression in the atria has been undertaken using two different assays. Expression of a reporter gene driven by ANF promoter sequences has demonstrated that 500 base pairs (bp) of the human gene9 and 3.4 kb of the rat gene10 are sufficient for ANF gene expression in the atria of transgenic mice. Assays that require the production of transgenic mice are laborious and difficult. Thus, a number of groups have used expression of a reporter gene (chloramphenicol acetyltransferase [CAT]) promoted by ANF sequences in primary cultures of neonatal cardiocytes as a more rapid test for identifying cis-acting regulatory sequences required for ANF gene expression.11–13 Studies by two groups11,13 have demonstrated that there is a distal regulatory element upstream of the rat ANF gene located more than 640 bp from the transcription initiation (CAP) site. This contrasts with studies of the human ANF gene in which all of the necessary signals for ANF promoter function are encoded on a 409-bp fragment.12 A regulatory element encoded between 332...
and 400 bp from the human ANF transcription initiation site appears able to bind a nuclear factor present in myocardial-derived nuclear extracts.\textsuperscript{14} The nucleotide sequence of this binding site is homologous to sequences located 1,200 bp from the rat ANF gene CAP site.\textsuperscript{14}

To elucidate whether important cis-acting regulatory elements are present in proximal rat ANF sequences (within 640 bp of the CAP site), we performed deletion analyses of this region. Sequences contained in the distal regulatory region have all been retained in these analyses. Transfection experiments of primary cultured neonatal rat atrial cells with ANF sequences directing CAT gene transcription have identified two proximal regulatory elements. These two elements lie within a 213-bp region located 609 bp upstream of the CAP site. Sequences that are homologous to the consensus recognition sites for activation protein-1 (AP-1) and a cyclic AMP–response element (CRE) are located in this region. We demonstrate here that the AP-1 binding proteins, the c-fos/c-jun proto-oncogene heterodimer, are able to bind the AP-1 site in the ANF gene but not the CRE binding site.

Nuclear extracts from neonatal atrial and ventricular cardiocytes were prepared to determine if proteins present in nonhypertrophied cardiocytes interact with AP-1 or CRE sequences identified in these regulatory elements. Specific binding was demonstrated to each regulatory element but not at the AP-1 or CRE site. We suggest that physiological expression of the ANF gene involves nuclear factors that interact with unique sequences within two proximal regulatory elements. We hypothesize that the c-fos/c-jun heterodimer may play a role in regulating ANF gene expression during states of increased proto-oncogene expression such as ventricular hypertrophy.

\section*{Methods}

\subsection*{Materials}

Restriction enzymes, calf intestinal alkaline phosphatase, T4 DNA ligase, polynucleotide kinase, and the Klenow fragment of DNA polymerase were obtained from Boehringer Mannheim or Bethesda Research Laboratories. Taq polymerase (Ampli-Taq) was obtained from Perkin-Elmer Cetus Corporation (Norwalk, Conn.). [\textsuperscript{32}P]-ATP and [\textsuperscript{35}S]chloramphenicol were obtained from New England Nuclear (Boston). Poly (dI-dC) and deoxynucleotides triphosphates were obtained from Pharmacia.

\subsection*{Plasmid Constructs}

The rat ANF gene was the generous gift of Peter L. Davies (Queen's University, Kingston, Canada).\textsuperscript{15} Preparation of constructs containing 5' ANF flanking sequence (0 position is CAP site) in the promoterless CAT vector p0CAT\textsuperscript{16} has been described previously.\textsuperscript{11} A construct containing 3.4 kb of ANF flanking sequences is called ANF-CAT (Figure 1). A derivative of ANF-CAT was produced by deletion of a 556-bp fragment lying between two HindIII restriction sites at positions -137 and -693 (the HindIII fragment) and is called \(\Delta\) H-ANF-CAT. The HindIII fragment was reinserted in a reverse, 3' to 5', orientation (RH-ANF-CAT). Three plasmids were also constructed in which the HindIII fragment was translocated to a novel position. Two are derived from a 3.5-kb fragment of \(\Delta\) H-ANF-CAT, isolated from Nde I restriction sites present in ANF sequences (position, -2.4 kb) and 3' of the thymidine kinase polyadenylation signal. This fragment was cloned into the Smal I site of Bluescript KS (+) (Stratagene Cloning Systems, La Jolla, Calif.), which contained the HindIII fragment inserted in the polylinker. The resulting constructs contain the HindIII fragment either 1.7 kb (T1-ANF-CAT) or 3.5 kb (T2-ANF-CAT) from its original position in an Nde I derivative of \(\Delta\) H-ANF-CAT. The CAT activity of a 2.4-kb Nde I derivative of ANF-CAT containing the HindIII fragment is equivalent to that of ANF-CAT.\textsuperscript{10,11} Construct T3-ANF-CAT was produced by inserting the HindIII fragment into the Kpn I site (-3.0 kb) of \(\Delta\) H-ANF-CAT to control for differences related to vector sequences.

\subsection*{Polymerase Chain Reaction}

Primer sequences were chosen by linking 5'-GGG-AAG-CTT-3' to 21 bp selected from the 5' flanking sequences of ANF.\textsuperscript{17} Polymerase chain reaction was performed in a Perkin-Elmer Cetus DNA Thermal Cycler using ANF-CAT as the template and the reaction conditions of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 30 seconds. Reactions were performed in 50 µl using 0.4 µg each primer, 200 mM each dNTP, 2.5 IU/assay Taq polymerase, and Cetus polymerase chain reaction buffer. Twenty-five or 30 cycles were performed, and the reaction products were digested with HindIII and isolated by electrophoresis from a 2% or 3% Nusieve/1% agarose gel depending on fragment size. The 5' ANF sequences were amplified with flanking HindIII sites to facilitate cloning into \(\Delta\) H-ANF-CAT (Figure 2). The orientation of inserted ANF sequences was determined by restriction mapping or by polymerase chain reaction using one internal and one external primer. Dideoxy sequence analysis of constructs ANF-CAT-3, ANF-CAT-5, and ANF-CAT-6 revealed no differences from the parent construct (ANF-CAT) in relevant amplified sequences.

\subsection*{Primary Cardiocyte Cultures, Transfection, and CAT Assays}

Atrial cardiocyte cultures were prepared from isolated neonatal atrial tissues as previously described.\textsuperscript{11} Studies have shown that more than 95% of cells prepared by this method react with antiumosin antisemum.\textsuperscript{11} Transfection was performed as previously described\textsuperscript{11} except that a Bio-Rad Gene Pulser Apparatus was used that was set at 450 mV and 125
μF. Transfected cells were cultured in 6 ml of RM medium (Dulbecco's minimal medium without glutamine, supplemented with 9% [vol/vol] heat-inactivated fetal calf serum, 0.6 mg/ml thymidine, 20 units/ml penicillin, 20 μg/ml streptomycin, and 20 μg/ml gentamicin) and incubated in an atmosphere of 7% CO₂/93% air. After 65 hours, cells were harvested, lysed, and pelleted in 450 μl of lysis buffer (0.5 M Tris, 1 mM EDTA). Then, 300 μl of the supernatant was assayed for CAT activity²⁸ for 3 hours. Reaction products were separated by thin-layer chromatography²⁸ and quantified. After subtracting background counts, absolute activity was taken as a ratio of counts from the acetylated forms of chloramphenicol to counts from the nonacetylated forms. To compare experiments, this number was normalized to the activity of the parent construct, ANF-CAT, which was arbitrarily defined as 100%. All constructs except T2-ANF-CAT were transfected in triplicate, and at least three separate experiments were performed with each. T2-ANF-CAT was analyzed in six independent transfections.

In Vitro Translation and Nuclear Extracts

As previously described,¹⁹ fos and jun were translated in vitro using reticulocyte lysates. Nuclear extracts were prepared from atrial and ventricular cardiocytes cultured as described above using a modification²⁰ of the standard Dignam²¹ protocol for small numbers of cells. This modification uses small volumes, microfuge centrifugation for reduced times, and cell lysis with a 1-ml hypodermic syringe but otherwise is similar to the standard procedure and has been shown to support efficient in vitro transcription and pre-mRNA splicing.²⁰ HeLa and bovine endothelial cell nuclear extracts were the generous gifts of Drs. A. Lee and T. Quertermous.

Preparation of ³²P-Labeled DNA Fragments

The DNA fragments containing the synthetic AP-1 site and a fragment identical except for a mutated AP-1 site (the nonspecific competitor [NS]) have been described.¹⁹ The self-complementary DNA fragment containing the consensus CRE site (sequence, 5'-CCCGGATGACGTCATCATC-CCGGG-3') was the generous gift of Dr. T. Hai.²² Synthetic DNA fragments of the ANF gene corresponding to positions −609 to −577 (fragment B), −576 to −556 with 5'-GGG-AAG-CTT-3' added to each end (fragment C), −447 to −427 with 5'-GGG-AAG-CTT-3' on each end (fragment D), and −497 to −468 (fragment E) were also prepared. Complementary oligonucleotides were annealed to generate double-stranded DNA and blunted with reverse transcriptase where necessary. Fragment A was produced by polymerase chain reaction. The sense primer was 5'-GGG-AAG-CTT-3' joined to ANF sequence −497 to −477. The antisense primer was 5'-GGG-AAG-CTT-3' joined to ANF sequence complementary to −427 to −447. Thirty cycles were carried out under the polymerase chain reaction conditions specified above, and the appropriate size product was isolated by electrophoresis from a 3% Nusieve/1% agarose gel. One hundred nanograms of each oligonucleotide was end-labeled using [γ³²-P]ATP and isolated from a 5% polyacrylamide gel as previously described.¹⁹

DNA Binding Assays

Binding of in vitro translated proteins and nuclear extracts to end-labeled fragment A (10,000 cpm) was performed as previously described¹⁹ except that the sense strand of the NS oligonucleotide was added to all binding reactions as a cold competitor for single-strand DNA binding proteins. Binding of nuclear extracts to end-labeled fragment B (10,000 cpm) was performed using the low ionic strength protocol.
ANF Gene

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>% CAT Activity</th>
</tr>
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<tbody>
<tr>
<td>ANF-CAT</td>
<td>100 ± 17</td>
</tr>
<tr>
<td>ΔH-ANF-CAT</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>ANF-CAT-1</td>
<td>78 ± 6</td>
</tr>
<tr>
<td>ANF-CAT-2</td>
<td>132 ± 7</td>
</tr>
<tr>
<td>ANF-CAT-3</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>ANF-CAT-4</td>
<td>14 ± 6</td>
</tr>
<tr>
<td>ANF-CAT-5</td>
<td>89 ± 32</td>
</tr>
<tr>
<td>ANF-CAT-6</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>ANF-CAT-7</td>
<td>22 ± 3</td>
</tr>
</tbody>
</table>

Figure 2. Schematic of effect of proximal atrial natriuretic factor (ANF) deletions on chloramphenicol acetyltransferase (CAT) activity showing 5' flanking sequence of rat ANF gene. For simplicity, sequences −800 to −2,400 and −2,500 to −3,000 are not drawn to scale. Locations of fragments A, B, C, D, and E used in gel-shift assays (Figures 3 and 4) are shown. Activator protein-1 (AP-1)–like and cyclic AMP–responsive element (CRE)–like sequences and relevant restriction enzymes sites are indicated. CAT constructs containing ANF sequences (black line) and deletions (diagonal stripes) are defined. Plasmids ANF-CAT-1 through ANF-CAT-4 contain deletions at position indicated to −693. Plasmids ANF-CAT-5 through ANF-CAT-7 contain two deletions: −609 to −693 and −137 to position indicated. Mean (±SD) CAT activity from representative experiments of atrial cells transfected with each construct is given relative to ANF-CAT (see “Methods”).

described by Chodosh with 2 μg of poly (dI/dC) added to inhibit nonspecific binding.

Statistical Analysis

Statistical comparison of CAT activity was performed with a two-tailed t test using STATVIEW II (Abacus Concepts, Berkeley, Calif.) on a Macintosh II (Apple) computer.

Results

cis-Acting Control Elements

To determine if proximal cis-acting sequences in the rat ANF gene are important for directing atrial transcription, deletion analyses were performed. The assay for ANF promoter activity involved introduction of putative ANF promoter CAT fusion genes into primary neonatal atrial cardiocytes by electroporation and then assay of CAT activity. Previous studies have demonstrated that ANF sequences contained in a 3.4-kb region (ANF-CAT) promoted atrial-specific gene transcription. An internal deletion of a 556-bp HindIII fragment (positions, −693 to −137) inactivated the ANF-CAT fusion gene (Figure 1, Δ H-ANF-CAT, p≤0.005), demonstrating that there are sequences required for ANF gene expression in this fragment.

To define the role of the regulatory elements on the 556-bp HindIII fragment, we questioned whether these sequences could function in a position- and orientation-independent fashion as seen with enhancer elements. The function of the nucleotide sequences on the HindIII fragment is independent of orientation with respect to the gene (compare ANF-CAT with RH-ANF-CAT in Figure 1). In contrast, the function of these sequences is affected by their location (compare ANF-CAT, T1-ANF-CAT, T2-ANF-CAT, and T3-ANF-CAT in Figure 1). All three positional modifications result in a loss of CAT activity to less than 20% of activity detected with ANF-CAT (p<0.0005). Thus, the regulatory elements encoded on the 556-bp HindIII fragment function in an orientation-independent, position-dependent fashion.

To more precisely define the elements within the HindIII fragment that are important for transcriptional activity, we constructed a series of ANF-CAT fusion genes that lack some of the sequences between positions −137 and −693. Two of the constructs—ANF-CAT-1 and ANF-CAT-2—deleted some or all of the sequence between residues −693 and −609 but retained full activity (Figure 2). ANF-CAT-2 actually manifested a modest increase in activity over the parent construct, ANF-CAT (p<0.03). Although we cannot exclude the possibility that ANF-CAT-2 deletes a negative regulatory element, we think this unlikely and, given the modest quantitative effect,
**Table 1. Binding Sites for Known trans-Acting Factors**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Sequence (consensus/actual)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
<td>(A)TGACTCTA</td>
<td>−496 to −489</td>
</tr>
<tr>
<td>CRE</td>
<td>AGCGGCA</td>
<td>−602 to −596</td>
</tr>
<tr>
<td>KE-2</td>
<td>CAGGTG</td>
<td>−437 to −431</td>
</tr>
<tr>
<td>PEA-2</td>
<td>GACGGCA</td>
<td>−461 to −467</td>
</tr>
<tr>
<td>SP-1</td>
<td>GGGCGG</td>
<td>−398 to −403</td>
</tr>
</tbody>
</table>

AP-1, activator protein-1; CRE, cyclic AMP-responsive element; KE-2, immunoglobulin κ-chain enhancer; PEA-2, polyclonal enhancer; SP-1, promoter-specific transcription factor.

have not pursued it further. However, deletions that extended to or beyond position −576 (ANF-CAT-3 and ANF-CAT-4) had significantly lower CAT activity than ANF-CAT (p < 0.0005), suggesting that part or all of a proximal regulatory region lies between residues −609 and −576 (compare ANF-CAT-2 with ANF-CAT-3 and ANF-CAT-4, Figure 2).

The 3' boundary of the regulatory elements contained within the HindIII fragment was defined by assays of three constructs. For all of these constructs, the sequences between −693 and −609 were deleted because this did not diminish CAT activity. Analysis of ANF-CAT-5 transfected cells demonstrated that 259 bp can be deleted from the 3' HindIII site without a significant loss of activity compared with the parent construct (Figure 2, p > 0.61). Deleting an additional 100 and 159 bp destroys approximately 60% and 78% of the transcriptional activity, respectively (for both compared with ANF-CAT, p < 0.0002; for ANF-CAT-5 versus ANF-CAT-6, p ≤ 0.006). The activity of construct ANF-CAT-7 is significantly greater than the background transcriptional activity of Δ H-ANF-CAT (p ≤ 0.005).

These analyses delineate the boundaries of a 213-bp region that is required for full function of the ANF promoter. The construct ANF-CAT-5 appears to retain the elements necessary for appropriate tissue-specific expression: Atrial transcription is maintained, but ventricular expression in 2-day-old cardiocytes is negligible (data not shown). Other previously identified cis-acting elements are less than 30 bp long. The transcriptional activity in this 213-bp region is sensitive to deletions at both ends. Together, this suggests that there are at least two regulatory elements in proximal sequences that control ANF gene expression.

**Trans-Acting Control Factors**

Further definition of the mechanisms that control ANF gene expression requires identification of nuclear or trans-acting factors that bind ANF regulatory sequences. To assess whether previously characterized trans-acting factors bind the 213-bp sequence identified in the ANF gene, we searched this sequence for homology to the consensus recognition sequences of known nuclear factors. Homology searches between consensus recognition sequences and the 213-bp ANF sequence were performed allowing for one base mismatch. Sequences homologous to binding sites for several known transcriptional activators, including AP-1 and CRE, were identified (Table 1). AP-1 is the binding site for the products of two proto-oncogenes—c-fos and c-jun, which form a heterodimer and can activate transcription. Because CRE and AP-1 sites are similar, this heterodimer also binds some but not all CRE sites.

Because cardiac expression of c-fos and the related gene r-fos change in parallel with ventricular ANF expression during development and hypertrophy, we questioned whether the putative AP-1 binding and CRE sites, identified by sequence homology, were recognized by cognate nuclear factors. To determine whether the fos/jun heterodimer binds these sites in the ANF gene, gel retardation assays were performed. Both c-fos and c-jun were translated in vitro and assayed for ability to bind two different ANF-DNA fragments (designated A and B in Figure 2). The fos/jun heterodimer binds to fragment A (encoding an AP-1-like recognition site) but not fragment B (encoding a CRE-like site), producing a shifted band similar to that seen with the synthetic AP-1 oligonucleotide (Figure 3) that encodes the functional AP-1 site found in the human myc gene. Furthermore, binding to fragment A is specific for the AP-1 sequence and effectively competed by either fragment A or the synthetic AP-1 oligonucleotide but not by an identical oligonucleotide containing a mutated AP-1 binding site (Figure 3, lanes 3–5). We conclude that the ANF AP-1-like binding site specifically binds the fos/jun heterodimer in vitro but the CRE-like site does not.

To test the hypothesis that sequences contained in fragments A and B bind trans-acting factors present in vivo, we prepared atrial and ventricular nuclear extracts from 4-day-old neonatal rat cardiocytes and used these in gel retardation assays. Proteins in nuclear extracts derived from atrial cardiocytes bind to fragments A and B, producing shifted bands (Figure 4). To determine if this binding was sequence specific, fragments C and D were used as competitors. Protein binding to fragments A and B can be competed by the identical unlabeled fragment but not by unrelated fragments from the same region of the promoter (Figure 4, arrows). Fragment A binding is not competed by oligonucleotide encoding AP-1 consensus sequence or by the actual ANF AP-1-like sequence (Figure 4, left panel, lanes 4 and 5). Thus, a factor other than the fos/jun heterodimer is responsible for the binding detected in atrial nuclear extracts. Fragment B binding is not competed by an oligonucleotide encoding a CRE sequence (Figure 4, right panel,
lane 5), suggesting that fragment B binding factors are functionally distinct from the previously described members of the CRE binding family that avidly bind this sequence.22

If the nuclear factors that bind these proximal regulatory elements are entirely responsible for the tissue specificity of ANF gene expression, they should be detected only in cells that produce ANF.
To assess the abundance of the binding proteins that interact with fragments A and B, nuclear extracts were prepared from HeLa cells, endothelial cells, and 4-day-old ventricular cardiocytes. These cells have no demonstrable ANF peptide hormone, and ANF mRNA levels are less than 0.1% of levels in atrial cells.\(^1\) Extracts from these cells contained factors that bind fragment B and produce a band shift similar to that seen with atrial extracts (data not shown). The nuclear proteins that interact with fragment A are more abundant in atrial than in ventricular extracts (Figure 4, left panel). This difference is probably not a result of defective ventricular extracts because the same extract produced a band shift comparable to atrial extracts with fragment B.
Discussion

Previously, we demonstrated that 3.4 kb of 5′ flanking sequence from the rat ANF gene is sufficient to direct high-level, atrial-specific transcription.11 The data presented here demonstrate that there is a 213-bp sequence located between −609 and −397 that is required for ANF promoter activity. We suggest that there are at least two cis-acting regulatory sequences within this region that are necessary for ANF expression. Earlier observations11 demonstrated that some regulatory sequences lie between −640 bp and −2.4 kb. These distal upstream sequences have been retained in all constructs analyzed here, as have very proximal promoter elements (e.g., TATAA). The results presented suggest that the organization of the rat ANF promoter is different from that of the human gene, where the proximal upstream 409 bp are sufficient to direct high-level, specific expression.12 At least three distinct regulatory elements are required for full expression of the rat ANF gene.

The regulatory elements located between residues −693 and −137 of the ANF gene have some features of an enhancer. They can function in either orientation with respect to the ANF gene. This orientation-independent nature of regulation is characteristic of classic enhancer elements. However, when these sequences were moved, they lost their ability to direct ANF promoter activity. This is unlike classic enhancer elements, which typically are also position independent. Why this region exhibits orientation-independent but position-dependent behavior is uncertain. Our data suggest that this region contains multiple regulatory elements. Although the region may harbor an enhancerlike element, it must contain at least one additional element that is unable to function at great distances from the promoter.

Our data demonstrate that a 213-bp sequence (−609 to −397) that is required for ANF promoter activity contains an AP-1 binding site that is functional in vitro. AP-1 sites are recognized by a heterodimer, one subunit of which is the product of the proto-oncogene fos and one of which is the product of the jun proto-oncogene.19,29–33 There are low (or undetectable) amounts of these proto-oncogenes in normal cardiac tissue.36,37,39 These data and our nuclear extract binding studies suggest that the AP-1 binding site does not play an important role in normal physiological expression of the ANF gene. Nevertheless, identification of a potential AP-1 site in fragment A is particularly intriguing. Expression of c-fos and other proto-oncogenes37,38,40 and ventricular ANF25,6 increases in pressure-overload cardiac hypertrophy. Furthermore, proto-oncogene induction precedes ventricular ANF gene expression.37 Demonstration that the c-fos/c-jun heterodimer binds a cis-acting element of the ANF gene provides a potential molecular mechanism linking increased proto-oncogene expression and an altered phenotype in cardiac hypertrophy.

Two other lines of evidence support the hypothesis that binding at this AP-1 site could play a role in ventricular ANF induction by hypertrophy. First, because binding at AP-1 sites mediates the transcriptional response to phorbol esters,23,29,30 one would expect treatment of cardiocytes with phorbol esters to increase ANF transcription if AP-1 binding is biologically relevant to increased ventricular expression. Treatment of neonatal rat cardiocyte cultures with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate produces a modest increase in ANF mRNA levels that, interestingly, is more marked for ventricular than atrial cardiocytes.41 Furthermore, it is likely that the response in these studies would have been more dramatic if serum-free media had been used because serum can increase basal AP-1 binding activity in cultured cells.32 Second, Knowlton and colleagues42 have studied ANF expression in cardiocytes treated with α-adrenergic agonists to produce an in vitro model of hypertrophy. The cis-acting elements necessary for the induction of ANF in this model appear to lie in the proximal upstream 638 bp, as does the AP-1 site identified here. At present, these data are consistent with but do not establish a biological role for AP-1 in hypertrophy in vivo. Further experiments will be required to determine the roles that the c-fos/c-jun heterodimer in general and this AP-1 site in particular might play in the inducible or basal expression of ANF. Studies involving transgenic mice carrying ANF-CAT constructs that delete the AP-1 site are currently under way to examine the biological significance of this binding site in vivo.

That nuclear factors from normal cardiocytes appear to bind fragments A and B independent of the AP-1 and CRE sites emphasizes two important points. First, given the complexity of transcriptional regulation, it is not surprising that multiple proteins interact with the same regulatory element. Second, the absence of detectable CRE or AP-1 binding activity in noninduced cardiocytes suggests that basal levels of these binding proteins are low in cardiocytes. These proteins could become important transcriptional regulators of ANF expression when induced or activated (e.g., by phosphorylation) during hypertrophy.

These studies demonstrate that there are at least three cis-acting regulatory elements contained in sequences 5′ of the rat ANF gene. The boundaries of two proximal regulatory elements have been identified and shown to specifically bind proteins contained in cardiac nuclear extracts. Binding proteins to one regulatory element are more abundant in atrial than ventricular nuclear extracts and may be involved in atrial-specific expression of the ANF gene. An AP-1 site present in one regulatory element binds a known transcriptional activator—the
fos/jun heterodimer— and could be important in induction of ANF expression during pathological states such as ventricular hypertrophy. Future studies will focus on defining the functional significance of proto-oncogenes in regulating pathological ANF expression and identifying the other trans-acting factors that bind these regulatory regions.

Acknowledgments

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**KEY WORDS**

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- *jun*
- proto-oncogenes
- genetics
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