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Divergent Regulation of the Human Atrial Natriuretic Peptide Gene by c-*jun* and c-*fos*

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Employing transient transfection analysis in neonatal rat cardiocytes, we have demonstrated that overexpression of c-*jun* results in a dose-dependent induction of the human atrial natriuretic peptide (hANP) gene promoter. Studies using a series of mutations in the hANP gene promoter identified a TRE-like, *cis*-acting regulatory sequence which conferred c-*jun* sensitivity. This same region was shown to interact with the c-*jun*/c-*fos* complex in an in vitro gel mobility shift assay. Selective mutation of this site suppressed basal activity of the hANP promoter and significantly reduced c-*jun*-dependent activation. Overexpression of c-*fos* had a biphasic effect on hANP gene promoter activity. At low levels, in concert with c-*jun*, it activated, while at higher levels it suppressed, transcription from the hANP gene promoter. This inhibition was both cell and promoter specific. hANP gene promoter sequences which mediate c-*fos*-dependent inhibition appear to be separable from those responsible for the induction. In addition, the protein domains on c-*fos* responsible for transcriptional activation and repression can be segregated topographically, with the inhibitory activity being localized to the carboxy-terminal domain. Thus, c-*fos* can activate or repress hANP gene expression through two separate functional domains that act on distinct regulatory elements in the hANP gene promoter. These data imply that the ANP gene may be a physiological target for c-*fos*- and c-*jun*-dependent activity in the heart and suggest a potential mechanism linking environmental stimuli to its expression.

The atrial natriuretic peptide (ANP) is a cardiac hormone with potent natriuretic, diuretic, and vasorelaxant properties (8, 11). Expression of the ANP gene in the adult animal is largely restricted to the cardiac atria. Expression in the cardiac ventricle is high in the fetal and early neonatal periods but decays to relatively modest levels in the adult, where it is confined to discrete subendocardial and perivascular locations (50). Ventricular expression can be increased dramatically by maneuvers which promote either pressure or volume overload of the ventricular chamber. The hypertrophy of ventricular myocardium provoked by these stimuli is accompanied by significant increments in tissue and plasma ANP and ventricular ANP mRNA accumulation (10, 22, 29). Thus, expression of this gene appears to parallel the hypertrophic process, suggesting that it may share with the structural contractile proteins sensitivity to a common set of regulatory stimuli.

Known regulators of the ANP gene include the glucocorticoids (10, 14, 16), thyroid hormone (15), a variety of neurotransmitters (9, 23) and peptide hormones (47), as well as physical stretch of myocardial tissue (29). At a mechanistic level, several factors from the latter groups (e.g., α_1-adrenergic agonists, endothelin, or physical stretch) are thought to act by increasing phospholipase C activity (31, 35), with subsequent generation of inositol trisphosphate and diacylglycerol, a potent activator of protein kinase C. In addition, a number of studies have shown that activation of protein kinase C with the tumor-promoting phorbol 12-myristate 13-acetate (TPA) results in an increase in secretion of ANP as well as expression of the ANP gene (26, 42), particularly in ventricular cardiocytes. TPA has been shown to activate target gene expression through a number of different mechanisms (3, 4). One of the best studied is that involving the transcription complex AP-1, a complex containing components of two proto-oncogene families related to c-*jun* and c-*fos*, respectively (5, 7, 43). TPA is believed to activate transcription by promoting the association of the AP-1 complex with its cognate recognition sequence, the so-called TRE (TGAC/GTCA) (5, 7, 40, 49). This activation is dependent on transcriptional as well as posttranscriptional mechanisms (2, 6, 36). TPA has been shown to augment expression of both the c-*jun* (25) and c-*fos* (36) genes. In addition, TPA has been shown to increase AP-1 activity by removing inhibitory phosphate residues near the DNA binding site of the c-*jun* protein (6). Taken together, these data raise the possibility that protein kinase may be an important regulator of ANP gene expression and suggest a potential mechanism whereby this regulation might occur. We have examined the available 5′ flanking sequence of the human ANP gene for sequence with potential homology to the consensus TRE; three sites were identified in the region lying between −357 and −222 relative to the transcription start site. Two of these showed partial homology (homologous at five of seven positions), while a third showed complete conservation of the consensus sequence. This study was designed to determine whether the components of the AP-1 complex (i.e., c-*jun* and c-*fos*), either alone or in combination, are capable of fostering the expression of the ANP gene and, if so, whether this regulation is effected, at least in part, through the consensus AP-1 target sequence described above.

MATERIALS AND METHODS

Restriction enzymes, calf intestinal alkaline phosphatase, T4 polynucleotide kinase, and T4 DNA ligase were purchased from Bethesda Research Laboratories or Boehringer Mannheim. ^{3}H-acetyl coenzyme A (200 mCi/mmol) was obtained from Amersham. TPA and 4αTPA were purchased

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from Biomol Research Laboratories. Other reagents were purchased from standard commercial suppliers.

Plasmids. Human ANP gene promoter-chloramphenicol acetyltransferase (hANPCAT) constructs (27, 51), RSVCAT (19), and Rous sarcoma virus (RSV) β-galactosidase (13) have been described previously. Nomenclature for the hAN PCAT constructs is based on the 5'-most nucleotide (relative to the transcription start site in the native gene) included in the construct. $pSV_{0L}CAT$ is a derivative of pSV_0CAT in which a polylinker sequence lying between the *Hin*dIII site and the *Sma*I site in pUC18 have been substituted for the *Nde*I-*Hin*dIII fragment in the parent plasmid. Lamin CAT is a promoterless construct in which a 400-bp fragment from the human lamin cDNA has been positioned in the *Hin*dIII site of $pSV_{0L}CAT$. −410 hANPCAT MUT was generated by polymerase chain reaction on the double-stranded −410 hANPCAT template (20), using oligonucleotides which contain nonconservative transversion mutations at each of the seven positions included in the consensus TRE (positions −241 to −235). Hence, the sequence of the coding strand in the native gene reads 5'-GGGAGCCTCTTGAGTCAAAT CAGTAAG-3' and that of the mutant reads 5'-GGGAGCCT CTGTCCGACAATCAGTAAG-3' across this region. Structures of the plasmids are otherwise identical. The human c-*fos* expression vector BK28 (FBJ LTR c-*fos*) was generously provided by I. Verma (43); the human c-*jun* expression vector (RSV-c-*jun*) (49) and GEM4-based rat c-*fos* (49) and human c-*jun* (49) cDNA clones were provided by V. Baichwal and R. Tjian. The c-*fos* mutant (Δfos) contained a deletion of DNA sequence lying between the *Nco*I and *Eco*RI sites of the parent vector BK28 and was generously provided by B. West. This deletion resulted in the elimination of 70 amino acids at the extreme carboxy terminus of the c-*fos* protein (44). Antiserum directed against c-*fos* was supplied by S. Sagar and has been described previously (46). The AP-1 TKCAT plasmid contained three copies of a 35-bp oligonucleotide harboring the consensus TRE from the human metallothionein gene upstream from a minimal herpes simplex virus thymidine kinase gene promoter (positions −32 to +45 relative to the transcription start site) linked to CAT (30) and was provided by D. Leitman.

Cell culture. Primary cultures of neonatal rat atrial or ventricular cardiocyte-enriched cells were established as described previously (15). In brief, 1-day-old Sprague-Dawley rat pups were sacrificed by decapitation, and the chests were opened; the upper 30% of the heart mass was used to prepare atrial cardiocytes, while the lower 70% of the heart was used for ventricular cardiocyte preparation. Myocytes were dispersed by alternating cycles of trypsin digestion (0.1%) and mechanical disruption (trituration through a 10-ml-wide bore pipette). After removal of cell debris, cells were preplated for 30 min at 37°C to allow nonmyocardial cells to attach to the cell culture plates. Myocardial cells were decanted from the plates, washed twice with phosphate-buffered saline (PBS) containing 0.1% glucose, and used for electroporation.

Myocyte-depleted mesenchymal cultures (i.e., nonmyocardial cells) were prepared from the rapidly adherent (30 min) fractions of ventricular or atrial cells. These cells were allowed to proliferate for 1 to 2 weeks in DME H-21 medium supplemented with 10% fetal calf serum, passaged once at a 1:5 dilution, and expanded to ~50% confluency. Secondary cultures were used to reduce the potential contribution of residual myocytes found in primary mesenchymal cultures. They were then removed from the plates with trypsin and electroporated as described below. GC cells were grown and passaged in DME H-21 containing 10% fetal calf serum.

Transfections and CAT assay. Cardiac myocytes and mesenchymal cells were transiently transfected with plasmid DNA by the electroporation method (51). Atrial cardiocytes (6×10^6 to 8×10^6), ventricular cardiocytes (10×10^6), or mesenchymal cells (15×10^6) were resuspended in 0.4 ml of PBS containing 0.1% glucose, 30 μg of either a promoterless reporter construct ($pSV_{0L}CAT$ or lamin CAT vector), or one of the hANPCAT reporter constructs. Where indicated, cells were cotransfected with one or both of the FBJ LTR human c-*fos* and RSV human c-*jun* expression vectors. The total amount of transfected DNA was always adjusted to 45 μg with pBR322 or pUC18. The myocardial cells were electroporated in a 1-ml electroporation cuvette at 280 V and 250 μF, using a Bio-Rad Gene Pulsar apparatus, while cardiac mesenchymal cells and GC cells were pulsed at 250 V and 960 μF. After a 5-min incubation at room temperature, 0.4 ml of PBS with 0.1% glucose was added to the cuvette. After an additional 5 min of incubation, electroporated cells were resuspended in 1 ml of DME H-21 medium containing 10% fetal calf serum and 1 mM sodium butyrate and plated at a density of 3×10^6 to 4×10^6 atrial or 5×10^6 ventricular cardiocytes, mesenchymal cells, or GC cells per 6-cm culture dish in 6 ml of the same medium. Medium was replaced every 24 h. At 67 to 72 h after transfection, the cells were washed twice in cold PBS, scraped from the plates, transferred to 1.5-ml microfuge tubes, and collected by centrifugation. The medium was aspirated, and the cell pellets were lysed with 200 μl of 0.25 M Tris-HCl (pH 7.6) containing 0.1% Triton X-100. Cellular lysates were assayed for CAT activity as described previously (33). The protein concentration of each cell extract was measured by using the Coomassie protein reagent (Pierce Biochemicals), and equal amounts of protein (100 μg) were used for all assays. To ensure reproducibility, experiments were repeated three to five times, using at least three different DNA preparations. Transfection efficiency, assessed from measurements of RSV-driven β-galactosidase activity (34), varied by less than 15% within a given experiment. For each cell preparation, expression of the hANPCAT constructs was compared with that of the positive control vector (i.e., pRSVCAT). The latter was extremely active in cardiocytes, necessitating a reduction in transfected plasmid (5 μg) relative to the ANP constructs (30 μg). At these concentrations, the ratio of −410 hANPCAT to pRSVCAT expression in atrial cardiocytes was 0.0426 ± 0.0103 (average from 10 experiments).

DNA binding assay. The rat c-*fos* plasmid was linearized with *Eco*RI, and the human c-*jun* plasmid was linearized with *Bam*HI. The capped mRNAs were synthesized by using T7 polymerase (Promega Riboprobe System) under conditions recommended by the vendor. RNA was treated with RNase-free DNase, extracted with phenol-chloroform, dissolved in H_2O, and stored at −70°C. c-*fos* and c-*jun* RNAs (20 to 40 ng/μl) were translated in a rabbit reticulocyte lysate system containing a methionine-free amino acid mixture and 20 μM unlabeled methionine. The translation reactions were incubated at 30°C for 60 min. Products were stored frozen at −70°C until use.

Binding reactions were carried out in a 40-μl volume that typically contained 5 μl of in vitro-translated c-*jun* and c-*fos*, 10×10^3 to 20×10^3 cpm of ^{32}P-end-labeled DNA, 1.5 μg of poly(dI-dC), and 20 μg of bovine serum albumin in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.8)–12% glycerol–1 mM EDTA–4 mM dithiothreitol–0.1% Nonidet P-40–3 mM $MgCl_2$. All binding reactions were

carried out for 120 min at room temperature. The target DNA was a 128-bp *Kpn*I-*Pvu*II fragment of the hANP gene (−337 to −208 relative to the transcription start site) containing a consensus AP-1 binding site (i.e., TGAGTCA) at positions −241 to −235. Specificity of binding was determined by competition analysis using increasing concentrations of the unlabeled 128-bp *Kpn*I-*Pvu*II fragment, three copies (head-to-tail orientation) of the human metallothionein TRE (5′-GAGCCGCAAGTGACTCAGCGCGGGGC GTGTGCAGG-3′), a double-stranded oligonucleotide containing the native TRE from the hANP gene (5′-GG GAGCCTCTTGAGTCAAATCAGTCCG-3′), or an oligonucleotide (5′-GGGAGCCTCTGTCCGACAATCAGTAAG-3′) harboring a selective mutation across the TRE. In selected experiments, in vitro-translated products were preincubated with an antiserum directed against the peptide sequence in c-*fos* prior to incubation with radiolabeled DNA. Binding reactions were analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels for 2 to 3 h at 160 to 180 V in a buffer containing 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA, pH 7.4. Shifts in mobility of the radiolabeled probe were detected by autoradiography.

RESULTS

We have previously demonstrated that the treatment of ventricular cardiocytes with the phorbol ester TPA results in a significant increase in steady-state ANP mRNA levels in these cells (26). To determine whether this effect resulted from enhanced transcription of the ANP gene, we transfected chimeric constructs containing 1,150 bp of hANP gene 5′ flanking sequence linked to a CAT reporter into neonatal ventricular cardiac myocytes. Sixty-seven hours later, the cells were exposed to increasing concentrations of the phorbol ester for a total duration of 5 h. Treatment with TPA resulted in a dose-dependent increase in reporter activity which reached the highest levels at a concentration of 10^{-7} M (Fig. 1). An equivalent concentration of the bioinactive phorbol ester 4αTPA had no effect on the expression of the reporter construct. Furthermore, neither TPA nor 4αTPA had a significant effect on the promoterless construct $pSV_{0L}CAT$.

Given the TPA sensitivity of the hANP gene promoter and the presence of the putative TRE described above, we examined whether overexpression of the individual components of the AP-1 complex, specifically c-*jun* and c-*fos*, would activate reporter constructs containing this promoter. As shown in Fig. 2, cotransfection of the −332 hANPCAT reporter with increasing concentrations of a c-*jun* expression vector (0.01 to 1.0 μg) resulted in a dose-dependent increase in reporter activity. This effect was seen in both atrial and ventricular cardiocytes, although the magnitude of the induction was more pronounced in the latter. In both cases, cotransfection with 5 μg of c-*jun* resulted in a decrease in reporter activity relative to the lower concentration (i.e., 1 μg). Somewhat surprisingly, cotransfection with a c-*fos* expression vector resulted in a dose-dependent inhibition of CAT activity in both atrial and ventricular cells. In this case, the effect was more pronounced in the atriocytes.

Since our cardiocyte primary cultures are uniformly contaminated with nonmyocardial cells (primarily fibroblasts), one possible explanation of the c-*jun* effect is that transfection of our cultures with the c-*jun* expression vector resulted in increased expression of a growth factor in the mesenchymal cells which, in turn, stimulated hANP gene promoter activity in adjacent myocardial cells. A number of growth

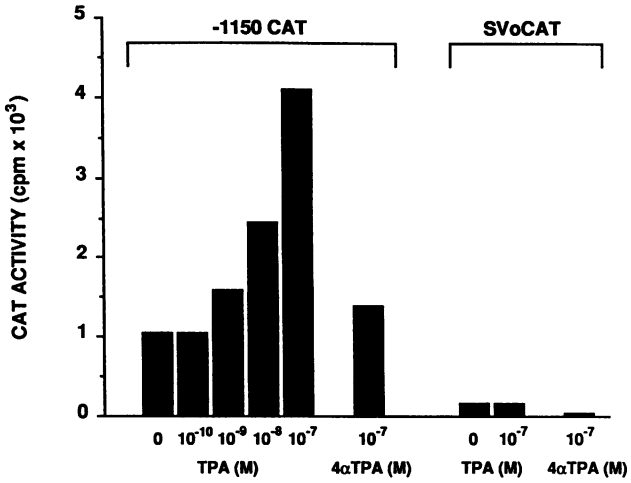

FIG. 1. TPA induction of an ANP-CAT fusion gene in neonatal rat ventricular cardiocytes. Neonatal rat ventricular cardiocytes were transfected with 30 μg of −1150 hANPCAT or 30 μg $SV_{0L}CAT$ as described in Materials and Methods. At 48 h after transfection, cells were washed and subjected to serum withdrawal for 19 h in serum-free DME H-21 medium. Cells were then incubated for 5 h in serum-free medium plus vehicle (control) or the indicated concentration of TPA or 4αTPA. Cell lysates were processed for CAT assay as described in Materials and Methods. CAT activity (^{3}H-acetylchloramphenicol produced) was standardized by using an equal amount of soluble protein (100 μg) for each reaction. A mock reaction containing no protein was included in each CAT assay to establish a baseline activity which, in turn, was subtracted from each experimental value. The values shown here and in Fig. 2 to 8 are from a representative experiment done in duplicate.

factors, including the acidic and basic fibroblast growth factors and transforming growth factor β, have been shown to activate expression of the endogenous ANP gene (38, 39). To address this issue in our system, we transfected cardiac mesenchymal cells with the c-*jun* expression vector and cardiac ventriculocytes with −1150 hANPCAT independently and then cocultured these cells for 67 h prior to collection. As shown in Table 1, transfection of mesenchymal cells with RSV-c-*jun* did not activate hANPCAT expression during the ensuing coculture period and, in fact, had a tendency to reduce reporter expression. These findings suggest that the c-*jun*-dependent activation takes place directly through the ANP gene-expressing cardiocytes and not through a paracrine intermediate.

In an attempt to define the location of the c-*jun*- and c-*fos*-sensitive elements in the hANP gene promoter, we compared the abilities of a number of 5′ deletion mutants to respond to overexpression of these factors in cotransfection experiments. As shown in Fig. 3, truncation of the hANP gene promoter from −2593 to −332 bp of 5′ flanking sequence reduced the overall level of basal expression but preserved c-*jun* sensitivity. However, further reduction from −332 to −217 completely eliminated the c-*jun*-dependent increase in CAT activity. Cotransfection with c-*fos*, on the other hand, resulted in an inhibition of reporter activity with each of the hANPCAT constructs tested. These findings suggest that the c-*jun*- and c-*fos*-responsive elements map to different regions of the hANP gene promoter.

Next, we examined whether cotransfection with c-*fos* would inhibit c-*jun*-dependent activation of the ANP gene promoter. As shown in Fig. 4, the addition of c-*fos* resulted in a dose-dependent reduction in c-*jun*-dependent activity.

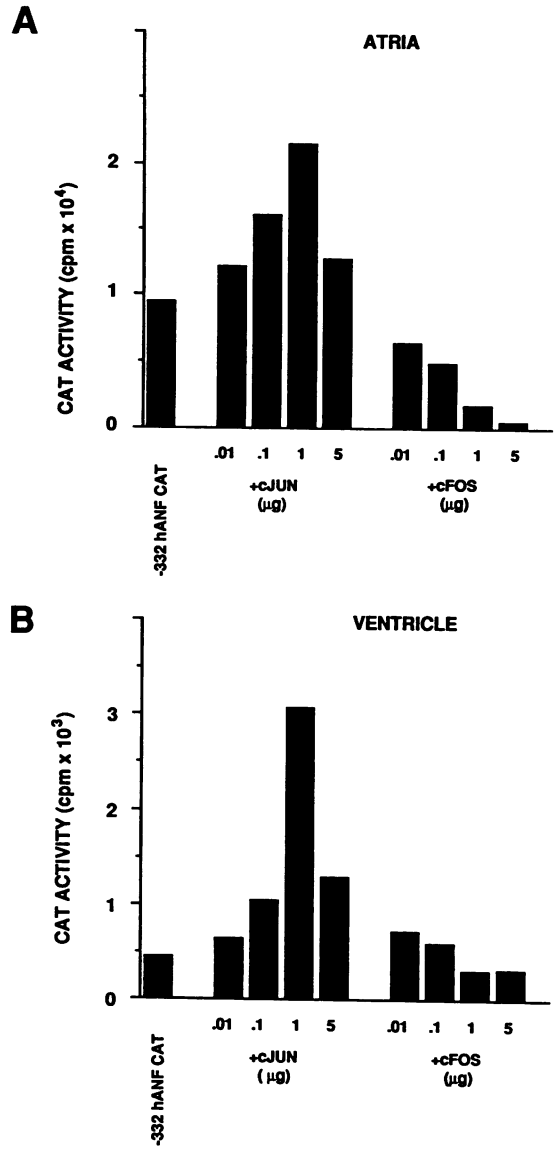

FIG. 2. Regulation by exogenous c-*jun* and c-*fos* of −322 hAN PCAT activity in neonatal atrial and ventricular cardiocytes. Atrial or ventricular cardiocytes were transfected with 30 μg of −332 hANPCAT and either 0.01, 0.1, 1, or 5 μg of the c-*jun* or c-*fos* expression vector. CAT activity was analyzed 72 h posttransfection.

Again, as noted above, the c-*fos*-dependent inhibition appeared to be more effective in atrial than in ventricular cardiocytes. The inhibition by c-*fos* did not result simply from a shift in the dose-response relationship linking c-*jun* to ANP reporter expression. As shown in Fig. 4C, the profile of c-*jun*-dependent activity was preserved in the c-*fos*-transfected cells (compare with Fig. 2) despite a global reduction in c-*jun*-dependent activity.

To provide assurance that this *fos*-dependent inhibition was not an anomaly of AP-1-dependent transcription in myocardial cells, we examined the effect of c-*jun* and c-*fos* on the expression of a reporter plasmid containing three copies of the TRE from the human metallothionein gene inserted upstream from TKCAT. Transfections were carried out in the presence of the c-*jun* or c-*fos* expression vector,

TABLE 1. Evidence that overexpression of c-*jun* in cardiac mesenchymal cells does not alter expression of the hANP gene promoter in cocultured ventricular myocytes[a]

Cells	CAT activity (cpm/100 μg of protein)	
	−c-*jun*	+c-*jun*
Control	1,599 ± 235	
+0.5 × 10^6 mesenchymal cells	1,268 ± 212	862 ± 109
+1 × 10^6 mesenchymal cells	1,062 ± 209	651 ± 111

[a] Neonatal ventricular cardiocytes were transfected with 30 μg of −1150 hANPCAT. Following transfection, 4 × 10^6 cardiocytes were plated in 10% fetal calf serum in the presence or absence (control) of 0.5 × 10^6 or 1.0 × 10^6 cardiac mesenchymal cells (primarily fibroblasts) independently transfected with 1 μg of pUC18 (−c-*jun*) or 1 μg of RSV-c-*jun* (+c-*jun*). Cells were cocultured for 66 h, at which point cell extracts were prepared and analyzed for CAT activity as described for Fig. 1.

either alone or in combination. As shown in Fig. 5, cotransfection with the c-*jun* expression vector alone resulted in roughly a threefold increase in reporter gene activity. Cotransfection with the c-*fos* vector alone increased reporter activity approximately 20-fold. Cotransfection with the combination of c-*jun* and c-*fos* resulted in a roughly additive effect with no evidence for inhibition, indicating that this TRE behaves in the expected fashion in myocardial cells.

As a further test of the specificity of the c-*fos*-dependent inhibition, we assessed the effect of overexpressing this proto-oncogene on hANPCAT reporter activity in a nonmyocardial cell population. Cardiac mesenchymal cells do not express the endogenous ANP gene (17) but will display low-level nonspecific expression of selected hANPCAT constructs (51). Levels of such nonspecific expression at times fall in the range of that seen with ventricular cardiac myocytes. GC cells are a rat pituitary tumor cell line with similar properties (i.e., mesenchymal cell-like) referable to ANP gene expression; because of their improved transfectability

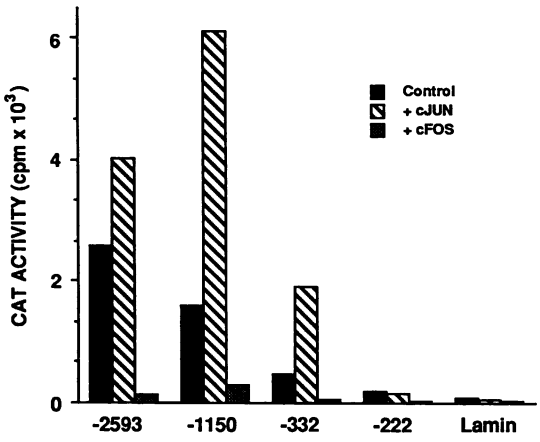

FIG. 3. Effects of exogenous c-*jun* and c-*fos* on transcriptional activity of human ANP 5′ deletion mutants in ventricular cardiocytes. Ventricular cardiocytes were cotransfected with 30 μg of the promotorless lamin CAT construct or 30 μg of the hANPCAT 5′ deletion mutants indicated together with either 1 μg of c-*jun* or 5 μg of c-*fos* expression vector. The ANP promoter constructs are identified by the 5′ border of the included hANP gene promoter sequence (see Materials and Methods). At 72 h after transfection, cell lysates were prepared and CAT activity was measured as described for Fig. 1.

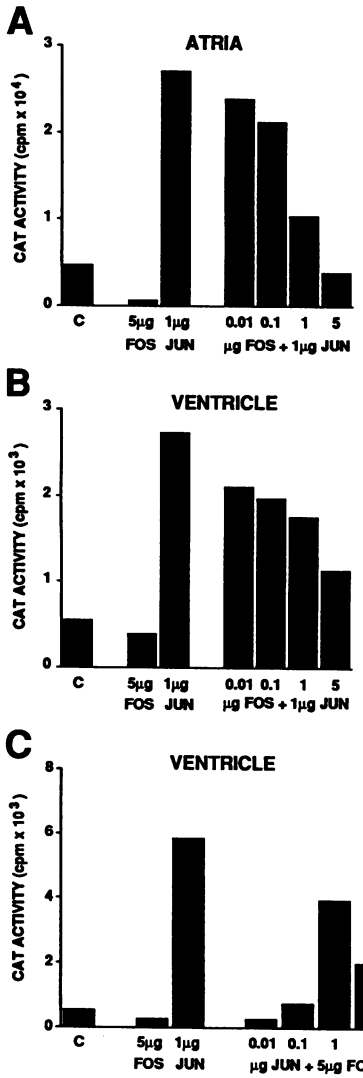

FIG. 4. Inhibition by c-*fos* of c-*jun*-dependent activation of −332 hANPCAT. Atrial (A) and ventricular (B) cardiocytes were cotransfected with 30 μg of −332 hANPCAT reporter alone (control) and either 1 μg of c-*jun*, 5 μg of c-*fos*, or a combination of 1 μg of c-*jun* and increasing amounts of c-*fos*. (C) Ventricular cardiocytes were cotransfected with 30 μg of −332 hANPCAT alone (control) or with 1 μg c-*jun*, 5 μg of c-*fos*, or a combination of 5 μg of c-*fos* and increasing amounts of c-*jun*. Extracts were prepared after 72 h.

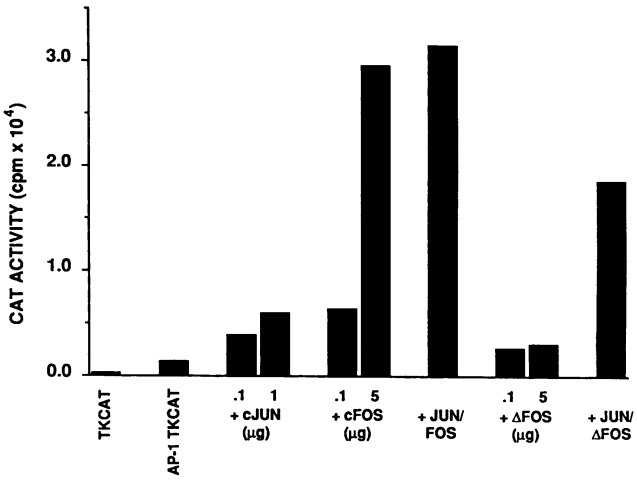

FIG. 5. Stimulation by c-*fos* protein of transcription from AP-1 TKCAT. Ventricular cardiocytes were transfected with 15 μg of AP-1 TKCAT (containing three copies of the human metallothionein TRE placed upstream from a minimal thymidine kinase gene promoter) alone or together with 0.1 or 1 μg of c-*jun*, 0.1 or 1 μg of c-*fos*, 0.1 or 1 μg of Δfos, or a combination of 1 μg of c-*jun* and 5 μg of c-*fos* or 5 μg of Δfos. Cell extracts were collected and processed for CAT assays as described in Materials and Methods.

relative to the cardiac cell primary cultures, levels of nonspecific hANPCAT expression can be significant. Most importantly, the qualitative profile of expression of 5′ deletion mutants is similar in cardiac mesenchymal cells and GC cells and quite distinct from that seen in atrial and ventricular myocytes (51). To assess the cellular specificity of the c-*fos*-dependent inhibition, we transfected cardiac mesenchymal cells and GC cells with several hANPCAT reporter constructs in the presence or absence of the c-*fos* expression vector. As shown in Fig. 6, expression of the reporter constructs, while reduced in atrial myocytes, was either unchanged or slightly increased by cotransfection with the c-*fos* vector in mesencyhmal or GC cells. These data indicate that the c-*fos*-dependent inhibition is not only promoter specific but cell type specific as well. In the same experiment (Fig. 6), we noted that c-*fos*-dependent inhibitory activity in atriocytes was lost as the hANP gene 5′ flanking sequence was reduced from 222 to 104 bp. This would imply that a c-*fos*-sensitive element is present in this region of the hANP gene.

As discussed below, selective inhibitory activity intrinsic to the c-*fos* protein has been demonstrated in other cell types (44, 45). In some cases, the inhibitory domain has been localized to the carboxy terminus of the protein. Full activation of the inhibitory activity appears to require a phosphorylation event, or series of events, in this region of the molecule (36). To evaluate the role of this inhibitory element in suppressing hANP gene expression, we used a c-*fos* deletion mutant in which 70 amino acids at the carboxy terminus of c-*fos* were replaced by a single termination codon. This particular mutation would be expected to eliminate the inhibitory domain of c-*fos* while preserving at least a portion of its activation function (36). This was confirmed with the metallothionein AP-1 TKCAT construct described above. As shown in Fig. 5, the mutant displayed only a fraction of the functional activity of the full-length c-*fos* protein when transfected alone or in combination with c-*jun*. More importantly, cotransfection of the c-*fos* deletion mutant, again with −332 hANPCAT, failed to reproduce the inhibitory effect seen with the c-*fos* molecule (Fig. 7). Cotransfection of the mutant with the c-*jun* expression vector resulted in a dose-dependent activation of the hAN PCAT construct. This effect was at least partially reversible if the wild-type c-*fos* expression vector was included in the transfection (Fig. 7). These findings indicate that the protein domain on the c-*fos* molecule responsible for suppression of hANP promoter activity is topographically separable from that responsible for its activation function.

The presence of discordant activity in the c-*fos* protein raised the possibility that each function, if truly independent, might have a unique dose-response relationship with regard to promoting its individual effect. To evaluate this possibility, we repeated the cotransfection experiments with much

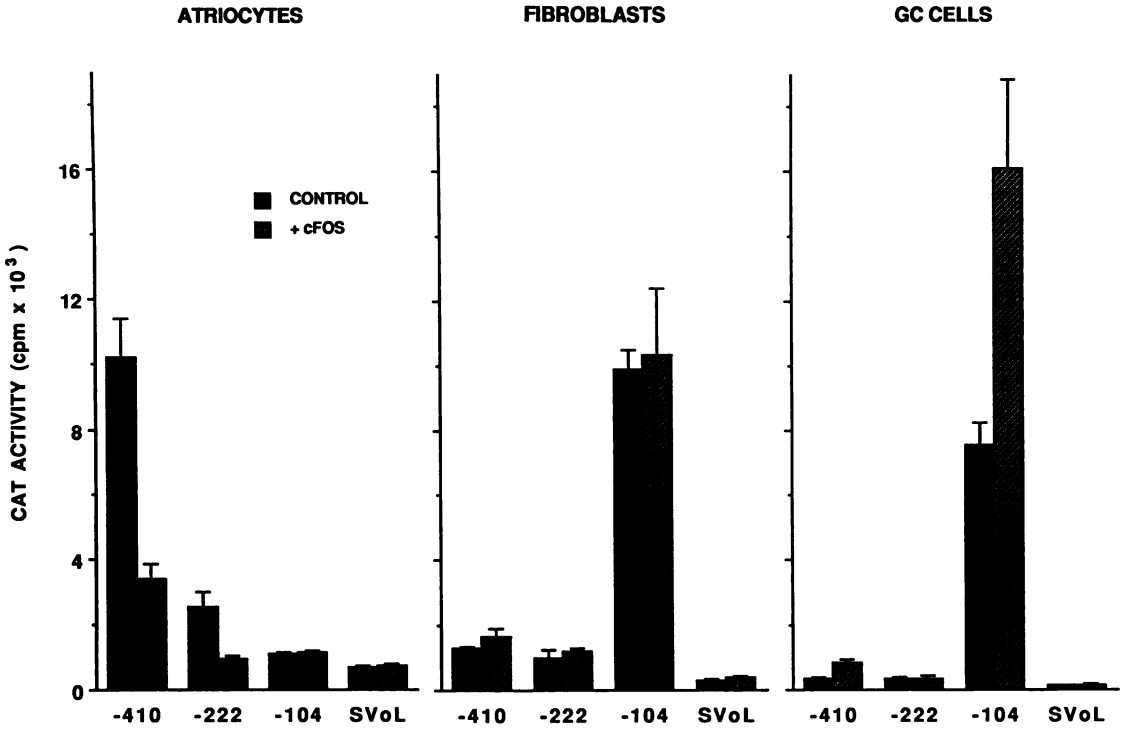

FIG. 6. Cellular specificity of c-*fos* repression. Atrial cardiocytes, subconfluent cardiac mesenchymal cells (primarily fibroblasts), or GC cells were cotransfected with 30 μg of the hANPCAT plasmid indicated in the presence or absence of 5 μg of c-*fos*. Extracts were prepared and assayed as for Fig. 1. The values are the means of three determinations ± standard deviation.

lower concentrations of the c-*fos* and c-*jun* expression vectors. A total of eight different combinations were tested. As shown in Fig. 8, transfection with 0.01 μg of c-*fos* had virtually no effect on the expression of the −332 hANPCAT reporter, while 0.1 μg of c-*jun* expression vector resulted in a modest stimulation. Cotransfection with c-*fos* and c-*jun* together, on the other hand, resulted in a dramatic increase in reporter activity. A similar result was obtained when a combination of 0.05 μg of c-*jun* and c-*fos* was used. These findings imply that (i) under certain specific conditions, the activation function of c-*fos* can predominate and (ii) the relative concentrations of c-*fos* and c-*jun* are important in determining the direction of the response. Overexpression of either one or the other above a certain threshold results in loss of the activation phenomenon.

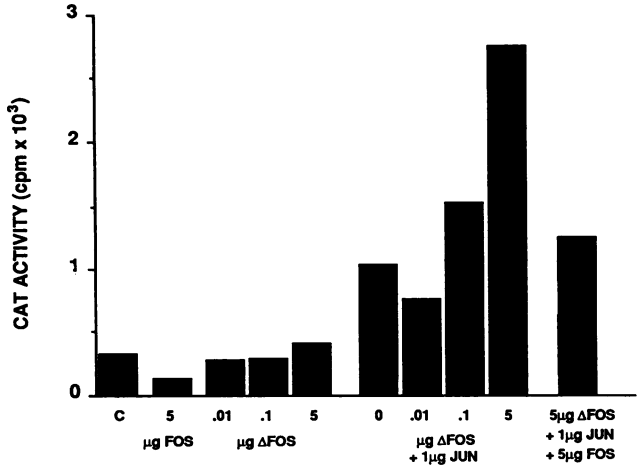

FIG. 7. Evidence that a truncated c-*fos* mutant stimulates rather than inhibits c-*jun*-dependent activation of hANPCAT. Thirty micrograms of −410 hANPCAT alone (C) or together with 5 μg of c-*fos* or the indicated concentrations of Δfos was cotransfected into ventricular cardiocytes. A second group of cells was transfected with 1 μg of c-*jun* in combination with the indicated concentrations of Δfos plus 1 μg of c-*jun*, 5 μg of Δ*fos*, and/or 5 μg of c-*fos*. Extracts were prepared and assayed 72 h later.

Next, to determine whether the stimulatory activity of the c-*jun* molecule was related to the consensus TRE located at positions −244 to −236 in the hANP gene promoter, we created a site-directed mutant in which each nucleotide of the consensus sequence was altered in a nonconservative fashion. As shown in Fig. 9, this mutation resulted in a modest suppression of basal activity relative to the wild type and a significant reduction in the c-*jun*-dependent activation of reporter activity in atriocyte cultures. Still unexplained is the fact that c-*fos*-dependent inhibition was relatively ineffective with the mutant promoter in ventriculocyte cultures.

Finally, using gel mobility shift analysis, we demonstrated that this region of the hANP gene promoter associates directly with the *jun/fos* complex in vitro. As shown in Fig. 10, a 128-bp restriction fragment that incorporates the putative TRE was found to associate with protein components of a programmed reticulocyte lysate in a *jun/fos*-dependent fashion. c-*fos* alone was devoid of binding activity, while c-*jun* demonstrated only a modest ability to associate with the radiolabeled probe. The *jun/fos* combination, on the other hand, provided a significant level of binding which exceeded that seen with the individual components by severalfold. These associations were suppressed with unla-

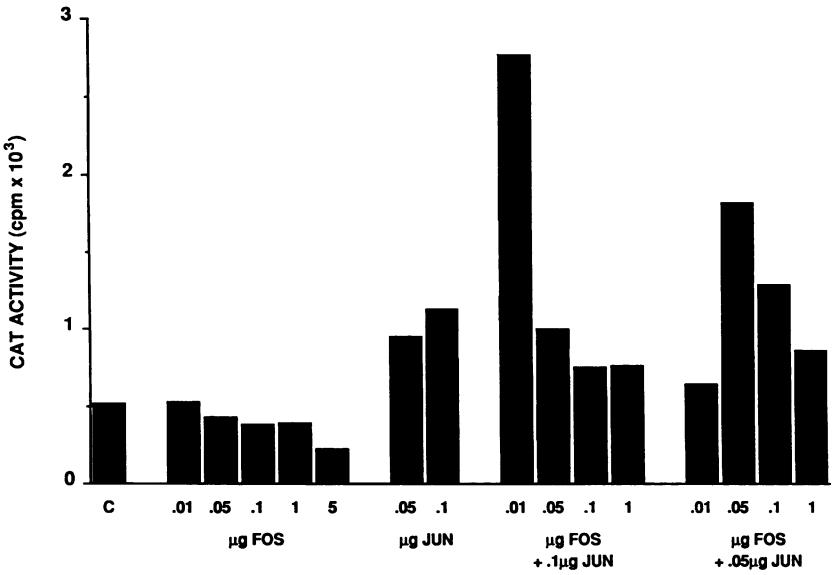

FIG. 8. Evidence that effects of c-*jun* and c-*fos* on the hANP gene promoter are dependent on the levels of *jun* and *fos* expression. −410 hANPCAT (30 μg) (C) was cotransfected into ventricular cardiocytes together with increasing concentrations of the c-*fos* or c-*jun* expression vector or with various concentrations of c-*jun* (0.1 or 0.05 μg) in the presence of increasing concentrations of c-*fos*. At 72 h after transfection, cell extracts were prepared and assayed for CAT activity.

beled 128-bp fragment as well as by oligonucleotides harboring the wild-type TRE sequence from either the hANP or metallothionein gene but not by oligonucleotides containing the mutated TRE or totally unrelated DNA sequence. Noteworthy is the fact that an antibody directed against the c-*fos* protein proved capable of disrupting the complex, supporting the specificity of the DNA-protein interaction.

DISCUSSION

The divergent regulatory effects of c-*jun* and c-*fos* at the level of the ANP gene promoter suggest that they act through at least partially different mechanisms. The c-*jun*-dependent activation appears to operate through a conventional AP-1-like mechanism, given the dependence on the TRE-like sequence in the hANP gene promoter for optimal activation and the demonstrated ability of the *jun/fos* heterodimers to bind to a DNA fragment containing this element in a sequence-specific fashion. The nature of the components in the intact cell with which c-*jun* might dimerize to bind to the TRE remains unclear. c-*jun* homodimers will associate with the TRE, although in most cases (1, 37, 40, 49), as here (Fig. 10), the interaction is relatively weak. The *jun/fos* complex represents the prototypic heterodimer capable of associating with this element, but it is also clear that overexpression of c-*fos* can have adverse effects on hANP gene expression independent of the TRE (see above). Basal c-*fos* levels in cardiac myocytes tend to be relatively low (21, 24), and it is conceivable that under physiological conditions subtle elevations in c-*fos* gene expression, approximating those produced by low-level transfection with FBJ LTR c-*fos*, could activate the hANP gene promoter through the TRE without triggering the inhibitory function. Alternatively, c-*jun* could form a functional dimer with another member of the *fos* family (e.g., *fosB*, *fra1*, or *fra2*) which possesses little or none of the inhibitory activity seen with c-*fos*. Such diversity would be expected to broaden the repertoire of transcriptional responses which environmental signals generate within the cardiac cell and thereby effect a more sensitive control of ANP gene expression.

As mentioned above, the effects of overexpression of the c-*fos* protein are considerably more complex than a simple AP-1-dependent mechanism would predict. The data presented in Fig. 6 suggest that the inhibition is most dependent on sequences lying between −222 and −104 in the hANP gene promoter, well downstream from the TRE. It is notable, however, that in ventricular but not atrial myocytes, mutation across the TRE reduced the inhibitory effect, suggesting that under some circumstances, this element may contribute to the c-*fos*-dependent suppression of hANP gene promoter activity. The reason behind this dichotomous behavior in atrial versus ventricular cardiocytes remains unexplained. c-*fos*-dependent inhibition is unlikely to result from a nonspecific squelching of core transcription factor activity, since similar concentrations of the c-*fos* expression vector were shown to stimulate activity of the AP-1 TKCAT reporter. c-*fos* has been shown to reduce transcription of selected target genes, including the native c-*fos* gene itself (41, 44, 45) as well as the *myoD* (28), *HSP70* (44), *egr-1* and *egr-2* (18), and *aP2* (12) genes. In the case of the c-*fos* gene, the location of the element responsible for the inhibition remains somewhat controversial (18, 41, 44) but appears to lie close to the serum response element in the promoter. The structurally similar CArG-box element has been implicated in the c-*fos* suppression of the *egr-1* gene. Analysis of sequence between −222 and −104 in the hANP gene failed to reveal regions with significant homology to either the CArG box or the more inclusive serum response element. Sassone-Corsi et al. (44) and Gius et al. (18) have shown recently that the inhibitory activity can be localized to the carboxy terminus of the c-*fos* molecule in a region harboring a significant number of serine residues. Subsequent studies have demonstrated that phosphorylation of this region is required for manifestation of inhibitory activity (36). The same phenomenon appears to be operative in the system described here, in that expression of a carboxy terminus-

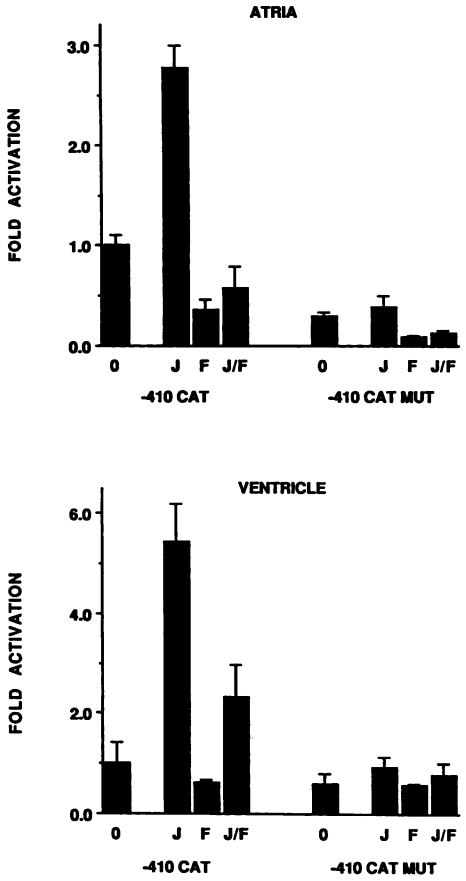

FIG. 9. Evidence that the TRE in the hANP gene promoter is a specific target for c-*jun*-dependent stimulation. Atrial and ventricular cardiocytes were transfected with 30 μg of −410 hANPCAT or 30 μg of a modified version of −410 hANPCAT (−410 CAT MUT) in which the TRE at positions −241 to −235 has been selectively mutated. These reporter plasmids were transfected alone (0) or together with 1 μg of c-*jun* (J), 5 μg of c-*fos* (F), or 1 μg of c-*jun* and 5 μg c-*fos* together (J/F). At 72 h after transfection, lysates were assayed for CAT activity. CAT activity has been normalized to expression of the control −410 hANPCAT construct. The values shown represent the means ± standard deviations of three different experiments.

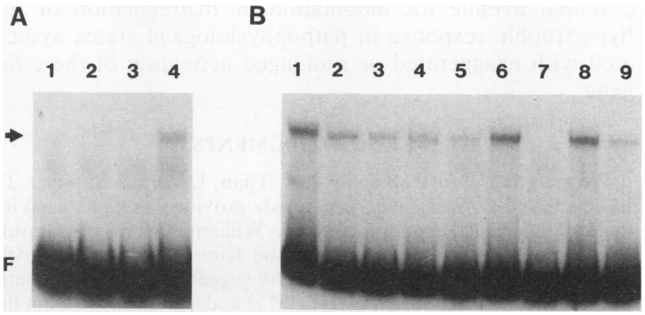

FIG. 10. Specific DNA binding activity of in vitro-translated c-*fos* and c-*jun*. A *Kpn*I-*Pvu*II fragment (corresponding to positions −337 to −208) of the hANP gene was end labeled with [γ-^{32}P]ATP and assayed for the ability to associate with the c-*jun*/c-*fos* proteins in vitro. c-*jun* and c-*fos* were made by in vitro transcription of their respective cDNAs followed by translation of the relevant RNA products in a reticulocyte lysate system. In vitro-translated c-*fos* and c-*jun* (4 μl of lysate for each) were preincubated together for 15 min at 30°C. Radiolabeled probe (20,000 cpm) was then added, and incubation was continued for 120 min at 23°C. Products of the binding reaction were separated on a nondenaturing polyacrylamide gel. (A) Lanes: 1, unprogrammed lysate; 2, c-*jun* programmed lysate; 3, c-*fos* programmed lysate; 4, c-*jun* plus c-*fos*. (B) Lanes: 1, c-*jun* plus c-*fos*; 2 and 3, 25 and 50 ng of unlabeled 128-bp *Kpn*I-*Pvu*II fragment; 4 and 5, 25 and 50 ng of oligonucleotide harboring the consensus TRE from the hANP gene promoter (corresponding to positions −251 to −225); 6, 50 ng of mutated oligonucleotide in which the hANP TRE gene has been eliminated; 7, 25 ng of a 110-bp fragment from the AP-1 TKCAT plasmid harboring three copies of human metallothionein TRE; 8, 50 ng of an unrelated 30-bp oligonucleotide derived from the 5′ flanking sequence of the human herpes simplex thymidine kinase gene (positions −53 to −28); 9, *jun*/*fos* complex preincubated with anti-c-*fos* antiserum for 15 min at room temperature prior to the DNA binding reaction. All binding reactions were carried out as described in Materials and Methods; sequences for the individual oligonucleotides are provided there. For competition experiments, competitor DNA was added to the incubation at the same time as radiolabeled probe. F, free probe; arrow indicates the position of the probe specifically associated with the *fos*/*jun* complex. Other bands could not be competed for by the unlabeled 128-bp fragment and are thought to represent nonspecific protein-DNA complexes.

deleted c-*fos* protein resulted in activation rather than suppression of the hANPCAT reporter. These findings also imply that the underlying stimulatory activity of the c-*fos* protein is masked or suppressed by the coexistent inhibitory function when c-*fos* is expressed at high levels.

The identification of an AP-1-sensitive TRE in the hANP gene promoter has important physiological implications. Activation of the hypertrophic phenotype in ventricular cardiac myocytes in vivo (22, 24, 32, 48) or following exposure to selected hormonal agonists in vitro (21, 23, 47, 48) results in the stimulation of at least two very important gene groups. Early on there is an increase in expression of the immediate-early gene repertoire (e.g., c-*jun*, c-*fos* or c-*myc*) followed by the reactivation of a collection of embryonic cardiac genes not normally expressed in adult heart. Included in the latter group are the genes for alpha skeletal actin, beta myosin heavy chain, and, notably, ANP. In fact, the dramatic changes which are effected in ventricular ANP gene expression suggest that it represents one of the most sensitive molecular indicators of the hypertrophic process. Until now, these two phenomena have been linked only inferentialy on the basis of their sequential appearance following application of the hypertrophic stimulus. The findings presented herein close an important mechanistic gap by linking early gene overexpression to functional activation of at least one component of the hypertrophic phenotype (i.e., ANP gene expression). The suppressive effect of high concentrations of c-*fos* was not predicted by the conventional AP-1-dependent model but would suggest that prolonged or exaggerated expression of c-*fos* in vivo might suppress the hypertrophic stimulus to expression of the ANP gene and perhaps of other genes in the embryonic repertoire. If such gene products reflect a truly adaptive response to hemodynamic overload, suppression could result in myocardial decompensation and cardiac failure.

In summary, we have identified an AP-1-sensitive element in the 5′ flanking sequence of the hANP gene. This element may represent a mechanistic link between immediate-early gene activation and the increased ANP gene expression seen in cardiac hypertrophy. A paradoxical suppression of ANP gene expression by high-level c-*fos* expression suggests a

potential avenue for modulation or malregulation of the hypertrophic response in pathophysiological states associated with exaggerated or prolonged activation of the c-*fos* gene.

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