Differential Effects of Protein Kinase C, Ras, and Raf-1 Kinase on the Induction of the Cardiac B-type Natriuretic Peptide Gene through a Critical Promoter-proximal M-CAT Element*

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The cardiac genes for the A- and B-type natriuretic peptides (ANP and BNP) are coordinately induced by growth promoters, such as α2-adrenergic receptor agonists (e.g. phenylephrine (PE)). Although inducible elements in the ANP gene have been identified, responsible elements in the BNP gene are unknown. In this study, reporter constructs transfected into neonatal rat ventricular myocytes showed that in the context of 2.5 kilobase pairs of native BNP 5′-flanking sequences, a 2-base pair mutation in a promoter-proximal M-CAT site (CAT-TCT) disrupted basal and PE-inducible transcription by more than 98%. Expression of constitutively active forms of Ras, Raf-1 kinase, and protein kinase C, all of which are activated by PE in cardiac myocytes, strongly stimulated BNP reporter expression. Isolated M-CAT elements conferred PE, protein kinase C, and Ras inducibility to a minimal BNP promoter, however, they did not confer Raf-1 inducibility. These results show that M-CAT elements can serve as targets for Ras-dependent, Raf-1-independent pathways, implying the involvement of c-Jun N-terminal kinase and/or p38 mitogen-activated protein kinases, but not extracellular signal-regulated protein kinase/mitogen-activated protein kinase. Moreover, the essential M-CAT element distinguishes the BNP gene from the ANP gene, which utilizes serum response elements and an Sp1-like sequence.

The A-, B-, and C-type natriuretic peptides (NPs)† are structurally related cardiac-derived peptides with vasorelaxant, diuretic, and natriuretic effects (1–7). Upon treatment with stimuli that can eventually lead to the hypertrophic growth of ventricular myocytes, several embryonic cardiac genes are reactivated, including those for ANP, BNP, β-myosin heavy chain, and skeletal α-actin (6–18). While the precise function of this recapitulation of embryonic cardiac gene expression is unclear, it can be speculated that increased NP production represents a compensatory endocrine response to stimuli that often increase blood pressure. Accordingly, a knowledge of the mechanisms by which the NPs are induced during the hypertrophic growth program will provide a better framework upon which to understand how the expression levels of the hormones are regulated under less severe, but nonetheless hemodynamically challenging physiological conditions.

A variety of studies have addressed the mechanisms responsible for ANP induction in primary neonatal rat cardiac myocytes (11, 12, 15, 17). Recent studies have demonstrated the importance of serum response elements (SREs) (18) as well as SP-1-like elements (16) in the transcriptional activation of ANP in response to α2-adrenergic agonists. Earlier reports have also indicated the probable involvement of AP-1-binding cis-sequences, also known as 12-O-tetradecanoylphorbol-13-acetate response elements (TREs), in regulating transcription of human ANP (19). Accordingly, it is believed that α2-adrenergic agonists, and perhaps other ANP inducers, stimulate myocardial cell signaling pathways, which eventually lead to the activation of serum response factor, an Sp1-like protein, and perhaps AP-1, each of which converge on the transcriptional enhancement of ANP.

Relatively little is known about the regulation of BNP expression. Phorbol esters and diacylglycerol increase BNP mRNA and peptide levels (20, 21), suggesting the involvement of PKC. Additionally, BNP promoter activity, measured using constructs containing approximately 2.5 kb of the rat BNP 5′-FS upstream of a luciferase reporter, is inducible by phorbol esters, serum, or the α-adrenergic agonist, phenylephrine (PE) (22). Thus, it is possible that in part the coordinated induction of ANP and BNP involves the convergence of intracellular signaling mechanisms upon cis-elements that are conserved between the genes. The present study was undertaken to test this hypothesis by mapping and identifying regions of the BNP 5′-FS that are critical for basal and inducible transcription in rat cardiac myocytes.

MATERIALS AND METHODS

Mutagenesis

Preparation of Truncated BNP/Luciferase Constructs—A 2.5-kb portion of the rat BNP 5′-FS was inserted into a luciferase reporter construct (pGGL2; Promega, Madison, WI), as described previously (22). Truncated versions of BNP/luciferase were created either by using native restriction sites, by synthesizing specific PCR primers, or by unidirectional deletion (Erase-a-Base, Promega), starting with BNP-2501GL, as described previously (18).

Preparation of Cluster and Point Mutated BNP/Luciferase Constructs—A series of 6-hp cluster mutations covering the BNP 5′-FS between −103 bp and −42 bp was created in full-length BNP-2501 by site-directed mutagenesis using Altered Sites (Promega), as described previously for ANP (18). Briefly, a fragment of the BNP 5′-FS from −116 to +80 bp was inserted into the pAlter vector and used as a template. Oligonucleotides containing the desired 6-hp mutation (mutants A–H in Fig. 1) flanked by 12 nucleotides of native BNP sequence on either side, were synthesized and used to prepare the mutant constructs using methods described by the manufacturer. Point mutations in the M-CAT site (ΔM-CAT) were prepared beginning with oligonu-
cleotides containing the changed nucleotides flanked on either side by native BNP sequences, extending on the 5' side to BNP-116, where there is a SacI site. Using these oligonucleotides as sense primers, and an oligonucleotide complementary to sequences in the 5'-region of the luciferase gene as a common antisense primer, PCR was carried out using BNP-116GL as the template. PCR products were then digested with SacI (BNP-116) and BamHI (BNP+80), and then cloned into pGL2 to create BNP-116GL possessing cluster mutations A–H and the ΔM-CAT mutation shown in Fig. 1.

To create multiple mutations (e.g. ΔM-CAT/GATA−95), a BNP-116GL construct possessing the appropriate GATA-directed point mutation(s), prepared previously (22), was used as the template and the sense primer, possessing the 2-nucleotide M-CAT mutation (see above), was coupled with the luciferase primer to prepare the appropriate PCR-generated product. As above, PCR products were then digested with SacI (BNP-116) and BamHI (BNP+80), and then cloned into pGL2 to create BNP-116GL possessing various combinations of mutations in the M-CAT and GATA sites (see Fig. 3, top, for M-CAT/GATA mutations).

To prepare the cluster and point mutations in the 2.5-kb BNP 5'-FS, we utilized the SacI sites located 5' of −2501 in the pGL2 multiple cloning site and at −116 in the BNP 5'-FS. Wild type BNP-2501GL was digested with SacI, and the fragment from −2501 to −116 was purified. This fragment was then cloned into SacI-digested BNP-116GL constructs possessing the mutations described above to restore the full-length BNP-2501.

Preparation of M-CAT'/BNP-81GL—Two synthetic oligonucleotides (see below) were designed so that after hybridization there is a tandem repeat of two canonical BNP M-CAT core elements (boxed) separated by 5 bp (see Sequence 1).

Some flanking BNP sequences were included such that the repeats represent BNP−112 to −97/BNP−109 to −97. The lowercase nucleotides at the ends are not native to the rat BNP 5'-FS and were added to provide PspbA1 sites. The double-stranded synthetic oligonucleotide was ligated into the PspbA1 site on the 5' boundary of the rat BNP sequences in BNP-81GL. Positive clones were sequenced and some clones contained one insert (i.e. 2XM-CAT/BNP-81GL), and others contained two inserts (i.e. 4XM-CAT/BNP-81GL). All plasmid constructions were verified by dideoxy sequencing.

Cell Culture and Transfections

Myocardial cells were prepared as described (18, 22). For transfections, freshly dissociated cells were resuspended at a density of 30 million cells/ml of minimal medium (Dulbecco's modified Eagle's medium/F-12 medium (Life Technologies, Inc.) containing 1 mg/ml bovine serum albumin). For each transfection, 300 μl, or 9 million cells, were mixed with 15 or 30 μg of BNP/luciferase (test reporter), and 9 μg of CMV-β-galactosidase (normalization reporter), and in some experiments, 45 μg of a PKC, Ras, or Raf-1 expression construct (see below).

Each 300-μl aliquot was then electropropared in a Bio-Rad Gene Pulser at 700 V, 25 microfarads, 100 ohms in a 0.2-cm gap cuvette. This procedure results in an approximate 30% viability (18); accordingly, the 3 million viable cells were plated into fibronectin-coated 35-mm wells, at 1 × 106 cells/well, or into 24-mm wells at 0.5 × 106 cells/well. Thus, the plasmid concentrations per 106 viable cells were 5 μg of BNP/luciferase (10 μg in some experiments), 3 μg of CMV-β-galactosidase, and in some experiments, 15 μg of PKC, Ras, or Raf-1 test construct.

Transfected cells were maintained in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal bovine serum for approximately 14 h after electroporation. The cells were then washed thoroughly, and the medium was replaced with minimal medium. Unless otherwise stated, 24 h later, the medium was again replaced with minimal medium ± 50 μM phenylephrine with 1 μM propranolol added to block β-adrenergic receptors. Luciferase and β-galactosidase assays were performed as described (18, 22). Luciferase activity was measured for 30 s on a Bio Orbit 1251 Luminometer (Pharmacia Biotech Inc.). Data are expressed as “relative luciferase” = arbitrary integrated luciferase units/β-gal units, representative of at least three independent experiments performed with two different plasmid preparations, and represent the mean and S.E. of triplicate 35- or 24-mm wells.

To assess the effects of PKC on BNP/luciferase reporter expression, PKCβAOP, which codes for the production of a catalytically inactive form of PKC (23) was used as a control, and PKAC, which codes for the expression of a constitutively active form of PKC-β (24), was used as described previously (25). To assess the effects of Ha-Ras on BNP/luciferase reporter expression, pDCR Ha-Ras1212, which codes for the production of a constitutively active form of Ha-Ras (26, 27) was used with pCEP4 as the empty vector control, as described (28). To assess the effects of Raf-1 kinase on BNP/luciferase reporter expression, pCEP4 ΔRaf-1:ER, an expression plasmid coding for an estrogen-activated form of Raf-1 kinase (29), was used and pCEP4 was used as the empty control vector. As a second test of the effects of Raf-1 kinase, Raf BXB, which encodes a constitutively active form of c-Raf-1 (30, 31), was used.

In all transfection experiments, three identically treated cultures were used for each treatment. Each experiment was replicated at least three times, and the average of three experiments is shown.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was carried out using nuclei from neonatal rat ventricular tissue obtained as described (18, 32). Briefly, probes were prepared by Klenow fragment-mediated filling of the sticky ends of double-stranded oligonucleotides. A typical binding assay contained 20,000 cpm double-stranded probe and 10 μg of nuclear extract protein in 1 × binding buffer (10 mM Hepes, pH 7.9, 70 mM KCl, 5 mM MgCl2, 0.4 mM EDTA, 250 mM NaCl, 1 mM dithiothreitol, 0.5% Nonidet P-40, and 0.1 μg of poly(dI-cI)). Bound complexes were visualized by autoradiography of a dried nitrocellulose filter after the addition of an excess of an unlabeled competitor.

Clustered Point Mutations

Fig. 1. Diagram of the rat BNP 5'-flanking sequence. The approximate locations of several putative transcriptional enhancer elements within the full-length, 2501-bp rat BNP 5'-flanking sequence are shown at the top. Also shown is an expanded view of the promoter-proximal 140 bp of the BNP 5'-FS and the nucleotide sequence from −140 to the TATA box at about −30 nucleotides. The canonical M-CAT and GATA sequences are boxed, and the mutated nucleotides are shown beneath the native sequence for each of the cluster mutations used in the early parts of this study. The numbering scheme and nucleotide sequences have been published previously (22).
0.1 mM EGTA, 5% glycerol, 0.5 mM dithiothreitol. After a 10-min preincubation of extract with 0.1 μg of nonspecific competitor (poly[dI-dC], Pharmacia) ± competitor, the probe was added. Binding was allowed to proceed at room temperature for 30 min prior to separation of bound and free probe on a 4% native polyacrylamide gel (29:1 bis/acrylamide) in 0.5 × Tris-borate-EDTA buffer at 4 °C at 150 V. DNA-protein complexes were detected by autoradiography. The autoradiograms of some gels in this report were scanned using a Molecular Dynamics Personal Densitometer, and the resulting image was imported to Adobe Photoshop and Claris MacDraw Pro II for final figure preparation.

RESULTS

A Promoter-proximal M-CAT Element Is Critical for BNP Transcription—In a previous study we cloned and sequenced approximately 2.5 kilobase pairs of the rat BNP 5'-FS (22). A search of this sequence revealed the presence of various putative regulatory cis-elements in this region of the rat BNP gene (Fig. 1, top). To begin mapping areas of the gene involved in regulating BNP transcription, a series of reporter constructs was prepared with various lengths of the BNP 5'-FS driving luciferase expression. When primary ventricular cardiac myocytes were transfected with these constructs, it was apparent that the removal of about 1.5 kb of 5'-FS, down to BNP-535GL, had very little effect on basal or PE-inducible reporter activity (Fig. 2A). Interestingly, the removal of 137 bp between −535 and −398 appeared to result in greater basal and inducible promoter activity, suggesting the presence of repressor elements in this region of the gene. While the physiological role of such repressor elements is unknown, this result is similar to that recently observed in similar truncation analyses of the human BNP gene (33). Further truncation resulted in a gradual decline of promoter activity such that BNP-140GL displayed about 75% of the basal and inducible activities as the full-length construct. Truncation beyond this point seemed to have a more severe effect, such that BNP-116GL possessed only about 15% of the promoter activity observed with BNP-2501GL, while BNP-81GL and BNP-58GL possessed only 8% and 1% of original activity.

A series of 6-bp cluster mutations (see Fig. 1) targeted at promoter proximal regions within the full-length, BNP-2501GL was prepared. Mutants B, C, G, and H each decreased basal and PE-inducible promoter activity by about 30–50%, mutant D had no effect, while mutants E and F increased PE-inducible promoter activity by as much as 25% (Fig. 2B). Strikingly, however, mutant A, which spans the BNP 5'-FS between −103 to −98, resulted in a drastic, 98% reduction of reporter activity, implying the presence of a critical regulatory sequence in this region.

A CATTCT, or M-CAT, consensus sequence lies between −109 and −102 nucleotides in the rat BNP gene (Fig. 1). CATTCT elements bind a family of skeletal- and cardiac muscle-specific proteins originally named M (muscle)-CAT-binding proteins (see Fig. 1; Ref. 34). M-CAT-binding proteins, which are present in high levels in cardiac myocytes, are related to a family of proteins called transcription enhancement factors e.g. TEF-1, also known as enhancers of SV40 transcriptional activity, GT-1IC (34). Additionally, M-CAT-binding proteins have been implicated in the transcription of other cardiac muscle genes, such as those for cardiac troponin C, α-skeletal actin and α-myosin heavy chain, and β-myosin heavy chain (34–38).

Accordingly, in the context of 2.5 kb of the BNP 5'-FS, a 2-bp double-point mutation was prepared (ΔM-CAT), which was predicted from previous studies to specifically disrupt the binding of M-CAT-binding protein (39). Basal and PE-inducible promoter activity from ΔM-CAT were reduced by at least 98%, as seen for mutation A (Fig. 2C), emphasizing the absolute requirement for an intact M-CAT element in the promoter-proximal region of the BNP gene.

The GATA family of transcription factors, which have been implicated as regulators of myocardial cell BNP gene expression (22, 40–42), bind to DNA possessing the consensus, WGTAR sequence (43, 44). Since the cluster mutations B and C displayed somewhat reduced basal and PE-inducible promoter activity (Fig. 2B), and since these mutations were located over GATA-binding protein consensus sequences (Fig. 1, lower), point mutations known to disrupt GATA binding...
were prepared in the context of BNP-2501GL. In agreement with our previous study, which tested similar mutations in reporter constructs possessing only 116 bp of BNP 5'-FS (i.e. BNP-116GL) (22), the mutation at GATA(-95) was of little consequence, while the mutation at GATA(-84) resulted in an approximate 20% decline in PE-inducibility (Fig. 3). However, when the mutations were combined (i.e. GATA(-95, -84)), there was a much greater than expected, 60% reduction in basal and PE-inducible reporter activity (Fig. 3). One explanation for this unexpected reduction is the possibility that these two GATA sites, and perhaps the protein(s) that bind there, are interactive, either with each other, or with other elements. For example, perhaps a GATA-binding protein (BP) must bind to the BNP gene somewhere between about -100 and -80 to confer optimal promoter activity. If a GATA-BP binds to the native BNP gene primarily at GATA(-84), which would leave enough room for an M-CAT-binding protein to bind at -106, one would expect the -95 mutation to be of little consequence. However, the -84 mutation might be expected to disrupt GATA-BP binding there, perhaps promoting GATA-BP binding to the alternate, and apparently less effective GATA site at -95. Then, mutating both -84 and -95 would completely disrupt all GATA binding, resulting in the unexpectedly low activity observed in the GATA(-95, -84) double mutant.

To test for possible interactions between the GATA and M-CAT elements, the various GATA-directed mutations were combined with the ΔM-CAT mutation. As expected, overall promoter activities of the constructs harboring the ΔM-CAT mutation were severely decreased, probably due to the disruption of the consensus M-CAT site. Additionally, the GATA(-95) mutation had no additional effect when combined with the ΔM-CAT mutation; however, the GATA(-84) and GATA(-95, -84) mutations further decreased promoter activity. The abilities of the GATA-directed mutations to decrease promoter activity in the ΔM-CAT constructs, as well as those possessing the native M-CAT site, suggests that the GATA sites behave independently of the M-CAT site.

A TEF-1-like Protein Binds to the BNP M-CAT Element—Due to its absolute requirement for basal and inducible BNP promoter activity, the properties of the M-CAT element in the BNP 5'-FS were studied further. To evaluate whether cardiac nuclear proteins could bind to the promoter-proximal M-CAT site in a manner consistent with the functional consequences of the mutations in the reporter genes, EMSA were carried out. A variety of oligonucleotides were prepared either synthetically or by restriction digestion of the appropriate BNP/luciferase reporters (Fig. 4); the BNP(-2113/(-95) oligomer was used as a labeled probe and a competitor, while the others were used as competitors.

In the absence of any competitor, cardiac nuclear proteins and the BNP probe formed a single, major complex observed by EMSA (Fig. 5, lane 2). While this complex was disrupted effectively using the unlabeled probe (Fig. 5, lane 3), or an oligonucleotide containing a canonical M-CAT element modeled after that in the chicken troponin gene (Fig. 5, lane 4), the BNP(-113/-95) oligomer, which mimics the double point mutation, ΔM-CAT, was an ineffective competitor (Fig. 5, lane 5). Oligonucleotides mimicking larger stretches of the promoter-proximal region of the BNP 5'-FS and containing the M-CAT site (e.g. BNP(-116/(-52) and BNP(-116/(-71)), were also effective competitors (Fig. 5, lanes 6 and 8). However, when
these larger oligonucleotides contained either the double-point mutation in the putative M-CAT site (i.e., BN(113/95-2)), or cluster mutation A (i.e., BN(113/95 mutant A)(Fig. 5, lanes 7 and 9), they served as ineffective competitors, consistent with a requirement for the binding of an M-CAT/TEF-1-related protein to this region of the gene. Oligonucleotides mimicking mutants B and D, which harbored 6-bp clustered changes in regions outside the putative M-CAT region, were effective competitors (Fig. 5, lanes 10 and 11), consistent with the relatively minor roles of these mutations on BNP promoter function. As further controls, it was shown that neither the human c-fos serum response element (SRE), the collagenase 12-O-tetradecanoylphorbol-13-acetate response element (TRE), nor the promoter-proximal rat ANF SRE (18) acted as competitors of the shifted complex (Fig. 5, lanes 12–14). Thus, the EMSA analyses are consistent with the required binding of a protein possessing the characteristics of M-CAT-binding protein, or TEF-1, to the putative M-CAT element in the BNP 5'-FS for optimal PE-inducible promoter activity.

Roles of PKC, Ras, and Raf-1 in BNP Inducibility—Further experiments were carried out to investigate whether the promoter-proximal sequences, such as the M-CAT element, might participate in BNP promoter activation by PE and by intracellular signals activated by PE. In cardiac myocytes, α1-adrener-
sequences residing both distal and proximal to BNP-2501GL (Fig. 6). Ever, it stimulated BNP-140GL only about 40% as well as Ha-Ras served as a potent activator of BNP-2501GL; however, Raf-1 induced reporter expression from BNP-140GL relatively poorly, by only about 25% as well as from BNP-2501GL (Fig. 6D). These results indicated that sequences lying proximal to −140 bp were responsible for significant levels of inducibility, although they displayed somewhat differential responsiveness to the stimuli, conferring relatively strong induction in response to PE, PKC, and Ha-Ras, but very weak induction in response to Raf-1. To explore further the importance of the promoter-proximal M-CAT element in basal and inducible reporter activity, the BNP-related M-CAT sequences were cloned upstream of position −81 in BNP-81GL, a construct that normally expresses very low reporter activity. In this context the M-CAT sequences were found to confer a significant recovery of basal as well as PE, PKC, and Ras inducibility to BNP-81GL, with the extent of basal and inducible reporter expression being approximately proportional to the number of M-CAT sites (Fig. 7).

These results supported the hypothesis that the promoter-proximal M-CAT element can contribute to BNP inducibility in response to PE, and two of its major effectors, PKC and Ha-Ras. This view is corroborated further by the lack of PE inducibility of ΔM-CAT/BNP-2501GL (Fig. 2C) and a decrease in Ha-Ras inducibility of this same construct by over 80% (data not shown). However, since Ras is a known activator of Raf-1 in the ERK/MAPK pathway, the low Raf-1 responsiveness of BNP-140GL was inconsistent with this hypothesis, implying that while they were responsive to Ha-Ras, sequences in BNP-140GL were poorly responsive to Raf-1. Accordingly, the ability of Raf-1 kinase to activate the M-CAT/BNP-81GL constructs was evaluated using the ΔRaf:ER expression construct. Consistent with its poor ability to activate BNP-140GL, Raf-1 did not enhance reporter expression significantly from either 2X- or 4X-M-CAT/BNP-81GL (Fig. 8A). As expected, BNP-2501GL was induced significantly by Raf-1, while BNP-140GL was induced by less than 15% compared to BNP-2501GL. The lack of Raf-1 responsiveness supports the view that the PE-, PKC- and Ras-mediated increases in luciferase expression from 2X- or 4X-M-CAT/BNP-81GL, shown in Fig. 7, represent induction above basal expression; thus, the M-CAT element is apparently required for basal transcription and it can mediate inducible transcription. To confirm the unexpected lack of responsiveness of the M-CAT element to Raf-1, a different Raf-1 expression construct, Raf BXB, was used in a similar experiment. Again, Raf-1 kinase was ineffective as an enhancer of reporter expression from 4X-M-CAT/BNP-81GL (Fig. 8B), but like ΔRaf:ER, it served as a strong inducer of reporter expression from BNP-2501GL and a relatively weak inducer of BNP-140GL.

**DISCUSSION**

The results of this study indicate that BNP transcription requires a promoter-proximal M-CAT element that can mediate transcriptional stimulation in response to PE, as well as PKC and Ras, both of which are activated by α1-adrenergic agonists. Interestingly, however, the M-CAT element does not appear to contribute to BNP induction in response to Raf-1 kinase, which is also activated by α1-adrenergic agonists. Instead, the Raf-1-inducible elements reside distally, between −2501 and −140 of the BNP 5′-FS, and are yet to be identified. Accordingly, this is the first report to suggest that in addition to conferring inducibility in response to PKC, which was previously shown for the β-MHC gene (37), M-CAT elements in muscle-specific genes might also be responsive to Ras-activated signals, but not those involving Raf-1 kinase.

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4 The mitogen-activated protein kinases (MAPKs) consist of three related protein kinases: ERK/MAPK, JNK, and the p38 MAPK (71, 72).
Although M-CAT elements have not been previously shown to mediate Ras inducibility, a large body of information supports a role for M-CAT sequences as determinants of cardiac and skeletal muscle-specific gene expression (35, 57, 58). Cardiac and striated muscle tissues are particularly enriched in M-CAT binding factors; however, they are also found in many other cell- and tissue types (39, 59–61). Nonetheless, it is

**FIG. 6. Effects of constitutively active PKC, Ha-Ras, and Raf-1 kinase on BNP promoter activity.** Myocardial cells were transfected with BNP-2501GL, BNP-140GL, or BNP-81GL and CMV-β-galactosidase. For each experiment, the relative luciferase values obtained with BNP-2501GL were set to 100% and the other values normalized accordingly. Values are means ± S.E., n = 3 cultures. **Panel A,** cultures were treated with PE as in Fig. 2. **Panel B,** in addition to the BNP/luciferase and CMV-β-galactosidase constructs, myocardial cells were also transfected with 15 μg of a control plasmid, pβOP (Con), which codes for the production of catalytically inactive PKC, or 15 μg of pPKAc (PKC), which codes for the production of a constitutively active form of PKC-β (24). After 48 h in minimal media, the cultures were extracted and assayed for reporter enzyme activities. **Panel C,** in addition to the BNP/luciferase and CMV-β-galactosidase constructs, myocardial cells were also transfected with 15 μg of a control plasmid, pCEP4 (Con), which contains no insert, or 15 μg of the plasmid Ha-RasV12 (H-Ras), which codes for the production of a constitutively active form of Ha-Ras (26, 27). After 48 h the cultures were extracted and assayed for reporter enzyme activities. **Panel D,** in addition to the BNP/luciferase and CMV-β-galactosidase constructs, myocardial cells were also transfected with 15 μg of pCEP4/Raf-1:ER (Raf-1), which codes for the production of an estrogen-inducible form of Raf-1 kinase (50). After 48 h of incubation in minimal medium (Con) or in minimal medium containing 1 μM estradiol (Raf), the cultures were extracted and assayed for reporter enzyme activities. In control experiments it was shown that estradiol (48 h) did not alter the relative luciferase values in cultures transfected with pCEP4 (data not shown; see also Ref. 50).

**FIG. 7. Effect of isolated M-CAT sequences on PE, PKC, and Ha-Ras-mediated activation of BNP-81GL.** Reporter plasmids were prepared so that either two or four tandem repeats of the M-CAT element located at −105 bp in the native BNP 5'-FS were cloned 5' of the BNP sequences in BNP-81GL (see “Experimental Procedures”). Myocardial cells were then transfected with BNP-81GL, 2XM-CAT/BNP-81GL, or 4XM-CAT/BNP-81GL and CMV-β-galactosidase. Cultures were maintained for 24 h in serum-free medium and then they were incubated for 6 h in serum-free medium ± 50 μM PE, 1 μM propranolol, followed by extraction and reporter enzyme assay (panel A), or they were also transfected with PKC expression constructs (panel B), or Ha-Ras expression constructs (panel C), as described in the legend for Fig. 6, maintained for 48 h, and then extracted and assayed for reporter enzyme activities. Values are means ± S.E., n = 3 cultures.

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structs to activate M-CAT-containing reporter genes for PKC (63). Indeed, the abilities of PKC expression constructs to alter growth factor-

skeletal growth factor-

expression in response to PKC activation remains unknown. For example, TEF-1 possesses known, PKC phosphorylation sites that could alter the ability of the protein to bind to M-CAT elements and thus confer transcriptional activation. However, it is also possible, if not probable, that in comparison to PKC's abilities to activate transcription through SRF or AP-1, it may indirectly activate TEF, perhaps by way of altering the activities of other kinases or phosphatases which would ultimately alter the phosphorylation state of TEF, or closely associated proteins. Another possibility is that PKC and Ras might converge on a single pathway to ultimately effect enhancement of transcription through TEF-1.

Since PKC and Ras can both activate Raf-1 and, thus, ERK/MAPK, it is tempting to speculate that ERK/MAPK might serve as a downstream effector through which PE can stimulate transcription via TEF-1. However, the finding in this study that the M-CAT element appears to participate in Ras- but not Raf-1-inducible BNP promoter activation suggests a role for Ras-dependent, ERK/MAPK-independent pathways. Consistent with this are recent results demonstrating that PD 098059, a specific MEK inhibitor (65), blocks PE-inducible ERK/MAPK in cardiac myocytes by 80%, but does not block PE-inducible BNP promoter activity, as measured with BNP-2501GL (66). Among the Ras-dependent, ERK/MAPK-independent pathways of possible interest are those converging on the activation of the JNK and p38 members of the MAPK family (67). It is possible, therefore, that p38 MAPK and/or JNK might ultimately affect transcriptional enhancement through TEF-1, or accessory factors. Further, it is reasonable to hypothesize that even though all three MAPK family members might be activated in cardiac myocytes in response to PE, they could differentially effect changes in transcription. Indeed, the MAPKs display some substrate selectivity amongst certain transcription factors. For example, while JNK appears to preferentially phosphorylate c-Jun, ERK/MAPK preferentially phosphorylates c-Myc, p38 MAPK preferentially phosphorylates ATF-2 and ERK/MAPK and p38 MAPK phosphorylate Elk-1 to similar extents (68). Thus, to further dissect the mechanism of Ras-induction through TEF-1, it will be of interest to evaluate the abilities of all three MAPK family members to enhance transcription through BNP promoter-proximal M-CAT element.

In summary, the results from this study add new information to our understanding of the cis-elements and the signaling mechanisms responsible for α1-adrenergic agonist-mediated induction of BNP and other cardiac genes. We have found that in comparison to the α-skeletal actin and β-MHC genes, a promoter-proximal M-CAT element is important for α1-adrenergic inducibility of the BNP gene, and that this induction could be mediated at least partly by PKC. The present study has added further to our knowledge of how TEF-1 might mediate cardiac gene expression, demonstrating that the M-CAT element confers Ras-dependent induction, but in a Raf-independent manner; this finding implies the involvement of p38 and/or JNK/MAPK-mediated events, and/or other Ras-activated pathways not yet clearly identified. The mechanisms by which TEF-1 responds to these, and perhaps other signaling pathways, remain unknown. And while the recent findings that there are multiple forms of the TEFs (69, 70) might also add potential complication to the mechanism, it is also possible that such multiple pathways provides the potential for somewhat independent induction of TEF-responsive genes to match a variety of physiological requirements. Future studies of how the various forms of the TEFs interact with other accessory proteins such as E-box-binding proteins (62) or nearby transcription factors such as GATA-binding proteins (22, 40–42) will be required to further our understanding of this complex gene induction mechanism.

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Fig. 8. Effect of Raf-1 kinase on M-CAT-mediated activation of BNP/luciferase. Myocardial cells were transfected with BNP-81GL, BNP-140GL, BNP-2501GL, 2XM-CAT/BNP-81GL, or 4XM-CAT/BNP-81GL, as shown in the figure, and CMV-β-galactosidase. In addition, cultures were transfected with Raf-1 kinase expression constructs (ΔRaf:ER, panel A; or Raf BXB, panel B), as described in the legend for Fig. 6. After 48 h in minimal medium (estradiol in panel A), the cultures were extracted and assayed for reporter enzyme activities. In control experiments it was shown that estradiol (48 h) did not alter the relative luciferase values in cultures transfected with pCEP4 (data not shown; see also Ref. 60). Values are means ± S.E., n = 3 cultures.
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