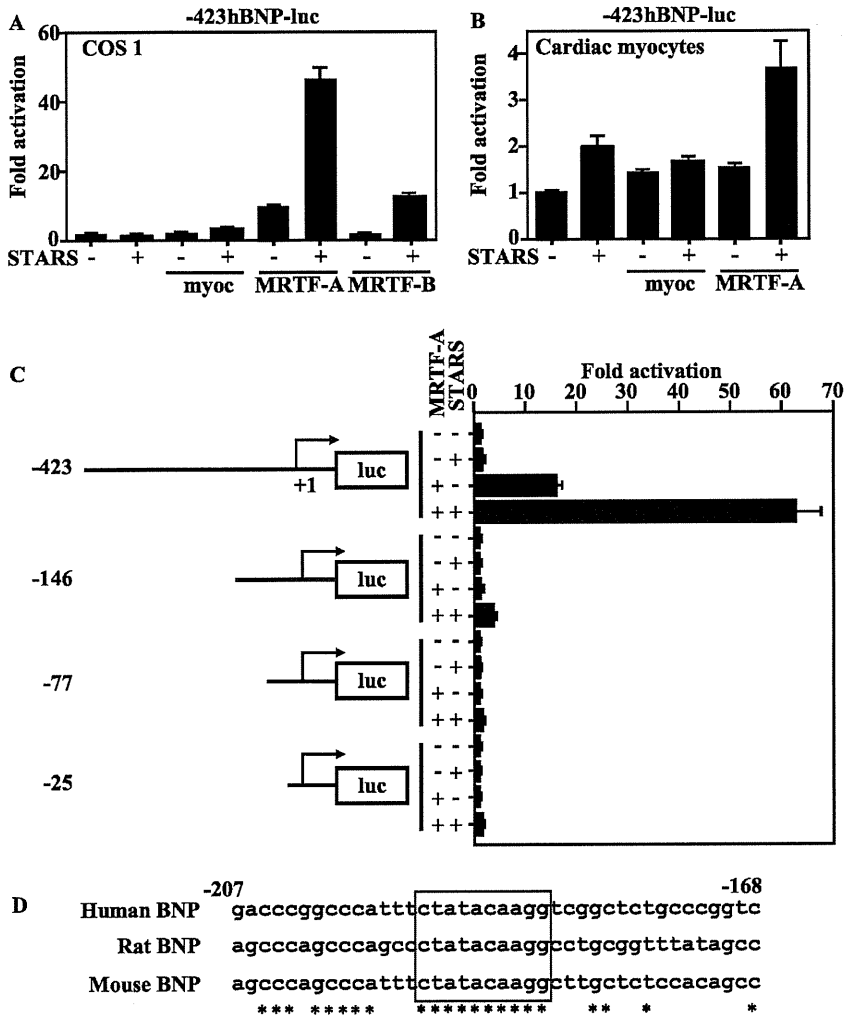

FIG. 4. Identification of the MRTF-A-responsive element in the BNP promoter. (A) MRTF-A and STARS activate −423BNP-luc in COS1 cells. COS1 cells were cotransfected with −423BNP-luc and expression vectors encoding STARS, myocardin (20 ng), MRTF-A (20 ng), or MRTF-B (20 ng). (B) Ventricular myocytes were cotransfected with −423BNP-luc and expression vectors encoding STARS, myocardin (20 ng), or MRTF-A (20 ng). (C) Ventricular myocytes were transfected with a luciferase gene driven by various DNA fragments from the BNP upstream region. Deletion from bp −423 to −146 abolished the response to MRTF-A and STARS in ventricular myocytes. A LacZ gene driven by the RSV promoter was also transfected as an internal control. (D) Sequences between bp −207 and −168 are shown. CArG box-like sequences completely conserved among humans, rats, and mice are shown in the red box.

din's transcription-activating domain is well conserved in MRTF-A, we tested whether p300 also participates in MRTF-A-induced activation of the BNP promoter. When we cotransfected cultured ventricular myocytes with a BNP-luciferase gene and expression vectors encoding MRTF-A, STARS, and/or wild type p300 or a dominant-negative p300 mutant (42), we found that wild-type p300 enhanced MRTF-A-mediated BNP promoter activation. In contrast, the dominant-negative p300 mutant significantly attenuated MRTF-A-induced activation of BNP gene transcription in both NIH 3T3 cells and cardiac myocytes (Fig. 6F and G). We also confirmed the physical interaction between MRTF-A and p300 (see Fig. S1A in the supplemental material). p300 thus appears to participate in MRTF-A-mediated activation of BNP gene transcription.

Mechanical stretch increases BNP promoter activity through SRF. We hypothesized that the stretch-induced nuclear accumulation of MRTF-A we observed contributes to the stretch-induced increase in BNP gene transcription. To test that idea, we examined whether CArG in the BNP promoter is responsible for stretch-induced BNP gene expression. We initially confirmed that expression of endogenous BNP and SM22α mRNA is upregulated in an *in vitro* model of mechanical stretch, as reported previously (Fig. 7A and B) (18). Consistent with stretch-induced expression of the endogenous gene, the BNP promoter was activated by mechanical stretch in neonatal ventricular myocytes (Fig. 7C). Mechanical stretch also increased SM22α promoter activity in an SRF binding site-dependent manner (Fig. 7D) and activated a luciferase reporter gene controlled by tandem CArG boxes (4× CArG-luciferase), confirming that myocardial stretch activates SRF-dependent transcription (Fig. 7E). Mutation of CArG within the BNP promoter significantly reduced the response to mechanical stretch, suggesting the CArG element transduces the signal initiated by mechanical stretch (Fig. 7C). Stretch-in-

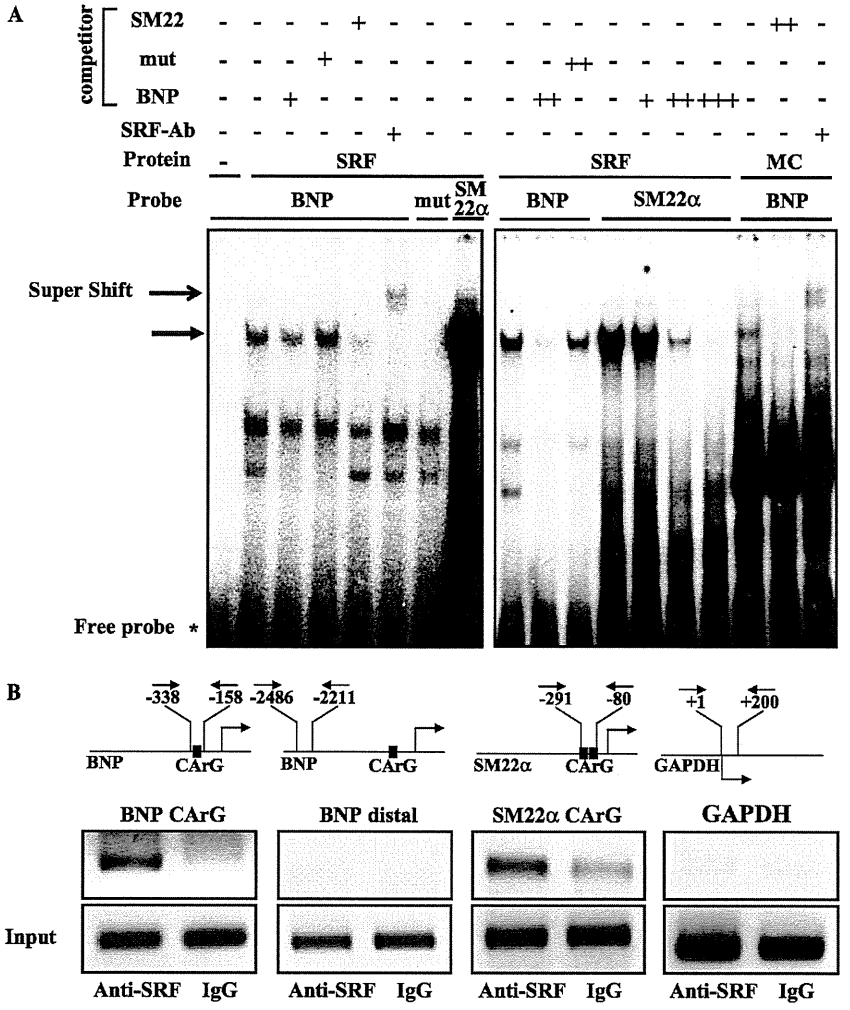

FIG. 5. SRF binds to the CArG-like region in the BNP gene. (A) EMSAs were performed using *in vitro*-translated SRF (indicated as SRF) or nuclear extract from cultured cardiac ventricular myocytes (indicated as MC) and the BNP CArG-box-like region, with (mut) or without mutation, or the SM22 CArG-box (SM22) as a probe. The unlabeled BNP CArG-box-like region, mutated BNP CArG box-like region, or SM22 CArG box was used as a cold competitor (+, 10-fold; ++, 100-fold; and +++, 1,000-fold excess). Anti-SRF antibody was used for supershift assays. Arrows indicate bands representing a specific DNA-SRF complex. The lowest of the EMSA bands was markedly weakened when the wild-type and mutant BNP CArG probes were applied in the presence of the wild-type and mutant BNP CArG cold probes but not the cold SM22 CArG probe, indicating that these bands represent a complex between the BNP probe and a nonspecific protein binding to a sequence other than the core CArG-like sequence in the BNP probe. Note that this band is not affected by anti-SRF antibody. (B) ChIP assays were performed with ventricular myocytes using anti-SRF antibody. PCR was performed with chromatin from around the CArG box in the BNP promoter region, around the region 2 kbp distal to the CArG box in the BNP promoter, around the two CArG boxes in the SM22 promoter region, and around the first exon of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Aliquots of chromatin obtained before immunoprecipitation were also analyzed as input. Representative PCRs with identical results in three independent experiments are shown. Schematic representations of the location of the PCR primers in each gene are shown in the upper panel.

duced BNP promoter activity was also diminished in the presence of latrunculin B or C3, which inhibit the nuclear translocation of MRTF-A (Fig. 7C and 1C), and in the presence of siRNA targeting MRTF-A, confirming that MRTF-A mediates stretch-induced BNP promoter activation (Fig. 7F).

MRTF-A mediates ET-1- and AngII-induced activation of BNP gene transcription. Since Rho activation is involved in cardiac hypertrophy induced by several humoral factors, including AngII, phenylephrine, and ET-1 (2, 20, 52), we also tested whether nuclear translocation of MRTF-A occurs following stimulation of cardiac myocytes with ET-1 or AngII. We found that both molecules enhanced nuclear accumulation of MRTF-A in ventricular myocytes (Fig. 8A and B) and that they activated the BNP promoter (Fig. 8C and D). Moreover, mutation of CArG significantly reduced the response, confirming that SRF activation plays an important role in ET-1- and AngII-induced activation of BNP gene transcription (Fig. 8C and D). ET-1-induced activation of the BNP promoter was blocked in the presence of a dominant-negative myocardin mutant that lacked a transcription-activating domain and inhibited the activities of all myocardin family members, including myocardin and MRTF-A and -B, which suggests their involvement in ET-1-induced, SRF-mediated activation of the BNP promoter (Fig. 8E). The dominant-negative myocardin

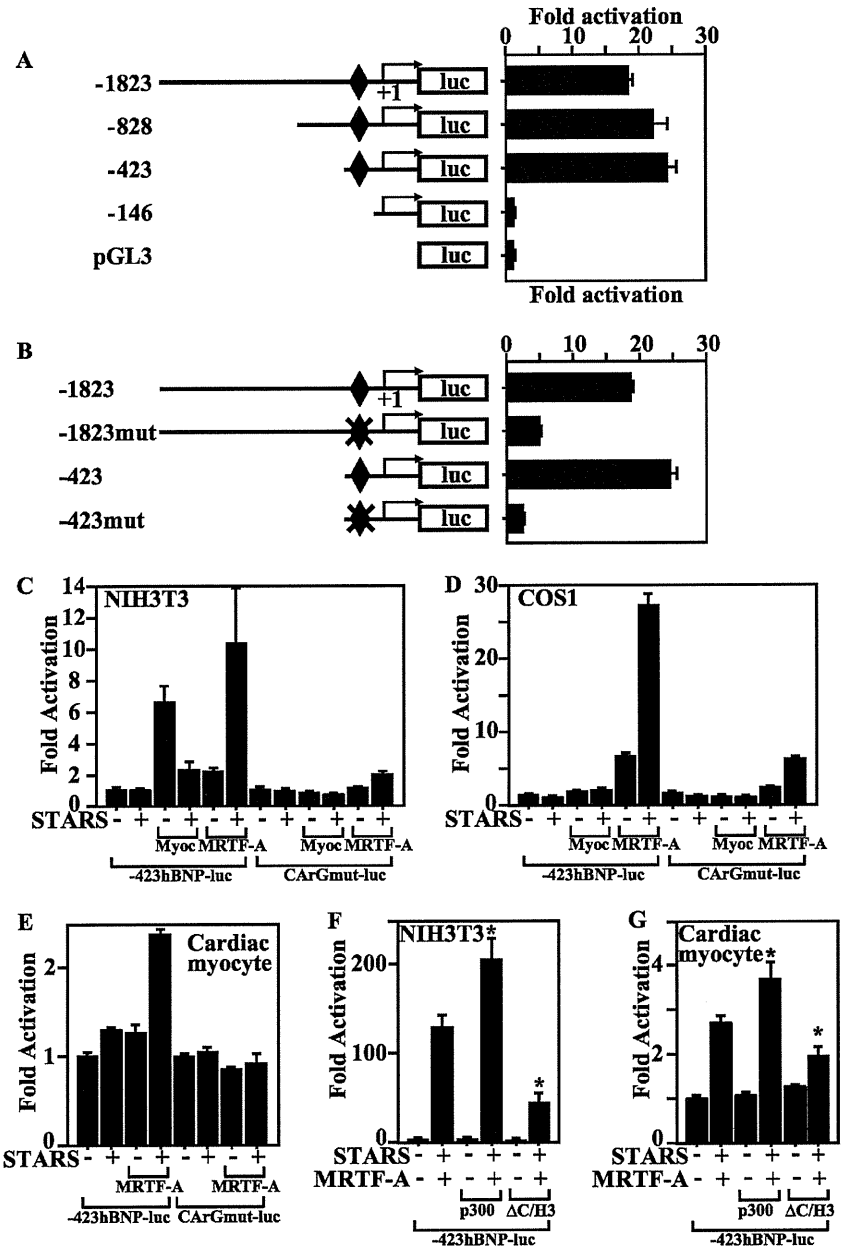

FIG. 6. MRTF-A directly activates the BNP promoter via the SRF-binding site. (A) COS1 cells were cotransfected with a luciferase gene driven by various DNA fragments from the BNP upstream region and an expression vector encoding the SRF-VP16 fusion protein. The SRF-binding site is shown as a diamond. (B) COS1 cells were cotransfected with a luciferase gene driven by 1,823 bp or 423 bp of the BNP upstream region, with (mut) or without mutation of the identified SRF-binding site, and an expression vector encoding the SRF-VP16 fusion protein. The SRF-binding site is shown as a diamond. (C and D) NIH 3T3 (C) or COS1 (D) cells were cotransfected with a luciferase gene driven by 423 bp from the BNP upstream region, with (CArGmut-luc) or without (−423hBNP-luc) mutation of the SRF-binding site, and an expression vector encoding STARS, myocardin, or MRTF-A. (E) Cardiac myocytes were cotransfected with −423hBNP-luc or CArGmut-luc and an expression vector encoding STARS or MRTF-A. (F and G) p300 contributes to MRTF-A-mediated activation of SRF. NIH 3T3 cells (F) or cultured cardiac myocytes (G) were cotransfected with −423hBNP-luc and expression vectors encoding STARS, MRTF-A, and wild-type p300 or a dominant-negative p300 mutant (ΔC/H3). Values are shown as means ± SEM (n = 4 each). *, $P < 0.05$ versus results with −423hBNP-luc with STARS and MRTF-A in the absence of p300.

mutant also inhibited ET-1-induced activation of a reporter gene controlled by tandem CArG boxes (4× CArG-luciferase) (Fig. 8F) and the ANP promoter (hANP-luciferase) (Fig. 8G), again suggesting the involvement of myocardin and MRTF family proteins. In addition, siRNA targeting MRTF-A but not myocardin inhibited ET-1-induced activation of the BNP promoter in ventricular myocytes, indicating that, as with mechanical stretch, nuclear translocation of MRTF-A mediates ET-1-induced hypertrophic signaling to activate BNP gene transcription (Fig. 8H). Finally, MRTF-A knockdown also inhibited ET-1-induced activation of 4× CArG-luciferase and hANP-luciferase (Fig. 8I and J), suggesting the general in-

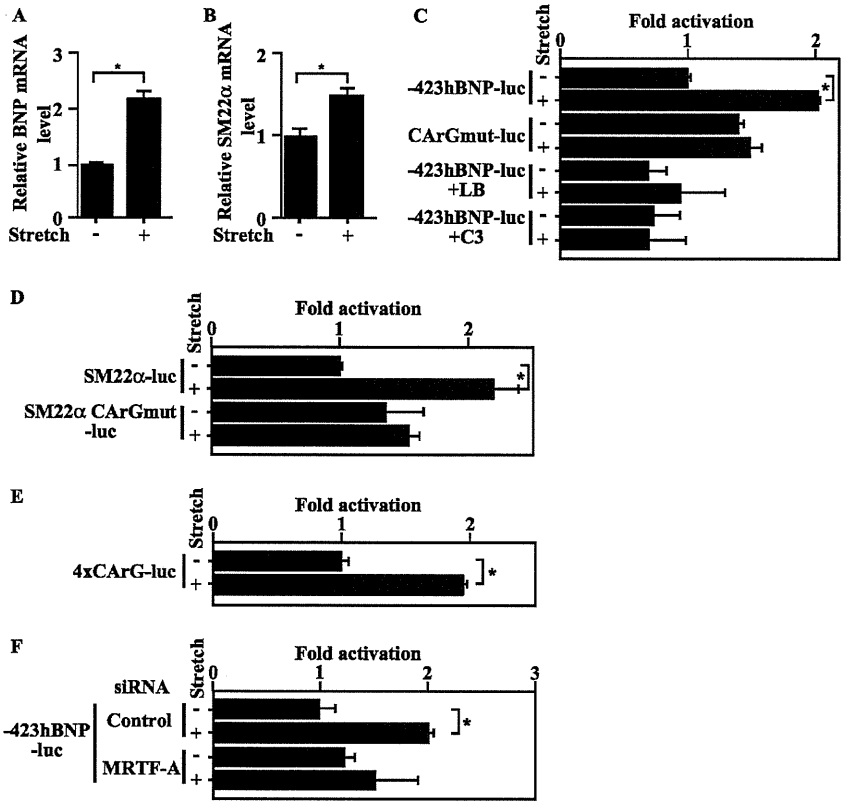

FIG. 7. Mechanical stretch activates the BNP promoter via the SRF-binding site. (A and B) Expression of BNP (A) or SM22α (B) mRNA was assessed using real-time RT-PCR in rat neonatal ventricular myocytes subjected to 20% mechanical stretch for 1 h. (C) Ventricular myocytes transfected with a luciferase gene driven by 423 bp from the BNP upstream region, with (CArGmut-luc) or without (−423hBNP-luc) mutation of the SRF-binding site, were subjected to mechanical stretch in the presence or absence of 0.5 μM latrunculin B (LB) or 2.5 μg/ml C3 exoenzyme (C3). (D) Ventricular myocytes transfected with a luciferase gene driven by the SM22 α upstream region, with (SM22α CArGmut) or without (SM22α-luc) mutation of SRF-binding sites, were subjected to mechanical stretch. (E) Ventricular myocytes transfected with 4× CArG-luc were subjected to mechanical stretch. (F) Ventricular myocytes transfected with −423hBNP-luc were subjected to mechanical stretch in the presence or absence of siRNA targeting MRTF-A. For all transfections, a LacZ gene driven by an RSV promoter was also transfected as an internal control. In all graphs, values are shown as means ± SEM; *, $P < 0.05$ versus results for the unstretched control.

volvement of MRTF-A in G protein-coupled receptor-induced increases in SRF-mediated gene transcription. MRTF-A knockdown also significantly attenuated ET-1- and AngII-induced increases in the size of cardiac myocytes (Fig. 8K; see also Fig. S1B in the supplemental material).

Reduced hypertrophic responses to chronic AngII treatment in MRTF-A$^{-/-}$ mice. We next examined the role of MRTF-A in chronic cardiac remodeling, a process in which neurohumoral factors are known to play pivotal roles. When we subcutaneously administered AngII for 2 weeks, systolic blood pressure (SBP) was similarly increased in wild-type and MRTF-A$^{-/-}$ mice (Fig. 9A). Under these conditions, cardiac hypertrophy, indicated by significant increases in HW/BW ratios, was observed in wild-type mice but not in MRTF-A$^{-/-}$ mice (Fig. 9B and C). Moreover, the expression of hypertrophy-related genes, including BNP and skeletal α-actin, induced by chronic AngII treatment was significantly weaker in MRTF-A$^{-/-}$ mice than in wild-type mice (Fig. 9D and E). This supports the notion that MRTF-A is necessary for chronic AngII-induced cardiac hypertrophy. In addition, echocardiographic analysis showed that AngII-induced increases in the thickness of the interventricular septum and left ventricular posterior wall, as well as the calculated left ventricular mass, were all significantly attenuated in MRTF-A$^{-/-}$ mice compared to findings for wild-type mice (Table 1). Levels of myocardin and MRTF-B mRNA were not significantly altered in wild-type or MRTF-A$^{-/-}$ mice, with or without AngII treatment (Fig. 9F and G), nor were levels of MRTF-A mRNA in wild-type mice (Fig. 9H). Taken together, these results demonstrate that MRTF-A is a crucial participant in cardiac hypertrophy signaling during the cardiac remodeling induced by AngII.

DISCUSSION

Mechanical stress is one of the earliest stimuli promoting the induction of cardiac hypertrophy, which is characterized in part by reactivation of the fetal cardiac gene program (e.g., ANP, BNP, skeletal α-actin, β-myosin heavy chain, SM22α, and smooth muscle α-actin) (4, 15, 25). Using an *in vitro* cardiac myocyte model, it has been shown that mechanical stretch activates a variety of intracellular signaling molecules, including PKC, MAPKs, p90 and p70 S6 kinases, Jak-STAT, and Rho family small G proteins (1, 16, 27, 47, 48, 51, 62). The precise molecular mechanism by which mechanical stretch is

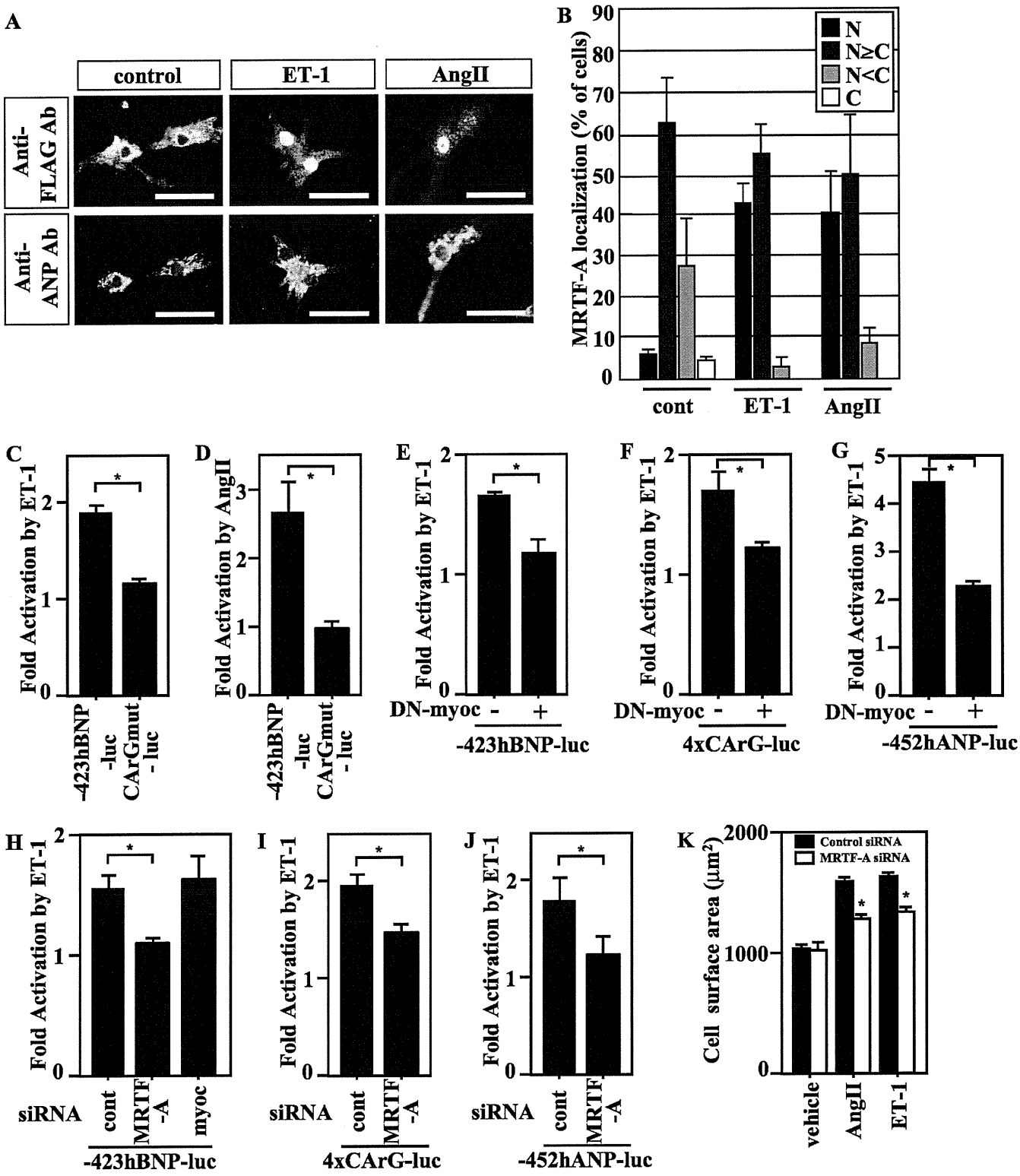

FIG. 8. ET-1 and AngII induce nuclear accumulation of MRTF-A. (A) Cultured neonatal rat ventricular myocytes infected with adenovirus encoding FLAG-tagged MRTF-A (Ad-MRTF-A) were treated with 100 nM ET-1 or 100 nM AngII for 1 h. The subcellular distribution of MRTF-A was determined by immunostaining of the FLAG epitope (green). Myocytes were positively stained with anti-ANP antibody (red). Bars represent 50 μm. (B) Graphs show the percent MRTF-A localization: N, exclusive staining of MRTF-A in the nucleus; N≥C, nuclear staining of MRTF-A was greater than or equal to the cytoplasmic staining; N<C, greater staining of MRTF-A in the cytoplasm than in the nucleus; C, exclusive staining of cytoplasmic MRTF-A (100 infected cells were counted). (C and D) Ventricular myocytes transfected with a luciferase gene driven by the human BNP upstream region, with (CArGmut) or without (−423hBNP-luc) mutation of SRF-binding sites, were treated with 100 nM ET-1 (C) or 100 nM AngII (D) for 48 h. (E to G) Ventricular myocytes cotransfected with a luciferase gene driven by the BNP upstream region

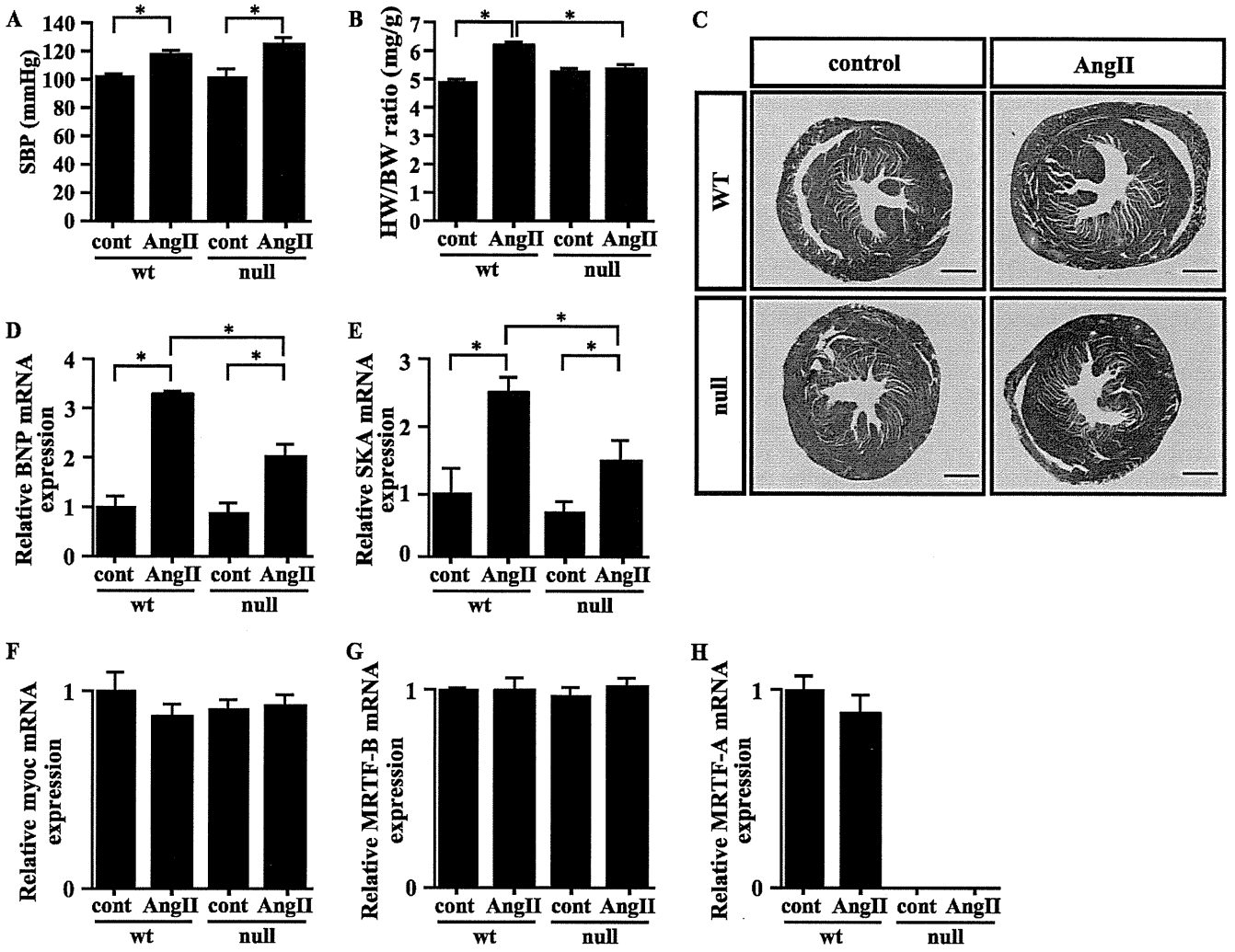

FIG. 9. MRTF-A participates in AngII-induced hypertrophic signaling *in vivo*. (A) SBP (mm Hg) in wild-type (wt) and MRTF-A$^{-/-}$ (null) mice, with or without AngII infusion for 2 weeks (n = 4 each for wild-type mice and 8 each for MRTF-A$^{-/-}$ mice). (B) HW/BW ratios (mg/g) in wt and MRTF-A$^{-/-}$ mice, with or without AngII infusion for 2 weeks (10 to 12 weeks of age; n = 4 each for wild-type mice and 8 each for MRTF-A$^{-/-}$ mice). (C) Hearts were sectioned coronally and stained with hematoxylin-eosin. Scale bar, 1 mm. (D to H) BNP (D), skeletal α-actin (SKA) (E), myocardin (myoc) (F), MRTF-B (G), or MRTF-A (H) gene expression was assessed by real-time RT-PCR using total RNA extracted from wt and MRTF-A$^{-/-}$ mice, with or without AngII for 2 weeks (n = 3 for wild-type mice without AngII, 4 for wild-type mice with AngII, 5 for MRTF-A$^{-/-}$ mice without AngII, and 6 for MRTF-A$^{-/-}$ mice with AngII). In all graphs, values are shown as means ± SEM; *, $P < 0.05$.

transduced to transcriptional activation remained unresolved, however. In the present study, we have shown that Rho- and actin treadmill-dependent nuclear accumulation of MRTF-A contributes to the transduction of mechanical stress to the transcriptional activation of SRF-dependent fetal cardiac genes in cardiac myocytes. In mice lacking MRTF-A, induction of BNP and other fetal cardiac genes in response to both acute and chronic pressure overload was significantly attenuated. We identified a functional SRF-responsive element in the 5′-flanking region of the BNP gene as a novel target of MRTF-A. In addition, we also showed the involvement of MRTF-A in chronic cardiac remodeling, a process in which neurohumoral factors play a pivotal role. Following stimulation with AngII or ET-1, MRTF-A was translocated into the nuclei of cardiac

(−423hBNP-luc) (E), by 4× CArG (4xCArG-luc) (F), or by the human ANP promoter (−452hANP-luc) (G), as well as an expression vector encoding a dominant-negative myocardin mutant (DN-myocardin), were treated with ET-1. (H) Ventricular myocytes cotransfected with −423hBNP-luc and siRNA targeting MRTF-A or myocardin were treated with ET-1. (I and J) Ventricular myocytes cotransfected with siRNA targeting MRTF-A and 4× CArG-luc (I) or −452hANP-luc (J) were treated with ET-1. In all luciferase experiments, a LacZ gene driven by the RSV promoter was also transfected as an internal control. The graphs show the fold activation by ET-1 in each group. In all graphs, values are shown as means ± SEM; *, $P < 0.05$. (K) Cultured ventricular myocytes in which MRTF-A expression was knocked down using siRNA were less susceptible to hypertrophic myocyte growth induced by ET-1 (100 nM) or AngII (100 nM) than control myocytes. Graphs show myocyte size (μm^2) in each group. Values are shown as means ± SEM; *, $P < 0.05$ versus results for control siRNA in each group.

myocytes, where it activated SRF. Moreover, MRTF-A$^{-/-}$ mice showed significantly weaker hypertrophic responses than their wild-type littermates. Collectively, these findings indicate that MRTF-A is a common mediator of mechanical stress- and neurohumoral stimulation-induced prohypertrophic signaling.

There are two distinct pathways leading to SRF activation: one involves the phosphorylation of ternary complex factors in Ets domain family proteins, while the other is controlled by Rho family small GTPases and actin dynamics (10, 14, 40, 56). MRTF-A is involved in the latter (35, 50). In the regulation of some immediate-early genes, Ets domain family proteins, such as Elk-1, which is phosphorylated by extracellular signal-regulated kinase (ERK), associate with and activate SRF independently of MRTF-A (14, 22, 40, 54, 61). The fact that mechanical stretch activates ERKs therefore suggests that during mechanical stress, the ERKs-Elk1 pathway contributes to the increased expression of several immediate-early genes through activation of SRF. Thus, the genetic response to mechanical stretch involves both MRTF-A-dependent and -independent SRF activation. Moreover, SRF reportedly interacts with two other cardiac transcriptional factors, GATA and NKX2.5, and with transcriptional regulators, such as HOP, which do not bind to DNA. This suggests that full activation of the hypertrophic cardiac gene program may require SRF to also work with transcriptional factors situated downstream of signaling pathways other than Rho-actin dynamics-dependent and ERK-dependent pathways (3, 17, 55).

The expression of the BNP gene is rapidly and dramatically upregulated in both *in vivo* and *in vitro* models of cardiac hypertrophy in response to hypertrophic stimuli, including mechanical stress and neurohumoral stimulation (11, 18, 41). Indeed, plasma BNP levels are a clinical marker used to detect and manage cardiac hypertrophy and heart failure in humans (38, 39, 60). Although several signaling pathways and transcriptional factors are known to be involved in the stretch- and neurohumoral stimulation-induced activation of the BNP promoter (27, 30, 37, 44), the entire molecular process governing the transcriptional activation of BNP has not yet been characterized. In that regard, expression of BNP mRNA is reportedly altered in SRF$^{-/-}$ cardiac myocytes (43), though the functional SRF-binding site had not been identified in the BNP gene. In the present study, we identified a functional but atypical CArG element within the 1,823-bp BNP promoter region. Deletion or mutation of this CArG box almost completely abolished the increase in transcription induced by SRF-VP16, suggesting this region is functionally the most important SRF-binding site, at least within the 1,823-bp BNP promoter. Indeed, this CArG element is completely conserved among humans, rats, and mice. We further showed that mutation of CArG reduced the response of the BNP promoter to two hypertrophic stimuli, mechanical stretch and neurohumoral stimulation. That the ANP promoter also has two CArG boxes that have been implicated in hypertrophic induction of ANP gene transcription (57) suggests ANP and BNP may be coordinately regulated by SRF.

Among the variety of signaling molecules activated within cardiac myocytes following mechanical stretch or neurohumoral stimulation, Rho family small GTPases, especially Rho A and Rac1, are known to be important regulators of cardiac hypertrophy (5, 24). For instance, the inhibition of Rho or ROCK (Rho kinase), a downstream target of Rho, has been shown to ameliorate pathological cardiac hypertrophy (5, 20, 29). Our study defines MRTF-A as a critical downstream mediator of Rho- and actin dynamics-associated prohypertrophic signaling in cardiac myocytes, while others have shown that in epithelial cells MRTF-A is also activated downstream of Rac (6). Two important events that occur downstream of Rho and Rac activation are alteration of actin cytoskeletal organization and gene transcription. It appears that MRTF-A is a key mediator of the latter. Consequently, diminishing MRTF-A-mediated transcriptional activation by inhibiting its nuclear translocation and/or its coactivator function, which would selectively block transcriptional pathways activated downstream of Rho family small GTPases, could be a safer and more specific therapeutic approach to preventing pathological cardiac remodeling without the potential side effects caused by disruption of the physiological organization of the actin cytoskeleton.

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We have no conflict of interest to report.

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REVIEW

Regulation and significance of atrial and brain natriuretic peptides as cardiac hormones

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Abstract. Atrial and brain natriuretic peptides (ANP and BNP, respectively) are cardiac hormones. During cardiac development, their expression is a maker of cardiomyocyte differentiation and is under tight spatiotemporal regulation. After birth, however, their ventricular expression is only up-regulated in response to various cardiovascular diseases. As a result, analysis of ANP and BNP gene expression has led to discoveries of transcriptional regulators and signaling pathways involved in both cardiac differentiation and cardiac disease. Studies using genetically engineered mice have shed light on the molecular mechanisms regulating ANP and BNP gene expression, as well as the physiological and pathophysiological relevance of the cardiac natriuretic peptide system. In this review we will summarize what is currently known about their regulation and the significance of ANP and BNP as hormones derived from the heart.

Key words: Natriuretic peptide, Cardiovascular endocrinology

ATRIAL and brain natriuretic peptides (ANP and BNP, respectively) are polypeptide hormones comprising the cardiac-derived natriuretic peptide system [1, 2]. ANP is usually synthesized in the atria, while BNP is primarily synthesized in the ventricles. These two peptides are markers of cardiac differentiation, and their expression is under tight spatiotemporal regulation during cardiac development. Indeed, analysis of the ANP and BNP promoters and their activity has contributed much to our present understanding of the transcriptional regulation of cardiac development. After birth, ventricular expression of both ANP and BNP is upregulated in several pathological conditions of the heart, and their plasma concentrations are markedly elevated in patients with cardiac hypertrophy or congestive heart failure (CHF) [3]. In fact, measurements of plasma ANP and BNP levels are used clinically to assist in the diagnosis of CHF, assess prognosis, and determine therapeutic strategies [2]. It thus appears that the molecular pathways involved in the reactivation of ANP and BNP gene expression are closely linked to adaptive or maladaptive cardiac signaling pathways induced in response to pathological stress. Because of the importance of these two hormones to the physiology and pathophysiology of the heart, many aspects of the molecular mechanism controlling ANP and BNP gene expression during cardiac development and in disease have been studied.

Upon their release, ANP and BNP act at multiple sites to exert diuretic, natriuretic and vasorelaxant effects [4]. Moreover, recent evidence indicates that ANP and BNP also act as paracrine factors, exerting antihypertrophic and antifibrotic effects in the heart. They exert both their hormonal and paracrine effects through activation of their common receptor, guanylyl cyclase-A (GC-A; also known as natriuretic peptide receptor-A), which is expressed in a variety of tissues, including kidney, blood vessel, adrenal gland and heart, and is coupled to an increase in the intracellular concentration of cGMP [4]. The significance of ANP/BNP-GC-A signaling in various physiological and pathophysiological settings has been examined in a number of studies using mice lacking genes encoding components of this signaling pathway. Here we review what is currently known about the transcrip-

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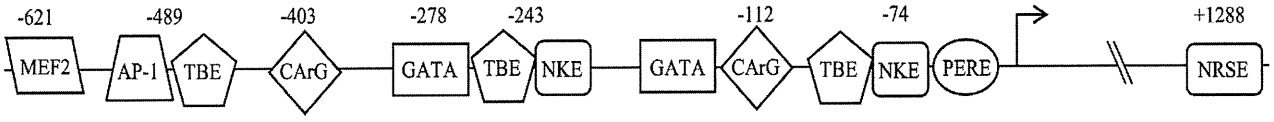

Fig. 1 Schematic representation of rat ANP promoter. Known cis-acting elements are shown.

tional regulation of ANP and BNP gene expression in the myocardium and the physiological and pathophysiological relevance of ANP and BNP as cardiac hormones.

A. Regulation of Natriuretic Peptides Gene Expression

I. Transcriptional regulation of ANP gene expression

Studies using transgenic mice carrying a 500-bp segment of the 5' flanking region (5'-FR) of the human ANP gene fused to a gene encoding SV40 large T antigen, a 2.4-kbp 5'-FR segment of human ANP gene fused to the chloramphenicol acetyltransferase gene or either a 638-bp or 3-kbp 5'-FR segment of the rat ANP gene fused to the luciferase gene have shown that these regions are sufficient to confer cardiac-restricted gene expression, with much higher expression in the atria than in the ventricles [5-7]. The ventricular activities of the 3-kbp, 2.4-kbp and 638-bp 5'-FR segments were down-regulated after birth, while the atrial activity remained high. These observations demonstrate that the proximal 5'-FR of the ANP gene is sufficient to recapitulate the spatial and temporal expression of the endogenous ANP gene and that the region contains sequences important for the regulation of ANP gene expression. Indeed, expression of a reporter gene driven by the proximal 5'-FR of the ANP gene in atrial or ventricular cardiac myocytes at different developmental stages showed that the region confers proper spatial and temporal activity to the ANP promoter [8, 9]. That said, there are some differences in the expression pattern between the proximal 5'-FR of the ANP gene and the intact endogenous ANP gene, which suggests the presence of regulatory elements outside the proximal 5'-FR [10]. It should be noted that in humans and mice, respectively, the ANP gene is located 8 kbp and 12 kbp downstream of the BNP gene on the same chromosome (human, chromosome 1; mouse, chromosome 4) [11, 12]. The proximal 5'-FR of the ANP gene contains two CArG boxes, two NKEs, three TBEs, two GATA sites, an A/T-rich element and a phenylephrine-responsive element (PERE), to which the transcriptional factors SRF, NKX2.5, Tbx5, GATA4/6, MEF2C and Zfp260 have been shown to bind (Fig. 1) [10, 13]. In addition, these elements have been shown to contribute singly or cooperatively to the basal and inducible activation of ANP promoter activity in cardiac cells [13-19]. NRSE, hypoxia-response element (HRE) and glucocorticoid responsive element (GRE), which are located outside the proximal promoter, also reportedly mediate inducible ANP gene transcription [20-23].

II. Transcriptional regulation of BNP gene expression

The 5'-FR of the BNP gene has also been studied so as to better understand the regulatory mechanisms governing the gene's cardiac-specific and inducible expression. A study using transgenic mice carrying a 5'-FR segment of the human BNP gene extending from -1818 to +100, or from -408 to +100, coupled to a luciferase gene (-1818hBNPluc and -400hBNPluc, respectively) showed that the proximal region of the human BNP promoter is sufficient to mediate ventricle-specific expression [24]. The luciferase activity of -1818hBNPluc was also higher in ventricular myocytes than in atrial myocytes [25]. BNP mRNA has a shorter half-life than ANP mRNA and has an AT-rich region in its 3'-UTR. This makes the gene unstable and implies post-transcriptional control of BNP gene expression [26-29]. In addition, deletion analysis showed that the region extending from -127 to -40 of the human BNP 5'-FR confers cardiac-specific expression [25]. This proximal region of the human BNP promoter contains potential GATA, M-CAT and AP-1/CRE-like elements, which are conserved among humans, rats and mice (Fig. 2) [25, 30, 31]. All of these elements are known to regulate cardiac-specific gene expression [31-38], and have been shown to mediate both basal and inducible BNP gene expression [31, 35, 38-43]. Other sites located in relatively distal regions of the human BNP 5'-FR, including NRSE (-552), SSREs (-652, -641 and 161), TRE (-1000) and NF-AT binding site (-927), have also been shown to par-

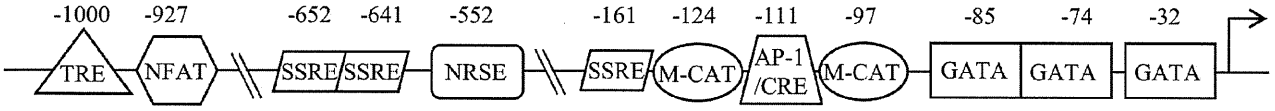

Fig. 2 Schematic representation of human BNP promoter. Known cis-acting elements are shown.

ticipate in the inducible activation of the human BNP promoter (Fig. 2) [44-47].

III. Transcription factor binding sites that regulate ANP and BNP promoter activity

GATA

The proximal human BNP promoter contains three potential sites for GATA binding (-85, -74 and -32) [12]. These sites are generally conserved among species, though the most proximal (-32) is a TATA-box in the mouse BNP promoter [31, 43], which is not seen in the human proximal BNP promoter. It is therefore likely that the -32 GATA site serves as a binding site for different transcription factors, including TATA-binding protein. Mutation of GATA sites in both the human and rat BNP promoter results in a marked reduction in BNP promoter activity in cultured cardiac myocytes [39, 42]. The transcription factor GATA4, which plays an important role in cardiac development [48, 49], can bind to the BNP GATA sites, thereby increasing promoter activity [31, 39, 50]. GATA sites are also present in the ANP proximal promoter, and GATA4 can activate ANP promoter activity [50]. Nonetheless, GATA4-null mice and mutant mice showing a 70% reduction of GATA4 express normal levels of ANP in the heart, suggesting GATA4 is dispensable for natriuretic peptide gene expression during normal cardiac development, though it is plausible that reductions in ANP reflecting the loss of GATA4 are masked by offsetting up-regulation induced by other factors, such as heart failure [48, 49]. Indeed, myocardial ANP gene expression was diminished in hearts with cardiac-restricted deletion of GATA4 accomplished using βMHC-CRE [51].

CArG box/Serum response element (SRE)

Two CArG boxes, also known as SREs, are present in the 5'-FR of the ANP gene, and were shown to mediate hypertrophic signaling [34]. The transcription factor serum response factor (SRF), which is known to play important roles in cardiovascular development and disease, binds to these sequences [52]. At least two signaling pathways are known to modulate SRF activity: one involving the phosphorylation of ternary complex factors in Ets domain family proteins and another controlled by Rho-family small GTPases and actin dynamics [53-56]. It was recently shown that stimulation of Rho- and actin dynamics-dependent signaling results in translocation of a novel SRF co-factor, myocardin-related transcription factor (MRTF)-A (also named as MAL or MKL1), from G-actin in the cytoplasm to the nucleus and in activation of SRF target genes [57, 58]. Although BNP gene expression is also reportedly under the control of SRF, a functional CArG box had not been identified in the proximal 5'-FR of the BNP gene [59]. Recently we have identified a conserved and functional SRF-binding site within the BNP promoter (unpublished observation, 2010).

T-box binding elements (TBE)

Three T-box binding elements (TBE) have been identified in the ANP promoter [60, 61], and the T-box transcription factors Tbx5, Tbx2, Tbx3 and Tbx20 are all thought to be involved in regulating ANP gene transcription [10]. Tbx5, whose mutation causes Holt-Oram syndrome, binds to TBE and interacts with NKX2.5 to synergistically activate ANP gene transcription [60, 61]. Tbx5 haploinsufficient mice showed marked reductions in ANP expression, while Tbx5-null mice expressed no ANP.

Myocyte enhancer factor (MEF) 2

MEF2A-D are MADS box transcription factors that bind to A/T-rich sequences in various muscle-specific genes. Mice lacking MEF2C do not express ANP and show impaired cardiac morphogenesis [62]. MEF2C reportedly binds to the low affinity A/T-rich sequence in the ANP promoter and then stimulates ANP gene transcription [18].

NK-homeobox binding element (NKE)

A homeodomain containing the transcription factor NKx2.5 plays a critical role in cardiac development and cardiac disease [32]. Two such NKEs have been identified in the ANP promoter.

M-CAT

The proximal human BNP promoter contains two M-CAT elements, of which the more proximal is conserved in mouse and rat. This proximal M-CAT site appears to be involved in regulating basal BNP promoter activity in both humans and rats, mediating β-adrenergic-induced human BNP promoter activity and phenylephrine-induced activation of rat BNP promoter activity [40, 42]. Although it is not yet certain which transcription factor binds to the proximal M-CAT site in the BNP promoter, it is likely that one or more TEF family transcription factors are involved, as is the case with the β-MHC and skeletal α-actin promoters [37]. Consistent with that idea, disruption of TEF-1 using a retroviral gene trap leads to the development of cardiac defects [63].

AP-1/CRE-like

The human BNP promoter contains multiple AP-1/CRE-like binding sites, of which the proximal -111AP-1/CRE-like binding site has been shown to play an important role in the basal regulation of both human and rat BNP promoter activity [38, 43]. AP-1 site is also identified in the ANP promoter, and a c-fos/c-jun complex was shown to bind to the element [64].

NF-AT

Molkentin *et al.* showed that the NF-AT family transcription factors act with GATA-4 to mediate phenylephrine-induced activation of the human BNP promoter. A functional NF-AT binding site is located at -972 in the human BNP promoter [46]. Mice in which both NF-ATC3 and -C4 were deleted die *in utero* due to abnormal cardiac development [65]. Recent studies have also shown that the TRPC1/3/6-calcineurin pathway is an upstream regulator of NF-AT-dependent transcription [66-69].

Neuron-restrictive silencer element (NRSE)

We have shown that the transcriptional repressor element NRSE, located at -552 in the human BNP promoter, represses basal BNP promoter activity and mediates the hypertrophic signaling evoked with extracellular matrix [70]. This NRSE is conserved in the rat and mouse BNP promoters. A transcriptional repressor, NRSF, binds to the element, thereby repressing promoter activity. Interestingly, NRSE is also located in the 3'UTR of the ANP promoter and is involved in basal and ET-1-inducible activation of the human ANP promoter [21]. That cardiac-restricted inactivation of NRSF through overexpression of a dominant-negative NRSF driven by the cardiac-specific α-MHC promoter leads to up-regulation of ANP and BNP gene expression in the ventricle, cardiomyopathy and sudden death confirms the importance of NRSF in the regulation of cardiac gene expression and cardiac function [22].

Shear stress-responsive element (SSRE)

Multiple SSREs are located in the human BNP promoter at -652, -533 and -162. Mechanical strain reportedly activates the BNP promoter activity via SSRE [71].

Thyroid hormone-responsive element (TRE)

Thyroid hormone (T3) and thyroid hormone receptor activate BNP gene transcription through TRE located at -1000 in the human BNP promoter. T3 and ET-1 act synergistically to stimulate human BNP promoter activity, and mutation of TRE reduces the response to both T3 and ET-1 [47].

IV. Regulation of ANP and BNP in cardiac myocytes

The expression and secretion of ANP and BNP is up-regulated in diseased hearts such as those showing cardiac hypertrophy or cardiomyopathy, and mechanical stress stimulates the synthesis and secretion of ANP and BNP in both atrial and ventricular cells [72-74]. In addition, neurohumoral factors, including ET-1 [75-78], thyroid hormone [79-81], α-adrenergic agonists [29, 82, 83], prostaglandins [84], glucocorticoids [81, 85] and angiotensin II [86], as well as various growth factors [87] and cytokines, including IL-1β [88, 89], LIF, CT-1 [90-92] and TNF-α [93], all stimulate ANP and BNP synthesis in cultured cardiac myocytes (see review in [72]). And multiple signaling pathways comprised of such MAPK family enzymes as ERK1/2, JNK, P38MAPK and ERK5, as well as CaMKII, PKCs, Jak-STATs, Rho-ROCK and calcineurin-NFATs, are reportedly involved in the up-regulation of ANP and/or BNP [43, 46, 78, 94-100]. Thus a broad spectrum of mediators and signaling pathways are thought to contribute to the increased synthesis and secretion of ANP and BNP observed under pathological conditions.

B. Physiological and Pathophysiological Significance of BNP, ANP and Signaling Through GC-A

The physiological functions of ANP and BNP and their receptor, GC-A, have been studied through their genetic ablation or by blocking GC-A. Genetic ablation of ANP leads to salt-sensitive hypertension in homozygous null mice [101]. In heterozygous and homozygous ANP-null mice maintained on low-salt diet, the natriuretic response to acute volume overload is diminished, as compared to wild type mice, suggesting ANP is required for the natriuretic response to volume expansion in animals on a low salt diet [102]. In rats, blockade of GC-A also results in a significant reduction in diuresis and natriuresis in response to acute volume overload [103]. Homozygous GC-A-deficient mice exhibit salt-insensitive hypertension and marked reductions in natriuresis and diuresis in response to acute volume overload [104-107]. All of these findings indicate ANP acts as a circulating hormone regulating systemic blood pressure and cardiorenal homeostasis through GC-A.

GC-A-null mice also show cardiac hypertrophy with extensive interstitial fibrosis [107, 108], and the cardiac hypertrophy observed in GC-A-null mice appears to be at least partially independent of the high blood pressure [109, 110]. Moreover, cardiac hypertrophy is observed in mice in which the CRE/loxP system was used to cardiac-specifically delete GC-A, despite preservation of all endocrine activity, including pressure- and volume-regulating effects [111]. These findings suggest ANP and BNP also act as local antihypertrophic regulators. Consistent with that idea, genetic ablation of angiotensin type IA receptor or blockade of angiotensin type I receptor significantly reduces cardiac hypertrophy and fibrosis in GC-A-null mice, suggesting the cardiac natriuretic peptide-GC-A system antagonizes the prohypertrophic signaling mediated by the angiotensin type I receptor [112]. Interestingly, there is a gender difference in the cardiac hypertrophic and fibrotic responses seen in GC-A-null mice, and androgen contributes to that gender difference in an angiotensin II type I receptor-dependent fashion [113].

In mice with smooth muscle cell (SMC)-specific deletion of GC-A accomplished using SM22-CRE, which reduced vascular GC-A gene expression by 80%, the vasodilatory effects of ANP on isolated vessels are abolished. These SMC GC-A knockout mice have normal arterial blood pressure; however, acute volume expansion, which normally causes release of ANP from the heart but does not affect blood pressure in control mice, evokes significant and rapid increases in blood pressure in SMC GC-A knockout mice. Thus SMC GC-A is apparently dispensable for chronic regulation of arterial blood pressure, but is critical for the acute regulation of the response to volume overload [114]. In addition, endothelial cell-specific deletion of GC-A accomplished using Tie2-CRE induces high blood pressure and cardiac hypertrophy, but the direct vasodilatory effect of ANP is preserved, suggesting endothelial GC-A also regulates vascular permeability and is critical for the regulation of the hypovolemic and hypotensive actions of ANP [115].

The role of GC-A in several pathological conditions has also been studied. In a mouse model of ischemia/reperfusion injury of the heart, both genetic loss of GC-A and its pharmacological blockade alleviate ischemia/reperfusion injury, suggesting GC-A plays a role in acute inflammatory responses in the heart [116]. Supporting that notion are the findings that BNP transgenic mice subjected to myocardial infarction show greater accumulation of neutrophils in the heart and a greater susceptibility to cardiac rupture than wild-type mice [117]. On the other hand, GC-A-null mice subjected to myocardial infarction show greater susceptibility to acute heart failure and exacerbation of chronic pathological cardiac remodeling, as compared to wild-type mice. These findings are indicative of the critical role played by GC-A in the regulation of volume during the acute phase after myocardial infarction, as well as the antihypertrophic and antifibrotic effects of GC-A during the chronic phase [118]. GC-A-null mice also show increased susceptibility to heart failure due to volume overload caused by aortocaval fictula, which further highlights the importance of GC-A for volume control under conditions of pathological volume overload [119]. Chronic hypoxia and aortic constriction worsen cardiac hypertrophy in GC-A-null and cardiac-specific GC-A knockout mice, respectively, which also reflects the antihypertrophic effect of GC-A signaling [111, 120]. Finally, BNP knockout mice do not exhibit hypertension or cardiac hypertrophy, but do develop cardiac fibrosis, suggesting BNP acts via GC-A to locally regulate cardiac remodeling [121].

C. Concluding Remarks and Perspective

Studies of ANP and BNP gene expression have led to discoveries of transcriptional regulators and signaling pathways involved in either cardiac differentiation or cardiac diseases. Still, much of molecular mediators via which the expression of ANP and BNP in myocardium is controlled remain to be characterized. It became apparent that the regulation of ANP and BNP gene expression is more complex than initially thought. More efforts to elucidate molecular mechanisms regulating ANP and BNP expression, in particular mechanisms regulating the perinatal suppression and the reactivation in diseased hearts of the expression may be necessary. Studies using genetically engineered mice have shed light on the physiological and pathophysiological relevance of the cardiac natriuretic peptide system and its downstream signaling. Continued efforts to identify molecular targets of natriuretic peptide system will further enhance our understanding of the role of natriuretic peptides in cardiovascular system and also of the molecular basis for the development and progression of cardiovascular diseases.

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