GATA-4 Is a Nuclear Mediator of Mechanical Stretch-activated Hypertrophic Program*

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In overloaded heart the cardiomyocytes adapt to increased mechanical and neurohumoral stress by activation of hypertrophic program, resulting in morphological changes of individual cells and specific changes in gene expression. Accumulating evidence suggests an important role for the zinc finger transcription factor GATA-4 in hypertrophic agonist-induced cardiac hypertrophy. However, its role in stretch-induced cardiomyocyte hypertrophy is not known. We employed an in vitro mechanical stretch model of cultured cardiomyocytes and used rat B-type natriuretic peptide promoter as stretch-sensitive reporter gene. Stretch transiently increased GATA-4 DNA binding activity and transcript levels, which was followed by increases in the expression of B-type natriuretic peptide as well as atrial natriuretic peptide and skeletal α-actin genes. The stretch inducibility mapped primarily to the proximal 520 bp of the B-type natriuretic peptide promoter. Mutational studies showed that the tandem GATA consensus sites of the proximal promoter in combination with an Nkx-2.5 binding element are critical for stretch-activated B-type natriuretic peptide transcription. Inhibition of GATA-4 protein production by adenovirus-mediated transfer of GATA-4 antisense cDNA blocked stretch-induced increases in B-type natriuretic peptide transcript levels and the sarcocure reorganization. The proportion of myocytes with assembled sarcomeres in control adenosine-fected cultures increased from 14% to 59% in response to stretch, whereas the values for GATA-4 antisense-treated cells were 6% and 13%, respectively. These results show that activation of GATA-4, in cooperation with a factor binding on Nkx-2.5 binding element, is essential for mechanical stretch-induced cardiomyocyte hypertrophy.

Cardiac hypertrophy is the primary adaptive mechanism for terminally differentiated cardiac myocytes to increased hemodynamic load. In addition to mechanical stretch, a number of humoral factors, such as G-protein-coupled receptor agonists angiotensin II and endothelin-1 (ET-1)1 as well as adrenergic receptor agonists released by the activated sympathetic nervous system participate in the adaptive process and modify the growth of cardiac myocytes in vivo (for review, see Refs. 1 and 2). When the cardiac overload is sustained, this initially compensatory mechanism fails, and the contractile function is impaired, leading to congestive heart failure (1).

The hypertrophic response in cardiac myocytes is characterized by morphologic changes that include increase in cell size and protein synthesis and enhanced sarcomere reorganization as well as specific changes in cardiac gene expression (2, 3). The early genetic response to hemodynamic overload is the activation of immediate early response genes such as c-fos and c-jun, components of the activator protein-1 (AP-1) transcription factor complex. This is followed by up-regulation of B-type natriuretic peptide (BNP) and reactivation of fetal genes such as atrial natriuretic peptide (ANP), β-myosin heavy chain, and skeletal muscle α-actin (skαA) (2, 4–6). The mechanisms regulating the genetic reprogramming in cardiac overload has been the subject of intensive research.

GATA-4 was originally shown to be a crucial regulator of cardiogenesis and cardiac-specific genes such as ANP and BNP (for review, see Refs. 7 and 8). Several lines of evidence suggest the involvement of GATA-4 in the development of cardiac hypertrophy. First, hemodynamic overload caused by aortic banding, AngII vasopressin infusion, or nephrectomy in vivo (9–11) as well as in vitro treatment of cultured neonatal rat cardiac myocytes with α1-adrenergic agonist phenylephrine, β-adrenergic agonist isoprenaline, or ET-1 (10, 12–19) activate GATA-4 binding on cis-acting elements of target genes. Second, hemodynamic overload in vivo induces transcription via GATA binding elements present in the regulatory region of the angiotensin type 1 receptor, β-myosin heavy chain, and BNP genes (9, 11, 20). Similarly, hypertrophic agonists phenylephrine and isoprenaline activate GATA-dependent transcription of ET-1, BNP, and ANP in vitro (12,14–16,21). Third, overexpression of GATA-4 in cultured neonatal cardiomyocytes by adenoviral gene transfer or in the hearts of transgenic mice was shown to

1 The abbreviations used are: ET-1, endothelin-1; ANP, atrial natriuretic peptide; AP-1, activator protein-1; BNP, B-type natriuretic peptide; rBNP, rat BNP; ds, double-stranded; EMSA, electrophoretic mobility shift assay; NF-AT, nuclear factor of activated T-cells; NKR, Nkx-2.5 binding element; skαA, skeletal muscle α-actin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum.
be sufficient to induce cardiomyocyte hypertrophy (13, 22). Finally, expression of dominant negative GATA-4 or antisense GATA-4 cDNA blocked GATA-4-directed transcriptional responses and features of cardiomyocyte hypertrophy induced by phenylephrine and ET-1 (13, 22). Although these observations have implicated GATA-4 as sufficient and, in the case of hypertrophic agonist-induced hypertrophy, a necessary mediator of trigger response, it is not known whether direct mechanical stretch also activates the hypertrophic program via GATA-4-dependent mechanism(s).

To directly address the role of GATA-4 and its potential cofactors in mechanical stretch-activated hypertrophic program, we employed an in vitro mechanical stretch model of cardiomyocytes. The model provides the advantage to dissect the mechanical component from the humoral and neural components of hemodynamic overload, which are always present in the whole animal. Stretch of cultured neonatal rat cardiomyocytes has been shown to stimulate BNP transcription (23), therefore providing a useful target gene to elucidate the mechanisms of stretch-induced gene expression in cardiac myocytes. Our present data show that GATA-4 is a critical regulator of transcriptional and morphological changes induced by mechanical stretching of cardiomyocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Specific antibodies raised against GATA-4 (C-20 and H-112), GATA-5 (M-20), GATA-6 (C-20), components of AP-1 complex (c-Fos (4G), c-Fos (K-25), c-Jun (N-1G), Jun B (N-17G), or Jun D (32G)-G), and Nkx-2.5 (P-25-G) were Santa Cruz Biotechnology (Santa Cruz, CA), and anti-rabbit IgG antibody linked to horseradish peroxidase was from New England Biolabs Ltd. (Hertfordshire, UK). FuGENE™ 6 transfection reagent was from Roche Molecular Biochemicals. ECL plus reagents, poly (dI-dC)/poly (dI-dC), [α-32P]dCTP, and RediGene™ 1 random sequence labeling system were from Rediprime (Oxford, UK). The BNP promoter and antisense as well as a 390-bp fragment of rat BNP cDNA probe (24) were gifts from Dr. Kazuwa Nagai, Kyoto University School of Medicine (Kyoto, Japan). Rat ANP cDNA probe Car-55 (25) was provided by Dr. Peter L. Davies, Queen’s University (Kingston, Canada). Cell culture reagents were from Life Technologies (Grand Island, NY). GATA-4 antibody (H-112) was a generous gift from R. P. McKee (The Victor Chang Cardiac Research Institute, Darlinghurst, Australia). Several candidate clones were sequenced using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Neurite outgrowth assay was performed according to the manufacturer. Oligonucleotide fragments were synthesized by the University of Ulster (Belfast, UK). Oligonucleotides were from Sigma Genosys (Cambridge, UK). For 5′ deletion assay, rat BNP (rBNP)-luciferase plasmids containing various rBNP promoter fragments were obtained by subcloning appropriate 5′ deletions of the BNP promoter (generated by restriction or by PCR) in pEF-FLAG-Nkx2.5 or pEF-FLAG-Nkx2.5 expressing a nuclear localization signal-lacZ and reporter genes were transfected with FuGENE™ 6 in fresh DMEM/F-12-FBS for 6–8 h. The total amounts of FuGENE™ 6 reagent and DNA were 6 and 3 μg/L.8 × 106 cells, respectively, unless otherwise indicated. To control the transfection efficiency, luciferase reporter plasmids were cotransfected with Rous sarcoma virus-β-galactosidase plasmids at the ratio of 3:1. Transfections were performed to myocyte cultures 4 h after switching to complete serum-free medium 24 h after plating. The composition of complete serum-free medium was similar to that described earlier (25), except that the concentration of triiodothyronine was 0.1 nM. In adenovirus infections, infections were performed to myocytes 4 h after switching to complete serum-free medium at a multiplicity of infection of 4 by adding the appropriate recombinant adenovirus to the culture media overnight. The media were replaced every 24 h. After experiments, the cells were washed twice with phosphate-buffered saline and quickly frozen at −70 °C. The reporter gene activities were measured by using the luciferase assay system (Promega) and the luminescent β-galactosidase detection kit II (Promega). Chemiluminescence (Promega) was detected using a cooled intensified charged-coupled device camera (Hamamatsu Photonics, Japan). Ratiometric analysis, 0.5 μg of pEF-FLAG-Nkx2.5 or pMT2 control plasmid with FuGENE™ 6 reagent. Forty-eight hours after transfection, cells were harvested and subjected to nuclear protein extraction.

**Application of Mechanical Stretch—**Stretch was introduced to attached myocytes after 18–24 h in complete serum-free medium by applying a computer-controlled vacuum suction under the flexible-bottomed Biolax collagen plates with Flexercell Strain Unit FX-3000 (Flexcell). The vacuum varied in two-second cycles at a level sufficient to promote 10–25% elongation of the cardiomyocytes at the point of maximal distension of the culture surface.

**Immunofluorescence—**The cells were washed twice with Hank’s balanced salt solution, fixed, and permeabilized with 4% paraformaldehyde for 15 min at room temperature and washed 3 times with phosphate-buffered saline. Filamentous actin was labeled with Alexa Fluor 488 phallolidin (1:20, Molecular Probes Inc, Eugene, OR). To assess for reorganized cells, random fields (minimum of 10 per well) were taken by laser confocal microscope (LSM 510, Zeiss) using green fluorescence channel. The number of cardiomyocytes harboring striated actomyosin fibers extending from one extremity of the cell to the other was counted and divided by the total number of cardiomyocytes in the same field. These observations were performed blindly.

**Western Blot—**Protein extracts were boiled in Laemmli buffer, resolved by SDS-PAGE, and transferred to Optitran BA-8 5 nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked in 5% nonfat milk and then incubated with GATA-4 antibody (H-112) at a 1:5000 dilution in 1% milk in Tris-buffered saline·Tween 20 overnight at 4 °C. Antibody binding was detected with an anti-rabbit horseradish peroxidase-IgG at a 1:2000 dilution and revealed using ECL+ Plus as described by the manufacturer.

**Isolation and Analysis of RNA—**Total RNA was isolated using the guanidium thiocyanate-CsCl method (30). For the RNA Northern blot analyses, 0.5–6 μg samples were separated by electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked in 5% nonfat milk and then incubated with GATA-4 antibody (H-112) at a 1:5000 dilution in 1% milk in Tris-buffered saline·Tween 20 overnight at 4 °C. Antibody binding was detected with an anti-rabbit horseradish peroxidase-IgG at a 1:2000 dilution and revealed using ECL+ Plus as described by the manufacturer.
Cyclic mechanical stretch activates genetic markers of cardiomyocyte hypertrophy. Cultured neonatal rat ventricular myocytes were stretched cyclically up to 24 h. The amplitude of stretch varied between 10 and 25% in 2-s cycles. After extraction, 4 μg of total RNA was separated on Northern gel. The blots were sequentially hybridized with specific cDNA probes complementary to rat A, B, ANP, C skaA and ribosomal 18 S RNA. Representative Northern blot analyses are shown on the bottom of each panel. The results in bar graphs are expressed as ratios of specific mRNA to ribosomal 18 S RNA as determined by Northern blot analysis. Open bars indicate control (n = 6–22), and solid bars indicate stretched cells (n = 6–23). Results are the mean ± S.E. from five independent cultures. The culture medium was collected, and immunoreactive rat B-type natriuretic peptide (ir-BNP) and atrial natriuretic peptide (ir-ANP) were measured by radioimmunoassay. The basal secretion of ir-BNP and ir-ANP were 180 ± 10 and 657 ± 76 fmol/ml/h, respectively. The results are expressed as the ratio of relative change in secretion in stretched cells to that in control cells. Open bars indicate control, and solid bars indicate stretched cells. Results are the mean ± S.E. from 12 independent cultures. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control (Student’s t test).

FIG. 1. Cyclic mechanical stretch activates genetic markers of cardiomyocyte hypertrophy. Luciferase (LUC) reporter constructs containing various lengths of the rBNP 5'-flanking region were co-transfected with Rous sarcoma virus-β-galactosidase plasmid (2 and 1 μg/35 mm well, respectively) into neonatal rat ventricular myocytes that were cyclically stretched for 24 h. The amplitude of stretch varied between 10 and 25% in 2-s cycles. Schematic presentation of the constructs is shown on the left. Deletion of the 5'-flanking region between −520 and −114 significantly decreased the inducibility of the rBNP promoter in response to stretch. This proximal region contains two GATA binding elements (GG) located at −373, and two NKE-like elements (NKE) located at −387 and −496. Open bars indicate non-stretched control, and solid bars indicate stretched cells. Basal promoter activities are shown in the middle relative to −60rBNP construct. Results are expressed as luciferase to β-galactosidase ratio ± S.E. of stretched cells relative to that of non-stretched control cells transfected with the same construct (n = 10–18 from 3–5 independent cultures). *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control (Student’s t test).
al. (31). Cells were washed and scraped with Tris-buffered saline. After centrifugation, cells were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, supplemented with 0.2 mM Na3VO4, 20 μg/ml leupeptin, 2 μg/ml pepstatin, 20 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM dithiothreitol, 6 μg/ml l-1-tosylamido-2-phenylethyl chloromethyl ketone, and 6 μg/ml 1-chloro-3-tosylamido-7-amino-2-heptanone) by gentle pipetting and allowed to swell on ice for 15 min. Cells were lysed by adding 25 μl of 10% Nonidet P-40 and vortexing vigorously for 10 s followed by centrifugation (12,000 rpm) for 30 s. Supernatant was saved as the cytosolic fraction. Pellets were resuspended in 25 μl of ice-cold buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, and 1 mM EGTA with supplements similar to those in buffer A) and rocked for 15 min. The samples were centrifuged at 12,500 rpm for 5 min. The supernatant was saved as the nuclear fraction. The entire procedure was carried out at 4°C. Probes were prepared by Klenow fragment-mediated filling of the sticky ends of double-stranded (ds) oligonucleotides and labeled with [γ-32P]dCTP. Binding reactions contained 3–6 μg of nuclear protein and 2 μg of poly-(dI-dC)(dI-dC) in a buffer containing 16 mM HEPES, pH 7.9, 120 mM NaCl, 1 mM MgCl2, 40 mM KCl, 1 mM dithiothreitol, 0.7 mM EDTA, 0.3 mM EGTA, 5% glycerol, 0.02% Nonidet P-40, 0.25 mM phenylmethylsulfonyl fluoride, and 1 μg/ml of each aprotinin, leupeptin, pepstatin, and when appropriate, various molar excesses of competitor DNAs. When designed, supershift experiments were performed by preincubating nuclear extract with 1 μg of appropriate antibody for 20 min at room temperature before performing the

![Fig. 3. Mechanical stretch transiently increases GATA binding activity and GATA-4 mRNA.](image-url)
binding reaction. After a 10-min preincubation of extract, the labeled probe was added, and binding was allowed to proceed at room temperature for 20 min. The reaction mixtures were then analyzed by electrophoresis on 5% polyacrylamide gel in 0.5 x Tris-borate-EDTA buffer at 4°C. After drying, the gel DNA-protein complexes were detected and quantitated as the Northern blot membranes.

Assay of Immunoreactive ANP and BNP—Radioimmunoassays of ANP and BNP were done as previously described (5, 32). The sensitivities of the BNP and ANP assays were 2 and 1 fmol/tube, respectively. 50% displacements of the respective standard curve occurred at 16 and 25 fmol/tube. The intra-assay and inter-assay variations were 10 and 15%, respectively.

Statistical Analysis—The results are expressed as the mean ± S.E. For multiple comparisons, data were analyzed with one-way analysis of variance followed by a least significant difference post hoc test. For comparison of two groups at each time point Student’s t test for unpaired data was used. A value of p < 0.05 was considered statistically significant.

RESULTS

Mechanical Stretch Induces a Hypertrophic Phenotype—To validate the experimental system we first analyzed the effect of mechanical stretch on the gene expression of BNP, ANP, and skαA, which represents genetic hallmarks of the stretch-induced cardiac hypertrophic program (23, 33–35). Cyclic mechanical stretch elevated BNP mRNA levels significantly after 4 h, peaking at 24 h (2.1-fold, Fig. 1A). Similarly, mechanical stretch caused significant increases in ANP and skαA mRNA levels, respectively (Fig. 1, B and C). Elevated natriuretic peptide mRNA levels were accompanied by increased peptide secretion from cardiomyocytes into the culture medium (Fig. 1, D and E). Conclusively, because of rapid response of the level of mRNA and the peptide released, BNP provides a sensitive target gene to further study the stretch-inducible signaling mechanisms.

Proximal 520 bp of rBNP 5′-Flanking Sequences Is Sufficient to Confer Stretch Inducibility—To identify the rat BNP promoter region that mediates the transcriptional activation by mechanical stretch, we transfected cardiomyocytes with luciferase reporter constructs driven by various lengths of the rBNP 5′-flanking sequence. Removal of about 1.2 kilobases of the −2200 rBNP 5′-flanking sequence down to −940 resulted in about a 30% decrease in basal reporter activity (Fig. 2). Further deletion down to −520 decreased basal promoter activity, in agreement with the suggested presence of repressor elements distally from −520 (21). Interestingly, deletion of the rBNP 5′-flanking region from −2200 to −520 did not significantly change the inducibility of the rBNP promoter in response to mechanical stretch. Yet truncation down to −114 reduced the basal as well as mechanical stretch-induced promoter activity by 70–80% compared with −520BPN. Further deletion to −60 completely abolished the stretch response. The −60 rBNP possessed only 3% of original basal promoter activity.

GATA-4 and AP-1 Are Activated by Mechanical Stretch—The tandem GATA elements within the −520-bp BNP promoter are well conserved between mammalian species (rat, human, and dog, Ref. 26). Moreover, these elements have been shown to mediate the activation of rBNP promoter in response to hemodynamic volume overload in vivo (11), suggesting an important role in stretch-activated BNP transcription. Therefore, we used 30-bp ds oligonucleotide probe containing the −90 GATA sites of rBNP (rBNP-90 GATA) as the probe in EMSA to analyze whether mechanical stretch activates nuclear protein binding on these sites. As shown in Fig. 3A, stretch transiently increased GATA DNA binding activity peaking at 1 h. Octamer-1 binding activity remained unaffected by stretch excluding unspecific effect on nuclear protein-DNA interaction (Fig. 3B). To determine the specificity of GATA binding activity, competition analyses were performed (Fig. 3D). The formation of complexes with the rBNP-90 GATA probe was dose-dependently inhibited by the unlabeled self DNA, but not by the mutated BNP GATA site, confirming the specific protein-DNA interaction. Super-shift analysis clearly showed complete antibody-induced supershift of the GATA-4 but not GATA-5 or -6 complexes (Fig. 3E).

To study the effect of mechanical stretch on GATA-4 gene expression, Northern blot analysis using rat GATA-4 cDNA probe was performed. The activation of GATA-4 binding was accompanied by a transient 1.6-fold increase in GATA-4 mRNA levels peaking at 4 h (Fig. 3F). Western blot analysis showed
that the levels of GATA-4 protein were almost undetectable in the cytosolic fraction. In the nuclear extracts of stretched cardiomyocytes GATA-4 protein accumulation appeared to increase; however, this change was not statistically significant (Fig. 3C).

Because previous studies suggest functional cooperation between GATA-4 and AP-1 in pressure overload-induced hypertrophy (20), we also studied nuclear protein binding on the AP-1 site by using ds oligonucleotide probe containing the rat BNP-373 AP-1 site (rBNP-373 AP-1). Stretch increased AP-1 binding activity, peaking at 1 h (2.5-fold increase) and sustaining for at least 24 h (Fig. 4A). Competition experiments demonstrated specificity of the complex formation, which was effectively inhibited by the unlabeled rBNP-373 AP-1 probe (self) (Fig. 4B), but not by the mutated BNP AP-1 site DNA. To identify the components of the AP-1 complex we performed supershift experiments using antibodies against different Fos and Jun family members. As shown in Fig. 4C, moderate supershifts were observed with JunD-specific antibody and nonspecific antibody against Fos family proteins. Instead, when using c-Fos-specific antibody, no supershift complex was seen, suggesting the presence of either Fos B, Fos-related antigen-1 or -2 but not c-Fos in the complex. Weak antibody-induced supershifts were observed also with c-Jun- and JunB-specific antibodies. Mechanical stretch did not change the supershift profile compared with nuclear extracts obtained of non-stretched cardiomyocytes (Fig. 4C).

**Stretch-induced BNP Promoter Activity Is Inhibited by Mutation of Proximal GATA Sites**—Next we studied whether the activation of GATA-4 had functional consequences on BNP transcription. We introduced site-directed mutations to the reporter construct containing the proximal 534-bp rBNP promoter that is sufficient to confer full stretch inducibility (−534rBNP, Fig. 5A). Mutation of the tandem −90 GATA sites resulted in 37% decrease in the stretch-induced reporter activity compared with intact −534rBNP promoter, which was activated by 2.2-fold (Fig. 5B). To study the possibility that stretch-induced rBNP transcription could be mediated by cooperation of GATA-4 with AP-1 as reported previously in pressure overload-activated angiotensin type 1A receptor transactivation of Proximal GATA Sites (36), we mutated the −373 AP-1 site of rBNP. Despite increased complex formation of cardiac nuclear proteins with rBNP-373 AP-1 (Fig. 4A), mutation of this site alone or in combination with the tandem GATA sites did not modify stretch-induced transcription of rBNP (Fig. 5B).

**−387 NKE-like Element Cooperates with GATA-4 in Stretch-induced BNP Transcription**—In addition to AP-1, GATA-4 has been shown to cooperate with several other transcription factors including Nkx-2.5 in regulation of cardiac-specific gene expression (36–38). Therefore, we utilized a computer-based search for cis-acting elements in a −534-bp rBNP promoter and identified two sequences with high homology to NKE located at −387 and −496. To confirm whether these NKE-like elements are bona fide binding sites for Nkx-2.5, we prepared nuclear extracts from COS-1 cells transfected with Nkx-2.5 expression vector. The nuclear extracts were incubated with labeled ds DNA probes corresponding to −387 and −496NKE-like sites of BNP and a previously described high affinity duplication NKE from the rat ANP gene (ANP NKE) (37, 38). As shown in Fig. 6 (see Table I), gel shift analysis demonstrated that the −387 NKE-like and the ANP NKE probes formed complexes that were specifically supershifted by an anti-Nkx-2.5 antibody; the affinity of the −387 NKE-like element for Nkx-2.5 was significantly weaker than that of the ANP NKE. Under the same conditions, the −496NKE-like site of BNP was not able to bind Nkx-2.5, in agreement with our recent study demonstrating that this site specifically binds the ETS transcription factor Elk-1 (39).

Given that the −387 NKE-like element proved to be the true binding target for Nkx-2.5 (hereafter referred as −387 NKE), we studied whether it cooperates with GATA element in stretch-induced transcription of BNP in cardiomyocytes. As Fig. 7 shows, mutation of −387 NKE alone had no effect on stretch-induced rBNP promoter activity. Interestingly, the inducibility by stretch was almost completely abolished when mutation of the −387 NKE was combined with the mutation of the tandem −90 GATA sites. Therefore, the requirement of −387 NKE in the stretch-induced BNP transcription was unmasked when the tandem −90 GATA sites were mutated, suggesting that Nkx-2.5 acts in cooperation with GATA-4. In support of this notion, mutation of −496NKE-like site, which is unable to bind Nkx-2.5 although it shares similar core sequence to −387 NKE, did not modify stretch-induced transcription of −534rBNP with intact or mutated GATA sites (Fig. 7).
GATA-4 Is Essential for Stretch-induced Sarcomere Reorganization—In addition to changes in gene expression, the hypertrophic phenotype of cultured cardiomyocytes is characterized by morphologic changes including increased reorganization of sarcomeres (3). To test whether the activation of GATA-4 is involved in the morphologic changes associated with stretch-induced hypertrophy, we specifically inhibited the production of GATA-4 protein by employing an adenovirus expressing antisense GATA-4 cDNA (GATA-4as). In agreement with previous studies (22, 28), GATA-4 protein levels decreased significantly in cells infected with GATA-4as compared with cells infected with control adenovirus expressing a nuclear localization signal-lacZ cDNA (LacZ) (Fig. 8A). Down-regulation of GATA-4 in cardiomyocytes blocked a stretch-induced increase in BNP mRNA levels (Fig. 8B) and inhibited sarcomere reorganization (Fig. 8C). Quantification of reorganized cardiomyocytes showed that stretch increased the number of cardiomyocytes that underwent sarcomere reorganization in LacZ-infected cultures by 4.1-fold \((p < 0.01)\) and that this effect was significantly smaller (2.0-fold) in GATA-4as-treated cells (Fig. 8D). GATA-4as treatment also decreased the basal sarcomere assembly in non-stretched cells compared with cells infected with LacZ. Collectively, the data demonstrate an essential role for GATA-4 in the genetic and morphologic response of cardiomyocytes to mechanical stretch.

DISCUSSION

Development of the in vitro stretch model for cultured neonatal rat cardiomyocytes has provided an established method for the study of the molecular mechanisms in stretch-activated changes in cardiomyocyte hypertrophy. These changes closely resemble those of cardiac overload-induced hypertrophy in vivo (for review, see Ref. 35). Experiments employing this model have led to increasing knowledge of cytosolic signal transduction pathways mediating the cellular responses to stretch. In the present study, we show for the first time that mechanical stretch per se activates GATA-4 binding on the well conserved proximal GATA element of BNP promoter. This element to-
cofactors in GATA-4-dependent hypertrophic responses has not been explored.

Interestingly, in the present study inhibition of GATA-4 production alone by GATA-4as adenovirus was sufficient to completely block the stretch-induced increase in BNP mRNA levels. On the contrary, the mutation of the −90 GATA sites in the context of −534rBNP resulted in about a 40% decrease in the stretch response (Fig. 5B), whereas the stretch response of −114 rBNP, still containing intact GATA sites, was minimal (Fig. 2). Because intact −534rBNP was sufficient to confer full stretch inducibility, these data suggest that other factor(s), which bind between −534 bp and −114 bp of rBNP promoter, are involved together with GATA in stretch-activated transcription. Also, the apparently conflicting results regarding necessity/sufficiency of GATA-4 in stretch response between transfection experiments and GATA-4as adenovirus experiments may be contributed to the interaction of GATA-4 with other cofactors. Down-regulation of GATA-4 protein may limit the complex formation between GATA-4 and its cofactors, whereas inhibition of GATA-4 DNA binding does not necessarily have an effect on the interaction with other factors that may recruit the complex on the promoter.

Xia et al. (48) report that GATA, NF-AT and myocyte enhancer factor elements were required for activation of adenylsuccinate synthetase 1 gene in electrical pacing-induced hypertrophy of cardiomyocytes (48). The previously described NF-AT binding element responsible for GATA-4/NF-AT3 synergy in activation of human BNP is located far distally (−927) in the promoter (41) and does not appear to be required for stretch response. Moreover, we were not able to detect specific binding activity on NF-AT consensus-like sites at −320 and −340 of rBNP 5′-flanking sequence (data not shown). In addition, 534 bp of 5′-flanking region of rat BNP gene contains no potential myocyte enhancer factor nor serum response factor binding elements, and the Yin Yang 1 element locates more proximally (between −80 to −60) of the rBNP 5′-flanking sequence (45), suggesting that these factors do not cooperate with GATA-4 in stretch-activated BNP transcription. The inducible expression of angiotensin type 1a receptor by pressure overload in vivo has been reported to be regulated by possible functional cooperation of GATA and AP-1 in adult rat heart (20). However, the inability of mutation of −373 AP-1 to modify stretch-induced reporter activity of −534rBNP with mutated tandem GATA sites indicates that AP-1 does not cooperate with GATA-4 or that the possible cooperation does not require AP-1 binding on the −373 AP-1 site in mechanical stretch-induced activation of rBNP.

A major finding of this study was that mutation of the tandem −90 GATA sites in combination with mutation of the NKE at −387 almost completely abolished the stretch inducibility of rBNP promoter, whereas mutation of −387 NKE alone had no effect on it. This suggests that GATA-4/NKx-2.5 interaction is targeted by stretch-induced signaling. Furthermore, GATA-4 may recruit NKx-2.5 on rBNP promoter via intact −90 GATA sites when Nkx-2.5 binding on its cognate binding site is disrupted by mutation, thereby enabling normal stretch response. Therefore, the involvement of Nkx-2.5 in stretch-induced rBNP transcription becomes evident only when −387 NKE is mutated and its recruitment on rBNP promoter is blocked by mutation of the tandem −90 GATA sites, resulting in further decrease in stretch inducibility compared with mutation in GATA sites only. Previously, the GATA-4/Nkx-2.5 interaction has been shown to be targeted by bone morphogenetic protein-2 and -4 signaling during the early stages of cardiogenesis (49, 50). Our results extend the relevance of the GATA-4/NKx-2.5 interaction to postnatal cardiomyocytes and implicate it in the response of cardiomyocytes to mechanical stretch.

| Stretch response (% of baseline) | 0 | 50 | 75 | 100 | 125 | 150 |
|--------------------------------|---|----|----|-----|-----|-----|
| mNKE387 | ** |     |    |     |     |     |
| mGATA |       | #  |    |     |     |     |
| mNKE387/mGATA | *** |     |    |     |     |     |
| mGATA/mNKE387 | * |     |    |     |     |     |
| mGATA/mNKE387 |     | # |    |     |     |     |

**Fig. 7.** Functional −387NKE cooperates with −90 GATA sites in mechanical stretch-induced rBNP transcription. Luciferase reporter constructs were cotransfected with Rous sarcoma virus-β-galactosidase plasmid (2 and 1 μg/35-mm well, respectively) into neonatal rat ventricular myocytes that were cyclically stretched for 24 h. Combining mutation of −387 NKE with mutation of −90 GATA (mGATA/mNKE387) almost completely abolished the stretch responsiveness of the BNP promoter, whereas the inhibitory effect of mutation of −90 GATA sites (mGATA) on stretch inducibility was not further potentiated by combination with mutation of −496 NKE (mGATA/NKE496). Mutation of either −387 NKE (mNKE387) or −496 NKE-like (mNKE496) elements alone had no effect on stretch-induced BNP transcription. Results are expressed as luciferase to β-galactosidase ratio relative to induction of intact −534 rBNP promoter (−534rBNP) (mean ± S.E. n = 6–14 from 3–5 independent cultures; p < 0.05 (**), p < 0.01 (***) are versus stretched cells transfected with −534rBNP, p < 0.05 (#) is versus stretched cells transfected with −534rBNP-containing mutation in −90 GATA sites (one way analysis of variance followed by least significant difference test).
In the previous studies hypertrophic response has been associated with a transient increase in GATA-4 DNA binding activity without significant change in total cellular GATA-4 protein levels, suggesting that GATA-4 activity is mainly regulated by posttranslational mechanisms (12, 13, 15, 20). Indeed, there is evidence that GATA-4-mediated changes in gene expression and cell morphology are controlled not only by interaction with other cofactors but also by phosphorylation of GATA-4 by specific protein kinases (16, 22, 51). ET-1 and phenylephrine activate small GTPase family member RhoA previously shown to activate sarcomere reorganization in cardiac myocytes (52). RhoA in turn activates GATA-4 via p38 mitogen-activated protein kinase-dependent phosphorylation on Ser-105, leading to genetic reprogramming and induction of sarcomere reorganization (18, 22). In addition to p38 mitogen-activated protein kinase, extracellular signal-regulated kinases have been shown to phosphorylate GATA-4 following enhanced binding and transactivation activity of GATA-4 (12, 51). Interestingly, mechanical stretch has been shown to activate both of these mitogen-activated protein kinase pathways (35, 40, 53). Moreover, Rho family members have been shown to play a critical role in mechanical stretch-induced activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase in cultured cardiomyocytes (54, 55), thereby providing a potential link between mechanical stretch and the GATA-4-dependent enhanced sarcomere assembly as well as BNP transcription observed in the present study.

Recently, Morisco et al. (16) reported that stimulation of cardiac myocytes with β-adrenergic agonists increased nuclear GATA-4 protein levels (16). The data suggest that activation of protein kinase B/Akt by isoprenaline led to inhibition of glycogen synthase kinase 3β, which phosphorylates the amino-terminal part of GATA-4, resulting in increased export from the nucleus. Previously, Akt has been shown to be activated by mechanical stresses in the ventricles of rats subjected to aortocaval shunt (56). Therefore, the possibility that increased GATA-4 protein accumulation in the nuclear extracts of stretched cells may partially result from decreased export via activation of the Akt-glycogen synthase kinase-3β pathway remains to be studied.

Previous studies have shown that autocrine/paracrine fac-
tors play a significant role in the development of load-induced cardiac hypertrophy. Mechanical stretch is coupled with the cellular release of angiotensin II and ET-1, which act as chemical mediators of stretch-induced myocyte hypertrophy in cultured rat cardiomyocytes (35, 57). Liang and Gardner (58) propose that stretch-triggered release of angiotensin II sequentially stimulates secretion of ET-1, which accounts for 50% of the stretch-induced human BNP gene transcription (58). In addition, ET-1 stimulates GATA-4 binding activity (10, 17, 23816). Recently, ET-1 stimulates GATA-4 binding activity (10, 17, 23816), thus raising the question of whether mutation of the tandem –90 GATA sites decreased the activation of rBNP promoter through endothelin-dependent component of stretch. Recently, we have shown that mutation of the tandem GATA sites at –90 of the rBNP promoter does not inhibit transcriptional activation by either ET-1 or angiotensin II (17). Furthermore, mutation of the –496 NKE-like site that is a functional target for Elk-1 was shown to specifically inhibit ET-1-induced BNP transcription (39), whereas it had no effect on stretch-induced promoter activity in the present study. Taken together these observations suggest that the role of GATA-4 in the stretch-induced response is ET-1-independent.

In summary, our results indicate that myocyte stretch, a major component of hemodynamic overload, activates a hypertrophic response in cultured neonatal cardiac myocytes, as demonstrated by enhanced sarcomere organization and activation of BNP transcription. Both responses occur via GATA-4-dependent mechanisms, indicating that GATA-4 is a nuclear mediator of mechanical stretch-activated hypertrophic program. Moreover, our results suggest that the GATA-4/NKx2.5 interaction is targeted by stretch-induced signaling, therefore extending the requirement for GATA-4/NKx2.5 interaction to postnatal cardiomyocytes and implicating its role in the response of cardiomyocytes to external stimuli.

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