The Neuron-Restrictive Silencer Element–Neuron-Restrictive Silencer Factor System Regulates Basal and Endothelin 1-Inducible Atrial Natriuretic Peptide Gene Expression in Ventricular Myocytes

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Induction of the atrial natriuretic peptide (ANP) gene is a common feature of ventricular hypertrophy. A number of cis-acting enhancer elements for several transcriptional activators have been shown to play central roles in the regulation of ANP gene expression, but much less is known about contributions made by transcriptional repressors. The neuron-restrictive silencer element (NRSE), also known as repressor element 1, mediates repression of neuronal gene expression in non-neuronal cells. We found that NRSE, which is located in the 5′ untranslated region of the ANP gene, mediated repression of ANP promoter activity in ventricular myocytes and was also involved in the endothelin 1-induced increase in ANP gene transcription. The repression was conferred by a repressor protein, neuron-restrictive silencer factor (NRSF). NRSF associated with the transcriptional corepressor mSin3 and formed a complex with histone deacetylase (HDAC) in ventricular myocytes. Trichostatin A (TSA), a specific HDAC inhibitor, relieved NRSE-mediated repression of ANP gene expression. Furthermore, in myocytes infected with recombinant adeno-virus expressing a dominant-negative form of NRSF, the basal level of endogenous ANP gene expression was increased and a TSA-induced increase in ANP gene expression was apparently attenuated, compared with those in myocytes infected with control adeno-virus. Our findings show that an NRSE-NRSF system plays a key role in the regulation of ANP gene expression by HDAC in ventricular myocytes and provide a new insight into the role of the NRSE-NRSF system outside the nervous system.

Cardiac hypertrophy is an adaptive response of the heart to mechanical stress, tissue injury or neurohumoral activation, but while hypertrophy initially acts as a compensatory mechanism, when prolonged it can lead to heart failure. In most forms of cardiac hypertrophy, there is an increase in the expression of embryonic genes, including those encoding natriuretic peptides and fetal contractile proteins (6). Expression of such embryonic genes in the ventricular myocardium normally decreases during the perinatal period and remains quiescent in the adult. For instance, the gene encoding atrial natriuretic peptide (ANP) is expressed in both the atrium and the ventricle during embryonic development, but shortly after birth its expression is downregulated in the ventricle, leaving the atrium as the primary site of ANP synthesis (56, 58). However, when ventricles are subjected to hemodynamic overload, expression of the ventricular ANP gene is reactivated (2, 29, 46). This induction of the ANP gene is a common feature of ventricular hypertrophy in all mammalian species and is a prognostic indicator of clinical severity (2, 9, 11, 15, 21, 25, 29, 46, 53). Accordingly, elucidation of the mechanisms regulating ANP gene expression should enable one to better understand the molecular mechanisms involved in the establishment and maintenance of the phenotypes of terminally differentiated cardiac myocytes.

Many of the features seen with hypertrophy in vivo can be duplicated using in vitro cardiomyocyte models, the most commonly used of which employ primary culture of neonatal rat cardiac myocytes (6). Using these systems, it has been shown that the 5′-flanking region (FR) of the ANP gene contains a number of cis-acting enhancer elements for several transcription factors, including Csx/Nkx2.5, GATA4, serum response factor (SRF), AP-1, and Sp-1, which play important roles in the regulation of ANP gene expression under various conditions (3, 10, 14, 16, 27, 30, 45, 51, 52). Nevertheless, its expression profile in the postnatal heart clearly suggests that ANP gene expression in ventricles depends on negative as well as positive transcriptional controls, but much less is known about the mechanisms which specifically mediate the repression of ANP gene transcription.

The neuron-restrictive silencer element (NRSE), also known as repressor element 1 (RE-1), has been defined as a negative-acting DNA regulatory element that prevents expression of neuronal genes in non-neuronal cell types and in undifferentiated neuronal cells (28, 34). To date, several studies have reported that NRSE-like sequences, present in the regulatory regions of multiple neuronal genes, are important for their neuron-specific expression (4, 12, 20, 22, 32, 33, 36, 42, 47, 48,
The neuron-restrictive silencer factor (NRSF), also known as the RE-1 silencing transcription factor (REST), has been identified as an NRSE-binding protein (7, 49). NRSF is a novel member of the Krüppel-like zinc finger transcriptional factor family and is widely expressed in most nonneuronal tissues, including the heart (7, 38, 48). NRSF is also strongly expressed in undifferentiated neuronal progenitors in the nervous system, and its downregulation during development enables the expression of neuron-specific terminal differentiation genes (5).

Not only neuron-specific genes but also some non neuronal genes have been reported to contain sequences similar to NRSE (48), but the function of NRSE in nonneuronal genes is not known yet.

In this study, we found that NRSE, which is located in the 3′ untranslated region (UTR) of the ANP gene, mediated repression of ANP expression in ventricular myocytes and was involved in endothelin 1 (ET-1)-induced reactivation of ANP gene expression. NRSE bound to the NRSE, and the repression was mediated in part through the association of NRSF with the transcriptional corepressor mSin3, resulting in the recruitment of histone deacetylase (HDAC). This is the first report showing the involvement of the NRSE-NRSF system and HDAC in the regulation of cardiac embryonic gene expression in ventricular myocytes. Furthermore, this study also provides new evidence that the NRSE-NRSF system, which is known to function as a silencer to determine cell-type-specific expression of neuronal genes, also participates in the regulation of nonneuronal gene expression as a transcriptional repressor in nonneuronal cells.

MATERIALS AND METHODS

Agents. Human ET-1 was purchased from Peptide Institute, Inc. (Osaka, Japan), and trichostatin A (TSA) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Plasmid constructs. A luciferase gene driven by the proximal enhancer-promoter of the ANP gene, −452hANPLuc, was generated by PCR using genomic DNA as a template: an upstream sense oligonucleotide (5′-GAGA TCTGG CTGCC TGCCCA TTTCC TCCCT TCCAC CCACT ACGGT GTT-3′), which incorporates a BglII site at the 5′ end, and a downstream antisense oligonucleotide (5′-GCGAG ATCTA CTAGA TGAAT GACG CTTCC-3′), which incorporates a BamHI site at the 3′ end of the luciferase gene in −ATAGT CGACA CTAGA TGAAT TAGGC CTCC-3′, which incorporates a ClaI site at the 3′ end and a downstream antisense oligonucleotide (5′-CAGAT CGATT ACTCG TTGCT GACGG CGTT-3′), which incorporates a ClaI site at the 3′ end, was used to amplify. The amplified products were verified by sequencing and then inserted into the ClaI site located upstream of the rabbit β-globin polyadenylation signal and downstream of the chicken β-actin promoter-cytomegalovirus enhancer (CAG promoter) (39) of the cosmid carrying the adenovirus vector.

A luciferase gene driven by the proximal enhancer-promoter of the ANP gene, −452hANPLuc, was generated by PCR using genomic DNA as a template: an upstream sense oligonucleotide (5′-GAGA TCTGG CTGCC TGCCCA TTTCC TCCCT TCCAC CCACT ACGGT GTT-3′), which incorporates a BglII site at the 5′ end, and a downstream antisense oligonucleotide (5′-GCGAG ATCTA CTAGA TGAAT GACG CTTCC-3′), which incorporates a BamHI site at the 5′ end, and a downstream antisense oligonucleotide (5′-GCGAG ATCTA CTAGA TGAAT GACG CTTCC-3′), which incorporates a BamHI site at the 3′ end. This fragment was cloned into the 5′-UTR and the 3′ FR, was obtained by PCR using an upstream sense oligonucleotide (5′-AAGGA GCTCG CAGTA CGTGA TAGCC CTCC-3′), which incorporates a SaeI site at the 5′ end, and a downstream antisense oligonucleotide (5′-CGCGG ATCTA CTAGA TGAAT GACG CTTCC-3′), which incorporates a SaeI site at the 3′ end. This fragment was cloned into the SaeI/BglII sites located upstream of the ANP promoter in −452hANPLuc, and the resultant plasmid was designated +1769 to +2228 of the ANP gene, which encompasses the 3′-UTR and the 3′ FR, was obtained by PCR using an upstream sense oligonucleotide (5′-TACGG ATCTG CAGTA CGTGA TAGCC CTCC-3′), which incorporates a BamHI site at the 5′ end, and a downstream antisense oligonucleotide (5′-TACGG ATCTG CAGTA CGTGA TAGCC CTCC-3′), which incorporates a BamHI site at the 5′ end, and a downstream antisense oligonucleotide (5′-TACGG ATCTG CAGTA CGTGA TAGCC CTCC-3′), which incorporates a SaeI site at the 3′ end. This fragment was cloned into the 3′-UTR and the 3′ FR, was obtained by PCR using an upstream sense oligonucleotide (5′-TACGG ATCTG CAGTA CGTGA TAGCC CTCC-3′), which incorporates a SaeI site at the 3′ end. This fragment was cloned into the SaeI/BglII sites located downstream of the luciferase gene in −452hANPLuc, and the resultant plasmid was designated −452 to +1769hANPLuc. Mutations in the ANP gene NRSE (NRSE−ANP) were generated by PCR using either wild-type +1769 to +2228 of ANP luciferase or −452 to +1769hANPLuc and their homologous NRSE mutants as templates. The sequence of the mutated NRSE−ANP was 5′-CGTCA GCACT ATTTA CAGAA GCAAA A-3′, which has been reported to result in the loss of NRSE binding to the sequence (34). The plasmids carrying the mutation were designated mTR/+1769/-452hANPLuc and mTR/-452/+1769hANPLuc. All plasmid constructions were verified by sequencing.

Plasmids encoding myc-tagged NRSF and a dominant-negative form of NRSF were kindly provided by David J. Anderson (California Institute of Technology, Pasadena). A GAL4-NRSF expression plasmid was constructed within the pBIND vector (Promega, Madison, Wis.). The full-length NRSE cDNA, constructed based on the plasmid encoding myc-tagged NRSF, was cloned in frame with the GAL4 DNA-binding domain of the pBIND vector (pBIND-NRSE).

Generation of recombinant adenovirus. The system used for introducing cDNA into the viral genome was described in detail by Kanegae et al. (23). In brief, a CDNA encoding myc-tagged dominant-negative NRSF was generated by PCR using the expression plasmid encoding myc-tagged dominant-negative NRSF as a template: an upstream sense oligonucleotide (5′-GAGAT CGATT AAAGG TATCG AGC-3′), which incorporates a ClaI site at the 3′ end and a downstream antisense oligonucleotide (5′-CAGAT CGATT ACTCG TTGCT GACGG CGTT-3′), which incorporates a ClaI site at the 3′ end, was used to amplify. The amplified products were verified by sequencing and then inserted into the ClaI site located upstream of the rabbit β-globin polyadenylation signal and downstream of the chicken β-actin promoter-cytomegalovirus enhancer (CAG promoter) (39) of the cosmid carrying the adenovirus vector. The recombinant virus, named AdDNNR, was purified and concentrated as described previously (24).

Ventricular myocyte culture and transfection. Neonatal (2- to 4-day-old) rat ventricular myocytes were prepared on a Percol gradient (37), and their transient transfection was carried out by electroporation at 280 V and 300 mF (26). When transfected with reporter plasmid only, 10 μg of plasmid and another 24 h of transfection with 10 μg of pRL-TK (TOYO INC Co., Ltd.), in which the herpes simplex virus thymidine kinase (TK) promoter was fused to the Renilla luciferase gene, was cotransfected and used to normalize luciferase activity. Transfected cells were initially plated for 24 h in gelatin-coated six-well plates (5 × 105 cells per well) in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). The medium was then changed to serum-free DMEM for 12 h and finally to serum-free DMEM containing 0.1% bovine serum albumin, with or without ET-1 or TSA, for 48 h. The cells were then harvested, lysed, and assayed for luciferase activity using a luminometer (Lumat LB 9507, Berthold, Wildbad, Germany) according to the manufacturer’s protocol (TOYO INC Co., Ltd.). In each experiment, aliquots of cell lysate from triplicate wells were assayed, and the luciferase activities were normalized to pRL-TK luciferase activities.

For immunoprecipitation experiments, ventricular myocytes were transfected with 10 μg of expression vector encoding myc-tagged NRSF alone or cotransfected with a combination of 10 μg of the vector encoding myc-tagged NRSF and FLAG-tagged mSin3B. Cells were then maintained with DMEM supplemented with 10% FCS for 72 h.

Adenoviral infection. One day after plating, ventricular myocytes were infected with adenovirus at a multiplicity of infection of five particles/cell for 24 h in DMEM with 10% FCS. The cells were cultured in serum-free medium for an additional 24 h. The efficiency of expression, examined by determining lacZ gene expression (Abbe & Co., Zürich, Switzerland) in cultured ventricular myocytes, is consistently more than 90% by this protocol.

EMSAs. Nuclear extract from P19 cells was prepared as previously described (8). Double-stranded oligonucleotides containing two copies of NRSE−ANP (5′-CAGTCAGGCA CCGATT AACG AACG-3′) or mutant NRSE−ANP (5′-CAGTCAGGCA CCGATT AACG AACG-3′) was synthesized and used as a probe for electrophoretic mobility shift assays (EMSA). DNA-protein binding reactions were carried out in a 20-μl final volume of reaction buffer containing 20 mM HEPES (pH 7.9), 125 mM KCl, 5 mM MgCl2, 10% glycerol, 125 μg of poly(dI-dC) per ml, and 1 mM dithiothreitol. The nuclear extract (15 μg of protein) was added to the reaction buffer and preincubated for 10 min on ice. Radiolabeled DNA probe was then added, and the nuclear extract was incubated for another 30 min at room temperature. Electrophoresis was then performed in 4% polyacrylamide gels (0.24 M-tris-borate-EDTA) at 1.1 to 150 V. For competition assays, 50× cold double-stranded NRSE−ANP, mutant NRSE−ANP, NRSE from the SCG10 promoter (NRSE−COMP), or the EBNA-binding sequence was coincubated in reaction mixtures with double-stranded radiolabeled NRSE−ANP probe as described above.

Monoclonal antibody raised against NRSF (3B7) used in the supershift experiment was generated using the NRSF N-terminal domain (residues 1 to 150) fused with glutathione S-transferase as an antigen and purified through BALB/c ascites.

Coimmunoprecipitation. Ventricular myocytes transfected only with expression vector encoding myc-tagged NRSF were cultured for 72 h with DMEM...
supplemented with 10% FCS, collected in lysis buffer, and sonicated. The cell debris was cleared by centrifugation, after which the supernatant (1 ml) was mixed overnight at 4°C with monoclonal anti-myc antibodies (9E7, 1:100 dilution; Santa Cruz Biotech, Inc., Santa Cruz, Calif.). Immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting using the following dilutions of antibodies as probes (Santa Cruz Biotech): anti-myc (1:100), anti-mSin3A (1:100), anti-mSin3B (1:100), anti-HDAC1 (1:100), and anti-HDAC2 (1:100).

Alternatively, the supernatant obtained from ventricular myocytes transfected with the vector combination encoding myc-tagged NRSF and FLAG-tagged mSin3B was mixed overnight at 4°C with anti-myc (1:100) or anti-FLAG (M2, 1:100; Sigma Chemical, St. Louis, Mo.) antibodies. Immunoprecipitates were subjected to SDS-PAGE and analyzed by Western blotting using anti-myc (1:100) and anti-FLAG (1:500).

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were carried out according to the protocols supplied by the manufacturer (Upstate Biotech, Lake Placid, N.Y.). Briefly, cells were fixed in 1% formaldehyde for 15 min at 37°C. They were then collected, resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1]) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, and 1 μg of pepstatin A per ml; sonicated three times for 10 s each time; and cleared by centrifugation. One aliquot of the lysate (10 μl) was removed and served as a control; the remainder was incubated overnight at 4°C in ChIP dilution buffer (16.7 mM Tris [pH 8.1], 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS, 1.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin per ml, and 1 μg of pepstatin A per ml) with 5 μl of anti-acetylated H4 antibody. Immunocomplexes were then recovered by adding 60 μl of salmon sperm DNA and a protein A-agarose bead suspension, followed by incubation at 4°C for 4 h. The beads were then sequentially washed for 5 min each time in 1 ml of buffer containing 150 mM or 500 mM NaCl plus 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl (pH 8.1), 2 mM EDTA, and Tris-EDTA (TE) (pH 8.0). The immunocomplexes were eluted by incubating the beads with 200 μl of 1% SDS plus 100 mM NaHCO3. After the addition of 5 mM NaCl, the eluates were heated to 65°C for 6 h to reverse any formaldehyde cross-linking. DNA was recovered using proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. The resultant pellets were resuspended in 50 μl of TE. Quantitative PCR was then carried out for 23 to 28 cycles using 3-μl samples of the DNA. Primers amplifying the DNA fragments encompassing the 3'-UTR of the human ANP gene and the PGV-B2 sequence were used to amplify −452/+1769ANP/Luc and −452/+1769αANP/Luc, while primers amplifying the DNA fragment encompassing the 3'-UTR and 3'-FR of the rat ANP gene were used to amplify the endogenous rat ANP gene. PCR products were resolved by electrophoresis and visualized with ethidium bromide. Images were recorded and quantified using NIH Image 1.5 Software. Aliquots of chromatin before immunoprecipitation were also analyzed by PCR (INPUT).

Northern blot analysis. Total RNA was prepared from ventricular myocytes using Trizol (Life Technologies, Inc., Rockville, Md.), and 10-μg samples were separated and transferred to Biodyne membranes (Pall Corp., Glen Cove, N.Y.). Randomly labeled ANP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were then hybridized to the membranes as previously described (13, 37).

RT-PCR analysis. Single-stranded cDNA was synthesized with the Thermoscript reverse transcription-PCR (RT-PCR) kit (Life Technologies, Inc.) using 3 μg of total RNA prepared from cultured ventricular myocytes incubated with or without 10 nM ET-1 for 24 h. The mRNA level of NRSF and REST4, an alternative spliced variant of NRSF, in cultured ventricular cells was determined by PCR using an upstream sense oligonucleotide (5′-GCTAC AGTTA TGGCC-3′) and a downstream antisense oligonucleotide (5′-AGAT AAGC GGCA GGGCA GTGTA-3′) for REST4 (40). The PCR products were analyzed on a 2% agarose gel.

RIA. The concentrations of ANP in the culture medium were measured by using our specific radioimmunoassay (RIA) as previously reported (13, 37).

Statistical analysis. Data are presented as the mean ± the standard error of the mean (SEM). Analysis of variance with post hoc Fisher's tests was used to determine significant differences. P values of <0.05 were considered significant.

RESULTS

NRSE<sup>ANP</sup> in the 3′-UTR mediates repression of ANP gene transcription in ventricular myocytes. Although the proximal enhancer-promoter region of the ANP gene is known to mediate cardiac myocyte-specific gene expression, it is not sufficient to regulate the expression of the ANP gene under several conditions (25, 44). To determine the regulatory roles of the ANP gene 3′-UTR and 3′-FR, the sequence from positions +1769 to +2288 of the ANP gene was inserted either upstream or downstream of a reporter gene driven by the ANP proximal enhancer-promoter (Fig. 1A). To our surprise, the fragment markedly repressed the activity of the ANP promoter in both cases (Fig. 1B). Using a computer search, we identified sequences in this region that were similar to NRSE. Such NRSE-like sequences (NRSE<sup>ANP</sup>) are well conserved in the ANP genes of mammalian species (Fig. 2A), and we therefore hypothesized that repression by ANP gene fragment from +1769 to +2288 is mediated by NRSE<sup>ANP</sup>.

To verify that NRSE<sup>ANP</sup> mediated the observed repression, we generated a mutant NRSE<sup>ANP</sup>, as previously reported (34), and examined the expression of the mutant constructs. As shown in Fig. 2B, introduction of a mutation into the NRSE<sup>ANP</sup> completely abolished repression mediated by the fragment from +1769 to +2288, confirming that NRSE<sup>ANP</sup> mediated the repressor activity of this fragment. The fact that the mutant constructs showed greater luciferase activities than the construct containing the proximal promoter alone suggests that other enhancer elements exist in the region whose activities are normally repressed in the presence of NRSE<sup>ANP</sup>. When the fragment from +1769 to +2288, with or without the mutation, was fused upstream of the TK or simian virus 40 (SV 40) promoter (mtNR/+1769αA-TKLuc or +1769αA-TKLuc [for TK] or mtNR/+1769αA-SV40Luc or +1769αA-SV40Luc [for SV40]), the reporter constructs containing the mutant NRSE<sup>ANP</sup> showed greater luciferase activities than those containing wild-type NRSE<sup>ANP</sup>, thus demonstrating that NRSE<sup>ANP</sup> can repress the activities of multiple promoters in ventricular myocytes (Fig. 2C and D).

To further confirm that NRSE is sufficient to repress the promoters in ventricular myocytes, we generated reporter genes in which only two copies of NRSE<sup>ANP</sup> were inserted upstream of −452ANP/Luc or TKLuc (2NR-hALuc and 2NR-TKLuc, respectively). As shown in Fig. 2E and F, the luciferase activities of 2NR-hALuc or 2NR-TKLuc were significantly lower than those of −452ANP/Luc or TKLuc, respectively.

NRSF represses transcription of the ANP gene through NRSE<sup>ANP</sup>. NRSF (or REST), a zinc finger transcriptional repressor protein, is known to bind to NRSE (7, 49). To test whether NRSF binds to NRSE<sup>ANP</sup>, EMSAs were carried out using nuclear extract from P19 cells, which is known to be abundant in NRSF, and a shifted band was observed when radiolabeled NRSE<sup>ANP</sup> was used as a probe (Fig. 3A, arrow, lane 2). The shift was completely blocked by the addition of cold NRSE<sup>ANP</sup> (Fig. 3A, lane 3) or NRSE from the SCG10 promoter (NRSE<sup>SCG10</sup>) (Fig. 3A, lane 4) but was unaffected by a cold probe for EBNA binding sequences (Fig. 3A, lane 5). When the mutant NRSE<sup>ANP</sup> was used as a probe, no shifted band was observed (Fig. 3B, lane 7, intact NRSE<sup>ANP</sup> as a probe, versus lane 8, mutant NRSE<sup>ANP</sup> as a probe). In addition, when intact NRSE<sup>ANP</sup> was used as a probe (Fig. 3B, lane 9), cold mutant NRSE<sup>ANP</sup> probe failed to block the band shift, in contrast to the cold intact NRSE<sup>ANP</sup> probe (Fig. 3B, lane 10). Furthermore, the band was supershifted in the presence of
an antibody raised against NRSF (Fig. 3C, arrowhead, lane 13). All of these results indicate that NRSF binds to NRSE ANP.

We next assessed whether the bound NRSF was responsible for the NRSE ANP-mediated repression of ANP expression. Ventricular myocytes were cotransfected with reporter genes and a dominant-negative NRSF mutant that lacked both the N- and C-terminal regions required for repressor activities (5). As shown in Fig. 3D, dominant-negative NRSF abolished the repression of the ANP promoter mediated by an intact 3'-UTR, whereas it had no effect on reporter gene expression by constructs carrying the mutant NRSE ANP. To further confirm that recruitment of NRSF is sufficient to repress ANP promoter activity, we generated 5UAS-hANPLuc, a reporter plasmid containing five copies of the GAL4 binding site inserted upstream of 2452hANPLuc, and cotransfected myocytes with 5UAS-hANPLuc and the plasmid encoding GAL4-NRSF fusion protein. The marked repression of the activities of 5UAS-hANPLuc in the presence of GAL4-NRSF shown in Fig. 3E clearly illustrates that NRSF recruited by the gene represses ANP promoter activity.

We further examined a function of NRSF in endogenous ANP gene expression by using a recombinant adenovirus expressing a dominant-negative form of NRSF (Ad/DNNR). As shown in Fig. 4A, the sequence spanning positions +1769 to +2228 of the ANP gene mediates repression on the ANP promoter. (A) Schematic representation of the reporter constructs. (B) A total of 10 μg of −452hANPLuc, +1769−452hANPLuc, or −452/+1769hANPLuc was cotransfected into ventricular myocytes with 4 μg of pRL-TK; +1769−452hANPLuc and −452/+1769hANPLuc contain the fragment from +1769 to +2228 inserted upstream or downstream, respectively, of −452hANPLuc. Transfected cells were plated in DMEM supplemented with 10% FCS for 24 h and then maintained in serum-free DMEM for 60 h. Luciferase activity was normalized to the activity of Renilla luciferase driven by the TK promoter; the relative luciferase activities of −452hANPLuc were assigned a value of 100. The bars represent the mean ± the SEM values of relative luciferase activities from at least three separate assays carried out in triplicate. * P < 0.05 versus −452hANPLuc.

FIG. 1. The sequence spanning positions +1769 to +2228 of the ANP gene mediates repression on the ANP promoter. (A) Schematic representation of the reporter constructs. (B) A total of 10 μg of −452hANPLuc, +1769−452hANPLuc, or −452/+1769hANPLuc was cotransfected into ventricular myocytes with 4 μg of pRL-TK; +1769−452hANPLuc and −452/+1769hANPLuc contain the fragment from +1769 to +2228 inserted upstream or downstream, respectively, of −452hANPLuc. Transfected cells were plated in DMEM supplemented with 10% FCS for 24 h and then maintained in serum-free DMEM for 60 h. Luciferase activity was normalized to the activity of Renilla luciferase driven by the TK promoter; the relative luciferase activities of −452hANPLuc were assigned a value of 100. The bars represent the mean ± the SEM values of relative luciferase activities from at least three separate assays carried out in triplicate. * P < 0.05 versus −452hANPLuc.

We further examined a function of NRSF in endogenous ANP gene expression by using a recombinant adenovirus expressing a dominant-negative form of NRSF (Ad/DNNR). As shown in Fig. 3F and G, endogenous ANP mRNA expression in ventricular myocytes infected with Ad/DNNR was markedly increased, compared with that in myocytes infected with the control adenovirus (Ad/lacZ). All of these lines of data indicate that NRSF represses ANP gene expression in ventricular myocytes through NRSE ANP.

The NRSE-NRSF system is involved in ET-1-induced expression of the ANP gene. ANP gene expression is reactivated in ventricular myocytes during cardiac muscle cell hypertrophy. We examined the role of NRSE ANP in hypertrophic stimulus-induced ANP gene expression using ET-1 as a hypertrophy-inducing agent (13, 37).

We first evaluated the effect of NRSE ANP mutation on the ET-1 inducibility of an ANP reporter gene. Figure 4A shows that the ET-1 inducibility of mtNR/−452/+1769hANPLuc or mtNR/+1769−452hANPLuc was about 50% lower than that of −452/+1769hANPLuc or +1769/−452hANPLuc, respectively. This suggests that NRSE ANP, at least in part, mediates the ET-1 inducibility of ANP gene expression.

To test whether NRSE ANP could mediate the ET-1 induction of other promoters, the ET-1 inducibility of an ANP reporter gene. Figure 4A shows that the ET-1 inducibility of mtNR/−452/+1769hANPLuc or mtNR/+1769−452hANPLuc was about 50% lower than that of −452/+1769hANPLuc or +1769/−452hANPLuc, respectively. This suggests that NRSE ANP, at least in part, mediates the ET-1 inducibility of ANP gene expression.

To test whether NRSE ANP could mediate the ET-1 induction of other promoters, the ET-1 inducibility of +1769hA-TKLuc was compared with that of mtNR/+1769hA-TKLuc. We found that the reporter activities of +1769hA-TKLuc were increased by ET-1 stimulation and that mutation of NRSE ANP within the construct (mtNR/+1769hA-TKLuc) markedly decreased the capacity of ET-1 to induce reporter activity (Fig. 4B). To further examine the role of NRSE ANP in mediating the induction of the ANP promoter by ET-1, we compared the ET-1 inducibility of 2NR-hALuc to that of −452hANPLuc. As shown in Fig. 4C, the response to ET-1 of 2NR-hALuc, in which only two copies of NRSE were inserted upstream of the
ANP promoter, was increased in comparison with that of 2452hANPLuc (Fig. 4C). When the effect of dominant-negative NRSF on the ET-1 inducibility of ANP reporter gene activity was examined, we found that cotransfection of dominant-negative NRSF with 11769/2452hANPLuc diminished the capacity of ET-1 to induce reporter gene activity, although cotransfection of dominant-negative NRSF with mtNR/11769/2452hANPLuc did not (Fig. 4D). It thus appears that the NRSF-NRSE system has a significant function in the ET-1-induced signaling pathways leading to the upregulation of ANP gene expression.

Because all these data suggest that ET-1 attenuates NRSE-mediated repression, we considered the possibility that ET-1 downregulates the level of NRSF in ventricular myocytes. However, RT-PCR analysis showed that treatment of ventricular myocytes with ET-1 did not decrease the level of NRSF mRNA (Fig. 4E). A recent study demonstrated that, in neuronal cells, REST4, a neuron-specific splice variant of NRSF, is induced in a protein kinase A-dependent manner and antagonizes the repressor function of NRSF (50). We next examined whether REST4 is induced by ET-1 in ventricular myocytes. As shown in Fig. 4F, however, REST4 mRNA was neither detected in basal condition nor induced by ET-1 in ventricular myocytes. These results suggest that ET-1 may modify the function of NRSF by posttranscriptional mechanisms.

NRSF associates with the mSin3-HDAC complex in ventricular myocytes. Recent studies have shown that NRSF associates with the mSin3-HDAC complex both in vivo and in vitro and that
Histone deacetylation is involved in the transcriptional repression mediated by the NRSE-NRSF system (18, 38, 44). We therefore examined whether the interaction between NRSF and mSin3-HDAC also occurs in ventricular myocytes. An expression plasmid encoding FLAG-tagged mSin3B was transfected into ventricular myocytes with 10 μg of either +1769/-452hANPLuc or mtNR/-1769/-452hANPLuc and 4 μg of pRL-TK. The luciferase activity was normalized to the activity of Renilla luciferase driven by the TK promoter; the luciferase activity of +1769/-452hANPLuc cotransfected with the control vector was assigned a value of 1.0. *P < 0.05 versus +1769/-452hANPLuc without DN-NRSF. (E) Recruitment of NRSF is sufficient to repress ANP promoter activity. 5UAS-hANPLuc, which contains five copies of the GAL4-binding site inserted upstream of -452hANPLuc, was cotransfected with the indicated doses of plasmid encoding GAL4-αNRSF fusion protein (pBIND-NRSF). The relative luciferase activity of 5UAS-hANPLuc transfected with only a control vector was assigned a value of 1.0. *P < 0.05 versus 5UAS-hANPLuc without pBIND-NRSF. For all experiments, the bars represent the mean ± the SEM value of the relative luciferase activities from at least three separate assays carried out in triplicate. (E) Expression of dominant-negative NRSF in ventricular myocytes infected with Ad/DNNR. Whole-cell lysates from ventricular myocytes infected with Ad/lacZ or Ad/DNNR were separated by electrophoresis and blotted with monoclonal anti-myc antibody (9E7). (F) A dominant-negative NRSF increased endogenous rat ANP gene expression in ventricular myocytes. Myocytes were infected with control Ad/lacZ or with Ad/DNNR at a titer of five particles/cell. Cells were incubated for 48 h and then harvested for analysis by Northern blotting. Shown are representative blots for ANP mRNA from three independent experiments with identical results.

To test whether NRSF associates with the endogenous mSin3-HDAC complex, coimmunoprecipitation was performed on whole-cell lysates from ventricular myocytes transfected with the expression vector for myc-tagged NRSF. The lysates were then incubated with either anti-myc antibody or control mouse immunoglobulin G (IgG). Immunocomplexes were separated by SDS-PAGE, subjected to Western blotting, and visualized using anti-mSin3A and -B or anti-HDAC1 and -2. As shown in Fig. 5B, endogenous mSin3A and -B and HDAC1 and -2 proteins were immunoprecipitated with introduced myc-tagged NRSF, indicating that, as previously reported in other cell types (18, 38), NRSF in ventricular myocytes recruits the mSin3-HDAC complex. These results suggest the involvement of HDAC in NRSE-NRSF-mediated repression of ANP gene transcription.
NRSE-NRSF represses ANP gene transcription through histone deacetylation. To determine whether HDAC is involved in NRSE-mediated repression of ANP gene transcription, we tested the effects of TSA, a specific inhibitor of HDAC, on ANP gene expression. Ventricular myocytes transfected with reporter plasmids containing intact or mutated NRSEANP were incubated with 10 ng of TSA per ml for 48 h, after which the cells were lysed and the luciferase activities were measured.
TSA increased the transcriptional activities of reporter genes containing intact NRSE to a greater degree than those containing mutated NRSE\(^\text{ANP}\) or those lacking NRSE\(^\text{ANP}\) altogether (Fig. 6A).

Then, to test whether NRSE mutation correlates with changes in the status of histone acetylation, ChIP assays were performed using primers spanning NRSE\(^\text{ANP}\). These assays revealed that NRSE\(^\text{ANP}\) mutation markedly increases the level of acetylated histones associated with the 3′-UTR of the ANP gene (Fig. 6B). Moreover, it was found that, in the endogenous ANP gene, the acetylation level of histones around NRSE\(^\text{ANP}\) is low under basal conditions (Fig. 7A) and that TSA treatment increases the acetylation level of the histones, suggesting that HDAC is largely responsible for maintaining basal hypoacetylation of histones around NRSE\(^\text{ANP}\). This TSA-induced increase in the acetylation level of histones around NRSE\(^\text{ANP}\) correlates with increased endogenous ANP gene expression (Fig. 7A and B). Consistent with the increased expression of ANP mRNA, the level of secreted ANP protein was also higher in TSA-treated myocytes than in untreated cells (Fig. 7C). TSA had no effect on the expression of the GAPDH gene, which is constitutively expressed in ventricular myocytes, indicating that its effect on ANP expression is specific. Furthermore, the TSA-induced increase in endogenous ANP gene expression in ventricular myocytes infected with Ad/DNNR was diminished compared with that in myocytes infected with control Ad/lacZ (Fig. 7D and E). These results clearly indicate that the NRSE-NRSF system represses ANP gene expression, at least in part, through the recruitment of HDAC.

Because we found that NRSE\(^\text{ANP}\) is involved in ET-1-induced ANP expression, our final experiments were designed to determine the effect of ET-1 on histone acetylation. As shown in the Fig. 7F, ET-1 increased the acetylation of histones associated with the 3′-UTR of the ANP gene, supporting the notion that attenuation of NRSE-mediated repression is an important component of the mechanism by which ET-1 induces ANP gene expression.

**DISCUSSION**

In the regulation of ANP gene expression in ventricular myocytes, a number of cis-acting enhancer elements have been shown to play central roles (3, 10, 16, 30, 51, 52). Ventricular ANP gene expression is elevated during embryonic life, but it remains low in the postnatal heart unless the ventricle is subjected to hemodynamic stress. This profile of ventricular ANP gene expression suggests that negative as well as positive transcriptional controls play an important role in ANP gene expression, but the contribution made by transcriptional repressors remains unclear. Here we show that NRSE, which is located in the 3′-UTR of the ANP gene, mediates repression of ANP gene expression in ventricular myocytes and is also involved in the ET-1-induced increase in ANP gene expression. The repression is conferred by NRSF, and HDAC is involved in the NRSE-mediated repression of ANP gene transcription. NRSE-mSin3-HDAC complex-mediated repression under the basal condition and several sources of hypertrophic stimulus-induced inhibition of the repression may contribute to the dynamic changes of ANP gene expression in ventricular myocytes. Our findings show a novel regulatory mechanisms of ANP gene expression in ventricular myocytes and provide a new insight into the role of the NRSE-NRSF system outside the nervous system.

The ANP 3′-UTR containing the NRSE repressed the ANP proximal promoter, whether it was situated upstream or downstream of the promoter. NRSE\(^\text{ANP}\) mutation completely abolished the repression by the 3′-UTR, confirming the role of NRSE in mediating the repression. NRSE\(^\text{ANP}\) also mediated repression of the TK and SV40 promoters, indicating that NRSE\(^\text{ANP}\) is capable of repressing multiple promoters in ventricular myocytes. In fact, two copies of NRSE\(^\text{ANP}\) were sufficient to repress both the ANP and TK promoters. Because NRSF is expressed in nonneuronal tissues, including the heart, and has been reported to bind to NRSE, we tested whether NRSF binds to the NRSE\(^\text{ANP}\). EMSAs carried out using radiolabeled NRSE\(^\text{ANP}\) as a probe demonstrated that NRSF does bind to the sequence. A dominant-negative mutant of NRSF introduced into ventricular myocytes with the ANP reporter gene restored the NRSE-mediated repression of ANP promoter activity. Furthermore, a GAL4-NRSF fusion protein significantly repressed the activities of an ANP promoter carrying the tandem GAL4-binding sites in its upstream region. Thus, recruitment of NRSF to the ANP gene is apparently responsible for NRSE\(^\text{ANP}\)-mediated repression of ANP gene expression.
FIG. 6. HDAC is involved in NRSE-mediated repression of the ANP promoter. (A) NRSE mutation decreases TSA inducibility of ANP reporter genes. A total of 10 μg of +1769−452hANP/Luc, −452+1769aNPLuc, −452hANPLuc, mtNR/+1769/−452hANPLuc, or mtNR/−452/+1769hANPLuc was cotransfected into ventricular myocytes with 4 μg of pRL-TK, after which the cells were incubated for 48 h with or without 10 ng of TSA per ml. The relative luciferase activities were normalized to the activity of Renilla luciferase driven by the TK promoter. The relative luciferase activity of each construct transfected into myocytes treated with TSA was expressed as the fold increase over the value for constructs transfected into myocytes not treated with TSA. The bars represent the mean ± the SEM values of relative luciferase activities from at least three separate assays carried out in triplicate. (B) NRSE mutation causes a marked increase in the level of acetylated histones associated with the 3′-UTR of the ANP gene. Soluble chromatin preparations from myocytes transfected with −452+1769mANPLuc or mtNR/−452/+1769hANPLuc were immunoprecipitated with an antibody raised against acetylated histone H3 or H4 (α-AcH3 or α-AcH4, respectively) and analyzed by quantitative PCR. Aliquots of chromatin obtained before immunoprecipitation were also analyzed (INPUT). Representative PCR results are shown in the lower three panels. The graphs show the relative amounts of PCR product coimmunoprecipitated with acetylated H3 or H4, corrected for the total amount of PCR product. The data are the mean ± the SEM values from three independent experiments carried out in duplicate. *, P < 0.05 versus mtNR/+1769/−452hANPLuc. The bars and numbers above the target genes represent the relative positions within the genes and the lengths of the products amplified in the PCR analysis.
expression. In fact, adenovirus-mediated expression of dominant-negative NRSF in ventricular myocytes increased endogenous ANP gene expression.

NRSE was initially identified as a silencer element regulating neuron-specific expression of the SCG10 and type II sodium channel (NaII) genes in rat (28, 34) and is now known to regulate neuron-specific expression of many neuronal genes (4, 12, 20, 22, 32, 33, 36, 42, 47, 48, 55). In addition, Schoenherr et al. described five nonneuronal genes that also contain functional NRSE (48). However, the functional role of NRSE in the expression of nonneuronal genes has not been elucidated. Since ANP gene expression and synthesis of the protein are predominantly higher in the heart than in other tissues, our data represent the first evidence that NRSE also functions as a repressor of nonneuronal genes, as well as of neuron-restricted genes, in certain tissues. Of particular interest to us is the fact that ANP is expressed in the same cardiac myocytes in which NRSF is expressed, suggesting that repression by NRSF can be

FIG. 7. HDAC is involved in the regulation of endogenous ANP gene expression in ventricular myocytes. (A) TSA induces the acetylation of histones associated with the 3′-UTR of the endogenous ANP gene. Soluble chromatin preparations from ventricular myocytes treated with or without TSA for 24 h were immunoprecipitated using an anti-acetylated histone H3 or H4 antibody (α-AcH3 or α-AcH4) and analyzed by quantitative PCR. Aliquots of chromatin obtained before immunoprecipitation were also analyzed (INPUT). Representative PCR results are shown in the lower three panels. The results shown in the top panel are expressed as the relative amount of PCR product coimmunoprecipitated with acetylated H3 or H4 corrected for the total amount of PCR product. The data are the mean ± the SEM values from three independent experiments carried out in duplicate. * P < 0.05 versus TSA (–). The bar and numbers above the target gene represent the relative position in the ANP gene and the length of the product amplified in the PCR analysis. (B) TSA induces the endogenous ANP gene expression in ventricular myocytes. Cells were incubated for 24 h with or without 10 ng of TSA per ml and then harvested for analysis by Northern blotting. Shown are representative blots for ANP and GAPDH (G3PDH) mRNA from two independent experiments with identical results. (C) TSA increased the secretion of ANP. Ventricular myocytes were incubated for 48 h with or without 10 ng of TSA per ml, and the concentration of ANP in the culture medium was measured by RIA. The data are the mean ± the SEM values from two independent experiments carried out in triplicate. * P < 0.05 versus the control. (D) TSA-induced increase in endogenous ANP gene expression is attenuated in ventricular myocytes infected with Ad/DNNR. Cells infected with control Ad/lacZ or Ad/DNNR were incubated for 24 h with or without 10 ng of TSA per ml and then harvested for analysis by Northern blotting. Shown are representative blots for ANP mRNA from two independent experiments with identical results. (E) The graphs show the relative ANP mRNA levels, which were determined by quantitation of the density of ANP mRNA bands using NIH image software and by correction with the amount of 28S. The relative ANP mRNA level in ventricular myocytes infected with Ad/lacZ and treated without TSA was assigned a value of 1.0. The data are the mean ± the SEM values obtained from two independent experiments carried out in triplicate. The numbers above each bar indicate the fold increase induced by TSA (10 ng/ml). (F) ET-1 increases the level of acetylation of histones associated with the 3′-UTR of the endogenous ANP gene. Soluble chromatin preparations from cells treated with or without ET-1 for 24 h were immunoprecipitated with α-AcH3 or α-AcH4 and analyzed by quantitative PCR. Aliquots of chromatin obtained before immunoprecipitation were also analyzed (INPUT). Representative PCR results are shown in the lower three panels. The results shown in the top panel are expressed as the relative amount of PCR product coimmunoprecipitated with acetylated H3 or H4, corrected for the total amount of PCR product. The data are the mean ± the SEM values obtained from three independent experiments carried out in duplicate. * P < 0.05 ET (–).
acts directly with core promoter factors. It has been proposed that whereas the mSin3-HDAC complex associates with the N-terminus repressor domain, the C-terminus repressor domain of NRSF acts by a mechanism unrelated to histone deacetylation (18, 38). In that regard, CoREST, a novel corepressor, was recently reported to interact with the C-terminal domain (1). CoREST contains two SANT (SW13/ADA2/NcoRTFIIB) domains, the same structures found in the Ncor/SMRT corepressors of steroid hormone receptors (17, 35, 41). It will be of great interest to delineate the mechanisms by which CoREST could function as a corepressor to completely resolve the mechanism by which NRSF represses ANP gene transcription in ventricular cells.

NRSF has been reported to contain repressor domains at both its N and its C termini (38, 54). mSin3 associates with the N terminus and forms a complex with HDAC, thereby mediating repression through histone deacetylation (18, 39). Since NRSF was shown in C6 glioma and NIH 3T3 cells to repress the expression of target genes in part via recruitment of the mSin3-HDAC complex (18, 39), we examined whether mSin3-HDAC is also involved in NRSE-NRSF-mediated repression in cardiac myocytes. Cotransfection and immunoprecipitation of myc-tagged NRSF and FLAG-tagged mSin3B showed that the two proteins do associate in ventricular myocytes. Further, an introduced NRSF associated with endogenous mSin3 and HDAC, which confirms that NRSF recruits mSin3-HDAC complex in ventricular myocytes and suggests that NRSF represses ANP gene transcription, at least in part, via histone deacetylation. Consistent with that idea, inhibition of HDAC using TSA increased the luciferase activity of ANP reporter constructs carrying intact NRSEANP to a significantly greater degree than those carrying mutant NRSEANP or lacking NRSEANP. Finally, ChIP assays showed that the level of acetylation of histones associated with a reporter gene containing intact NRSEANP was lower than that of histones associated with the gene containing mutant NRSEANP.

We also assessed the level of acetylation of the histones around the NRSE of the endogenous ANP gene. In this case, ChIP assays revealed that the level of histone acetylation was low under basal conditions and was significantly increased by TSA. Corresponding Northern blots showed that the expression of the endogenous ANP gene, but not the GAPDH gene, was upregulated with TSA. The upregulation of endogenous ANP gene expression by TSA was attenuated in ventricular myocytes infected with Ad/DNNR. In summary, these results are consistent with a scenario in which the NRSE-NRSF system represses ANP gene expression in ventricular myocytes through the recruitment of HDAC and the deacetylation of histones.

Although HDAC is certainly involved in the mechanism by which NRSF-NRSE represses ANP gene expression, the details of the mechanism by which recruited NRSF represses the ANP promoter is not well understood. Relocation of the NRSF-mSin3-HDAC complex to the core promoter region, which enables the deacetylation of histones and, maybe, the transcription factors bound there, would be essential for repression by the complex (19). It is known that mSin3A is able to bind the general transcription factor TFIIIB, indicating the possibility that mSin3 may recruit the NRSF repressor complex to the core promoter region (57). Another possibility is that factors associating with the C-terminus region of NRSF interact directly with core promoter factors. It has been proposed that whereas the mSin3-HDAC complex associates with the N-terminus repressor domain, the C-terminus repressor domain of NRSF acts by a mechanism unrelated to histone deacetylation (18, 38). In that regard, CoREST, a novel corepressor, was recently reported to interact with the C-terminal domain (1). CoREST contains two SANT (SW13/ADA2/NcoRTFIIB) domains, the same structures found in the Ncor/SMRT corepressors of steroid hormone receptors (17, 35, 41). It will be of great interest to delineate the mechanisms by which CoREST could function as a corepressor to completely resolve the mechanism by which NRSF represses ANP gene transcription in ventricular cells.

NRSE is also apparently involved in the reactivation of ANP gene expression during hypertrophic responses, since NRSE mutation or expression of a dominant-negative NRSF substantially reduced the capacity of ET-1 to induce ANP gene expression. This suggests that attenuation of NRSE-mediated repression of the ANP gene is an important component of the ET-1-induced increase in ANP gene expression. Indeed, ET-1 was found to increase the level of acetylation of histones around NRSEANP, supporting the notion that ET-1 inhibits NRSE-NRSF-mediated repression of ANP gene transcription. The fact that ET-1 did not completely abolish NRSE-NRSE-mediated repression means its inhibition of NRSF is incomplete. The details of the mechanisms by which ET-1 attenuates the repressor function of NRSF remain to be determined, however. In neuronal cells, REST4, an alternatively spliced variant of NRSF, was recently reported to be induced dependent on protein kinase A and to act in a dominant-negative fashion (50). However, because we could not detect REST4 mRNA expression in ventricular myocytes in both basal and ET-1-stimulated condition, it is unlikely that ET-1 induces REST4 and inhibits the repressor function of NRSF in ventricular myocytes (Fig. 4F). In addition, ET-1 did not decrease the expression level of NRSF mRNA in ventricular myocytes (Fig. 4E). ET-1 may regulate the function of NRSF by post-transcriptional mechanisms. In that regard, it was reported that Eve, a transcriptional repressor, is negatively regulated by phosphorylation (31). Phosphorylated Eve was unable to interact with the TATA-binding protein, a target for repression. ET-1 may phosphorylate NRSF and attenuates its repressor function through the allosteric interference with a protein-protein or protein-DNA interaction.

That ANP reporter constructs carrying the mutant NRSEANP were still induced indicates that ET-1 also exerts effects on other regions. As shown in this study and by others, the ANP promoter region can also mediate ET-1-inducible ANP gene expression. The ANP promoter region contains elements for several transcriptional enhancers, including SP-1 and SRF, which were reportedly involved in the induction of ANP gene expression under various conditions (3, 16, 52). Thus, ET-1 appears to induce ANP gene expression through both upregulation of enhancer activities and downregulation of repressor activities.

NRSE also exists in several other cardiac embryonic genes, including those for BNP, skeletal α-actin, and the Na,K-ATPase α3 subunit (Fig. 8). Although the role of NRSE in the expression of the Na,K-ATPase α3 subunit gene is controversial (42, 48), we recently confirmed that NRSE acts as a repressor of BNP gene expression (E. Ogawa and Y. Saito,
unpublished data), and others have shown that, when translated in vitro, NRSF binds to the NRSE in the skeletal α-actin gene (48). Thus, NRSF may be an important regulator of the expression of various cardiac embryonic genes. Indeed, by controlling the expression of several embryonic genes, NRSF may participate in the establishment and maintenance of the terminally differentiated phenotype of ventricular myocytes.

In conclusion, our results demonstrate the involvement of the NRSE-NRSF system in the regulation of basal and inducible ANP gene expression in ventricular myocytes. This study also provides a new insight into a potential contribution of the NRSE-NRSF system in the normal physiology and pathophysiology of nonneuronal tissues.

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