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The Atrial Natriuretic Factor Promoter Is a Downstream Target for Nkx-2.5 in the Myocardium

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The recently described NK2 family of homeodomain proteins are key developmental regulators. In *Drosophila melanogaster*, two members of this family, bagpipe and tinman, are required for visceral and cardiac mesoderm formation, respectively. In vertebrates, *tinman* appears to represent a family of closely related NK2 genes, including Nkx-2.5, that are expressed at an early stage in precardiac cells. Consistent with a role for Nkx-2.5 in heart development, inactivation of the Nkx-2.5 gene in mice causes severe cardiac malformations and embryonic lethality. However, little is known about the molecular action of Nkx-2.5 and its targets in cardiac muscle. In this paper, we report the identification and characterization of a functional and highly conserved Nkx-2.5 response element, termed the NKE, in the proximal region of the cardiac atrial natriuretic factor (ANF) promoter. The NKE is composed of two near-consensus NK2 binding sites that are each able to bind purified Nkx-2.5. The NKE is sufficient to confer cardiac cell-specific activity to a minimal TATA-containing promoter and is required for Nkx-2.5 activation of the ANF promoter in heterologous cells. Interestingly, in primary cardiocyte cultures, the NKE contributes to ANF promoter activity in a chamber- and developmental stage-specific manner, suggesting that Nkx-2.5 and/or other related cardiac proteins may play a role in chamber specification. This work provides the identification of a direct target for NK2 homeoproteins in the heart and lays the foundation for further molecular analyses of the role of Nkx-2.5 and other NK2 proteins in cardiac development.

The homeodomain is a 60-amino-acid motif that folds into a very stable helix-turn-helix structure which is able to bind DNA with high affinity. Both genetic and biochemical data have shown that homeodomain proteins are sequence-specific transcriptional regulators most often involved in developmental processes such as body segmentation, cell differentiation, and organogenesis. The homeodomain itself is a very conserved unit found in all eukaryotes from yeasts and protozoa to higher vertebrates. Moreover, specific members of the homeodomain superfamily are conserved throughout evolution in both primary sequence and function (17, 18, 48).

Recently, amino acid sequence comparison and low-stringency cloning unraveled a new family of homeoproteins termed NK2 whose members are found from planarians (Dth-1 and Dth-2) (15, 16) to leeches (lox10) (38), *Drosophila melanogaster* (NK2, NK3, and NK4) (25), *Caenorhabditis elegans* (Ceh-22) (41), and vertebrates (Nkx-2.1 to -2.6) (20, 29, 45). The homeodomain of NK2 proteins is characterized by the presence of a very conserved tyrosine at position 54; in other homeoproteins such as Antennapedia (Antp), this residue, a methionine, is known to directly contact DNA bases in the binding site (43). This amino acid difference between NK2 and Antp homeodomains may be responsible for the divergent target sites of NK2 proteins which have been found either through promoter analysis of target genes, as for Nkx-2.1 (20), or by in vitro site selection, as for Nkx-2.5 (8) and Nkx-2.1/TTF-1 (12). Outside the homeodomain, NK2 proteins possess a conserved segment of hydrophobic residues called the conserved peptide found just C terminal of the homeodomain. This conserved peptide may be involved in protein-protein interaction, or it may serve as a transcriptional inhibitory domain since its deletion in Nkx-2.5 and Nkx-2.1 enhances their transactivation properties (8, 10).

Similar to other homeoproteins, NK2 proteins control key steps in development. In *C. elegans*, Ceh-22 is involved in pharyngeal muscle development (41). In *D. melanogaster*, NK2 (or vnd) is thought to be involved in early neurogenesis by regulating the *achaete-scute* complex (22), NK3 (or bagpipe) has been shown to be required for visceral muscle formation (5), and NK4 (tinman) is required for precardiac mesoderm formation (6, 29). In vertebrates, Nkx-2.1 is a key regulator of thyroid and lung gene expression (7, 20) and is also essential for thyroid and lung organogenesis (26) and function (1). The presumptive mammalian homolog of tinman, Nkx-2.5, is expressed in the primordial spleen, the pharyngeal mesoderm, and the precardiac mesoderm, where its expression precedes that of other cardiac markers (27, 29). Consistent with a role for Nkx-2.5 in the heart, targeted disruption of the Nkx-2.5 gene arrests heart development at the looping stage, causing severe morphogenetic defect of the heart and embryonic lethality (31). This phenotype, which is less severe than the tinman phenotype, may be due to genetic redundancy between Nkx-2.5, Nkx-2.6, and Nkx-2.3, which are all present in vertebrate myocardia (11, 29, 51). Elucidating the molecular mechanisms of action of these factors and identification of their target genes in the heart are critical to understanding their respective roles in myocardial development.

In this report, we present data showing that the cardiac atrial natriuretic factor (ANF) promoter (4) is a target for Nkx-2.5

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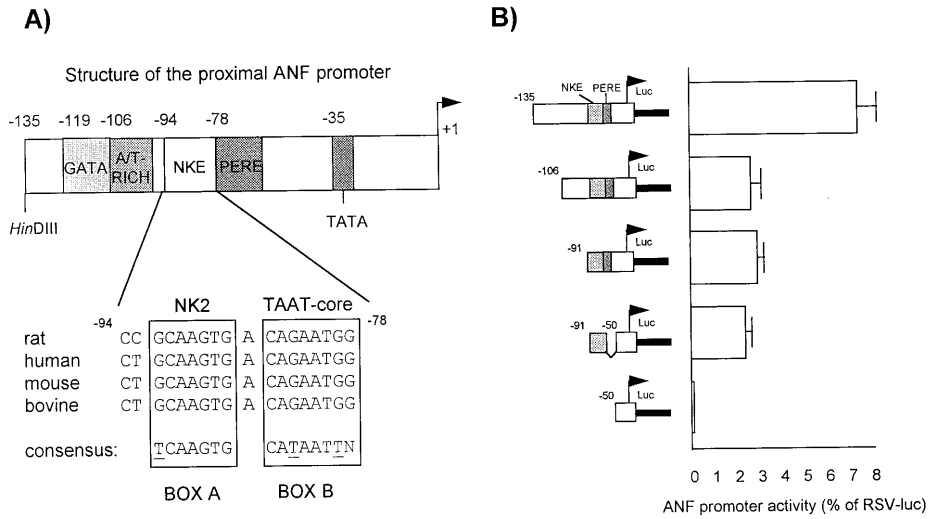

FIG. 1. Localization of an NK2-like binding site in the proximal ANF promoter. (A) Structural organization of the proximal ANF promoter. Putative regulatory elements of the ANF promoter are boxed, and their locations relative to the transcription start site are indicated. The PERE corresponds to the α_1-adrenergic response sequence (2). The Nkx-2.5 consensus sequence is based on the in vitro site selection studies of Chen and Schwartz (8). This structure is identical for rat, mouse, human, and bovine ANF promoters. (B) The NKE is critical for ANF promoter activity, as determined by transient transfection of recombinant ANF-luciferase (Luc) vectors in primary cardiocyte cultures derived from dissected ventricular tissues of 1-day-old rats. The results are expressed relative to the activity of the Rous sarcoma virus-luciferase (RSV-luc) promoter and represent the means ± standard deviations of at least four independent determinations. The shaded box represents the putative NKE (bp −91 to −78).

and contains an Nkx-2.5 response element termed the NKE. The NKE, which harbors two near-consensus binding sites for Nkx-2.5, interacts specifically with purified Nkx-2.5 and represents one of the first identified natural binding sites for Nkx-2.5 on a cardiac promoter.

MATERIALS AND METHODS

Cell cultures and transfections. Primary cardiocyte cultures were prepared from 1- or 4-day-old Sprague-Dawley rats and kept in serum-free medium as described previously (3). L cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were carried out by using calcium phosphate precipitation 24 h after plating. At 36 h posttransfection, cells were harvested and luciferase activity was assayed with an LKB luminometer, and the data were recorded automatically. In all experiments, pRSV-hGH was used as internal control, and the amount of reporter was kept at 3 μg per dish; the total amount of DNA was kept constant (usually 8 μg). Unless otherwise stated, the results reported were obtained from at least four independent experiments with at least two different DNA preparations for each plasmid.

Plasmids. ANF-luciferase promoter constructs were cloned in the PXP-2 vector as described by Argentin et al. (3). Mutations were generated by PCR and sequenced. The internal deletion of nucleotides −105 to −84 was constructed by inserting an oligonucleotide encompassing bp −135 to −106 in the *Hin*dIII site of the −50 construct. Plasmids containing the NKE sites upstream of the ANF −50 minimal promoter were generated by polymerizing one or multiple copies of a *Bam*HI-*Bgl*II NKE synthetic oligonucleotide in the *Bam*HI site of the −50 ANF-luciferase plasmid. The maltose-binding protein (MBP)–Nkx-2.5 and pEMSV-Nkx-2.5 plasmids are described in reference 8.

Recombinant protein and antibody production. After transformation of *Escherichia coli* BL21(DE3) with the MBP fusion vectors derived from pMalc-2 (New England Biolabs), individual colonies were picked and grown in 50 ml of 2XYT to an optical density of 0.6. Isopropyl thiogalactopyranoside (IPTG) was then added at a final concentration of 0.5 mM, and bacterial cultures were grown at 37°C for another hour. The cultures were centrifuged, and the bacteria were lysed by sonication. Purification on amylose columns was performed according to the manufacturer's instructions. An anti-Nkx-2.5 polyclonal antibody was generated by immunization of rabbits with purified MBP–Nkx-2.5 according to standard procedures. Immune serum was affinity purified and characterized by Western blotting (immunoblotting).

DNA binding assays. Whole-cell extracts were prepared from cardiac myocyte cultures as previously described (2). Protein concentration was assayed by the Bradford assay. Binding reactions for electrophoretic mobility shift assays (EMSAs) were performed on ice for 20 min in the presence of 500 ng of poly(dA-dT) and 500 ng of poly(dI-dC) in 60 mM KCl–6 mM $MgCl_2$–10 mM Tris-Cl (pH 7.9)–1 mM dithiothreitol–12% glycerol–20,000 cpm of double-stranded synthetic oligonucleotides and using typically 6 to 8 μg of whole-cell extract or a fractional nanogram amount of affinity-purified MBP fusion protein. For antibody blocking assays, typically 200 ng of affinity purified anti-Nkx-2.5 or an unrelated antibody was incubated in the presence of the protein extract 1 h prior to addition of the probe. Footprinting experiments using whole-tissue nuclear extracts were performed as previously described (3).

RESULTS

The ANF proximal promoter harbors an NK2-like cardiac element. The ANF gene, which encodes the heart major secretory product, is an early marker of cardiomyocyte differentiation (53). ANF transcription is tightly regulated spatially during cardiac development. Previous studies in our laboratory have identified within the first 700 bp of the ANF promoter two regions necessary for proper spatiotemporal expression of the gene (3). One, located between bp −50 and −135 is required for basal activity and for the function of the cardiac cell-specific enhancer located between bp −135 and −700. A complex array of molecular pathways converges at the ANF promoter, as exemplified by the identification of chamber- and stage-specific regulatory elements in the ANF enhancer (3, 32) and the presence of an inducible α_1-adrenergic or phenylephrine response element (the PERE) within the proximal promoter (2). However, except for GATA-4, which activates the ANF promoter (19) via the GATA binding site found in the proximal promoter (7a), the identities of the *trans*-acting factors that modulate ANF transcription remain largely unknown.

Since the PERE appeared to contribute little basal activity in cardiomyocytes (2), we carried out deletion analysis of the proximal promoter in order to identify the DNA elements and their cognate nuclear proteins that account for transcriptional activity of the −135 to −50 bp region. As shown in Fig. 1, sequence analysis reveals the presence of a number of consensus motifs, including a GATA binding site juxtaposed to an AT-rich element and a region of high homology to newly identified NK2 binding sites (8, 19). This organization is identical between ANF promoters from different species. Results of the deletion analysis reveal that the GATA and AT-rich element as well as the putative NKE site contribute to the

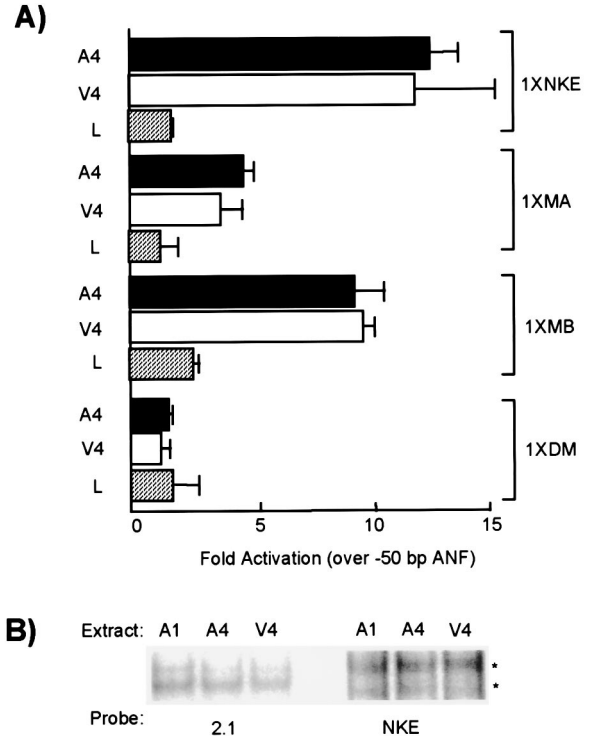

FIG. 2. The NKE requires both A and B boxes to be fully active. (A) An oligonucleotide containing one copy of the NKE is sufficient to confer cardiac specificity to a minimal ANF promoter in myocytes from 4-day-old atria (A4) or ventricles (V4) but is inactive in fibroblasts L cells. MA, MB, and DM correspond to mutant NKEs described in Table 1. Transfections were carried out as detailed in Materials and Methods, and the results ($n = 4$) are expressed as fold activation over that of the minimal ANF −50 construct (± standard deviation). (B) Nkx-2.5-like binding activity remains the same in different stage- and chamber-specific myocyte cultures. Whole-cell extracts derived from 1- or 4-day-old atrial myocytes (A1 and A4) and 4-day-old ventricular myocytes (V4) were assayed for the ability to bind the NKE or the 2.1 probe. The panel represents the Nkx-2.5-related binding and corresponds to the complex abolished by the Nkx-2.5 antibody and by an unlabeled high-affinity Nkx-2.5 binding site as shown in Fig. 4C. The doublet (asterisks) likely corresponds to different Nkx-2.5 isoforms which are also obtained in L cells transfected with the Nkx-2.5 expression vector.

transcriptional activity of the proximal promoter in 1-day-old ventricular cardiomyocytes (Fig. 1B). Indeed, deletion of sequences up to bp −106 removes both GATA and AT-rich elements and leads to a 2.5-fold drop in promoter activity. Furthermore, deletion of sequences between −91 and −50 decreases promoter activity by over 12-fold. Sequences between −91 and −78 account for most of this effect since, consistent with previous work (2), internal deletion of sequences between −78 and −57 which harbor the PERE reduces promoter activity only modestly. Interestingly, the −91 to −78 region contains two highly conserved motifs with homologies to consensus NK2 binding sites (Fig. 1). An oligonucleotide spanning this region is able to activate the minimal TATA-containing ANF promoter 10- to 15-fold in atrial or ventricular cardiomyocytes but not in noncardiac cells (Fig. 2A).

The relevance of the NKE to ANF promoter activity was also assessed in the context of the maximally active −700 promoter in both atria and ventricles of 1- and 4-day-old rats. We had previously shown that different transcription pathways were active in these stage-specific cultures (3). As shown in Fig. 3A, the NKE contributes similarly to ANF promoter activity in 1-day-old atrial and ventricular cardiomyocytes. Unexpectedly, this effect is lost in more differentiated ventricular myocytes and in fibroblast L cells, in which removal of the NKE leads to consistent 2- to 10-fold activation, respectively (Fig. 3B and C). In contrast, promoter activity is most dependent on the NKE in differentiated atrial myocytes (Fig. 3B) prepared simultaneously from the same 4-day-old rat hearts. These changes in promoter activity, including the marked upregulation in L cells, are not seen with a similar internal deletion that removes sequences upstream of the NKE (data not shown). Thus, it is unlikely that the gain of activity is simply the consequence of moving positive elements closer to the transcription initiation site; rather, it appears that the NKE itself harbors overlapping negative elements. The presence of such negative regulators within tissue-specific elements has already been observed (2, 3). Thus, the NKE, in collaboration with other regulatory elements of the ANF promoter, contributes to cardiac transcription in a chamber- and stage-specific manner.

Nkx-2.5 binds and activates the NKE. Next, we analyzed the interaction of this element with cardiac nuclear extracts and with purified Nkx-2.5 protein. DNase I footprinting revealed the presence of two contiguous footprints between −56 to −76 and −77 to −94 that encompass the previously characterized PERE and the NKE, respectively (Fig. 4A). The NKE footprint was found in cardiac but not liver extracts (Fig. 4A), indicating the presence of a tissue-specific DNA binding activity. Because the NKE contains homologies to NK2 binding sites, the footprint might correspond to binding of cardiac NK2 proteins.

Nkx-2.5 is presently the only well-characterized NK2 protein in mammalian heart. In vitro site selection studies have revealed that Nkx-2.5 binds with high affinity to sequences corresponding to the consensus: TCAAGTG and, with moderate affinity, *Antp*-like binding sites which contains the typical TAAT core (8). The NKE possesses two putative NK2 binding sites (Fig. 1); the first one, CCAAGTG (box A), diverges at a single nucleotide from the high-affinity consensus TCAAGTG, while box B consists of a modified TAAT core (CAGAATGG instead of CATAATTN). In EMSA, oligonucleotide probes containing both boxes or either box (Table 1) form specific complexes with bacterially expressed or in vitro-translated Nkx-2.5 (Fig. 4B and data not shown) which are competed for by the unlabeled high-affinity synthetic Nkx-2.1 binding site (Fig. 4B). Interestingly, direct binding and displacement studies indicate that the two boxes within the NKE can independently interact with Nkx-2.5, and only mutation of both sites completely abolishes this interaction (Fig. 4B and data not shown).

EMSAs were also carried out with cardiac extracts. As shown in Fig. 4C, incubation of atrial myocyte extracts with the NKE produced two major retarded complexes, one of which was completely abolished in the presence of an Nkx-2.5-specific antibody. Moreover, this complex was effectively abrogated in the presence of excess unlabeled oligonucleotides that retained the ability to bind purified Nkx-2.5 (Fig. 4B) but was unaffected by the NKE mutant (DM) that no longer interacted with Nkx-2.5. Together, these results strongly suggest that Nkx-2.5 or a highly related protein present in cardiomyocytes interacts with the NKE. Furthermore, and consistent with the high-level activity of the NKE in atrial and ventricular myocytes, the Nkx-2.5-related complex was present at similar levels in both atrial and ventricular myocyte extracts.

To ascertain the functional relevance of Nkx-2.5 to ANF transcription, ANF reporter constructs were cotransfected in heterologous cells with an Nkx-2.5 expression vector. As shown in Fig. 5A, Nkx-2.5 is a potent activator of the full-length but not the minimal ANF promoter. This dose-dependent activa-

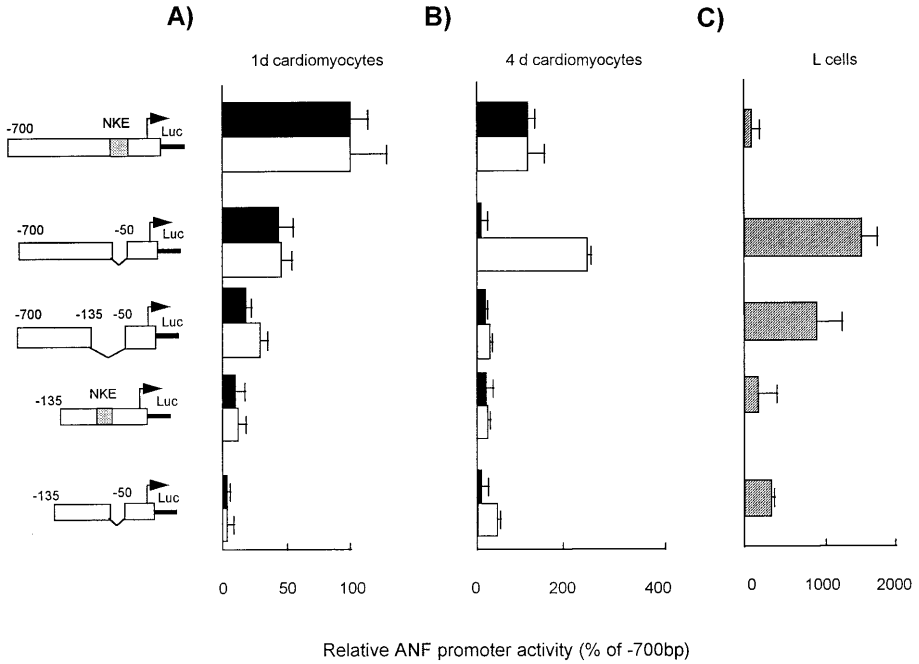

FIG. 3. The NKE contributes to ANF promoter activity in a developmental stage- and chamber-specific manner. Transfections were carried out in primary cardiomyocytes derived from 1-day-old (1d) or 4-day-old (4d) rats as described in Materials and Methods. Open bars represent ventricular myocytes, and black bars represent atrial myocytes. Note that the NKE appears to be most critical to ANF promoter activity in 4-day-old atria (B), whereas it has little or even an inhibitory effect in 4-day-old ventricles (B) and in fibroblasts (C). The results are expressed relative to the activity of the −700 promoter, taken as 100% for each cell type. For cardiomyocytes, $n = 8$; for L cells, $n = 4$.

tion is seen with 0.25 μg of expression vector and reaches 10- to 15-fold when 2.5 to 5 μg of Nkx-2.5 vector is used. The closely related Nkx-2.1 protein was also able to transactivate the ANF promoter; other homeodomain proteins expressed or not expressed in the heart, including Gax, Mhox, Otx1, Oct1 and Pit1, were also tested, but none could activate the ANF promoter (data not shown). The inductive effect of Nkx-2.5 was observed with all ANF promoters that retained the NKE but not with constructs lacking the NKE (Fig. 5B and data not shown). Interestingly and consistent with the binding data (Fig. 4), the presence of either site A or site B was sufficient for NKE transactivation by Nkx-2.5; however, this transactivation was abolished with the mutant NKE in which both sites are mutated and which is no longer able to bind Nkx-2.5 (Fig. 5C). Together, the data presented indicate that the ANF promoter is a target for Nkx-2.5 and possibly other NK2-related proteins in the heart.

DISCUSSION

The NK2 class of homeodomain proteins plays a central role in cardiac development. However the molecular circuitry underlying the action of NK2 proteins in the myocardium remains unknown. The present work provides data showing that Nkx-2.5 transactivates the cardiac ANF promoter via a novel cardiac-specific *cis* element, the NKE, which interacts specifically with recombinant Nkx-2.5 and Nkx-2.5-related antigens in cardiac extracts. Together, the data suggest that the ANF gene may be a direct downstream target for NK2 proteins in the myocardium.

The NKE is a new cardiac cis element. At the level of the ANF promoter, the site where Nkx-2.5 binds harbors the characteristics of a new cardiac specific *cis* element. So far only a limited number of such elements have been identified within cardiac cell-specific promoters; they include the WGATAR (19, 21, 35), M-CAT (36, 47, 50), CArG (3, 33, 47, 52), and Mef2 (23, 36, 39, 46) motifs that are known to participate in cardiac-specific transcription. Interestingly, most cardiac promoters contain several of these elements, suggesting that cardiac transcription is controlled by a complex regulatory network. For example, the ANF promoter contains, in addition to the NKE, WGATAR, CArG, and AT-rich/Mef2 elements that may function in different combinatorial pathways to direct proper spatial and stage-specific transcription of the ANF gene (discussed below). A search for different cardiac promoters revealed the presence of NKE-like elements within the upstream regulatory regions of the cardiac myosin light-chain 2 (MLC2) (40) and β-myosin heavy-chain (βMHC) genes (28) (Table 2). Incidentally, expression of these two genes is altered in mice homozygous for a null Nkx-2.5 allele, which display defective chamber specification (31). However, whether MLC2 or βMHC is a direct target for Nkx-2.5 will need to be tested. Nevertheless, it is intriguing that the ANF NKE and the putative NKEs on the MLC2 and βMHC promoters all contain an E-box motif, CAAGTG, that can interact with some basic helix-loop-helix (bHLH) factors (10a). In skeletal muscle, myogenic bHLH factors are key regulators of tissue-specific transcription and cell differentiation (42). Although no myocardium-specific bHLH factors have been identified, mutational analyses of some cardiac promoters have revealed the importance of regulatory elements containing E-box motifs for their activity in primary cardiomyocyte cultures (34, 37). Given the data for the ANF NKE, it is possible that these E-box motifs lie within functional NKEs. It is also possible that overlapping NKEs and E boxes reflect competitive regulation of cardiac transcription by NK2 and bHLH factors during development. The identification of a transcriptional target for NK2

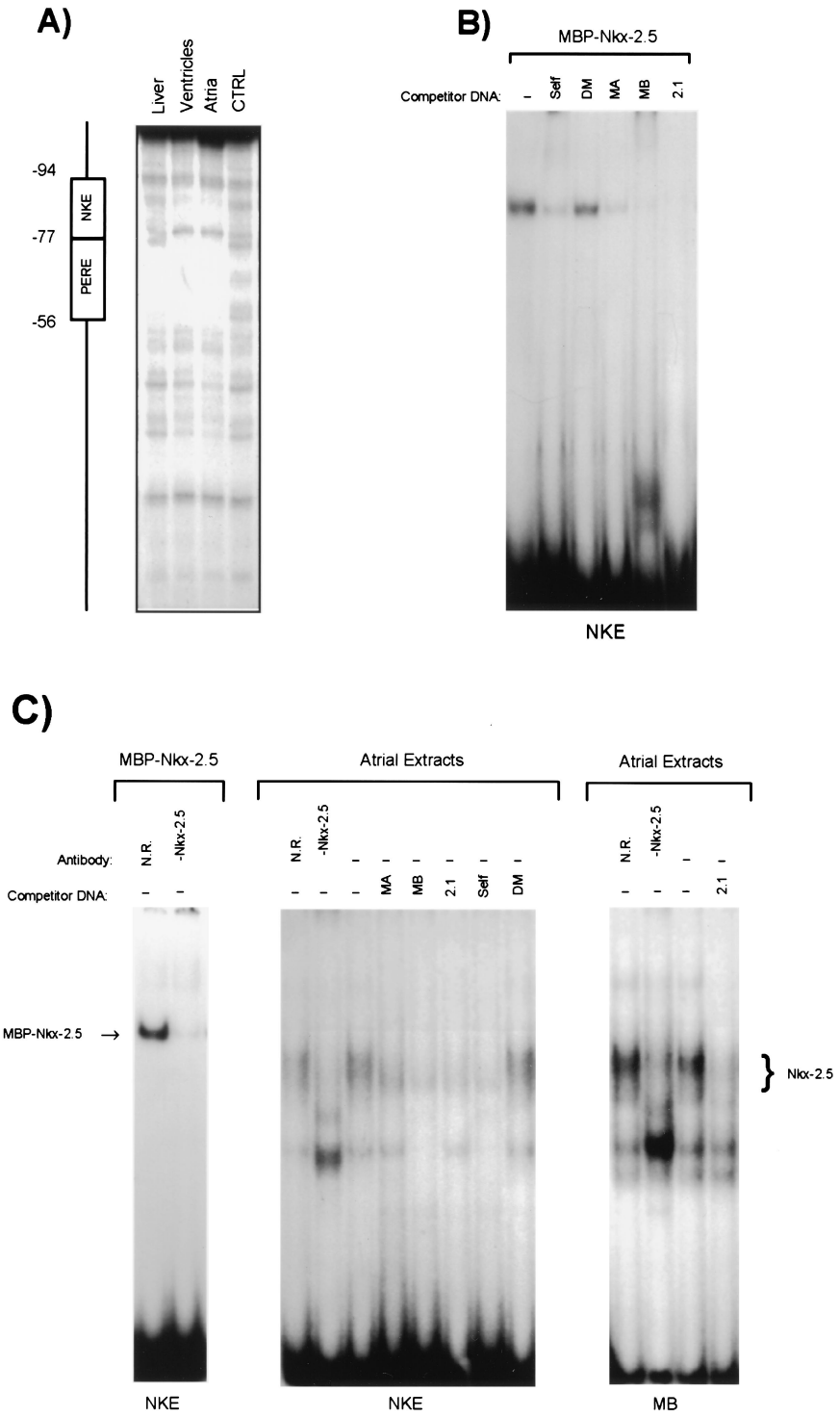

FIG. 4. Interaction of cardiac and recombinant proteins with the NKE. (A) DNase I footprinting experiments were carried out with a 5′-end-labeled 200-bp recombinant ANF (−135 to +50) fragment and 10 μg of tissue nuclear extracts prepared from the indicated tissues of 1-day-old rats as described by Argentin et al. (3). Positions of the protected regions were assigned on the basis of the simultaneously run Maxam-Gilbert sequencing reaction (G track). The −56 to −77 footprint (PERE) corresponds to the α_1-adrenergic response element previously characterized (2), while the −77 to −94 footprint covers the NKE. CTRL, control. (B) Bacterially produced Nkx-2.5 can bind to the ANF NKE. Nkx-2.5 fusion protein production and binding conditions are described in Materials and Methods. Nkx-2.5–DNA interaction was assayed over the NKE probe. Unlabeled competitor oligonucleotides were used at 250-fold molar excess. Sequences of the oligonucleotides used are given in Table 1. (C) Endogenous Nkx-2.5 or Nkx-2.5-related antigens present in atrial extracts bind the NKE. Whole-cell atrial myocyte extracts were assayed for the ability to form complexes with the NKE or a mutant oligonucleotide which retains box A (MB). Both purified MBP-Nkx-2.5 and atrial extracts were preincubated with an affinity-purified anti-Nkx-2.5 antibody or an unrelated purified antibody (N.R.). Preincubation with the anti-Nkx-2.5 antibody abolished the formation of the MBP–Nkx-2.5–DNA complex and the upper complex formed in atrial extracts. This upper complex was also abolished by unlabeled competitor oligonucleotides that bind Nkx-2.5 (MA, MB, and 2.1) but not by a mutant NKE site (DM) that no longer binds Nkx-2.5. In all cases, unlabeled oligonucleotides were used at 150-fold molar excess.

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Reference
NKE	GCCGCCG**CAAGTGACAGAATG**GGA	This study
MA	GCCGCCGCATATGA**CAGAATG**GGA	This study
MB	GCCGCCG**CAAGTG**ACAGCCAGGGA	This study
DM	GCCGCCGCATATGACAGCCAGGGA	This study
2.1	GCCCAG**TCAAGT**GCA	20

[a] NKE motifs are in boldface; mutations are underlined.

TABLE 2. NKE-like motifs in cardiac promoters

Promoter (reference)	NKE motif	Positions
ANF (4)	GCAAGTG, CAGAATG	−92, −84
βMHC (28)	CCAAGTG, TCAAGTG,	−1737, −1257
	CAGAATG, CAGAATG	−1691, −1545
MLC2 (40)	(A/G)CAAGTC, GCAAGTG	−190, −1194

factors in the myocardium will now allow direct testing of this hypothesis.

Finally, it is noteworthy that on the ANF promoter, the NKE is located within the first 100 bp of upstream sequences, similar to the two functional Nkx-2.1 binding sites in the thyroglobin (20) and lung surfactant B (7) promoters. In addition, in all three cases, the NKE is composed of a tandem of two sites that can independently bind Nkx-2.1 or -2.5 protein. In the case of the ANF NKE, mutation of either site decreases NKE activity in cardiomyocytes, with mutation of the A site resulting in the most significant loss of activity (30% of the wild-type level); however, only mutation of both sites completely abolishes the NKE activity. Thus, the presence of both sites may be required for productive interaction with cofactors or with components of the transcriptional machinery; alternatively, it may simply ensure an adequate local concentration of Nkx-2.5 or may enhance binding to the protein. In this respect, it should be noted that the ANF NKE is an imperfect match with the high-affinity sites selected in vitro (8) and has a lower apparent affinity for Nkx-2.5 than these sites. This, in turn, might provide added in vivo specificity in discriminating between structurally related proteins that are coexpressed in the myocardium but that may serve distinct developmental functions as previously suggested from in vivo studies of the conserved low-affinity binding sites for the fushi tarazu homeoprotein (17).

Stage and chamber specificity of NKE activity. One of the unexpected results of this study was the finding that NKE activity within the ANF promoter parallels the stage and chamber specificity of the endogenous ANF gene and the transfected ANF promoter; i.e., the contribution of the NKE to promoter activity was highest in differentiated atria and lowest in differentiated ventricles. Few cardiac *cis* elements identified so far possess such properties. Many cardiac genes are expressed in a developmental stage- and chamber-specific manner (reviewed by Lyons [30]), and some cardiac promoters, including the ANF promoter, display spatial specificity in transgenic mice (13, 14, 24, 44, 49). However, neither the regulatory elements nor the transcription factors that are responsible for chamber-specific expression have been identified. The mechanism underlying chamber-specific activity of the NKE is not readily understood, since the pattern of expression of Nkx-2.5 within the heart does not show such specification; nevertheless, inactivation of the Nkx-2.5 gene revealed a role for Nkx-2.5 in heart regionalization (31). Given that the transcriptional activity of the NKE per se does not differ between atrial and ventricular myocytes (Fig. 2B), it is likely that the chamber-specific activity of the NKE reflects interactions be-

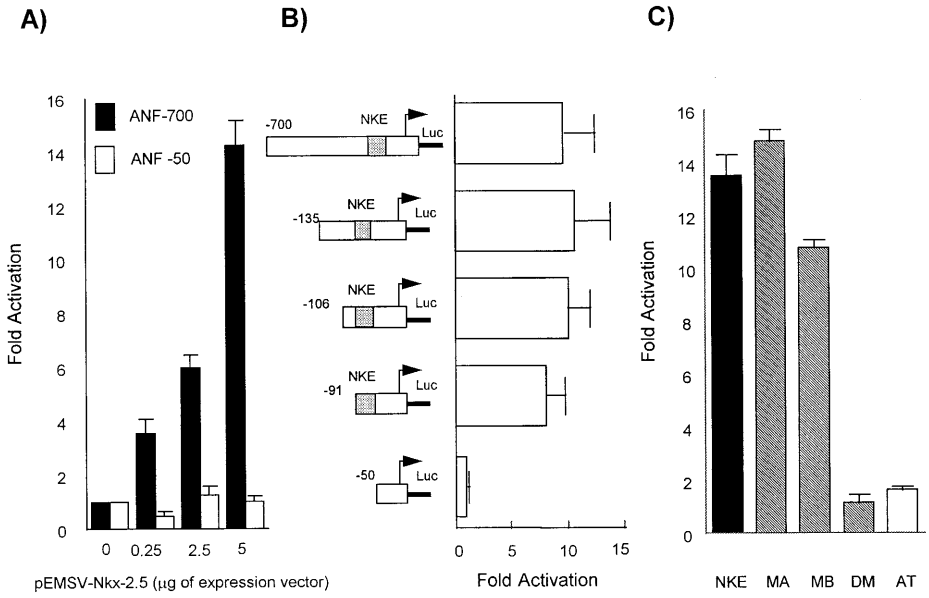

FIG. 5. (A) Nkx-2.5 activates the rat ANF −700 promoter in a dose-dependent manner. Transient cotransfection experiments were done in L cells by the calcium phosphate method as described in Materials and Methods. The results represent the means ± standard deviations of at least four independent experiments. (B) The Nkx-2.5-responsive region maps to the NKE. Cotransfection experiments were done in L cells, using 2 μg of pEMSV-Nkx-2.5 as described above. The results represent the means ± standard deviations of at least four independent experiments. Luc, luciferase. (C) Nkx-2.5 can transactivate the NKE. Cotransfection experiments were done in L cells, using 5 μg of the expression vector and 3 μg of various reporter constructs corresponding to different oligonucleotides (Table 1) fused in one copy upstream of the −50 ANF promoter. MA and MB correspond to mutations of the A and B boxes of the NKE, whereas DM is a double mutant of both. Not all TAAT core-containing sequences are responsive to Nkx-2.5, since an AT-rich region encompassing nucleotides −600 to −565 of the ANF promoter (AT) is unaffected by Nkx-2.5. Solid bars represent the ANF minimal promoter.

tween Nkx-2.5 and other factors bound to neighboring promoter elements. In this respect, Chen et al. have shown cooperative interactions between Nkx-2.5 and serum response factor, a MADS box factor, a MADS box factor, over the cardiac α-actin promoter (9). This interaction occurs at the serum response element and does not seem to be dependent on Nkx-2.5 binding. Perhaps serum response factor or another MADS box factor, Mef2, bound at upstream elements can interact with Nkx-2.5 and account for developmental and/or spatial specificity. Alternatively, the interaction of Nkx-2.5 with chamber-specific cofactors (activators or repressors) might result in the observed spatial specificity of the NKE. It is also noteworthy that the NKE is located near a functional GATA binding site and an AT-rich region which can interact with members of the MADS box family. Further studies will need to examine the interesting possibilities of cooperative interaction between Nkx-2.5 and other cardiac factors such as GATA-4 or Mef2 and their integration at the level of the ANF promoter and other cardiac promoters.

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