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Transgenic analysis of the *atrialnatriuretic factor (ANF)* promoter: Nkx2-5 and GATA-4 binding sites are required for atrial specific expression of *ANF*

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Abstract

The *atrial natriuretic factor (ANF)* gene is initially expressed throughout the myocardial layer of the heart, but during subsequent development, expression becomes limited to the atrial chambers. Mouse knockout and mammalian cell culture studies have shown that the *ANF* gene is regulated by combinatorial interactions between Nkx2-5, GATA-4, Tbx5, and SRF; however, the molecular mechanisms leading to chamber-specific expression are currently unknown. We have isolated the *Xenopus ANF* promoter in order to examine the temporal and spatial regulation of the *ANF* gene in vivo using transgenic embryos. The mammalian and *Xenopus ANF* promoters show remarkable sequence similarity, including an Nkx2-5 binding site (NKE), two GATA sites, a T-box binding site (TBE), and two SRF binding sites (SREs). Our transgenic studies show that mutation of either SRE, the TBE or the distal GATA element, strongly reduces expression from the *ANF* promoter. However, mutations of the NKE, the proximal GATA, or both elements together, result in relatively minor reductions in transgene expression within the myocardium. Surprisingly, mutation of these elements results in ectopic *ANF* promoter activity in the kidneys, facial muscles, and aortic arch artery-associated muscles, and causes persistent expression in the ventricle and outflow tract of the heart. We propose that the NKE and proximal GATA elements serve as crucial binding sites for assembly of a repressor complex that is required for atrial-specific expression of the *ANF* gene.
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Keywords: ANF; Nkx2-5; Atrium; Heart; Transcription factor; Transgenesis; *Xenopus laevis*

Introduction

The morphological events involved in formation of the vertebrate heart have been well described, but much less is understood about the molecular mechanisms regulating gene expression during cardiogenesis. One class of transcription factors that are known to be important for heart development are homeodomain proteins of the tinman family. In *Drosophila,* absence of tinman function completely abolishes formation of the dorsal vessel, the insect equivalent of the vertebrate heart (Bodmer, 1993; Azpiazu and Frasch, 1993). In vertebrates, a small family of tinman-related genes are expressed in the developing heart. The best-characterized member of the vertebrate tinman family is Nkx2-5 (also called Csx), which is among the very earliest markers of the cardiac lineage (Harvey, 1996; Komuro and Izumo, 1993). Nkx2-5 continues to be expressed throughout the myocardial layer during subsequent heart development and in the adult organ. Mice in which Nkx2-5 function has been ablated die at approximately E9.5 due to cardiac insufficiency (Lyons et al., 1995; Tanaka et al., 1999). Although a linear heart tube is formed and beating myocardial tissue is present, the heart tube fails to undergo the looping and morphogenetic movements associated with heart maturation. In addition, a number of cardiac genes fail to be expressed, or are expressed at much reduced levels, including *MLC-2v, CARP, eHand,* and *atrial natriuretic factor (ANF)* (Lyons et al., 1995; Tanaka et al., 1999). The relevance of Nkx2-5 function for human cardiac development is evidenced by the fact that a number of congenital heart abnor-

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malities, including atrial septal defects, AV conduction defects, and valvular dismorphogenesis, appear to be associated with haploinsufficiency of Nkx2-5 expression (Schott et al., 1998; Rosenthal and Harvey, 1999; Biben et al., 2000).

Nkx2-5 consensus binding sites (NKEs) have been identified in the promoter regions of a number of cardiac-expressed genes, including *GATA6* (Molkentin et al., 2000), *cardiac α actin* (Chen and Schwartz, 1996; Sepulveda et al., 1998), *cardiac troponin I* (Bhavsar et al., 2000), and *ANF* (Durocher et al., 1996). These genes may therefore be direct targets of Nkx2-5 regulation. In support of this hypothesis, in vitro studies using rat cardiomyocytes, CV1 fibroblast cells, and HeLa cells have shown that Nkx2-5 is able to activate transcription from the *ANF* and *cardiac α actin promoters* (Durocher et al., 1997; Sepulveda et al., 1998). Related cell culture studies strongly imply that Nkx2-5 cooperates with other transcription factors to efficiently activate cardiac gene transcription. For example, examination of the *ANF* and *cardiac α actin* promoters show the presence of GATA sites and SRF consensus binding sites (SREs) in close proximity to the Nkx2-5 binding sites. Cotransfection of both GATA4 and Nkx2-5 into cardiomyocytes, HeLa cells, CV1 cells, or 293 cells results in a synergistic activation of *ANF* and *cardiac α actin* promoter activity (Durocher and Nemer, 1998; Sepulveda et al., 1998; Lee et al., 1998). Furthermore, immunoprecipitation experiments suggest a direct physical interaction between the GATA4 and Nkx2-5 proteins. In an extension of these cell culture studies, cotransfection of SRF with GATA4 or Nkx2-5 also shows a synergistic upregulation of *cardiac α actin* promoter expression and the highest levels of promoter activity, approximately 200-fold over background, are observed when all 3 factors are present (Sepulveda et al., 2002). Overall, these studies strongly suggest that cooperative interactions between the Nkx2-5, GATA4, and SRF proteins are required for maximal activity of the *ANF* and *cardiac α actin* promoters and probably other cardiac genes.

Transcription factors of the T-box (Tbx) family have also been shown to play an important role in in cardiogenesis (Bruneau, 2002). Tbx5 mutations are associated with Holt-Oram syndrome, a human disease which results in cardiac and limb defects (Basson et al., 1997, 1999; Li et al., 1997) and mice heterozygous for Tbx5 display cardiac defects resembling those of Holt-Oram syndrome (Bruneau et al., 2001). Furthermore, when Tbx5 is ectopically expressed throughout the entire heart in chicken embryos, chamber development proceeds abnormally (Liberatore et al., 2000). Binding sites for Tbx proteins (TBEs) have been identified in a number of cardiac-expressed genes, including *ANF* and *cx40* (Bruneau et al., 2001; Habets et al., 2002). Combinatorial interactions apparently can occur between Nkx2-5 and Tbx5 proteins, and synergistic activation of the *ANF* and *cx40* promoters by these factors has been observed (Bruneau et al., 2001). In addition, Tbx2 has been implicated in repression of *ANF* expression in the AV canal in transgenic mice (Habets et al., 2002).

Despite these extensive in vitro studies, the relative importance of the Nkx2-5, GATA4, Tbx5, and SRF proteins for *ANF* regulation has not been systematically examined in transgenic animals. To address this issue, we have isolated the *ANF* gene from the frog, *Xenopus laevis,* and studied its regulation in transgenic frog embryos. Alignment of the frog and mammalian promoter sequences shows a remarkable degree of conservation of transcription factor binding sites, including two SREs, two GATA sites, a TBE and an NKE. The presence of these conserved sequence elements suggests that the transcriptional mechanisms regulating *ANF* expression have been highly conserved in evolutionarily diverse organisms. Our transgenic studies show that the proximal GATA site and the NKE are relatively unimportant for the overall efficiency of expression of the *ANF* promoter, but play an essential role in regulating restriction of *ANF* expression to the atrium.

Materials and methods

Isolation of the Xenopus ANF gene and construction of GFP transgenes

A 400-bp *Pst*I fragment from the 5′ terminus of the *X.laevis ANF* coding region was used to probe a *Xenopus* Lambda genomic library (courtesy of Mike King). Approximately 10^6 phage were screened at high stringency ($0.2 \times$ SSC/0.1% SDS at 65°C). Positive phage were purified and phage DNA was isolated from liquid culture by cesium chloride gradient, restriction digested with *Eco*RI and subcloned for analysis. Restriction mapping and analysis by Southern blotting against the 5′ region of the *Xenopus ANF* cDNA isolated an *Eco*RI fragment that contains 3.4 kb of 5′ flanking sequences.

For *Xenopus* transgenics, 3.4-kb and 625-bp fragments of the *ANF* 5′ regulatory sequences were amplified by using Pfu polymerase and a 5′ primer containing a *Sac*I and *Eco*RI linker, respectively: 5′-TCCGGAGCTCGGCTGTCAGTCT-CTGGATAA-3′ and 5′-GGAATTCCCAGTGAGCGATAT-TTGC-3′, and a 3′ primer containing a *Bam*HI linker: 5′-GTCTGTAAAGATATCACCGCCCTAGGGC-3′.

Both the 3.4-kb and 625-bp regulatory sequences were subcloned into the *Sac*I/*Bam*HI or *Eco*RI/*Bam*HI sites, respectively, of a modified pEGFP1 vector (Clontech).

The pEGFP-625*ANF* clone was used as a template for site-directed mutagenesis using Pfu polymerase. The pEGFP -NKE, -GATAp, -GATAd, -SREp, -SREd and -TBE constructions were created by using the following oligonucleotides. Mutated regions are underlined NKE-*Sma*I: 5′ TCCCCCGGGCTGCTCCGAAGGCGGGCTTCATTC-CTCTGC-3′ and 5′-TCCCCCGGGGCATGATAAAGA-ATCTGATTG-3′; GATAp-ApaI: 5′-GTGGGCCCC-TTTAAAAGGGAATCTTCTG-3′ and 5′-GTGGGCCCTGCCCTCCACATTTCCACAG-3′; GATAd: 5′-TGCTTTTACACCTGCATTCCTTATTAGT-3′ and 5′-GATGGGTCAGTTTATCCTTTTCAC; SREp: 5′-

TTATCCTGCCCTCCACATTTCCAC-3′ and 5′-CAAGTGTATAGAATCTTCTGTTGGCCTTCAATCAGA-3′; SREd: 5′-CCAAAGCTTTATTTAATAAATATGACG and 5′-TCAAACCCTCTGTTGGATTTACATTA-3′; TBE: 5′-TTCGGATGTAATGTGGAGGGCAGGAT-3′; and 5′-TTTCCAGGAGGTGCCTTATCC-3′.

Transgenesis

ANF promoter plus GFP reporter sequences were excised from the plasmid vector by digestion with *Eco*RI/*Pme*I, and the transgene was purified by using a QIAEX II gel extraction kit (Qiagen). The transgene was then used to generate transgenic *Xenopus* embryos using previously described protocols (Kroll and Amaya, 1996; Sparrow et al., 2000a). Since greater than 90% of *Xenopus* embryos that are transgenic for one construction also coexpress a second construction (Huang et al., 1999; Marsh-Armstrong et al., 1999), *γ-crystallin*–GFP expression in the eye was used as a control transgene to assay for insertion of DNA into the genome (Bronchian et al., 1999). Reporter gene expression was detected by fluorescence in live embryos using a Leica Fl-II microscope and MagnaFire digital camera or, in fixed embryos, by whole-mount in situ hybridization for GFP transcripts (Harland, 1991).

In presenting the results of transgenic experiments, we are cautiously proposing a system in which activity of different mutant promoters is expressed as a proportion of wild type (wt) 625-bp *ANF* promoter activity. This seems reasonable for the following reasons. First, examination of a large number of transgenic embryos has established that detectable expression of the GFP reporter is only observed in 49% (90 of 184) of embryos transgenic for the wt *ANF* promoter. Levels of GFP fluorescence vary widely among the 49%, but it is likely that any promoter mutation that reduces transgene expression will correspondingly increase the proportion of embryos failing to reach the threshold of detectable GFP fluorescence. Second, transgene expression is likely to be influenced by several factors, including the copy number of transgenes inserted, the relative head/tail orientation of transgene insertions, and the effects of chromosomal location. However, these effects will average out for both wt and mutant promoters, providing that a sufficient number of independent transgenic embryos are examined for each construction. In order to discourage too much emphasis on the precision of these estimates, relative promoter activity have been stated to the nearest 5%.

Results

Conservation of transcription factor binding sites in the mammalian and frog ANF promoters

In order to study the spatial and temporal regulation of cardiac gene expression, we have isolated a 4.4-kb DNA fragment containing the 5′ flanking sequences of the *Xenopus atrial natriuretic factor (ANF)* gene. Fig. 1 shows the proximal promoter region of the *Xenopus ANF* gene together with an alignment of the sequence elements conserved in the human *ANF* gene. The first 625 bp of 5′ flanking sequences contains a number of transcription factor binding sites, and both the sequence and the spacing of these putative regulatory elements is highly conserved between mammals and *Xenopus.* In particular, two SRF binding sites (SRE), two GATA sites, a Tbx factor binding site (TBE), and an NK2 factor binding site (NKE) are located within 350 bases of the start site of transcription. The proximal SRE-like domain, which differs from a consensus SRE by 2 out of 10 bases, and the distal consensus SRE are identical in the human and *Xenopus* genes. The Nkx2-5 binding region (NKE) is 12 of 16 bp conserved between human and *Xenopus.* The *Xenopus* 5′ flanking region also contains the proximal and distal GATA binding sites that are present in the mammalian *ANF* promoters and a TBE, although this last element is not precisely conserved in sequence or spacing. Based on the high level of conservation of promoter sequences, it seems likely that very similar regulatory mechanisms will be involved in controlling *ANF* transcription in these evolutionarily divergent organisms.

Spatial and temporal regulation of the ANF promoter

During mammalian development, *ANF* is initially expressed in both the atrial and ventricular regions of the myocardium of the developing heart (Zeller et al., 1987; Habets et al., 2002). Later during development, *ANF* expression becomes limited to the atrial chambers of the heart, with limited expression also observed in trabeculae of the ventricle. In the rat, this restriction occurs at approximately the time of birth (Zeller et al., 1987; Gardner et al., 1986; Argentin et al., 1994). A very similar pattern of *ANF* expression is observed during frog cardiogenesis (Small and Krieg, 2000), with *ANF* initially expressed throughout the myocardium and in the developing outflow tract before becoming restricted to the atrial chambers (Fig. 2A and B). This restriction commences at approximately stage 47 of development, corresponding to about 2 weeks after fertilization (Fig. 2B). Unlike the mammalian expression pattern, no expression of *ANF* is visible in the trabeculated ventricular myocardium of the frog heart (Small and Krieg, 2000). At no time is expression of *ANF* observed outside of the heart.

In order to study the regulation of *ANF* transcription in vivo, we have used the *Xenopus* transgenesis system (Kroll and Amaya, 1996; Sparrow et al., 2000a). Preliminary experiments compared the expression of a GFP reporter gene in constructions containing either 3.4 kb or 625 bp of *ANF* 5′ flanking sequence. Comparison of more than 20 individual transgenics for each construction showed that the expression levels and profiles were indistinguishable, and both were identical to the expression profile of the endogenous

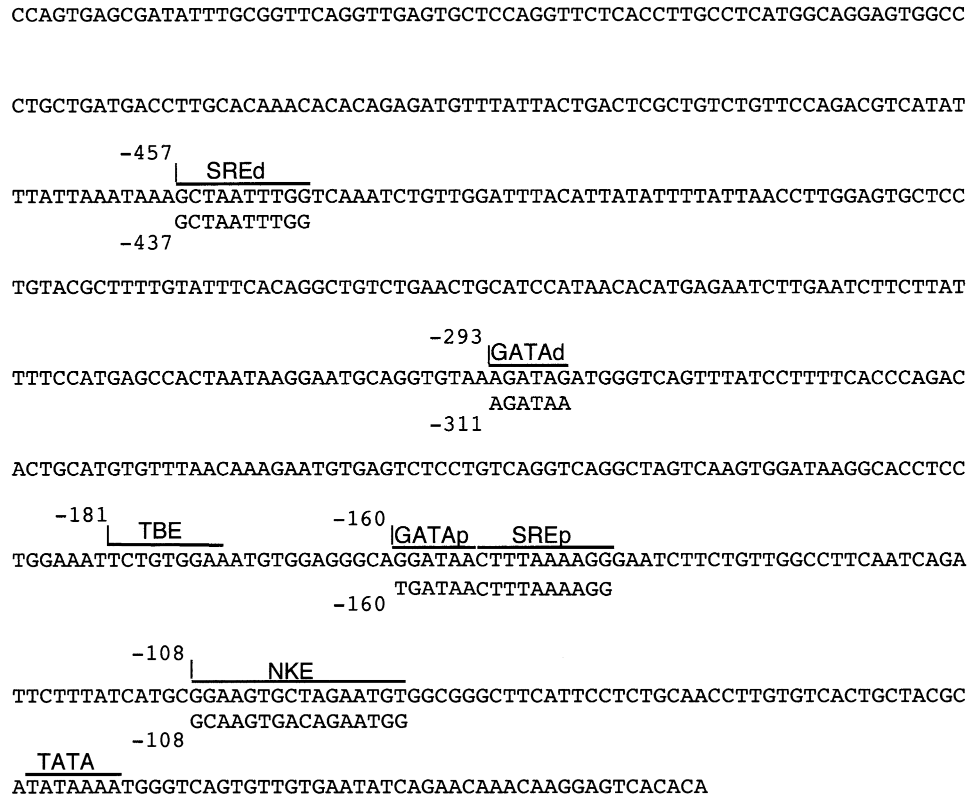
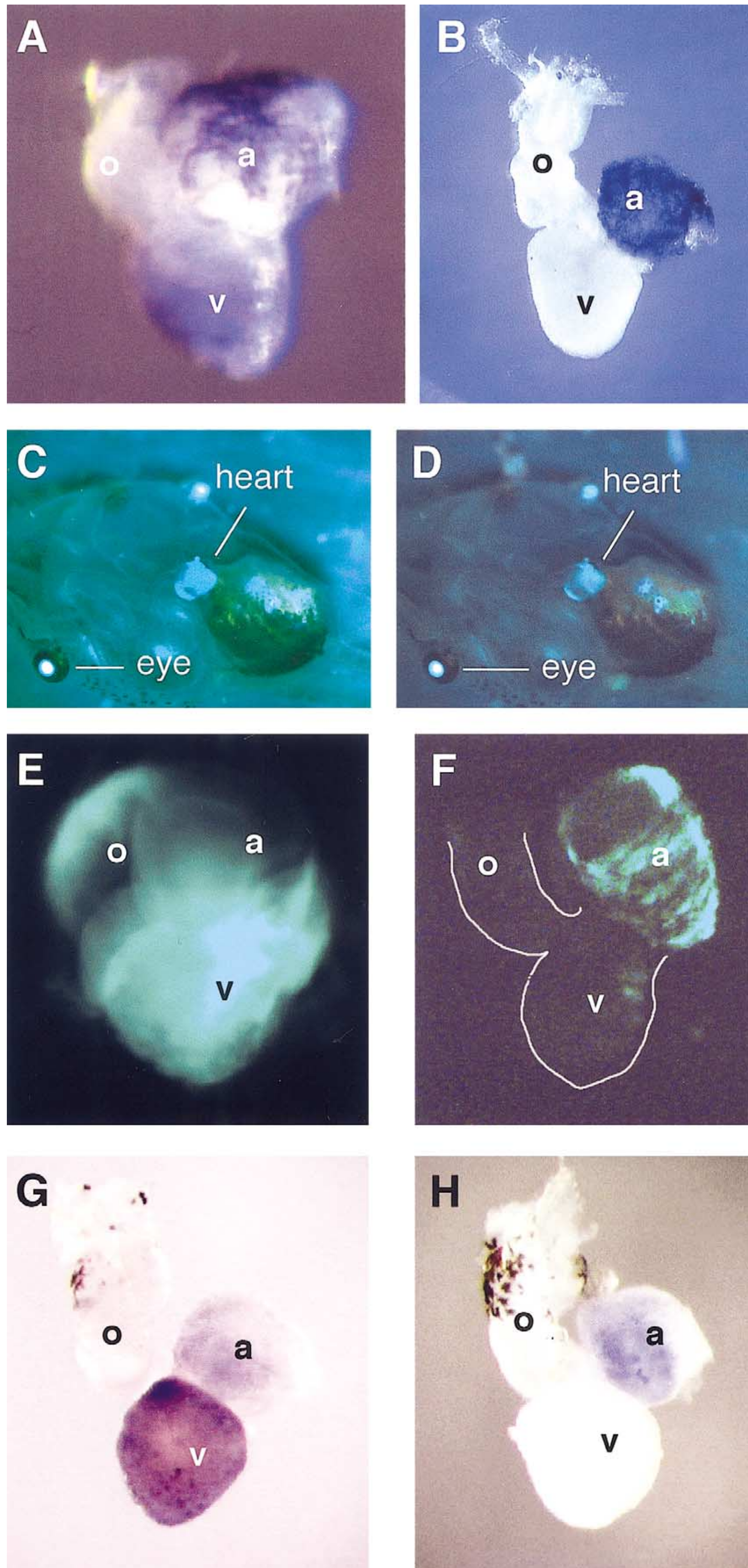


Fig. 1. The 5′ regulatory sequence of the *Xenopus ANF* promoter. The sequence of elements conserved in the human *ANF* promoter is aligned beneath the corresponding frog sequence. The promoter contains an extended Nkx2-5 binding site (NKE) that is identical in 12 of 16 bases. Also conserved are a proximal GATA (GATAp), distal GATA (GATAd), and two SRF binding sites (SREs) termed the proximal SRE (SREp) and distal SRE (SREd). The SREp and SREd are both identical in the human and frog sequences. In addition to the primary sequence, the relative spacing of these binding sites is also highly conserved between the frog and human promoters. Finally, the frog sequence contains a single Tbx binding site (TBE) that is not conserved in location compared with the human promoter.

ANF gene (data not shown). Based on these observations, the shorter *ANF* promoter construction was used in all subsequent studies. We have compared the pattern of expression of the wild type 625 bp *ANF* promoter-driven GFP transgene with expression of the endogenous *ANF* gene (Fig. 2). Transgene expression is first detected by fluorescence in the heart at approximately stage 34/35 (data not shown). During subsequent development, the level of GFP fluorescence increases and is prominent throughout both the atrial and ventricular regions of the myocardium, as shown in Fig. 2C–E. Expression of *ANF* in the atria always appears much weaker than ventricular expression because the atrial myocardial layer is only one or two cells across, whereas the ventricular myocardium is very thick (Small and Krieg, 2000). Starting at approximately stage 47, GFP fluorescence in the ventricle and the outflow tract begins to decrease and expression becomes completely restricted to the atria at about stage 49 (Fig. 2F and H). This restriction of *ANF* expression to the atria occurs over a period of approximately 5 days under standard growth temperature conditions (22°C). Observations using GFP fluorescence as the reporter can be directly confirmed by in situ hybridization

Fig. 2. The 625-bp wild-type *Xenopus ANF* promoter fragment recapitulates the endogenous *ANF* expression pattern. (A) In situ hybridization analysis of stage 45 nontransgenic heart showing *ANF* expression throughout the atrium, ventricle, and outflow tract. (B) Stage 49 nontransgenic Xenopus heart showing restriction of ANF transcripts to the atrial myocardium. (C) Stage 44 transgenic embryo showing GFP reporter expression driven by the wild-type 625-bp *ANF* promoter. This is a ventrolateral view of a living embryo under combined UV and visible light. GFP fluorescence can be observed in the eye, driven from the control γ-*crystallin* promoter and throughout the heart. (D) Fluorescent image of embryo in (C), highlighting GFP reporter expression. (E) Heart of living stage 44 transgenic embryo, with GFP expression driven from the wild-type 625-bp *ANF* promoter. Fluorescence is visible throughout the atrium, ventricle, and outflow tract. (F) Fluorescent image of dissected stage 48 transgenic heart, showing that GFP reporter expression is now limited to the atria. (G) In situ hybridization analysis of dissected stage 45 transgenic heart, showing distribution of GFP transcripts throughout the heart. (H) In situ hybridization analysis of GFP transcripts in dissected stage 49 transgenic heart. Note restriction of transgene expression to the atria, confirming the expression pattern observed using fluorescence. Abbreviations: o, outflow tract; a, atria; v, ventricle.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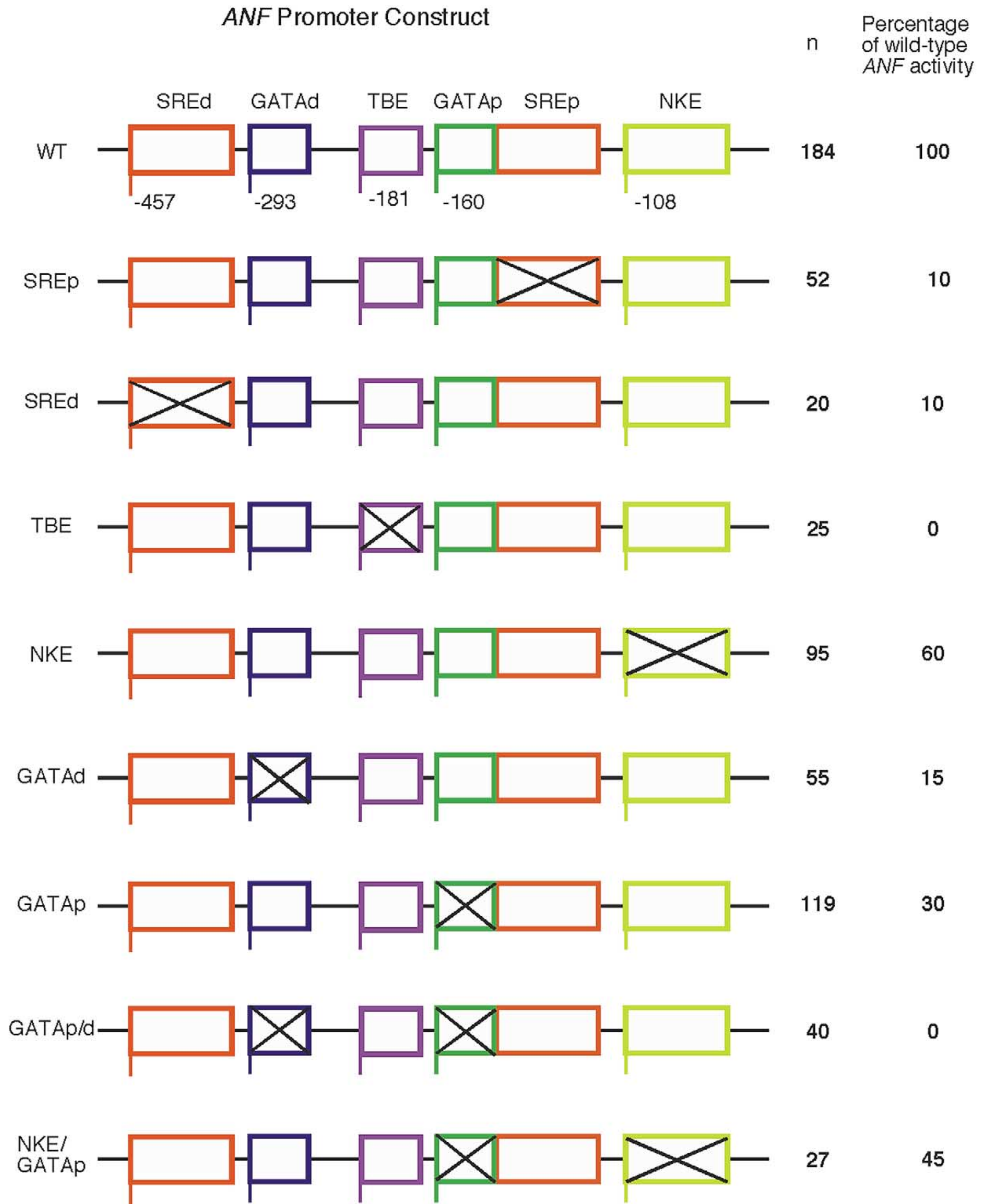


Fig. 3. Mutational analysis of the *Xenopus ANF* promoter in transgenic embryos. The different *ANF* promoter constructions are illustrated at the left of the figure. Numbers on the top line indicate the distance of the regulatory element upstream of the transcription start site. The number of independently generated transgenic embryos is indicated in the first column and the relative activity of the mutant constructions (expressed to the nearest 5%) is presented in the second column. This number is calculated as the proportion of mutant transgene embryos showing visible reporter expression relative to the number of wild type transgene embryos showing reporter expression (see Materials and methods).

against GFP mRNA sequences (Fig. 2G and H), indicating that the transgene expression pattern is not being misrepresented due to GFP protein stability or different transparency of the ventricular or atrial myocardial tissues. Finally, we note that reporter gene expression from the wild type 625-bp *ANF* promoter is restricted to the heart at all stages

of development, indicating that the tissue specificity of the promoter has been retained. Overall, the results with the *Xenopus ANF* promoter are consistent with previous transgenic experiments utilizing mammalian promoters (Field, 1988; Knowlton et al., 1995; Habets et al., 2002). These studies showed that all sequences required for cardiac specific expression are located within approximately 600 bp upstream of the transcription start site.

The presence of two SRF binding sites is required for efficient expression of the ANF promoter

Using expression from the wild type 625-bp *ANF* promoter as a reference, we have undertaken a mutational analysis to determine the relative importance of the different promoter regulatory elements in controlling the efficiency and tissue specificity of *ANF* expression in transgenic embryos. We commenced this analysis with mutations of the SREs, which act as binding sites for SRF. Constructions were prepared in which either the proximal SRE (SREp) or the distal SRE (SREd) were mutated, while taking care to maintain precise spacing between regulatory elements. Using GFP fluorescence as the readout, more than 20 independent F0 transgenic embryos were analyzed for each construction. As shown in Fig. 3, mutation of either the proximal or distal SRE results in a dramatic reduction in expression from the *ANF* promoter, to approximately 10% of the level observed from the wild type promoter. Since the SRE mutant promoters resulted in very weak transgene expression levels, no information on the requirements of these sites for temporal or spatial regulation of *ANF* expression was obtained by using our assay.

The TBE is essential for efficient expression of the ANF promoter

Mutation of the single TBE completely eliminates detectable GFP reporter expression during early myocardial development. By stage 45, about 5 days after the normal onset of expression, GFP became visible in the hearts of 30% of transgenic embryos, albeit at much reduced levels relative to wild type ($n = 18$; data not shown). Determination of atrial restriction in these mutants was not possible due to the very low levels of GFP. Based on these results, it appears that a functional TBE is essential for the correct temporal activation of *ANF* expression and also for normal efficiency of the promoter.

A functional Nkx2-5 binding site is required for restriction of ANF expression to the atrium

The *ANF* promoter contains a conserved Nkx2-5 binding site, the NKE, centered at approximately 110 bp upstream of the transcriptional start site. The sequence and spacing of this element, relative to other promoter motifs, is highly conserved between the *Xenopus* and the human *ANF* promoters. As shown in Fig. 3, mutation of the NKE results in a surprisingly minor reduction of reporter gene activity in transgenic frog embryos. Analysis of 95 independent transgenic embryos shows that elimination of the Nkx2-5 binding site reduces detection of GFP expressing embryos to approximately 60% of wild type levels. Apart from this reduction in promoter activity from the NKE mutant promoter, the time of onset of cardiac GFP expression and the initial distribution of GFP fluorescence throughout the atrial and ventricular myocardium appears identical to the wild type sequence (data not shown).

The most striking aspect of the NKE mutant transgenics is the appearance of GFP reporter expression in non-cardiac tissues of the embryo. Ectopic expression is observed in the developing kidneys, facial muscles, and in a group of muscles adjacent to the aortic arch arteries, but never at any other location (Fig. 4). GFP expression outside of the heart was never observed in transgenics carrying the wild type promoter ($n = 184$) and so it seems extremely unlikely that this expression is due to copy number or position effects at the integration site. In NKE mutant transgenics, expression in developing facial muscles is first detected at approximately stage 32 by visible fluorescence (Fig. 4A) and by in situ hybridization (Fig. 4B). This is earlier than expression in the heart, which is first detected at about stage 34. Later in development, when muscle morphology is more pronounced, it is possible to identify the facial muscles as the musculus interhyoideus, musculus quadratohyangularis, and orbitohyoideus (Sokol, 1977) (Fig. 4C–E). Ectopic transgene expression is also observed in the interbranchialis I and II, adjacent to the third and fourth aortic arch arteries, and the subarcualis obliquus (Fig. 4C and D). Ectopic expression from the NKE mutant promoter also occurs in the kidneys, as shown by fluorescence in a stage 44 embryo (Fig. 4F).

Significantly, the NKE mutant also shows aberrant expression of the GFP reporter during later stages of heart development. As described above, expression from the wild type transgene becomes completely restricted to the atrial myocardium by stage 49 of development, identical to expression of the endogenous *ANF* gene (Fig. 5A and B). In the case of the NKE mutant, GFP fluorescence persists in the outflow tract and is maintained at high levels in the ventricular myocardium (Fig. 5C). The ventricular expression has never been observed to decrease in the NKE mutants and persists for at least 3 weeks after expression is restricted to the atria in wild type controls (Fig. 5B). Failure of restriction was confirmed by in situ hybridization against GFP mRNA (data not shown).

The distal GATA site is required for basal promoter activity while the proximal GATA site is required for restriction of ANF expression to the atrium

The *ANF* promoter contains two conserved GATA sites at −160 and −293 designated proximal GATA

(GATAp) and distal GATA (GATAd), respectively, in Fig. 1. To examine the requirement of these elements for *ANF* promoter function in vivo, we have generated promoter constructions containing mutations of either the GATAp, or GATAd alone, or mutations in both elements. Once again, the mutations have preserved the correct spacing of these elements relative to other regulatory sequences. The results of our transgene experiments indicate that the GATAp and GATAd sites have distinct functions in the regulation of *ANF* promoter activity in the frog embryo (Fig. 3). First, the distal GATA site appears to be required for efficient expression from the *ANF* promoter. Mutations in the GATAd element strongly reduce expression of the GFP transgene, to about 10% of wild type levels. This is approximately equivalent to the degree of reduction observed when either SRE was mutated. In all cases where GFP fluorescence was visible, expression from the GATAd mutant promoter was limited to the developing heart. On the other hand, the proximal GATA site appears to be involved in regulation of tissue-specific expression of *ANF*. Mutation of GATAp reduced detectable GFP expression to approximately 30% of wild type numbers. Unlike GATAd mutant expression, mutations in the GATAp element resulted in ectopic expression of the GFP reporter in a subset of tissues apparently identical to those observed for the NKE mutant. Furthermore, initial expression of the GATAp mutant in the heart appeared normal, but GFP fluorescence failed to restrict to the atria after stage 47 (Fig. 5D). Again, this is indistinguishable from the expression observed with the NKE mutant promoter. Finally, as might be expected, mutation of both the proximal and distal GATA sites resulted in a complete absence of detectable GFP transgene expression. Overall, these results indicate that the proximal and distal GATA sites are both required for efficient *ANF* expression, but that the proximal GATA element is also required for regulation of correct spatial expression.

Since mutation of either the proximal GATA site or the NKE, individually, does not abolish the GFP transgene expression, we have tested the activity of a construction containing a double mutation of the NKE and GATAp sites. Surprisingly, embryos transgenic for the NKE and pGATA double mutant promoter still exhibit a relatively high level of GFP transgene expression. As shown in Fig. 3, expression from the NKE/GATAp double mutant promoter is observed at approximately 45% the frequency of the wild type control. Note that this is similar to the expression levels observed for either the NKE or the GATAp single mutations. Similarly, the cardiac and ectopic expression pattern of GFP fluorescence driven by the NKE/GATAp double mutant is indistinguishable from that previously observed for the NKE or GATAp single mutants (data not shown).

Discussion

Mutation of SRF binding sites in the ANF promoter

Our first transgenesis experiments focused on the conserved SRF binding sites (SREs) in the *ANF* promoter, since previous studies have suggested that these elements play a central role in muscle gene regulation (Hines et al., 1999; Wang et al., 2001a). Although the proximal SRE is not a perfect consensus site, previous studies have shown that both the proximal and distal SREs are capable of binding SRF protein. Furthermore, although SRF binds the nonconsensus SREp with slightly lower affinity, functional analyses have demonstrated that this site is indeed SRF-inducible in cardiac myocytes (Hines et al., 1999). Our transgenic studies showed that mutation of either the proximal or the distal SRE resulted in a severe reduction in expression from the *ANF* promoter, to about 10% of normal levels. These observations are in complete agreement with a model in which SRF proteins form the foundation of a transcription complex containing myocardin, a powerful transcriptional activator that does not itself possess DNA binding activity (Wang et al., 2001a). Cell culture experiments using several different cardiac-expressed promoters demonstrate that two SRF binding sites are required for optimal activation by myocardin (Wang et al., 2001a). With specific reference to activity of the *ANF* promoter, however, our results disagree with a previous study using *ANF* promoter constructions in transfected primary cardiac muscle cells, which suggested that the proximal SRE element played only a minor role in basal promoter efficiency (Hines et al., 1999). These differences may reflect species specific variation in the importance of the proximal SRE between mammals and frogs, or may be due to differences between the in vitro and transgenic assays.

A functional Tbx binding site is essential for correct ANF expression

The mammalian *ANF* promoters contain three potential TBEs. Although the sequence and location of the single TBE in the *Xenopus ANF* promoter is not conserved with any of those in the mammalian promoters, our results demonstrate an important role for both the timing and efficiency of *ANF* promoter activity. Mutation of the TBE results in total absence of detectable GFP in the hearts of transgenic embryos at the normal time of activation. Approximately 5 days later, GFP reporter expression becomes detectable in about one-quarter of the transgenics, but at very low levels. These results suggest that a functional TBE is essential for regulation of both the time of activation and the efficiency of expression of the *ANF* promoter. This finding is consistent with previous studies showing that Tbx5 potently activates the *ANF* promoter in CV1 cells (Bruneau et al., 2001). Furthermore, levels of Tbx5 seem to be crucial for *ANF*

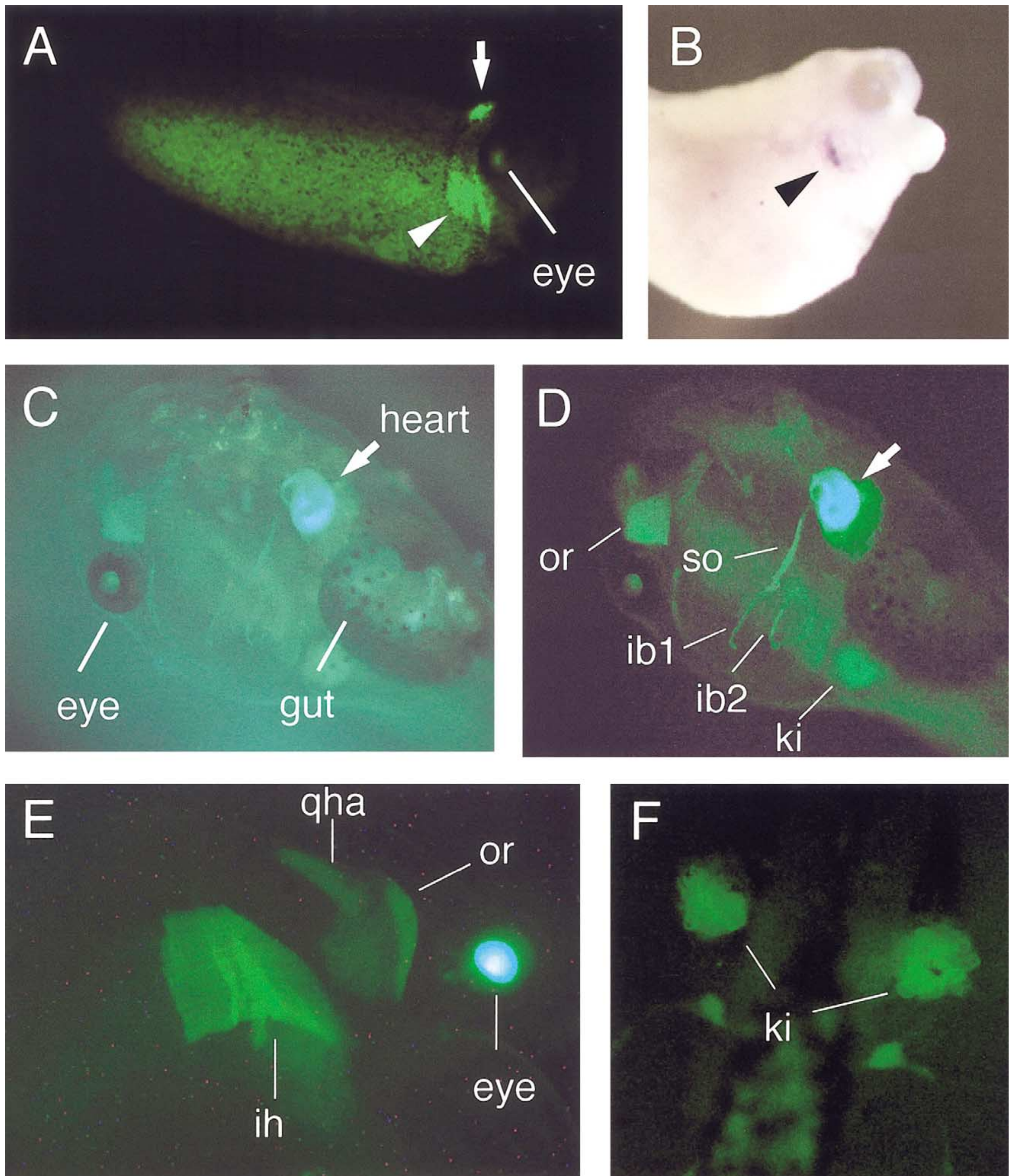


Fig. 4. Mutation of the NKE within the *ANF* promoter results in ectopic transgene expression outside of the heart. All transgenic embryos shown are mutant for the NKE, but identical results are observed for the GATAp mutant promoter. (A) Fluorescent image of a living stage 32 transgenic embryo. Ectopic expression of reporter GFP is visible in the developing facial muscles (indicated by an arrow head). Expression in the brain, from the control γ *crystallin* promoter, as indicated by an arrow. Apparent fluorescence in the flank of the embryo is background due to autofluorescence of yolk granules. Cardiac expression of the transgene has not commenced at this stage. (B) In situ hybridization detection of GFP reporter gene expression in stage 32 embryo also shows transcripts in developing facial muscles (arrowhead). (C) Combined brightfield and UV image of stage 45 transgenic embryo. Ventrolateral view shows ectopic reporter expression in facial muscles and muscles associated with the third and fourth aortic arch artery. (D) Fluorescent image of the tadpole in (C) showing ectopic GFP expression in the interbranchialis I and II (ib1 and ib2) adjacent to the third and forth aortic arch arteries, the subarcualis obliquus (so) muscle, and the orbitohyoideus (or) muscle. Expression is also observed in the kidneys (ki). Transgene expression is strong in the heart (arrow). (E) Ventral view of the head of a different transgenic embryo shows additional facial muscles expressing GFP. The orbitohyoideus (or) associated with the musculus quadratohyangularis (qha) and the musculus interhyoideus (ih) are indicated. Control γ-*crystallin* promoter-driven GFP is observed in the eye. (F) Dorsal-view of stage 45 transgenic embryo showing ectopic GFP expression in the kidneys (ki).

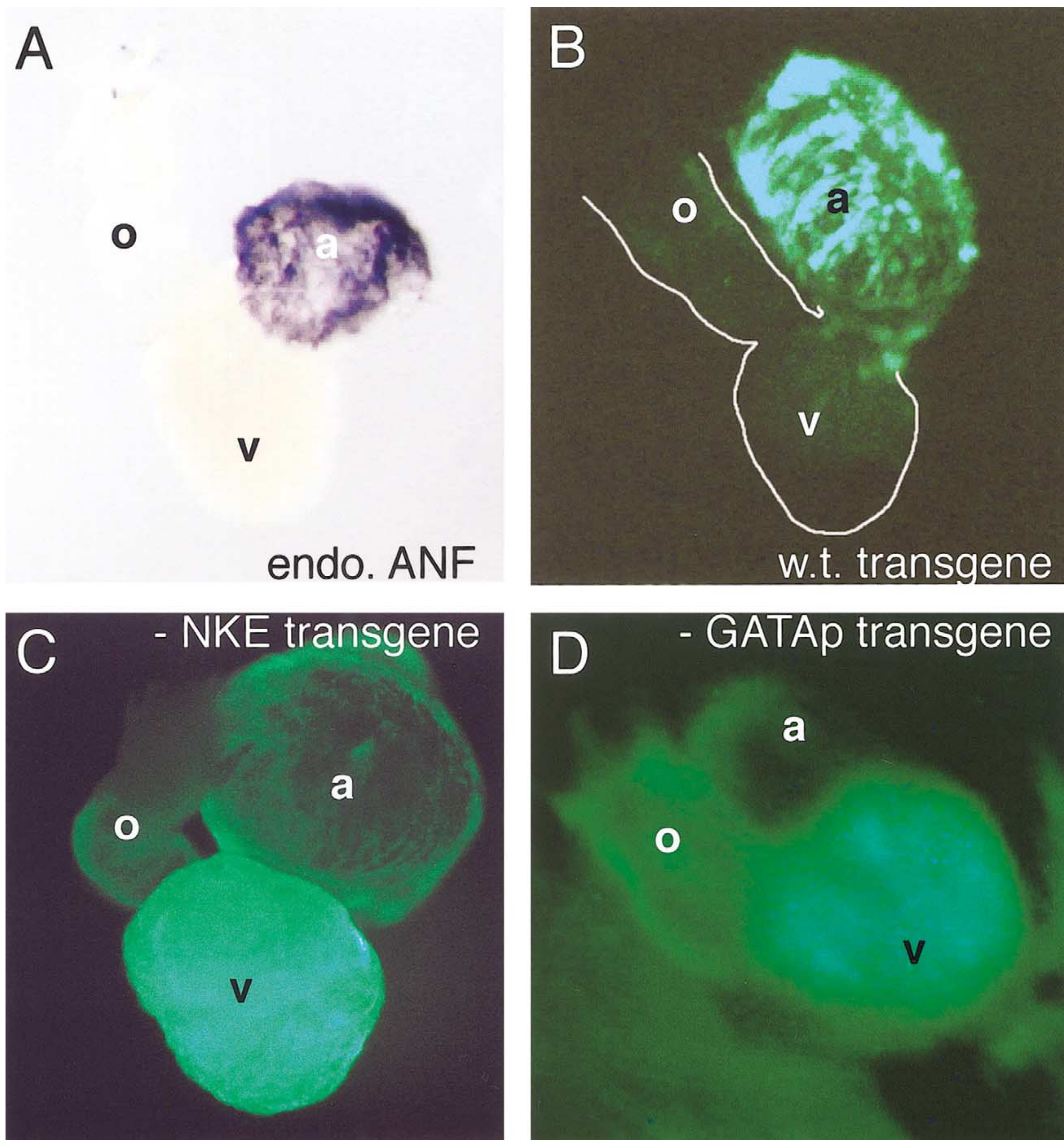


Fig. 5. Mutations of the NKE or proximal GATA (GATAp) element within the *ANF* promoter result in persistent transgene expression throughout the ventricle and outflow tract. (A) In situ hybridization analysis of dissected nontransgenic stage 49 heart, showing restriction of endogenous *ANF* transcripts to the atrium. Note patchy expression of endogenous gene. (B) Fluorescent image of GFP expression in dissected stage 49 heart transgenic for the wild-type *ANF* promoter. Reporter expression is restricted to atria and also shows patchy expression. (C) Fluorescent image of dissected stage 52 transgenic heart. Mutation of the NKE within the *ANF* promoter results in persistent expression of the GFP reporter throughout the ventricle, and outflow tract. This ectopic expression persists for at least 3 weeks after expression of endogeneous *ANF,* or the wild-type *ANF* transgene, has restricted to the atria. (D) Fluorescent image of GATAp mutant transgene expression in the heart of a living stage 50 embryo. Expression is visible throughout the ventricle and outflow tract, exactly as observed with the NKE mutant transgene.

expression. Mice heterozygous for Tbx5 show reduced levels of *ANF* transcripts, while Tbx5 null mice completely lack *ANF* expression (Bruneau et al., 2001). Other studies in mice have reported that Tbx2 is able to cooperate with Nkx2-5 to inhibit AV canal expression of *ANF*. This is believed to be mediated via the TBE at −259, which is adjacent to an NKE (Habets et al., 2002). This pairing of a TBE and NKE is not present in the *Xenopus ANF* promoter.

Mutation of the NKE causes only a slight reduction in ANF promoter activity

The sequence of the single NKE element is almost identical in the *Xenopus* and mammalian *ANF* promoters, suggesting that the regulatory function of this element has been conserved in evolutionarily distant organisms. In transgenic *Xenopus* embryos, mutation of the NKE resulted in only a relatively minor reduction in the number of embryos expressing GFP in the heart, to approximately 60% of wild type levels. This result was initially very surprising, because it appears to contradict the observation that *ANF* expression is greatly reduced or completely absent in the Nkx2-5 knockout mouse (Harvey et al., 1999; Tanaka et al., 1999). One explanation might be that, whereas Nkx2- 5 protein is essential for *ANF* promoter activity, direct binding of the protein to its target site is not. For example, it is possible that protein–protein interactions with either GATA- 4 or SRF are sufficient to tether Nkx2-5 to the transcription complex, independent of direct binding to the NKE. This interpretation is in general agreement with several in vitro studies of the *cardiac α actin* and *ANF* promoters, which have suggested that the absence of the NKE does not result in a great reduction in promoter activity (Chen and Schwartz, 1996; Durocher and Nemer, 1998). Indeed, a nearly identical 50% reduction in promoter activity is observed when an ANF promoter with a mutated NKE is transiently transfected into primary cardiac myocytes (Durocher and Nemer, 1998). Other experiments have shown that the DNA binding domain of Nkx2-5 is not required for activity of the *cardiac α actin* promoter, provided that SRF is present (Chen and Schwartz, 1996). Together, these studies suggest that Nkx2-5 is indeed an important transcriptional activator for *ANF* expression, but that direct interaction of Nkx2-5 protein with DNA is largely dispensable. It may not be appropriate to generalize this result too far, however, since transgenic studies have shown that an Nkx2-5 binding site is essential for GATA-6 promoter activity (Molkentin et al., 2000). Overall, these transgenic and cell culture studies suggest that the effect of NKE mutation may be promoter-specific. In particular, the consequences for transcriptional activity may be dependent on the presence of binding sites for specific cofactors that could form a transcription complex with Nkx2- 5 and help to modulate its activity. Finally, the most striking effect of NKE mutation in transgenic embryos is activation of *ANF* promoter activity in several different tissues outside of the developing heart and the persistence of *ANF* expression in the ventricle and outflow tract. These observations will be discussed below.

Mutation of the GATA binding sites in the ANF promoter

Our transgenic studies indicate that the two GATA sites in the *ANF* promoter exhibit distinct regulatory activities. First, the distal GATA element is essential for efficient *ANF* promoter activity, since mutants lacking this site showed greatly reduced reporter gene expression (approximately 15% of wild type activity). This result is not consistent with a previous cell culture study which suggested that the distal GATA exerts only a modest effect on *ANF* promoter activity (Morin et al., 2001). On the other hand, the proximal GATA element plays a more subtle role in modulating *ANF* activity. Elimination of this site results in a moderate reduction in cardiac promoter activity, but also allows ectopic expression of the GFP reporter in the facial muscles and in the developing kidneys, apparently identical to the aberrant pattern of expression observed for the NKE mutant. During subsequent development of the heart, expression of the proximal GATA mutant never restricted to the atria, again corresponding to the pattern of the NKE mutant.

Mutation of both the proximal GATA and the NKE sites

Dual mutation of the proximal GATA site and the NKE within the *ANF* promoter reduces reporter expression to approximately half of wild type activity (Fig. 3). The expression of the double mutant is indistinguishable from both the single NKE mutant, or the proximal GATA site mutant. This result indicates that mutation of both binding sites causes no further reduction in promoter activity or tissue specificity than mutation of either site independently. These results are in apparent disagreement with *ANF* promoter studies in cultured cells, which suggest that binding of both Nkx2-5 and GATA-4 is required for efficient transcription (Lee et al., 1998). In fact, mutation of both the GATA and NKE sites nearly eliminated promoter activity in primary cardiac myocytes (Durocher and Nemer, 1998). Additional cell culture studies indicated that GATA-4 and Nkx2-5 synergize strongly to activate *ANF* expression, resulting in a transcriptional activation of between 25-fold and 200-fold over expression levels observed with one of the factors alone (Durocher et al., 1997; Lee et al., 1998). Our studies in the transgenic embryo do not contradict a possible synergy between the Nkx2-5 and GATA proteins, but they strongly suggest that the corresponding binding sites are not required for this activation. Based on previous studies (Grueneberg et al., 1992; Treisman, 1994; Chen and Schwartz, 1996; Belaguli et al., 2000), the most likely explanation is that the SRF protein, binding to the essential proximal and distal SRE sites, facilitates the assembly of a transcription complex that includes both the GATA and Nkx2-5 proteins. This would be similar to the proposed role for SRF in providing a platform for recruitment of the non-DNA binding transcriptional activator, myocardin, to the promoter of cardiac-specific genes (Wang et al., 2001a).

Tissue-specific regulation of ANF expression

An unexpected result of our transgene experiments was the observation that mutation of either the NKE or the proximal GATA element results in ectopic *ANF* promoter

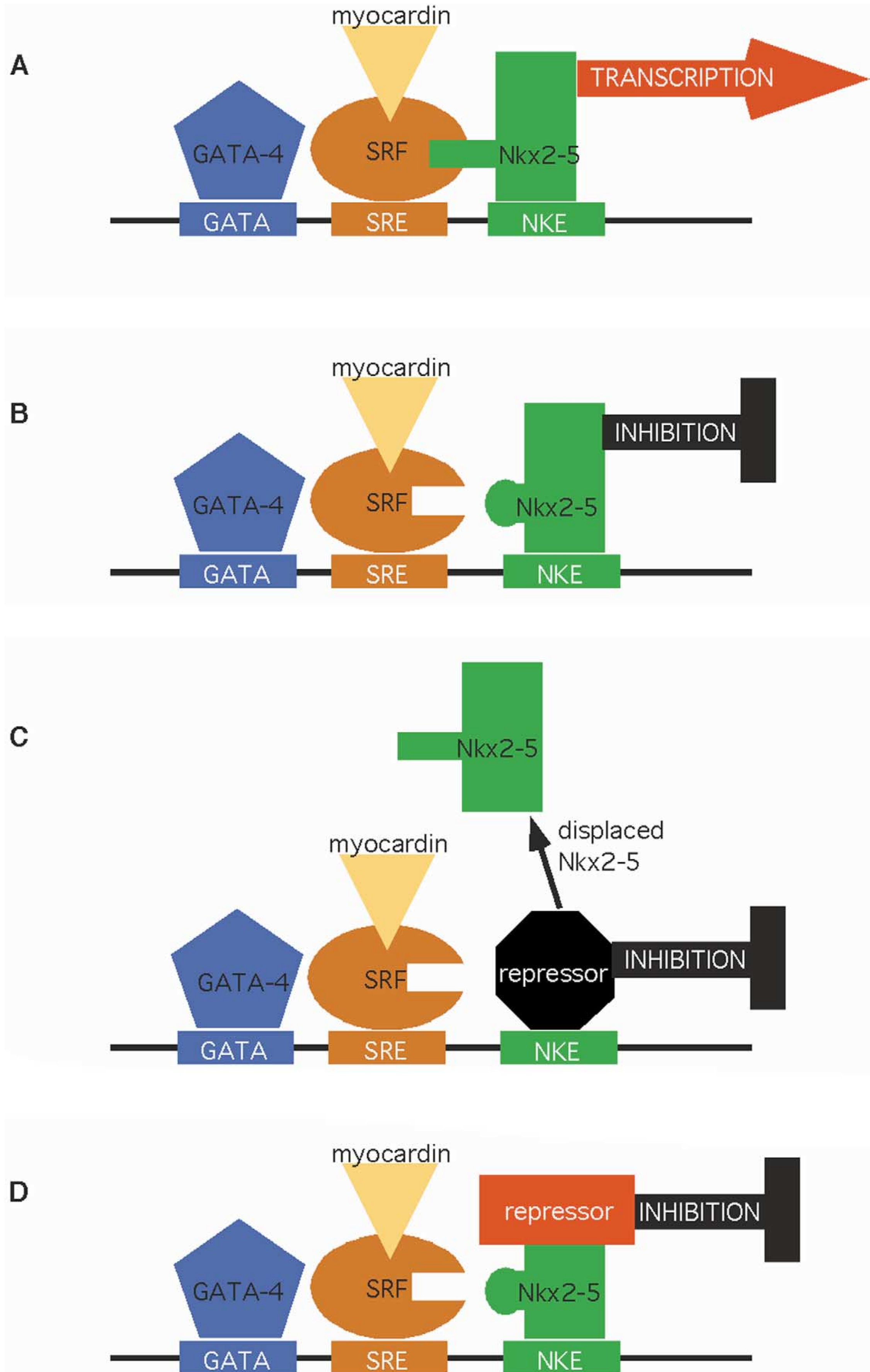
activity. In the wild type promoter, therefore, it appears that the NKE and the proximal GATA can play an inhibitory role, preventing *ANF* expression in ectopic tissues. Such an inhibitory role for the NKE is consistent with previous studies, suggesting that Nkx2-5, acting together with Tbx2, inhibits *ANF* expression in the atrioventricular canal (AVC) region of the mouse heart (Habets et al., 2002). We note, however, that the NKE implicated in the AVC inhibition is located at about −350 in the mouse *ANF* promoter and this element is not conserved in the frog sequence. How might the inhibitory function of the NKE be explained? Within cardiac tissues, it is possible that Nkx2-5 undergoes a post-translational modification that converts it from an activator to a repressor of transcription (Fig. 6B). To explain the observed pattern of *ANF* expression, the modification would be limited to the ventricle and outflow tract tissues. A necessary corollary of this model is that the posttranslational modification would also prevent assembly of Nkx2-5 into the SRF transcription complex. If this were not the case, then mutation of the NKE sequences would not have any effect on *ANF* regulation in the transgenics. This model has the advantage that the known interactions between GATA and Nkx2-5 proteins (Durocher et al., 1997; Sepulveda et al., 1998) may continue to be relevant for the inhibitory mechanism.

An alternative explanation for these observations is that, in kidney, facial muscles, and the ventricle, the NKE and/or the GATA sites serve as the recognition sequence for a distinct repressor protein (Fig. 6C). Activity of this putative inhibitor in the ventricle and the outflow tract would result in restriction of *ANF* expression in the atria. Mutation of the inhibitor recognition sequence would be expected to prevent inhibitor binding and thereby permit continued *ANF* transcription. Since Nkx2-5 and GATA proteins continue to be expressed throughout all myocardial tissues during development and in the adult heart (Lints et al., 1993; Kasahara et al., 1998; our unpublished observations), this model assumes that the inhibitor would either displace Nkx2-5 from the SRF-based transcription complex (Fig. 6C), or bind directly to Nkx2-5 and completely inhibit its activation properties (Fig. 6D). In general support of this model, Takimoto et al. (2000) have shown that persistent overexpression of Nkx2-5 throughout the myocardium increases expression of *ANF* in the ventricle after restriction, possibly by competing with the inhibitor protein for NKE binding. Furthermore, Durocher et al. (1996) showed that mutation of the NKE caused an upregulation of *ANF* promoter activity in ventricular myocytes, consistent with an inhibitory role in this tissue.

Somewhat surprisingly, a relatively large number of proteins capable of binding to the NKE have been reported, and several of these may have the potential to act as inhibitors. First, it appears that all members of the NK2 family of homeodomain proteins, and also the NK3 (bagpipe) family proteins, show affinity for a binding site closely related to the 5′-AAGTG-3′ sequence present in the *ANF* NKE (Guazzi et al., 1990; Okkema and Fire, 1994; Chen and Schwartz, 1995; Carson et al., 2000; Steadman et al., 2000; Sparrow et al., 2000b). All known NK2 proteins contain a sequence N-terminal to the homeodomain that has similarity to the engrailed repressor (Smith and Jaynes, 1996), although there is currently no evidence that this domain serves an inhibitory function in vivo. On the other hand, it is clear that NK3 (bagpipe) family proteins may act as transcriptional inhibitors (Steadman et al., 2000). Similarly, it has been reported that the transcription factor COUP-TF1, and homeodomain proteins of the Hmx family, can recognize the Nkx2-5 binding site and may act as transcriptional repressors by opposing the activity of Nkx2-5 (Amendt et al., 1999; Guo et al., 2001). The expression patterns of most of these potential inhibitors have not been reported during the later stages of heart development, although the hmx1 orthologue in chicken, GH6, appears to be specifically expressed in the ventricular myocardium (Stadler and Solursh, 1994). Expression of the putative repressor may either be restricted to nonatrial tissues, or alternatively, the factor might undergo posttranslational modification to assume inhibitor activity in the nonatrial tissues. At present, it is not possible to determine whether the same repressor that is acting in the heart is also responsible for inhibiting ectopic *ANF* expression in the facial muscles and kidney, or whether different repressors are acting in the different tissues.

The observation that mutation of the GATA and NKE elements results in persistent expression of *ANF* in the ventricle and outflow tract may help to more generally illuminate the mechanisms by which atrial-specific expression of genes is achieved during normal development. In all species examined, *ANF* is initially expressed throughout developing atrial and ventricular myocardium and later becomes restricted to the atria (Zeller et al., 1987; Akizuki et al., 1991; Seidman et al., 1991; Small and Krieg, 2000; Houweling et al., 2002; Habets et al., 2002). Similarly, a number of other atrial-specific genes, including the quail

Fig. 6. A model of *ANF* transcriptional regulation by Nkx2-5, GATA-4, and SRF. (A) In the early myocardium and in the mature atria activation of *ANF* transcription is proposed to be regulated by a complex of Nkx2-5, SRF and cofactor myocardin, and GATA-4. (B) Inhibition of *ANF* transcription in the ventricle may occur due to posttranslational modification of Nkx2-5, which results in exposure of an inhibitory domain within the protein. This modification must also abolish Nkx2-5–SRF interactions since mutation of the Nkx2-5 binding site within the *ANF* promoter results in loss of inhibition in the ventricle. (C) A second inhibitory model relies on a hypothetical repressor protein that becomes expressed (or activated) in the ventricle after stage 47. This repressor protein would bind to the NKE and displace Nkx2-5 from the *ANF* promoter. (D) A third possible inhibitory mechanism results from the binding of a ventricle-specific repressor protein directly to Nkx2-5. This interaction between Nkx2-5 and the candidate repressor must abolish the ability of Nkx2-5 to interact with SRF, and therefore, to be tethered to the promoter in the absence of a functional NKE.


A
myocardin
TRANSCRIPTION
GATA-4
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B
myocardin
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SRE
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GATA-4
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slow MyHC3, mouse *MLC2a* and *MLC1a* (Wang et al., 1996; Kubalak et al., 1994; Lyons, 1994), are initially expressed throughout the heart but later become limited to the atrial chambers. In the case of *slow MyHC3,* atrial restriction is proposed to require the inhibitory properties of the homeodomain protein Irx4 (Wang et al., 2001b), which is ventricle-specific in all species examined (Bao et al., 1999; Bruneau et al., 2000; Garriock et al., 2001). The repression of *slow MyHC3* by Irx4 in the ventricle is thought to be mediated by protein–protein interactions with a Vitamin D–Retinoid X receptor complex. This complex tethers Irx4 to the vitamin D receptor binding element (VDRE) (Wang et al., 2001b). Since the time of restriction to the atrium varies widely between different cardiac genes, it is unlikely that the same factors are involved in regulation of all atrial-specific genes. However, we propose that inhibition by chamber-specific factors is a general mechanism of restriction of cardiac gene expression. The same families of inhibitory factors mentioned above might be considered as candidates for the ventricular repressors involved in regulation of other atrial-specific genes. In the case of the homeodomain proteins, the inhibitors may interact with GATA proteins to stabilize DNA binding.

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