The regulation of chamber-specific gene expression in the developing heart
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CHAPTER IV

Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation


ABSTRACT

During heart development chamber myocardium forms locally from the embryonic myocardium of the tubular heart. The atrial natriuretic factor (ANF) gene is specifically expressed in this developing chamber myocardium and is one of the first hallmarks of chamber formation. We investigated the regulatory mechanism underlying this selective expression. Transgenic analysis shows that a small fragment of the ANF gene is responsible for the developmental pattern of endogenous ANF gene expression. Furthermore, this fragment is able to repress cardiac troponin I (cTnI) promoter activity selectively in the embryonic myocardium of the atrioventricular canal (AVC). In vivo inactivation of a T-box factor (TBE)- or NK2-homeobox factor binding element (NKE) within the ANF fragment removed the repression in the AVC without affecting its chamber activity. The T-box family member Tbx2, encoding a transcriptional repressor, is expressed in the embryonic myocardium in a pattern mutually exclusive to ANF, thus suggesting a role in the suppression of ANF. Indeed, Tbx2 formed a complex with Nkx2.5 on the ANF TBE-NKE, and was able to repress ANF promoter activity. Our data provide a potential mechanism for chamber restricted gene activity in which the cooperative action of Tbx2 and Nkx2.5 inhibits expression in the AVC.
INTRODUCTION

The vertebrate heart is first formed as a linear tube, which subsequently loops and transforms into the definitive four-chambered heart. The events that lead to the formation of the mature heart have been described (Fishman and Chien 1997; Srivastava and Olson 2000) but the mechanisms that underlie the formation of the chambers are still largely undefined. The linear heart tube is patterned along three body axes and has an embryonic phenotype (i.e., ability to spontaneously dipolarise (automaticity), slow contraction, poor intercellular coupling and poorly developed sarcoplasmic reticulum and sarcomeres). Positional information guides the localized development of different components of the heart. At specific sites of the looping tubular heart trabeculated ventricular and atrial chamber myocardium is formed from this embryonic myocardium. In contrast to the embryonic myocardium the chamber myocardium has lost its automaticity, has a fast contraction pattern reminiscent of the working myocardium of the mature heart and is well coupled intercellularly (Moorman et al. 1998). Indeed, the chamber myocardium specifically initiates the expression of gap-junction genes connexin (Cx) 40 and Cx43 required for intracellular coupling (Delorme et al. 1997), and other genes including ANF and Chisel (Christoffels et al. 2000; Palmer et al. 2001). Thus, chamber formation requires the localized initiation of a transcriptional differentiation program. The smooth-walled myocardium of the inflow tract (IFT), atrioventricular canal (AVC), inner curvature and outflow tract (OFT) retains the embryonic myocardial phenotype longer, and concomitantly does not express Cx40, Cx43, ANF and Chisel. These regions are crucial for septation and they also contribute to the formation of the nodal components of the conduction system (i.e., sino-atrial node, atrioventricular node and atrioventricular junction myocardium), which share phenotypic characteristics with the embryonic myocardium (Moorman et al. 1998; Davis et al. 2001). As many cardiac malformations find their origin in the incorrect development of these embryonic regions, knowledge regarding the mechanisms behind the regulation of the site-specific differentiation program...
is essential. The \( ANF \) gene is ideal to analyse the molecular mechanisms that may underlie the localized formation of atrial and ventricular chamber myocardium within the linear heart tube. First, although in the mature heart \( ANF \) gene expression is restricted to the atrial auricles, during development its expression is specific for the forming ventricular and atrial chambers. It therefore serves as a marker gene for the chamber myocardium (Christoffels et al. 2000). Second, the regulation of the \( ANF \) gene has been well characterized and serves as a paradigm for the regulatory mechanisms that control cardiac gene expression. Previously, a 0.7 kbp upstream fragment of the \( ANF \) gene was shown to be sufficient for cardiac-specific gene expression in cultured cardiomyocytes and transgenic mice (Field 1988; Argentin et al. 1994; Knowlton et al. 1995), although the developmental pattern of the transgene was not reported. A number of general and cardiac-enriched transcription factors were shown to interact with this fragment. Of these, the NK2 homeobox factor Nkx2.5 and T-box factor Tbx5 were shown to be required for \( ANF \) gene expression \( \textit{in vivo} \) (Lyons et al. 1995; Tanaka et al. 1999; Bruneau et al. 2001). Inactivation of either factor in \( \textit{Xenopus} \) and mouse results in severely affected heart development. Moreover, mutations in the genes encoding these factors in human and mouse result in congenital cardiac malformations including septum defects and conduction disease (Basson et al. 1997, Li et al. 1997, Schott et al. 1998; Bruneau et al. 2001). \( \textit{In vitro} \) studies demonstrated that Tbx5 and Nkx2.5 associate and synergistically activate the \( ANF \) regulatory fragment (Hiroi et al. 2001; Bruneau et al. 2001). Although these studies have greatly advanced our understanding of the regulation of heart-specific gene expression, the mechanism for the chamber-specificity remained unclear. In this study we demonstrate that the 0.7 kb \( ANF \) fragment is responsible for the developmental pattern of the \( ANF \) gene. A part of this fragment was able to repress the activity of a \( \textit{cardiac troponin I} (\textit{cTnI}) \) promoter fragment specifically in the AVC. \( \textit{In vivo} \) inactivation of an NK-2 homeobox factor binding element (NKE) or T-box factor binding element (TBE) within the \( ANF \) fragment did remove the repression in the embryonic myocardium of the AVC, while the activity in the chamber myocardium was not affected.
Additional analysis showed that Tbx2 gene expression is restricted to the embryonic areas of the developing heart in a pattern complementary to ANF. Indeed, Tbx2 and Nkx2.5 formed a complex on the TBE-NKE site within the ANF fragment, and Tbx2 was able to repress the activity of the ANF fragment. Our data suggest a novel mechanism for the site-specific formation of chamber myocardium by localized repression of the differentiation program within the embryonic heart.

MATERIALS & METHODS

Transgene construction
All constructs used to generate transgenic mice (Table 1) contain a chimeric intron from the pCI vector (Promega), lacZ with a nuclear localisation signal (nlacZ) and the polyadenylation signal from the bovine growth hormone gene. The ANF construct contains the -638/+70 bp ANF regulatory region, the cTnI construct contains the -230/+126 bp cTnI promoter region. ANF-cTnI is a chimeric construct in which the -638/-138 ANF sequence is fused in front of the -230/+126 cTnI promoter region. In the MLC2V-cTnI construct the -250/-40 MLC2V sequence is fused to the -230/+126 cTnI promoter region. ANFmutNKE-cTnI is identical to the ANF-cTnI construct, with the exception of a four base-pair substitutional mutation of the NKE located at position -250 of the ANF promoter region (NKE: TTGAAGTGGG, NKEmut: TTGCCTCGGG) (Shiojima et al. 1999). The ANFmutTBE-cTnI construct is identical to the ANF-cTnI construct with the exception of a four base-pair substitutional mutation of the TBE located at position -259 of the ANF promoter region (TBE: TCTCACACCTT, TBEmut: TCTCTTTGCTT) (Sinha et al. 2000). The mutations were generated using the QuickChangeTM Site-Directed Mutagenesis kit (Stratagene, USA). With exception for the ANF-nlacZ construct, all constructs were flanked by a 1.2 kb SalI-BamHI tandem repeat of a chromosomal insulator sequence from the 5’ region of the chicken β-globin gene kindly provided by G. Felsenfeld (Chung et al. 1993).
Generation, identification and analysis of transgenic mice
After removal of the vector sequences, the transgene constructs were injected into the pronuclei of zygotes of FVB mice and the injected zygotes were reimplanted into pseudopregnant foster mothers using standard techniques (Hogan et al. 1994). Animal care was according to guidelines as described (Christoffels et al. 1995). Constructs were analysed on lines (ANF), F0 embryos (ANFmutNKE-cTnI and ANFmutTBE-cTnI), or both F0 and lines (cTnI, ANF-cTnI and MLC2V-cTnI). Positive embryos were scored by Southern blot and PCR on DNA prepared from the yolk sac. For Southern blot analysis we used the nlacZ reporter gene (2kb Nd/ScI fragment) as a probe (Sambrook et al. 1989). For PCR analysis, primers specific to the nlacZ sequences were used (lacZ+, GCA TCG AGC TGG GTA ATA AGC GTT GGC AAT and lacZ-, ACT GCA ACA ACG CTG CTT CGG CCT GGT AAT) according to standard procedures (Sambrook et al. 1989). Embryos were stained for β-galactosidase activity as described (Franco et al. 2001).

Plasmid constructs and transfections
Cultures of primary atrial and ventricular cardiomyocytes were prepared from E17.5 Wistar rats as described (van Wamel et al. 2000). Cos-7 cells were grown under standard culture conditions in DMEM/F12 (Gibco BRL, The Netherlands) supplemented with 10% fetal calf serum. Cells were transfected with 4.4 μg of reporter construct, 10-1000 ng of expression plasmid or empty vector for compensation and 200 ng of luciferase expression vector (CMV-Luc) as an internal control per 6-cm dish, using the calciumphosphate method (Sambrook et al. 1989). Cell extracts and luciferase assays were performed as described (Christoffels et al. 1995). β-galactosidase activity was measured using the Galacto-Light kit (Tropix, Bedford) according to the manufacturers instructions. Light emission was measured in a Turner TD-20/20 luminometer. All results are from one representative experiment (out of 3) done in duplicate. Full-length mouse FLAG-Nkx2.5, kindly provided by Dr. R. Harvey, was cloned into pCI (Promega).
FLAG-Nkx2.5-L176P was generated by PCR and subcloned into pCI (Promega). Full-length human TBX5 (Basson et al. 1999), kindly provided by Dr. C. Basson, was cloned into pcDNA3.1 (Clontech). Full-length human TBX2 and TBX2-delRD were cloned in pcDNA3.1 as described (Jacobs et al. 2000). TBX2R122E/R123E was generated by PCR and subcloned into pcDNA3.1 (Clontech).

**Non-radioactive in situ hybridisation**

Whole mount *in situ* hybridisation and non-radioactive *in situ* hybridisation on sections were performed as described (Moorman et al. 2001). The cDNA probes used were ANF (Zeller et al. 1987), Tbx2, Tbx5 (Chapman et al. 1996) and Cx40 (Delorme et al. 1997).

**Electromobility shift assays**

Nuclear extracts were prepared from HEK cells transfected with expression vectors for TBX2, TBX2-delRD, TBX2-R122E/R123E, TBX5, FLAG-Nkx2.5 and FLAG-Nkx2.5-L176P. Double stranded oligonucleotides were synthesised and labelled with [α-32P]dATP using Klenow polymerase. Labelled probes were incubated and used in binding reactions as described and resolved on a 6% polyacrilamide gel (Espinas et al. 1994). Oligonucleotides used (complementary strand not shown, mutations underlined): Wild type (WT) TCTGCTCTTCTCACACCTTTGAAGTGGGGGCCTCTTG, TBE mutated (TBEmut) TCTGCTCTTCTCTATTGCTTTGA AGTGGGGGGCCTCTTG and NKE mutated (NKEmut) TCTGCTCTTCTCACACCTTTGCGGGGGGCCTCTTG.

**Western-blot analysis**

Western-blot analysis was performed according to standard methods (Sambrook et al. 1989). Primary antibodies were a rabbit polyclonal raised against the amino terminus of human TBX2 (Jacobs et al. 2000), and an anti-FLAG antibody from ABR, USA.
RESULTS

The ANF regulatory region is active in atrial and ventricular chamber myocardium

We first assessed whether the ANF regulatory region is capable of driving reporter gene expression specifically in the atrial and ventricular chamber myocardium of the developing heart. Therefore, we generated transgenic mice harbouring this ANF regulatory region (-638/+70) coupled to the nlacZ reporter gene. Heart-specific reporter gene expression was analysed by whole mount X-gal staining of mouse (E9.5 and E11.5) embryos (Figure 1).

FIGURE 1: The 0.7 kb ANF regulatory region is responsible for the developmental pattern of the endogenous ANF gene.
(A) A lateral view of the endogenous ANF gene expression in the heart of E9.5 mouse embryo.
(B /C) A lateral view of the nlacZ reporter gene expression in the heart of E9.5 transgenic embryos.
(D /F) A ventral (D) and dorsal view (F) of the endogenous ANF expression at E11.5.
(E /G) A ventral (E) and dorsal (G) view of ANF transgene expression at E11.5. ift: inflow tract, la: left atrium, ra: right atrium, avc: atrioventricular canal, lv: left ventricle, rv: right ventricle, oft: outflow tract, lscv: left superior caval vein, rscv: right superior caval vein, as: aortic sac.

At E9.5, expression of the reporter gene was observed in the atrial and ventricular chamber myocardium while expression was absent from the
embryonic myocardium of AVC, inner curvature and OFT (Table 1 and Figure 1B and 1C). At stage E11.5 *lacZ* expression is still present in both atria and both ventricles while the expression is higher in the LV as compared to the RV (Figure 1E and 1G). At both stages the transgene expression pattern is comparable to that of the endogenous ANF gene (Figure 1A, 1D and 1F). The only exceptions were the right and left superior caval veins that express the transgene, but not the endogenous gene (Figure 1F and 1G). Therefore, the 0.7 kb ANF regulatory region mimics the endogenous developmental expression pattern in the mouse heart and selectively demarcates the atrial and ventricular chamber myocardium.

**TABLE 1:** Reporter gene expression data of ANFcTnI, ANF-cTnl, MLC2V-cTnl, ANF mutNKE-cTnl, ANFmutTBE-cTnl and ANFmutTBE/NKE transgenic mice at E10.5.

An arbitrary scale of intensity was assigned with +++++ indicating very strong expression, +/- very weak expression and - as having no detectable staining. * indicates the result from multiple embryos of a transgenic mouse line. Each embryo from a line showed an identical expression pattern. Others are single embryos derived from F0 screens. With exception of the ANF construct, all constructs were flanked by insulators. ift: inflow tract, la: left atrium, ra: right atrium, avc: atrioventricular canal, lv: left ventricle, rv: right ventricle, oft: outflow tract.

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The \textit{cTnl} regulatory region is active in the embryonic myocardium of the atrioventricular canal

The observed absence of expression of the ANF transgenes in the embryonic myocardium of the AVC and OFT could result from lack of activation or from active repression in these regions. To discriminate between these two mechanisms we searched for a minimal cardiac promoter region that is predominantly active in the embryonic myocardium. Coupled to the regulatory sequences of the \textit{ANF} gene, this minimal cardiac promoter could be used as a read-out for lack of activation or active repression in the embryonic myocardium. The \textit{cTnl} gene is expressed in the entire myocardium (Vallins et al. 1990; Ausoni et al. 1991). The 356 bp promoter region (-230/+126), analysed in transgenic mice, however, showed a variable pattern of expression, which always included the myocardium of the AVC (Di Lisi et al. 1998; Di Lisi et al. 2000).
Furthermore, only six out of sixteen transgenic mice showed expression (R. Di Lisi and S. Schiaffino, personal communications). To protect the small cTnl promoter region from position effects it was flanked by insulator sequences from the chicken β-globin locus (Chung et al. 1993; Chung et al. 1997; Bell et al. 1999), which did not affect the activation of the cTnl promoter by various transcription factors in transient transfection assays (data not shown). As shown in Table 1, all insulated mouse lines and transgenic embryos expressed the transgene in the heart and transgene expression was always present in the AVC (Figure 2A). In addition, in 9 out of 10 transgenic embryos expression was extended to the RA and LV (Table 1 and Figure 2A). None of the transgenic embryos showed expression in the myocardium of IFT and OFT. Application of insulator sequences appeared to stabilise the transgene expression pattern and strongly increased the proportion of expressing transgenic mice (Z-test; p=0.013, insulated versus non insulated). Therefore, insulators flanked all further constructs used in this study.

The 0.5 kb ANF regulatory region extinguishes cTnl promoter activity in the atrioventricular canal

The -638/-138 bp region of the ANF promoter (Durocher and Nemer 1998) was placed upstream of the otherwise identical cTnl construct and transgenic embryos were generated. All ANF-cTnl transgenic embryos showed a similar expression pattern in the heart (Table 1). At E10.5 transgene expression was observed in the chamber myocardium of the RA, LA, LV and RV, with lower expression levels in the RV as compared to the LV. No transgene expression was observed in the myocardium of the IFT, AVC, inner curvature and OFT (Figure 2B). The expression pattern of these ANF-cTnl transgenics is very similar to the expression pattern of the transgenics that harbour the full 0.7 kb ANF regulatory region (compare Figure 1G and 2B). These observations suggest that the characteristic cTnl promoter activity in the AVC (Figure 2A), is actively repressed by the presence of the 0.5 kb fragment of the ANF promoter. This, in turn, would require the presence of a repressor mechanism that is active in the AVC but not in the atrial and ventricular chamber.
myocardium. To determine whether the repressive effects of the ANF regulatory sequences were specific, a third chimeric construct (MLC2V-cTnI) was made in which we replaced the 0.5 kb ANF regulatory region by a 0.2 kb region (-250/-42) of the MLC2V promoter. This MLC2V promoter region confers right ventricular and OFT expression to a lacZ reporter gene in vivo (Ross et al. 1996), and also in our vector backbone (data not shown). Both MLC2V-cTnI transgenic lines gave similar expression patterns in the heart (Table 1). At E10.5 expression of the transgene was restricted to the RA, AVC and LV, identical to the pattern of the cTnI transgenes (compare Figure 2A and 2C). This indicates that the 0.2 kb MLC2V promoter region is not capable of imposing its activity onto the cTnI promoter or of extinguishing expression in the myocardium of the AVC. Therefore, the repression of AVC activity is specific for the ANF fragment.

Inactivation of a high affinity NKE in the ANF regulatory region removes repression in the atrioventricular canal

Nkx2.5 is important in the control of ANF expression (Lyons et al. 1995; Durocher et al. 1996; Tanaka et al. 1999), and interacts with multiple binding elements (NKEs) within the ANF regulatory fragment, including a high affinity NKE at position -250 bp (Durocher et al. 1997; Lee et al. 1998; Durocher and Nemer 1998; Shiojima et al. 1999; Hiroi et al. 2001). To analyse whether this NKE is involved in the repressive activity of the ANF fragment, we generated transgenic embryos with the ANF-cTnI construct in which the NKE is inactivated by mutation. All transgenic embryos with the NKE mutation (ANFmutNKE-cTnI) did show nlacZ expression in the AVC (Table 1 and Figure 2D). Additionally, they showed expression in the RA, LA, LV and RV similar to ANF (Figure 1G) and ANF-cTnI transgenes (Figure 2B). These results show that in vivo the NKE is not required for activation of expression in the chambers but for repression in the AVC. It is not likely that the specific repression in the AVC is solely explained by the function of Nkx2.5, because this transcription factor is expressed in the entire heart (Lints et al. 1993; Komuro and Izumo 1993; Kasahara et al. 1998).
FIGURE 2: Localization of transgene expression in E10.5 mouse hearts.

(A) The cTnl transgene is predominantly expressed in the primary myocardium of the AVC.

(B) The ANF-cTnl transgene is solely expressed in the chamber myocardium. No transgene expression is present in the AVC myocardium.

(C) MLC2V-cTnl transgenics show predominant expression in the primary myocardium of the AVC, similar to the pattern of the cTnl transgenes.

(D) Mutation of the NKE at position -250 bp in the ANF regulatory region removes the repression in the AVC.

(E) Mutation of the TBE at position -259 bp in the ANF regulatory region also removes the repression in the AVC.

(F) Mutation of both the TBE and NKE removes the repression in the AVC as well. ift: inflow tract, la: left atrium, ra: right atrium, avc: atrioventricular canal, lv: left ventricle, rv: right ventricle, oft: outflow tract.
Therefore, we assumed that the observed effect of the NKE mutation reflects an interaction between Nkx2.5 and other factors bound to neighbouring elements.

Inactivation of a T-box binding element adjacent to the NKE removes repression in the atrioventricular canal

The ANF regulatory region contains a T-box binding element (TBE) in close vicinity (position -259 bp) to the NKE. This TBE is conserved between species, homologous to a T-half site (Kispert et al. 1995) and required for the activation by Tbx5 and Nkx2.5 in transfection assays (Hiroi et al. 2001; Bruneau et al. 2001). We generated transgenic embryos that have an inactivating mutation (Sinha et al. 2000) in the TBE within the ANF-cTnI transgene construct (ANFmutTBE-cTnI). Nine transgenic embryos were analysed at E10.5 and revealed a similar transgene expression pattern in the heart (Table 1). Similar to the ANFmutNKE-cTnI transgenes, expression was present in the AVC as well as in the RA, LA, LV and RV (Figure 2E). These results demonstrate that the TBE is essential for the repression by ANF regulatory sequences in the embryonic myocardium of the AVC, but is not essential for activity in the chamber myocardium. Both TBE and NKE were essential for the synergistic activation of the ANF fragment by Tbx5 and Nkx2.5 in transfection assays. However, inactivation of neither element visibly affected chamber activity in vivo. To investigate whether in vivo the TBE and NKE are redundant for ANF activity, transgenic embryos were generated in which both elements were inactivated (ANFmutTBE/NKE-cTnI). Three transgenic embryos were analyzed at E10.5 and revealed a similar transgene expression pattern in the heart (Table 1). Similar to the ANFmutNKE-cTnI and ANFmutTBE-cTnI transgenes, expression was present in the AVC as well as in the RA, LA, LV and RV (Figure 2F). These results demonstrate that both elements are dispensable for chamber activity.
The transcription factor Tbx2 is expressed in the embryonic myocardium

Tbx2 is a T-box factor family member that acts as a transcriptional repressor (Carreira et al. 1998; Sinha et al. 2000; Jacobs et al. 2000), and is expressed in the AVC of the chicken and mouse heart (Gibson-Brown et al. 1998; Yamada et al. 2000). To explore its possible involvement in ANF gene regulation we analysed the pattern of Tbx2 mRNA in the developing mouse heart by non-radioactive in situ hybridisation on serial sections. At E8.75, the Tbx2 gene was present in the embryonic myocardium of the IFT and AVC (Figure 3D). At this stage, ANF is selectively expressed in the ventricular myocardium and absent from the IFT (Figure 3A).

FIGURE 3: Non-radioactive in situ hybridisation on serial sections shows complementary expression of endogenous ANF and Tbx2 and Tbx5 mRNA.

(A-C and G) ANF expression at E8.75 (A), E9.5 (B and C) and E11.5 (G).
(D-F and H) Tbx2 expression at E8.75 (D), E9.5 (E and F) and E11.5 (H).
At E9.5, *Tbx2* is expressed in the IFT, AVC, inner curvature and in the OFT (Figure 3E and 3F). No *Tbx2* expression could be observed in the atrial and ventricular chamber myocardium (Figure 3E and 3F). The pattern of *ANF* is strictly complementary to that of *Tbx2*, and is restricted to the chamber myocardium of both atria and ventricles and absent from the embryonic myocardium of the IFT, AVC, inner curvature and OFT (Figure 3B and 3C). At E11.5, *Tbx2* is expressed in the AVC and OFT (Figure 3H). The pattern is complementary to the pattern of *ANF* that is expressed in the atrial appendages and the LV (Figure 3G). At E11.5, the *Tbx5* gene, encoding a transcriptional activator involved in *ANF* gene regulation (Hiroi et al. 2001; Bruneau et al. 2001; Ghosh et al. 2001), showed expression in the myocardium of RA, LA, AVC and LV (Figure 3I). Expression of both *Tbx5* and *ANF* is virtually absent from the RV and OFT. The *Tbx5* gene expression pattern overlaps that of *ANF*, *Tbx5* being additionally expressed in the embryonic myocardium of the IFT, AVC, inner curvature and in the atrial septum (Figure 3G and 3I). *Cx40* is, like *ANF*, also under control of *Tbx5* (Bruneau et al. 2001). The expression of *Cx40* (Delorme et al. 1997) is similar to the *ANF* expression pattern, being also complementary to the pattern of *Tbx2* (data not shown).

**Tbx2 and Nkx2.5 form a ternary complex with the ANFTBE-NKE**

The requirement of the TBE and NKE for repression in the AVC and the complementarity in expression pattern between *Tbx2* and *ANF* prompted us to study the interaction of *Tbx2* and Nkx2.5 with the NKE-TBE site using electromobility shift assay (EMSA) experiments. Oligonucleotide probes were used that correspond to *ANF* promoter sequences -273 to -236 that harbour both the TBE and NKE (WT), an intact TBE and a mutated NKE (NKEmut), or a mutated TBE and an intact NKE (TBEmut). Nkx2.5 as well as Tbx2 bound to the WT probe and could be supershifted using specific antibodies (Figure 4A). Nkx2.5 binding was abolished by the NKE mutation (NKEmut probe) while Tbx2 binding was not affected (Figure 4A). Tbx2 binding was abolished by the mutation in TBE (TBEmut probe) (Figure 4A) while Nkx2.5
binding was not affected (not shown). Incubation of both Nkx2.5 and Tbx2 with the WT probe produced a larger ternary complex in addition to the Nkx2.5- and Tbx2-DNA complexes (Figure 4A).

**FIGURE 4:** Tbx2 and Nkx2.5 form a ternary complex with the ANFTBE-NKE.

(A) EMSAs were performed using nuclear extracts from HEK cells expressing Nkx2.5 or Tbx2. The WT probe contains the TBE-NKE, the NKEmut probe contains the TBE and the mutated NKE as used in the transgene construct. The TBEmut probe contains the NKE and the mutated TBE as used in the transgene construct. Both Nkx2.5 and Tbx2 bind to the WT probe. Nkx2.5 does not bind the NKEmut probe and Tbx2 does not bind the TBEmut probe. When mixing together Nkx2.5 and Tbx2 extracts, an additional ternary complex is formed on the WT and to a lesser extend on the NKEmut probe, while the complex is absent when using the TBEmut probe.

(B) Replacing Nkx2.5 for Nkx2.5-L176P (NKx2.5-LP) or Tbx2 for Tbx2-R122E/R123E (Tbx2-RE/RE) shows that on the WT probe the DNA binding ability of both Nkx2.5 and Tbx2 is necessary for complex formation. The carboxy terminal region of Tbx2 is not required for DNA binding (Tbx2-delRD). Tbx5 is also able to bind the WT probe.

(C) Once formed, the Nkx2.5/Tbx2 complex is stable. The WT probe was incubated with nuclear extracts and cold competitor oligonucleotides (100 and 1000 fold excess) as indicated at the top of the figure. Whereas a 100 fold excess of WT probe was sufficient to disrupt the complex, a 100 fold excess of NKEmut and a 1000 fold excess of TBEmut were not sufficient.

(D) Western-blots of nuclear extracts of HEK cells expressing FLAG-Nkx2.5, FLAG-Nkx2.5-LP, Tbx2, Tbx2-delRD and Tbx2-RE/RE.
Mutation of the TBE abolished ternary complex formation while this ternary complex was still weakly present when the NKE was mutated (Figure 4A). These results indicate that the TBE, and to a lesser extend the NKE are necessary for ternary complex formation. Nkx2.5-L176P, which contains a leucine to proline substitution within the Nkx2.5 DNA binding domain that inactivates its DNA binding ability (Grow and Krieg 1998), did not bind to the WT probe and did not form a ternary complex with Tbx2 (Figure 4B). Tbx2-R122E/R123E in which amino acids involved in DNA interaction were substituted, did not bind the WT probe (Figure 4B) and did not form a complex with Nkx2.5 (not shown). These results indicate that binding to the DNA of both Nkx2.5 and Tbx2 is necessary for ternary complex formation. Tbx2-delRD also shows binding to the TBE indicating that the portion C-terminal to the T-box that is involved in repression, is not required for DNA binding (Figure 4B). Compared to the full length protein, binding of the truncated protein to the TBE is more efficient. A similar observation was made for C-terminal truncated versions of the Tbx5 protein, which were found to increase the affinity for the DNA (Ghosh et al. 2001). To test whether the ternary complex, once formed, was stable, competition assays were performed. A 100-fold excess of unlabelled WT probe successfully competed the ternary complex (Figure 4C). In contrast, even a 1000-fold excess of NKEmut probe produced weak competition and no competition was observed using the TBEmut probe as competitor (Figure 4C). These results indicate that the ternary complex, once formed, is stable and is not disrupted by competition for binding with one of the two factors. Western-blot analysis demonstrated that nuclear extracts contained TBX2, TBX2-delRD, TBX2-R122E/R123E, FLAG-tagged Nkx2.5 and Nkx2.5-L176P protein (Figure 4D).

The ANF promoter is a functional target of Tbx2
To study whether the ANF regulatory region is a functional target of Tbx2, co-transections were performed with the 0.7 kb ANF promoter reporter construct. In atrial cultures, co-transfection of full length Tbx2 resulted in a two fold decrease of ANF promoter activity,
while co-transfection of Tbx2 without its repressor domain (RD) and fused to the transactivation domain of VP16 (VP16-Tbx2-delRD) gave a two fold increase in ANF promoter activity. Although the effect of co-transfecting these factors is similar in atrial and ventricular cardiomyocyte cultures, the differences are more pronounced in the ventricular cultures (Figure 5A). In Cos-7 cells, ANF promoter activity decreased three fold upon co-transfection of Tbx2 (Figure 5B). Tbx2-delRD was unable to repress the ANF promoter indicating that the repressor domain is essential for the observed repression (Figure 5B). Co-transfection of VP16-Tbx2-delRD resulted in a drastic increase in activity, which became even more pronounced when VP16-Tbx2 was used (Figure 5C) which contains the full length Tbx2 cDNA coupled to VP16. VP16-Tbx2-R122E/R123E was not able to activate the ANF promoter demonstrating that DNA binding of Tbx2 is essential for regulation of the ANF promoter (Figure 5C). Together, these data demonstrate that the 0.7 kb ANF regulatory region is a target for Tbx2-mediated repression. To address whether Tbx2 and Nkx2.5 mediate ANF gene regulation via the TBE and NKE, respectively, an ANF reporter construct containing point mutations in the TBE at -259 bp was transfected in Cos-7 cells (Figure 5D). Loss of the TBE site diminished the VP16-Tbx2 induced ANF promoter activity. Additional mutation of the TBE located at -485 bp did not further decrease promoter activity. The residual activation of the mutated ANF promoters possibly results from VP16-Tbx2 activation via a potential T-half site located at -90 bp (Bruneau et al. 2001). This site is however not present in the ANF-cTnI transgene constructs. Co-transfection of Nkx2.5 resulted in a three fold activation of the 0.7 kb ANF promoter (Figure 5E). Mutation of the NKE located at -250 did not influence the inducibility of the ANF promoter by Nkx2.5. Possibly, this response is mediated by additional low affinity NKEs located at -242 bp and -80 bp in the ANF promoter (Lee et al. 1998, Shiojima et al. 1999). Previous studies have demonstrated that the TBE is a functional binding site for the transcriptional activator Tbx5 and that the TBE-NKE is involved in synergistic activation of the ANF promoter by Tbx5 and Nkx2.5 (Hiroi et al. 2001; Bruneau et al. 2001).
FIGURE 5: The 0.7 kb ANF promoter is a functional target for Tbx2.

(A) Transient transfections were carried out with the 0.7 kb ANF promoter in primary atrial and ventricular cardiomyocytes. Co-transfections show that Tbx2 repressed ANF promoter activity whereas VP16-Tbx2-delRD activated the ANF promoter. The results are from one representative experiment (out of 3) done in duplicate. Error bars represent the difference between the duplicates.

(B) Tbx2 mediated repression requires the Tbx2 repressor domain and interaction with the DNA. Co-transfection experiments were carried out with the 0.7 kb ANF promoter in Cos-7 cells.

(C) Fusion of the VP16 transactivation domain to either the full length Tbx2 (VP16-Tbx2) or to Tbx2, from which the carboxy terminal end that includes the repression domain, was removed (VP16-Tbx2-delRD) resulted in strong activation of the ANF promoter region. VP16-Tbx2-R122E/R123E did not activate the ANF promoter region.

(D) Co-transfection using point mutations of the 0.7 kb ANF promoter in Cos-7 cells show that VP16-Tbx2 activates the ANF regulatory region via the TBE. The basal values of the mutated constructs were comparable to the control construct.

(E) Nkx2.5 activation of the ANF promoter does not require the NKE. Synergistic activation of the ANF promoter by Tbx5 and Nkx2.5 requires an intact TBE and NKE. The basal values of the mutated constructs were comparable to the control construct.

(F) Synergistic activity of Nkx2.5 and Tbx5 is reduced by Tbx2 in a dose dependent manner, indicating that Tbx2 can efficiently compete with Tbx5 in the regulation of the ANF promoter. All results are from one representative experiment (out of 3) done in duplicate. Error bars represent the difference between the duplicates. N: NKE located at position -250 bp, T2: TBE located at position -259 bp, T3: TBE located at position -485 bp.
Because both $Tbx5$ and $Tbx2$ are expressed in the AVC we tested whether Tbx2 can compete with Tbx5. The ANF promoter was transfected in Cos-7 cells and co-transfected with Tbx5, Nkx2.5 and increasing amounts of Tbx2 (Figure 5F). As expected, Tbx5 and Nkx2.5 synergistically activated the ANF promoter (Figure 5F). The activation depended on both the TBE and NKE (Figure 5E). Adding as little as 10 to 100 ng of Tbx2 compared to 400 ng of Tbx5 and Nkx2.5 resulted in a loss of induction, indicating that Tbx2 can efficiently compete with Tbx5 in the regulation of ANF promoter activity (Figure 5F). Adding larger amounts of Tbx2 resulted in an even higher reduction (Figure 5F). The competition of Tbx2 is specific as both the Tbx2-R122E/R123E and the unrelated factor Irx5 were unable to interfere (Figure 5F).

**DISCUSSION**

Chimeric regulatory regions reveal active repression in the atrioventricular canal

In the developing heart ANF displays a chamber-restricted pattern of expression that is recapitulated by the proximal 0.7 kb ANF regulatory region. Since the AVC activity of the $cTnI$ promoter was extinguished in the ANF-cTnI transgenics, we conclude that the ANF regulatory region actively imposes repression on the $cTnI$ promoter. By studying the 0.7 kb ANF regulatory region itself, and not in the context of chimeric constructs, this property would not have been revealed, and would not have prompted us to search for the repressor function within this region. Dysfunction of the $cTnI$ promoter due to the composition of the chimeric construct is unlikely for a number of reasons. The combined promoter is active in the chambers, similar to the ANF promoter, showing that the construct is transcriptionally competent. When $MLC2V$ sequences were placed upstream of the $cTnI$ promoter, no interference with $cTnI$ promoter activity was detected. When single site mutations were made in the ANF-cTnI construct,
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The activity in the AVC was restored. Therefore, the AVC-specific extinction of transcription by ANF sequences can be attributed to an intrinsic repressor function.

The NKE and TBE are essential for repression in the atrioventricular canal

The removal of repression in the AVC by inactivation of the NKE or TBE site revealed that an NK2 factor, probably Nkx2.5, and a T-box factor are components of an inhibitory pathway. The pattern of Tbx2 gene expression, and the ability of Tbx2 to repress the ANF promoter and to bind to the ANF TBE-NKE site together with Nkx2.5 indicate that this TBE is a target for Tbx2. Based on these findings we propose that Nkx2.5 and Tbx2 cooperatively repress the ANF promoter in the AVC. Preliminary data showed that the MLC2v promoter, active in the OFT and RV, is extinguished in the OFT by the 500 bp ANF regulatory region, suggesting that this pathway is also active in the OFT (our unpublished observations). In this repression mechanism, Nkx2.5 functions as a cardiac accessory factor for Tbx2, which in turn represses the ANF promoter in the AVC. The accessory function of Nkx2.5 is in line with the ability of this factor to cooperate with members of several classes of factors, including GATA factors, SRF and Tbx5 in the regulation of cardiac genes (Grepin et al. 1994; Durocher et al. 1996; Durocher et al. 1997; Morin et al. 2000; Morin et al. 2001; Bruneau et al. 2001; Hiroi et al. 2001). The hypothesis that cardiac compartment specific gene expression/repression results from cooperativity between cardiac factors and compartment-restricted factors is strongly supported by our in vivo observations. Nkx2.5 and Tbx5 were shown to be essential components of the activation pathway of the ANF gene in vivo (Lyons et al. 1995; Tanaka et al. 1999; Bruneau et al. 2001). Both factors activate transcription through multiple binding sites present within the ANF promoter (Lee et al. 1998; Shiojima et al. 1999; Hiroi et al. 2001; Bruneau et al. 2001). Furthermore, Nkx2.5 and Tbx5 were shown to activate the ANF promoter in synergy in transfection assays (Hiroi et al. 2001; Bruneau et al. 2001).
Inactivation of the -259 bp TBE or -250 bp NKE, required for this synergy in transfections, did not visibly affect chamber activity, suggesting that neither site is essential for ANF promoter activity in vivo. Therefore, Nkx2.5 and Tbx5 achieve activation of the ANF promoter through the remaining elements, or through an indirect activation pathway. Our transient transfection results support a role for the remaining elements in activation because the constructs in which either the NKE, TBE or both were mutated could still be partially stimulated by VP16-Tbx2 or by Nkx2.5 and Tbx5 (Figure 5D and 5E). The repressive activity of Tbx2 on cardiac gene expression in the AVC might be relevant for the mechanism underlying the pathogenesis in Holt-Oram patients, Tbx5 mutant mice and humans with a mutation in the NKX2.5 gene, which all have conduction disease including AV block (Basson et al. 1997, Li et al. 1997, Schott et al. 1998; Bruneau et al. 2001). The AV node and AV junctional myocardium are derived from the AVC (Moorman et al. 1998; Davis et al. 2001) that express Nkx2.5, Tbx5 and Tbx2. Besides affecting directly downstream gene expression in the AVC, reduction of Tbx5 or Nkx2.5 levels might cause an imbalance in the interaction with Tbx2 to regulate downstream genes. This in turn could affect the formation of the AV conduction system. The role of Tbx2 in formation of the conduction system merits further investigation. Furthermore, the wide variation in phenotype within Holt-Oram patients and patients with an NKX2.5 mutation suggests that polymorphic variations in the Tbx2 gene may contribute to this variable phenotype.

**A potential mechanism for site-specific chamber formation: local repression of differentiation**

To understand what role the inhibitory Tbx2/Nkx2.5 pathway might have in the formation of the four-chambered heart it is important to appreciate that regional differences in differentiation within the tubular heart exist. The linear heart tube is patterned along A-P, D-V and L-R axis and has a 'nodal' phenotype (high automaticity, slow contraction, slow conduction). Atrial and ventricular chamber myocardium forms at specific sites within the tubular heart during and after looping (de Jong et al. 1992; Christoffels et al. 2000).
FIGURE 6: A potential mechanism for site-specific chamber formation by local repression of differentiation.

Schematic representation of the transcriptional mechanisms involved in chamber formation. As part of an ongoing chamber formation program, Tbx5 and Nkx2.5 stimulate cardiac genes. Specific regions in the linear heart tube remain embryonic and do not develop into chamber myocardium due to the presence of Tbx2 in those regions. Nkx2.5 and Tbx2 form a repressor complex that suppresses genes that are part of the chamber differentiation program. The Tbx5 triangle and Nkx2.5 rectangle indicate Tbx5 and Nkx2.5 expression in the linear heart tube, respectively. Tbx2 is expressed in the primary myocardium of the inflow tract, atrioventricular canal and outflow tract (light gray), while ANF is expressed in the chamber myocardium (dark gray). ift: inflow tract, la: left atrium, avc: atrioventricular canal, lv: left ventricle, oft: outflow tract.

This chamber myocardium obtains a more mature phenotype (low automaticity, fast contraction, well-coupled cells and a well-developed sarcoplasmic reticulum). The myocardium of the IFT, AVC, inner curvature and OFT retains the nodal phenotype of the myocardium of the embryonic heart tube. These observations indicate that a transcriptional program responsible for differentiation is activated at specific sites in the tubular heart to form chamber myocardium. The IFT, AVC, inner curvature and OFT escape the differentiation program until later in development and play an
important role in the alignment of the chambers, in septation and in the formation of the conduction system. Genes for ANF, Chisel and gap-junction proteins Cx40 and Cx43 are part of this differentiation program because they are specifically expressed in the forming chamber myocardium (Delorme et al. 1995; van Kempen et al. 1996; Delorme et al. 1997; Christoffels et al. 2000; Palmer et al. 2001). ANF and Cx40 were shown to be targets of Tbx5 (Bruneau et al. 2001; Hiroi et al. 2001) and also Cx43 was shown to be a target for Tbx factors (Chen et al. 2001). Indeed, in regions where Tbx5 is (almost) absent, that is the OFT and, later in development, the RV, none of the downstream genes are expressed. The regions of the looped heart that express Tbx2, which functions as a repressor of transcription (Carreira et al. 1998; Sinha et al. 2000; Jacobs et al. 2000), remain embryonic irrespective of whether they express Tbx5. It is therefore tempting to speculate that Tbx2 expression in the IFT, AVC, inner curvature and OFT is needed to escape the differentiation program (Figure 6). The fact that Tbx2 and Tbx5 are co-expressed in the IFT, AVC and inner curvature indicates that Tbx2 successfully competes with Tbx5 in the regulation of downstream genes. This implication is strengthened by our observation that Tbx2 forms a ternary complex with Nkx2.5 and the TBE-NKE site (Figure 4A) and efficiently counteracts the synergistic activation by Tbx5 and Nkx2.5 (Figure 5F). We propose that Tbx5 is involved in enforcing the chamber specific transcription program, whereas Tbx2 counteracts the positive regulatory function of Tbx5 in specific regions of the heart. Both Tbx5 and Tbx2 cooperate with Nkx2.5, which functions as an accessory factor to restrict the T-box factor activities to cardiac genes.

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