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

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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*. 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.

[*Key Words*: Heart development; chamber formation; transgenic mice; ANF; Tbx2; Nkx2.5]

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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). The chamber myocardium specifically initiates the expression of gap-junction genes *connexin* (*Cx*) *40* and *Cx43* required for intercellular 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), and 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 junc-

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tion 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 analyze 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-kb 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 in vivo (Lyons et al. 1995; Tanaka et al. 1999; Bruneau et al. 2001). Inactivation of either factor in *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). In vitro studies showed that Tbx5 and Nkx2.5 associate and synergistically activate the *ANF* regulatory fragment (Bruneau et al. 2001; Hiroi 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 show 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 *cardiac troponin I* (*cTnI*) promoter fragment specifically in the AVC. 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, whereas 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*. 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.

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 harboring this *ANF* regulatory region (–638/+70) coupled to the *nlacZ* reporter gene. Heart-specific reporter gene expression was analyzed by whole-mount X-gal staining of mouse (E9.5 and E11.5) embryos (Fig. 1). At E9.5, expression of the reporter gene was observed in the atrial and ventricular chamber myocardium, whereas expression was absent from the embryonic myocardium of AVC, inner curvature, and OFT (Table 1; Fig. 1B,C). At stage E11.5, *nlacZ* expression is still present in both atria and both ventricles, whereas the expression is higher in the LV as compared with the RV (Fig. 1E,G). At both stages, the transgene expression pattern is comparable with that of the endogenous *ANF* gene (Fig. 1A,D,F). The only exceptions were the right and left superior caval veins that express the transgene, but not the endogenous gene (Fig. 1F,G). Therefore, the 0.7-kb *ANF* regulatory

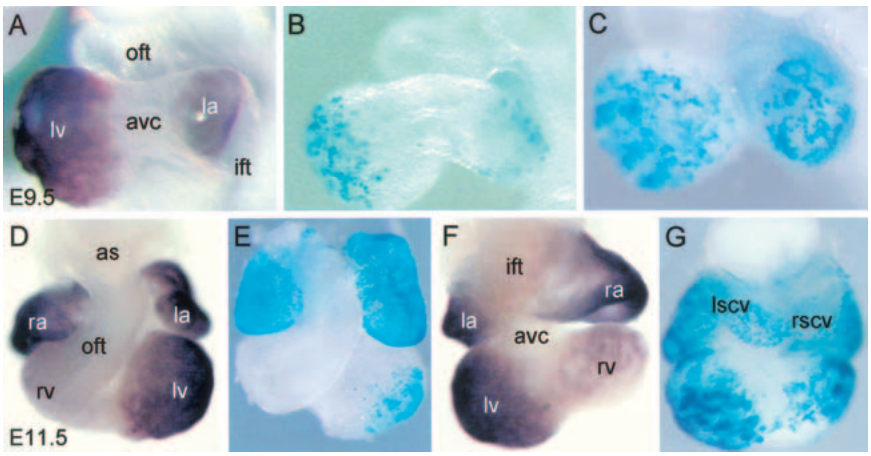

Figure 1. The 0.7-kb *ANF* regulatory region is responsible for the developmental pattern of the endogenous *ANF* gene. (*A*) A lateral view of the endogenous *ANF* gene expression in the heart of E9.5 mouse embryo. (*B*,*C*) A lateral view of the *nlacZ* reporter gene expression in the heart of E9.5 transgenic embryos. (*D*,*F*) A ventral (*D*) and dorsal (*F*) view 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.

Table 1. *Reporter gene expression data of ANFcTnI, ANF-cTnI, MLC2V-cTnI, ANFmutNKE-cTnI, ANFmutTBE-cTnI, and ANFmutTBE/NKE transgenic mice at E10.5*

Construct	IFT	LA	RA	AVC	LV	RV	OFT	Ectopic expression
ANF 1[a]	++	+++	+++	–	++	–	–	Yes
ANF 2[a]	+	+++	+++	–	+++	++	–	Yes
ANF 3[a]	–	–	–	–	–	–	–	Yes
cTnI[a]	–	–	+/–	+	–	–	–	No
cTnI[a]	–	–	+/–	++	–	–	–	No
cTnI[a]	–	+	++	++	++	+	–	Yes
cTnI	–	–	–	+	–	–	–	Yes
cTnI	–	–	+	+	+/–	–	–	No
cTnI	–	–	+	++	–	–	–	Yes
cTnI	–	+/–	++	++	+	–	–	Yes
cTnI	–	–	+	++	+	–	–	No
cTnI	–	–	++	+++	++	–	–	No
cTnI	–	–	++	+++	++	–	–	No
ANF-cTnI[a]	–	+	+	–	+	–	–	No
ANF-cTnI[a]	–	++	++	–	+	–	–	No
ANF-cTnI[a]	–	++	++	–	++	+/–	–	No
ANF-cTnI[a]	–	+++	+++	–	+++	+/–	–	No
ANF-cTnI	–	++	++	–	++	–	–	Yes
ANF-cTnI	–	+++	+++	–	+++	++	–	No
MLC2V-cTnI[a]	–	–	++	+++	++	–	–	No
MLC2V-cTnI[a]	–	–	++	++	+	–	–	Yes
MLC2V-cTnI	–	+/–	+	+	+	–	–	Yes
MLC2V-cTnI	–	++	+++	+++	+++	–	–	Yes
ANFmutNKE-cTnI	–	+	+	+	+	–	–	No
ANFmutNKE-cTnI	+	++	++	++	++	–	–	No
ANFmutNKE-cTnI	+/–	+++	+++	+++	+++	–	–	No
ANFmutNKE-cTnI	+	++++	++++	++++	++++	+	+	Yes
ANFmutNKE-cTnI	–	–	–	–	–	–	–	Yes
ANFmutTBE-cTnI	–	+/–	+/–	+	+/–	–	–	No
ANFmutTBE-cTnI	–	+	+	++	+	–	–	No
ANFmutTBE-cTnI	–	++	++	++	++	+	–	No
ANFmutTBE-cTnI	–	++	++	++	++	+	–	No
ANFmutTBE-cTnI	–	++	++	++	++	+	–	No
ANFmutTBE-cTnI	–	++	++	++	++	+	–	No
ANFmutTBE-cTnI	–	+++	+++	+++	+++	+++	–	No
ANFmutTBE-cTnI	–	+++	+++	+++	+++	++	+/–	No
ANFmutTBE-cTnI	–	+++	+++	+++	+++	+++	+/–	No
ANFmutTBE/NKE-cTnI	–	+++	+++	+++	+++	+++	–	No
ANFmutTBE/NKE-cTnI	–	++	++	+++	++	++	–	Yes
ANFmutTBE/NKE-cTnI	–	+	+	++	+	–	–	Yes

An arbitrary scale of intensity was assigned. (++++) Very strong expression; (+/–) very weak expression; (–) no detectable staining.
[a]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 F_0 screens. With the 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.

region mimics the endogenous developmental expression pattern in the mouse heart and selectively demarcates the atrial and ventricular chamber myocardium.

The cTnI *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 *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 *cTnI* gene is expressed in the entire myocardium (Vallins et al.

1990; Ausoni et al. 1991). The 356-bp promoter region (–230/+126), analyzed in transgenic mice, however, showed a variable pattern of expression, which always included the myocardium of the AVC (Di Lisi et al. 1998, 2000). Furthermore, only 6 of 16 transgenic mice showed expression (R. Di Lisi and S. Schiaffino, pers. comm.). To protect the small *cTnI* promoter region from position effects, it was flanked by insulator sequences from the chicken β-*globin* locus (Chung et al. 1993, 1997; Bell et al. 1999), which did not affect the activition of the *cTnI* 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 (Fig. 2A). In addition, in 9 of 10 transgenic embryos, expression was extended to the RA and LV (Table 1; Fig. 2A). None of the transgenic embryos showed expression in the myocardium of IFT and OFT. Application of insulator sequences appeared to stabilize the transgene expression pattern and strongly increased the proportion of expressing transgenic mice (Z-test; $P = 0.013$, insulated vs. noninsulated). Therefore, insulators flanked all further constructs used in this study.

The 0.5-kb ANF *regulatory region extinguishes* cTnI *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 *cTnI* construct and transgenic embryos were generated. All *ANF–cTnI* 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 with the LV. No transgene expression was observed in the myocardium of the IFT, AVC, inner curvature, and OFT (Fig. 2B). The expression pattern of these *ANF–cTnI* transgenes is very similar to the expression pattern of the transgenics that harbor the full 0.7-kb *ANF* regulatory region (cf. Figs. 1G and 2B). These observations suggest that the characteristic *cTnI* promoter activity in the AVC (Fig. 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 (Fig. 2, cf.

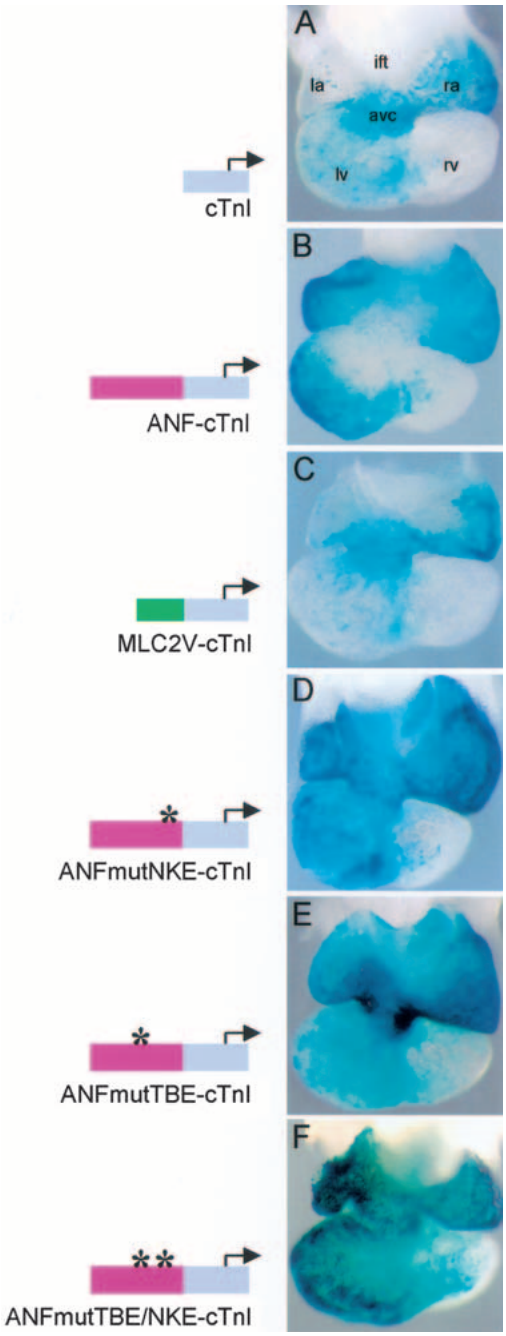

Figure 2. Localization of transgene expression in E10.5 mouse hearts. (*A*) The *cTnI* transgene is predominantly expressed in the primary myocardium of the AVC. (*B*) The *ANF–cTnI* transgene is solely expressed in the chamber myocardium. No transgene expression is present in the AVC myocardium. (*C*) *MLC2V–cTnI* transgenics show predominant expression in the primary myocardium of the AVC, similar to the pattern of the *cTnI* 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.

A and C). 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; Durocher and Nemer 1998; Lee et al. 1998; Shiojima et al. 1999; Hiroi et al. 2001). To analyze 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; Fig. 2D). Additionally, they showed expression in the RA, LA, LV, and RV similar to *ANF* (Fig. 1G) and *ANF–cTnI* transgenes (Fig. 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 (Komuro and Izumo 1993; Lints et al. 1993; Kasahara et al. 1998). Therefore, we assumed that the observed effect of the NKE mutation reflects an interaction between Nkx2.5 and other factors bound to neighboring 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 (Bruneau et al. 2001; Hiroi 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 analyzed 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 (Fig. 2E). These results show 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 (Fig. 2F). These results show 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; Jacobs et al. 2000; Sinha 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 analyzed the pattern of *Tbx2* mRNA in the developing mouse heart by nonradioactive in situ hybridization on serial sections. At E8.75, the *Tbx2* gene was present in the embryonic myocardium of the IFT and AVC (Fig. 3D). At this stage, *ANF* is selectively expressed in the ventricular myocardium and absent from the IFT (Fig. 3A). At E9.5, *Tbx2* is expressed in the IFT, AVC, inner curvature, and in the OFT (Fig. 3E,F). No *Tbx2* expression could be observed in the atrial and ventricular chamber myocardium (Fig. 3E,F). 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 (Fig. 3B,C). At E11.5, *Tbx2* is expressed in the AVC and OFT (Fig. 3H). The pattern is complementary to the pattern of *ANF* that is expressed in the atrial appendages and the LV (Fig. 3G).

At E11.5, the *Tbx5* gene, encoding a transcriptional activator involved in *ANF* gene regulation (Bruneau et al. 2001; Ghosh et al. 2001; Hiroi et al. 2001), showed expression in the myocardium of RA, LA, AVC, and LV (Fig. 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 (Fig. 3G,I). *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 ANF *TBE–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 inter-

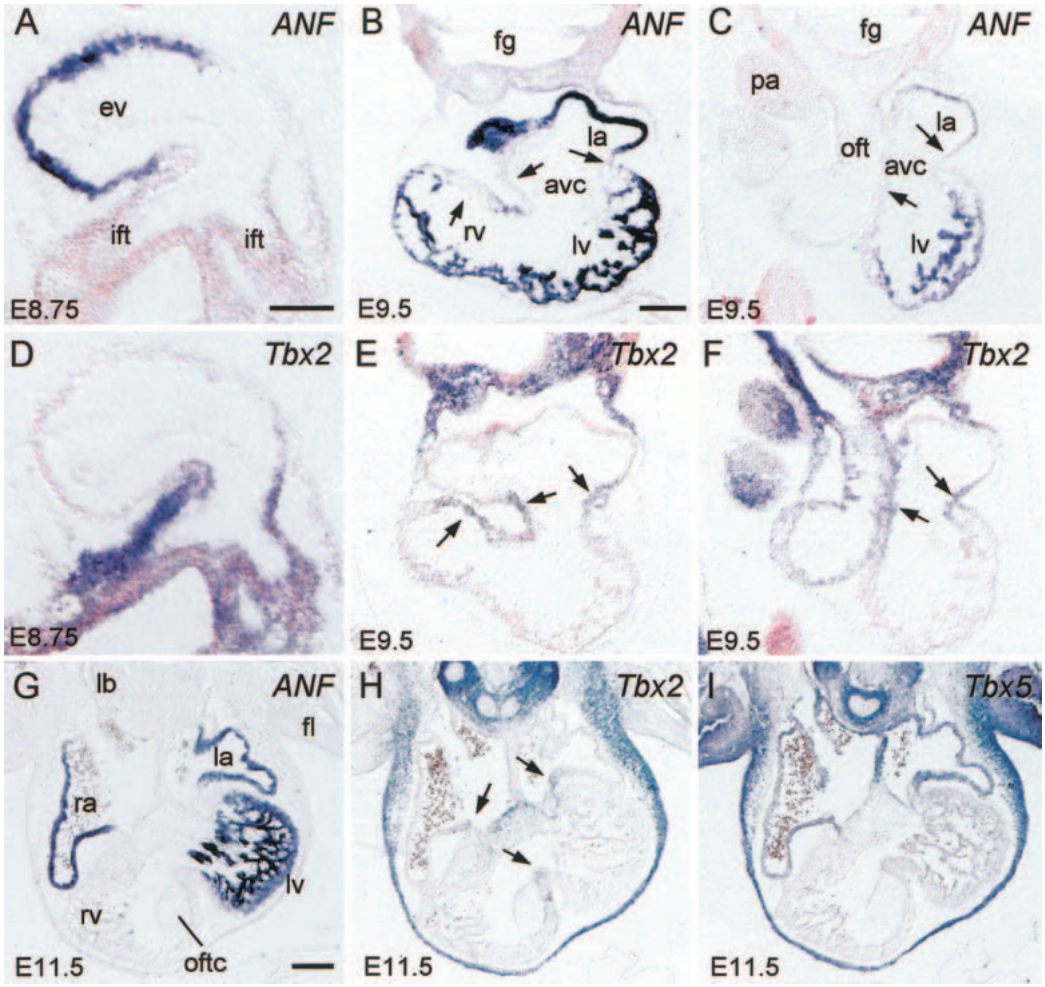

Figure 3. Nonradioactive in situ hybridization on serial sections shows complementary expression of endogenous *ANF*, *Tbx2*, and *Tbx5* mRNA. (*A–C,G*) *ANF* expression at E8.75 (*A*), E9.5 (*B,C*), and E11.5 (*G*). (*D–F,H*) *Tbx2* expression at E8.75 (*D*), E9.5 (*E,F*), and E11.5 (*H*). (*I*) *Tbx5* expression at E11.5. Note the mutually exclusive pattern of expression of *ANF* and *Tbx2*. Arrows in *B* and *E* indicate the AVC and OFT region that is continuous at the inner curvature. Arrows in *C*, *F*, and *H* indicate the AVC region. (ift) Inflow tract; (la) left atrium; (ra) right atrium; (avc) atrioventricular canal; (lv) left ventricle; (rv) right ventricle; (oft) outflow tract; (ev) embryonic ventricle; (fg) foregut; (pa) pharyngeal arch; (oftc) outflow tract cushion; (lb) lung bud; (fl) forelimb. Bar, 100 μm.

action 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 harbor both the TBE and NKE (wild type), 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 wild-type probe and could be supershifted using specific antibodies (Fig. 4A). Nkx2.5 binding was abolished by the NKE mutation (NKEmut probe), whereas Tbx2 binding was not affected (Fig. 4A). Tbx2 binding was abolished by the mutation in TBE (TBEmut probe; Fig. 4A), whereas Nkx2.5 binding was not affected (data not shown). Incubation of both Nkx2.5 and Tbx2 with the wild-type probe produced a larger ternary complex in addition to the Nkx2.5 and Tbx2–DNA complexes (Fig. 4A). Mutation of the TBE abolished ternary complex formation, whereas this ternary complex was still weakly present when the NKE was mutated (Fig. 4A). These results indicate that the TBE, and to a lesser extent, 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 wild-type probe and did not form a ternary complex with Tbx2 (Fig. 4B). Tbx2–R122E/R123E, in which amino acids involved in DNA interaction were substituted, did not bind the wild-type probe (Fig. 4B), and did not form a complex with Nkx2.5 (data 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 carboxy-terminal to the T-box that is involved in repression is not required for DNA binding (Fig. 4B). Compared with the full-length protein, binding of the truncated protein to the TBE is more efficient. A similar

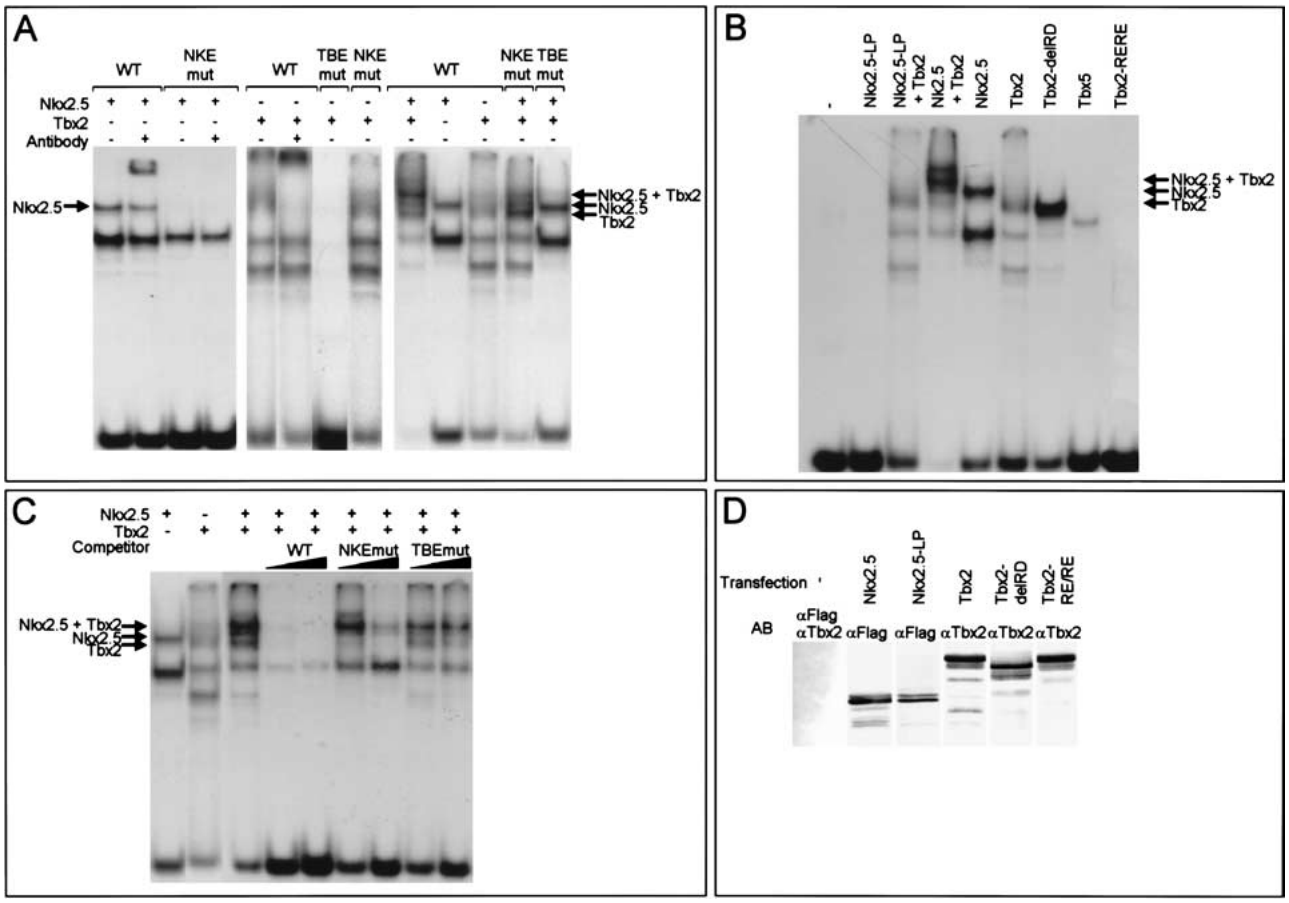

Figure 4. Tbx2 and Nkx2.5 form a ternary complex with the *ANF* TBE–NKE. (*A*) EMSAs were performed using nuclear extracts from HEK cells expressing Nkx2.5 or Tbx2. The wild-type probe contains the TBE–NKE, the NKEmut probe contains the TBE, and the mutated NKE as used in the transgene construct. The TBEmut probe contains the NKE and the mutated TBE as used in the transgene construct. Both Nkx2.5 and Tbx2 bind to the wild-type 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 wild type, and to a lesser extent, on the NKEmut probe, whereas 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 wild-type 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 wild-type probe. (*C*) Once formed, the Nkx2.5/Tbx2 complex is stable. The wild-type probe was incubated with nuclear extracts and cold competitor oligonucleotides (100- and 1000-fold excess) as indicated at *top*. Whereas a 100-fold excess of wild-type 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.

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 unlabeled wild-type probe successfully competed the ternary complex (Fig. 4C). In contrast, even a 1000-fold excess of NKEmut probe produced weak competion, and no competition was observed using the TBEmut probe as competitor (Fig. 4C). These results indicate that the ternary complex, once formed, is stable and is not disrupted by competion for binding with one of the two factors. Western blot analysis showed that nuclear extracts contained TBX2, TBX2-delRD, TBX2-R122E/R123E, FLAG-tagged Nkx2.5, and Nkx2.5-L176P protein (Fig. 4D).

The ANF *promoter is a functional target of Tbx2*

To study whether the *ANF* regulatory region is a functional target of Tbx2, cotransfections were performed with the 0.7-kb *ANF* promoter reporter construct. In atrial cultures, cotransfection of full-length Tbx2 resulted in a twofold decrease of *ANF* promoter activity, whereas cotransfection of Tbx2 without its repressor domain (RD) and fused to the transactivation domain of VP16 (VP16-Tbx2-delRD) gave a twofold increase in *ANF* promoter activity. Although the effect of cotransfecting these factors is similar in atrial and ventricular cardiomyocyte cultures, the differences are more pronounced in the ventricular cultures (Fig. 5A). In Cos-7 cells, *ANF* promoter activity decreased threefold upon cotransfec-

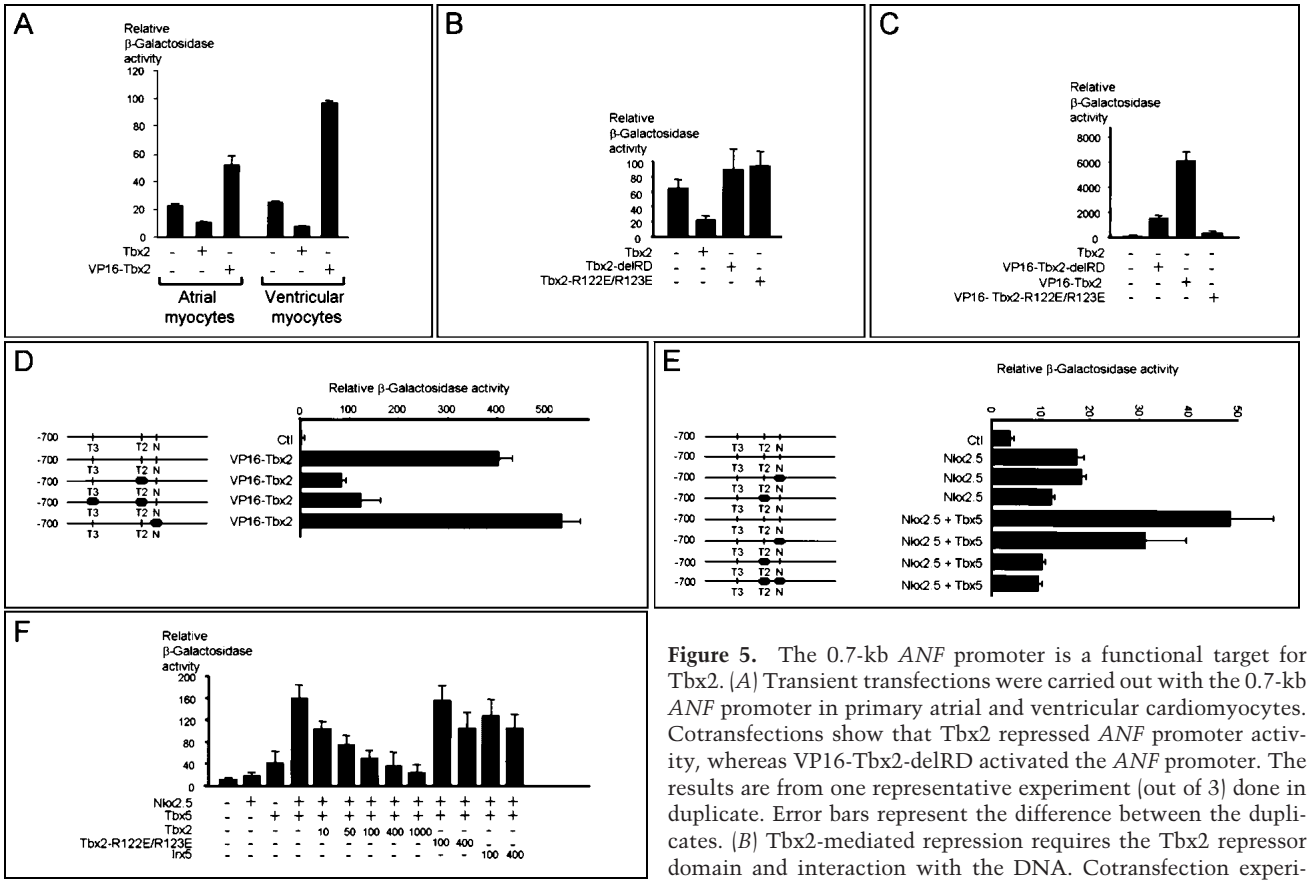

Figure 5. The 0.7-kb *ANF* promoter is a functional target for Tbx2. (*A*) Transient transfections were carried out with the 0.7-kb *ANF* promoter in primary atrial and ventricular cardiomyocytes. Cotransfections show that Tbx2 repressed *ANF* promoter activity, whereas VP16-Tbx2-delRD activated the *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. Cotransfection 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*) Cotransfection, using point mutations of the 0.7-kb *ANF* promoter in Cos-7 cells, shows that VP16-Tbx2 activates the *ANF* regulatory region via the TBE. The basal values of the mutated constructs were comparable with 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 with 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.

tion of Tbx2 (Fig. 5B). Tbx2-delRD was unable to repress the *ANF* promoter, indicating that the repressor domain is essential for the observed repression (Fig. 5B). Cotransfection of VP16-Tbx2-delRD resulted in a drastic increase in activity, which became even more pronouced when VP16-Tbx2 was used (Fig. 5C) that contains the full-length Tbx2 cDNA coupled to VP16. VP16-Tbx2-R122E/R123E was not able to activate the *ANF* promoter, showing that DNA binding of Tbx2 is essential for regulation of the *ANF* promoter (Fig. 5C). Together, these data show 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 (Fig. 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). However, this site is not present in the ANF–cTnI transgene constructs.

Cotransfection of Nkx2.5 resulted in a threefold activation of the 0.7-kb *ANF* promoter (Fig. 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 and –80 bp in the *ANF* promoter (Lee et al. 1998; Shiojima et al. 1999).

Previous studies have shown 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 (Bruneau et al. 2001; Hiroi et al. 2001). 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 cotransfected with Tbx5, Nkx2.5, and increasing amounts of Tbx2 (Fig. 5F). As expected, Tbx5 and Nkx2.5 synergistically activated the *ANF* promoter (Fig. 5F). The activation depended on both the TBE and NKE (Fig. 5E). Adding as little as 10 to 100 ng of Tbx2 compared with 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 (Fig. 5F). Adding larger amounts of Tbx2 resulted in an even higher reduction (Fig. 5F). The competition of Tbx2 is specific as both the Tbx2-R122E/R123E and the unrelated factor Irx5 were unable to interfere (Fig. 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. Because 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, 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. On the basis of 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 (P.E.M.H. Habets, A.F.M. Moorman, and V.M. Christoffels, unpubl.). 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, 1997; Morin et al. 2000, 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; Bruneau et al. 2001; Hiroi et al. 2001). Furthermore, Nkx2.5 and Tbx5 were shown to activate the *ANF* promoter in synergy in transfection assays (Bruneau et al. 2001; Hiroi 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 (Fig. 5D,E).

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*. Beside 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-cham-

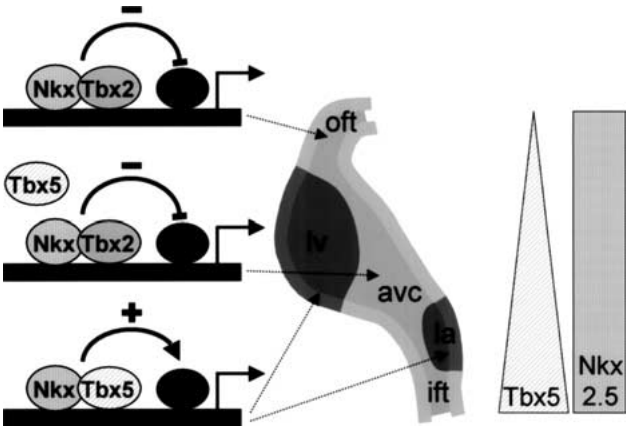

Figure 6. A potential mechanism for site-specific chamber formation by local repression of differentiation. Schematic representation of the transcriptional mechanisms involved in chamber formation. As part of an ongoing chamber formation program, Tbx5 and Nkx2.5 stimulate cardiac genes. Specific regions 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), whereas *ANF* is expressed in the chamber myocardium (dark gray). (ift) Inflow tract; (la) left atrium; (avc) atrioventricular canal; (lv) left ventricle; (oft) outflow tract.

bered heart, it is important to appreciate that regional differences in differentiation within the tubular heart exist. The linear heart tube is patterned along the 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). 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, 1997; van Kempen et al. 1996; 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). In regions in which *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; Jacobs et al. 2000; Sinha 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 (Fig. 6). The fact that Tbx2 and Tbx5 are coexpressed 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 (Fig. 4A) and efficiently counteracts the synergistic activation by Tbx5 and Nkx2.5 (Fig. 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.

Materials and 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 localization 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 to 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 4-bp 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 4-bp substitutional mutation of the TBE located at position –259 of the *ANF* promoter region (TBE, TCTCA CACCTT; TBEmut, TCTCTTTGCTT) (Sinha et al. 2000). The mutations were generated using the QuickChangeTM Site-Directed Mutagenesis kit (Stratagene). With the exception of the ANF–nlacZ construct, all constructs were flanked by a 1.2-kb *Sal*I–*Bam*HI 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 by use of standard techniques (Hogan et al. 1994). Animal care was according to guidelines as described (Christoffels et al. 1995). Constructs were analyzed in lines (ANF), F_0 em-

bryos (ANFmutNKE–cTnI and ANFmutTBE–cTnI), or both F_0 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 (2-kb *Nco*I/*Sac*I fragment) as a probe (Sambrook et al. 1989). For PCR analysis, primers specific to the *nlacZ* sequences were used (*lacZ*+, GCATCGAGCTGGGTAATAAGC GTTGGCAAT and *lacZ*–, ACTGCAACAACGCTGCTTCG GCCTGGTAAT) 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) 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 3-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) according to the manufacturer's 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 (The Victor Chang Cardiac Research Institute, Darlinghurst, Australia), 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).

Nonradioactive in situ hybridization

Whole-mount in situ hybridization and nonradioactive in situ hybridization 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 synthesized and labeled with [α-^{32}P]dATP using Klenow polymerase. Labeled probes were incubated and used in binding reactions as described and resolved on a 6% polyacrylamide gel (Espinas et al. 1994). Oligonucleotides used (complementary strand not shown, mutations underlined): Wild-type, TCTGCTCTTCTCACACCTTT GAAGTGGGGGCCTCTTG, TBE mutated (TBEmut), TCT GCTCTTCTC<u>TTTG</u>CTTTGAAGTGGGGGCCTCTTG, and NKE mutated (NKEmut) TCTGCTCTTCTCACACCTTT G<u>CCTC</u>GGGGGCCTCTTG.

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.

Acknowledgments

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