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Control of early cardiac-specific transcription of *Nkx2-5* by a GATA-dependent enhancer

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SUMMARY

The homeobox gene *Nkx2-5* is the earliest known marker of the cardiac lineage in vertebrate embryos. *Nkx2-5* expression is first detected in mesodermal cells specified to form heart at embryonic day 7.5 in the mouse and expression is maintained throughout the developing and adult heart. In addition to the heart, *Nkx2-5* is transiently expressed in the developing pharynx, thyroid and stomach. To investigate the mechanisms that initiate cardiac transcription during embryogenesis, we analyzed the *Nkx2-5* upstream region for regulatory elements sufficient to direct expression of a *lacZ* transgene in the developing heart of transgenic mice. We describe a cardiac enhancer, located about 9 kilobases upstream of the *Nkx2-5* gene, that fully recapitulates the expression pattern of the endogenous gene in cardiogenic precursor cells from the onset of cardiac lineage specification and throughout the linear and looping heart tube. Thereafter, as the atrial and ventricular chambers become demarcated, enhancer activity becomes restricted to the developing right ventricle. Transcription of *Nkx2-5* in pharynx, thyroid and stomach is controlled by regulatory elements separable from the cardiac enhancer. This distal cardiac enhancer contains a high-affinity binding site for the cardiac-restricted zinc finger transcription factor GATA4 that is essential for transcriptional activity. These results reveal a novel GATA-dependent mechanism for activation of *Nkx2-5* transcription in the developing heart and indicate that regulation of *Nkx2-5* is controlled in a modular manner, with multiple regulatory regions responding to distinct transcriptional networks in different compartments of the developing heart.

Key words: *Nkx2-5*, GATA, Cardiac lineage, Transcription, Mesoderm

INTRODUCTION

The heart is the first organ to form during mammalian embryogenesis (reviewed in Olson and Srivastava, 1996; Fishman and Chien, 1997). Heart formation begins at about embryonic day (E) 7.5 in the mouse, when cells within bilaterally symmetric regions of the anterior lateral plate mesoderm become committed to a cardiogenic fate in response to inductive signals from adjacent endoderm (reviewed in Nascone and Mercola, 1996). Cardiogenic precursor cells from this region, known as the cardiac crescent, converge along the ventral midline of the embryo to form the linear heart tube at about E8.0. Soon thereafter, the heart tube initiates rhythmic contractions and undergoes rightward looping, followed by chamber specification, septation and valvulogenesis.

The embryologic events involved in heart formation have been carefully documented in a variety of organisms but, until recently, little has been known of the transcriptional networks that control cardiac myogenesis or morphogenesis. However, with the realization that several key cardiac transcription factors are structurally and functionally conserved in vertebrates and invertebrates, it has become possible to think about heart development in the context of an evolutionarily conserved transcriptional pathway and to gain insights into the complex events of cardiogenesis in vertebrates from studies of simpler, genetically more tractable organisms, such as *Drosophila* (reviewed in Bodmer, 1995; Fishman and Olson, 1997). Of particular interest in this regard is the *Drosophila* NK-type homeobox gene *tinman*, which is expressed specifically in the cardiac lineage (Bodmer et al., 1990; Bodmer, 1993; Azpiazu and Frasch, 1993), and several related genes from vertebrates, which also show cardiac-restricted expression (reviewed in Harvey, 1996).

The heart-like organ in *Drosophila*, the dorsal vessel, is derived from a subset of dorsal mesodermal cells that become committed to a cardiac fate in response to the TGF-β-like growth factor, Decapentaplegic (Dpp), secreted by ectodermal cells adjacent to the cardiogenic mesoderm (Staehling-Hampton et al., 1994; Frasch, 1995; Yin and Frasch, 1998). *Tinman* is the earliest marker for dorsal mesodermal cells fated to form the dorsal vessel and in *tinman* mutant embryos, the cardiac lineage is not specified, indicating that *tinman* is required at an early step in the cardiogenic pathway to establish cardiac cell fate (Bodmer et al., 1990; Azpiazu and Frasch,

1993; Bodmer, 1993). Later, Tinman also controls cardiac cell differentiation by directly activating transcription of the myogenic regulatory gene *D-mef2*, encoding a MADS-box transcription factor that switches on downstream muscle structural genes (Gajewsky et al., 1997).

Five *tinman*-related NK-type homeobox genes, *Nkx2-3* (Evans et al., 1995; Buchberger et al., 1996), *Nkx2-5* (Komuro and Izumo, 1993; Lints et al., 1993), *Nkx2-6* (Biben et al., 1998), *Nkx2-7* (Lee et al., 1996) and *Nkx2-8* (Reecy et al., 1997; Brand et al., 1997), are expressed in cardiac lineages in vertebrates (reviewed in Harvey, 1996). Among these, *Nkx2-5* (also called *csx*) is expressed earliest and its expression is maintained in the heart throughout prenatal and postnatal life. In addition to being expressed in the developing heart, *Nkx2-5* and other cardiac NK-type homeobox genes show overlapping expression patterns in pharyngeal precursors and their derivatives, as well as in thyroid and stomach. The overlapping expression patterns of these genes have led to the concept of an 'Nkx code', in which cell fates are specified by unique combinations of these NK-homeodomain proteins (Reecy et al., 1997).

Nkx2-5 is the only cardiac NK-homeobox gene to be knocked out thus far in mice. In mice homozygous for an *Nkx2-5* null allele, cardiomyocytes differentiate and the linear heart tube forms normally, but looping morphogenesis is disrupted (Lyons et al., 1995). This relatively late function for *Nkx2-5* in cardiac morphogenesis contrasts with the early function of *tinman* in specification of cardiac cell fate and suggests either that *Nkx2-5* and *tinman* are not functionally conserved or, more likely, that other cardiac-expressed NK homeobox genes substitute for the absence of *Nkx2-5*. In this regard, Nkx2-5 and Tinman can bind the same target genes in *Drosophila*, but Nkx2-5 cannot substitute for Tinman because it lacks an essential cardiogenic regulatory domain found at the N terminus of Tinman that appears to mediate interaction with specific accessory factors (Ranganayakulu et al., 1998). Forced expression of *Nkx2-5* in zebrafish or frog embryos expands the heart field and accelerates cardiac gene expression, consistent with an early role for *Nkx2-5* in cardiomyocyte specification or differentiation (Fu and Izumo, 1995; Chen and Fishman, 1996; Cleaver et al., 1996).

The regulatory mechanisms that activate *Nkx2-5* transcription in cardiac mesoderm are of particular interest because they represent the initial step in cardiogenesis. Thus, understanding how *Nkx2-5* expression is controlled specifically in cells destined to form heart should yield insight into how the cardiac lineage is specified. To begin to address this issue, we analyzed the 5′-flanking region of the mouse *Nkx2-5* gene for early cardiac regulatory elements. Here we describe a powerful cardiac-specific enhancer upstream of *Nkx2-5* that is activated concomitant with the endogenous gene in the cardiac crescent and continues to be active throughout the linear and looping heart tube. As chamber formation occurs, activity of this enhancer becomes restricted to the developing right ventricle. This enhancer contains an essential high-affinity binding site for the cardiac-restricted zinc finger transcription factor GATA4, which is coexpressed with *Nkx2-5* early in the cardiac lineage. These results reveal interdependent roles for GATA4 and Nkx2-5 in early heart formation and demonstrate that *Nkx2-5* transcription is controlled in a modular way, with distinct transcriptional codes acting through separate enhancers in different compartments of the heart.

MATERIALS AND METHODS

Cloning, mapping and sequencing

Three independent genomic clones of *Nkx2-5* were isolated by screening a SV129 mouse genomic library (Stratagene) using a 1.2 kb mouse Nkx2-5 cDNA, kindly provided by Robert Schwartz (Baylor College of Medicine). Positive clones were purified through secondary and tertiary screening and phage DNA was isolated, digested with *Not*I and subcloned into pBluescript for sequencing. The longest genomic *Nkx2-5* clone was about 18 kb in length and the other two were about 11 kb. All three clones contained an 8 kb *Eco*RI fragment containing the two coding exons.

Generation of transgenic mice

Different fragments of the *Nkx2-5* 5′ flanking region were cloned into the hsp68lacZ reporter gene (Kothary et al., 1989). DNA for pronuclear injection was gel-purified and eluted using a QIAEX II kit (QIAGEN). Fertilized eggs from B6C3F1 female mice were collected for pronuclear injection. Injected eggs were implanted into ICR female mice and foster mothers were killed to collect F_0 embryos. The yolk sacs of embryos were kept for genotyping. For E9.5 and later stage embryos, genotyping was done by Southern blot using a *lacZ* probe obtained by PCR from the *lacZ* gene. Primer sequences are available on request. For genotyping of stable transgenic lines, tails of 2-week-old pups were cut for DNA preparation and transgenes were detected by Southern blot.

β-Galactosidase staining and histology

Methods for analysis of transgenic mice were described previously (Cheng et al., 1993). Briefly, embryos were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS on ice for 10 to 90 minutes depending on the size of the embryos, washed twice with PBS and stained in staining solution (5 mM ferrocyanide, 5 mM ferricyanide, 2 mM $MgCl_2$, 1 mg/ml X-Gal in dimethylformamide) for 3 hours to overnight. For sections, the embryos were postfixed overnight in 4% formaldehyde after washing off X-Gal. Embryos were dehydrated with 2, 2-dimethoxypropane (DMP), cleared in light mineral oil, embedded in paraffin, sectioned at 5 μm, rehydrated and stained with Nuclear Fast Red (Moller and Moller, 1994).

Gel mobility shift assays

The consensus GATA site within fragment –9435/–9277 was tested for its ability to bind GATA4. GATA4 protein was translated in vitro with a TNT T7-coupled reticulocyte lysate system (Promega), using pcDNAI GATA4 as DNA template. Two complementary oligonucleotides containing the consensus GATA site (5′-GGCGGGGAAGGGAGATAAGATGACATAC and 5′-GGTATGTC-ATCTTATCTCCCTTCCCCG) were annealed and labeled using a random priming kit (Gibco). Gel shift assays were performed as described previously (Brennan and Olson, 1990). Briefly, (0.5×10^5 cts/minute) of labeled probe was incubated with in vitro translated GATA4 protein in buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 1 mM EDTA, 5% (v/v) glycerol, 2 mM DTT), and 1 μg poly(dI-dC).poly(dI-dC) for 20 minutes at room temperature. Unlabeled competitor and mutant competitor (5′-GGCGGG-GAAGGGACCCGGGATGACATAC) in 10-, 30- and 100-fold excess were added to the mixture to test for specificity of DNA binding. The DNA-protein complex was separated by electrophoresis on a 5% nondenaturing polyacrylamide gel in 0.5× TBE.

Site-directed mutagenesis

The consensus GATA-binding site at –9362 was mutated to a *Sma*I site in the context of fragment –9700/–7353. PCR-based mutagenesis was performed using two primer pairs (AccUP: 5′-CACTATAGGGCGTCGACTCGATCC, Gm10: 5′-TGGTATGTC-ATCCCGGGTCCCTTCCCCGCTG and Gm9: 5′GGGGAAG-GGACCCGGGATGACATACCAGAGC, H3P: 5′-TCACACGC-

CAAGCTTTCAAGGTGC). PCR was performed for 10 cycles of 94°C, 30 seconds; 50°C, 30 seconds; 72°C, 90 seconds and 20 cycles of 94°C, 30 seconds; 55°C, 30 seconds; 72°C, 90 seconds using 20 ng fragment –9700/–8922 as template. The two PCR products were gel-purified and denatured at 72°C for 15 minutes and annealed to be the template for the second-round PCR using AccUP and H3P as primers. The final PCR product was digested with *Sma*I and sequenced to confirm the presence of the mutation and no other mutations introduced by PCR. The PCR fragment was subcloned back to the hsp68-lacZ containing the fragment –9700/–7353 to replace the original fragment.

RESULTS

Analysis of the *Nkx2-5* upstream region for transcriptional regulatory regions

As a first step toward defining the initial steps in cardiogenic specification, we isolated and characterized three mouse *Nkx2-5* genomic clones. Sequencing and restriction mapping of the clones revealed the two coding exons and the 275 nucleotide 5′ untranslated region from exon 1, as reported previously (Lints et al., 1993). A consensus TATA box was also located in the vicinity of the major transcription start site.

In an effort to identify *cis*-acting sequences that control *Nkx2-5* transcription in the developing heart, we created a series of *lacZ* reporter genes that contained the hsp68 basal promoter linked to DNA sequences upstream of the mouse *Nkx2-5* gene. The first construct that we created contained a DNA fragment extending from 9700 bp upstream of the reported transcription start site (Lints et al., 1993) to nucleotide +369 in the first exon (fragment 1, Fig. 1). In five of five F0 transgenic mouse embryos harboring this transgene, we observed *lacZ* expression in the heart, pharynx, thyroid primordium and stomach at E11.5 (Fig. 2A). Thus, this upstream region appeared to contain regulatory elements sufficient to direct expression in the same sites as the endogenous *Nkx2-5* gene at this stage of development (Lints et al., 1993).

Sections of E11.5 embryos bearing the fragment 1-lacZ transgene are shown in Fig. 2. β-gal staining was observed within cells in the floor of the pharynx (Fig. 2B). The endogenous *Nkx2-5* gene is also expressed in these cells (Lints et al., 1993). Subsequently, expression in the pharynx became localized to the midline region and ultimately to the thyroid primordium, which forms by invagination of the pharyngeal floor between the first and second branchial pouches. *lacZ* expression was also seen in the third branchial pouch (data not shown). The endogenous *Nkx2-5* gene shows an identical expression pattern to the transgene in this region at these stages (Lints et al., 1993).

In contrast to the endogenous *Nkx2-5* gene, which is expressed throughout the heart at E11.5, the transgene was expressed in the outflow tract and right ventricle of the heart at this stage (Fig. 2C-E). *lacZ* expression in the right ventricle

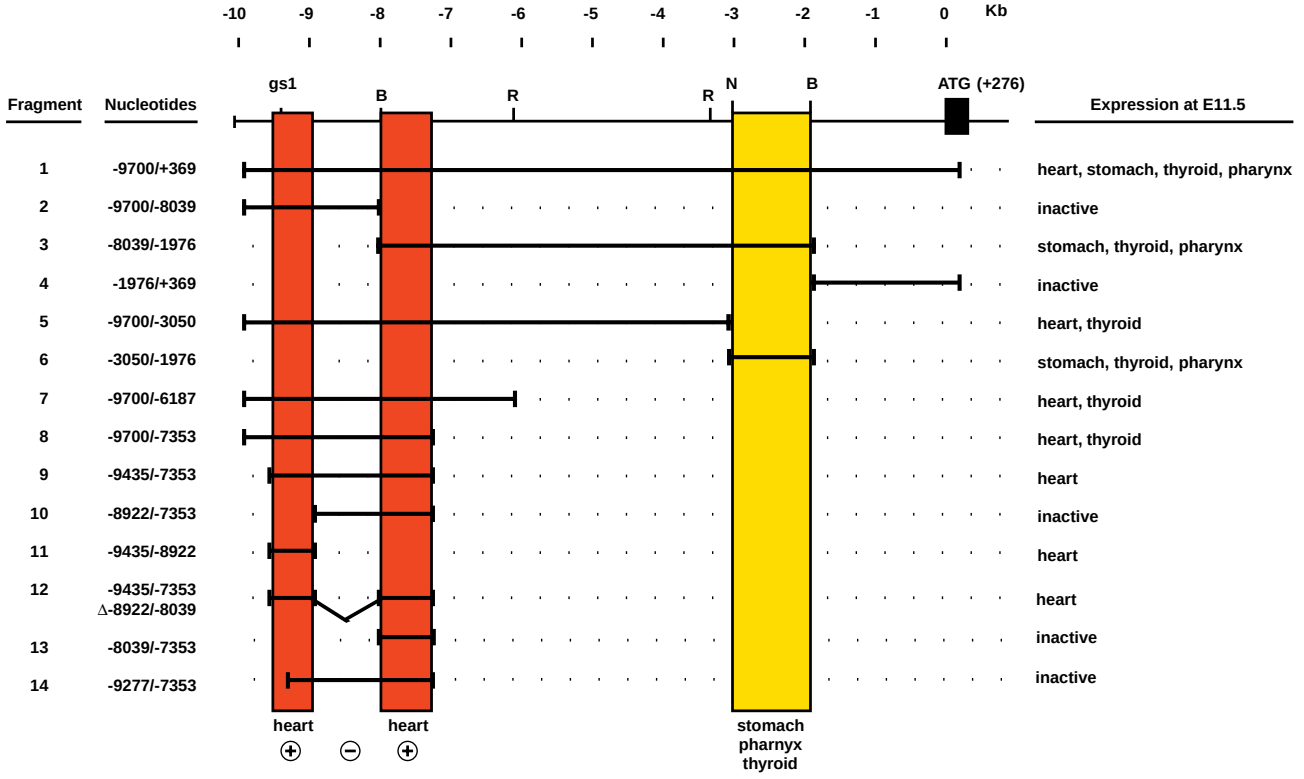

Fig. 1. Transgenes used to identify the *Nkx2-5* cardiac enhancer. The structure of the 5′ region of the mouse *Nkx2-5* gene is shown at the top, with exon 1, as reported previously (Lints et al., 1993), shown in black. Restriction sites are: B, *Bam*HI; N, *Not*I; R, *Eco*RI. Regions of *Nkx2-5* 5′ flanking DNA used in *lacZ* transgenes are shown and sites of expression are indicated. Regions that contribute to cardiac expression are indicated in red and the region that directs expression in stomach, pharynx and thyroid in yellow. The location of the essential high-affinity GATA-binding site (gs1) at –9362 is indicated at the top. Positive and negative cardiac regulatory regions are indicated as + and –, respectively, at the bottom.

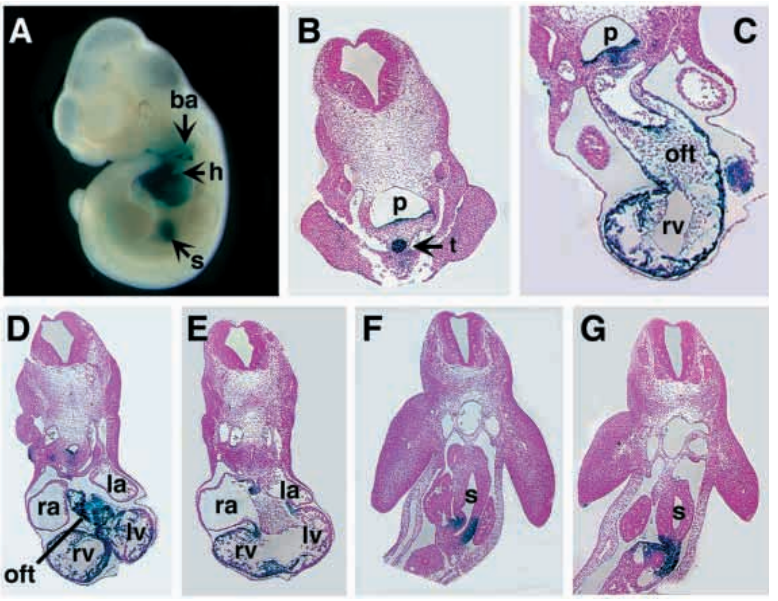

Fig. 2. LacZ expression at E11.5 from the *Nkx2-5* upstream region. Founder transgenic mice bearing a transgene containing fragment 1 (–9700/+369) linked to *lacZ* were analyzed at E11.5. (A) Whole-mount staining. *lacZ* expression is apparent in the heart (h), stomach (s) and branchial arch (ba) region. (B-G) Transverse sections of the embyro in A stained with Nuclear Fast Red. (B) Section through the head reveals β-gal staining in the floor of the pharynx (p) and the thyroid (t). (C) Section through the anterior region of the heart reveals β-gal staining in the floor of the pharynx, the outflow tract (oft) and right ventricle (rv). (D,E) Sections through the four chambers of the heart reveal β-gal staining in the primitive atrial septum, ventricular chambers and outflow tract. (F,G) Sections through the abdominal region reveal β-gal staining in the gastroduodenal junction and pancreatic primordium.

was detected in both compact myocardial and trabecular layers, but not in the endocardium. Occassionally, a few *lacZ*-positive cells were observed in the left ventricle, but no expression was seen in the atria. Like the endogenous *Nkx2-5* gene, the fragment 1-lacZ transgene was also expressed in a localized region at the distal end of the developing stomach and duodenum, as well as in the pancreatic primordium in the adjacent mesentery (Fig. 2F,G).

Localization of stomach and thyroid enhancers

To begin to localize the upstream DNA sequences that directed cardiac expression of *Nkx2-5*, we constructed a series of deletions of fragment 1 and analyzed their abilities to direct *lacZ* expression from the hsp68 basal promoter in F_0 transgenic mice at E11.5. The presence of all transgenes was tested by Southern analysis of yolk sac DNA.

Separation of the 9700 bp upstream region into three fragments, –9700/–8039 (fragment 2), –8039/–1976 (fragment 3), and –1976/+369 (fragment 4), revealed that fragment 3 contained elements for gastric/duodenal, thyroid and pharyngeal expression, whereas none of the three fragments were able to direct cardiac expression (Fig. 3A). The loss of cardiac expression when the 9700 bp upstream region was subdivided suggested either that the cardiac element spanned the junction of two of the subfragments or that it was influenced by complex interactions between multiple regions.

In an effort to further localize the cardiac regulatory region, we tested the region from –9700 to –3050 (fragment 5), which spanned the junction of fragments 2 and 3, for enhancer activity. This upstream region possessed cardiac, as well as thyroid, regulatory elements, but it lacked stomach and pharyngeal elements (Fig. 5F,K). These results suggested that expression of *Nkx2-5* in stomach and pharynx required the 3′-most region of fragment 3, which was missing from fragment 5 (nucleotides –3050 to –1976). Consistent with this conclusion, fragment *6*, (–3050/–1976) was able to direct transgene expression in stomach and pharynx, as well as thyroid (Fig. 3B-E). Since our primary interest was to map the regulatory elements responsible for early cardiac transcription of *Nkx2-5*, we did not attempt to further delimit the boundaries of the stomach, pharyngeal or thyroid enhancer(s).

Mapping the cardiac enhancer

To further delimit the boundaries of the cardiac enhancer, we created a series of 5′ and 3′ deletions of the –9700/–3050 region (fragment 5) and tested them for cardiac activity in F_0 transgenic mice at E11.5. Deletion from the 3′ end to –6187

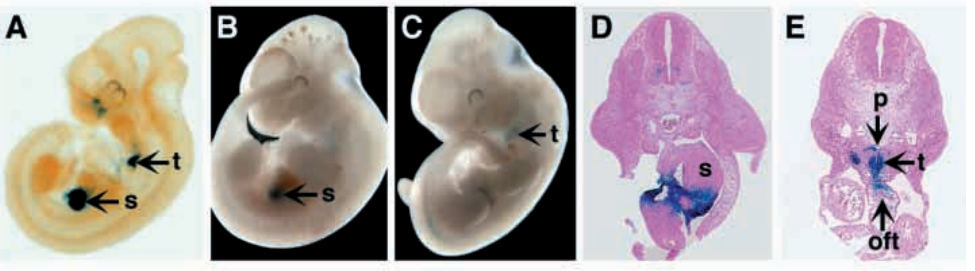

Fig. 3. Localization of the *Nkx2-5* stomach and thyroid enhancers. Founder transgenic mice bearing a transgene containing fragment 3 (–8039/–1976) (A) or fragment 6 (–3050/–1976) (B-E) linked to *lacZ* were analyzed at E11.5. The whole-mount embryo in A was cleared to show more clearly the internal sites of expression. β-gal staining can be seen in the stomach (s) and thyroid (t). Staining in the head is ectopic. (B,C) Whole-mount embryos in different planes of focus, revealing expression in stomach (B) and thyroid (C). (D,E) Sections of the embryo in B stained with Nuclear Fast Red. (D) Staining can be seen in the distal region of the stomach (s) and associated mesentery. A few *lacZ*-positive cells, representing ectopic expression, can also be seen in the ventral neural tube. (E) Staining can be seen in the pharynx (p), thyroid primordium, and third branchial pouch.

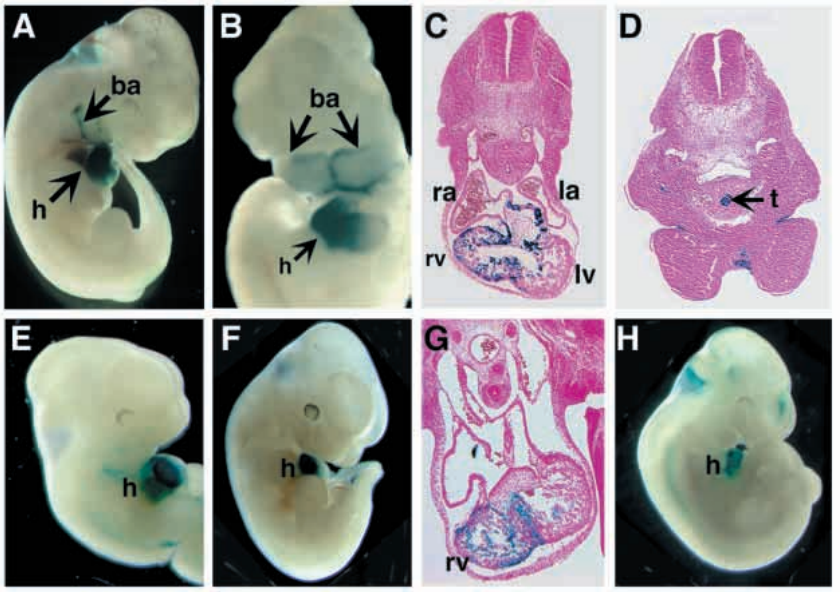

Fig. 4. Localization of the *Nkx2-5* cardiac enhancer. Founder transgenic mice bearing a transgene containing fragment 7 (–9700/–6187) (A-D), fragment 8 (–9700/–7353) (E), fragment 9 (–9435/–7353) (F,G) or fragment 11 (–9435/–8922) (H) linked to *lacZ* were analyzed at E11.5. (A,B) Whole-mount staining, with expression in the heart (h) and epithelium of branchial arches (ba). (C,D) Transverse sections of the embyro in A and B stained with nuclear fast red. (C) Section through the heart reveals β-gal staining in right ventricle (rv), but not in the left ventricle (lv) or atria (ra, la). (D) Section through the pharyngeal region reveals β-gal staining in the thyroid (t) and regions of the branchial pouches. (E,F,H) Whole-mount staining in the heart. Expression with fragments 8 and 9 (E,F, respectively) was reproducibly stronger than with fragment 11 (H). (G) Section through the heart reveals β-gal staining in the right ventricle, but not in thyroid. Staining in the forebrain and hindbrain in H is ectopic.

and –7353 (fragments 7 and 8; Fig. 1) did not eliminate cardiac expression (Fig. 4A-E). Since fragment 2, encompassing nucleotides –9700 to –8039 was inactive, these results indicated that the region from –8039 to –7353 was essential for cardiac activity (see Fig. 1).

A 5′ deletion fragment extending from –9435 to –7353 (fragment 9) also retained cardiac activity (Fig. 4F,G), whereas further deletion from the 5′ side to –8922 (fragment 10) eliminated cardiac expression (not shown). The loss of activity of fragment 10 suggested that cardiac enhancer activity required the region between –9435 and –8922. We therefore tested this region alone (fragment 11) and found it to exhibit cardiac activity; however, the level of expression from this region was lower than fragments 8 and 9 (Fig. 4H). Comparison of fragments 2, 8 and 11 suggested to us that another positive-acting cardiac element lay between –8039 and –7353, and a negative element between –8922 and –8039. To test this hypothesis, we created an internal deletion mutant

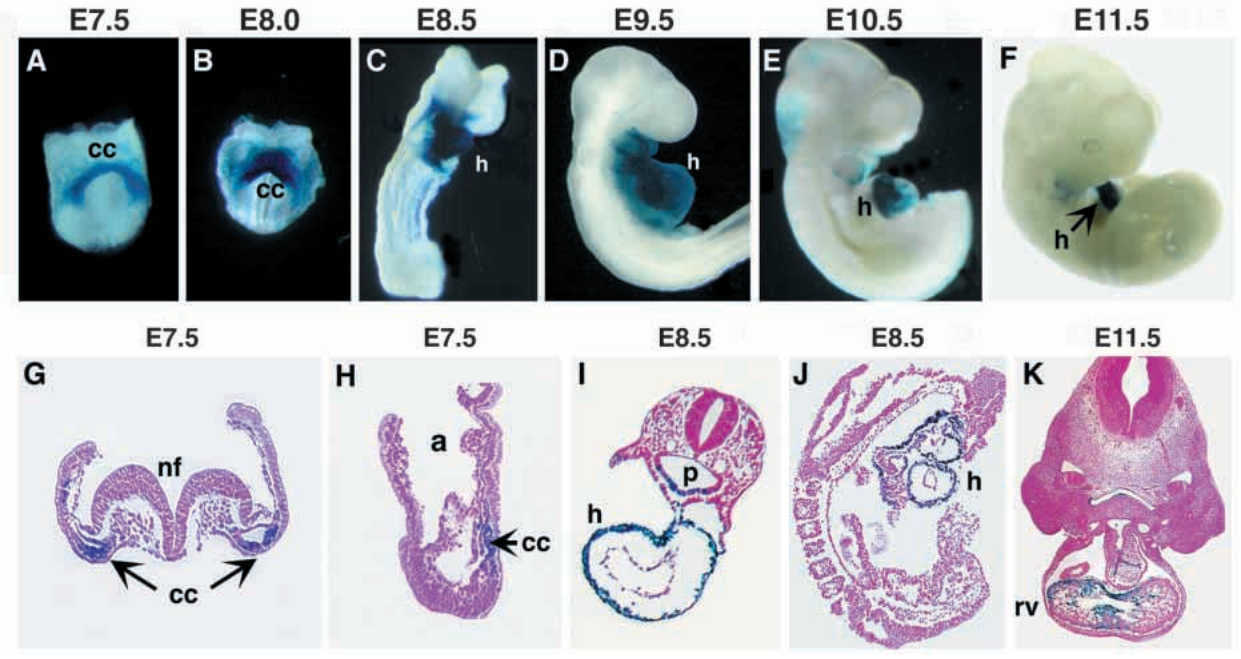

Fig. 5. Expression of the *Nkx2-5* cardiac enhancer throughout mouse development. A transgenic mouse line bearing fragment 5 (–9700/–3050) linked to *lacZ* was analyzed for *lacZ* expression at the indicated days of embryogenesis. (A-F) Whole-mount embryos and (G-K) sections stained with Nuclear Fast Red. β-gal staining is evident in the cardiac crescent (cc) at E7.5 (A,G,H) and E8.0 (B) and in the linear and looping heart tube between E8.5 and 10.5 (C-E, I,J). By E10.5 (E,F,K), *lacZ* expression becomes restricted to the right ventricle. Sections in G,I and K are transverse and sections in H and J are sagittal. a, amnion; h, heart; nf, neural folds; p, pharynx; rv, right ventricle.

(fragment 12), in which the –8922/–8039 region was deleted from within fragment 9 (Fig. 1). This fragment showed strong cardiac expression comparable to that of fragment 9 (data not shown). Together, the above results suggested that cardiac activity was influenced by two separable positive regions; one between –9435 and –8922, the other between –8039 and –7353 (Fig. 1).

To determine whether the region between –8039 and –7353 could act alone to confer cardiac expression, we tested this region for activity (fragment 13). It showed no activity at E11.5. Thus, while it enhances the activity of the more 5′ cardiac enhancer in fragment 11, it does not exhibit autonomous activity.

We also observed thyroid expression with constructs 5, 7 and 8 (Fig. 4D). Since constructs 7 and 8 did not overlap with construct 6, which also showed thyroid expression, this indicated the existence of at least two separate enhancers active in thyroid (see Fig. 1). Deletion of the 5′ end of fragment 8 to yield fragment 9 resulted in a loss of thyroid expression without affecting cardiac expression (Figs 1, 4F,G). This result suggests that the more 5′ thyroid enhancer requires the region between –9700 and –9435 and is therefore separable from the adjacent cardiac regulatory region.

Temporospatial expression pattern of the early cardiac-specific enhancer

The complete temporospatial expression pattern of the cardiac enhancer was determined using a stable transgenic mouse line bearing fragment 5 (–9700/–3050). The cardiac enhancer directed *lacZ* expression in the cardiogenic mesoderm beginning at E7.5 (Fig. 5A). Transverse and sagittal sections through β-gal-stained embryos showed intense staining within the bilaterally symmetric cardiogenic precursors at this stage and in no other regions of the embryo (Fig. 5G,H). By E8.0, the cardiac enhancer directed high levels of *lacZ* expression throughout the linear heart tube (Fig. 5C) and expression remained homogeneous throughout all cardiac cells until E8.5-9.5, during looping morphogenesis (Fig. 5C,D,I,J). The upstream enhancer fully recapitulated the expression of the endogenous *Nkx2-5* gene through E9.5. However, by E10.5, transgene expression became restricted to the developing right ventricular region (Fig. 5E). We also examined *lacZ* expression in stable transgenic lines bearing the fragment 8-lacZ transgene and observed the same expression pattern as with fragment 5 (data not shown).

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-9432   TCTGGGTCCTAATGCGGGTGGCGTCTCTCTTGACAGGCAGCGTTTGGGGA

-9382   CAACAGCGGGGAAGGGAGATAAGATGACATACCAGAGCAGATTTGGTGTG
                            gs1

-9332   CGCGCTGATACTCCTGGCCCGACAGGAAACTCGGAGCTATTTAAAAAGGC

-9282   CCTATCGATTACTTTATCTTCCCCGGAGGAAATCTTGCCGAGAGACAAAA
                      gs2

-9232   GATGTCCCCCTACCTAAAGATACAAGGCCACACAGCAGAGGGTTTGTACA

-9182   GGCGACGCGCGAGTAGATCCCTGGGAGCGCAGAGACGCCTTTCTACCGGC

-9132   AGAGACTGAAGTTTGACTGGAGCGAGGCGGGGCGGAGCCACCTCCCGGTC

-9082   CCCGTGCACTCCGGAATTGTGAACGCTCCCGCAAAGTCCCCGCGAGTGTT

-9032   GTGTTCACACTAATTGGTGTGCCTCTGAGTTCCATCCGTACTCCCCCCCC

-8982   CCCAAGTTTAAATGCTCCTTTTAAGGGCTTGAGTGTCTGCAGCCGTCATG
                                                  E-box

-8932   TGCACCTTGA
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Fig. 6. Sequence of the minimal cardiac enhancer region. The sequence of the minimal cardiac enhancer (–9432/–8922) is shown. GATA sites, gs1 and gs2, are indicated.

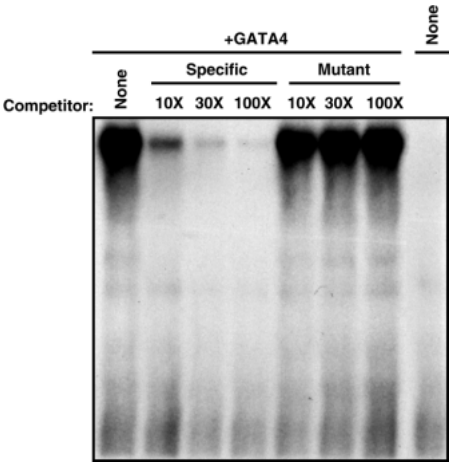

Fig. 7. Binding of GATA4 to the *Nkx2-5* cardiac enhancer. A ^{32}P-labeled oligonucleotide probe encompassing the GATA4 site (gsl) from the *Nkx2-5* enhancer was used in gel mobility shift assays with GATA4 translated in vitro, as described in Materials and Methods. Free probe was run off the gel. The GATA4-DNA complex was competed specifically by excess unlabeled probe, but not by a mutant probe. Fold-excess of competitor DNA compared to labeled probe is shown. No DNA-protein complex was observed with lysate alone (left lane).

Previous studies have shown that *Nkx2-5* is expressed homogeneously throughout the heart prenatally and postnatally (Lints et al., 1993; Komuro and Izumo, 1993; Kasahara et al., 1997). Thus, it appears that the upstream enhancer contains the elements sufficient to recapitulate *Nkx2-5* expression during early stages of cardiogenesis.

Regulation of the cardiac enhancer by GATA4

We inspected the cardiac regulatory region (–9435/–8922) for binding sites of transcription factors that might be involved in cardiac gene expression and noted several potential binding sites for GATA-type transcription factors, A/TGATAA/G (Ko and Engel, 1993; Merika and Orkin, 1993) (Fig. 6). Previous studies have demonstrated important roles for GATA factors in activation of a variety of cardiac muscle structural genes (reviewed in Evans, 1997). To begin to assess the significance of these sites, we created a further deletion (fragment 14) that removed two of the sites, designated gs1 and gs2 (Fig. 1). This fragment, encompassing the region from –9277 to –7353 lost all cardiac expression at E11.5.

To determine whether the gs1 or gs2 sites bind GATA4, we performed gel mobility shift assays using these sequences as probes and GATA4 translated in a rabbit reticulocyte lysate. As shown in Fig. 7, GATA4 bound avidly to the 5′-most GATA

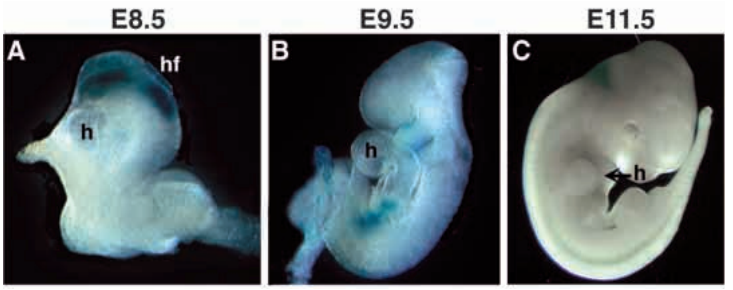

Fig. 8. Mutation of GATA site gs1 abolishes cardiac enhancer activity. Founder transgenic mice bearing a *lacZ* transgene containing fragment 8 (–9700/–7353) in which GATA site gsl was mutated were analyzed at E8.5, 9.5 or 11.5, as indicated. The total number of F_0 transgenic embryos analyzed was 3 at E8.5, 5 at E9.5 and 10 at E11.5. No β-gal staining was observed in the heart at any stage. However, ectopic β-gal staining was observed in noncardiac regions of certain embryos as seen in A and B. h, heart; hf, head fold.

site (gs1) and binding was competed by the cognate site, but not by a mutant site. In contrast, GATA4 showed only weak binding to the other GATA site (gs2) (data not shown).

To further define the potential role of GATA4 site gs1 for cardiac activity of the *Nkx2-5* enhancer, we mutated this site in the context of the –9700/–7353 (fragment 9) enhancer region and tested this mutant enhancer for its ability to direct *lacZ* expression in combination with the hsp68 basal promoter. Examination multiple of F_0 transgenic embryos at E8.5, E9.5 and E11.5 failed to show *lacZ* expression (Fig. 8). In some embryos, ectopic expression of *lacZ* was observed, reflecting transcriptionally active sites of transgene integration, but no heart expression was detected. These results demonstrate that GATA site gs1 is essential for cardiac activity of this distal enhancer.

Expression of the cardiac enhancer in *Nkx2-5* null embryos

Recent studies have shown that *tinman* is subject to positive autoregulation during *Drosophila* embryogenesis (Xu et al., 1998). By analogy, we tested whether the cardiac enhancer might also be regulated by Nkx2-5 through an autoregulatory loop. To address this question, we crossed transgenic mice bearing the fragment 5-lacZ transgene with mice heterozygous for an *Nkx2-5* null mutation (Lyons et al., 1995). Subsequent heterozygous intercrosses yielded *Nkx2-5* null embryos with the *lacZ* transgene. As shown in Fig. 9, the enhancer was expressed normally in *Nkx2-5* null embryos. These results indicate that Nkx2-5 does not positively autoregulate its expression, at least through the upstream enhancer that we have characterized.

DISCUSSION

Expression of *Nkx2-5* marks the specification of cardiac cells in vertebrate embryos and is the earliest known indicator of cardiogenic commitment. Thus, elucidation of the mechanisms that activate *Nkx2-5* transcription will provide insights into the initial steps in establishment of cardiac cell identity. Our results identify a cardiac-specific enhancer that fully recapitulates the expression of *Nkx2-5* from the onset of cardiogenesis in the cardiac crescent and heart tube through the stage of looping. During later stages of cardiac development, activity of this enhancer becomes right ventricular chamber-specific. These findings suggest that *Nkx2-5* regulatory elements are modular, with other enhancers controlling expression of the gene in the developing left ventricle and atrial chambers.

Control of *Nkx2-5* transcription by GATA factors

Our deletion studies demonstrate that the region from –9435 to –9277 upstream of *Nkx2-5* is required for cardiac enhancer activity and contains an essential high-affinity GATA-binding site. GATA4, GATA5 and GATA6 are expressed in the developing heart in patterns overlapping that of *Nkx2-5* (Arceci et al., 1993; Laverriere et al., 1994; Morrisey et al., 1996, 1997; Jiang and Evans, 1996). However, these GATA factors are also expressed in cells in which the *Nkx2-5* enhancer is inactive. At the cardiac crescent stage, for example, the expression of GATA4 extends posteriorly beyond the boundaries of the *Nkx2-5* expression pattern (Schultheiss et al., 1996). GATA4, GATA5 and GATA6 are also expressed in endodermally derived regions of the gut, where the *Nkx2-5* cardiac enhancer is inactive (Arceci et al., 1993; Laverriere et al., 1994). Moreover, these GATA factors are expressed homogeneously in the heart after E11.5, when activity of the enhancer becomes restricted to the right ventricle. Thus, while GATA factors are necessary, they cannot be sufficient to activate the *Nkx2-5* cardiac enhancer. It is likely that GATA factors act together with other cardiac-restricted factors to activate the enhancer or that there are negative factors that inhibit enhancer activity in noncardiogenic cells.

Because the different GATA factors exhibit the same DNA-binding specificity (Ko and Engel, 1993; Merika and Orkin, 1993), the present studies do not allow us to determine whether they can each regulate the *Nkx2-5* cardiac enhancer or whether there might be selectivity for one particular GATA factor. However, the fact that *Nkx2-5* is expressed in the heart of *GATA4* null (Molkentin et al., 1997; Kuo et al., 1997) and *GATA5* null (J. Molkentin and E. N. O., unpublished) mouse embryos suggests functional redundancy among these factors with respect to *Nkx2-5* enhancer activation.

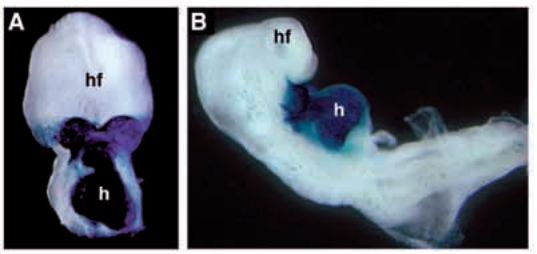

Fig. 9. Expression of the *Nkx2-5* cardiac enhancer in *Nkx2-5* null embryos. Expression of the transgene containing fragment 5 (–9700/–3050) linked to *lacZ* in frontal (A) and lateral (B) views of *Nkx2-5* null embryo at E9.5 is shown. The enhancer is active in the mutant.

GATA4 has been shown to bind essential sites in the control regions of a variety of cardiac muscle structural genes, including those encoding α-myosin heavy chain (Molkentin et al., 1994), cardiac troponin C (Ip et al., 1994), atrial natriuretic factor (ANF) (Grepin et al., 1997), b-type natriuretic peptide (Thuerauf et al., 1994) and troponin I (Murphy et al., 1997). Activation of *ANF* transcription by GATA4 is dependent on physical interaction with Nkx2-5, which binds an adjacent site in the promoter (Durocher et al., 1997). Thus, these two classes of transactivators converge on certain downstream cardiac genes to cooperatively activate transcription. Moreover, activation of the *GATA6* promoter in the developing heart is dependent on a binding site for Nkx2-5 in a distal upstream enhancer (J. Molkentin and E. N. O., unpublished results). Considered together with the present results, it appears that GATA4 and Nkx2-5 function within a mutually reinforcing transcriptional network to control cardiac gene expression.

Activation of *Nkx2-5* expression requires multiple transcriptional inputs. In contrast to the homogeneous expression of the *Nkx2-5* cardiac enhancer throughout the cardiac crescent and heart tube, activity of the enhancer becomes restricted to the right ventricle following cardiac looping. The failure of this enhancer to be expressed in the developing left ventricle and atria, suggests the existence of other regulatory elements that control these aspects of *Nkx2-5* expression.

Of note, at least two other regulatory regions that direct *Nkx2-5* expression in the developing heart have been identified by others. An enhancer located about 3 kb upstream of the gene has been shown to direct expression in a portion of the cardiac crescent and later, in the anterior heart tube, outflow tract and right ventricle (Searcy et al., 1998). The expression of this enhancer in the cardiac crescent and heart tube appears to be much more restricted than expression of the enhancer that we have characterized, which fully mirrors the expression of the endogenous gene until the looping stage. In our study, we detected only stomach, thyroid and pharynx expression, but no cardiac expression with DNA fragments spanning this more downstream region (fragments 3 and 6). Another cardiac enhancer that controls expression in the cardiac crescent and right ventricle has been identified between −3400 and −4800 (J. Reecy and R. Schwartz, personal communication) upstream of the gene. We also failed to detect cardiac enhancer activity in this region (our fragment 3). These discrepancies could be explained by the existence of negative regulatory elements contained within the fragments that we tested and underscore the complexity of *Nkx2-5* regulation.

The influence of negative regulatory elements on *Nkx2-5* transcription is illustrated by our analysis of the distal cardiac enhancer. The region from −9435 to −8922 (fragment 11) shows cardiac activity on its own, but when combined with the region between −8922 and −8039 (fragment 13), the cardiac enhancer is completely inactive (fragment 2). Thus, this region immediately 3′ of the cardiac enhancer appears to function as a negative element. Interestingly, the region 3′ of the negative element, from −8039 to −7353, can overcome the silencing effect of the negative element and restore full cardiac activity to the upstream enhancer (fragment 8) (Fig. 1). However, this 3′ positive element shows no enhancer activity on its own. Together, these results support the conclusion that the upstream cardiac region that we have characterized is bipartite, with two activating regions separated by a region that interferes with transcriptional activity.

Although *Nkx2-5* is preferentially expressed in the heart, expression is seen at other sites, including thyroid, tongue, pharynx, stomach and spleen. Our results localize the enhancer required for expression in pharynx and stomach to the region between −3050 and −1976. There appear to be two separate enhancers that direct thyroid expression of *Nkx2-5*. One maps to the same general region as the pharynx and stomach enhancers and the other maps to the vicinity of the upstream cardiac enhancer. However, this more distal thyroid enhancer requires sequences immediately 5′ of the cardiac enhancer which can be deleted without affecting cardiac transcription (fragment 11). Thus, cardiac and thyroid activity of the distal region are separable.

Modular regulation of cardiac transcription

The modular mode of regulation of *Nkx2-5* transcription in which different enhancers control transcription of the gene at different times and in different regions of the developing heart is consistent with recent studies of more downstream genes in the cardiac pathway (reviewed in Firulli and Olson, 1997). The *SM22* (Li et al., 1996), *desmin* (Kuisk et al., 1996), *myosin light chain-2V* (Ross et al., 1996) and *myosin light chain-3F* (Kelly et al., 1995; Franco et al., 1997) genes, for example, have been shown to be governed by multiple *cis*-acting sequences that show spatially restricted patterns of expression in the heart. Our results indicate that compartment-specific programs of gene transcription are established even at the earliest stages of cardiogenesis to regulate the regulatory genes themselves.

The mechanisms that confer compartment-specific regulation to cardiac genes are presently unclear. The only chamber-restricted transcription factors identified to date are the bHLH factors dHAND and eHAND, which are expressed in the right and left ventricular chambers, respectively (Cserjesi et al., 1995; Srivastava et al., 1995; Firulli et al., 1998; Biben and Harvey, 1997). The MADS-box transcription factor MEF2C has also been shown to be important for right ventricular gene expression and development (Lin et al., 1997; Ross et al., 1996; Kuisk et al., 1996), despite the fact it is not expressed in a compartment-specific manner (Edmondson et al., 1994). Similarly, GATA factors, which our results indicate are essential for right ventricular expression of *Nkx2-5*, are expressed throughout the developing heart. How widely expressed cardiac transcription factors control chamber-restricted cardiac enhancers remains to be determined.

The control of *Nkx2-5* transcription by multiple, independent cardiac enhancers is reminiscent of *tinman* regulation during *Drosophila* embryogenesis. The complete expression pattern of *tinman* in the early mesoderm and cardiogenic lineage is controlled by five different transcriptional enhancers, which are influenced by negative elements (Yin et al., 1997; Li et al., 1997; Lee et al., 1997; Xu et al., 1998). This type of modular regulation may allow these early cardiogenic control genes to independently integrate diverse inputs throughout development and provides for precise control of gene transcription.

Nkx2-5 as a target for cardiogenic inductive signals

The signaling systems that initiate cardiogenesis in the mesoderm appear to be conserved from *Drosophila* to vertebrates. Formation of the cardiac lineage in *Drosophila* is

dependent on Dpp and Wingless signaling (Frasch, 1995; Wu et al., 1995; Park et al., 1996). Activation of *tinman* transcription in response to Dpp signaling is mediated by binding of Tinman itself and the Smad4 homolog Medea to a dorsal mesoderm-specific enhancer (Xu et al., 1998). Initiation of cardiogenesis in anterior lateral plate mesoderm of vertebrate embryos requires signaling from adjacent endoderm (Schultheiss et al., 1995, 1996). Several peptide growth factors have been shown to possess cardiac-inducing activity, including bone morphogenetic proteins (BMPs), activin and fibroblast growth factors (FGFs) (Sugi and Lough, 1995; Lough et al., 1996; Schultheiss et al., 1996; Andree et al., 1998). The *Nkx2-5* cardiac enhancer described here is activated in the cardiac crescent at a time when *Nkx2-5* expression has been shown to be dependent on signaling from adjacent endoderm (Schultheiss et al., 1996). The identification of this enhancer opens the way to studies of the initial events of cardiogenic specification. Whether this enhancer is the primary target for initiation of *Nkx2-5* transcription by inductive signals and how its activity relates to that of other *Nkx2-5* cardiac enhancers are particularly intriguing issues for the future.

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REFERENCES

Andree, B., Duprez D., Vorbusch B., Arnold H. H. and Brand T. (1998). BMP-2 induces ectopic expression of cardiac lineage markers and interferes with somite formation in chicken embryos. *Mech. Dev.* **70**, 119-131.

Arceci, R. J., King, A. A. J., Simon, M. C., Orkin, S. H. and Wilson, D. B. (1993). Mouse GATA-4: A retinoic acid-inducible GATA-binding transcription factor expressed in endodermally-derived tissues and heart. *Mol. Cell Biol.* **13**, 2235-2246.

Azpiazu, N. and Frasch, M. (1993). *tinman* and *bagpipe*: Two homeobox genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev.* **7,** 1325-1340.

Biben, C. and Harvey, R. P. (1997). Homeodomain factor Nkx2-5 controls left/right asymmetric expression of the bHLH gene eHAND during murine heart development. *Genes Dev.* **11**, 1357-1369.

Biben, C., Hatzistavrou, T. and Harvey, R.P. (1998). Expression of *NK-2* class homeobox gene *Nkx2-6* in foregut endoderm and heart. *Mech. Dev.* **73**, 125-127.

Bodmer, R. (1993). The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* **118,** 719-729.

Bodmer, R. (1995). Heart development in *Drosophila* and its relationship to vertebrates. *Trend Card. Med.* **5,** 21-28.

Bodmer, R., Jan, L.Y., and Jan, Y. N. (1990). A new homeobox-containing gene, *msh-2*, is transiently expressed early during mesoderm formation in *Drosophila*. *Development* **110,** 661-669.

Brand, T., Andree, B., Schneider, A., Buchberger, A. and Arnold, H.-H. (1997). Chicken Nkx2-8, a novel homeobox gene expressed during early heart and foregut development. *Mech. Dev.* **64,**53-59.

Brennan, T. J. and Olson, E. N. (1990). Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization. *Genes Dev.* **4**, 582-595.

Buchberger, A., Pabst, O., Brand, T., Seidl, K. and Arnold, H.-H. (1996). Chick Nkx2.3 represents a novel family member of vertebrate homologues to the *Drosophila* homeobox gene *tinman*: differential expression of cNKx2.3 and cNKx2-5 during heart and gut development. *Mech. Dev.* **56,** 151-163.

Chen, J.-N. and Fishman, M. C. (1996). Zebrafish *tinman* homolog demarcates the heart field and initiates myocardial differentiation. *Development* **122,** 3809-3816.

Cheng, T. C., Wallace, M. C., Merlie, J. P. and Olson, E. N. (1993). Separable regulatory elements governing myogenin transcription during mouse embryogenesis. *Science* **261**, 215-218.

Cleaver, O. B., Patterson, K. D. and Krieg, P. A. (1996). Overexpression of the tinman-related genes *XNkx2-5* and *XNkx2-3* in *Xenopus* embryos results in myocardial hyperplasia. *Development* **122,** 3549-3556.

Cserjesi, P., Brown, D., Lyons, G. E. and Olson, E. N. (1995). Expression of the novel basic-helix-loop-helix gene eHAND in neural crest derivatives and extraembryonic membranes during mouse development. *Dev. Biol.* **170**, 664-678.

Durocher, D., Charron, F., Warren, R., Schwartz, R. J. and Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J.* **16**, 5687-5696.

Edmondson, D. G., Lyons, G. E., Martin, J. F. and Olson, E. N. (1994). *Mef2* gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* **120**, 1251.

Evans, S. M., Yan, W., Murillo, P. M., Ponce, J. and Papalopulu, N. (1995). *tinman*, a *Drosophila* homeobox gene required for heart and visceral mesoderm specification, may be represented by a family of genes in vertebrtaes: XNkx2.3, a second vertebrate homologue of *tinman*. *Development* **121,** 3889-3899.

Evans, T. (1997). Regulation of cardiac gene expression by GATA-4/5/6. *Trend Card. Med.* **7**, 75-83.

Firulli, A. B. and Olson, E. N. (1997). Modular regulation of muscle gene transcription: A mechanism for muscle cell diversity. *Trends Genet.* **13**, 364-369.

Firulli, A. B., McFadden, D. G., Lin, Q., Srivastava, D. and Olson, E. N. (1998). Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand 1. *Nat. Gen.* **18**, 266-270.

Fishman, M. C. and Chien K. R. (1997). Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124**, 2099-2117.

Fishman, M. C. and Olson, E. N. (1997). Parsing the heart: genetic modules for organ assembly. *Cell* **91**, 153-156.

Frasch, M. (1995). Induction of visceral and cardiac mesoderm by ectopic Dpp in the early*Drosophila* embryo. *Nature* **374,** 464-467.

Franco, D., Kelly, R., Lamers, W. H., Buckingham, M. and Moorman, A. F. M. (1997). Regionalized transcriptional domains of myosin light chain 3f transgenes in the embryonic mouse heart: morphogenetic implications. *Dev. Biol.* **188**, 17-33.

Fu, Y. and Izumo S. (1995). Cardiac myogenesis: overexpression of *XCsx2* or *XMef2A* in whole *Xenopus* embryos induces the precocious expression of XMHCα gene.*Roux's Arch. Dev. Biol.* **205,** 198-202.

Gajewski, K., Kim, Y., Lee, Y. M., Olson, E. N. and Schulz, R. A. (1997). *D-Mef2:* A target for tinman activation during *Drosophila* heart development. *EMBO J.* **16**, 515-522.

Grepin, C., Nemer, G. and Nemer, M. (1997). Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development* **124**, 2387-2395.

Harvey, R. P. (1996). *NK-2* homeobox genes and heart development. *Dev. Biol.* **178,** 203-216.

Ip, H. S., Wilson, D. B., Heikinheimo, M., Tang, Z., Ting, C. N., Simon, M. C., Leiden, J. M. and Parmacek, M. S. (1994). The GATA4 transcription factor transactivates the cardiac muscle-specific troponin C promoter-enhancer in non-muscle cells. *Mol. Cell. Biol.* **14**, 7517-5126.

Jiang, Y. and Evans T. (1996). The Xenopus GATA4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. *Dev. Biol.* **174**, 258-270.

Kasahara, H., Bartunkova, S., Martina, M., Tanaka, M. and Izumo, S. (1997). Cardiac and extracardiac expression of Csx/Nkx2.5 homeodomain protein. *Circ Res.* **82**, 936-946.

Kelly, R., Alonso, S., Tajbakhsh, S., Cossu, G. and Buckingham, M. (1995). Myosin light chain 3F regulatory sequences confer regionalized cardiac and skeletal muscle expression in transgenic mice. *J. Cell Biol.* **129**, 383-396.

Ko, L. J. and Engel, J. D. (1993). DNA-binding specificities of the GATA transcription factor family. *Mol. Cell Biol.* **13**, 4011-4022.

Komuro, I. and Izumo, S. (1993). *Csx*: A murine homeobox-containing gene specifically expressed in the developing heart. *Proc. Natl Acad. Sci. USA* **90,** 8145-8149.

Kothary, R., Clapoff, S., Darling, S., Perry, M. D., Moran, L. A. and Rossant, J. (1989). Inducible expression of an hsp68-lacZ hybrid gene in transgenic mice. *Development* **105**, 707-714.

Kuisk, I. R. Li, H., Tran, D. and Capetanaki, Y. (1996). A single MEF2 site

governs desmin transcription in both heart and skeletal muscle during mouse embryogenesis. *Dev. Biol.* **174**, 1-13.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., Soudais, C. and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev*. **11**, 1048-1060.

Laverriere, A. C., NacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B. E. and Evans, T. (1994). GATA-4/5/6: A subfamily of three transcription factors expressed in developing heart and gut. *J Biol. Chem*. **269**,23, 177-23, 184.

Lee, K. H, Xu, Q. and Breitbart R. E. (1996). A new tinman-related gene, nkx2.7, anticipates the expression of nkx2.5 and nkx2.3 in zebrafish heart and pharyngeal endoderm. *Dev. Biol*. **180,** 722-731.

Lee, Y. M., Park, T., Schulz, R. A. and Kim, Y. (1997). Twist-mediated activation of the NK-4 homeobox gene in the visceral mesoderm of *Drosophila* requires two distinct clusters of E-box regulatory elements. *J. Biol. Chem*. **272**, 17531-17541.

Li, L., Miano, J. M., Mercer B. and Olson, E. N. (1996). Expression of the SM22 α-promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J. Cell Biol*. **132**, 849-859.

Lin, Q., Schwarz, J., Bucana, C. and Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276**, 1404-1407.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I. and Harvey, R. P. (1993).*Nkx2-5*: A novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* **119,** 419-431.

Lough, J. M., Barron, M., Brogley, M., Sugi, Y., Bolender, D. L. and Zhu, X. L. (1996). Combined BMP-2 and FGF-4, but neither factor alone, induces cardiogenesis in non-precardiac embryonic mesoderm. *Dev. Biol*. **178,** 198-202.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L. and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene *Nkx2-5*.*Genes Dev.* **9,** 1654-1666.

Merika, M. and Orkin, S. H. (1993). DNA-binding specificity GATA family transcription factors. *Mol. Cell Biol*. **13**, 3999-4010.

Moller, W. and Moller, G. (1994). Chemical dehydration for rapid paraffin embedding. *Biotechnic & Histochemistry*. **69**, 289-290.

Molkentin, J. D., Kalvakolanu, D. V. and Markham, B. E. (1994). Transcription factor GATA4 regulates cardiac muscle-specific expression of the alpha-myosin heavy-chain gene. *Mol. Cell Biol.* **14**, 4947-4957.

Molkentin, J. D., Lin, Q., Duncan, S. A. and Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev*. **11**, 1061-1072.

Morrisey, E. E., Ip, H. S., Lu, M. M. and Parmacek, M. S. (1996). GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Dev. Biol*. **177**, 309-322.

Morissey, E. E., Ip, H. S., Tang, Z., Lu, M. M. and Parmacek, M. S. (1997). GATA-5: a transcriptional activator expressed in a novel temporally and spatially-restricted pattern during embryonic development. *Dev. Biol*. **182**, 21-36.

Murphy, A. M., Thompson, W. R., Peng, L. F. and Jones, L. 2nd (1997). Regulation of the rat cardiac troponin I gene by the transcription factor GATA-4. *Biochem. J.* **322**, 393-401.

Nascone, N. and Mercola, M. (1996). Endoderm and cardiogenesis: New insights. *Trends Card. Med.* **6,** 211-216

Olson, E. N and Srivastava, D. (1996). Molecular pathways controlling heart development. *Science* **272,** 671-676.

Park, M., Wu, X., Golden, K., Axelrod, J. D. and Bodmer, R. (1996). The wingless signaling pathway is directly involved in *Drosophila* heart development. *Dev. Biol*. **177**, 104-116.

Ranganayakulu, G., Elliott, D., Harvey, R. and Olson, E. (1998). Divergent roles for Nk-2 class homeobox genes in cardiogenesis in flies and mice. *Development* **125**, 3037-3048.

Reecy, J.M., Yamada, M., Cummings, K., Sosic, D., Chen, C.Y., Eichle, G., Olson, E. N. and Schwartz, R.J. (1997). Chicken Nkx2-8: A novel homeobox gene expressed in early heart progenitor cells and pharyngeal pouch-2 and -3 endoderm. *Dev. Biol*. **188,** 295-311.

Ross, R. S., Navankasattusas, S., Harvey, R. P. and Chien, K. R. (1996). An HF-1a/HF-1b/MEF combinatorial element confers cardiac ventricular specificity and established an anterior-posterior gradient of expression. *Development* **122**, 1799-1809.

Schultheiss, T. M., Burch, J. B. E. and Lassar, A. B. (1996). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev.* **11,** 451-462.

Schultheiss, T. M., Xydas, S. and Lassar, A. B. (1995). Induction of avian cardiac myogenesis by anterior endoderm. *Development* **121**, 4203-4214.

Searcy, R. D., Vincent, E. B., Liberatore, C. M. and Yutzey, K. E. (1998). A GATA-dependent *nkx-2.5* regulatory element activates early cardiac gene expression in transgenic mice. *Development* **125**, 4461-4470.

Srivastava, D., Cserjesi, P. and Olson, E. N. (1995). New subclass of bHLH proteins required for cardiac morphogenesis. *Science* **270**, 1995-1999.

Staehling-Hampton, K., Laughon, A. and Hoffmann, F. (1995). A *Drosophila* protein related to the human zinc finger transcription factor PRDII/MBPI/HIV-EP1 is required for dpp signalling. *Development* **121**, 3393-3403.

Sugi, Y., and Lough, J. (1995). Activin-A and FGF-2 mimic the inductive effects of anterior endoderm on terminal cardiac myogenesis *in vitro*. *Dev. Biol.* **169**, 567-574.

Thuerauf, D. J., Hanford, D. S. and Glembotski, C. C. (1994). Regulation of rat brain natriuretic peptide transcription. A potential role for GATA-related transcription factors in myocardial cell gene expression. *J. Biol. Chem.* **269**, 17772-17775.

Yin, Z. and Frasch, M. (1998). Regulation and function of *tinman* during dorsal mesoderm induction and heart specification in *Drosophila*. *Dev. Genet.* **22**, 187-200.

Yin, Z., Xu, X. -L. and Frasch, M. (1997). Regulation of the Twist target gene tinman by modular cis-regulatory elements during early mesoderm development. *Development* **124**, 4871-4982.

Xu, X., Yin, Z., Hudson, J. B., Ferguson, E. L. and Frasch, M. (1998). Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the *Drosophila* mesoderm. *Genes Dev.* **12**, 2354-2370.

Wu, X., Golden K. and Bodmer R. (1995). Heart development in *Drosophila* requires the segment polarity gene *wingless*. *Dev. Biol.* **169,** 619-628.