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REVIEW ARTICLE

Building the heart piece by piece: modularity of *cis*-elements regulating *Nkx2-5* transcription

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SUMMARY

Heart formation in *Drosophila* is dependent on the homeobox gene *tinman*. The homeobox gene *Nkx2-5* is closely related to *tinman* and is the earliest known marker for cardiogenesis in vertebrate embryos. Recent studies of *cis*-regulatory elements required for *Nkx2-5* expression in the developing mouse heart have revealed an extraordinary array of independent cardiac enhancers, and associated negative regulatory elements, that direct transcription in distinct regions of the embryonic heart. These studies demonstrate the modularity in cardiac transcription, in which different regulatory elements respond to distinct sets of transcription factors to control gene expression in different compartments of the developing heart. We consider the potential mechanisms underlying such transcriptional complexity, its possible significance for cardiac function, and the implications for evolution of the multichambered heart.

Key words: Heart, Transcription, *cis*-element, *Nkx2.5*

INTRODUCTION

The heart is the first organ to form in mammals. Cardiogenic lineages originate from paired regions of anterior lateral mesoderm, the cardiac crescent, soon after gastrulation and develop into parallel cardiac primordia that fuse to form the primitive heart tube along the ventral midline of the embryo. Subsequent events of looping, chamber maturation and alignment with the vasculature give rise to the mature multichambered heart (Olson and Srivastava, 1996; Fishman and Chien, 1997). Based on the phenotypes of mouse and zebrafish mutants lacking cardiogenic regulatory genes, it has been proposed that the heart develops as a modular organ, such that each anatomical region is controlled by a distinct transcriptional regulatory program (Fishman and Olson, 1997). Consistent with this notion, the heart tube can be divided into segments that form the atria, left ventricle, right ventricle and ventricular outflow tract. Precursors of these regions of the heart appear to originate from separate lineages that develop according to their positions along the anteroposterior axis of the embryo (Yutzey and Bader, 1995). Several recent studies have revealed *cis*-regulatory elements that direct cardiac transcription specifically in the left or right ventricular chambers and atria, and even within subdomains within the chambers. Whether this regional specificity of transcription is important for the physiologic and functional differences of the chambers of the adult heart and how these transcriptional territories are established and maintained are issues of intense interest.

The homeobox gene *Nkx2-5* (also called *Csx*) (Lints et al., 1993; Komuro and Izumo, 1993) is the earliest known marker of vertebrate heart development and has been identified as a potential vertebrate homologue of *tinman*, a homeobox gene required for cardiac development in the *Drosophila* embryo (Azpiazu and Frasch, 1993; Bodmer, 1993; reviewed in Harvey, 1996). Deciphering the regulatory mechanisms that activate *Nkx2-5* transcription in cardiac mesoderm is an important problem because it will provide a window into the cellular circuitry that specifies cardiac cell fate and may ultimately provide opportunities for cardiac regeneration. Recently, a series of papers has described *cis*-acting regulatory elements that control *Nkx2-5* expression during heart development in the mouse. These studies have revealed surprising complexity in *Nkx2-5* regulation, with multiple enhancers acting in distinct populations of cardiomyocytes during development. Here we describe a model for cardiac development based on the modularity of transcriptional units that control *Nkx2-5* and suggest a potential role for this modularity in evolution of the multichambered heart.

CONTROL OF EARLY CARDIOGENESIS BY NK-2 HOMEOBOX GENES

Nkx2-5 genes are highly conserved across vertebrate species

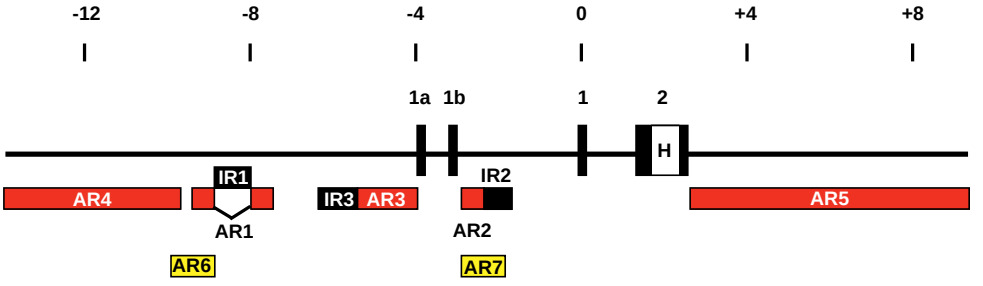
and are expressed in early cardiac progenitor cells prior to cardiogenic differentiation and through adulthood in mice (Lints et al., 1993; Komuro and Izumo, 1993), zebrafish (Chen and Fishman, 1996), frogs (Tonissen et al., 1994), and chickens (Schultheiss et al., 1995). In addition to the heart, *Nkx-2-5* is also expressed in developing pharyngeal arches, spleen, thyroid, stomach and tongue. Four other *tinman* related NK-type homeobox genes, *Nkx2-3* (Evans et al., 1995; Buchberger et al., 1996), *Nkx2-6* (Biben et al., 1998), *Nkx2-7* (Lee et al., 1996) and *Nkx2-8* (Reecy et al., 1997; Brand et al., 1997), are also expressed in vertebrate cardiac lineages and 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 a 'Nkx code', in which cell fates are specified by unique combinations of these NK-homeodomain proteins (Reecy et al., 1997).

Nkx2-5, tinman and related factors bind DNA and recognize novel NKE sequence elements 5′-NAAGTG-3′ (Chen and Schwartz, 1995; Damante et al., 1994). Intact NKEs are required for expression of the myogenic regulatory gene *D-MEF2* in *Drosophila* (Gajewski et al., 1998) and for transcription of a variety of vertebrate target genes, including *cardiac α-actin* (Chen and Schwartz, 1995) and *atrial natriuretic factor* (Durocher et al., 1996). Recent cotransfection assays in fibroblasts demonstrate that Nkx2-5 activity may require combinatorial interactions with other cardiac-restricted factors, such as serum response factor (Chen and Schwartz, 1996) and GATA-4 (Sepulveda et al., 1998; Durocher et al., 1997). Although Nkx2-5 and tinman bind the same DNA sequence, Nkx2-5 cannot substitute for tinman to control cardiac development in *Drosophila* (Ranganayakulu et al., 1998; Park et al., 1998). Mutagenesis of tinman has revealed a novel 43-amino acid domain at the extreme N terminus that acts together with the homeodomain to regulate dorsal vessel formation (Ranganayakulu et al., 1998). If this domain is tethered to Nkx2-5, it confers the ability to induce heart formation in *Drosophila* embryos. It is likely this domain interacts with an essential cardiogenic cofactor.

The phenotype of mice lacking *Nkx2-5* reveals an important early role for *Nkx2-5* in cardiac gene expression and morphogenesis. Embryos homozygous for mutant *Nkx2-5* display defects in looping morphogenesis of the heart, but cardiomyocytes are properly specified and able to differentiate (Lyons et al., 1995; Tanaka et al., 1999a). This relatively late function for *Nkx2-5* suggests either that *Nkx2-5* and *tinman* are not functionally equivalent or that other cardiac-expressed *NK-2* homeobox genes are functionally redundant in the early stages of cardiogenesis. To address this issue, two groups have injected dominant negative Nkx2-5 mutants into early *Xenopus* blastomeres (Fu et al., 1998; Grow and Kreig, 1998). Both resulted in a complete absence of heart formation and cardiac gene expression, revealing an essential role for *NK-2* homeobox genes in early cardiac development in vertebrates. Recent studies have also shown that mutations in *Nkx2-5* are responsible for congenital cardiac malformations and atrioventricular conduction abnormalities in humans (Schott et al., 1998).

SIGNALING SYSTEMS THAT INDUCE *NKX2-5* EXPRESSION IN CARDIOGENIC MESODERM

The initial step in heart formation in vertebrates involves commitment of cells from anterior lateral mesoderm to a cardiogenic fate. This has been shown to be dependent on signals from adjacent endoderm (Schultheiss et al., 1995). Bone morphogenetic proteins (BMPs)-2 and -4 (Schultheiss et al., 1997), as well as fibroblast growth factor (Lough et al., 1996) and activin (Ladd et al., 1998), are among the peptide growth factors that can induce cardiogenesis in anterior lateral mesoderm, but the exact combinations of factors that perform this function during embryogenesis have not been fully defined. *Nkx2-5* is expressed concomitant with cardiac specification and is the earliest known marker of the cardiac lineage, making it likely that regulatory elements associated



AR1: cardiac crescent, entire linear heart tube, outflow tract, right ventricle

AR2: anterior cardiac crescent, anterior linear heart tube, outflow tract, right ventricle

AR3: outflow tract, right ventricle

AR4: left ventricle?

AR5: right ventricle?

AR6: thyroid

AR7: thyroid, spleen, pharynx, stomach

Fig. 1. Schematic diagram of mouse *Nkx2-5* genomic region with positions of regulatory elements. Positions of exons and homeobox (H) are shown. Activating regions (AR) for cardiac and noncardiac cell types are indicated in red and yellow, respectively, and inhibitory regions (IR) in black. Question marks designate regulatory regions inferred from deletion analyses, but not yet shown to be sufficient alone for expression. Distances in kb are shown at the top. AR1 is bisected by IR1; the left portion of AR1 has activity alone that is augmented by the right portion. AR4 has not been shown to have activity alone, but its activity is inferred by the left ventricular expression of the entire 14 kb upstream region and the lack of left ventricular expression by regions downstream of –9 kb.

with the gene respond directly to the signaling pathways activated by cardiogenic inducers.

In *Drosophila*, activation of *tinman* transcription in dorsal mesoderm is dependent on signaling by the BMP family member decapentaplegic (Dpp), which acts together with wingless to induce the cardiac lineage (Frasch, 1995; Park et al., 1996). Thus, the role of BMP signaling in cardiogenic induction appears to be evolutionarily conserved. However, a role for wingless proteins in vertebrate cardiogenesis has not yet been established.

TRANSCRIPTIONAL CONTROL OF *NKX2-5* EXPRESSION

Four recent reports have investigated the regulation of *Nkx2-5* gene expression during mouse embryogenesis (Searcy et al., 1998; Lien et al., 1999; Reecy et al., 1999; Tanaka et al., 1999b). The results are summarized in Fig. 1. Within 23 kb of DNA surrounding the *Nkx2-5* gene, seven different activating regions and three repressor regions were identified or inferred. Some *Nkx2-5* enhancers are active in the same regions of the heart and some direct expression patterns that demarkate distinct subpopulations of cardiomyocytes within cardiac compartments, but none can account for the complete expression pattern of the gene during embryonic development and after birth.

The protein-coding region of *Nkx2-5* is contained in two exons, which can be spliced to either of two alternative exons (exons 1a and 1b) located 3-4 kb upstream. Most transcripts contain only the two 3′ coding exons and encode a protein of 318 amino acids. The upstream exons contain 5′ untranslated and coding sequences, which, by alternative splicing to different 3′ acceptor sites, result in a variety of different *Nkx2-5* transcripts (Reecy et al., 1999; Tanaka et al., 1999b). Although used rarely in normal development, exon 1a can be spliced into the *Nkx2-5*-coding region in several ways, thereby potentially creating novel Nkx2-5 protein isoforms, whose transcriptional activity is greatly diminished compared to wild-type Nkx2-5 (Reecy et al., 1999).

Through serial deletions of 14 kb of 5′ and 6 kb of 3′ *Nkx2-5* flanking sequence linked to a *lacZ* reporter gene, a surprisingly complex array of regulatory elements has been identified (Reecy et al., 1999; Searcy et al., 1998; Lien et al., 1999; Tanaka et al., 1999b). With 14 kb of 5′ flanking DNA, *lacZ* expression was observed in the cardiac crescent at E7.5, and later in the outflow tract, interatrial groove, atrioventricular canal, right and left ventricles, as well as in pharynx, thyroid primordia and stomach at E10.5 (Tanaka et al., 1999b). However, in the adult heart, this region was active only in the atrioventricular junction and subendocardium of the ventricular septum, despite the fact *Nkx2-5* is expressed homogenously throughout all compartments of the adult heart. Dissection of the upstream region identified a cardiac enhancer (activating region 1), located about 9 kilobases upstream of the gene, that recapitulates the expression pattern of *Nkx2-5* in cardiogenic precursor cells from the onset of cardiac lineage specification and throughout the linear and looping heart tube, when combined with the heat-shock protein 68 basal promoter (Lien et al., 1999). Thereafter, as the atrial and ventricular chambers become demarcated, activity of this enhancer becomes restricted to the developing right ventricle, with no expression in the left ventricle or atria (Fig. 2). The region immediately 3′ of this minimal cardiac enhancer (inhibitory region 1) strongly inhibits enhancer activity, whereas the adjacent downstream region overcomes this inhibition, but has no transcriptional activity on its own. Thus, activating region 1 acts as a bipartite regulatory module with two positive regulatory regions separated by an intervening negative element. Thyroid expression is also seen with cardiac activating region 1 combined with immediately adjacent 5′ sequences (referred to as activating region 6).

A second cardiac enhancer is located between –3 and –2.5 kb upstream of the gene (activating region 2) (Searcy et al., 1998). Activity of this enhancer is restricted to the anterior region of the cardiac crescent and primitive heart tube before becoming localized to the developing outflow tract and future right ventricle of the looped heart tube. This enhancer is repressed by an immediately adjacent inhibitory element (inhibitory region 2) (Lien et al., 1999). Activating region 2 also directs expression in the developing spleen anlage at least 24 hours before the earliest reported spleen marker and in the pharyngeal pouches and their derivatives including the thyroid. In the presence of inhibitory region 2, cardiac activity of activating region 2 is inhibited, but expression in spleen, pharynx and thyroid is unaffected. Sequences within 887 bp upstream of exon 1a (activating region 3) direct expression in the outflow tract, but this region is inactive when combined with an additional 750 bp of sequence immediately upstream (inhibitory region 3) (Reecy et al., 1999). Because 14 kb of 5′ flanking DNA gives right and left ventricular expression (Tanaka et al., 1999b), but none of the cardiac enhancers downstream of –9 kb gives left ventricular expression, by inference, this expression is likely to be dependent on the region between –14 and –9 kb (activating region 4). An additional positive regulatory region responsible for complete expression in the right ventricle and compact layer of the lateral walls also appears to lie within 6 kb 3′ of the gene (activating region 5) and may act in concert with the intron between exons

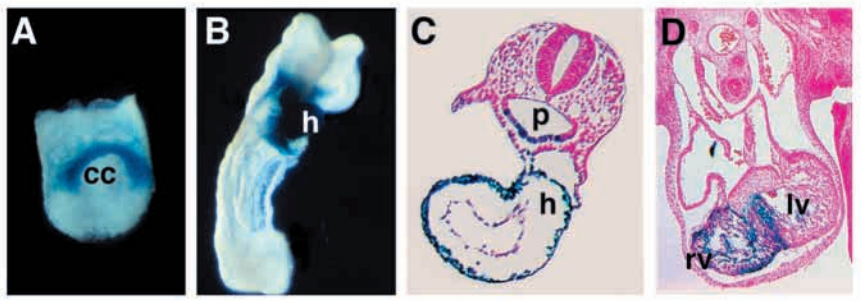


Fig. 2. Expression of a *lacZ* transgene under control of activating region 1. Mice bearing a *lacZ* transgene under control of AR1 (See Fig. 1) were stained for *lacZ* expression at E7.5 (A), E8.5 (B,C) and E11.5 (D). C and D show transverse sections. Expression is cardiac-specific in all figures except for cells in the floor of the pharynx in C. (D) *lacZ* expression can be seen to be localized to the right ventricle. cc, cardiac crescent; h, heart; lv, left ventricle; p, pharynx; r, right ventricle. Figures from Lien et al. (1999).

1a and 1b (Tanaka et al., 1999b). No atrial regulatory elements have yet been identified for the gene. Identification of these atrial enhancers and how they interact with other regulatory regions will be of great interest in the future.

A key question is the identity of *trans*-acting factors that regulate the multiple *Nkx2-5* enhancers. The distal cardiac enhancer (activating region 1) contains a high-affinity binding site for GATA factors. Mutation of this site abolishes activity in heart as well as thyroid (Lien et al., 1999). Similarly, mutation of paired GATA sites within activating region 2 eliminates gene activation in the heart, pharynx and spleen primordia of transgenic embryos (Searcy et al., 1998). GATA4, GATA5, and GATA6 are coexpressed with Nkx2-5 in the developing heart (Laverriere et al., 1994). However, since GATA factors are also expressed more broadly than these enhancers, there must be other factors that contribute to enhancer activity. Interestingly, *Nkx2-5* also seems to be negatively regulated by its own gene product, because when *lacZ* was 'knocked-in' to replace the entire coding exons, *lacZ* expression was much higher in the hearts of homozygous mutant embryos than in heterozygotes (Tanaka et al., 1999b). Whether this involves direct binding of Nkx2-5 protein to gene regulatory sequences or reflects an indirect mechanism remains to be determined.

While activating regions 1 and 2 direct expression throughout the cardiac crescent at E7.75 and appear to be activated concomitant with the endogenous *Nkx2-5* gene, it remains unclear whether these enhancers are the actual transcriptional targets for cardiac inductive signals. The dependence of these elements on GATA factors raises questions in this regard since GATA factors are expressed more broadly than *Nkx2-5* and there is no evidence that their expression precedes expression of *Nkx2-5*. On the contrary, BMP-7 has been shown to induce *Nkx2-5* expression in isolated chick embryos without inducing GATA4 (Schultheiss et al., 1997). It remains possible, therefore, that the initial signal for *Nkx2-5* gene activation is directed at a different enhancer and that GATA factors are involved immediately thereafter in an amplification mechanism or that elements other than GATA sites in the cardiac crescent enhancers mediate *Nkx2-5* induction in response to endodermal signals. It is also interesting to point out that Nkx2-5 and GATA factors have been shown to cooperate to activate transcription of downstream genes (Durocher et al., 1997; Sepulveda et al., 1998) and recent studies have shown that *GATA6* transcription is dependent on a cardiac enhancer controlled by Nkx2-5 (J. Molkentin and E. N. O., unpublished data). Thus, Nkx2-5 and GATA factors act within a mutually reinforcing transcriptional network to control cardiac gene expression.

Transcriptional activation in response to BMP signaling is mediated by Smad proteins, which translocate to the nucleus following cell surface receptor activation and bind DNA in combination with other factors (reviewed in Heldin et al., 1997). The specific DNA sequences bound by Smad proteins depend on their protein partners. Like *Nkx2-5*, *tinman* is controlled by a complex array of positive and negative *cis*-regulatory elements (Yin et al., 1997; Lee et al., 1997), one of which confers responsiveness to Dpp through binding of the vertebrate Smad4 homolog Medea (Xu et al., 1998). There are also sequences resembling Smad binding sites in activating regions 1 and 2 of *Nkx2-5*, but their potential roles in transcriptional activation in cardiac mesoderm have not been investigated nor have potential cardiogenic partner proteins for Smads been identified.

MODULARITY OF *CIS*-REGULATORY ELEMENTS AS A FOUNDATION FOR EVOLUTION OF THE HEART

Like *Nkx2-5*, several downstream muscle structural genes, including those encoding myosin light chains 2 and 3f (Franco et al., 1997; O'Brien et al., 1993), SM22 (Li et al., 1996) and desmin (Kuisk et al., 1996), have been shown to be controlled by modular regulatory elements (reviewed in Firulli and Olson, 1997). However, the complexity of modules for *Nkx2-5* is far greater than for any other cardiac gene described thus far. The diversity of transcriptional regulatory elements for *Nkx2-5* and other cardiac genes suggests a potential mechanism for regionalization of cardiac development. Indeed, since *NK-2* homeobox genes are essential for development of heart-like organs in *Drosophila* and *C. elegans*, as well as vertebrates (Harvey, 1996), it is possible that acquisition of novel *cis*-regulatory modules by a primordial member of this family resulted in expansion of the heart in a modular way by progressive addition of segments or compartments, such as atrial and ventricular chambers (Fishman and Olson, 1997). Alternatively, the evolved complexity of *Nkx* gene regulatory elements could reflect subdivisions of previous cardiac zones, for further specification of function, rather than addition of segments in a developmental sense.

It is unknown whether gene expression in subdomains of the developing heart is controlled by combinations of cardiac-restricted and widely expressed transcription factors or whether chamber-restricted transcriptional activators and repressors control these gene expression patterns. The basic helix-loop-helix transcription factors dHAND and eHAND are expressed in the right and left ventricular chambers, respectively (Cserjesi et al., 1995; Srivastava et al., 1997; Firulli et al., 1998; Biben and Harvey, 1997), and are therefore potential regulators of regional gene expression patterns in the heart. Loss-of-function mutations in the genes encoding these factors result in severe defects in cardiac morphogenesis, supporting the notion that they regulate chamber-specific gene expression and morphogenesis (Srivastava et al., 1997; Firulli et al., 1998; Riley et al., 1998).

The homeobox gene, *Irx4*, distinct from the *NK-2* class, is expressed specifically in the ventricular chambers, but not in atria or outflow tract of the heart (Bao et al., 1999). Ventricle-specific expression of *Irx4* is observed in the prospective ventricular region at the linear heart tube stage and is maintained throughout development and adulthood. Misexpression of *Irx4* in the atria results in activation of ventricle-specific genes and, conversely, expression of a dominant negative *Irx4* mutant in ventricle can upregulate atrial myosin heavy chain expression. Thus, Irx4 is a potential regulator of region-specific gene expression in the heart, but its specific target genes have not yet been identified.

These studies raise several interesting questions about *Nkx2-5* regulation and the logic of cardiac development. Why are the transcriptional control elements for *Nkx2-5* so complex? Why are there multiple independent enhancers that seemingly direct transcription in the same cell types of the heart? Are these

enhancers functionally redundant? Do the different transcriptional territories of the heart reflect specific cardiac lineages that have gone previously unrecognized? How do these specific patterns of transcriptional activity get established? Do they reflect cell autonomous gene regulatory programs or are they responsive to extracellular signals or hemodynamic influences within the developing heart? Answers to these questions promise to yield insight into the fundamental mechanisms for development, disease and evolution of the heart.

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