Identification of upstream regulatory regions in the heart-expressed homeobox gene Nkx2-5

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SUMMARY

Nkx2-5 marks the earliest recognizable cardiac progenitor cells, and is activated in response to inductive signals involved in lineage specification. Nkx2-5 is also expressed in the developing foregut, thyroid, spleen, stomach and tongue. One approach to elucidate the signals involved in cardiogenesis was to examine the transcriptional regulation of early lineage markers such as Nkx2-5. We generated F0 transgenic mice, which carry Nkx2-5 flanking sequences linked to a lacZ reporter gene. We identified multiple regulatory regions located within the proximal 10.7 kb of the Nkx2-5 gene. In addition to a proximal promoter, we identified a second promoter and a novel upstream exon that could participate in the regulation of Nkx2-5 transcription. Although used rarely in normal development, this novel exon could 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 as compared to wild-type Nkx2-5. An enhancer that directs expression in pharynx, spleen, thyroid and stomach was identified within 3.5 kb of exon 1 between the coding exon 1 and the novel upstream exon 1a. Two or more enhancers upstream of exon 1a were capable of driving expression in the cardiac crescent, throughout the myocardium of the early heart tube, then in the outflow tract and right ventricle of the looped heart tube. A negative element was also located upstream of exon1a, which interacted in complex ways with enhancers to direct correct spatial expression. In addition, potential autoregulatory elements can be cooperatively stimulated by Nkx2-5 and GATA-4. Our results demonstrate that a complex suite of interacting regulatory domains regulate Nkx2-5 transcription. Dissection of these elements should reveal essential features of cardiac induction and positive and negative signaling within the cardiac field.

Key words: Heart, Nkx2-5, Mouse, Transcriptional regulation

INTRODUCTION

Recent studies in Drosophila suggest that members of the NK-2 class of homeodomain proteins play an essential role in the establishment of myogenic lineages during development. NK4/tinman is first expressed throughout the presumptive mesoderm, then later during mesodermal subdivision in the dorsal-most cells from which muscles of the dorsal vessel and midgut are derived. In tinman mutants, mesoderm invagination and dorsal spreading appear normal; however, the dorsal vessel and visceral muscle lineages fail to form (Bodmer et al., 1990; Azpiazu and Frasch, 1993). These observations suggest that tinman is involved in mesodermal patterning, as well as specification and differentiation of the muscles of the heart and gut.

Two inductive signals regulating tinman expression have been identified. Decapentaplegic (dpp) expression in ectoderm is required to maintain tinman expression in dorsal mesoderm (Frasch, 1995). In addition, wingless (wg) expression in ectoderm and/or mesoderm is essential for subdivision of the dorsal mesoderm into segmentally reiterated cardiac precursor pools (Park et al., 1996). Elimination of wg function shortly after gastrulation, at a time when tinman expression is becoming restricted to the dorsal mesoderm, results in the selective loss of myocardial progenitor cells with little effect on other mesodermal lineages. Recently, Lee et al. (1997) and Yin et al. (1997) reported on the identification of four cis-acting elements that regulate tinman expression in the developing Drosophila embryo. The basic helix-loop-helix factor twist, which sits at the head of the mesoderm regulatory cascade, as well as tinman itself, directly transactivates the tinman gene (Lee et al., 1997; Yin et al., 1997)

In all vertebrates, the myogenic and endocardial lineages of the heart develop from anterior lateral plate mesoderm. Myocardial precursors of Amphibia and Aves are induced firstly by signals that emanate from the organizer or node, an
early embryonic patterning center, and then from anterior endoderm (Sater and Jacobson, 1989, 1990; Gilinka et al., 1998; Schultheiss et al., 1995; Nascone and Mercola, 1995). Schultheiss et al. (1997) and Andree et al. (1998) demonstrated that ectopic placement of bone morphogenetic factors, BMP2, BMP4 and BMP7, medial to the cardiac crescent of chick embryos is capable of inducing heart markers and/or a beating phenotype. There may be a conserved role for BMPs in induction of NK-2 genes, since, as noted above, inductive signaling by the BMP family member dp4 is required for maintenance of tinman expression in dorsal mesoderm of the fly embryo (Frasch, 1995).

Several vertebrate NK-2 class homeobox genes related to tinman have been isolated. To date, seven members are known in the mouse (Harvey, 1996; Pabst et al., 1997) and at least two of these are expressed in the heart. Furthermore, Nkx2-5 cognates have also been identified in the Xenopus (Tonissen et al., 1994), chicken (Schultheiss et al., 1995), zebrafish (Lee et al., 1996) and humans (Shiojima et al., 1996). All Nkx2-5 genes isolated to date are highly related in sequence and expression pattern. Murine Nkx2-5 is expressed in early heart progenitor cells, as well as in pharynx, tongue, stomach and spleen (Komuro and Izumo, 1993; Lints et al., 1993). Nkx2-6 is expressed from a later time in heart development, and only in the inflow and outflow regions (Biben et al., 1998).

Studies on NK-2 homeodomain factors have indicated that they are DNA-binding proteins capable of directly activating transcription (Mizuno et al., 1991; Damante et al., 1994; Chen and Schwartz, 1995; Reecy et al., 1997). DNA-binding assays show that Nkx2-5 and its relative Nkx2-1 bind to the novel sequence element 5'-TNAAGTG-3', which is different from the 5'-TAA T-3 motif recognized by Hox family members (Damante et al., 1994; Chen and Schwartz, 1995; Ray et al., 1996). In vitro assays also demonstrate that Nkx2-5 binds to serum response elements (SREs) within the alpha cardiac actin gene, as well as to an “NKE” element within the atrial natriuretic factor (ANF) promoter (Chen and Schwartz, 1996; Durocher et al., 1996). These elements are essential for high-level cardiac-specific promoter activity. Thus, Nkx2-5 can functionally and physically interact with SRF and GATA4 to regulate these target genes (Chen and Schwartz, 1996; Durocher et al., 1996; Sepulveda et al., 1998). Furthermore, NKE-like sites are also required for tinman activation of Drosophila MEF2 gene expression (Gajewski et al., 1997).

An attractive hypothesis from analysis of the Nkx2-5 expression profile and transfection experiments is that the Nkx2-5 homeodomain factor functions high in a phylogenetically conserved regulatory pathway for cardiac muscle development. However, whether Nkx-2.5 plays a role in specification of the cardiac lineages is still not known. Targeted mutagenesis of the endogenous murine Nkx-2.5 gene results in embryonic lethality around 9 days post coitum (Lyons et al., 1995), but a beating heart tube still develops, even though looping morphogenesis, a critical determinant of its form, is disrupted. Furthermore, several genes encoding myofilament proteins appeared to be expressed normally. However, expression of genes encoding ventricle-specific myosin light chain-2 (MLC-2v), ANF, the actin cross-linking protein SM-22/transgelin, and transcription factors CARP and eHAND, were abolished or spatially deranged (Lyons et al., 1995; Biben et al., 1997a,b; Zou et al., 1997). These experiments demonstrate an essential role for Nkx2-5 in normal heart morphogenesis and function, and implicate it as a component of the genetic circuitry controlling myogenic specialization of the ventricles.

Each of the known vertebrate NK-2 family members are expressed in unique spatiotemporal patterns in the developing embryo (Lazzaro et al., 1991; Price et al., 1992; Lints et al., 1993; Pabst et al., 1997; Nikolova et al., 1997; Biben et al., 1998). Other transcription factors expressed in the developing heart, including GATA4, SRF and MEF2 (Lints et al., 1993; Arceci et al., 1993; Edmondson et al., 1994; Croissant et al., 1996; Molkentin et al., 1996), are also expressed in a range of other tissues. These observations suggest that a mechanistic understanding of cardiac myocyte specification and determination will require detailed information on the molecular mechanisms controlling transcription of these key regulatory genes. In this study, we present our initial characterization of the regulatory sequences associated with the Nkx2-5 locus. Our functional analysis of the proximal 10.7 kb of 5' sequence has revealed the presence of two Nkx2-5 promoters, as well as two or more right ventricle-specific enhancers, which appear to work in concert, and an enhancer regulating expression in spleen, thyroid and pharyngeal endoderm. An inhibitory domain, which refines the spatial aspects of Nkx-2.5 expression, was also revealed. These sequences are capable of directing early expression of Nkx-2.5 in the bilateral heart field and the linear heart tube. Furthermore, Nkx2-5 and GATA4 were shown to cooperatively transactivate the Nkx2-5 regulatory region, suggesting collaboration in a positive autoregulatory loop.

**MATERIALS AND METHODS**

**Cloning of the mouse Nkx2-5 promoter**

A genomic fragment that contained the Nkx2-5 locus was isolated from a 129SVJ mouse genomic library which was cloned into the Lambda Fix II vector (Stratagene, La Jolla, CA). A 13 kb SalI fragment was cloned into the SalI site of pBluescript-SK to generate pBS-Nkx2-5 clone 2. This DNA fragment contained 10.7 kb of 5’ flanking sequences, the first exon and intron and part of exon 2. Approximately 2 kb of 3’ flanking sequences were kindly provided by Katherine Yutzey.

**Nkx2-5 mRNA analysis**

Total RNA was isolated with TRIzol™ (Life Technologies, Gaithersburg, MD) as previously described (Reecy et al., 1997).

5’ Rapid amplification of cDNA ends

First-strand cDNA synthesis was performed with 5 μg of total RNA and random primers (Gibco BRL). Ligation-anchored PCR was used to extend the Nkx2-5 cDNA in the 5’ direction as previously described Reecy et al. (1997) with the following primers: anchor-specific primer 5’-GGGCGCTTTTATTAAAACCTCCTACTAAA-3’, reverse gene-specific primer 1 5’-CTTTTGGCCAGTCTCACAGTGGTGAATG-3’ and reverse gene-specific primer 2 5’-TTATTCCGCCCGAGGTTCTTGTG-3’.

**RNase protection analysis**

The RNase protection probe was generated by PCR, in order to add additional non-transcribed sequences to the 5’ end of the probe, with the following primer pairs: Exon 1a – 5’-ATGGAATTTGCGAC-TATGGTGAATG-3’ and 5’-CAGTCTAGAAACGCGTGATC-3’, Exon 1 – 5’-GCTCAAAGACATCCTGGAACCTG-3’ and 5’-CCTCGGT-GTCTAGAGTGAC-3’. PCR reactions (50 μl; 10 mM Tris-HCl, pH
RT-PCR analysis

Exon 1a- and exon 1-specific primers were used in combination with a common reverse primer for RT-PCR analysis: exon 1a (5'-GAATCCGGTTCCTCCT TTG-3'), exon 1 (5'-CCAATGGCAG-GCTGAATCC -3'), and Nkx2-5 Rev (5'-TTATCCCGCCGAGGTCT-TTTG-3'). PCR products were digested with cDNA generated from 0.5 μg of total RNA (Gibco, BRL), which was isolated from the indicated tissues. RT-PCR products were visualized by hybridization with a radiolabeled Nkx2-5 cDNA probe, subcloned into pCRRII (Invitrogen, Carlsbad, CA) and sequenced.

Section in situ hybridization

Sequence in situ were performed as described by Albrecht et al. (1997). 35S-labelled RNA probes were synthesized from a full-length Nkx2-5 cDNA by restriction endonuclease digestion with NotI and transcribing with T7 RNA polymerase (antisense), or restriction transgenes were removed from vector sequences by digestion and T3 RNA polymerase. RNase protection assays were performed according to manufacturer’s protocol (Ambion, Austin, TX).

Generation of Nkx2-5 promoter constructs

To generate the −10765D and −7238U Nkx2-5 reporter constructs, pBS-Nkx2-5 clone 2 was digested with XhoI or partially digested with Xba restriction endonuclease, respectively. The lacZ and Luciferase cDNA from pPD46.21 (Fire et al., 1990) and pGL3-Basic (Promega, Madison, WI) were subcloned in-frame into the XhoI and XbaI sites of pBS-Nkx2-5 clone 2 after modification. Deletion constructs were generated by restriction endonuclease digestion. To produce −10765D-Rev, −10765D was restriction endonuclease digested with NotI and ligated back together. Reverse orientation of the insert was determined by restriction endonuclease digestion.

Generation, collection and identification of transgenic embryos

lacZ transgenes were removed from vector sequences by SalI-XhoI restriction endonuclease digestion followed by size separation on agarose gels. DNA fragments were purified from the agarose gel with Qiaex beads (Qiagen, Chatsworth, CA). DNA fragments were eluted with 0.1x TE. Transgenic embryos were produced by pronuclear injection of 1-cell-stage embryos (Hogan et al., 1986). Embryos were harvested 7.25 to 13.5 d.p.c and stained for β-gal activity (Behringer et al., 1993). Paraffin-embedded embryos were sectioned (10 μm), dehydrated and the nuclei were counter stained with nuclear fast red. Sections were hybridized overnight at 58°C and washed at 64°C. Sections were processed for emulsion autoradiography and were post-stained with Hoechst 33258 and visualized by epifluorescence and dark-field microscopy.

RESULTS

Identification and characterization of a novel 5′ Nkx2-5 exon

The Nkx2-5 gene contains two coding exons (Lyons et al., 1995). However, the presence of an additional 5′ exon was suggested by the DNA sequence of a single Nkx2-5 cDNA clone isolated from an adult mouse heart library (see Lints et al., 1993). Although this clone appeared to be a head-to-tail fusion between two separate cDNA fragments, the Nkx2-5 portion contained sequences at its 5′ end that could not be found in continuity with the first coding exon in corresponding genomic sequences (data not shown). A fragment of the unique sequence was also present in the cDNA published by Komuro and Izumo (1993), but in reverse orientation, consistent with our view that this cDNA contains an inversion. An oligonucleotide probe specific to the putative exon detected homologous sequences approximately 3.5 kb upstream of the Nkx2-5 initiation methionine (data not shown), suggesting that they represented a genuine exon. To clarify the nature of this exon further, we performed 5′ RACE analysis on total RNA isolated from adult mouse heart. Sequence analysis of 5′ RACE fragments and comparison with Nkx2-5 genomic sequences confirmed the presence of a novel exon of at least 89 bp (exon 1; Fig. 1A). This exon contained a putative initiation ATG and, in fragments isolated by 5′ RACE, was spliced in frame into exon 1 of the Nkx2-5 gene. Translation of this mRNA splice variant would result in a Nkx2-5 protein that contained a novel amino terminus that lacks part of the evolutionarily conserved TN-domain (Fig. 1A). However, ribonuclease protection analysis (RPA) with a probe that spans part of exon 1 (bp +40 to +178, relative to the translation start site), and spans all of exon 1a and some sequences 5′ of exon 1a (bp −3781 to −3504), revealed that the splice variant was not
expressed at significant levels in the hearts of 15-16 d.p.c. embryos (Fig. 1B) or adults (data not shown). We next examined whether there was a tissue-specific distribution of exon 1a by RT-PCR analysis (Fig. 1C). The results showed that exons 1 and 1a were transcribed with a similar distribution, although some differences were noted. We found expression of Nkx2-5 in the previously unrecognized sites of testis, lung and thymus (see Lints et al. 1993); among these, exon 1a was only detected in thymus (Fig. 1C). Although the RT-PCR analysis was not quantitative, both exons were found most abundantly in heart.

In RT-PCR reactions with exon 1a-specific primers, two to three bands were generally detected on agarose gels (Fig. 1C and data not shown). Upon sequencing of RT-PCR products, we found that exon 1a could be alternatively spliced into coding exons 1 or 2 in at least four different configurations (Fig. 1D). Three of the products had spliced exon 1a in frame with the protein-coding portion of Nkx2-5. In one case exon 1a was spliced into exon 1 as described above, in the second exon 1a was spliced in 260 bp 3¢ of the exon1 ATG, in the other exon 1a was spliced into the splice acceptor site of exon 2 just upstream of the homeodomain. Due to the presence of a potential translation start site in exon 1a, alternative splicing may generate mRNAs that encode Nkx2-5 proteins with different biological activities. However, our analysis suggests that these alternative versions of Nkx2-5 mRNA are rare in hearts in the course of normal development, and probably in all other tissues in which Nkx2-5 is expressed.

Transcriptional activity of Exon 1a-containing Nkx2-5 proteins

To assess the function of Nkx2-5 proteins that contain Exon 1a translated sequences, we performed transient transfection experiments with an NKE reporter construct (Fig. 2). Translation of the Nkx2-5-155 protein is terminated 57 bp 3¢ of the exon 1 splice acceptor site; however, it still contains the wild-type translation start site. The protein(s) translated from this expression construct was weakly capable of transcriptional activation. Similarly, the splicing of exon 1a into exon 1 (+40 and +260 relative to the wild-type Nkx2-5 translation start site) or exon 2 decreased the ability of Nkx2-5 to transactivate transcription. Thus, the presence of exon 1a amino acid sequences destroyed the ability of Nkx2-5 to transactivate an NKE reporter construct.

Two promoters direct tissue-specific Nkx2-5 gene expression

Before testing for regulatory elements in the Nkx2-5 promoter, we performed in situ hybridization experiments to examine the tissue distribution of Nkx2-5 expression at 12.5 d.p.c. (Fig. 3), which is the approximate age at which expression from promoter constructs was to be analyzed (see below). Robust expression was found in heart, thyroid, tongue, stomach and spleen, which confirm the data of Lints et al. (1993). Expression was not detected in lung or thymus, in which transcripts were found by RT-PCR (see Fig. 3C).

We tested the ability of Nkx2-5/lacZ transgenes to recapitulate the endogenous Nkx2-5 expression pattern in F0 transgenic embryos. At 13.5 d.p.c., a transgene construct, which contained 10.7 kb of DNA upstream of the Nkx2-5 initiator methionine (−10765D), was highly expressed in the atrial-ventricular canal, interventricular septum and the trabeculae of the right ventricle and outflow tract of the heart, spleen primordia, pyloric region of the stomach, thyroid and ventral endoderm of the pharynx (Fig. 3B-F). In contrast, endogenous Nkx2-5 was normally expressed throughout the heart myocardium, as well as in the tongue (Fig. 2). Thus, high-level Nkx2-5/lacZ transgene expression was not observed in the right and left ventricle free walls or atria. We conclude that sequences within the proximal 10.7 kb of the Nkx2-5 5¢ flanking sequences are capable of driving transgene reporter expression.
expression in a subset of the normal Nkx2-5 expression domains.

To determine where the Nkx2-5 regulatory domains were located, we first constructed two lacZ transgenes, −3512D and −7238U, which contained lacZ fused in-frame to exons 1 or 1a, respectively (Fig. 4A). In −3512D transgenic embryos, lacZ expression was detected in the outflow tract of the heart, pharynx, thyroid, spleen primordium and stomach (Fig. 4G-K). In addition, ectopic expression was detected in the telencephalon, branchial arch arteries and pericardial sac (Fig. 4L,K). In contrast, the −7238U transgene was expressed exclusively in the atrial-ventricular canal, interventricular septum and outflow tract of the heart (Fig. 4L,M). In this series, three other constructs were analyzed (Fig. 4A). In −1626D transgenic mice, no β-gal activity was detected (data not shown). However, in embryos carrying a minimal region upstream of exon 1a (−887U), expression was seen in the outflow tract of the heart but not in the right ventricle (Fig. 4G-K). Thus, there appears to be two promoters capable of directing high level expression of the Nkx2-5 gene, as well as enhancers that direct expression to the right ventricle and outflow tract of the heart and several extracardiac sites.

Interacting regulatory regions direct expression in right ventricle

Does the right ventricular/outflow tract enhancer work exclusively with the promoter associated with exon 1a? To address this issue, we reversed the orientation of the sequences located between −10765 and −3512, while maintaining sequences between −3511 and exon 1 in their normal orientation (−10765D-Rev; Fig. 5A). In this construct, transcription initiated from exon 1a would not result in expression of the lacZ gene (Fig. 5A). In −10765D-Rev transgenic embryos, lacZ expression was detected in pharynx, spleen primordia, and stomach, identical to the pattern seen in −3512D embryos. In addition, robust lacZ expression was detected throughout the right ventricular and outflow tract myocardium (Fig. 5B-F). Thus, the right ventricular/outflow tract enhancer was capable of regulating the exon 1 promoter, consistent with our finding that exon 1a is utilized only rarely in embryonic and adult hearts (see Fig. 1B). To further delineate the cardiac element(s), we generated a series of deletion mutants that lacked 5′ flanking sequences (−8554D, −5870D, −5011D, −4434D and −4059D; Fig. 5A). lacZ expression was lost in the atrial-ventricular canal, interventricular septum and right ventricle when sequences 5′ of −8554 were deleted. However, the cis-acting element located 5′ of −8554 was incapable of directing high level expression in the right ventricle when analyzed in conjunction with the downstream Nkx2-5 promoter (Fig. 5L,M). Thus, an essential enhancer would appear to be located between −10765 and −8554. While no expression in heart was seen with −5870D, lacZ expression was again detected, albeit sparsely, in the right ventricle when sequences 5′ of −5011 were deleted (Fig. 5A,G-K). In this case, however, ectopic expression was detected in brain and the costal region, in either rib cartilage or intercostal myoblasts (Fig. 5G-K). Furthermore, it appears that this proximal right-ventricular enhancer works in conjunction with the distal enhancer (−10765 to −8554) to override the negative cis-acting element located between −5870 and −5011 to direct high level transgene expression in the atrial-ventricular canal, interventricular septum and right ventricular trabecula (Fig. 5N,O). Thus, multiple cardiac elements are modified in a complex way by an inhibitory domain located between −5870 and −5011. Deletion of this inhibitory domain also leads to ectopic Nkx2-5 expression, suggesting that it works in collaboration with the cardiac elements to ensure spatial specificity.

Nkx2-5 transgene expression in the cardiac crescent and early heart tube

At 7.25 d.p.c., the −10765D transgene directed expression exclusively in cardiac primordia in a bilaterally symmetrical manner (Fig. 6A,B). In comparison to the endogenous Nkx2-5 expression pattern, our transgene did not appear capable of directing lacZ expression throughout the whole crescent. However, in the early looping heart tube, expression was detected in both right and left ventricles and in the developing common atrium (Fig. 6D), although expression was stronger in the cranial segments. This contrasts the situation in older hearts, in which expression was absent from left ventricle and atria (see above). These findings suggest that one or more cardiac enhancers located within the −10765 region can drive Nkx2-5 expression in all primitive chambers of the heart tube, but can only maintain expression in the cranial segments. In early looping hearts of embryos which carry the −3512D transgene, myocardial expression at 13.5 d.p.c. was not observed; expression was seen primarily in the ventral floor of the pharynx and in a portion of the inflow and outflow tracts of the heart adjacent to the dorsal mesocardium. The majority of the heart tube itself was devoid of expression (Fig. 6E,F).

Nkx2-5 and GATA4 cooperatively transactivate the Nkx2-5 gene

Nkx2-5 and GATA4 are required for normal heart development (Kuo et al., 1997; Molkentin et al., 1997; Lyons et al., 1995). In addition, they can cooperatively transactivate the ANF and
cardiac α-actin promoters (Durocher et al., 1996; Sepulveda et al., 1998). Sequence analysis revealed multiple potential binding sites for Nkx2-5 and GATA scattered throughout the identified Nkx2-5 cis-acting elements. To investigate the possible role of these two cardiac-specific transcription factors in regulation of Nkx2-5, we tested their ability to transactivate the Nkx2-5 gene. Individually, neither Nkx2-5 nor GATA4 were capable of transactivation (Fig. 7). However, the combination of Nkx2-5 and GATA4 increased Nkx2-5 promoter activity 3- to 5-fold. In addition, a C-terminal deletion of Nkx2-5 lacking the conserved NK-2-specific domain (Harvey, 1996), which shows increased transcriptional activity on synthetic reporter genes in vitro (Chen and Schwartz, 1995) only transactivated the upstream Nkx2-5 promoter. Thus, Nkx2-5 and GATA4 may act in combination in an autoregulatory feedback loop supporting Nkx2-5 expression.

DISCUSSION

Nkx2-5 is a vertebrate member of the NK-2 class of homeobox genes, which appear to function during the early period of
organogenesis in the developing embryo. Nkx2-5 is expressed in early heart myocardiocyte progenitors and developing heart, as well as in the pharynx, spleen, stomach and tongue (Lints et al., 1993). Gene knockout studies have demonstrated an important role for Nkx2-5 in heart and pharyngeal patterning (Lyons et al., 1995; Biben and Harvey, 1997). In addition, in vitro studies have shown that the protein can directly transactivate cardiac promoters (Sepulveda et al., 1998; Chen et al., 1996; Durocher et al., 1996).

The Nkx2-5 protein sequence, as predicted from the DNA sequence of cDNA clones (Lints et al., 1993; Komuro and Izumo, 1993), is encoded by two genomic exons (Lyons et al., 1995). However, as reported here, examination of other cDNA clones and 5' RACE products, as well as mapping of the natural Nkx2-5 mRNAs by RT-PCR analysis, has revealed the presence of a

![Diagram](image)

**Fig. 5.** The downstream Nkx2-5 promoter is regulated by putative right ventricular enhancers located between bp –10765 and –8554, and –5011 and –4434. (A) Schematic representation of the Nkx2-5 enhancer constructs analyzed in F0 day 12.5 embryos and a summary of the transgene expression patterns. The orientation of Nkx2-5 exons (red) and their relationship to the lacZ marker gene (blue) are shown. (i) Sequences –10765 to –3512 were reversed so that exon 1a initiated transcripts would not result in lacZ mRNA transcription. No β-gal activity was detected in the right ventricle of –8554D, –5867D, –4434D and –4059D transgenic animals (data not shown). (B-F) –10765D-Rev transgenic lacZ expression. (G-K) –5011D transgenic lacZ expression. lacZ activity was detected in the brain in an inconsistent pattern (data not shown). (L,M) –6439/–3615D transgenic lacZ expression. (N,O) –8554/–5870D transgenic lacZ expression. At, atrium; G, Gonad; Lb, Limb bud; LTG, Laryngeal Tracheal Groove; LV, Left Ventricle; OB, Omentum Bursa; Ph, Pharynx; RV, Right Ventricle; St, Stomach.

![Image](image)

**Fig. 6.** Nkx2-5 transgene expression in 7.25-9.5 d.p.c. mouse embryos. The expression pattern of the –10765D transgene in day 7.25 (A,B) and day 8-8.5 (C,D) mouse embryos. (A) Lateral view. (B) Ventral view. –10765D transgene expression is observed in the bilateral heart fields (arrows). (C) Lateral view. β-galactosidase activity is observed in the looping heart tube and underlying tissues. (D) Frontal section at the level indicated in C. β-gal staining is present throughout the heart tube. The expression pattern of –3512D transgene in day 9-9.5 mouse embryos (E,F). (E) Lateral view. β-galactosidase activity was observed in the inflow and outflow tracts of the heart. (F) Transverse section at the level indicated in E. β-gal staining was observed in the dorsal mesocardium and ventral pharyngeal endoderm. Ec, Endocardium; HT, Heart tube; Mc, Myocardium; NT, Neural Tube; Ph, Pharynx.
We have begun to analyze the cis-acting elements that regulate Nkx2-5 expression: identification of two transcripts could play an important role. Or under certain physiological or pathological conditions, these adults heart. It is formally possible that they result from cryptic low in abundance in the hearts of mid-gestation embryos and RNase protection analysis suggests that their mRNAs are very possibilities for novel Nkx2-5 proteins are intriguing, our were ineffectual in transfection assays. While these splicing of exon 1a were detected by RT-PCR analysis (see Fig. 4A). The exon 1-proximal sequences in −3512D (the ‘downstream’ sequences) were able to drive expression in outflow tract, ventral pharynx, thyroid, spleen and stomach at 12.5-13.5 d.p.c., but not in the right ventricle trabecula and free wall. A shorter construct from this downstream region (−1626D) was unable to direct lacZ gene expression at all. The exon 1a-proximal sequences in −7238U (‘upstream’ sequences), on the other hand, directed expression in the atrial-ventricular canal and interventricular septum, but not in the right ventricle free wall or trabecula or other tissues. A minimal region proximal to exon 1a (−887U) could, however, drive expression in a portion of the outflow tract. These results demonstrate the existence of functional promoters associated with exons 1 and 1a, and multiple cis-acting elements that drive expression in the right ventricle of the heart and other tissues. To directly demonstrate that the cis-acting elements that direct transgene expression in right ventricle work through the downstream promoter as suggested by our RPA analysis, we generated a transgene in which sequences between −10765 and −3512 were reversed (−10765D-Rev; see Fig. 5A), which placed the exon 1a transcriptional start site distal to the exon 1 lacZ reporter, and in an opposite orientation. The lacZ expression pattern in the right ventricle in the −10765D-Rev embryos was similar to that seen in normal −10765D embryos, which indicates that a right ventricle-specific cardiac enhancer is located between −10765 and −3512. Furthermore, this enhancer can function robustly through the downstream promoter. Thus, even though the upstream promoter can work efficiently in the context of some constructs, it appears that in normal heart development, the right ventricular enhancer works primarily through the downstream promoter.

Nkx2-5 regulatory sequences: identification of two promoters

We have begun to analyze the cis-regulatory regions of the Nkx2-5 locus in F0 transgenic mice that carry Nkx2-5/lacZ reporter constructs. The first question addressed relates to the issue of whether functional promoters could be found in the proximity of exons 1 and 1a. We analyzed a lacZ reporter construct which contained 10.7 kb of Nkx2-5 5’ flanking region (−10765D). This transgene could drive expression in a tissue-specific and temporal-specific manner, recapitulating some aspects of the endogenous Nkx2-5 expression pattern (Komuro and Izumo, 1993; Lints et al., 1993). The −10765D transgene directed lacZ expression at the earliest time points of cardiac development in bilaterally symmetrical progenitor pools in anterior lateral plate mesoderm, then in the linear heart tube and eventually the right ventricle and outflow tract myocardium. It also directed expression in the spleen, pharynx, thyroid and stomach. However, the transgene could not direct expression to the tongue, or maintain robust expression in the right and left ventricular free wall and atria of the heart (see below), sites where Nkx2-5 is normally expressed (Komuro and Izumo, 1993; Lints et al., 1993).

To test for promoter function, we physically separated the region upstream of exon 1a from that upstream of exon 1. Construct −3512D linked lacZ to all sequences between exon 1a and exon 1, but did not include the transcription start site of exon 1a, while construct −7238U contained sequences upstream of exon 1a, including its transcriptional start site (Fig. 4A). The exon 1-proximal sequences in −3512D (the ‘downstream’ sequences) were able to drive expression in outflow tract, ventral pharynx, thyroid, spleen and stomach at 12.5-13.5 d.p.c., but not in the right ventricle trabecula and free wall. A shorter construct from this downstream region (−1626D) was unable to direct lacZ gene expression at all. The exon 1a-proximal sequences in −7238U (‘upstream’ sequences), on the other hand, directed expression in the atrial-ventricular canal and interventricular septum, but not in the right ventricle free wall or trabecula or other tissues. A minimal region proximal to exon 1a (−887U) could, however, drive expression in a portion of the outflow tract. These results demonstrate the existence of functional promoters associated with exons 1 and 1a, and multiple cis-acting elements that drive expression in the right ventricle of the heart and other tissues.

A single enhancer directs Nkx2-5 expression in ventral pharynx, stomach, spleen and thyroid, but requires upstream sequences for full specificity

The cis-acting elements that regulate Nkx2-5 expression in the ventral pharynx, thyroid, stomach and spleen primordia are located within the sequences located between exons 1 and 1a. Deletion analysis places an enhancer between −1.7 and −3.5

![Fig. 7.](image-url)
Nkx2-5 expression in the developing heart is subject to complex transcriptional regulation

We have demonstrated the presence of cis-regulatory elements that control Nkx2-5 expression in the lateral plate mesoderm, linear heart tube and right ventricle. The −10765D lacZ reporter transgene was expressed from the earliest time in cardiac development, in bilaterally symmetrical cell groups within the anterior lateral plate mesoderm. It is interesting to note, however, that this transgene appeared unable to activate reporter gene expression throughout the whole cardiac crescent yet could direct some expression in all chambers of the early heart tube. Expression in the left ventricle and atria, could not be maintained after that point. Thus, in addition to the domains that confer spatial regulation in heart, temporal-specific enhancers and autoregulatory domains may be missing from our transgene constructs. Oka et al. (1997) recently presented in vitro evidence for an autoregulatory region within the first intron of CSX1, the human cognate of Nkx2-5.

While we have not precisely delimited the number and position of the cardiac regulatory elements within the proximal 10.7 kb, our deletion analysis indicated the presence of at least two but possibly more cardiac enhancers within sequences upstream of exon 1a. We have also detected an inhibitory domain that interacts with the enhancer elements. The −10765D transgene gave robust expression in the right ventricle, although the shorter transgene −8554D gave no such pattern. However, further deletion re-established the right-ventricular expression pattern. Whether there are two or more right ventricular enhancers is not yet clear, but it is apparent that complex interaction occur between positive and negative elements within the Nkx2-5 regulatory domains.

Nkx2-5 is expressed uniformly throughout the heart myocardium. One theoretical way to accomplish this would be to have a single element that controls Nkx2-5 expression in this tissue layer. However, the lack of evidence for a master gene regulatory mechanism for cardiac myogenesis (Evans et al., 1995), and the results of our transgenic analysis, favor a model in which distinct but interactive positive and negative cis-acting elements control regional expression of Nkx2-5 in the heart. This model is consistent with the compartmental-specific expression of a growing number of transgenes and endogenous genes in the developing heart (Ross et al., 1996; Franco et al., 1997; He and Burch, 1997). Why should a transcription factor gene expressed throughout the whole heart myocardium have such an elaborate transcriptional control mechanism? Based on genetic and evolutionary considerations, it has been suggested that the heart is a modular organ, with individual modules that represent unique and separate innovations that were added to the heart in a stepwise manner during evolution. This model accounts for the null phenotypes of several heart-expressed genes, in which individual modules were deleted with little effect on overall patterning (Fishman et al., 1997a,b).

In the developing Drosophila embryo, Lee et al. (1997) and Yin et al. (1997) have recently reported the identification of four cis-acting elements that regulate tinman expression. Each of these elements was differentially utilized throughout development in a cell-type-specific and temporal-specific manner. In addition, they responded to different signaling molecules. By analogy, the control of Nkx2-5 transcription may also be highly modular, with individual elements able to respond to different developmental cues. For example, bone morphogenetic proteins (BMPs) appear to have an early role in signaling the formation of heart mesoderm in vertebrate embryos (Schultheiss et al., 1997; Andree et al., 1998). We can expect that sequences upstream of the Nkx2-5 locus will include those capable of directing transgene expression in the lateral plate mesoderm under BMP control.
Cardiac restricted transcription factors can cooperatively transactivate Nkx2-5 promoter activity

The presence of NK-2 homeodomain and GATA factor DNA-binding site in the Nkx2-5 regulatory sequences raised the possibility that Nkx2-5 transcription is controlled via direct transactivation by Nkx2-5 and GATA4. Both Nkx2-5 and GATA4 can transactivate cardiac promoters (Molkentin et al., 1994; Chen and Schwartz, 1995, 1996; Durocher et al., 1996; Sepulveda et al., 1998). We found, however, that neither Nkx2-5 nor GATA4 alone were capable of transactivating Nkx2-5 promoter/enhancer constructs in vitro (Fig. 7). In contrast, a combination of Nkx2-5 and GATA4 transactivated to a modest degree, although coactivation was not as robust as that seen with other promoters (i.e., ANF and cardiac α-actin). Thus, activation and maintenance of Nkx2-5 expression most likely involves other factors in addition to Nkx2-5 and GATA4. Recently, Xu et al. (1998) reported that Tinman was required in combination with the Dpp signaling pathway to induce Tinman transcription, which suggests that Nkx2-5 like tinman may function in a feedback loop to regulate their own transcription. Further analysis of the regulatory elements highlighted in this study may help to identify such factors.

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REFERENCES


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