A GATA-dependent nkar-2.5 regulatory element activates early cardiac gene expression in transgenic mice

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SUMMARY

nkx-2.5 is one of the first genes expressed in the developing heart of early stage vertebrate embryos. Cardiac expression of nkx-2.5 is maintained throughout development and nkx-2.5 also is expressed in the developing pharyngeal arches, spleen, thyroid and tongue. Genomic sequences flanking the mouse nkx-2.5 gene were analyzed for early developmental regulatory activity in transgenic mice. Approximately 3 kb of 5' flanking sequence is sufficient to activate gene expression in the cardiac crescent as early as E7.25 and in limited regions of the developing heart at later stages. Expression also was detected 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. The observed expression pattern from the ~3 kb construct represents a subset of the endogenous nkx-2.5 expression pattern which is evidence for compartment-specific nkx-2.5 regulatory modules. A 505 bp regulatory element was identified that contains multiple GATA, NKE, bHLH, HMG and HOX consensus binding sites. This element is sufficient for gene activation in the cardiac crescent and in the heart outflow tract, pharynx and spleen when linked directly to lacZ or when positioned adjacent to the hsp68 promoter. Mutation of paired GATA sites within this element eliminates gene activation in the heart, pharynx and spleen primordia of transgenic embryos. The dependence of this nkx-2.5 regulatory element on GATA sites for gene activity is evidence for a GATA-dependent regulatory mechanism controlling nkx-2.5 gene expression. The presence of consensus binding sites for other developmentally important regulatory factors within the 505 bp distal element suggests that combinatorial interactions between multiple regulatory factors are responsible for the initial activation of nkx-2.5 in the cardiac, thyroid and spleen primordia.

Key words: nkx-2.5, GATA, Heart, Spleen, Transgenic analysis, Mouse

INTRODUCTION

The heart is the first organ to function in vertebrate embryos and cardiac lineages are among the first to be specified during development. Regulatory factors responsible for establishing and maintaining cardiac lineages are present in the anterior lateral heart forming regions of mid to late gastrulation stage embryos. Nk-x-2.5 (Csx-1), a homologue of the Drosophila gene tinman (tin), is one of the first genes to be expressed specifically in the heart-forming region of vertebrate embryos (Lints et al., 1993; Komuro and Izumo 1993). Loss of tin expression in Drosophila embryos results in the complete absence of cardiac and visceral muscle lineages (Azpiazu and Frasch, 1993; Bodmer, 1993). Similarly, targeted mutation of nkx-2.5 in mice produces lethal cardiovascular defects but the precise cause of death in these embryos is unknown since early formation of the primitive heart tube proceeds normally (Lyons, et al., 1995). Other related NK-2 homeobox proteins are temporally and spatially regulated during cardiac and pharyngeal development and may partially compensate for the mutation of nkx-2.5 in these mice (Lints et al., 1993; Komuro and Izumo, 1993; Buchberger et al., 1996; Brand et al., 1997; Reecy et al., 1997). The examination of the regulatory mechanisms that govern the temporal and spatial restriction of nkx-2.5 gene expression will provide important insights into the regulatory networks involved in early cardiac lineage determination and differentiation.

In the mouse, nkx-2.5 is one of the initial markers of the cardiogenic region and is expressed in the heart throughout development and in the adult. It is the first NK-2 family member to be expressed in the heart primordia of vertebrate embryos (Lints et al., 1993; Tonissen et al., 1994; Schultheiss et al., 1995). Additional NK-2 family members, including nkx-2.3 and nkx-2.8 are expressed in the chick heart with the initiation of cardiomyogenic differentiation (Buchberger et al., 1996; Brand et al., 1997; Reecy et al., 1997). Nkx-2.5 and other related NK family members are expressed in distinct but overlapping
patterns in the developing foregut and pharyngeal arches and their derivatives. An NK-2 code has been described whereby nkx-2 family members have distinct but related roles in cardiac and pharyngeal differentiation and patterning (Reecey et al., 1997). The analysis of regulatory elements controlling the restricted patterns of NK-2 homeobox gene expression likely will reveal molecular interactions involved in the early establishment of these cell lineages. After midgestation, obvious sites of nkx-2.5 gene expression are the tongue, thyroid and spleen (Lints et al. 1993; Kasahara et al., 1998). The complexity of nkx-2.5 gene expression throughout development is likely achieved through temporally and spatially distinct regulatory pathways acting through multiple cis-acting regulatory elements.

Analysis of cis-acting regulatory elements controlling tin gene expression in Drosophila embryos indicates that at least four distinct enhancer elements are responsible for activation of gene expression in the head, trunk mesoderm, cardioblasts and dorsal mesoderm respectively (Yin et al., 1997). Each of these elements is under the control of distinct regulatory pathways. Expression in dorsal mesoderm which develops into cardioblasts and visceral muscle is sensitive to decapentaplegic (dpp) signaling and cardioblast-specific tin expression is responsive to wingless (wg) signaling (Yin et al., 1997). Wg signaling has been shown to be required for cardiogenesis in Drosophila, however a direct interaction between wg signaling molecules and tin gene expression has not been demonstrated (Wu et al., 1995; Park et al., 1996). In vertebrates, the heart forming region is in contact with cells expressing BMPs, which are the vertebrate homologues of dpp, and with Wnts, the vertebrate homologues of wg (Kispert et al., 1996; Monley et al., 1996; Schultheiss et al., 1997; Andree et al., 1998). In chick embryos, early expression of nkx-2.5 in anterior mesoderm has been shown to be activated by BMPs (Schultheiss et al., 1997; Andree et al., 1998). Additional early regulators of cardiogenesis are MEF-2C and GATA4, 5 and 6 which are required for the expression of a number of cardiac-specific genes and are expressed with nkx-2.5 in the cardiogenic mesoderm prior to heart formation (reviewed in Olson and Srivastava, 1996). The regulatory pathways controlling heart development in vertebrates are dependent on these factors but the hierarchical relationships between them are not well understood.

In order to identify regulatory interactions important for early embryonic gene expression, the cis-acting elements controlling nkx-2.5 gene expression during mouse development were examined. Nkx-2.5 flanking sequences were linked to a lacZ reporter gene and tested in transgenic mice. Approximately 3 kb of 5′ flanking sequence is sufficient to activate expression in the heart forming region prior to cardiomyogenic differentiation and in the outflow tract and right ventricle of the heart, pharynx and spleen later in development. A 505 bp distal element activates gene expression during early cardiac, pharynx and spleen development and recapitulates the expression observed with the 3 kb construct. The distal element contains multiple consensus binding sites for regulators of cardiogenesis including NK and GATA factors (Ko and Engel, 1993; Merika and Orkin, 1993; Chen and Schwartz, 1995). Gene activation by the distal regulatory element in the heart, pharynx and spleen is dependent on paired GATA sites since mutation of these sites eliminates gene activity. The presence of multiple consensus binding sites for a variety of developmentally important regulatory factors in the nkx-2.5 distal element suggests that complex factor interactions govern distinct temporal and spatial regulation of nkx-2.5 gene expression during development.

**MATERIALS AND METHODS**

**Isolation of the mouse nkx-2.5 gene and construction of lacZ transgenes**

The mouse nkx-2.5 (cxs-1) cDNA (gift from Dr Seigo Izumo) was used to probe a J129 mouse genomic library (Matzuk et al., 1992; Komuro and Izumo 1993). Approximately 10^7 pfu were plated and phage DNA was transferred to nitrocellulose filters using standard procedures (Mamiatis et al., 1982). A 982 bp cxs-1 fragment, purified after excision from pBluescript with EcoRI, was radiolabeled using the High Prime labeling system (Boehringer) in the presence of [α-32P]dCTP. Filters were hybridized at 60 °C in 5x SSC, 5x Denhardt’s, 0.1 mg/ml denatured herring testis DNA, 0.2% SDS overnight. Hybridized filters were washed twice at room temperature in 2x SSC/0.1% SDS and twice at 60 °C in 0.2x SSC/0.2% SDS. Positive phage were purified through secondary and tertiary screening. Phage DNA was isolated from liquid lysates, restricted with NotI and subcloned into pBluescript for further analysis. The entire reported nkx-2.5 coding sequence is present in an 8.1 kb NotI fragment (Lyons et al., 1995). Both DNA strands of this fragment were subjected to sequence analysis using primers within the vector and primers synthesized from internal nkx-2.5 sequences. An additional 6 kb of 5′ flanking sequence was identified in the original lambda clone by Southern blot analysis. The orientation of the additional nkx-2.5 5′ flanking genomic sequence was confirmed by sequence analysis after comparison to the original isolate.

Constructs for transgenic analysis were generated with −959 bp, −3059 bp or −9 kb of nkx-2.5 flanking sequence 5′ to the reported transcriptional start site (Lyons et al., 1995). The lacZ reporter gene including the ATG initiation codon was isolated from pAC CMV/pLplA (Gomez-Foix et al., 1992) and linked to the SV40 small t-intron and poly(A)+ addition site derived from pCXIZ (Mikawa et al., 1991). An XhoI site was inserted at +223 of nkx-2.5 exon 1 which lies 5′ to the translational initiation site of nkx-2.5. The −3059nkx-2.5/lacZ transgene construct was generated by using the NotI site at −3059 of the original genomic fragment and the XhoI site at −959 of the XhoI site at +223. The additional 6 kb of 5′ flanking sequence was linked to the −3059nkx-2.5/lacZ transgene at the 5′ endogenous NotI site to generate the −9000nkx-2.5/lacZ transgene. The −3059−2554 distal nkx-2.5 regulatory element corresponds to a NotI/DraI restriction fragment which was linked directly to the lacZ/SV40 UTR transgene in pBluescript. The NotI/DraI nkx-2.5 distal element (−3059 to −2554) was inserted into the SalII/HindIII sites 5′ to the mouse inducible heat shock promoter hsp68 adjacent to lacZ (Kothary et al., 1989). The junctions of all constructed plasmids were confirmed by sequence analysis prior to generation of transgenic mice.

**Generation and analysis of transgenic mice**

DNA restriction fragments containing the nkx-2.5 flanking sequence linked to the lacZ reporter gene were separated from plasmid sequences by gel electrophoresis and purified. Transgenic mice were generated after pronuclear microinjection of FVB/N mouse fertilized eggs. Founder animals were identified by PCR analysis of genomic DNA using primers in the lacZ gene (Colbert et al., 1996). For F0 analysis of lacZ expression, embryos were isolated from implanted dams 7-10 days after microinjection. For established transgenic lines, embryos were isolated after timed matings of confirmed transgenic with nontransgenic mice. Embryonic ages were calculated based on noon of the morning of a copulation plug being E0.5. In some cases, in the absence of copulation plugs, the ages of embryos were estimated based on comparison with characteristic developmental landmarks (Kaufman, 1992). lacZ expression was analyzed with X-gal detection of β-galactosidase activity as described by Sanes et al. (1986). Stained...
embryos were post-fixed, infiltrated with sucrose and embedded in OCT or gelatin prior to cutting of frozen sections (10 μm) (Ster, 1993).

**In situ hybridization**
Embryos were isolated after timed matings of nontransgenic FVBN mice at E7-0-9.0 and fixed overnight in 4% paraformaldehyde/PBS, dehydrated in ethanol and stored at −20°C. Nkx-2.5 antisense digoxigenin-labeled RNA probe was synthesized as previously described (Lints et al., 1993) and in situ hybridizations were performed essentially as described by Wilkinson (1993). Embryos were treated with 10 μg/ml proteinase K for 5 minutes at room temperature prior to hybridization in 1 μg/ml probe at 70°C. Staining reactions with NBT/BCIP were stopped after 1-3 hours and hybridized embryos were post-fixed in 4% paraformaldehyde in PBS prior to photography.

**Site directed mutagenesis of the distal regulatory element**
Consensus sites for GATA protein binding located between −2975 and −2775 were mutated within the context of the distal regulatory element. Base pairs within the consensus sequences that have been determined to be critical for GATA binding (Molkentin et al., 1994) were mutated using the QuikChange Site-directed mutagenesis Kit (Stratagene). 5-10 ng of the distal element within pBluescript was used for 16 PCR cycles of 95°C, 30 seconds; 55°C, 60 seconds; 68°C, 7 minutes. Oligos used for mutagenesis of one GATA site followed by mutagenesis of the second GATA site were: 5’- GCGAGGCCTTTGTTTGAATGCAAAGCTACGGATAACGTCG-3’ and 5’- GAGAAAGCTACGGATAACGCCGTGC-3’ (GATA sites underlined). Oligos were 32P end-labeled with T4 polynucleotide kinase (BMB), purified over a G50 spin column and quantitated by liquid scintillation counting. Double stranded oligonucleotide duplexes were formed by the addition of a five-fold excess of the unlabeled complementary strand in the presence of 40 mM KCl. 32P-labeled oligonucleotide (40 fmols, usually 40,000 cts/minute) was incubated with GATA-4 protein in 40 mM KCl, 15 mM HEPES pH 7.9, 5 mM DTT, 50% w/v glycerol, 1.5 μg poly(dI-dC)poly(dI-dC) (Pharmacia) in a volume of 20 μl for 15 minutes at room temperature either alone or in the presence of cold competitor (10- to 250-fold excess). Confirmed GATA-4 binding sites in the rat α-MHC promoter were used as a GATA-specific competitor (Molkentin et al., 1994). Additional competing mutant GATA site oligonucleotides were the same as those used for mutagenesis of these sites described above. Bound and unbound oligonucleotides were separated by electrophoresis through a 5% non-denaturing polyacrylamide gel in 0.5x TBE running buffer. Gels were dried and exposed to X-ray film (Fuji film).

**RESULTS**

**Comparative analysis of expression from nkkx-2.5/lacZ transgenes**

The isolation and analysis of the mouse nkkx2.5 gene was initiated to study the regulatory pathways of early cardiogenesis and to identify early cardiovascular regulatory elements. The mouse nkkx2.5 (Csx-1) cDNA was used to probe a mouse genomic library (Matzuk et al., 1992; Komuro and Izumo 1993). Sequence analysis of the isolated genomic clone shows that 3 kb of 5’ flanking sequence, the entire reported nkkx2.5 coding region and 2.1 kb of 3’ flanking sequence are contained in an 8.1 kb NotI restriction fragment (Fig. 1A and 1B).

- **A.**

  - **-3059**
  - **-9000**

- **B.**

  - **-9000nkkx2.5/lacZ**
    - 2(5)
    - 7-8dpc
    - cardiac crescent
    - 12+dpc
    - heart OT/RV
    - spleen

  - **-3059nkkx2.5/lacZ**
    - 3(9)
    - 7-8dpc
    - cardiac crescent
    - 12+dpc
    - heart OT/RV
    - spleen

  - **-959nkkx2.5/lacZ**
    - 0(12)
    - 7-8dpc
    - cardiac crescent
    - 12+dpc
    - heart OT/RV
    - spleen
Lints et al., 1993; Lyons et al., 1995). The *nkx-2.5* coding sequence in this clone is >96% identical to the published *nkx2.5* cDNA sequence and consensus TATA and CAAT boxes are present in close proximity to the reported transcriptional start site (Lyons et al., 1995). The ability of *nkx2.5* flanking sequences to activate gene expression during embryogenesis was tested in transgenic mouse lines. Three gene constructs were analyzed which contain approximately −9 kb, −3 kb or −1 kb of 5′ flanking sequences linked to a *lacZ* reporter gene (Fig. 1A). Each transgene construct contains the reported transcriptional start site plus 223 bp untranslated region from exon I linked to *lacZ*. Multiple transgenic lines were generated for each construct and embryonic *lacZ* gene expression was analyzed after timed matings of confirmed transgenic with nontransgenic mice. *lacZ* expression was scored in the cardiac crescent and primitive heart tube at E7.5-8, and in the definitive heart and spleen at E12-13 for each transgenic line (Fig. 1B).

Similar patterns of transgene expression were observed with −9 or −3 kb of *nkx-2.5* flanking sequence linked to *lacZ*. At E7.5-8.0, *lacZ* expression was detected only in the anterior region of the cardiogenic crescent and primitive heart tube. Consistent outflow tract and right ventricular expression was apparent later in development. Each of these constructs also was expressed in the developing spleen for all expressing transgenic lines. No expression in the developing heart or spleen was observed at any stage in the twelve lines of mice generated with 1 kb of 5′ flanking sequence. These data demonstrate that critical control elements are located between −1 kb and −3 kb. Representative transgenic (E9.5-E10) embryos for each construct are shown in Fig. 2. In situ hybridizations indicate that the endogenous *nkx-2.5* gene is expressed throughout the heart, in the pharyngeal arches and in the spleen primordium at E9.5. Expression of *lacZ* in transgenic embryos is evident in the developing outflow tract, pharyngeal regions and spleen primordium for the −3 kb and −9 kb constructs but not with −1 kb construct. Significantly, no
additional domains of expression within the heart were observed for the −9 kb versus the −3 kb constructs at any time during development suggesting that the observed expression is regulated by sequences within −3 kb for transgenic lines generated with either construct. The reduced pattern of expression from the −9 kb construct suggests that a negative regulatory element is present between −3 kb and −9 kb of nkx-2.5 flanking sequence. Comparison of transgenic mice generated with either −3 kb or −1 kb of nkx2.5 flanking sequence indicates that regulatory elements responsible for early gene expression are present in the deleted distal sequence between −959 and −3059. However, neither the −9 kb of the −3 kb construct completely recapitulates the full endogenous pattern of expression suggesting that nkx-2.5 is subject to developmentally restricted modulatory regulatory mechanisms.

Developmental expression of the −3059 nkx-2.5/lacZ transgene

lacZ expression from −3059 nkx2.5/lacZ is activated in the heart primordia prior to cardiomyogenic differentiation (Fig. 3). Nkx-2.5 is one of the first genes activated in the cardiogenic region of the embryo and its expression is first detected by in situ hybridization at 7.25 days of embryonic development. Expression from −3059nkx-2.5/lacZ also is evident in the heart forming region at E7.25 (Fig. 3A). Thus regulatory elements sufficient for the initial activation of nkx-2.5 in cardiac progenitors are present within −3059 of nkx-2.5 flanking sequence. At E7.5 lacZ expression is evident in the anterior cardiac crescent which may reflect the initial activation in a subset of the cardiac progenitors (Fig. 3B). With formation of
the primitive heart tube at E8, lacZ expression is restricted to the cranial portions of the primitive heart tube that ultimately develop into the outflow tract and right ventricle (Fig. 3C). Analysis of sectioned embryos demonstrates that lacZ is expressed in the myocardial layer of the anterior primitive heart and also in the pharyngeal endoderm (Fig. 4A,B). No transgene expression was observed in the endothelial layer or in the posterior segments of the primitive heart. All three –3 kb transgenic lines expressed lacZ in the left lateral mesoderm posterior to the heart which may represent the initial spleen anlage. At E10.5, –3059nkx2.5/lacZ is expressed strongly in the outflow tract and right ventricle of the heart, the aortic sac and in the clefts between pharyngeal arches 2 and 4 (Fig. 4C). The transgene is not expressed in the caudal regions of the heart including the left ventricle, atria and sinus venosus. Histological analysis shows expression in the outer myocardial layer of the outflow tract, pharyngeal endoderm and in the third and fourth pharyngeal pouches (Fig. 4D,F). Towards the venous end of the heart, the transgene is expressed in the inner trabeculated layer but not in the exterior compact layer of the right ventricle. Additional strong expression is obvious in the mesenchyme underlying the stomach which includes the spleen primordium at this stage (Fig. 4E).

At E14.5, –3059 nkx-2.5/lacZ expression is evident in derivatives of positively staining structures seen at E10.5 and earlier (Fig. 5A-D). Strong transgene expression persists in the conotruncal region of the heart. In sectioned tissue, lacZ expression is evident in the inner trabeculated layer of the right ventricle and in the myocardial layer of the conus surrounding the pulmonary and aortic valves, although no expression was observed either in the valves or in the major arteries. No lacZ expression was detected in other regions of the transgenic hearts. This staining pattern in the conotruncal region and right ventricle also was observed in hearts isolated from neonatal and adult animals (Fig 5E,F). In addition to being expressed in the heart, the –3059 nkx-2.5/lacZ transgene also is expressed at high levels in the spleen which, at this stage, lies along the caudal surface of the stomach within the dorsal mesogastrium. However, no transgene expression was observed in the tongue which expresses the endogenous nkx-2.5 gene at this stage of development (Lints et al., 1993). Thus regulatory sequences within –3059 nkx-2.5/lacZ are sufficient to activate cell type-specific gene expression at early critical stages of lineage determination and to maintain expression during organogenesis in a subset of nkx-2.5-expressing tissues.

Identification of a distal nkx-2.5 regulatory element

The nkx-2.5 proximal promoter sequence contained in –959 nkx-2.5/lacZ is not sufficient for gene activation in transgenic mice. Thus regulatory elements between –3059 and –959 are critical for the observed gene expression pattern. Sequence analysis of this region revealed a cluster of transcription factor consensus binding sites present within 505 bp at –3059 to –2554 (Fig. 6). Among these sites are several for known regulators of cardiac gene expression including eight GATA sites, two NKE sites and an E-box (Murre et al., 1989; Ko and Engel, 1993; Merika and Orkin, 1993; Chen and Schwartz, 1995). Other potentially important developmentally regulated sites include two Hox sites and an HMG site (Waterman et al., 1991; Kalionis and O’Farrell, 1993). In comparison, the remaining 1.5 kb between –2554 and –959 contains one GATA site, one Nkx site, one combination NKE/GATA site and no Hox or HMG sites. Other cardiac regulatory elements such as MEF-2, SRF and TEF-1 sites were not identified in the 3 kb flanking the nkx-2.5 gene (reviewed by Robbins, 1996). The presence of these clustered consensus binding sites for multiple developmentally important regulatory factors within –3059 to –2554 suggests that this region may be important for early cardiac gene activation.

In addition to the consensus sequences for regulatory factor binding, the distal element also contains potential TATA box consensus sequences at –2625 and –2695. The ability of the –3059 to –2554 region to act as a promoter was demonstrated in chick cardiac cells transfected in culture with the distal element linked directly to lacZ (data not shown). Promoter activity was assessed in vivo in transgenic mice generated with the –3059/–2554 nkx-2.5/lacZ construct. Five confirmed transgenic F0 embryos were analyzed at a developmental stages corresponding to E7.5 and E10.5 (Fig. 7A-C). At E7.5 lacZ expression was detected in the cardiac crescent prior to cardiomyogenic differentiation. Transgenic embryos at E10.5 exhibited lacZ expression in the outflow tract of the heart, pharyngeal region and spleen primordium similar to that

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**Fig. 6.** Multiple consensus binding sites for known regulators of cardiogenesis are present within a distal regulatory element –3059 to –2554 of the reported nkx-2.5 transcriptional start site. Clustered GATA, Nkx, HOX, HMG and bHLH consensus binding sites present in the distal region of the nkx-2.5 transgene are indicated. This sequence was linked directly to the lacZ reporter gene for the generation of transgenic mice. The paired GATA sites at –2798 to –2770 which represent the closest match to known GATA target sequences were mutated (bases indicated by stars) and tested for GATA binding and gene activity within the context of the distal regulatory element.
observed for the −9 kb or −3 kb n_{kx-2.5}/lacZ constructs. Northern, RACE and PCR analyses of n_{kx-2.5} transcripts present in E14.5 heart and spleen mRNA did not identify transcripts initiated from the distal element (data not shown). The ability of the −3059/−2554 element to act as an enhancer when linked to the heterologous hsp68 promoter was tested in two transgenic lines (Fig. 7D,E). lacZ expression was observed in the primitive heart tube at E8.5 and in the developing outflow tract of the heart, pharyngeal clefts and spleen later in development. Thus the −3059/−2554 n_{kx-2.5} sequence has enhancer activity in vivo when linked to the hsp68 promoter but the expression pattern is not identical to the −3059n_{kx-2.5}/lacZ expression pattern which may be indicative of specific n_{kx-2.5} promoter/enhancer interactions.

**GATA sites in the distal element are required for n_{kx-2.5} gene activation**

Among the GATA consensus sequences in the −3059/−2554 element, the paired GATA sites at −2795 to −2775 have the optimal spacing, orientation and consensus sequences of known GATA target genes (Ko and Engel, 1993; Merika and Orkin, 1993; Molkentin et al., 1994; Grepin et al., 1994; Ip et al., 1994; McGrew et al., 1996). The ability of these sequences to bind GATA-4 protein in vitro was tested by gel mobility shift assay (Fig. 8). Specific DNA binding of GATA-4 to these sites was evident in a shifted complex which was eliminated in the presence of excess unlabeled GATA site competitor oligonucleotides. The GATA site competitors consisted of either a confirmed GATA binding site from the α-myosin heavy chain promoter or the original (−2795 to −2775) GATA site oligo (Molkentin et al., 1994). However, competition was reduced with an oligo in which one of the GATA sites was mutated and no competition was observed with an oligo with both GATA sites mutated. These assays indicate that the −3059 to −2554 region is a candidate downstream target for GATA regulation via these paired GATA sites. The importance of the paired GATA sites for n_{kx-2.5} element gene activation was confirmed in vivo by transgenic analysis. The two GATA sites at −2795 to −2775 were mutated within the context of the distal element (−3059 to −2554) and the mutated element was linked directly to lacZ. A total of eight confirmed transgenic F_{0} embryos were analyzed at E9.5 and E10.5 (Fig. 7F). No lacZ staining was observed in the heart, pharynx or spleen in any of the embryos at either stage. Thus the presence of the intact paired GATA sites at −2795 to −2775 is required for gene activation of the n_{kx-2.5} element at the early stages of embryogenesis.

**DISCUSSION**

Regulatory elements of the n_{kx-2.5} gene were analyzed in transgenic mice. 3 kb of 5’ flanking sequence relative to the reported transcriptional start site is sufficient to activate gene expression at the earliest stages of cardiac development. lacZ expression driven by these sequences is activated in the cardiogenic region concurrently with the initial activation of n_{kx-2.5} gene expression. In the differentiated heart, cardiac expression from these elements is confined to the anterior outflow tract and right ventricular segments throughout embryonic development and in the neonate. These same regulatory elements activate gene expression in the developing pharynx and spleen beginning at the earliest stages of organogenesis. The distal 505 bp (−3059 to −2554) are sufficient to activate anterior heart, pharynx and spleen expression and the nucleotide sequence of the distal element contains consensus binding sites for developmentally important regulatory factors. DNA/protein binding studies show that GATA factors bind specifically to the n_{kx-2.5} distal element in vitro. Mutation of the paired GATA sites results in the loss of gene expression in the heart, pharynx and spleen which is indicative of a regulatory interaction between GATA factors and the critical n_{kx-2.5} distal element. The presence of a variety of consensus binding sites in the distal element suggests that multiple regulatory interactions are responsible for the complex expression pattern of n_{kx-2.5} during development.

Flanking sequences within 3 kb of the reported transcriptional start site of n_{kx-2.5} are sufficient to recapitulate a subset of n_{kx-2.5} gene expression during development. Endogenous n_{kx-2.5} gene expression is activated in the cardiac region during the early stages of lineage commitment and differentiation and n_{kx-2.5} is expressed throughout the heart during development and in the adult (Lints et al., 1993; Komuro and Izumo, 1993). N_{kx-2.5} gene expression is not heart-specific and other regions of expression include the pharyngeal endoderm and the developing tongue, thyroid and spleen (Kasahara et al., 1998). In general, n_{kx-2.5} expression is activated in the organs where it is expressed during the early stages of differentiation and morphogenesis and is downregulated later in development. Transgenic mice generated with −3059n_{kx-2.5}/lacZ express lacZ in the anterior segments of the heart, regions of the pharynx including the thyroid and in the spleen primordium but expression was never detected in the caudal regions of the heart or in the developing tongue. The initial activation of lacZ expression in the heart forming region coincides with the earliest stage of n_{kx-2.5} gene activation. In the differentiated heart, the n_{kx-2.5} transgene is positionally restricted to the anterior segments of the cardiac crescent and the primitive heart tube. In the looped heart, these n_{kx-2.5} elements activate gene expression in the developing outflow tract and right ventricle. The positional restriction of transgene expression in the developing cardiogenic region indicates that distinct regulatory modules are responsible for n_{kx-2.5} gene expression in the different segments of the heart. The consistent anterior restriction of the n_{kx-2.5} transgene throughout cardiac development, starting from the cardiac crescent stage, is clear evidence that restricted patterns of cardiac gene expression are determined prior to cardiomyogenic differentiation. These observations support studies in the chick which demonstrated that positional information is specified in the heart-forming fields soon after gastrulation (Yutzey et al., 1995). The inability of these elements to activate gene expression in the venous regions of the heart including the left ventricle, atria or sinus venosus which express the endogenous n_{kx-2.5} gene indicates that other regulatory elements not present within 9 kb of 5’ flanking sequence are responsible for gene expression in these regions. These studies are consistent with other reports of modular expression of cardiac regulatory elements in transgenic mice and may reflect cardiac compartment-specific regulatory pathways (Ross et al., 1996; Franco et al., 1997; He and Burch, 1997).
The activation of \( nkx-2.5 \) expression in the developing spleen precedes the activation of \( hox11 \), the earliest reported marker of spleen development by at least 2 days (Dear et al., 1995). \( Hox11 \) is expressed in a similar pattern to \( nkx-2.5 \) in the pharyngeal arches, outflow tract of the heart and tongue (Dear et al., 1995; Raju et al., 1993). Targeted mutagenesis of \( hox11 \) results in complete loss of the spleen in otherwise apparently normal embryos (Roberts et al., 1994). The similarities in the patterns of expression of \( hox11 \) and \( nkx-2.5 \) suggest a potential for regulatory interactions between these factors. Interestingly the \( Drosophila \) homologue of \( hox11 \), \( 31G \), is genetically linked to the cluster of NK-homeobox genes and is expressed in a pattern similar to \( tin \) (Dear and Rabbitts, 1994). The expression of \( nkx-2.5 \) in the heart and spleen in the days preceding \( hox11 \) gene activation suggests that \( hox11 \) could be a downstream target of \( nkx-2.5 \). This is supported by the presence of multiple \( nkx-2.5 \) binding sites in the flanking sequence of the \( hox11 \) gene (Arari et al., 1997). Deletion of the 300 bp which include these sites results in loss of all \( hox11 \) promoter activity in F9 cells. Although the development of the spleen anlage is not well characterized, the expression of the \( nkx-2.5 \) transgene in the left lateral mesoderm posterior to the heart is consistent with the region of the embryo fated to become the spleen. Taken together these studies support a critical role for \( nkx-2.5 \) in the earliest events of spleen lineage specification and development.

The restricted expression of the \( nkx-2.5 \) transgenes examined suggests that multiple regulatory elements activate \( nkx-2.5 \) gene expression during development. Four distinct regulatory elements have been characterized for the \( tin \) gene in \( Drosophila \) that are responsible for its spatially and temporally restricted expression pattern (Yin et al., 1997). Each of these elements is activated by a specific regulatory pathway at distinct stages and in distinct cell types during development. A similar and potentially even more complex set of regulatory interactions appears to be responsible for \( nkx-2.5 \) gene expression in vertebrates. The genetic elements responsible for \( nkx-2.5 \) gene expression in the left ventricle, atria and sinus venosus of the heart and in the tongue have not yet been

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**Fig. 7.** The \(-3059/-2554\) element activates gene expression in the heart, pharynx and spleen and is dependent on paired GATA sites for activity. (A-C) Transgenic mouse embryos generated with \(-3059/-2554\)\( nkx-2.5 \) linked to \( lucZ \) express \( \beta \)-gal in the cardiac crescent (arrows) at E7.5 and in pharyngeal region, outflow tract of the heart and spleen primordium at E10.5. Transgenic F0 mouse embryos were assayed 7 (A) or 10 (C-D) days after microinjection for \( \beta \)-gal activity. A total of 5 transgenic embryos were obtained and all expressed \( lucZ \) in the represented patterns. (D-E) The \(-3059/-2554\) \( nkx-2.5 \) sequence exhibits enhancer activity when linked to \( hsp68/lacZ \). Two transgenic lines were analyzed and \( lucZ \) expression was observed in the primitive heart tube at E8.5 and in the pharyngeal clefts (asterisks), outflow tract of the heart and spleen primordium at E10.5. (F) Mutagenesis of paired GATA sites at \(-2798\) to \(-2770\) eliminates gene activity in transgenic embryos. The loss of all gene activity was observed in 8 F0 confirmed transgenic embryos.

**Fig. 8.** GATA-4 binds to sequences within the distal element of \( nkx-2.5 \) and mutations that eliminate gene activity result in loss of GATA-4 binding. Gel mobility shift assays were performed with GATA-4 protein translated in vitro and a \( ^{32}P \)-labeled oligonucleotide containing GATA consensus binding sites located at \(-2798\) to \(-2770\) of the \( nkx-2.5 \) distal element. Specificity of GATA-4 binding was confirmed by competition with unlabeled sequences at 10× to 250× the labeled oligo concentration. Oligos corresponding to GATA sites within the mouse \( \alpha \)-myosin heav chain promoter (GATA) or to the original \( nkx-2.5 \) sequence (Wt) competed for GATA-4 binding. Oligonucleotides containing the indicated mutations in the GATA binding sites competed less well (Mu1) or did not compete (Mu2) with the Wt oligonucleotide for GATA-4 binding. The distal element with both GATA sites mutated was used to generate the transgenic embryo shown in Fig. 7F.
identified. These studies are indicative of a multiplicity of regulatory elements, some of which may be active concurrently, that are responsible for the complex expression pattern of the nkrx-2.5 gene during development.

Regulatory interactions necessary for early gene expression in the cardiogenic region act through the distal activating domains of the nkrx-2.5 promoter region (~3059 to ~2554). Among the consensus binding sites in this region are several GATA and NKE sites, which suggests that there are autoregulatory and cross regulatory interactions between GATA and NKX family members. Mutagenesis of the paired GATA sites in the distal nkrx-2.5 element results in loss of gene activity in the pharynx, heart and spleen. Many genes expressed in the differentiated heart including α-myosin heavy chain, cardiac troponin C, atrial natriuretic factor (ANF) and B-type natriuretic peptide (BNP) contain at least one GATA site in the regulatory elements responsible for cardiac-specific gene activation (Ip et al., 1994; Grepin et al., 1994; Molkenstein et al., 1994). GATA factors interact synergistically with NKX-2.5 in the activation of ANF expression, and similar interactions also may occur on adjacent NKE/GATA consensus sites within the nkrx-2.5 distal element (Durocher et al., 1997). NK and GATA factors are coexpressed in the cardiac, pharyngeal and splenic regions where the nkrx-2.5 distal element is activated (Morrissey et al., 1996; Jiang and Evans, 1996; Morrissey et al., 1997). Loss of functional GATA-4 in homozygous mutant mice does not eliminate cardiac-specific gene expression of nkrx-2.5 but increased levels of GATA-6 may compensate for the loss of GATA-4 (Molkenstein et al., 1997). The dependence of the nkrx-2.5 distal element on the presence of GATA consensus binding sites suggests that nkrx-2.5 is regulated by GATA factors. Since nkrx-2.5 and GATA factors are coexposed in the undifferentiated heart primordia, they may be involved in reinforcing regulatory networks that promote cardiac differentiation.

The factors responsible for the initial activation of nkrx-2.5 gene expression in the anterior lateral mesoderm are not well-characterized. Nkrx-2.5 has been shown to be activated with application of ectopic BMPs in the anterior mesoderm of primitive streak stage chick embryos (Schultheiss et al., 1997; Andree et al., 1998). The sensitivity of nkrx-2.5 to BMP signaling is also observed in Drosophila with the activation of tin gene expression by dpp signaling (Frasch, 1995). A DNA target site of dpp-regulated gene activation in Drosophila has been identified as a cAMP responsive element (CRE) (Eresh et al., 1997). The CRE consensus sequence is not present within the distal nkrx-2.5 regulatory element suggesting that this element may not be directly regulated by BMPs via a cAMP-dependent pathway. An alternative mechanism for BMP-mediated gene regulation involves Smads factors and partial consensus sequences for Smads DNA binding are present within the ~3059 to ~2554 nkrx-2.5 sequence (Graff et al., 1996; Denlinger et al., 1998; Zawal et al., 1998). Tin expression in cardioblast cells in Drosophila embryos is dependent on wg signaling (Wu et al., 1995; Park et al., 1996). The presence of an HMG site consensus sequence for Wnt-responsive gene expression in the distal element suggests that nkrx-2.5 might be a direct target for Wnt-activated gene expression in the cardiogenic region (McKendry et al., 1997). The activation of both Wnt and BMP regulatory pathways has been observed in a number of developmental systems at the critical stages of patterning and cell lineage commitment (reviewed by Hogan; 1996; Cadigan and Nusse, 1997). The identification of regulatory elements that are activated at the earliest stages of cardiomyogenesis, such as the nkrx-2.5 distal regulatory element, will facilitate the examination of these critical regulatory interactions in early heart development. It seems likely that multiple intersecting developmental pathways and combinatorial interactions between regulatory factors such as the GATA and Wnt- or BMP-responsive elements are responsible for the spatially and temporally restricted activation of nkrx-2.5 gene expression during development.

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