Mef2c is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development

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Summary

The vertebrate heart forms initially as a linear tube derived from a primary heart field in the lateral mesoderm. Recent studies in mouse and chick have demonstrated that the outflow tract and right ventricle originate from a separate source of mesoderm that is anterior to the primary heart field. The discovery of this anterior, or secondary, heart field has led to a greater understanding of the morphogenetic events involved in heart formation; however, many of the underlying molecular events controlling these processes remain to be determined. The MADS domain transcription factor MEF2C is required for proper formation of the cardiac outflow tract and right ventricle, suggesting a key role in anterior heart field development. Therefore, as a first step toward identifying the transcriptional pathways upstream of MEF2C, we introduced a lacZ reporter gene into a bacterial artificial chromosome (BAC) encompassing the murine Mef2c locus and used this recombinant to generate transgenic mice. This BAC transgene was sufficient to recapitulate endogenous Mef2c expression, and comparative sequence analyses revealed multiple regions of significant conservation in the noncoding regions of the BAC. We show that one of these conserved noncoding regions represents a transcriptional enhancer that is sufficient to direct expression of lacZ exclusively to the anterior heart field throughout embryonic development. This conserved enhancer contains two consensus GATA binding sites that are efficiently bound by the zinc finger transcription factor GATA4 and are completely required for enhancer function in vivo. This enhancer also contains two perfect consensus sites for the LIM-homeodomain protein ISL1. We show that these elements are specifically bound by ISL1 and are essential for enhancer function in transgenic embryos. Thus, these findings establish Mef2c as the first direct transcriptional target of ISL1 in the anterior heart field and support a model in which GATA factors and ISL1 serve as the earliest transcriptional regulators controlling outflow tract and right ventricle development.

Key words: Mef2c, transgenic mouse, cardiac development, transcription, secondary heart field, anterior heart field, ISL1 (ISL-1), GATA4

Introduction

The heart is the first organ to form and function in vertebrates. Cardiac development is a complex process that requires myogenesis and morphogenesis to occur simultaneously with contractility, and distinct cell populations have to integrate with each other in a temporally and spatially precise fashion. The primary heart field forms initially as a crest from two bilaterally symmetrical regions of anterior lateral mesoderm, which are specified during gastrulation. At about 8.0 days post coitum (dpc) in the mouse, the myocardial cells in the precardiac mesoderm are folded together ventrally, allowing fusion of the two cardiac primordia into a linear heart of cardiac myocytes surrounding an endothelial tube. Shortly thereafter, the immature, linear heart becomes contractile and undergoes rightward looping to create an asymmetrical, curved tube (Brand, 2003; Kelly and Buckingham, 2002; Yutzey and Kirby, 2002). As the embryo continues to develop, chamber maturation and septation occur to give rise to a functional four-chambered heart (Brand, 2003).

Embryological studies in chick and mouse suggested that the conotruncal region of the heart was likely to arise from a separate population of myogenic precursor cells (Kelly and Buckingham, 2002; Yutzey and Kirby, 2002). Indeed, very recent studies have identified that the outflow tract and outlet region of the right ventricle are derived from a precursor population that is distinct from the cells of the primary heart field (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). This population of cells, referred to as the anterior, or secondary, heart field, is derived from mesoderm anterior to the primary heart field and appears to be added to the heart at the time of cardiac looping (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). The identification of at least two distinct populations of cells contributing to the heart provides a likely explanation for the spatial restriction of gene expression and function that has often been observed in the heart (Kelly and Buckingham, 2002; Schwartz and Olson, 1999). For example, the cardiac restricted T box gene Tbx5 plays an important role in the formation of the atria and left ventricle but is not required for proper formation of the right ventricle or outflow tract (Bruneau et al., 2001), suggesting that it may not play a prominent role in the anterior heart field. It is also clear that a number of cardiac genes are controlled by
multiple, separate enhancers that direct spatially restricted expression within the developing heart (Schwartz and Olson, 1999).

The observations that the amniote heart forms from multiple progenitor populations and that a number of genes and transgenes exhibit spatially restricted expression within the heart suggest that there are likely to be important differences in the transcriptional pathways governing gene expression between the two heart fields. Interestingly, many of the key transcriptional regulators required for cardiac development are expressed in both the anterior and primary heart fields, and thus it remains unclear how spatial restriction within the heart is controlled. The homeodomain transcription factor NKX2.5 and the zinc finger transcription factor GATA4 are the first cardiac restricted transcription factors to be expressed during development, and both are expressed throughout the precardiac mesoderm, including the anterior heart field (Arceci et al., 1993; Chen and Fishman, 2000; Harvey, 1996; Lints et al., 1993; Parmacek and Leiden, 1999). The functions of GATA4 and NKX2.5 are essential for cardiac development (Kuo et al., 1993; Parmacek and Leiden, 1999). The two factors function as part of a combinatorial transcriptional complex to cooperatively activate cardiac gene expression (Durocher et al., 1997; Lee et al., 1998; Sepulveda et al., 1998; Sepulveda et al., 2002). However, the role of these transcription factors in gene expression within the anterior heart field, and how these factors fit together into transcriptional pathways with other key transcriptional regulators, remains to be determined.

Very recent studies by Evans and colleagues demonstrated a critical role for the LIM-homeodomain transcription factor ISL1 (also known as ISL-1) in anterior heart field development (Cai et al., 2003). ISL1 was first identified as a key regulator of the rat insulin I gene enhancer and expression was observed in endocrine cells of the pancreas (Karlsson et al., 1990). During embryonic development, however, Isl1 is also expressed in the crescent-shaped pattern of the anterior heart field, medial and dorsal to the MLC2a (Myl7 – Mouse Genome Informatics) expressing cells in the primary heart field (Cai et al., 2003). By mid-gestation, Isl1 is expressed broadly outside the anterior heart field, but expression in the cardiogenic region is restricted to the pharyngeal mesoderm of the anterior heart field, and fate mapping studies demonstrated that the Isl1 expressing cells from the anterior heart field contribute to the vast majority of cells in the outflow tract and right ventricle in the embryo (Cai et al., 2003). Targeted disruption of Isl1 results in embryonic lethality between 9.5 and 10.5 dpc (Pfaff et al., 1996). Mice lacking Isl1 have severely misshapen, unlooped hearts at 9.5 dpc and the right ventricle and outflow tract fail to form in these embryos, demonstrating an essential role for Isl1 in anterior heart field development (Cai et al., 2003). While these studies demonstrated a critical and previously unappreciated role for ISL1 in cardiac development, no direct targets of ISL1 have been identified in the anterior heart field.

Members of the myocyte enhancer factor 2 (Mef2) transcription factor family also play key roles in cardiac myogenesis. There are four mef2 genes in vertebrates and a single mef2 gene in Drosophila (Black and Olson, 1998). In mice, Mef2c is among the earliest markers of the cardiac lineage with transcripts evident in the developing heart beginning at about 7.5 dpc, shortly after the expression of the Nkx2.5 and Gata genes (Arceci et al., 1993; Edmondson et al., 1994; Harvey, 1996; Lin et al., 1997; Lints et al., 1993; Morrisey et al., 1996; Morrisey et al., 1997; Parmacek and Leiden, 1999). Despite the early expression of Mef2c in the heart, MEF2 activity is not required for muscle specification; instead, it appears to play a key role in differentiation. This notion is strongly supported by work in Drosophila in which inactivation of the single Mef2 gene results in a complete loss of muscle differentiation in all three muscle lineages with no apparent defect in myoblast specification (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995). The role of MEF2 factors in vertebrate muscle is supported by the observation that a dominant negative form of MEF2 is sufficient to block differentiation in cultured mouse myoblasts (Ornatsky et al., 1997). The essential role for MEF2 activity in vertebrate cardiac development is apparent from genetic studies in mice, in which disruption of the Mef2c gene results in embryonic lethality at 9.5 dpc due to cardiac and vascular defects (Bi et al., 1999; Lin et al., 1997; Lin et al., 1998). The cardiac phenotype in Mef2c null embryos is nearly identical to the heart phenotype observed in Isl1 null embryos (Cai et al., 2003; Lin et al., 1997). The hearts of Mef2c null embryos fail to undergo looping, they have gross abnormalities in the outflow tract, and the right ventricle fails to form (Lin et al., 1997), suggesting a key role for Mef2c in the transcriptional pathways regulating anterior heart field development. However, despite the importance of Mef2c in cardiac development, nothing has been elucidated regarding its transcriptional regulation in the heart.

In the present study, we used in vivo bacterial artificial chromosome (BAC) recombination to generate a Mef2c-lacZ BAC that recapitulates the endogenous pattern of Mef2c expression in transgenic embryos. By comparing the human and mouse genomic sequence in the region encompassed by the mouse Mef2c-lacZ BAC, we identified multiple regions of conservation in Mef2c noncoding regions. One of these regions of conservation represents an enhancer that is sufficient to direct transcription to the anterior heart field, but not to other regions where endogenous Mef2c is expressed. We show that this Mef2c enhancer begins to function by 7.5 dpc in the precardiac mesoderm and continues to direct expression to the anterior heart field and its derivatives in the outflow tract and right ventricle throughout embryonic and fetal development. This enhancer contains two evolutionarily conserved GATA binding sites that are bound by GATA4 with high affinity and are completely required for enhancer function in vivo. We also show that the Mef2c anterior heart field enhancer is a direct transcriptional target of ISL1 via two evolutionarily conserved, perfect consensus ISL1 binding sites, which are essential for enhancer function in vivo. Thus, these studies identify Mef2c as the first direct target of ISL1 in the anterior heart field and support a model in which ISL1 and the GATA family of transcription factors serve as the earliest regulators of the transcriptional program controlling the development of the outflow tract and right ventricle.

Materials and methods

In-vivo BAC recombination

The bacterial lacZ reporter gene was introduced into BAC clone GS133, which encompasses the 5’ end of the mouse Mef2c gene, using
A previously described system for in vivo BAC recombination (Yang et al., 1997). GS133 contains the first six exons of the mouse Mef2c gene and extends from 74.0 kb upstream to 46.4 kb downstream of the transcriptional start site. The lacZ gene was recombined into clone GS133 in the first coding exon of Mef2c such that the Mef2c start codon was deleted but the exon boundaries were preserved. This strategy for recombination was designed to allow translational initiation from lacZ instead of from Mef2c but to allow transcriptional initiation to occur from the endogenous Mef2c start sites present in the BAC. To create the BAC recombinant, two Mef2c-homology arms were amplified from GS133 using the following primers: 5′ arm/prime 1, 5′-CAACCGGGCTTCAGACATCCTCTGGCTGTAACAA-3′; 5′ arm/prime 2, 5′-CTTACTAGTTCTCTGCTGCAAAAATTATG-3′; 3′ arm/prime 1, 5′-GGAGAATTCAAAAGATTCCAGATTCGAGC-3′; 3′ arm/prime 2, 5′-CAAGTCGACACCTGATATTCAAC-3′. Both arms were cloned into the promoterless lacZ reporter plasmid AUG-β-gal (McFadden et al., 2000), and the entire cassette containing the Mef2c-homology arms flanking the lacZ-polyA cassette in AUG-β-gal was cloned into plasmid pSV1.RecA (Yang et al., 1997) as a Sall fragment. The resulting plasmid, pSV1.RecA-Mef2c-lacZ was used as the recombination vector to create co-integrates in GS133 as previously described (Yang et al., 1997). Co-integrates were resolved using fusaric acid, and recombinants were identified by colony hybridization with a lacZ probe. The junctions at the site of recombination and at the ends of the recombinant BAC, termed Mef2c-L8-lacZ, were confirmed by sequence analysis on both strands. The entire recombined insert was purified from the plasmid backbone for use in oocyte microinjection by digestion with NotI and purification of the band by pulse field gel electrophoresis. DNA was extracted from the low melting point agarose gel slice by melting at 65°C then incubating at 37°C for 5 minutes in Q Buffer (Qiagen) containing 235 mg/ml urea. The DNA insert was then purified using a Qiagen tip-20 plasmid miniprep kit according to the manufacturer’s directions.

Cloning and mutagenesis
A 6142 bp fragment of the mouse Mef2c gene and eight additional deletion fragments were generated by PCR with primers designed to correspond to the nucleotide numbers given for each enhancer construct in this study (see Fig. 3A). Each Mef2c enhancer fragment was cloned into the transgenic reporter plasmid HSP68-lacZ (Kothy et al., 1989) except for Mef2c-F6/Frag3, which uses an endogenous promoter from Mef2c and was cloned into the promoterless lacZ reporter plasmid AUG-β-gal (McFadden et al., 2000). Mutations were introduced into the wild-type Mef2c-F6/Frag2 cardiac enhancer fragment (nucleotides 1-3970) by PCR as described (Dodou et al., 2003) to create the following mutant sequences: mGATA-p, 5′-AAGTCACCCCGTTGCTAGCCAGGGAAGC-3′; mGATA-d, 5′-CTAAGAATTTCTGCGCAAGTTCTGTC-3′; mISL-p, 5′-GGTTAATGCTAGTTACCTCGGATAAAG-3′; mISL-d, 5′-GGTCAGG- GGAGCCTAGTCTTTTGGG-3′. For the double GATA mutant, mGATA-p/d, the GATA-p mutation was introduced into the GATA-d mutant background to create the double mutant enhancer. For the double ISL mutant, mISL-p/d, the ISL-p mutation was introduced into the ISL-d mutant background to create the double mutation. The entire sequence of each mutant fragment was confirmed by sequencing on both strands. The GenBank accession number for the sequence of the mouse Mef2c anterior heart field enhancer described in these studies is AY3324098.

Generation and analysis of transgenic mice
Transgenic reporter fragments were digested away from the plasmid backbone with Xhol/NotI (for plasmid constructs) or NotI (for the BAC construct), gel purified, and suspended in 5 mM Tris-Cl, 0.2 mM EDTA (pH 7.4) at a concentration of 2 ng/μl for pronuclear injection as described previously (Hogan et al., 1994). Injected embryos were implanted into pseudopregnant CD-1 females, and embryos were collected at the indicated times for F0 analysis or were allowed to develop to adulthood for establishment of stable transgenic lines. β-galactosidase expression from lacZ transgenic embryos or tissues was detected by X-gal staining as described previously (Dodou et al., 2003). For sections, embryos were collected at either 9.5 or 11.5 dpc, fixed, stained with X-gal, and fixed again. Following staining, embryos were dehydrated with ethyl alcohol and xylene and mounted in paraffin. Transverse sections at a thickness of 5 μm were cut using a Leica RM 2155 microtome and were counterstained with Nuclear Fast Red to visualize embryonic structures as described (Anderson et al., 2004). Genotypes were determined by Southern blot as described previously (Dodou et al., 2003). For Mef2c-L8-lacZ transgenic embryos, the BAC ends were checked by PCR to confirm that the intact BAC sequence was integrated into the mouse genome.

Electrophoretic mobility shift assay (EMSA)
DNA-binding reactions were performed as described previously (Dodou et al., 2003). Briefly, double-stranded oligonucleotides were labeled with 32P-CTP using Klenow to fill in overhanging 5′ ends and purified on a nondenaturing polyacrylamide-TBE gel. Binding reactions were pre-incubated at room temperature in 1× binding buffer (40 mM KCl, 15 mM HEPES pH 7.9, 1 mM EDTA, 0.5 mM DTT, 5% glycerol) containing 2 μg of each recombinant CAT4A, recombinant ISL1, or unprogrammed reticulocyte lysate. 1 μg of poly dI-dC, and competitor DNA (100-fold excess where indicated) for 10 minutes prior to probe addition. Reactions were incubated an additional 20 minutes at room temperature after probe addition and electrophoresed on a 6% nondenaturing polyacrylamide gel. Recombinant GATA4 and a truncated version of the ISL1 cDNA containing the homeodomain were generated from plasmids pCITE-GATA4 and pCITE-ISL1, respectively, using the TNT Quick Coupled Transcription/Translation System as described in the manufacturer’s directions (Promega). pCITE-GATA4 was generated by cloning the complete Rattus norvegicus GATA4 coding region into the translational enhancement vector pCITE-2A (Novagen). pCITE-ISL1 was generated by cloning an EcoRI fragment from the hamster Mesocricetus auratus Isl1 cDNA (Wang and Drucker, 1994) encoding the C-terminal 231 amino acids of ISL1 as an in-frame fusion into the translational enhancement vector pCITE-2A (Novagen). This fragment of the ISL1 cDNA has had the N-terminal 118 amino acids removed. These residues include the ISL1 LIM domains, which have been shown previously to inhibit DNA binding in vitro (Sanchez-Garcia and Rabbitts, 1993). The Nks2.5 g1 control GATA4 binding site and the mutant g1 (M1) oligonucleotides have been described (Lien et al., 1999). The sense strand sequence of the wild-type and mutant ISL1 site control oligonucleotides from the InsulinI promoter were generated based on previously published studies (Karlsson et al., 1987) and were: In, 5′-GCCCTTGTATAAATCTAATACCTCAG-3′; mIn, 5′-GCCCTTGTCAAGATCGTTACCTCAG-3′. The sense strand sequences of the Mef2c oligonucleotides used for EMSA were: GATA-p, 5′-GGTCACCCGGTTATCATCAGGGCCG-3′; GATA-d, 5′-GGTAAGAGTTCTTATCGTAGGCTC-3′; mGATA-p, 5′-GGTCACCCGGTTATCATCAGGGCCG-3′; mGATA-d, 5′-GGTAAGAGTTCTTATCGTAGGCTC-3′; ISL-p, 5′-GGTTTACTGCTG GGACCTCAGTCTTTTGGG-3′. For the double GATA mutant, mGATA-p/d, the GATA-p mutation was introduced into the GATA-d mutant background to create the double mutant enhancer. For the double ISL mutant, mISL-p/d, the ISL-p mutation was introduced into the ISL-d mutant background to create the double mutation. The entire sequence of each mutant fragment was confirmed by sequencing on both strands. The Results

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expression in heart, skeletal muscle, vasculature, neural crest, and pharyngeal mesoderm (Edmondson et al., 1994) (B.B. and E.D., unpublished observations). The very complex temporal and spatial expression pattern of Mef2c suggested that it might be regulated by multiple, independent transcriptional enhancers that each control expression in a single lineage. This notion was consistent with earlier studies that identified multiple transcriptional enhancers, which were sufficient to direct expression to distinct subsets of the endogenous Mef2c gene that was preserved (Fig. 1A). This BAC recombinant (Mef2c-L8-lacZ) was used to generate transgenic embryos to determine if the BAC sequence contained transcriptional enhancers sufficient to direct expression of lacZ in vivo. Expression directed by the BAC transgene could be observed first at 7.5 dpc in the anterior heart field (Fig. 1C), a crescent-shaped region of splanchnic mesoderm adjacent and medial to the primary heart field (Cai et al., 2003; Kelly et al., 2001). Similarly, expression at 8.0 dpc was readily apparent in the pharyngeal mesoderm at the anterior end of the linear heart tube (Fig. 1D). By 8.5 dpc, expression was still readily apparent in the anterior heart field, but other regions known to express endogenous Mef2c, including neural crest and vascular endothelium, began to be marked by lacZ expression (Fig. 1E). By 9.5 dpc, all developing tissues known to express endogenous Mef2c (Edmondson et al., 1994), including pharyngeal mesoderm, heart, somites, branchial arches (BA), pharyngeal mesoderm, outflow tract and heart. RV, right ventricle. (C-F) Representative X-gal stained transgenic embryos at each stage. Arrowheads point to pharyngeal mesoderm/anterior heart field. Two F0 transgenic embryos and embryos collected from an independent BAC transgenic line displayed identical patterns of expression at all stages.

**An intronic enhancer from the Mef2c gene is sufficient to direct expression to the anterior heart field**

Based on the notion that conservation occurs preferentially in functionally important sequences, we tested each of the Mef2c conserved, noncoding sequences for enhancer activity in transgenic embryos by cloning each of the conserved regions into the transgenic reporter plasmid HSP68-lacZ (Kothary et al., 1989) as depicted in Fig. 1B. These analyses identified multiple transcriptional enhancers, which were sufficient to direct lacZ expression to distinct subsets of the endogenous
Mef2c pattern, including skeletal muscle (Dodou et al., 2003), neural crest (M.V and B.B., unpublished observations), and vascular endothelium (J.A. and B.B., unpublished observations). In addition, we identified a 6142 bp conserved enhancer that was sufficient to direct lacZ expression to the anterior heart field in transgenic mouse embryos (Fig. 2). Expression directed by this conserved enhancer element mimicked a subset of the expression seen with the recombinant Mef2c BAC transgene (Fig. 1) with expression apparent exclusively in the anterior heart field, including pharyngeal mesoderm, right ventricle, and outflow tract (Fig. 2). This conserved cardiac enhancer encompassed the third untranslated exon (Fig. 1A). Thus, the enhancer resided between 16.3 kb and 22.5 kb downstream of the first untranslated exon and between 48.9 kb and 55.1 kb upstream of the first translated exon of mouse Mef2c (Fig. 1A,B).

We examined expression of lacZ directed by the 6142 bp Mef2c anterior heart field enhancer throughout development (Fig. 2). The expression of lacZ was first apparent between 7.5 and 7.75 dpc and was restricted to a region corresponding to the anterior heart field (Fig. 2A) (Cai et al., 2003; Kelly et al., 2001). By 8.0 dpc, expression of the Mef2c-lacZ transgene became much more robust and was restricted to the anterior heart field (Fig. 2A) (Cai et al., 2003; Kelly et al., 2001). At 9.5 and 11.5 dpc, expression was observed only in the pharyngeal mesoderm, right ventricle, and outflow tract (Fig. 2). No expression was detected in the left ventricle, the atria, or any other cardiac structures outside the anterior heart field at 9.5 dpc (Fig. 2C-E) or 11.5 dpc (Fig. 2F-H). The expression of lacZ directed by this Mef2c enhancer was also examined in the fetal and adult heart (Fig. 2J). Expression in the fetal heart was largely restricted to the outlet region of the right ventricle near the base of the heart and to the right ventricular outflow (pulmonary artery), although weaker expression could also be observed in other regions of the right ventricle (Fig. 2I). No expression of lacZ was observed in the adult heart (Fig. 2J), and no β-galactosidase activity could be detected outside of cardiac lineages at any embryonic, fetal or adult stage. These results indicate that the function of this conserved Mef2c enhancer is restricted to the anterior heart field during development and that it does not function in the adult heart.

The Mef2c anterior heart field regulatory region contains an endogenous promoter and is controlled by a small, evolutionarily conserved enhancer module

Our screen of all the conserved sequences from the Mef2c locus utilized a strategy in which each conserved region was fused to the HSP68 heterologous promoter to determine if a given region conferred specific enhancer activity. Because the anterior heart field regulatory region from the Mef2c gene encompasses the third untranslated exon from the Mef2c gene (depicted in Fig. 1A), we reasoned that this regulatory module might contain its own, endogenous promoter. To determine if promoter sequences were present within the Mef2c anterior heart field regulatory module, we cloned nucleotides 1-3970, which includes the upstream region of the module and the first 76 nucleotides of untranslated exon 3, into the promoterless transgenic reporter plasmid AUG-β-gal (McFadden et al., 2000) and tested for activity in transgenic embryos (Fig. 3A, Frag #3). This region of the enhancer directed robust expression in transgenic embryos when fused to the HSP68 promoter (Fig. 3A, Frag #2; Fig. 3B). Similarly, this region of the Mef2c gene also directed strong expression to the anterior heart field in the absence of a heterologous promoter (Fig. 3C), indicating the presence of an endogenous promoter immediately upstream of the third untranslated exon (Fig. 3A).

Next we wanted to define the minimal region of the enhancer that was required for expression in vivo as a first step toward
defining a transcriptional pathway upstream of \( Mef2c \) in the anterior heart field. Toward this goal, we tested a series of enhancer deletion fragments for the ability to direct cardiac specific expression in transgenic embryos (Fig. 3A). These analyses identified a 1.4 kb fragment (Fig. 3A, Frag #6) that was sufficient to direct robust expression of \( \text{lacZ} \) exclusively to the anterior heart field (Fig. 3D). By contrast, a 1.1 kb fragment (Fig. 3A, Frag #7) was completely incapable of directing any expression in vivo (Fig. 3E). Thus, these results indicate that the 286 bp region between nucleotides 2584 and 2869 is essential for cardiac expression and that one or more critical \( \text{cis} \)-acting elements must reside in that region. Based on these observations, we tested the 286 bp region for enhancer function in vivo to determine if this region of the \( Mef2c \) gene was sufficient for enhancer activity (Fig. 3A, Frag #7). In five independent transgenic events, this region of the enhancer was never sufficient to direct \( \text{lacZ} \) expression (Fig. 3F), indicating that additional sequences were required for enhancer function in vivo. Importantly, addition of 163 nucleotides (Fig. 3A, Frag #10) conferred strong anterior heart field specific expression (Fig. 3G). These results indicated that additional \( \text{cis} \)-acting elements required for anterior heart field expression must also reside within the 163 bp between nucleotides 2869 and 3032. Taken together, these deletional analyses identified a 449 bp minimal enhancer element (nucleotides 2584-2869) sufficient for anterior heart field specific expression in vivo, which contains two smaller regions that are each required for enhancer function.

**The \( Mef2c \) anterior heart field enhancer is dependent on essential GATA and ISL1 sites**

To identify specific transcription factor binding sites within the minimal sufficiency region of the enhancer, we analyzed it for evolutionarily conserved sequences that might serve as potential \( \text{cis} \)-regulatory elements. These analyses identified several putative candidate binding sites for cardiac gene expression. Among these were three conserved candidate binding sites for the GATA family of transcription factors that resided between nucleotides 2584 and 2869. The proximal GATA site (GATA-p) consisted of a conserved, perfect consensus element; the medial GATA site (GATA-m) consisted of a conserved, imperfect GATA element; and the distal GATA site (GATA-d) represented a conserved, perfect consensus GATA sequence (Fig. 4). These analyses also identified two conserved perfect consensus elements for the LIM-homeodomain transcription factor ISL1 that were present between nucleotides 2869 and 3032 (Fig. 4). In addition, we identified two candidate E boxes, representing potential binding sites for HAND factors, and a putative NKE binding site for NK class factors such as NKX2.5 (Fig. 4). As a first
Fig. 4. Alignment of conserved mouse and human sequence in the necessary and sufficient region of the MeF2c anterior heart field enhancer. Three conserved candidate GATA sites (red shading), two conserved ISL sites (green shading), two candidate E boxes (yellow shading) and a conserved NKE (black box) are noted. The three GATA sites are contained within Fragment 9, while the two ISL sites and the GATA sites are included in Fragment 10. Numbers at the right represent the nucleotide positions within the mouse MeF2c-F6/Frag2 sequence depicted in Fig. 3 and deposited in GenBank (accession number AY324098).

test to determine if these candidate sites might represent bona fide binding sites, we tested each of these elements for the ability to bind to their respective candidate transcription factors by EMSA. Among all the sites in the enhancer, robust binding by putative transcriptional regulators was only detected for two of the GATA elements and for both the ISL sites. GATA4 bound specifically to the GATA-p (Fig. 5, lanes 9-14) and GATA-d (lanes 15-20) sites but was completely unable to bind to the GATA-m element (data not shown). We compared the ability of GATA4 to bind to the MeF2c GATA sites to its ability to bind to a control GATA site (gs1) from Nkx2.5 (Lien et al., 1999) (Fig. 5, lanes 1-8). GATA4 bound robustly to the Nkx2.5 gs1 site (Fig. 5, lane 2), and binding to this site was completely ablated by unlabeled MeF2c GATA-d and GATA-p (Fig. 5, lanes 5 and 7), indicating that the MeF2c sites represented bona fide GATA elements. By contrast, mutant versions of the GATA-d and GATA-p sites were unable to compete for binding to the Nkx2.5 gs1 (Fig. 5, lanes 6 and 8). GATA4 binding to the MeF2c GATA-p site (Fig. 5, lane 10) was specific, since it was competed by an excess of unlabeled self-probe or by an excess of unlabeled Nkx2.5 gs1 (Fig. 5, lanes 11 and 13). Mutant versions of each of these oligonucleotides were unable to compete for GATA4 binding to GATA-p, even at a 100-fold excess (Fig. 5, lanes 12 and 14). Similarly, the MeF2c GATA-d site was bound efficiently by GATA4 (Fig. 5, lane 16) and this binding was specific since it was competed by excess unlabeled oligonucleotides representing the GATA-d site itself or the Nkx2.5 gs1 site (Fig. 5, lanes 17 and 19). Excess mutant Nkx2.5 gs1 or MeF2c GATA-d failed to compete for GATA4 binding to the MeF2c GATA-d site (Fig. 5, lanes 18 and 20). Taken together, all the results from Fig. 5 demonstrate that the MeF2c GATA-p and GATA-d elements represent robust and specific binding sites for GATA4.

Similarly, we tested the ability of ISL1 to bind to each of the two conserved, perfect consensus ISL sites in the enhancer (Fig. 6). ISL1 bound specifically to the ISL-p (Fig. 6, lanes 9-14) and ISL-d (Fig. 6, lanes 15-20) sites as well as it bound to the bona fide control Insulin I ISL1 site (Fig. 6, lanes 1-8). Binding of ISL1 to each of the ISL sites in the MeF2c enhancer (Fig. 6, lanes 10 and 16) was specific, since it could be competed by excess unlabeled probe (Fig. 6, lanes 11 and 17) but not by a 100-fold excess of unlabeled mutant probe (Fig. 6, lanes 12 and 18). In addition, binding to both sites was competed by unlabeled
control Insulin I probe (Fig. 6, lanes 13 and 19) but not by a 100-fold excess of a mutant version of that probe (Fig. 6, lanes 14 and 20). Similarly, unlabeled ISL-d (Fig. 6, lane 5) and ISL-p (Fig. 6, lane 7) probes each efficiently competed for the binding of ISL1 to the control ISL1 site from the Insulin I gene, but mutant versions of those sites were unable to compete for binding (Fig. 6, lanes 6 and 8, respectively). Taken together, the results from Fig. 6 demonstrate that each of the conserved ISL1 sites in the critical region of the Mef2c anterior heart field enhancer are bound specifically by ISL1.

To test the function of the Mef2c GATA and ISL sites in vivo, we introduced mutations into each site in the context of the Mef2c-F6/Frag2-lacZ transgene (Fig. 3, Frag #2) and determined the effect of those mutations on enhancer function at 9.5 dpc (Fig. 7). The introduced mutations were identical to those used in the EMSA analyses, which were shown in Figs. 5 and 6 to completely ablate GATA4 and ISL1 binding, respectively. The wild-type Mef2c fragment directed strong expression of lacZ in the pharyngeal mesoderm, outflow tract and right ventricle at this stage (Fig. 7A). Double mutation of the Mef2c GATA sites completely ablated all transgene expression in every transgenic founder embryo examined (Fig. 7B). Likewise, mutation of the two ISL binding sites in the enhancer also completely disrupted lacZ expression in transgenic embryos (Fig. 7C). We also examined the effect of the ISL and GATA double mutations at 8.0 dpc and 11.5 dpc, and we observed no X-gal staining at any stage (data not shown). Single mutation of each of the individual GATA sites in the enhancer had a dramatic, but not complete, effect on enhancer function. Mutation of the GATA-p element only almost completely ablated activity of the enhancer, although very weak expression could sometimes be seen in the pharyngeal mesoderm; mutation of the GATA-d site resulted in a dramatic reduction in β-galactosidase activity, but the pattern of lacZ expression directed by this mutant was unchanged, suggesting that this site has a quantitative effect on expression in vivo (data not shown).

In addition to the ISL and GATA sites in the enhancer, we also tested the candidate NKE and the candidate E boxes to determine if these putative sites might be targets for NKX2.5 and HAND proteins, respectively. The candidate NKE in the Mef2c enhancer failed to bind to any detectable amount of the cardiac restricted transcription factor NKX2.5 under conditions in which NKX2.5 bound to the NKE from the Gata6 enhancer. Furthermore, the candidate NKE

![Image](image-url)

**Fig. 6.** The ISL sites in the Mef2c anterior heart field enhancer are specifically bound by ISL1. Isl1 was transcribed and translated in vitro and incubated with radiolabeled, double-stranded oligonucleotides spanning the Mef2c ISL-p site (lanes 9-14), the Mef2c ISL-d site (lanes 15-20) or a consensus ISL1 binding site from the rat Insulin I gene as a control (lanes 1-8). ISL1 efficiently bound to all three ISL sites (lanes 2, 10 and 16). Binding of ISL1 to the ISL-p site was specifically competed by excess unlabeled Mef2c ISL-p (I-p) (lane 11) and by excess unlabeled Insulin I ISL1 site (In) (lane 13) but not by a 100-fold excess of mutant ISL-p site (mI-p) (lane 12) or by a 100-fold excess of a mutant Insulin I site (mln) (lane 14). Likewise, the binding of ISL1 to the ISL-d site was specifically competed by excess unlabeled ISL-d (I-d) (lane 17) and by excess unlabeled Insulin I site (lane 19) but not by a 100-fold excess of either mutant ISL-d (ml-d) (lane 18) or mln control (lane 20). The Mef2c ISL-p and ISL-d sites also efficiently competed for ISL1 binding to the control ISL1 site from the Insulin I promoter (lanes 5 and 7), but mutant versions of the ISL-p and ISL-d sites were unable to compete for binding to the Insulin I site (lanes 6 and 8). In samples where in vitro translated ISL1 protein was not included (denoted by a minus sign in lanes 1, 9 and 15), an equal amount of unprogrammed reticulocyte lysate was included.

![Image](image-url)

**Fig. 7.** The Mef2c anterior heart field enhancer is dependent on GATA and ISL sites for function in vivo. The wild-type Mef2c enhancer transgene construct, Mef2c-F6/Frag2-lacZ, or transgenes carrying mutations in either the two GATA sites or the two ISL sites in that context, were used to generate transgenic mice. Representative transgenic embryos at 9.5 days post coitum (dpc) are shown. The wild-type fragment directed strong expression to the pharyngeal mesoderm, outflow tract and right ventricle (A). Mutation of the GATA-p element nearly completely eliminated transgene expression in vivo, although very weak expression could sometimes be seen in the pharyngeal mesoderm. Mutation of the GATA-d site had a dramatic impact on the level of transgene expression, although the pattern of expression remained unchanged. Mutation of both GATA sites in the enhancer (mGATA) completely eliminated lacZ expression (as shown in B). For the double GATA mutant, eight independent transgenic events were analyzed, and none showed any expression in the heart. Mutation of both ISL sites (mISL) in the enhancer completely eliminated all transgene expression (C) in all six independent transgenic events analyzed. The wild-type 3970 bp enhancer construct directed robust lacZ expression, as shown in (A), in 16 of 17 transgenic lines. Arrowheads denote expression in the pharyngeal mesoderm/anterior heart field. RV, right ventricle.
in the Mef2c enhancer was completely dispensable for enhancer function in vivo (data not shown). Similarly, we tested whether either of the E boxes in the Mef2c anterior heart field enhancer were bound by HAND proteins. Because HAND proteins have been shown to bind as homodimers or as heterodimers with each other and with E proteins (Firulli et al., 2000), we tested the ability of dHAND/E12, eHAND/E12, dHAND/dHAND, and dHAND/eHAND dimers to bind to each of the candidate E boxes in the enhancer. In no case were we ever able to detect any binding by these candidate factors to either of the sites in the enhancer under conditions in which a control site from the ANF gene was bound efficiently by dHAND/E12 heterodimers in EMSA (data not shown).

Taken together, the results of the EMSA and mutational analyses demonstrate that the Mef2c anterior heart field enhancer is bound by GATA factors and by ISL1 and that the evolutionarily conserved binding sites for these factors are completely required for enhancer function in vivo. These results also suggest that Mef2c expression in the anterior heart field is not dependent on direct activation by Nkx2.5 or HAND proteins. Overall, the results presented in this study support a model for anterior heart field expression of Mef2c that is dependent on cardiac restricted GATA transcription factors and ISL1 through direct binding and activation of a discrete anterior heart field enhancer module.

Discussion

Embryos lacking Mef2c have severe defects in the outflow tract and fail to form a right ventricle (Lin et al., 1997), which is consistent with the strong expression of Mef2c in the anterior heart field. At the time of cardiac looping, Mef2c transcripts are present in the left ventricle and atria but are much more robustly expressed in the right ventricle, outflow tract and pharyngeal mesoderm (Fig. 1A) (Edmondson et al., 1994). These observations suggest a key role for MEF2C in the transcriptional pathways controlling myoblast differentiation in the anterior heart field. Thus, identification of the transcription factors functioning upstream of Mef2c represents a critical step toward understanding the regulation of gene expression in the anterior heart field and the function of Mef2c in the embryonic heart. In this study, we identify a transcriptional enhancer from the Mef2c gene that is sufficient to direct robust expression to the anterior heart field, and we show that this enhancer is a direct target for activation by the LIM-homeodomain protein ISL1 and the GATA family of zinc finger transcriptional regulators. The observation that Mef2c is a direct transcriptional target of ISL1 in vivo is consistent with the observations that disrupted expression of either of those genes results in nearly identical phenotypes. Mice lacking either Mef2c or Isl1 exhibit gross abnormalities in the heart, which fails to loop, and have severely affected or absent outflow tract and right ventricle (Cai et al., 2003; Lin et al., 1997). The nearly identical defects in the outflow tract and right ventricle demonstrate the essential role of each of these transcription factors in anterior heart development and further support the observations presented here that Mef2c is a direct target of ISL1. These observations, taken together with the results of the work presented here, strongly support a role for MEF2C as a key downstream effector of ISL1 in the anterior heart field.

A GATA- and ISL1-dependent model for transcriptional activation in the anterior heart field

The Gata4/S6 genes are among the earliest markers of the cardiac lineage in the developing mouse, with expression appearing throughout the precardiac mesoderm as early as the expression of Nkx2.5 (Arceci et al., 1993; Parmacek and Leiden, 1999). This very early expression suggests that GATA transcription factors are likely to be involved in the initial specification of cardiomyocytes, which is supported by studies in embryonal carcinoma stem cells that showed that expression of GATA4 dominantly induced precocious cardiac differentiation (Grepin et al., 1995). Furthermore, expression of a dominant negative form of GATA4 in those cells blocked cardiac differentiation (Grepin et al., 1997). Additional evidence for a role for GATA factors in cardiac specification comes from studies in zebrafish, which demonstrated that GATA5 could dominantly induce cardiogenesis and the expression of Nkx2.5 (Reiter et al., 1999). Likewise, misexpression of murine GATA4 in Drosophila results in expansion of cardiac cells in the dorsal mesoderm (Gajewski et al., 1999).

The expression directed by the Mef2c enhancer described here is restricted to the anterior heart field throughout development. This enhancer contains two conserved GATA sites, which are bound by GATA4 with high affinity and are required for enhancer activity. These results indicate that Mef2c is a direct target of GATA factors and support a model in which GATA factors play a crucial role in the specification of cardiomyocytes in the anterior heart field. Additional support for this model comes from studies showing that Nkx2.5 and dHAND also appear to be direct transcriptional targets of GATA factors in the anterior heart field (Lien et al., 1999; McFadden et al., 2000; Searcy et al., 1998). Importantly, the cardiac restricted GATA transcription factors, GATA4/S6, are expressed more broadly than the anterior heart field restricted pattern of the Mef2c-lacZ transgene described in this study (Arceci et al., 1993; Morrisey et al., 1996; Morrisey et al., 1997; Parmacek and Leiden, 1999). The broader expression pattern of the GATA factors suggests that additional regulators must be required to restrict enhancer function to the anterior heart field. Indeed, in this study, we show that the Mef2c anterior heart field enhancer is also a direct target of ISL1. As is the case for the GATA factors, ISL1 expression within the embryo is broader than the pattern directed by the Mef2c enhancer described here, but expression of ISL1 within cardiogenic lineages appears to be limited to the anterior heart field (Cai et al., 2003). Thus, these data suggest a model for anterior heart field-specific transcriptional activation dependent on the combined activities of ISL1 and GATA transcription factors.

The Mef2c enhancer described here is the first identified direct transcriptional target of ISL1 in the anterior heart field. It will be interesting to determine if other anterior heart field restricted genes or enhancers are also dependent on ISL1 for activation. Notably, the GATA-dependent cardiac enhancer from the Nkx2.5 gene, which appears to direct expression to the anterior heart field, also contains a perfect consensus site for ISL1 in close proximity to the essential GATA binding site, although the function of the ISL1 site in that enhancer was not investigated (Lien et al., 1999). GATA factors have been shown to function cooperatively with other transcription factors expressed in the heart, including SRF, Nkx2.5, dHAND and...
MEF2C (Dai et al., 2002; Durocher et al., 1997; Lee et al., 1998; Morin et al., 2000; Sepulveda et al., 2002), and it will be interesting to determine if GATA factors function as part of a cooperative transcriptional complex with ISL1 as well.

**Distinct transcriptional hierarchies in the primary and secondary heart fields**

A GATA- and ISL1-dependent transcriptional cascade in the anterior heart field contrasts with the hierarchy present in the primary heart field and in the Drosophila heart. In Drosophila, the Nkx2.5 ortholog, tinman, is the earliest marker of the heart (Frasch, 1999) and is thought to be the initial transcriptional activator responsible for cardiac specification (Cripps and Olson, 2002; Frasch, 1999). In the fly heart, the GATA transcription factor gene, pannier, is a direct downstream target of Tinman, which also directly activates the transcription of Mef2 (Gajewski et al., 1997; Gajewski et al., 2001). A similar NKK dependent hierarchy appears to exist in the primary heart field in vertebrates as well. NKK2.5 is among the earliest known cardiac transcription factors expressed in the primary heart field in vertebrates (Brand, 2003; Harvey, 1996; Lints et al., 1993), and some evidence suggests that NKK2.5 may play a role in cardiomyocyte specification (Yamagishi et al., 2001). Furthermore, recent studies of the Nkx2.5 gene have identified several enhancers regulating expression in the heart (Schwartz and Olson, 1999). One of these enhancers directs expression to the cardiac crescent and primary heart tube and was shown to be dependent on SMAD proteins in vivo (Liberatore et al., 2002; Lien et al., 2002). These observations suggest that Nkx2.5 may be a direct target of BMP-inducing activity, and that this enhancer may serve as the initiator of cardiac specification in the primary heart field (Liberatore et al., 2002; Lien et al., 2002). Taken together, all these observations suggest that the transcriptional hierarchies for myocyte development in the two vertebrate heart fields might be divergent, with NKK2.5 as the critical factor in the primary heart field and GATA factors, along with ISL1, as the key transcriptional regulators in the anterior heart field.

**Modular regulation of Mef2c expression**

An important question that remains to be defined is how the cells derived from the anterior and primary heart fields are integrated during cardiac morphogenesis. Cells appear to be added progressively from the pharyngeal mesoderm into the conotruncal region of the heart at the time of cardiac looping, and evidence suggests that this addition ultimately contributes to the outflow tract and much of the right ventricle (Kelly et al., 1998; Morin et al., 2000; Sepulveda et al., 2002), and it will be interesting to determine whether these enhancers function as truly independent modules or whether they operate together in the context of the entire Mef2c locus.

The expression pattern of Mef2c during mouse development is complex. Transcripts are abundant in heart, skeletal muscle, vasculature and neural crest (Edmondson et al., 1994; Leifer et al., 1993; Lyons et al., 1995a). The studies presented here have defined for the first time a transcriptional enhancer from Mef2c sufficient to direct expression only to the anterior heart field, and previous studies have identified a transcriptional enhancer from Mef2c that functions only in skeletal muscle (Dodou et al., 2003; Wang et al., 2001). The identification of these discrete enhancer modules strongly suggests that the expression of Mef2c is controlled by separate transcriptional enhancers in each lineage where it is expressed. This type of modular model for transcriptional regulation dependent on multiple, separate enhancers has been proposed previously as a mechanism for regulatory diversity (Firulli and Olson, 1997). This seems to be the case for Mef2c, as we have recently identified additional enhancers from the Mef2c BAC (Fig. 1A) that are capable of independently directing lacZ expression to vascular endothelium, smooth muscle and neural crest (M.V., J.A. and B.B., unpublished observations). It will be interesting to determine whether these enhancers function as truly independent modules or whether they operate together in the context of the entire Mef2c locus.

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**References**


Cai, C. L., Liang, X., Shi, Y., Chu, P. H., Pfaff, S. L., Chen, J. and Evans, S. (2003). Isl1 identifies a cardiac progenitor population that proliferates...
prior to differentiation and contributes a majority of cells to the heart. Dev. Cell 5, 877-889.


Yamagishi, H., Yamagishi, C., Nakagawa, O., Harvey, R. P., Olson, E. N. and Srivastava, D. (2001). The combinatorial activities of Nkx2.5 and dHAND are essential for cardiac ventricle formation. Dev. Biol. 239, 190-203.
