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An HF-1a/ HF-1b/MEF-2 combinatorial element confers cardiac ventricular specificity and establishes an anterior-posterior gradient of expression

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SUMMARY

The molecular determinants that direct gene expression to the ventricles of the heart are for the most part unknown. Additionally, little data is available on how the anterior/posterior axis of the heart tube is determined and whether the left and right atrial and ventricular chambers are assigned as part of this process. Utilizing myosin light chain-2 ventricular promoter/β-galactosidase reporter transgenes, we have determined the minimal *cis*-acting sequences required for ventricular-specific gene expression. In multiple independent transgenic mouse lines, we found that both a 250 base pair myosin light chain-2 ventricular promoter fragment, as well as a dimerized 28 bp sub-element (HF-1) containing binding sites for HF1a and HF1b/MEF2 factors, directed ventricular-specific reporter expression from as early as the endogenous gene, at day 7.5-8.0 post coitum. While the endogenous gene is expressed uniformly throughout both ventricles, the transgenes were expressed in a right ventricular/conotruncal dominant fashion, suggesting that they contain only a subset of the elements which respond to positional information in the developing heart tube. Expression of the transgene was cell autonomous and its temporospatial characteristics not affected by mouse strain/methylation state of the genome. To determine whether ventricular-specific expression of the transgene was dependent upon regulatory genes required for correct ventricular differentiation, the 250 base pair transgene was bred into both *retinoid X receptorα* and *Nkx2-5* null backgrounds. The transgene was expressed in both mutant backgrounds, despite the absence of endogenous myosin light chain-2 ventricular transcript in *Nkx2-5* null embryos. Ventricular specification, as judged by transgene expression, appeared to occur normally in both mutants. Thus, the HF-1 element, directs chamber-specific transcription of a transgene reporter independently of *retinoid X receptorα* and *Nkx2-5*, and defines a minimal combinatorial pathway for ventricular chamber gene expression. The patterned expression of this transgene may provide a model system in which to investigate the cues that dictate anterior-posterior (right ventricle/left ventricle) gradients during mammalian heart development.

Key words: cardiogenesis, morphogenesis, homeobox genes, transcriptional regulation, retinoic acid receptor, embryonic patterning

INTRODUCTION

Cardiogenesis is one of the earliest and most-critical steps during vertebrate embryogenesis. Defects in cardiac ventricular chamber growth, maturation and morphogenesis are often associated with embryonic lethality, as evidenced in a growing number of gene-targeting studies in murine embryos (Kastner et al., 1994; Yang et al., 1995; Lyons et al., 1995; Sucov et al., 1994).

The morphogenic changes that give rise to a fully mature heart begin early during embryonic development, when paired primordia arise in anterior splanchnic mesoderm of the presomitic embryo and cluster in a region termed the cardiogenic plate (Davis, 1927). These cells give rise to a distinct cardiogenic crescent, which grows in size, and condenses to form a midline heart tube. Fusion and subsequent differentiation of these progenitor cells occurs in a craniocaudal direction, suggestive of a distinct anterior/posterior (A/P) organization at this early time. Subsequently, the bilaterally symmetrical aspects of this process are disrupted with the rightward and ventrocaudal looping of the tube, the first evidence of left-right asymmetry in the developing murine embryo (Levin et al., 1995; Hoyle et al., 1992). The primitive ventricular chamber then undergoes expansion of the compact zone and trabeculation, followed by formation of a muscular septum, distinguishing distinct right (RV) and left ventricular (LV) chambers.

Currently, our understanding of the molecular cues that confer the ventricular phenotype or regional specificity of genes within these ventricular chambers, is limited. In addition,

only rudimentary data is available as to which molecules might orchestrate differential gene expression and morphogenesis in the left/right axis of the developing embryo and heart tube. While gene-targeting studies have implied that a number of factors may be involved in these processes, the precise role of any one, is unclear. Furthermore, evidence is now accumulating that ventricular myogenic specification occurs through a combinatorial pathway, as distinct from a master-regulatory mechanism (Evans et al., 1994).

To define the molecular cues that guide early stages of ventricular chamber specification, maturation and morphogenesis, our laboratory has employed the myosin light chain-2 ventricular gene (*MLC-2v*) as a model system (Chien et al., 1993). The *MLC-2v* gene encodes a contractile protein which is expressed in both cardiac and slow skeletal muscle. Within the heart, MLC-2_v is the earliest known ventricular-specific marker in vertebrate cardiogenesis (O'Brien et al., 1993) displaying bilaterally symmetrical expression in a restricted zone of the cardiogenic crescent prior to fusion of the progenitors in the midline at day E7.5-8.0 (Lyons et al., 1995). Chamber-specific expression of *MLC-2v* is seen throughout embryonic and postnatal development. Previous studies have established that a 250 base pair (bp) MLC-2_v promoter fragment can confer ventricular specificity in transgenic mice (Lee et al., 1992). However, the precise combinatorial pathways which confer chamber specificity, remain unclear.

The current study provides evidence that a 28 bp element termed HF-1, which is composed of adjacent HF-1a and HF-1b/MEF-2 sites, is sufficient to confer ventricular specific expression of a β-galactosidase (*lacZ*) reporter gene during early stages of murine cardiogenesis. A duplex HF-1 oligonucleotide completely recapitulated the pattern of expression seen with the intact 250 bp MLC-2_v promoter fragment. In contrast to the even biventricular expression of the endogenous gene, both of these transgenes are initially expressed bilaterally in the embryo, and subsequently dominant expression is seen in the conotruncal/right ventricular (RV) regions.

To determine whether other mutations associated with ventricular chamber defects intersect with this HF-1 dependent pathway, transgenic mice were crossed into genetic backgrounds that were deficient in the Retinoid X Receptor α (*RXRα*) or the homeobox *Nkx2-5* gene, where down-regulation of endogenous *MLC-2v* expression is found (Lyons et al., 1995). In both null backgrounds, it was apparent that ventricular specification, as well as development of graded positional information, had occurred normally in their hearts, as judged by MLC-2_v/lacZ transgene expression.

MATERIALS AND METHODS

Cloning procedures

To generate the wild-type 250 bp MLC-2_v/lacZ expression vector, a full-length *E. coli lacZ* reporter cDNA was excised from the plasmid pCMVβ (Clontech) and inserted into the unique *Not*I site in a parent vector which contains the 250 bp MLC-2_v promoter (Hunter et al., 1995). Orientation of the insert was confirmed by restriction endonuclease and sequencing analyses. For generation of constructs containing mutations in the HF-3 and MLE1 sites, the wild-type MLC-2_v promoter was excised and replaced with mutant promoters (Zhu et al., 1991; Navankasattusas et al., 1994).

For 'minimal promoter' constructs, oligonucleotides were synthesized (Pharmacia Gene Assembler) with 5′ *Hin*dIII and 3′ *Spe*I ends

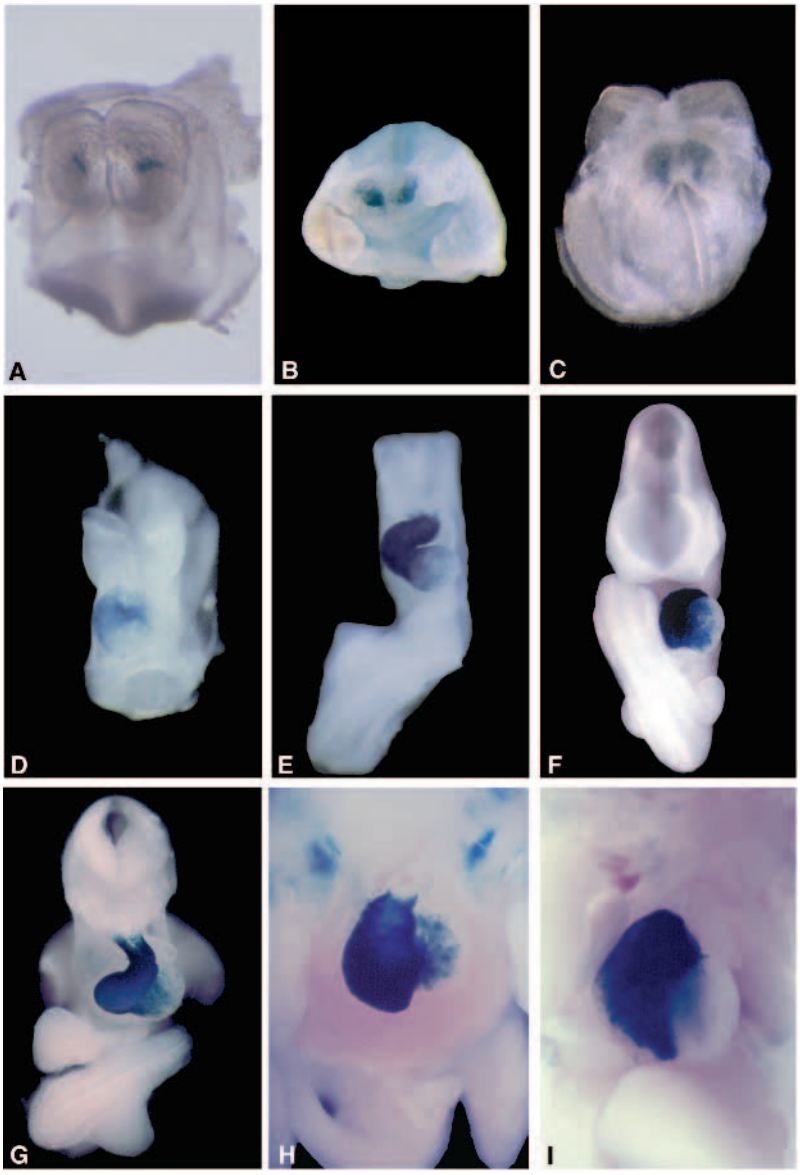

Fig. 1. Timed transgenic MLC-2_v/lacZ embryos (line 54) stained in whole mount for β-galactosidase activity. (A) Day 7-7.5 p.c. (early headfold stage). (B) Day 7.5-8 p.c. (C) Day 7.5-8 p.c. (D) Day 8-8.5 p.c. (E) Day 9-9.5 p.c. (F) Day 9.5-10 p.c. (G) Day 11-11.5 p.c. (H) Day 12-12.5 p.c. and (I) day 16-16.5 p.c. β-gal staining was initially evident in a bilaterally symmetrical manner at the earliest time points of cardiogenesis (A-C) in presumptive cardiac precursor cells flanking the midline. By day 8.0 p.c. as the linear heart tube forms (D), asymmetric β-gal staining was evident with dominant expression of the transgene in the conotruncal and bulboventricular sections of the forming heart. This pattern persisted through the remainder of cardiogenesis (E-I) and was clearly defined in the septated heart by day 12 p.c. (H).

to allow for cloning into unique sites in the parent *lacZ* expression vector after excision of the wild-type 250 bp MLC-2_v fragment:

(a) MLC-2_v TATA: $^{-41}$CCAGGGAAGAGGTATTTATTGTTC-CACAGCAGGGG^{-7},

(b) HF-1b/MLC-2_v TATA: $^{-57}$GGGGTTATTTTTAACC^{-42} + MLC-2_v TATA sequence (above),

(c) HF-1/MLC-2_v TATA: $^{-72}$GCCAAAAGTGGTCATGGGGT-TATTTTTAACC^{-42} + MLC-2_v TATA sequence,

(d) MLE1/HF-1b MLC-2_v TATA: $^{-178}$GGTCTCACGTGTCC^{-165} AAGCTT $^{-57}$GGGTTATTTTTAACC^{-42} + MLC-2_v TATA sequence,

(e) PRE B/HF-1b MLC-2_v TATA: $^{-317(\alpha MHC)}$GGGCCAT-

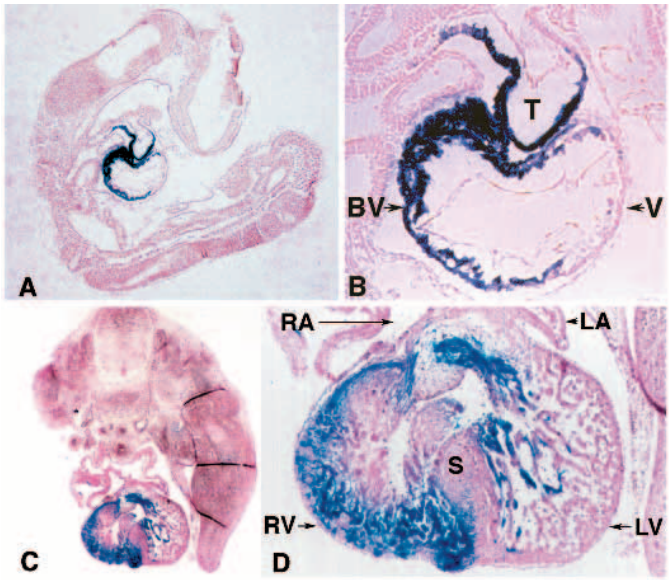

Fig. 2. Representative histological sections of MLC-2_v/lacZ transgenic embryos stained for *lacZ* expression as whole mounts and subsequently sectioned. (A,B) Parasagittal sections of a day 9-9.5 p.c. embryo. β-gal staining was evident in an asymmetric manner with dominant expression in the conotruncus and bulboventricular locations. (C,D) Transverse sections of a day 12-12.5 p.c. embryo. Expression was seen only in the ventricular chamber with none in the atria and dominant expression in the right ventricle. Sections were counterstained with 1% eosin. BV, bulboventricular; LA, left atrium; LV, left ventricle; RA, right atrium, RV, right ventricle; S, septum; T, truncus; V, ventricle.

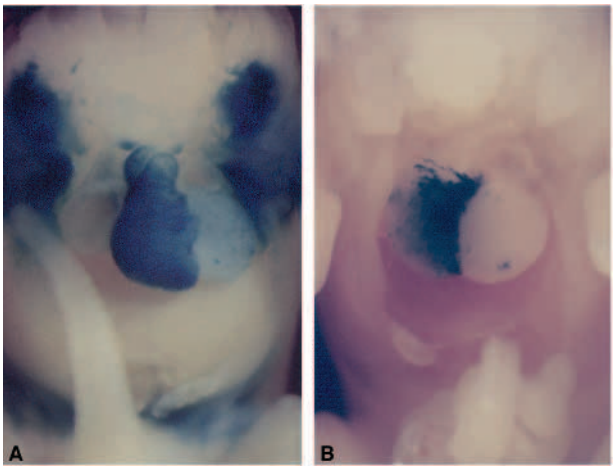

Fig. 3. Whole-mount staining of representative embryos from HF-3 mutant MLC-2_v/lacZ transgenic lines. (A) Day 12 p.c. embryo of HF-3 line 9. (B) Day 12 p.c. embryo of HF-3 line 41. Ectopic as well as skeletal muscle staining is seen in one line (A) while another representative line showed no staining in any location but the heart where an RV dominant pattern of expression is detected (B).

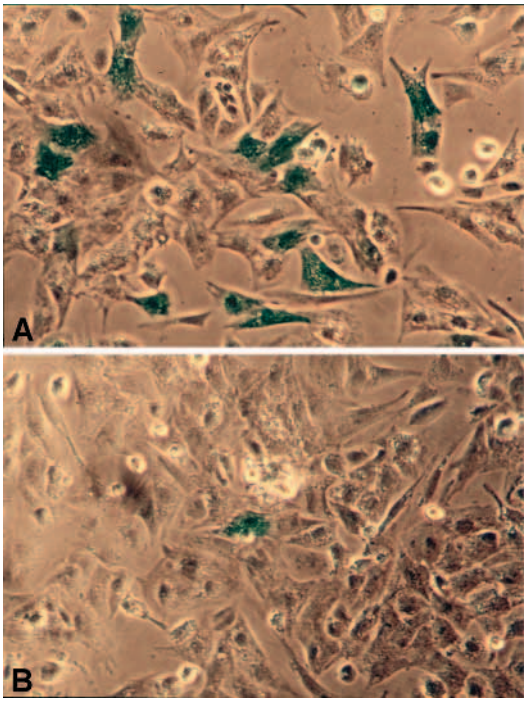

Fig. 4. *lacZ* expression in ventricular myocytes is cell autonomous. (A) RV myocytes, (B) LV myocytes. Day 15 p.c. embryonic ventriculomyocytes from MLC-2_v (250 bp)/lacZ transgenic animals displayed cytoplasmic β-gal staining following 48 hours in culture with significantly less staining of LV-derived cells. Numerous cells within every microscopic field of RV myocytes showed easily detectable β-gal staining, while only a rare blue LV myocyte could be found.

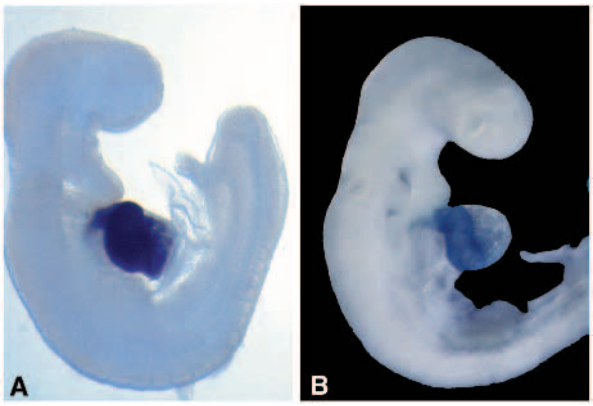

Fig. 5. Whole-mount staining of embryos from a wild-type MLC-2_v/lacZ transgenic line following 3 generations of outcrossing onto the DBA background (A) and in the C57BL/6J genetic background (B). Outcrossing of the C57BL/6J founder animals into the DBA background caused significant increases in qualitative β-gal staining in this and other lines, yet the gradient of expression of the transgene was unchanged.

GTGGG^{-306} TA $^{-57}$GGGTTATTTTTAACC^{-42} + MLC-2_v TATA sequence,

(f) 2× HF-1/MLC-2_v TATA: $^{-72}$GCCAAAAGTGGTCATGGGGTTATTTTTAACC^{-42} CCT $^{-72}$GCCAAAAGTGGTCATGGGGTTATTTTTAACC^{-42} + MLC-2_v TATA sequence.

For the 2× HF-1/thymidine kinase (TK) TATA construct, an *SSTI*/*Bgl*II fragment which contains the TK TATA box (Nordeen, 1988), was cloned into pBluescript SK$^+$ (pBS) (Statagene). The 2× HF-1 sequence (AGCTTGCCAAAAGTGGTCATGGGGTTATTTTTAACCCCTGCCAAAAGTGGTCATGGGGTTATTTTTAACCCCAGCT) was inserted 5′ of this sequence in pBS. A *Hin*dIII/*Spe*I fragment containing the HF-1/TK TATA element was excised from pBS and inserted into the *lacZ* expression vector above. The sequence listed in (f) was utilized in construction of the 2× HF-1 *lacZ* transgenic animals.

Generation, screening and breeding of transgenic mice

DNA utilized for transgenic mouse production was digested with *Hin*dIII and *Kpn*I which cut immediately 5′ of the promoter and 3′ of the poly adenylation signal site, respectively. In this manner, no contaminating plasmid vector sequences were included with the injected DNA. Subsequent DNA preparation and transgenic animal generation was performed as has been previously described (Lee et al., 1992) using standard methodology (Hogan et al., 1986) and fertilized eggs of the CB6 strain. Founder animals and offspring were identified using a combination of genomic Southern blotting and polymerase chain reaction (PCR) using tail biopsies as described previously (Lee et al., 1992). PCR analysis utilized a sense primer homologous to the MLC-2_v promoter, either (a) 5′GGCCCCAGCCACTGTCTCTT3′ for wild-type, HF-3 and MLE1 transgenics or (b) 5′TTTTAACCCCTGCCAAAAGTGG (2× HF-1 construct), and an antisense primer located in the *lacZ* portion of the transgene (5′CTCCTTGCTGGTGTCCAGAC3′). PCR generated a 365 or 232 bp product, depending upon the transgene being analyzed. Confirmation of the genotype of the *RXRα* or *Nkx2-5* null animals or embryos was performed as described previously (Lyons et al., 1995; Sucov et al., 1994).

For breeding, founder animals were crossed initially with C57BL/6J mice, and subsequently by outcrossing C57BL/6J transgenic or founder CB6 animals, with commercially available DBA mice, for at least three generations.

Whole-mount and histological analysis of transgenic embryos

Matings were performed and the presence of vaginal plugs assessed. Embryos were aged as 0.5 day postcoitum (p.c.) at midday of the morning that the vaginal plug was observed. Embryos were analyzed at times ranging from 6.5-20 days p.c. Additional animals were analyzed postnatally and as adults. Embryos or postnatal hearts were fixed and stained using standard techniques (Sanes et al., 1986). Extensive thoracotomy was performed in embryos older than day 11.5 p.c. so that the heart was efficiently penetrated with X-gal substrate during color development. Adult hearts were excised and perfused with solutions using a modified Langendorff apparatus.

Whole embryos or tissues were analyzed and photographed on a Zeiss SV-6 dissecting microscope. For paraffin-based histological analysis, specimens were postfixed in 4% formaldehyde, dehydrated and embedded in paraffin. Sections were cut at 5-8 μm and counterstained with 1% eosin.

Immunostaining and in situ hybridization procedures

For immunostaining and postembedding histochemistry, specimens were fixed in 4% paraformaldehyde for 15-30 minutes, cryoprotected in 30% sucrose/PBS overnight at 4°C and embedded in Aquamount (Lerner)/OCT (Miles) (50:50 volume/volume) mounting medium. Specimens were cryosectioned at 10-15 μm and utilized for immunostaining or histochemistry. Immunostaining was performed using a standard protocol (Harlow and Lane, 1988) and mouse-monoclonal anti-β-galactosidase (Promega) or rabbit-polyclonal anti-MLC-2_V antibodies (Shubeita et al., 1992). Immunofluorescence was observed and photographed using a Nikon Diaphot microscope with epifluorescent optics. Histochemistry was performed by incubation of fixed sections in X-gal substrate overnight at 30°C in a humidified chamber. In situ hybridization protocols were as described by Lyons et al. (1995).

Culture and transfection of cardiac myocytes

Neonatal rat ventricular, and fetal murine myocytes, were cultured as previously described (Kubalak et al., 1995; Zhu et al., 1991). Calcium phosphate transfection, luciferase and cellular *lacZ* assays were performed as has been previously described (Zhu et al., 1991). All transfections were performed in duplicate, with *lacZ* activity being normalized to luciferase activity expressed from a cotransfected control plasmid.

RESULTS

A myosin light chain-2 ventricular (250 bp) promoter fragment confers ventricular-specificity and graded anterior-posterior expression of a lacZ transgene

From 99 mice in the founder generation, 16 were identified to carry the transgene. Founder lines were screened for transgene (*lacZ*) expression by histochemical staining of F_1, F_2 or F_3 embryos at developmental time points between 9 and 12 days p.c. Five of the 16 independent lines from the wild-type MLC-2v/lacZ construct expressed the transgene in a dominant cardiac-specific manner (Table 1). Two additional lines revealed detectable *lacZ* expression at non-cardiac sites: one in developing skeletal muscle (with no expression in the heart) and a second with ectopic staining in the forebrain. Nine lines had no detectable reporter expression.

Several lines displaying a high level of *lacZ* expression were chosen for detailed analysis. The MLC-2_v 250 bp promoter directed cardiac-specific expression from the earliest time points of cardiac differentiation, when cardiac primordia are arranged in a bilaterally symmetrical manner

Table 1. Founder lines and relative expression of wild-type and mutant MLC-2_v/lacZ transgenes

Line	Aortic sac	Cono-truncus	Right ventricle	Left ventricle	Right atrium	Left atrium
MLC-31	–	++++	++++	–	–	–
MLC-54	–	+++	++	+	–	–
MLC-356	–	+++	++	+	–	–
MLC-375	–	+++++	++++	+++	–	–
MLC-M34	–	+++	++	+	–	–
HF3-9	–	++++	+++	++	–	–
HF3-39	–	++	+	–	–	–
HF3-41	–	++++	+++	+	–	–
MLE-1 (10 lines)	–	–	–	–	–	–

Several independent lines for each of the wild-type MLC and mutant HF3 and MLE-1 transgenic lines were evaluated for β-gal staining in the developing heart and great vessels. An arbitrary scale was assigned by intensity of staining with +++++ being the most intense staining noted within a line, – having no detectable staining. Relative intensity within a line can then be noted by the scale across a row. MLC - line 54 embryos are shown in Fig. 1; HF-3 lines 9 and 41 embryos in Fig. 3.

(Fig. 1A-C). This was in agreement with the onset of expression of the endogenous *MLC-2v* gene (Lyons et al., 1995). Once cardiogenesis had proceeded to the linear heart tube stage (Fig. 1D), transgene expression remained cardiac specific, and within the heart, exclusive to the ventricular chamber, with no detectable expression in the atria, aortic sac, conotruncal ridges, or endocardial cushions. A majority of expressing lines revealed an anterior-posterior (RV>LV) gradient of expression. (Table 1). Expression was highest in the cono and bulboventricular regions, and tapered to the lowest detectable level in the primordial LV (Fig. 1D-I). This pattern of expression was maintained throughout development as well as postnatally and in the adult heart (data not shown). Findings from whole-mount staining for β-gal were confirmed with histological sectioning (Fig. 2).

Point mutations in a negative regulatory element (HF-3) do not affect ventricular chamber specificity while mutations in an E-box regulatory element (MLE1) abolishes transgene expression in multiple transgenic lines

To examine the role of discrete *cis*-elements on ventricular regulation and establishment of the A/P gradient of expression, additional transgenic lines were generated which harbored mutations in specific *cis*-regulatory elements. Previous studies have identified a conserved element, HF-3, which acts as a negative regulator of an MLC-2_v/luciferase transgene in adult mice (Lee et al., 1994), as mutations in the HF-3 site result in augmented reporter expression in heart and other muscle tissues. This element had no apparent effect on developmental expression of a lacZ transgene, as three HF-3 mutant MLC-2_v/lacZ lines (Table 1) revealed patterned expression similar to wild-type MLC-2v (250 bp)/lacZ transgenics (Fig. 3). Like the wild type promoter lines, expression of the mutant HF3/MLC-2v/lacZ transgene remained ventricular chamber-specific and the A/P gradient of expression was maintained.

Transient assays in neonatal rat ventricular myocytes (NRVM) have documented the importance of a positive *cis* regulatory element (MLE1) in the maintenance of MLC-2_v promoter activity. This site contains a core E-box consensus site and is identical to the binding site for upstream stimulating factor (USF) first identified in the adenovirus major late promoter (Navankasattusas et al., 1994). To evaluate the importance of this site on chamber specificity and in establishing the A/P gradient of transgene expression, MLC-2_v/lacZ transgenic animals were constructed with a substitution mutation in two base pairs within the MLE1 region. Of 10 founder lines which transmitted the transgene to at least the F_3 generation, no detectable *lacZ* expression was identified, confirming our in vitro data.

The MLC-2_v (250 bp)/lacZ expression is cell autonomous and is not strain dependent

Two types of experiments were performed to determine the mechanism which leads to the A/P gradient of expression of

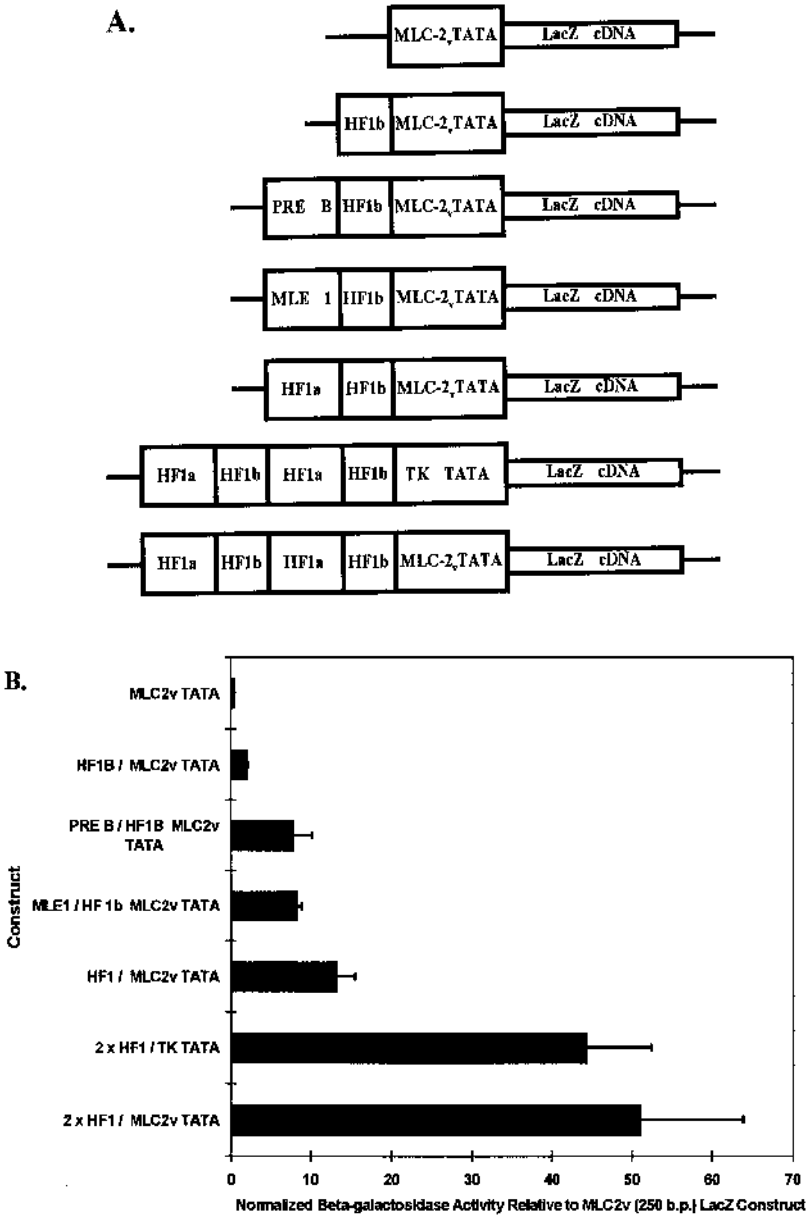

Fig. 6. Constructs utilized for in vivo or in vitro experiments and their respective *lacZ* activity in transient transfection studies of NRVM. (A) Block diagrams of constructs utilized in transfection experiments. (B) β-galactosidase activity of transfected constructs relative to the 250 bp MLC-2_v/lacZ construct. The HF-1 element was seen to have moderate *lacZ* activity while the HF-1b site alone had relatively low activity. Substitution of the HF-1a site with either the PRE B or MLE1 elements conferred moderate activity of expression on the HF-1b core construct. Significant increases in expression were seen when the HF-1 element was dimerized, either in the context of the endogenous MLC-2_v TATA or with the TK TATA in place of the MLC-2_v TATA. *lacZ* activity of each cellular extract was normalized to luciferase activity from cotransfected RSV-luciferase plasmid. Relative activity is expressed as a percentage of the of the wild-type 250 base pair MLC-2_v/lacZ construct. Values are expressed as means ± s.e.m. MLC-2_v TATA, endogenous MLC-2_v TATA box; TK, thymidine kinase.

the MLC-2_V/lacZ transgene. First, to evaluate whether *lacZ* expression was cell autonomous, ventricular cells from day 15 p.c. embryos were cultured. *lacZ* activity remained positive for more than 48 hours in cultured ventricular myocytes. Greater numbers of RV than LV myocytes appeared to express the transgene ex vivo (Fig. 4).

The activity of other transgenes has been shown to be dependent upon methylation state, which in turn can be influenced by genetic background (Engler et al., 1991). Further, graded expression of transgene reporters driven by other muscle-specific promoters, specifically MLC 1/3 fast, have been shown to be governed by the methylation state of the transgene (Donoghue et al., 1991; Grieshammer et al., 1992). The C57BL/6J background, in which initial transgene analysis was performed, is known to be a highly methylated strain. Alternatively, the DBA strain has a relatively hypomethylated genome. Given that a hypomethylated state can be affected by outcrossing a hypermethylated strain into a hypomethylated background, the strain dependence of the MLC-2_v/lacZ transgene gradient was examined.

A number of transgenic lines previously shown to express the *lacZ* reporter in a cardiac-specific/RV-dominant manner, as well as numerous non-expressing founder lines in the C57BL/6J background (including all lines of the HF-3 and MLE1 mutant construct) were examined. Lines that had been originally outcrossed for several generations onto the C57BL/6J background, were outcrossed three or more generations with DBA breeder animals. Embryos were then examined for β-gal staining. Although outcrossing into the hypomethylated DBA strain was associated with a visible gross increase in the intensity of transgene expression, the A/P gradient of expression within the ventricular chambers was not affected (Fig. 5). No strain dependent effect was noted on expression of the MLE1 mutant transgene, as all 10 lines bred into the DBA background still had no detectable expression of the *lacZ* reporter (Table 1). However, one line of the wild-type MLC-2_v/lacZ lines, which showed no expression of the transgene in the C57BL/6J background, expressed it strongly in a cardiac-specific manner when outcrossed three generations into the DBA strain. This was the only line (either wild-type or HF-3 mutant) which showed *lacZ* expression equally in the LV and RV. Two other lines which showed no transgene expression in the C57BL/6J background, developed ectopic expression of *lacZ* in the DBA background. Taken together, these results suggest that the A/P gradient of expression is not a function of genetic background nor methylation state of the genome.

Two copies of a conserved 28 bp element (HF-1) containing HF-1a/ HF-1b/MEF-2 sites confer ventricular specificity and maintain an anterior-posterior (RV/LV) gradient of expression in vivo

Previous work from this laboratory has determined that a conserved 28 bp sequence within the MLC-2_v promoter (HF-1), composed of discrete HF-1a and HF-1b/MEF-2 sites, is required for maximal activity of this promoter in transient assays of cultured ventriculocytes (Zhu et al., 1991). Mutagenesis of either the HF-1a or HF-1b sites in the context of the 250 bp MLC-2_v promoter significantly reduced expression of

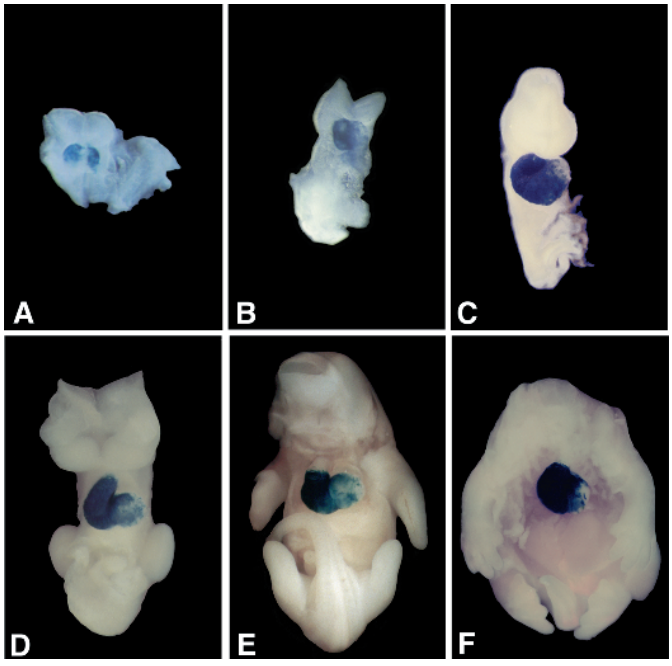

Fig. 7. Whole-mount staining of representative timed 2× HF-1/lacZ embryos. (A) Day 7-7.5 p.c. (late headfold stage/early foregut pocket). (B) Day 8-8.5 p.c. (C) Day 9-9.5 p.c. (D) Day 10-10.5 p.c. (E) Day 12-12.5 p.c. (F) Day 14-14. 5 p.c. Transgene expression was restricted to the heart and displayed in an anterior/posterior gradient similar to the MLC-2_v (250 bp)/lacZ transgenics.

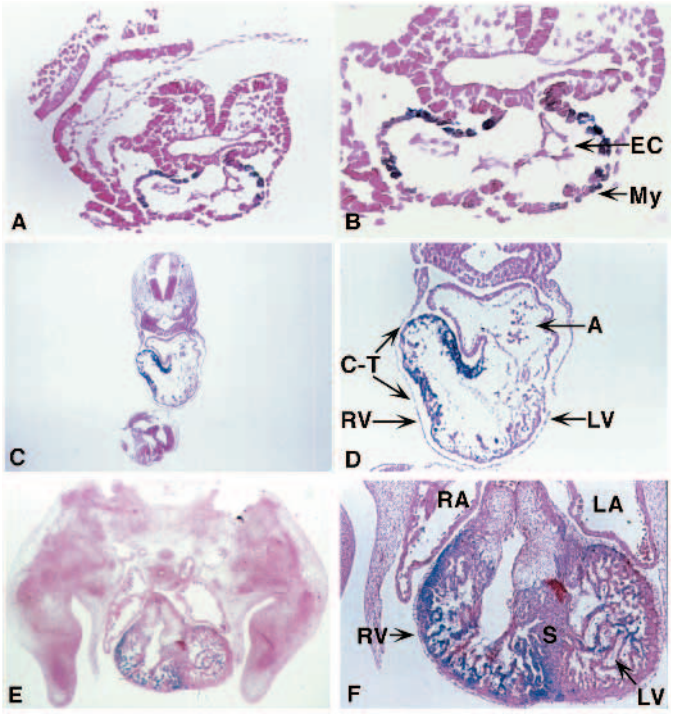

Fig. 8. Representative histological sections of timed 2× HF-1/lacZ transgenic embryos. (A,B) Transverse sections of day 7.5-8 p.c. embryos. (C,D) Parasagittal sections at day 9-9.5 p.c. (E,F) Transverse section at day 12-12.5 p.c. EC, endocardial cells; My, myocardial cells; C-T, conotruncus; LV, primordial left ventricle; RV, primordial right ventricle; A, atria; RA, right atrium; LA. left atrium.

a luciferase reporter gene in adult transgenic mice (Lee et al., 1994). Given these results, we next sought to evaluate whether these elements would be sufficient, in the context of a minimal promoter, to confer ventricular chamber specificity and whether they could establish the A/P gradient of transgene expression during murine cardiogenesis.

We first directly assessed the relative strength of expression of various minimal promoter/lacZ constructs in transient assays in cultured NRVM. Previous studies which first identified the MLE1 site (Navankasattusas et al., 1994), determined that the factor USF could bind multiple cardiac muscle-specific control elements: the MLE1 as well as HF-1a sites of *MLC-2v*, and the PRE B site of the α-myosin heavy-chain (α-MHC) gene. Given these data, transient transfection studies were performed in NRVM utilizing a minimal construct consisting of the MLC-2_v TATA box fused to a *lacZ* reporter gene. As shown in Fig. 6, this unmodified construct had essentially background activity. The HF-1b site in combination with the TATA construct increased activity of the reporter only minimally, but further addition of an HF-1a site led to a substantial increase in activity. Interestingly, significant reporter activity was still maintained by substitution of the HF-1a site with other USF binding sites such as the MLE1 or PRE B sites, above. These results suggest that a combinatorial *trans*-acting factor pathway can function to direct significant ventricular expression of a minimal promoter, in vitro. Finally, by increasing the copy number of the HF-1 site from one to two, an approximate 4-fold increase in expression of the reporter was seen in the NRVM, whether the dimer was in the context of the native MLC-2_v TATA, or a heterologous TATA derived from the TK gene.

Given these results, that the endogenous TATA of MLC-2_v was interchangeable with the TK-TATA in the transient transfection studies, a minimal promoter transgene construct was made containing a dimer of the 28 bp HF-1 element, upstream of the endogenous MLC-2_v TATA sequence, controlling a *lacZ* reporter gene. We term this construct 2× HF-1/lacZ. Of 12 founder lines which transmitted the transgene, 6 lines had no detectable *lacZ* expression, and 6 were found to express the transgene in a cardiac-specific manner. Of the expressing lines, 4 of the 6 had β-gal staining in a ventricular chamber-specific manner with no detectable expression in the atrial muscle, aortic sac, conotruncal ridges, or endocardial cushion. Further, this minimal promoter transgene could direct expression of the reporter from an early time point in murine cardiogenesis (Fig. 7A), prior to formation of the linear heart tube or looping morphogenesis. As heart tube formation proceeded, the majority of these transgenic lines, as with the MLC-2_v wild-type and HF-3 mutant/lacZ lines, showed highest expression levels in the cono- and bulboventricular portions of the forming heart, and lowest levels in the left ventricular chamber (Fig. 7B-F). Expression of the transgene was confirmed in sections of whole-mount embryos to be expressed firstly in a bilaterally symmetrical manner, then dominantly in the primordial RV and outflow tract (Fig. 8A-F). Immunostaining of a day 12 p.c. HF-1 transgenic embryo confirmed this dominant expression pattern as distinct from that of the endogenous *MLC-2v* gene (Fig. 9). Two of the HF-1/lacZ lines showed high level reporter gene expression throughout both atrial and ventricular chambers, with no obvious gradient of expression, and no expression in other locations in the embryo. This less common expression pattern was most likely due to modification of the transgene by regulatory sequences at the insertion site within the murine genome. Taken together, the above results suggest that the HF-1a and HF-1b/MEF-2 *cis*-elements act combinatorially to confer chamber specificity and can establish an A/P pattern of expression during murine cardiac morphogenesis. Since the endogenous *MLC-2v* gene is expressed uniformly throughout the ventricular chambers, presumably other elements/factors are required for maximal expression in the left ventricle.

Use of the MLC-2/lacZ transgene as a ventricular marker in murine null mutants that have abnormal ventricular development

By crossing these MLC-2_v/lacZ transgenic mice into other

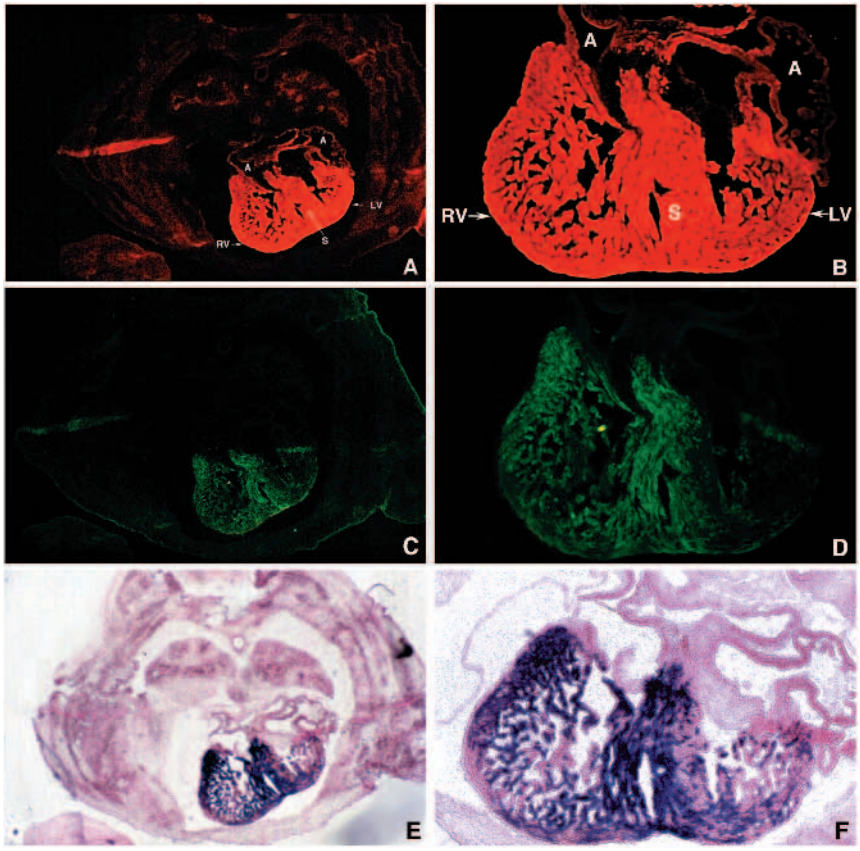

Fig. 9. Immunofluorescent and histochemical staining of a 2× HF-1/lacZ day 12 p.c. embryo. All panels are transverse sections. (A,B) Lissamine-rhodamine staining of endogenous MLC-2_v expression using a polyclonal rabbit anti-rat MLC-2_v antibody. (C,D) FITC staining of the *lacZ* transgene in the same sections as shown in (A,B). (E,F) Histochemical staining for β-galactosidase activity. Transgene expression is clearly discordant with the endogenous gene and dominantly expressed in the right ventricle. A, atria; LV, left ventricle; RV, right ventricle; S, septum.

genetic backgrounds, it should be possible to evaluate whether the HF-1a/ HF-1b/MEF-2 combinatorial pathway intersects with other genetic pathways that are important for normal ventricular formation. Recent gene targeting studies have identified the importance of the *retinoid X receptor* α and cardiac homeobox gene (*Nkx2-5*) in ventricular chamber morphogenesis (Lyons et al., 1995; Sucov et al., 1994). The *RXR*α null mutation causes embryonic lethality between E13.5 and 16.5 p.c. due to ventricular chamber defects manifested as a lack of proliferation in the compact zone, and decreased ventricular trabeculation. The *Nkx2-5*$^{-/-}$ embryos die at an earlier stage, (days 9-10 p.c.) and display profound defects in cardiac looping and ventricular differentiation. Interestingly, the *Nkx2-5* null phenotype includes a marked selective decrease in expression of the *MLC-2v* gene, despite the continued expression of other ventricular-specific marker genes, such as β-MHC and cyclin D2. To examine ventricular specification in these mutants, the MLC-2_v (250 bp)/lacZ transgene was bred into both the *RXR-*α and *Nkx2-5* backgrounds. Double heterozygous offspring (*MLC-2v*$^{+/-}$/(*Nkx2-5*$^{+/-}$ or *RXR*$\alpha^{+/-}$) were identified by PCR analysis and used for further analysis.

Timed matings of the double heterozygote MLC-2_v (+/–)/*RXR*α (+/–) animals were performed and embryos at days 11.5-14.5 p.c. obtained for analysis. PCR of DNA extracted from yolk sacs confirmed the presence of *RXR*α null mutant embryos within the litters that expressed the MLC-2_v/lacZ transgene. In the *RXR*α mutant hearts, which showed the typical ventricular defects, the MLC-2_v/lacZ transgene was expressed at high levels and in an A/P gradient of expression (RV/LV), similar to wild-type or *RXR*$\alpha^{+/-}$ littermates (Fig. 10A).

As shown previously (Lyons et al., 1995), endogenous MLC-2_v transcripts are expressed at very low to near undetectable levels in the heart of *Nkx2-5*$^{-/-}$ embryos (Fig. 10B,C). However in whole-mount analysis of 4 embryos from 3 separate matings, the MLC-2_v/lacZ transgene expressed at high levels in the *Nkx2-5* null background and in an apparent ventricular chamber specific manner (Fig. 10D-F). Although staining *Nkx2-5* mutant hearts for *lacZ* activity suggested that an A/P gradient of expression was also established in this background, it was not possible to confirm by direct comparison of RV and LV chambers, since they are indistinguishable in the malformed organ. Nevertheless, the data suggest that the combinatorial action of HF-1a and HF-1b/MEF2 pathways which confer ventricular-specification and an A/P gradient of expression, occur independently of *RXR*α and *Nkx2-5*.

DISCUSSION

HF-1a/MEF-2 is a minimal combinatorial pathway which directs ventricular chamber specific expression in vivo

The molecular mechanisms that dictate ventricular-specific gene expression in the mammalian heart are for the most part unknown. The MLC-2_v gene has served as an excellent model system for the study of this important aspect of cardiogenesis. Utilizing MLC-2_v/lacZ transgenes, the present study has provided the first identification of a minimal combinatorial element, composed of known *trans*-acting factor binding sites, which can confer ventricle-specific transgene expression during murine cardiogenesis. Two copies of a 28 bp *cis*-regulatory element (HF-1) found in the endogenous 250 bp MLC-2_v promoter, can alone confer ventricular chamber specificity. HF-1 contains adjacent positive regulatory elements that bind two distinct factors termed HF-1a and HF-1b/MEF2. Both of these elements of HF-1 are critical for maintenance of 250 bp MLC-2_v promoter activity in transient assays, as well as in transgenic mice (Lee et al., 1994; Zhu et al., 1991). The HF-1 region also shows essentially complete conservation between vertebrate species as diverse as chick and mouse (Henderson et al., 1989) further highlighting the likelihood that it plays a key role in regulation of the *MLC-2v* gene.

The *trans*-acting factors which bind HF-1, are neither, ventricular nor cardiac chamber specific. The HF-1a factor has been found to be ubiquitously expressed as assessed by gel electrophoretic mobility shift assays (Navankasattusas et al., 1992). This HF-1a site effectively binds the broadly expressed factor EFI_A, the rat homologue of human YB-1 (Ozer et al., 1990) that associates with a 30×10^3 M_r protein in extracts from cardiac cells (Zou and Chien, 1995). Another ubiquitous factor, USF, can act as a negative regulator at the HF-1a site (Navankasattusas et al., 1994). The importance of the HF-1a site in directing cardiac-specific expression is emphasized by the fact that another cardiomyocyte *trans*-factor (binding factor 2) binds the PRE-B of the MHCα promoter, as well as the HF-1a sequence of MLC-2_v (Molkentin et al., 1993). The PRE-B site of MHCα, like HF-1a, is immediately adjacent to an MEF-2 site. Our in vitro studies (Fig. 6) which show that the PRE-B can substitute for HF-1a in combining with an MEF2 site to drive significant expression of the reporter gene in cardiac myocytes, suggest that cooperative interaction between MEF-2 and other factors may therefore be a common mechanism for conferring chamber specificity of gene expression within the heart.

The HF-1b site of MLC-2_v is composed of a consensus MEF-2 binding site which appears critical for the maintenance of cardiac and muscle specificity of a number of muscle promoters (Yu et al., 1992). MEF-2 is essential for the differentiation of three distinct muscle lineages (skeletal, cardiac, and smooth muscle) in *Drosophila*. Furthermore, the MEF-2C gene is one of the earliest regulatory genes detected in the developing vertebrate heart (Lilly et al., 1995). While expression of MEF2 is induced in non-muscle cells by expression of myogenic determination genes (Cserjesi and Olson, 1991) MEF2 expression alone does not dominantly activate a cardiac phenotype. Heterokaryons which contain equal numbers of fibroblast and cardiac nuclei, have little expression of cardiac-cell markers (Evans et al., 1994). These findings, together with the fact that an MEF-2 site is insufficient in itself to maintain significant expression and cardiac specificity of the *MLC-2v* gene, suggest that a paradigm must be considered whereby the combinatorial activity of *trans*-factors is required to specify the ventricular muscle gene program. To understand the role of HF-1 further, it will be necessary to identify the cofactors which interact with HF-1a and MEF-2, to examine their tissue distribution and to assess their essential role by gene targeting studies.

While a combinatorial action of *trans*-factors on the HF-1 site appears a plausible mechanism to direct chamber-restricted *MLC-2v* expression during murine cardiogenesis, there are

undoubtedly other factors which influence the overall level of expression and that may confer more subtle regulation. Indeed, mutation of the MLE1 site of the MLC-2_v promoter completely abolished expression of the lacZ transgene in 10 independent founder lines, despite transmission of the transgene to at least the F_3 generation. In vitro (Fig. 6), the MLE1 element can substitute for HF-1a in acting coordinately with the HF-1b/MEF-2 element, to direct expression of a lacZ reporter. Thus, the MLE1 site could be a key element in fine regulation of the MLC-2_v promoter and generation of embryos which are deficient in the MLE1 binding factor (USF-1, USF-2) will be an informative experiment. Interestingly, mutation of another MLC-2_v *cis*-element, HF-3, found to be a negative regulator of promoter activity in adult mice (Lee et al., 1994), had no significant effect on developmental expression of transgenes examined in this study. While this suggests that HF-3 is not required for the ventricular specificity of the 250 bp MLC-2_v promoter fragment, it remains a possibility that HF-3 may be important in the modulation of the endogenous *MLC-2v* gene as noted in earlier studies in transgenic mice (Lee et al., 1994). The recent identification of an HF-3 binding factor should provide further insight into this question.

HF-1a/ HF-1b/MEF-2 pathways confer an anterior-posterior gradient of transgene expression during ventricular chamber morphogenesis

The formation of distinct right and left ventricular chambers is a critical step during cardiac morphogenesis. While these two chambers develop in a similar fashion, there are clear morphogenic, physiological and molecular differences between them. For example, the RV chamber may ultimately provide cues for outflow tract development and formation of the conotruncal ridges, while LV may provide the appropriate environment at the atrioventricular junction for endocardial cushion morphogenesis. Since the primitive RV is positioned anterior to the LV, understanding the developmental cues which establish A/P patterning of the cardiac progenitors, will provide key insights into cardiac morphogenesis. Indeed, it may be the process of A/P patterning in the early heart that establishes identity of heart chambers and the particular myogenic programs that they express. The MLC-2_v/lacZ transgenes not only directed ventricle-specific transgene expression, but also expression in a distinct A/P gradient. Expression of both the MLC-2_v (250 bp) promoter lacZ, and the 2× HF-1/lacZ constructs, was first visualized in a bilaterally symmetrical manner in the presumptive cardiac primordia at the headfold stage of embryonic development (see Fig. 7). However, conotruncal and RV dominant expression was seen at, and following, the linear heart tube stage, a pattern that is distinct from that of the endogenous *MLC-2v* gene which is uniformly expressed throughout the entire ventricular portion of the myocardium. While LV *lacZ* expression was still present, it was significantly reduced in comparison to the expression in these other regions.

Numerous studies in recent years have identified genes that are expressed in a spatially and temporally restricted fashion during cardiogenesis. These genes include several that encode sarcomeric proteins including MHCα, MLC-1_{Atrial} and tropomodulin (Lyons et al., 1990). At day 8 p.c. restricted sub-populations of myocytes can be distinguished on the basis of their selective gene expression, e.g. atrial natriuretic factor and MLC-2_v (O'Brien et al., 1993; Zeller et al., 1987). Spatial restriction of cardiac gene transcription can also be restricted with time, as evidenced by the downregulation of MHCβ expression in atrial myocytes by day 9.5 of murine gestation and of MHCα expression in the outflow tract, at this same time (Lyons, 1994). Gradients of expression within cardiac chambers have also been documented. For example, muscle-specific creatine kinase transcripts are most highly expressed in the epicardium, and also preferentially in one ventricular chamber (Lyons, 1994). An example of RV/LV gradients of expression was evidenced by preliminary studies with mouse desmin promoter/lacZ transgenic mice which express the transgene in the heart in a pattern similar to those seen in the present study (Kuisk et al., 1995). Additionally, a chimeric MLC-1/3 fast transgene, composed of the MLC-3 promoter and downstream enhancer, expressed a *lacZ* reporter in both skeletal muscle and in heart, where expression was found predominantly in the LV and atrial chambers (Kelly et al., 1995). Little is known about the mechanisms that dictate these unique patterns of expression in the heart.

One potential mechanism which could influence the patterned expression of endogenous genes or transgenes is DNA methylation. One study (Engler et al., 1991) evaluated immunoglobulin gene rearrangement in transgenic animals and revealed that site-specific recombination of the transgene would occur primarily when it was in an unmethylated state in a DBA background, as opposed to the highly methylated C57BL/6J genetic background. The rostrocaudal gradient of expression of a MLC-1/3 fast chloramphenicol acetyl-transferase (CAT) transgene in murine skeletal muscle has also been analyzed (Donoghue et al., 1991; Grieshammer et al., 1992). While discordant with expression of the endogenous gene, the expression of this transgene inversely correlated with a gradient of transgene methylation along the body axis, with higher CAT expression found in muscle with lower degrees of methylation. We thus investigated whether the RV/LV gradient of the MLC-2_v/lacZ transgene could be influenced by strain-dependent methylation. Numerous expressing lines of the wild-type 250 bp promoter transgene, and all HF-3 and MLE1 mutant lines were crossed from the C57BL/6J background into the hypomethylated DBA strain. While the relative intensity of reporter expression was increased after this cross, there was no apparent effect on the gradient of transgene expression in the heart. The 2× HF-1/lacZ transgene, which lacks sequences on which methylation could occur, showed more frequent expression in the native C57BL/6J background and was sufficient to confer the A/P gradient of transgene expression. Although direct proof that methylation is uninvolved in the graded expression of transgenes, these results suggest that HF-1a/ HF-1b/MEF-2 pathways themselves are the components essential for establishing this graded positional information within the heart.

One further question was whether the transgene gradient was a cell autonomous event, i.e. whether the gradient was stably specified at an early time in development and not dependent on the immediate local environment of the forming heart. Cardiocytes continued to express *lacZ* for at least 48 hours in culture. This stability in reporter gene expression is similar to that observed in ex vivo cultures from myotomes of the MLC-1-CAT transgenic mice, mentioned above. A more detailed analysis of this event is currently being explored via direct

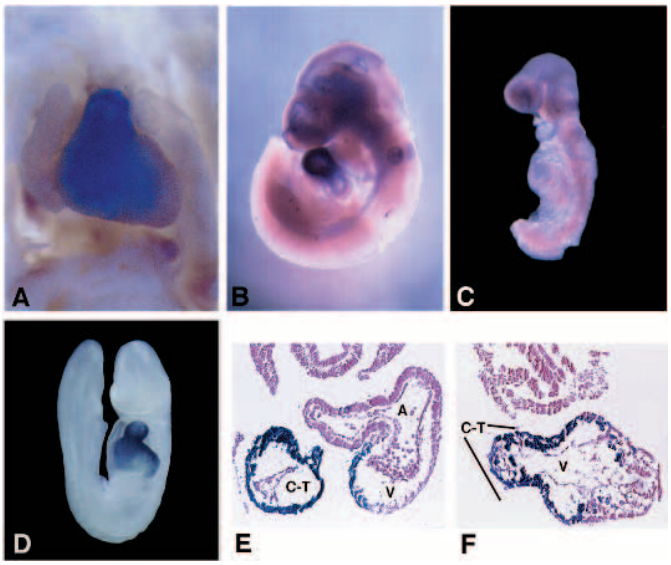

Fig. 10. MLC-2_v/lacZ transgene expression occurs in an *RXRα* and *Nkx2-5* independent manner. (A) Day 14.5 p.c. embryo with *RXRα*$^{-/-}$, MLC-$2_v^{-/+}$ genotype shows patterned *lacZ* expression in the ventricle in a manner similar to normal embryos (compare with Fig. 1H). (B,C) Day 9.5 p.c. *Nkx2-5* +/+ and –/– embryos, respectively, analyzed by whole-mount in situ hybridization with an MLC-2_v probe. The *Nkx2-5*$^{+/+}$ embryo (B) shows significant expression of MLC-2_v (brown-black color) in the ventricle, while none is detected in the –/– littermate (C). Growth retardation of the null embryo is as previously described (Lyons et al., 1995). Control riboprobes showed no ventricular signal. (D) Whole mount analysis of β-gal staining of a day 9.5 p.c. *Nkx2-5*$^{-/-}$, MLC-$2_v^{+/-}$ embryo, shows significant ventricular expression in the primitive ventricle and outflow tract. (E,F) Transverse sections of an *Nkx2-5* null embryo expressing the MLC-2/lacZ transgene in the malformed ventricle. No atrial expression was seen. C-T, conotruncus; V, primordial left ventricle; A, atria.

injection of RV-derived ventriculocytes into murine LV and vice versa.

Chamber-restricted MLC-2_v/lacZ transgene expression occurs in an *RXRa* and *Nkx2-5* independent manner

To determine whether ventricular specification is influenced by genes known to be crucial for normal ventricular development, we analyzed MLC-2_v/lacZ transgene expression in two mutant backgrounds: those deficient in the retinoid receptor gene *RXRα* and the homeobox gene *Nkx2-5*. Our results show that in these mutant backgrounds transgene expression was substantially normal, showing both chamber specificity and graded A/P expression. It is interesting and perhaps paradoxical that the transgene was expressed in the *Nkx2-5* null background, since *Nkx2-5* mutant embryos express only very low levels of the endogenous *MLC-2v* gene (Lyons et al., 1995). Although the molecular basis for the dependence of MLC-2_v expression on *Nkx2-5* is not understood, MLC-2_v transgenes may escape this dependence due to the fact that transgenes are integrated as multiple copies. Such release of dependence was also seen in relation to the MLE1 element, essential in the context of the 250 bp MLC-2_v promoter transgene, but evidently dispensable for expression of the 2× HF-1 promoter transgene. Restricted expression of the transgene in *Nkx2-5* mutant hearts strongly supports the suggestion that ventricular specification has occurred normally in these embryos, despite the fact that the earliest known marker of ventricular differentiation (MLC-2_v) is not expressed. These data also support the notion that the HF-1a/ HF-1b/MEF2 pathways that confer chamber specificity and an A/P gradient on transgene expression in the heart, do so in an *RXRα* and *Nkx2-5* independent manner. Undoubtedly, these MLC-2_v/lacZ transgenic animals will prove useful for evaluation of the importance of other genes in effecting ventricular/cardiac expression, and how the pathways that lead to ventricular specification may be dissected from those affecting differentiation and morphogenesis.

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