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Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube

(transgenic mice/cardiogenesis/*in situ* hybridization)

TERRENCE X. O'BRIEN*, KEVIN J. LEE, AND KENNETH R. CHIEN†

†Department of Medicine, Center for Molecular Genetics, *Veterans Administration Medical Center, and American Heart Association–Bugher Foundation Center for Molecular Biology, University of California, San Diego, School of Medicine, La Jolla, CA 92093-0613

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ABSTRACT **To study the process of ventricular specification during cardiogenesis, we examined the *in situ* expression of cardiac ventricular myosin light chain 2 (MLC-2v) mRNA during murine embryogenesis. As assessed by hybridization with a specific MLC-2v riboprobe, mRNA expression can be found in the ventricular region at day 8.0 postcoitum (pc). MLC-2v expression is high in the ventricular portion of the heart tube, with no detectable expression in the atrial or sinus venosus regions. The proximal outflow tract of the heart tube also expresses MLC-2v mRNA at minimally detectable levels at this time but then displays a temporally and spatially distinct pattern with expression well established in the proximal outflow tract region adjacent to the ventricular segment by days 9–10 pc, eventually reaching levels comparable to the trabeculated ventricular myocardium. By day 11 pc, prior to the completion of septation, expression then becomes restricted to the ventricular region at and below the level of the atrioventricular cushion. Transgenic mice harboring a 250-base-pair MLC-2v promoter fragment fused to a luciferase reporter gene demonstrate reporter gene activity from at least day 9 pc. Ventricular region-restricted expression of the luciferase reporter in the embryonic heart, as assessed by immunofluorescence and direct assay of reporter activity in microdissected atrial and ventricular muscle specimens, was confirmed from at least day 15 pc on. Taken together, this provides evidence for early positional specification of MLC-2v gene expression in the primitive heart tube and indicates regional specification of part of the ventricular muscle gene program can precede ventricular septation during mammalian cardiogenesis. Since the 250-base-pair promoter fragment is active developmentally in transgenic mice, this establishes it as a molecular target for the process of ventricular specification in the developing heart tube.**

The primitive heart tube originates from the lateral plate mesoderm and initially appears as a linear structure that rapidly acquires a looped configuration prior to a complex series of morphologic events (1). Relatively little is known concerning the positional and/or molecular cues that lead to the regional specification of cardiac muscle cells and the concomitant acquisition of distinct electrophysiologic, contractile, endocrine, and biochemical properties of the various chambers and specialized tissues within the heart (26). Whether regional gene specification occurs early in the primitive heart tube remains an open question. All of the currently described mammalian atrial and ventricular chamber-specific genes are coexpressed throughout the early looped heart tube, and regional specificity is acquired relatively late during cardiogenesis and, in certain cases, after parturition (2–4).

In this regard, previous studies have established the rat cardiac ventricular isoform of the myosin light chain 2 (MLC-2v) gene as a valuable model system for the identification of molecular signaling pathways that control the cardiac muscle gene program during its growth and development (5, 6). MLC-2v is the phosphorylatable, regulatory ventricular myosin light chain isoform of the myosin unit. Although MLC-2v is also expressed in slow-twitch skeletal muscle, within the adult rodent heart MLC-2v mRNA is expressed exclusively in the ventricular chamber and is not detectable in the atrium under normal conditions (7). The question arises as to whether this ventricular marker is coexpressed in all regions of the embryonic heart tube or, rather, reflects an early regional specification to the ventricular region. Accordingly, this study examines expression of cardiac MLC-2v mRNA during murine embryogenesis and in transgenic mice harboring a 250-base-pair (bp) MLC-2v promoter fragment fused to a luciferase reporter gene (MLC250L) that has been shown to confer ventricular-specific expression in adult transgenic mice (7).

MATERIALS AND METHODS

C57BL/6JXSJL mice (Simonson Laboratories, Gilroy, CA) and MLC250L transgenic mice were used for timed breeding with females being separated out [considered day 1 postcoitum (pc) at the 24-hr point] and sacrificed at specific times. For luciferase activity analysis, the whole embryo or, after day 15 pc, the microdissected embryonic heart, atria, or ventricles were immediately placed in liquid nitrogen, stored at −70°C, and measured as described (7). For *in situ* hybridization and immunofluorescence, the embryos were placed in 4% paraformaldehyde in phosphate-buffered saline and processed as described (7). Additional staged, fixed normal mouse sections were obtained from Novagene, then defatted, and dehydrated prior to hybridization. Two independent MLC250L transgenic mouse lines were constructed by inserting a 250-bp MLC-2v promoter fragment upstream of a promoterless luciferase reporter gene (7). Confirmed male founder animals were propagated by mating with breeder female mice with embryos sacrificed at specific times.

For *in situ* hybridization studies, riboprobes were generated and labeled with UTP[^{35}S] (7). Template DNA consisted of a full-length 640-bp rat MLC-2v cDNA (8) subcloned into pBluescript II SK (Stratagene) with a 200-bp rat MLC-2v antisense riboprobe generated from the 3′ untranslated and coding region. Comparison of the rat and mouse MLC-2v cDNA sequences used yielded 100% homology at the amino acid level and 94% at the nucleotide level. RNase protection studies utilizing riboprobes derived from either species resulted in large protected fragments with cardiac RNA from

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Abbreviations: MLC, myosin light chain; MLC-2v, ventricular MLC 2; pc, postcoitum; OFT, outflow tract; MHC, myosin heavy chain; ANF, atrial natriuretic factor.

both species (data not shown). For controls, a 116-bp sense MLC-2v riboprobe and 131-bp antisense and 187-bp sense riboprobes from a full-length 658-bp rat atrial natriuretic factor (ANF) cDNA (9) were generated. All riboprobes were hybridized, exposed, and photographed as described (7). All *in situ* experiments were repeated in duplicate in three separate experiments. Immunofluorescence experiments used a primary polyclonal rabbit anti-luciferase antibody (10) and were viewed with a Nikon microscope equipped with epifluorescence optics (7).

RESULTS

MLC-2 Gene Expression Is Restricted to the Ventricular and Proximal Outflow Regions of the Murine Heart Tube. To determine expression at early stages of heart tube development, the cardiac MLC-2v riboprobe was hybridized with sections of staged murine embryos. Fig. 1*A* is a bright-field image of a day 8 pc embryo transversely exposing the ventricular region of the linear heart tube. In the dark-field image of the same section (Fig. 1*B*), hybridization with the (antisense) MLC-2v riboprobe resulted in an intense signal in the ventricular region, with no levels above background elsewhere in the developing heart. Multiple embryos examined at day 7 pc (prior to heart tube formation) showed no MLC-2v hybridization above background (data not shown). At day 8.5 pc (Fig. 1*C*) a midsagittal section reveals the heart tube to be an "S"-looped structure, corresponding to the inflow (sinus venosus and atrial), ventricular, and OFT regions along the heart tube as labeled. The OFT takes its origin from the distal ventricular segment of the heart tube (what will become the right ventricle) and is involved in the formation of the base of the aortic and pulmonary vessels, the semilunar valves, the pulmonary conus, and the aortic vestibule (i.e., the truncoconal region). Fig. 1*D* shows a dark-field image of the same section as Fig. 1*C*, displaying intense hybridization in the ventricular region and less intense signal in the proximal OFT, whereas in the inflow tract (including the atrial region) there is no detectable hybridization above background. At day 9 pc, the heart tube becomes more convoluted with well-defined OFT, ventricular, atrial, and sinus venosus regions. A parasagittal section exposes the ventricular region, the junction with the atrial segment, and a portion of the sinus venosus (Fig. 1*E*). Hybridization with the MLC-2v riboprobe displays a strong signal in the ventricular region, with a clear demarcation at the junction with the atrial region, which itself does not express any detectable levels above background (Fig. 1*F*). Another sagittal section at this stage (Fig. 1 *G* and *H*) provides a view of the atrial, ventricular, and OFT areas with a markedly positive signal in the ventricular region and the proximal OFT immediately adjacent to the ventricular region. A different sagittal section at day 9.0 pc also shows MLC-2v expression restricted to the ventricular and OFT regions (Fig. 1 *I* and *J*). The expression of ANF is used as a control, since it has been shown to be coexpressed in the atrial and ventricular compartments in the embryonic heart from day 7.5 pc to just prior to birth (11). Hybridization of an adjacent section to Fig. 1*I* with an ANF antisense riboprobe reveals a faint signal in the atrial and ventricular compartments but not the OFT (Fig. 1*K*). In contrast, hybridization of adjacent tissue sections with sense MLC-2v and ANF riboprobes results in no detectable signal above background (data not shown).

As the midsagittal view in Fig. 2*A* reveals, by day 10 pc, the developing atrioventricular cushions are prominent and the ventricular muscle is extensively trabeculated. Hybridization with the MLC-2v riboprobe results in an intense signal in the ventricular myocardium as well as a now more intense signal further into the OFT but not its cushion structures (Fig. 2*B*). By day 11 pc, the process of ventricular septation is initiated. In Fig. 2 *C* and *D* the atrial and OFT regions are separated from the ventricular compartment by the atrioventricular cushions. Hybridization is seen in the superior aspect of the ventricular septum (i.e., the region that lies above the

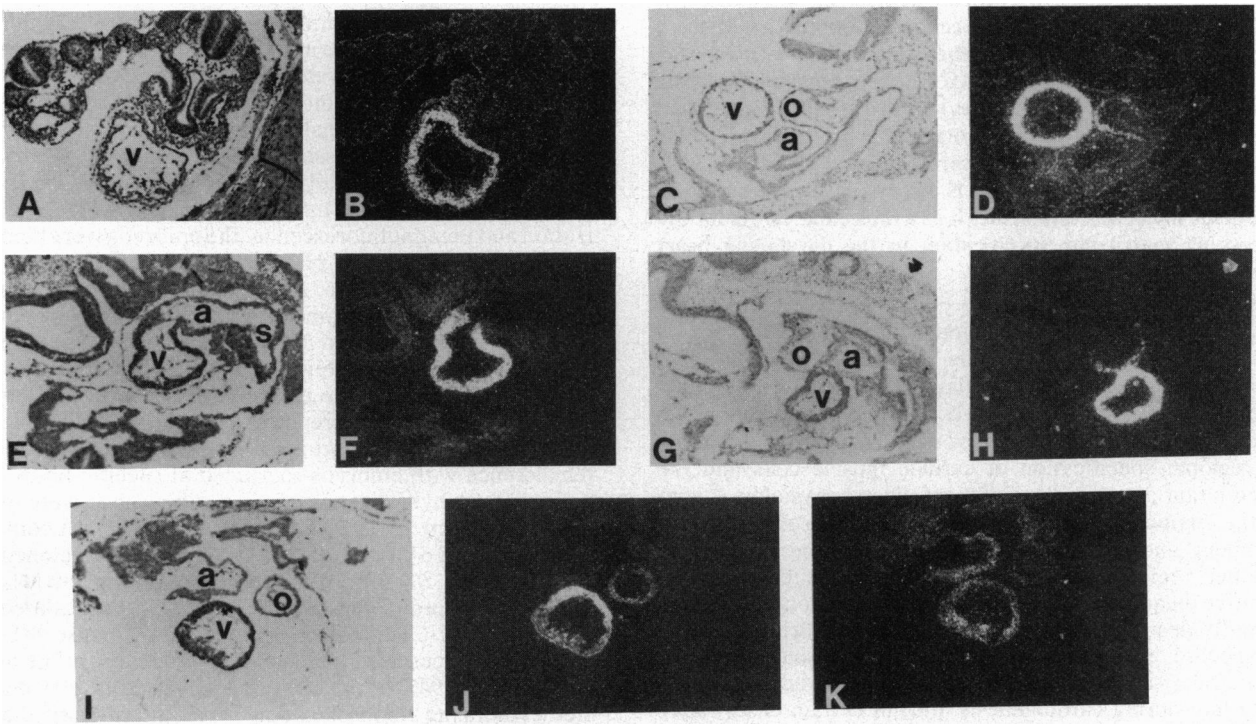

FIG. 1. Paired murine embryonic images. (×100.) Abbreviations for all figures are as follows: a, atrial; v, ventricular; o, outflow tract (OFT); s, sinus venosus. (*A*) Day 8 pc sagittal. (*B*) Dark-field image of *A* with MLC-2v riboprobe hybridization. (*C*) Day 8.5 pc midsagittal. (*D*) Dark-field image of *C* with the MLC-2v riboprobe. (*E*) Day 9 pc parasagittal. (*F*) Dark-field image of *A* with the MLC-2v riboprobe. (*G*) Day 9 pc sagittal. (*H*) Dark-field image of *G* with MLC-2v riboprobe hybridization. (*I*) Additional day 9 pc sagittal. (*J*) Dark-field image of *I* with MLC-2v riboprobe. (*K*) Adjacent section to *I* with the ANF riboprobe.

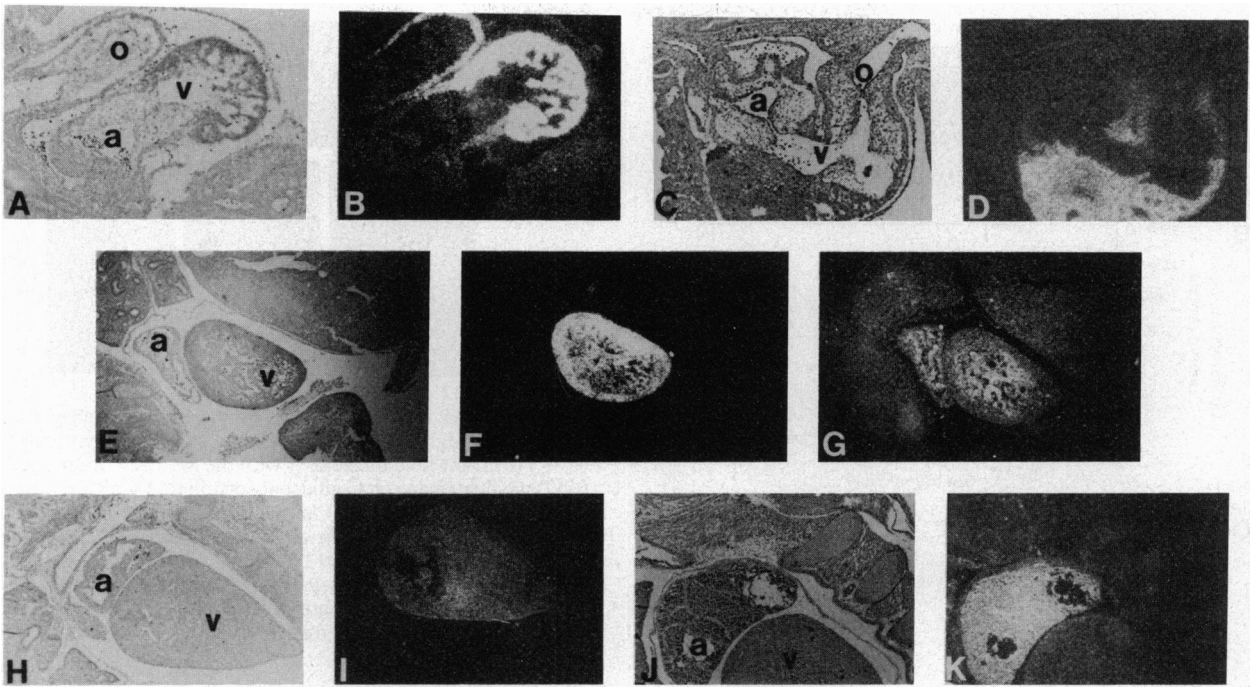

FIG. 2. (*A*) Day 10 pc midsagittal. (×100.) (*B*) Dark-field image of *A* with MLC-2v hybridization. (×100.) (*C*) Day 11 pc parasagittal. (×100.) (*D*) Dark-field image of *C* with MLC-2v signal. (×100.) (*E*) Day 13 pc sagittal. (×50.) (*F*) Dark-field image of *E* with MLC-2v hybridization. (×50.) (*G*) Adjacent section to *E* hybridized with the ANF riboprobe. (×50.) (*H*) Day 15 pc through atrium and ventricle. (×50.) (*I*) Dark-field image of *A* with MLC-2v signal. (×50.) (*J*) Day 16 pc section viewing atrium and ventricle. (×50.) (*K*) Dark-field image of *C* with ANF riboprobe signal. (×50.)

"V" label in Fig. 2*C*), which will later grow down to fuse with the inferior aspect of the septum. Interestingly, the myocardium adjacent to the OFT cushions (the outflow ridges being below the "O" label) now has barely detectable hybridization. Moreover, the proximal OFT (labeled "O") now has no detectable expression. Thus, between days 10 and 11 pc, the MLC-2v mRNA in the OFT becomes progressively restricted to the ventricular region at and below the atrioventricular cushion. As seen in the parasagittal section in Fig. 2 *E* and *F*, by day 13 pc, septation is complete and the expression of MLC-2v mRNA is restricted to the ventricular compartment. Hybridization with an ANF riboprobe in Fig. 2*G* reveals expression in both atrial and ventricular chambers (particularly the subendocardium), consistent with published results (12). Examining later stages, Fig. 2*H* is a parasagittal day 15 pc section, with Fig. 2*I* showing ventricular MLC-2v expression. Fig. 2*J* is a day 16 pc section, with the ANF hybridization seen in Fig. 2*K* now localizing predominantly to the atrium.

A 250-bp Promoter Fragment of the MLC-2v Gene Confers Ventricular-Specific Expression During Murine Cardiogenesis. Previous studies (7, 13, 14) have identified a 250-bp promoter fragment of the MLC-2v gene that can confer cardiac-specific and inducible expression of a luciferase reporter gene in rat neonatal myocytes and adult transgenic mice (for a depiction of the transgene, see Fig. 3*A*). Therefore, to examine reporter gene expression developmentally, staged embryonic sections were made from the offspring of MLC250L transgenic males and breeder females. Luciferase activity was measured in extracts derived from whole embryos (days 6–13 pc) (Fig. 3*B*) and from isolated embryonic heart tissue (day 15 pc through postpartum day 1) (Fig. 3*C*). Luciferase activity was first detectable at day 9 pc and steadily increased during embryonic development. As expected, nontransgenic littermates displayed no detectable reporter activity. To confirm that the expression of MLC-2v mRNA was not altered in these transgenic lines, the *in situ* hybridization patterns of MLC-2v (Fig. 4*B*) and ANF (Fig. 4*C*) mRNAs at day 13 pc were shown to be identical to the endogenous nontransgenic pattern.

Embryonic sections were examined with immunofluorescence studies using a polyclonal luciferase antibody. At day 13 pc, the ventricular myocardium expresses the luciferase protein at significantly higher levels than the surrounding pericardium (Fig. 4*D*), whereas a nontransgenic littermate (Fig. 4*E*) demonstrates no significant expression over the background level seen in a negative control (Fig. 4*F*). Since the thin-walled nature of the primitive atrium (and other organs with thin free edges on the section) created a significant background "edge effect" in the day 13 pc and earlier embryos that decreased the immunofluorescent sensitivity, the regional luciferase expression was examined in embryos at days 15 and 18 pc and day 1 postpartum embryos by the direct analysis of microdissected atrial and ventricular specimens. In each of the five embryos examined, the luciferase activity of the ventricular sample was comparable to the levels seen in the positive transgenic embryonic hearts of the same age (i.e., activity similar to that in Fig. 3*B*), whereas all atrial extracts were at nondetectable background levels (data not shown).

DISCUSSION

The Expression of the MLC-2v Gene in the Primitive Murine Heart Is Specified Developmentally. The developmental cues that regulate the acquisition of regional differences in myocardial structure and function are unknown. Previous murine studies (2–4) have shown that the cardiac-specific isoforms that regionally specify do so at least after the completion of ventricular septation (day 13 pc). For example, two distinct alkali MLC-1 genes are expressed in either the atrial (MLC-1a) or ventricular (MLC-1v) compartments of the adult mouse heart. In the embryonic heart, the MLC-1a and MLC-1v genes are coexpressed in the atrial and ventricular chambers throughout cardiogenesis. The down-regulation in the nonexpressing chamber (MLC-1a in the ventricle;

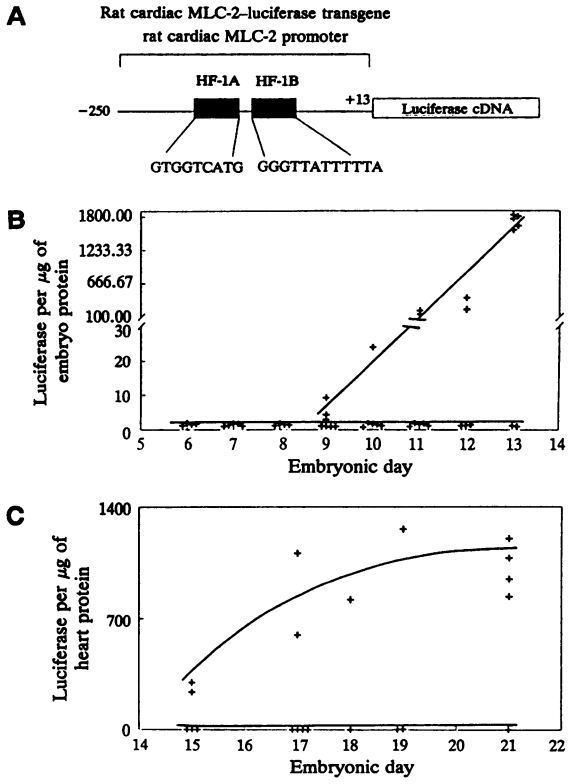

FIG. 3. (*A*) Diagram of the 250-bp rat MLC-2v promoter fragment ligated into the promoterless luciferase expression vector pSVOALΔ5′ used to generate two transgenic mouse lines (7). Highly conserved sequences are labeled within the 250-bp promoter as HF-1A and HF1-B (15). (*B*) Luciferase activity per μg of protein in the isolated embryos at each stage generated by the mating of male MLC250L transgenic mice to wild-type females. Each day represents the results of assaying each whole embryo from a single litter. (*C*) Luciferase activity per μg of protein in the isolated hearts of day 15 pc through postpartum day 1 (= day 21 pc) of similar MLC250L transgenic mice offspring. Lines are drawn through the nontransgenic littermates in *B* and *C* that had only background activity.

MLC-1v in the atrium) leading to regional specification does not occur until parturition. Similarly, murine myosin heavy chain (MHC) isoform patterns do not take their adult phenotype until postpartum. The atrium and ventricle initially coexpress β-MHC. By day 9.5 pc, β-MHC is restricted to the ventricle but does not achieve its adult phenotype (for the rodent) until expression ceases completely by day 7 postpartum (α-MHC is continuously coexpressed). ANF does not localize completely to the atrium until postpartum (12). Since cardiac regional specification occurs after the formation of the distinct chambers, it has been speculated that gene expression in mammalian species may be influenced by developmental cues (e.g., physiologic stimuli) that in some way vary between the chambers. However, immunohistologic studies of avian heart development have suggested that regional specification can be an early event (11, 16, 17). In human cardiogenesis, there is early β-MHC immunohistological localization to the ventricle and OFT with expression still seen in the atrioventricular canal and sinoatrial node (18). Also, components of the ventricular conduction system express a specific neural antigen prior to ventricular septation (19, 20).

This present study provides clear evidence that MLC-2v expression is established early in the primitive heart tube and suggests the mechanisms that restrict expression to ventricular cardiomyocytes may be already in place at the stages of heart tube formation examined. However, it has not been determined whether such a mechanism involves the early

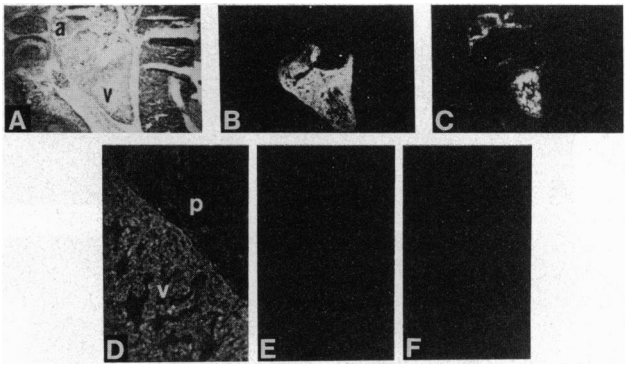

FIG. 4. (*A*) *In situ* analysis of day 12 pc transgenic mice. Parasagittal bright-field image including atrial and ventricular areas. (×40.) (*B*) Dark-field image of *A* with the MLC-2v riboprobe. (×40.) (*C*) Dark-field image of an adjacent section to *A* with ANF riboprobe activity. (×40.) (*D*) Indirect immunofluorescence of luciferase antibody expression in the ventricle of day 13 pc MLC250L transgenic mice. Ventricular region (v) positively stained with anti-luciferase antibodies with adjacent negative pericardial sac (p). (×170.) (*E*) Negative transgenic littermate of *A* with no signal to antiluciferase antibodies. (*F*) Same embryo as *A* exposed to nonspecific rabbit IgG antibody.

specification to the ventricular region or negative regulation to the atrial inflow region. Therefore, studies employing the MLC-2v gene may prove to be of utility in the identification of molecular and/or developmental cues that regulate positional specification within the mammalian heart tube.

Ventricular Specification Can Precede Septation. These data indicate regional specification of a ventricular region-specifying cardiac muscle gene can occur prior to septation in the mammal and may not require the presence of physiologic stimuli associated with a more developed ventricular chamber. The contrasting expression patterns of the MLC-1v and MLC-2v genes provide evidence for the existence of divergent pathways for the regulation of regionally specific gene expression of the cardiac muscle gene program. Since specification of the MLC-2v gene is coincident with the earliest stages of cardiogenesis, the question can be raised as to whether there may be a direct commitment to the ventricular muscle cell lineage from mesodermally derived progenitor cells, with no antecedent cardiac muscle "stem" cell. Alternatively, atrial and ventricular muscle cells might arise from a common progenitor muscle cell, or regional specificity may arise from positional cues.

A Temporally and Spatially Distinct Pattern of MLC-2v Gene Expression Occurs in the Ventricular and Proximal OFT Regions. At day 8.5 pc, the levels of OFT MLC-2v mRNA expression are barely detectable above background and are confined to proximal areas immediately adjacent to the ventricular segment and do not include the OFT cushions (Fig. 1*D*). As the heart tube then develops there is a progressive increase in the level of MLC-2v expression throughout the proximal OFT, excluding the cushions, such that by day 10 pc these regions express a level of MLC-2v mRNA qualitatively comparable to that of the ventricle (Fig. 2*B*), which by day 11 pc then has decreased markedly (Fig. 2*B*). This region of the OFT expresses β-MHC continuously through at least embryonic day 18 pc in the rat (corresponding to about days 16–17 pc in the mouse) (2) and at least days 56–60 in the human (11). By these time points, the withdrawal of previously coexpressing α-MHC is also noted in the rat and human. Similarly, cardiac troponin I has been found to be expressed continuously in the heart and OFT of the rat during late embryonic development (21). Also, between day 10.5 and 15.5 pc in the mouse, MLC-1v transcripts gradually disappear from the OFT (3). The temporal delay and the gradient of the

proximal OFT MLC-2v expression might reflect a preestablished gradient in the molecular signals that regulate MLC-2v expression and/or the migration or presence of ventricular myocytes into the OFT. Taken together, these data suggest that a temporal gradient of expression between the ventricular and proximal OFT regions may be a generalized feature of a subset of cardiac muscle genes and that regulatory mechanisms are able to coordinate transcriptional and morphological events.

An MLC250L Transgene Provides a Molecular Target for Ventricular Specification During Mammalian Cardiogenesis. The 250-bp MLC-2v promoter fragment is active during cardiogenesis in a manner which parallels that of endogenous MLC-2v mRNA. Luciferase activity specifies the ventricle from day 15 pc by microdissection, but since day 9–13 pc activity is derived from whole embryos, similar specificity is not addressed. However, these studies imply that the transacting factors that directly bind to cardiac muscle cis regulatory elements within the 250-bp promoter fragment are candidate regulatory factors for ventricular specification during cardiogenesis. A combination of mutagenesis, gel-shift, and methylation interference studies has led to the identification of a conserved 28-bp cis regulatory element (HF-1) within the 250-bp MLC-2v promoter region that can confer cardiac muscle-specific and inducible expression during cardiac muscle growth and hypertrophy (15). Two cardiac nuclear factors bind to the HF-1 site: a ubiquitous factor binds to a GGTAC core region in HF-1 (HF-1a), and a distinct factor binds to an (A+T)-rich sequence at the 3′ end of the 28-bp sequence (HF-1b) (21). Interestingly, a similar (A+T)-rich sequence has been found to be essential for the cardiac-specific expression of the troponin T gene in avian cardiac muscle cells (22). Recent studies have identified a zinc finger protein (23) and serum response factor-related proteins (RSRF) (24, 25) that bind to the HF-1b site. The question arises as to whether the HF-1b zinc finger or RSRF proteins serve as commitment factors during the early ventricular specification of the MLC-2v gene or play a role in establishing the expression pattern of MLC-2v in the primitive heart tube.

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