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Subdivision of the Cardiac Nkx2.5 Expression Domain into Myogenic and Nonmyogenic Compartments

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Nkx2.5 is expressed in the cardiogenic mesoderm of avian, mouse, and amphibian embryos. To understand how various cardiac fates within this domain are apportioned, we fate mapped the mesodermal XNkx2.5 domain of neural tube stage *Xenopus* embryos. The lateral portions of the XNkx2.5 expression domain in the neural tube stage embryo (stage 22) form the dorsal mesocardium and roof of the pericardial cavity while the intervening ventral region closes to form the myocardial tube. XNkx2.5 expression is maintained throughout the period of heart tube morphogenesis and differentiation of myocardial, mesocardial, and pericardial tissues. A series of microsurgical experiments showed that myocardial differentiation in the lateral portion of the field is suppressed during normal development by signals from the prospective myocardium and by tissues located more dorsally in the embryo, in particular the neural tube. These signals combine to block myogenesis downstream of XNkx2.5 and at or above the level of contractile protein gene expression. We propose that the entire XNkx2.5/heart field is transiently specified as cardiomyogenic. Suppression of this program redirects lateral cells to adopt dorsal mesocardial and dorsal pericardial fates and subdivides the field into distinct myogenic and nonmyogenic compartments.

INTRODUCTION

Broad regions of potency to form particular organs have been mapped in avian and amphibian embryos and are termed morphogenetic fields (reviewed in Jacobson and Sater, 1988). Classically, morphogenetic fields were identified based on the ability of explanted or grafted tissue to differentiate autonomously. By this criterion, the amphibian heart field at the neural tube stages has been mapped to a crescent of ventroanterior mesoderm extending dorsally toward the hindbrain (Ekman, 1925; Jacobson, 1960; Sater and Jacobson, 1990a). The initial studies showed that mesoderm at the dorsolateral margins of the field in newts was capable of differentiating into rhythmically beating tissue if transplanted to ectopic sites (Ekman, 1925) or *in situ* following removal of the ventral region (Copenhaver, 1926). Similar experiments using chick embryos (Rawles, 1936; DeHaan, 1965) generalized the conclusion that a broad region of heart potency exists in early embryos. In addition to prospective myocardium, neural plate and/or folds (amphibians, chick), somitic tissues (amphibian), and notochord (zebrafish, amphibian) have all been implicated as sources of signals that suppress autonomous differentiation of beating heart tissue (Jacobson, 1960; Fautrez and Amano, 1961; Orts-Llorca, 1964; DeHaan, 1965; Rosenquist and DeHaan, 1966; Jacobson and Duncan, 1968; Sarasa and Climent, 1987; Sater and Jacobson, 1990a; Goldstein and Fishman, 1998).

The traditional interpretation of these data has been that such signals redirect lateral portions of the heart field to adopt noncardiac fates. The consequence of this would be to narrow the size of the entire field, which comprises both myocardial and nonmyocardial cells, thereby initiating a process that has been termed heart field restriction (Sater and Jacobson, 1990a). However, no data have yet been provided showing the fate of the lateral cells that apparently lose heart-forming potency. Thus, it has not been possible to distinguish the notion that the entire heart field narrows from the alternate view that it is subdivided such that cells in the lateral portion are respecified to form nonmyocardial

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tissues of the heart. Nonmyocardial tissue (e.g., mesocardium or pericardium) might not have been recognized in the classical studies since they relied on beating and lumen formation as indicators of heart specification.

In *Xenopus,* the heart field is specified in mesoderm flanking Spemann's organizer by interactions with the organizer and underlying deep endoderm and is largely complete by early gastrulation (stage 10.5; Sater and Jacobson, 1989; Nascone and Mercola, 1995). *Xenopus* heart induction may incorporate aspects of early inductive interactions with hypoblast (Yatskievych *et al.,* 1997) and definitive endoderm (Schultheiss *et al.,* 1995) known to occur in the chick. During gastrulation, the paired heart primordia first migrate anteriorly until they underlie the prospective hindbrain and then migrate laterally to fuse at the ventral midline by stage 20 (neural tube stage). Between stages 28 and 30 (late tail-bud stage), the sheet of cardiac cells folds to form a tube connected to the pericardial cavity by the dorsal mesocardium. The heart begins beating at stages 33–36 and subsequently loops and fuses to form the three-chambered heart (reviewed in Icardo, 1988; Fishman and Chien, 1997).

Vertebrate heart induction correlates with expression of homologues of the *Drosophila tinman* gene. Of these, Nkx2.5 is expressed in heart tissue before the induction of heart-specific contractile protein genes and may be involved, at least indirectly, in their regulation (reviewed in Chien *et al.,* 1993; Chen and Schwartz, 1995; Fu and Izumo, 1995; Lyons *et al.,* 1995; Chen and Fishman, 1996; Harvey, 1996; Durocher and Nemer, 1998; Lee *et al.,* 1998). It has been proposed that XNkx2.5 expression in the lateral portion of the heart field may be down-regulated during postneurula to tail-bud stages and that this may explain what has been considered a loss of heart potency in this region (Tonissen *et al.,* 1994; Drysdale *et al.,* 1997). However, in the absence of fate-mapping data, it is not clear if the lateral heart field cells express XNkx2.5 and, if so, whether expression is lost upon subsequent development. Furthermore, the apparent suppression of heart differentiation in the lateral field (described as medial in chick or zebrafish) is not well characterized in general and has never been demonstrated in *Xenopus* (Jacobson, 1960; Fautrez and Amano, 1961; Orts-Llorca, 1964; DeHaan, 1965; Rosenquist and DeHaan, 1966; Jacobson and Duncan, 1968; Sarasa and Climent, 1987; Sater and Jacobson, 1990a; Goldstein and Fishman, 1998). In addition, ectopic overexpression of XNkx2.5 in *Xenopus* and zebrafish increased the number of cells expressing contractile protein mRNA only slightly, consistent with the existence of overriding signals capable of blocking cardiomyogenesis downstream of XNkx2.5 (Cleaver *et al.,* 1996).

In this paper, we present lineage, 3D reconstruction, and a series of explant experiments showing that the lateral portion of the XNkx2.5 domain coincides with the lateral portion of the heart field as classically defined. We find that the entire heart/XNkx2.5 field is specified initially to form myocardium but that the lateral portion becomes respecified to form dorsal mesocardium and the roof of the pericardium. We go on to show that myocardial differentiation in the lateral heart/XNkx2.5 field is inhibited by interactions with flanking ventral (prospective myocardium) and dorsal (neural tube, notochord, and somitic) tissues. Of the latter, the neural tube in particular appears to have potent anticardiomyogenic activity. Importantly, XNkx2.5 expression is not lost upon respecification of the lateral heart field but only appears to become constricted because of the tissue morphogenesis of tube closure. Consistent with this observation, cardiomyogenesis in the lateral region in intact embryos and explants is suppressed at a point downstream of XNkx2.5. We present a new model for heart field morphogenesis in which the heart/XNkx2.5 field is first specified as cardiomyogenic but becomes subdivided into distinct dorsal peri/mesocardial and myocardial compartments.

MATERIALS AND METHODS

Culture of Embryos

Xenopus eggs, fertilized *in vitro,* were dejellied with a 2% cysteine solution (pH 7.8) and cultured in 0.1× Marc's modified Ringer's solution (MMR). Staging was according to Nieuwkoop and Faber (1967).

Histology

Embryos were fixed in MEMFA (0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 1 h, dehydrated through a graded series of alcohols, and embedded in Fibrowax (Difco). Seven-micrometer transverse sections were stained with Feulgen/Light Green/Orange G triple stain and images captured using a Kodak DCS420 digital camera attached to a Zeiss Axiophot microscope. 3D reconstruction was performed using SURFdriver 2.0 software package (www.surfdriver.com). No independent reference points were available for aligning successive images or correcting distortions in individual sections. Alignment was, therefore, based upon the position of the notochord since whole-mount *in situ* data and parasagittal sections indicate that this can be considered approximately linear over the region that includes the heart primordium. Expression domains for myocardial markers and for XNkx2.5 were established individually, using serial 7-μm wax sections from several equivalently staged embryos stained by RNA whole-mount *in situ* hybridization. These data were used to define the equivalent regions on the reference section series used for 3D model construction. The resulting models therefore represent idealized composites rather than morphologically precise representations of individual embryos.

Fate Mapping

Stage 22 embryos were microinjected with Cell Tracker CM-DiI (Molecular Probes) at a concentration of 0.5 mg/ml in a 0.1% sucrose and 10% ethanol solution. Small, multicellular patches in three different regions of the heart field (as in Fig. 3) were labeled. Labeled embryos were cultured in 0.1× MMR to stage 28–31, fixed in MEMFA for 1–2 h at 20°C, and then transferred to 1× PBS with

sodium azide. Embryos were mounted in gelatin (Bober *et al.*, 1994), and 30-μm transverse Vibratome sections of the heart region were prepared. CM-DiI was detected using an epifluorescence or fluorescence confocal microscope.

Explants and Extirpations

Stage 22 or 28 embryos were transferred to 0.75× MMR and devitellinized as necessary (stage 22). Microsurgical manipulations were performed on a bed of 1% agarose in 0.75× MMR. All tissues or embryos were allowed to heal for at least 30 min. The explants were cultured at 20°C in 0.75× MMR with 20 μg/ml gentamicin sulfate (Sigma) until fixed 1–2 h in MEMFA at room temperature. Explants were cultured until stages 30–35 to assay XNkx2.5 or stages 40–42 to assay for all other markers and beating. Sandwich recombinants between ventral lateral heart field tissues and potential suppressive tissues were performed at stage 22 in 0.75× MMR and were maintained in this medium until scored for beating when sibling embryos had reached stage 42.

In Situ Hybridization

Explants, along with their respective whole embryo controls, were processed for whole-mount *in situ* hybridization as described (Harland, 1991) using digoxygenin-labeled cRNA probes except that the SSC wash solutions following hybridization contained 0.4% SDS. cRNA probes included cardiac troponin I ($XTn\text{-}I_c$) (Drysdale *et al.*, 1994), cardiac actin (AC100; gift from Doug Melton), XMLC-2 (gift from Sylvia Evans), XNkx2.5 (Tonissen *et al.*, 1994), and XMHC-α (Logan and Mohun, 1993). Following the alkaline phosphatase color reaction of 3 to 30 h, samples were postfixed in MEMFA for at least 1 h and dehydrated into methanol. The explants were viewed and scored on a dissecting microscope in methanol or cleared in benzyl alcohol:benzyl benzoate (1:2).

Immunocytochemistry

Tissues were stained for $Tn\text{-}T_c$ with the monoclonal antibody CT3 (Developmental Hybridoma Studies Bank) at a 1:10 dilution following methods previously reported (Hemmati-Brivanlou, 1990). CT3 expression was secondarily detected with a goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma).

Statistical Methods

Statistical differences between data sets collected from beating and molecular marker expression assays were determined by JMP version 3.1 (SAS Institute, Inc.). We treated all data as nominal and performed Fisher's two-tailed exact tests.

RESULTS

Expression of Molecular Markers during Heart Tube Formation

We first used whole-mount RNA *in situ* hybridization to compare the domains of expression of XNkx2.5 with those of various myocardial differentiation markers. Expression of the *Xenopus tinman* homologues XNkx2.3 and XNkx2.5 is first detected during gastrulation and, by neurula stages, transcripts are localized in bilateral domains that eventually merge into a single anteroventral "collar" (Tonissen *et al.*, 1994; Evans *et al.*, 1995; Fu and Izumo, 1995). By the early tail-bud stage, sections through this region of the embryo demonstrate that XNkx2.5 staining is present both in the ventral mesoderm that contains the cardiac progenitors and in adjacent foregut endoderm (Fig. 1).

At the onset of myocardial differentiation (Fig. 1A), expression of the cardiac muscle-specific markers XMLC-2a and XMHCα is confined to bilateral regions within the cardiac mesoderm, posterior to the cement gland. Identical spatial patterns of expression are observed in the hearts for all contractile markers examined and none were ever observed to be expressed more dorsally by prospective mesocardial or pericardial tissue. In contrast, XNkx2.5 is expressed in a broader domain that encompasses the differentiating myocardial tissue and extends much farther dorsally. Such an apparent difference could result if cardiac muscle differentiation in the lateral portions of the XNkx2.5 domain was delayed relative to the ventral region. However, even after a single domain of myocardial differentiation has formed on the ventral midline (Fig. 1B), XNkx2.5 continues to be expressed in a much broader region of tissue. Transverse sections confirm that the lateral domain consists of mesodermal tissue adjacent to and contiguous with the ventral mesoderm undergoing myocardial differentiation (Fig. 1C).

The spatial relationships between the mesodermal XNkx2.5 expression domain and the differentiating myocardial tissue were examined using 3D models based on serial transverse sections through the heart region of tail-bud embryos (Fig. 2). At stage 28, the myocardial tissue has extended ventrally into the pericardial cavity around a single endocardial tube which bifurcates at its posterior end (the future sinus venosus). While the dorsal aspect of the heart tube has not yet closed, it is progressively more constricted along its AP axis (Figs. 1C and 2A). Strikingly, the point where the sheet is constricted coincides precisely with the boundary of myocardial differentiation. By stage 32, constriction is complete and the linear heart tube is closed in all but the most anterior portion. The anterior end of the endocardial tube adopts a more vertical orientation, extending dorsally in the region that forms the outflow tract (Fig. 2B). The XNkx2.5 expression domain extends into the more lateral mesoderm throughout the length of the heart tube both before and after dorsal closure. The precise dorsal edge of this domain is difficult to establish from transverse sections since staining intensity declines progressively in more lateral tissue. It is, however, evident that after closure of the heart tube, the dorsal mesocardium and the roof of the pericardial cavity both express XNkx2.5. This is illustrated most clearly by 3D models of the posterior portion of the heart tube from stage 32 embryos (Fig. 2C).

Together, these data suggest a model for heart tube morphogenesis in which a broad domain of XNkx2.5-expressing mesoderm becomes subdivided into ventral and

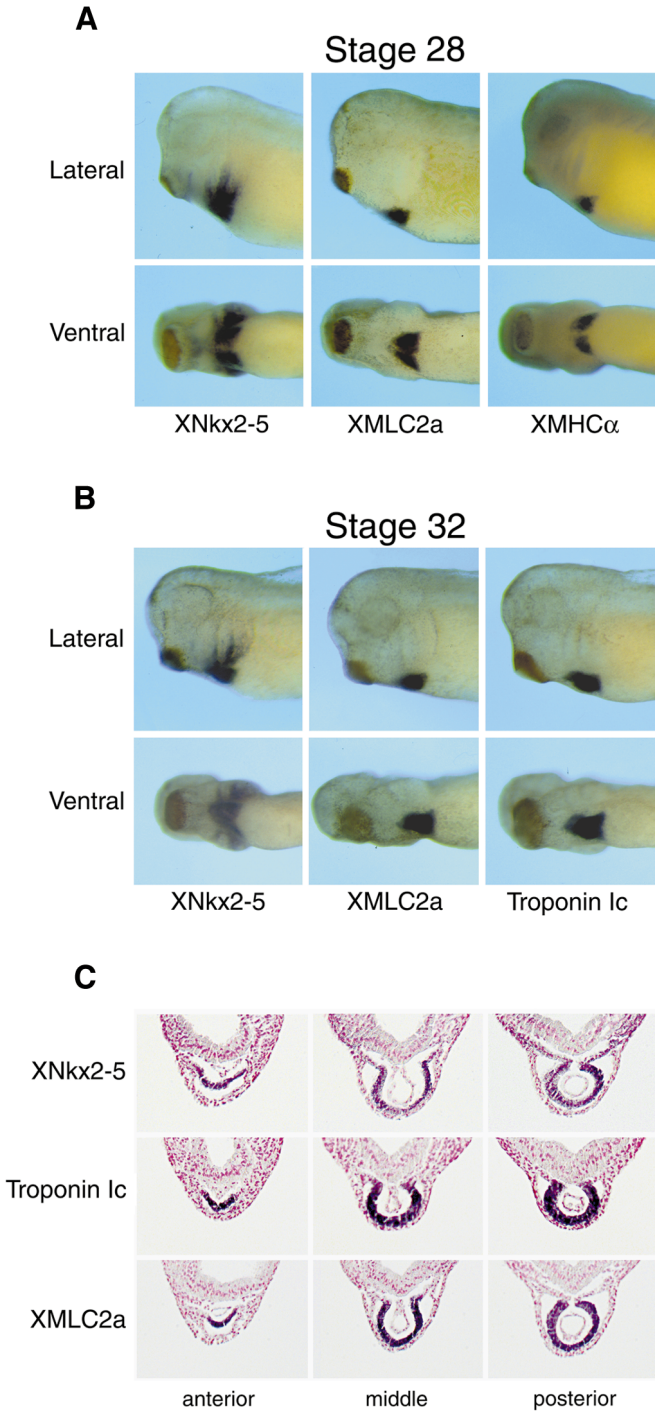


FIG. 1. Expression domains of XNkx2.5 and myocardial differentiation markers during heart tube formation. (A, B, and C) Embryos were stained for expression of XNkx2-5, XMLC-2a, XMHCα, and XTn-I$_c$ genes at stages 28 (A) and 32 (B) by RNA whole-mount *in situ* hybridization. Stained embryos were embedded in paraffin wax and (C) shows representative transverse sections through the anterior, middle, and posterior portions of a stage 28 heart region.

lateral domains. The former gives rise to myocardial tissue while the adjacent lateral domain forms the dorsal mesocardium and the pericardial tissue to which it is attached. To test this possibility, we used dye labeling to follow the fate of cells originating in the ventral and lateral regions of the heart morphogenetic field.

Fate Map of the XNkx2.5 Expression Domain

Stage 22 embryos were microinjected with CM-DiI to label small patches of cells within three different regions of the mesodermal XNkx2.5 expression domain. As diagrammed in Fig. 3, sites A, B, and C were arrayed in a line extending dorsally from the ventral midline at the posterior edge of the cement gland. The position of site A on this line was aligned with the bottom of the eye vesicle as visible externally, site B was aligned with the dorsal margin of the cement gland, and site C was just off the ventral midline. Site A approximated the border between XNkx2.5-expressing and -nonexpressing cells, whereas sites B and C labeled the lateral and ventral regions of the XNkx2.5 field, respectively (Figs. 3A, 3B, and 3C). For comparison with the functionally defined heart morphogenetic field, sites A and B span the lateral portion of the heart field which apparently loses myocardial potency during postneurula stages (Sater and Jacobson, 1990a), whereas site C corresponds to the region where myocardial potency persists (see following section and Fig. 4).

We evaluated the positions of the progeny of the labeled cells at stages 28 to 31. Progeny of cells labeled at site C contributed primarily to myocardial and endocardial tissues (Table 1 and Figs. 3F and 3G). Occasionally, pharyngeal endoderm cells would be labeled due to penetration of the needle through the thin layer of ventral mesoderm at stage 22. Cells labeled at site A did not contribute to the myocardium, endocardium, nor mesocardial tissues. Label was seen most frequently at the dorsolateral edge of the dorsal pericardium (Table 1 and Figs. 3D and 3G) corresponding to the dorsolateral boundary of XNkx2.5 expression at stage 28 (Fig. 3C). Since site A also corresponded to the dorsolateral edge of the XNkx2.5 domain at stage 22, it is clear that the lateral portion of the heart field maintains XNkx2.5 expression as the cardiac sheet folds to form the heart tube and contiguous structures. Cells labeled at site B showed marked contribution to dorsal mesocardium and the roof of the pericardium and, less frequently, to the abutting region of the myocardial tube (Table 1 and Figs. 3E and 3G). Occasionally, overlying and underlying tissue (presumably surface ectoderm and foregut endoderm) at sites A and B were labeled by CM-DiI microinjection as the needle punctured the tissue to label the intervening mesoderm. By stage 28–31, labeled mesodermal cells had become displaced ventrally relative to the injection site (Figs. 3D and 3E). The lineage analysis reinforces the idea that the broad domain of XNkx2.5 expression at stage 22 marks a contiguous sheet of cardiac progenitors which descends into the pericardial cavity, apparently as a consequence of folding and closure of

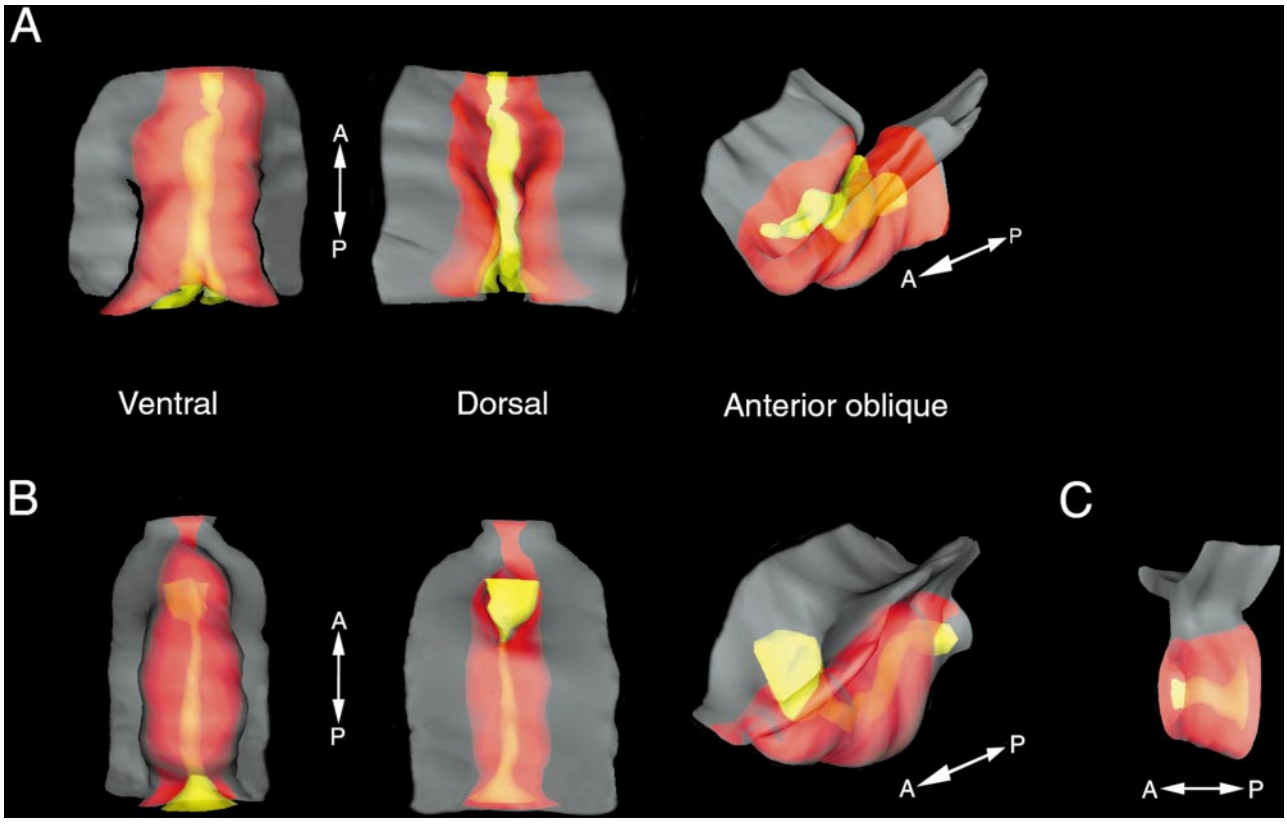


FIG. 2. 3D models of linear heart tube formation. 3D models showing the expression domains for XNkx2.5 (gray) and myocardial differentiation markers XMLC-2a and XTn-I_c (red) during heart tube formation. Endocardial tissue (yellow) was identified solely by histological criteria and is most prone to distortion during the wax embedding procedure. The dorsal boundary of XNkx2.5 expression is necessarily imprecise because of the low levels of transcript (see Fig. 1C and text), while the boundaries of myocardial differentiation are relatively precise. (A) Stage 28 embryo showing progressive dorsal constriction of the XNkx2-5 domain along the AP axis; tissue ventral to the constriction expresses cardiac muscle markers. (B) Stage 32 embryo showing dorsal closure of the linear heart tube. (C) A posterior portion of the Stage 32 model, seen from a lateral oblique view to show attachment of the heart tube to the pericardial roof via the dorsal mesocardium.

the heart tube. In addition, we did not detect mixing of labeled and unlabeled cells, suggesting that there is no significant movement of cells relative to their neighbors.

We conclude that cells in the lateral portions of the cardiac sheet form the roof of the pericardial cavity and contiguous dorsal mesocardium while ventral cells give rise to myocardium. Endocardial cells also appeared to arise within the ventral region. However, it is not clear if they and myocardial cells arise from a common precursor since small patches, rather than single cells, were labeled. Importantly, mesodermal expression of XNkx2.5 is maintained in the myocardium, mesocardium, and pericardial roof throughout heart tube morphogenesis (compare Figs. 2 and 3). Thus, the apparent restriction visible in whole-mount stained embryos between stages 22 and 28 arises as the sheet of cells folds to form the myocardial tube and contiguous mesocardium and pericardial roof.

Myocardial Differentiation in the Lateral Region of the XNkx2.5 Domain Is Suppressed by Signals from Adjacent Ventral and Dorsal Tissues

The fate mapping demonstrated that the lateral portion of the XNkx2.5 domain forms cardiac tissue dorsal to the heart tube. We then asked if this region coincides with the lateral portion of the heart field characterized by classical studies to lose heart potency during postneurula stages. To test this possibility, we designed a series of explant experiments to see if the lateral XNkx2.5 domain is capable of cardiomyocyte differentiation when isolated in culture and to identify the source(s) of suppressing signals which could act in the intact embryo.

We first assayed the ability of the tissue from the lateral portion of the XNkx2.5 domain to differentiate into myocardial tissue following the removal of the presumptive

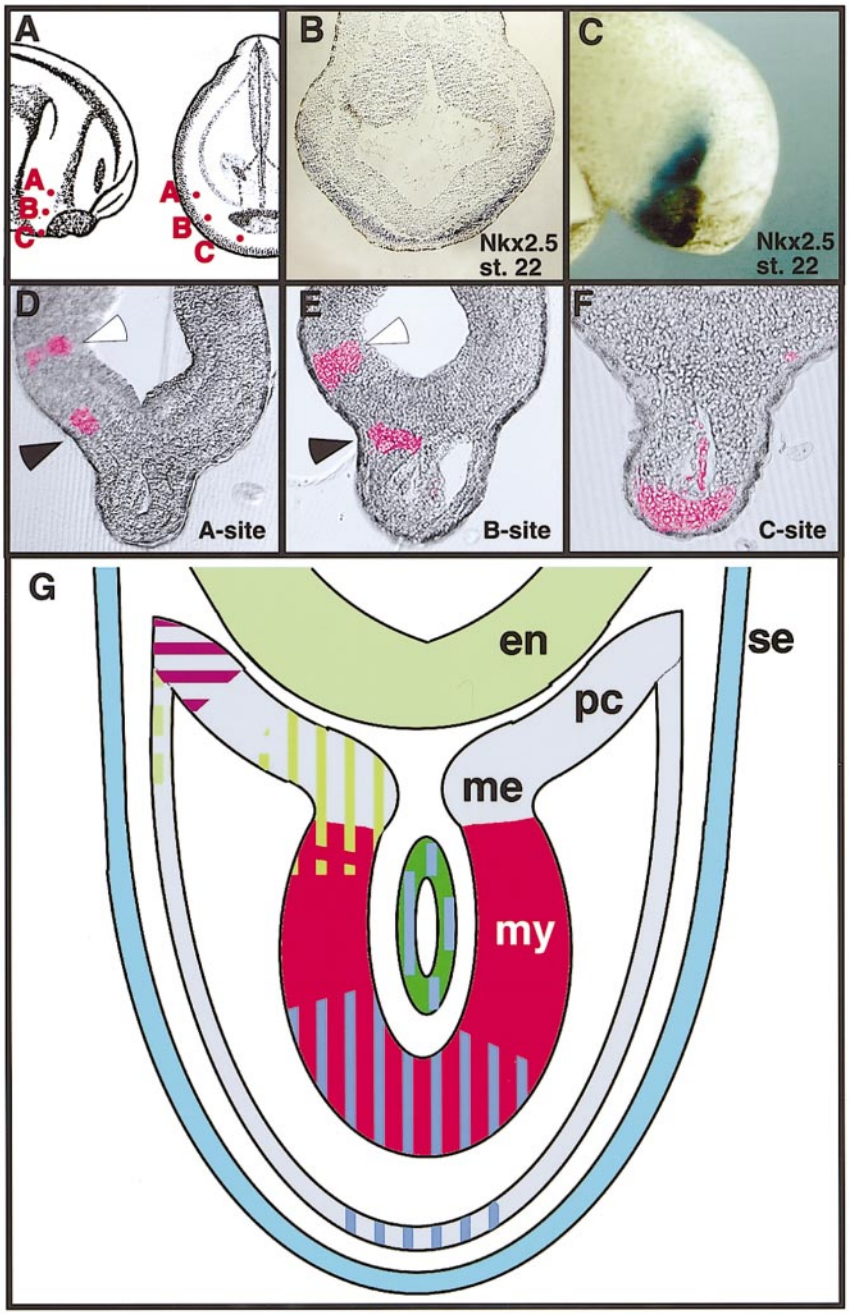


FIG. 3. Fate map of the XNkx2.5 expression domain. (A and B) Three small multicellular patches within the XNkx2.5 domain of early tail-bud stage (stage 22) embryos were microinjected with the lipophilic dye, CM-DiI. Injections were positioned in a line extending dorsally from the posterior margin of the cement gland with site A aligned with the bottom of the eye vesicle as visible externally, site B aligned with the dorsal border of the cement gland, and site C just off the ventral midline. (C) XNkx2.5 mRNA in a stage 22 embryos. Site A corresponded to the dorsal border of the stage 22 Nkx2.5 domain, site B corresponded to the lateral region, and site C was near the ventral midline. (D, E, and F) The positions of cells labeled at the three sites were examined at stage 28 in transverse Vibratome sections. (G) The data set ($n = 7$ for site A, $n = 15$ for site B, and $n = 12$ for site C) is summarized in the schematic (also in Table 1). Purple, yellow, and blue hatchlines represent locations of cells injected at sites A, B, and C, respectively. Labeling of overlying ectoderm and underlying endoderm occasionally occurs and can serve as a reference point for the injection site (white arrowheads in D and E). Note that mesodermal cells labeled by A- and B-site injections (black arrowheads in D and E) shift ventrally between stages 22 and 28 such that cells from site A contribute to the dorsolateral edge of pericardial roof while cells from site B contribute to dorsal mesocardium and the adjoining portion of the pericardial roof (and, infrequently, the adjoining myocardium). Site C cells, in contrast, form myocardium and endocardium. Thus, cells within the lateral portion of the XNkx2.5 domain at stage 22 (sites A and B) shift ventrally but do not lose XNkx2.5 expression as the sheet of cardiac cells folds to form the heart tube. Dorsal is up in all images. Abbreviations: en, endoderm; me, mesocardium; my, myocardium; se, surface ectoderm; pc, pericardium.

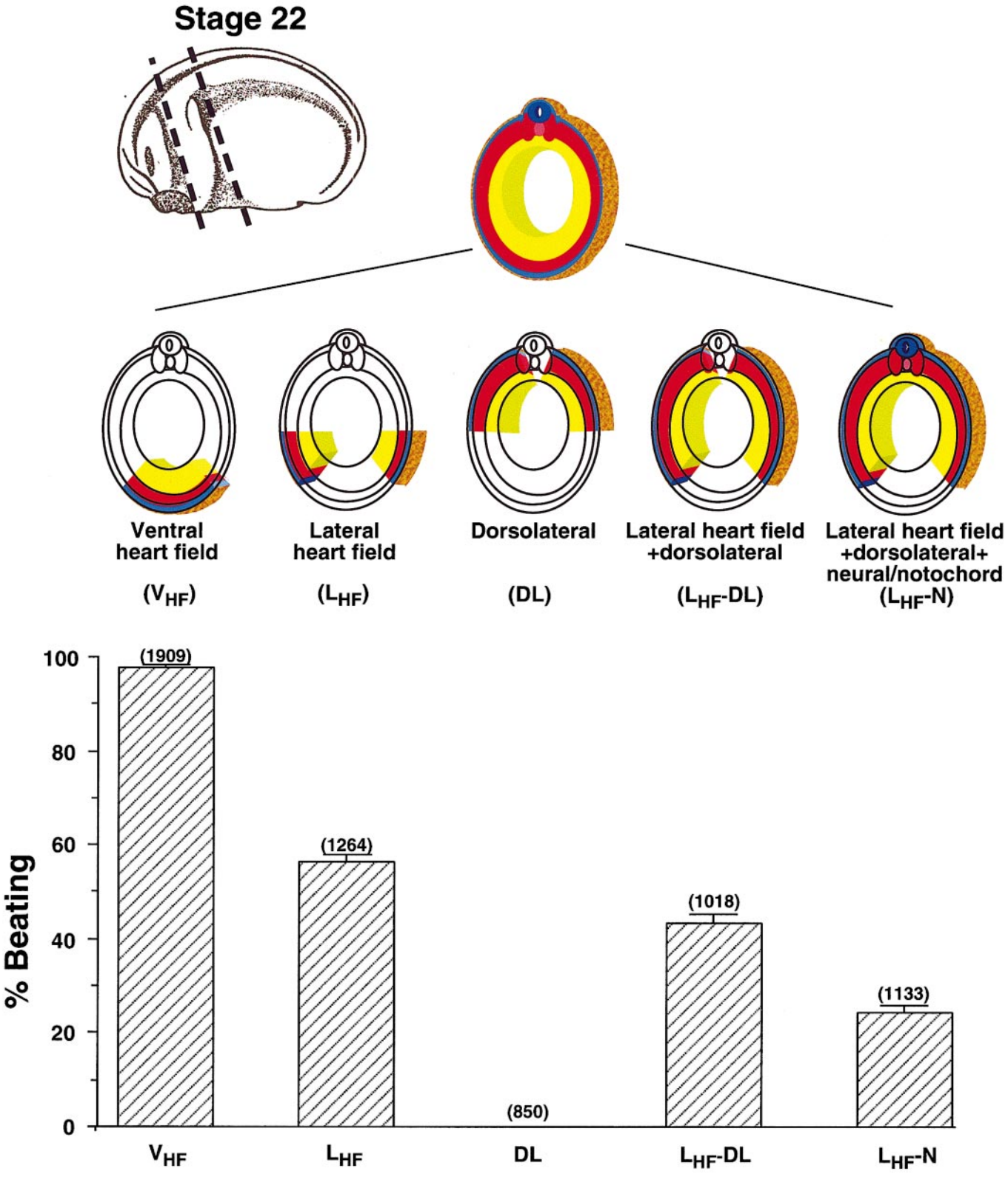


TABLE 1
Fate Mapping of the XNkx2.5 Field

Injection site	Number of experiments	Number of embryos with label in cardiac tissue at stage 28–31				
		Dorsolateral edge of pericardial roof	Pericardial roof	Dorsal mesocardium	Myocardium	Endocardium
A	7	4	0	0	0	0
B	15	3	9	12	3	1
C	12	0	0	0	9	11

Note. Embryos were injected with CM-DiI to label small patches of cells within the XNkx2.5 domain at stage 22 and were fixed and sectioned by Vibratome at stage 28–31 to examine location of labeled cells (see Fig. 3 and Materials and Methods). Sites of A, B, and C injections were aligned with external features as described in the text. Of the seven site A experiments (which targeted the dorsolateral edge of the XNkx2.5 domain at stage 22), three labeled cells outside the cardiac mesoderm (not tabulated). All site B and C experiments labeled cardiac mesoderm.

myocardium, similar to experiments done by Copenhaver using *Ambystoma* (1926). Prospective myocardial tissue (tissue extending ventrally from the B-site microinjections) was extirpated from stage 22 embryos and heart regeneration was scored at stage 40. Rhythmically beating cardiac tissue in the lateral mesoderm was seen in nearly all cases (90 ± 3.4%, $n = 79$). A substantial proportion of these embryos (22.5%) regenerated two hearts, indicating incomplete fusion at the ventral midline. Hearts that formed from single sides were smaller and resembled those formed by the lateral heart field explants (see below). This suggests that prospective myocardial tissue suppresses myocardial differentiation in the lateral region of the XNkx2.5 domain.

We next examined myocardial differentiation of the lateral XNkx2.5/heart field tissue when isolated in explant culture. Figure 4A diagrams a series of explants taken from different positions within a transverse slice of tissue at the level of the heart field of a stage 22 embryo. For reference, the ventral (V_{HF}) and lateral (L_{HF}) heart field explants together comprised the entire XNkx2.5 expression domain; the L_{HF} explant extended from site A to just ventral to site B (Fig. 3), whereas the V_{HF} explant contained tissue labeled by site C injections. In striking contrast to its fate *in situ*, 56 ± 1.5% of the L_{HF} region explants isolated at stage 22 went on to beat in culture (Fig. 4B), indicating a strong capacity for autonomous myocardial differentiation and confirming the earlier results of Sater and Jacobson (1990a). Importantly, explants of tissue located just outside the XNkx2.5 domain (DL explants, Fig. 4B) never formed beating tissue ($n = 850$). Together, these results indicate that the dorsal boundary of the classically defined heart field corresponds well to that of the XNkx2.5 domain.

To examine the source of suppressing signals, adjacent tissues were included in the explants. Inclusion of tissue immediately dorsolateral to the heart field (L_{HF}-DL explants) reduced the beating incidence to 43 ± 1.4% and the addition of neural and notochordal tissue (L_{HF}-N explants) further reduced the incidence to 25 ± 1.3% (Fig. 4B, both data sets are statistically significant to $P < 0.05$). Taken together with the ventral extirpations, we conclude that myogenesis of the lateral XNkx2.5/heart field cells is inhibited by interactions with ventral (prospective myo- and endocardium) and dorsal (somitic, neural, and notochord) tissues.

Each of the dorsal tissues present in the explants (somites, neural tube, notochord) was assayed for inhibitory activity individually by recombining with stage 22 lateral heart field tissues (Fig. 5). Of these tissues, the neural tube reduced beating frequency. Somites, notochord, and control posterior ventral (noncardiogenic) tissue had no statistically significant effect. Ventral heart field explants were not sensitive to inhibition, possibly reflecting commitment by stage 22.

FIG. 4. Myocardial differentiation in lateral heart field (L_{HF}) mesoderm is suppressed by dorsal tissue. Explants were dissected from stage 22 embryos, cultured to stage 41, and scored for rhythmic beating. For reference to the dye injection sites (Fig. 3), the border between the ventral heart field (V_{HF}) and the L_{HF} explants was positioned just ventral to the site B microinjections such that L_{HF} explants contained cells labeled by microinjection at site B (prospective dorsal pericardium and dorsal mesocardium) and V_{HF} explants encompassed site C (prospective myocardium). Note that stage 22 L_{HF} explants formed beating tissue efficiently when maintained in isolation from dorsal and ventral tissues. When explants included dorsolateral somitic tissue (L_{HF}-DL) or somitic, notochordal, and neural tissues (L_{HF}-N) myocardial differentiation was significantly reduced. Importantly, tissue just dorsal to the XNkx2.5 domain never formed beat in culture (DL explants), indicating that it resides outside the classically defined heart field. Numbers of explants are given in parentheses and error bars indicate standard error of the mean.

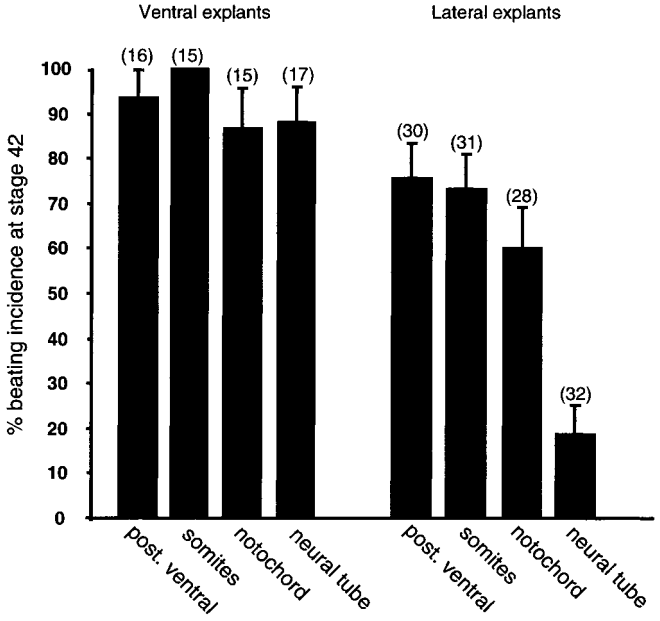


FIG. 5. Suppression of L_{HF} myogenesis by individual dorsal tissues in recombinants. Ventral and lateral heart field/Nkx2.5 field explants at stage 22 were recombined with dorsal tissues as indicated and beating incidence is presented. Note that neural tubes showed strong anticardiomyogenic activity when recombined with lateral heart field tissues. Lack of an effect on ventral field explants may reflect commitment by this stage. Numbers of recombinants are given in parentheses and error bars indicate standard error of the mean.

Differential Suppression of Heart Muscle Genes by Dorsal and Lateral Tissues

The above results suggest that cardiomyogenesis in lateral heart field cells is inhibited downstream of XNkx2.5. To examine this further, we assayed the effect of ventral (prospective myo- and endocardium) and dorsal (prospective neural tube, notochord, and somitic) tissues on expression of XNkx2.5 and cardiac contractile protein genes in cells of the lateral heart field. Explants as in Fig. 4 were cultured and assayed by *in situ* hybridization. Figure 6 summarizes the entire data set graphically. Each histogram plots the incidence of expression of the markers for a particular type of explant and the horizontal dashed line represents the beating incidence for that type of explant. Figure 7 shows examples of the most commonly observed expression patterns for the various markers.

As expected from the lineage data, XNkx2.5 transcripts were present at a high incidence in nearly all cases, regardless of explant type (Fig. 6). Moreover, inclusion of dorsal or ventral tissue did not appear to affect the size of the XNkx2.5 expression domain [compare expression in isolated L_{HF} explants to that in L_{HF}-N explants (Fig. 7) or intact embryos (Fig. 1)]. We investigated this further and found that the level of XNkx2.5 and XNkx2.3 mRNA by RT-PCR remained constant in L_{HF} explants cultured from stage 22 over 2 days as cardiomyogenesis occurred (not shown), consistent with the maintenance of expression seen in intact embryos (Figs. 1–3). This supports the model that suppression of the myogenic program in the lateral heart field occurs downstream of XNkx2.5.

Contractile protein markers were differentially regulated. Two genes, XTn-I_c and cardiac actin, were ectopically expressed at high levels in all explants of the lateral heart field following removal of ventral tissue. For cardiac actin, only heart and not somitic (skeletal muscle) expression was scored. All other markers are cardiac specific. As shown in Fig. 6, their incidences of expression well exceeded beating incidences in all cases. This discordance was most pronounced when the lateral heart field was isolated together with adjacent neural tube, notochord, and somitic tissues (L_{HF}-N) in which cardiac actin and XTn-I_c mRNAs were expressed by 94 ± 2.3 and 72 ± 3.1% of the explants, respectively, but only 25% beat (Fig. 6A). Ectopic contractile gene expression in the lateral mesoderm of these nonbeating explants often marked structures which were outwardly similar in appearance to beating hearts (Fig. 7, red arrows) and contained lumens lined with endothelial cells (Fig. 8). We conclude that: (1) ventral tissue (prospective myocardium) suppresses XTn-I_c and cardiac actin gene expression in the lateral heart field of intact embryos since removal of ventral tissue results in their expression and (2) dorsal tissue is less capable of suppressing XTn-I_c and cardiac actin genes since ectopic transcripts were detected frequently in nonbeating L_{HF}–N explants.

XMHC-α and XMLC-2a mRNAs and Tn-T_c protein were also ectopically expressed in the lateral heart field following removal of ventral tissues, but with lower frequencies [L_{HF}-N explants, Figs. 6 and 7 (*Xenopus* Tn-T_c cDNA is not available; protein was examined using the monoclonal antibody CT3, see Materials and Methods)]. Thus, their expression is also inhibited by the ventral signal(s). But unlike XTn-I_c and cardiac actin, their expression incidences in the lateral region explants increased significantly when dorsal tissues (neural tube and dorsal mesoderm) were removed (compare L_{HF}–N to L_{HF} explants, Figs. 6A and 6B). XMLC-2a mRNA showed the greatest sensitivity to suppression by dorsal tissues (35 ± 4.1% in L_{HF}-N explants, approximating the beating frequency). XMHC-α mRNA and Tn-T_c protein were intermediate (49 ± 3.2 and 45 ± 4.2%, respectively, nearly double the beating frequency). Results are significant to $P < 0.05$.

We conclude that suppression of myogenic potency in the lateral heart field in intact embryos normally occurs downstream of XNkx2.5 and either upstream or at the level of contractile gene expression. The explant assays revealed that each of the contractile genes is sensitive to suppression by signals from the prospective myocardium. Expression of XMLC-2a is particularly sensitive to suppression by signals from dorsal tissue (most likely the neural tube), whereas XTn-I_c and cardiac actin genes are relatively insensitive. MHC-α mRNA and Tn-T_c protein appear intermediate

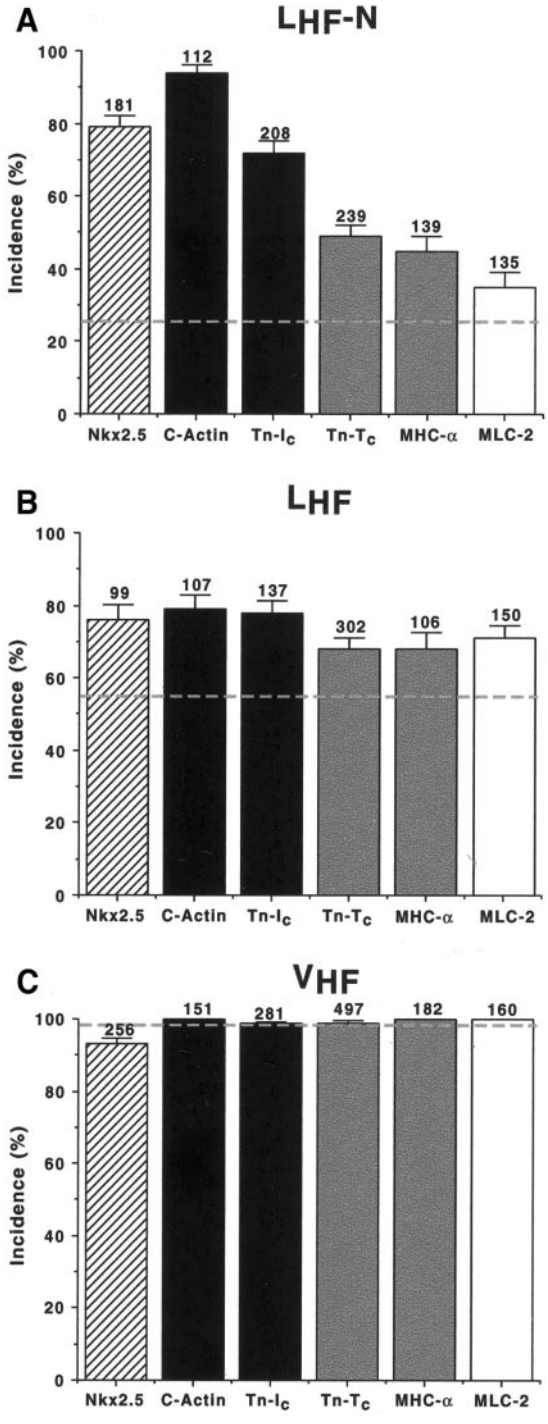


FIG. 6. Differential regulation of contractile gene expression by dorsal tissue. (A–C) Explants were isolated at stage 22, cultured, and subsequently assayed for gene expression and beating (see Materials and Methods). Contractile protein mRNA expression is represented by the bars in the histogram. The beating frequency is indicated by the dashed horizontal line. XNkx2.5 is normally expressed in the L_{HF} region in intact embryos (Figs. 1–3) and is likewise expressed in the corresponding tissue when explanted either with (A) or without (B) dorsal tissues (neural tube and dorsal mesoderm). Contractile protein genes are not normally expressed in L_{HF} cells (Figs. 1–3). Absence of ventral (prospective myocardial) tissue caused expression of contractile protein genes ectopically in the L_{HF} region (L_{HF}-N explants, A), suggesting that signals from the prospective myocardium suppress myocardial genes in the lateral portion of the heart field. Note that the fraction of explants expressing Tn-I_c and cardiac actin transcripts exceeded beating incidence, while the fraction expressing XMLC-2a coincided with beating incidence. Removal of neural and dorsal mesodermal tissue in L_{HF} explants (B) increased the incidence of expression of genes (XTn-T_c, XMHCα, and XMLC-2a) such that all markers were expressed in a large proportion of the explants. Beating incidence also increased. (C) Control explants of prospective myocardium (V_{HF}) generally beat and expressed all markers. Expression of all markers, with the exception of cardiac actin, was in the L_{HF} region (see Fig. 7). For cardiac actin, only heart and not somitic expression was scored. Results are statistically significant ($P < 0.05$).

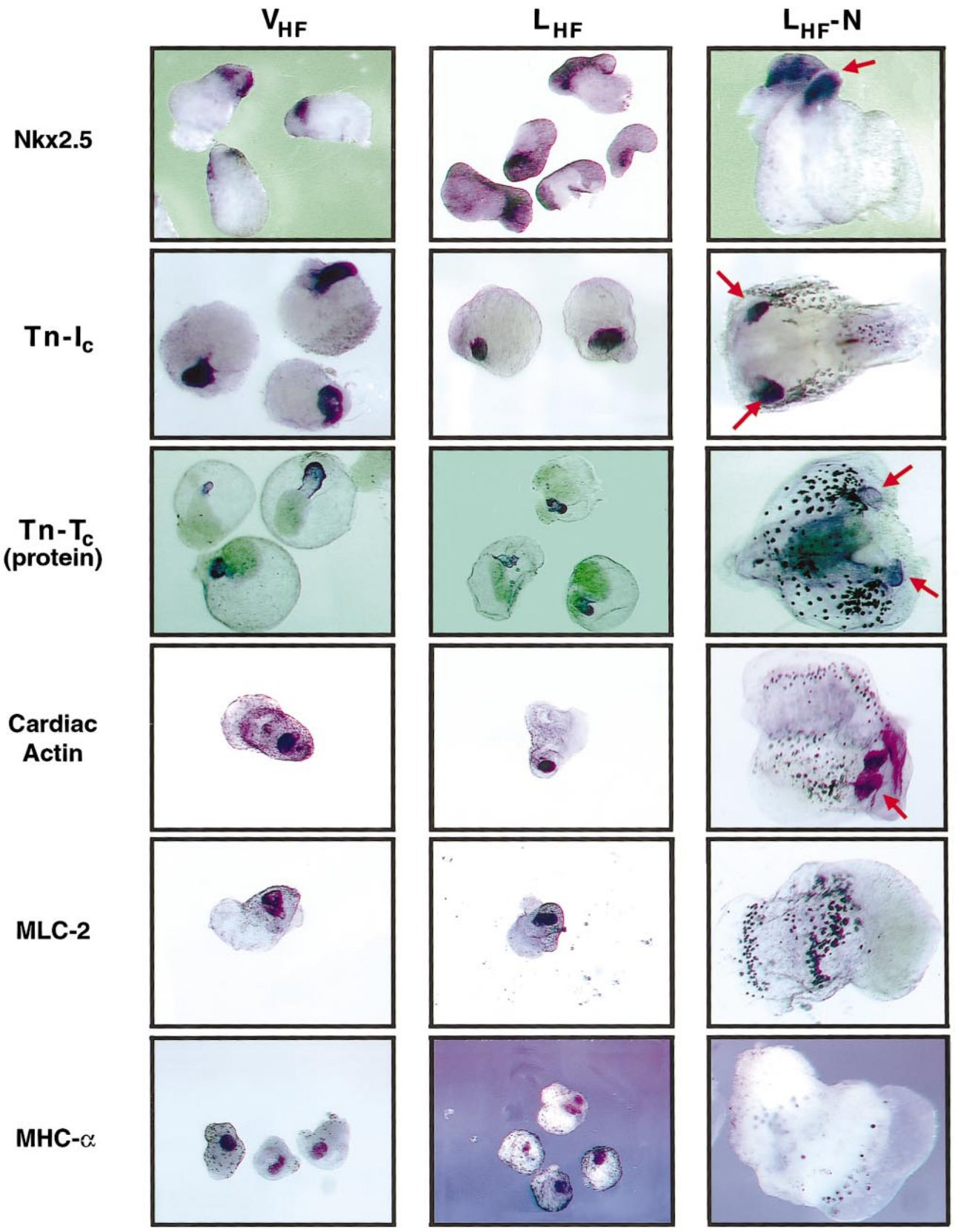
between these two extremes. Whether the differential suppression of cardiac genes reflects distinct dorsal and ventral inhibitors or a graded response to a common inhibitory signal is discussed below.

DISCUSSION

XNkx2.5 and the Heart Field

We found that the lateral boundary of XNkx2.5 expression is maintained in the sheet of cardiogenic precursors as it folds to form the heart tube. The lateral portions of the XNkx2.5 domain form the dorsal mesocardium and the roof of the pericardial cavity while the intervening region, which spans the ventral midline, gives rise to the heart tube (Figs. 1–3). Although fated to form nonbeating tissue, the lateral portions of the XNkx2.5 expression domain (L_{HF} explants), when cultured, autonomously differentiated into beating heart tubes with lumens (Figs. 4 and 8). Since tissue located just dorsal to the XNkx2.5 domain lacks heart-forming potential in culture (DL explants, Fig. 4), we conclude that the dorsolateral edge of the XNkx2.5 domain coincides with that of the classically defined heart field in the neurula stage embryo (Figs. 3 and 4). This is probably similar to the situation in chicks in which the posterior and lateral boundaries of the heart field also coincide with those of the Nkx2.5 expression domain (Ehrman and Yutzey, 1999).

These results help clarify the nature of heart field restriction. The concept of a broad heart morphogenetic field was drawn from extirpation, transplantation, and explantation experiments which suggested that the tissue capable of autonomous heart differentiation initially extends beyond the region that eventually contributes to the heart tube (Ekman, 1925; Copenhaver, 1926; Rawles, 1936; Jacobson, 1960; Sater and Jacobson, 1990a). Early studies of the



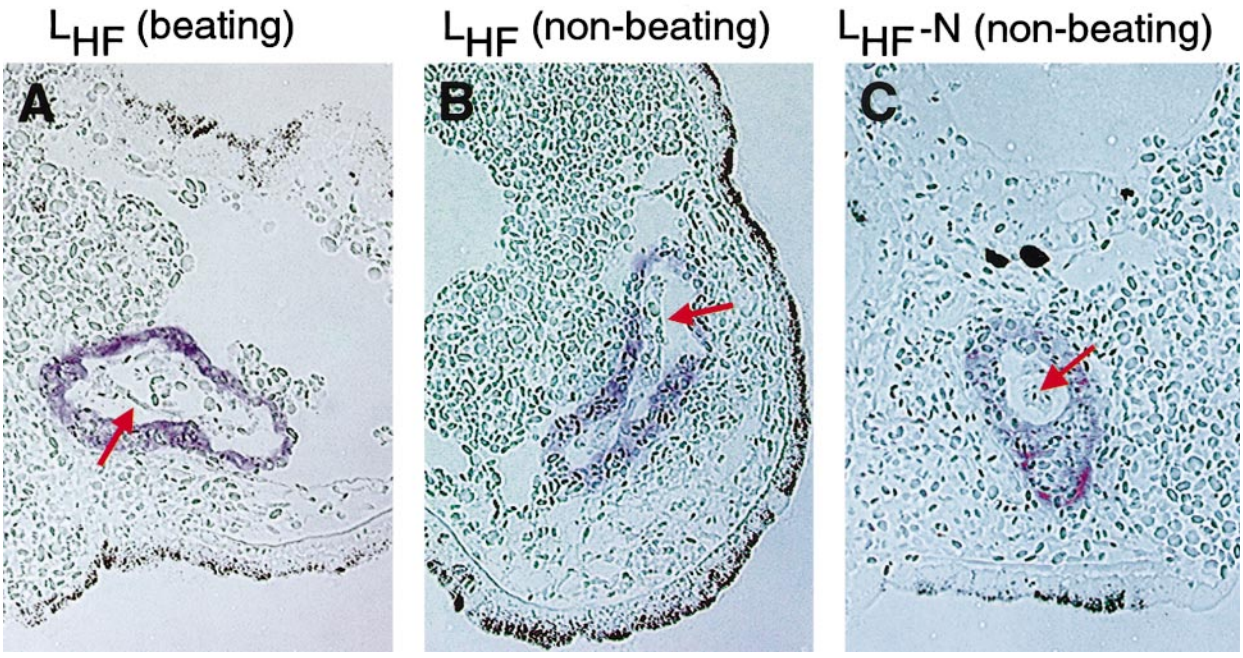


FIG. 8. Nonbeating L_{HF} and L_{HF}-N explants form tubes with lumens lined by endothelial cells. Explants were removed at stage 22, cultured to stage 40, and subsequently separated into beating and nonbeating classes at which time they were probed for XTn-I_c mRNA (blue stain) by *in situ* hybridization. Beating L_{HF} (A) as well as nonbeating L_{HF} (B) and L_{HF}-N (C) explants formed a myocardial tube lined with unstained, endothelial cells (arrows) that resembles a normal heart tube in cross section.

amphibian heart field showed that the capacity for autonomous differentiation within the lateral portion of the field (L_{HF} in our studies) is lost during postneurula stages (Copenhaver, 1926; Jacobson, 1960; Fautrez and Amano, 1961; Jacobson and Duncan, 1968; Sater and Jacobson, 1990a). Performed before cardiac molecular markers became available, these studies relied on presence of beating tissue and/or formation of heart tubes to score heart differentiation. For this reason, and because a fate map of the heart field was not available, cells within the lateral portion of the field were predicted to form nonheart tissue, implying a narrowing, or restriction, of the entire field (see discussion in Sater and Jacobson, 1990a). Instead, we found that the lateral portion, while transiently specified as myocardial, becomes respecified to form dorsal mesocardium and roof of the pericardial cavity between stages 22 and 28. Thus, the classical studies recorded the loss of *myogenic* potency that occurs as part of this respecification. Furthermore, the apparent narrowing of XNkx2.5 between stages 22 and 28 visible in whole mount (Fig. 1) is caused by the ventral shift and folding of the sheet of cardiac precursors during heart tube closure, rather than by a loss of expression (Figs. 2 and 3). Maintenance of expression during differentiation of mesocardial and pericardial roof tissues suggests that the loss of myogenic potency in the lateral heart field occurs downstream of XNkx2.5.

Suppressive Signals Subdivide the XNkx2.5/Heart Field into Myocardial and Dorsal Peri/Mesocardial Domains

The explant, extirpation, and recombinant studies show that both prospective myocardium and dorsal tissues (in particular the neural tube) inhibit myogenesis in cells of the lateral heart field (Figs. 4 and 5). Removing these signals caused lateral heart field tissue at stage 22 to express contractile protein genes and form heart tubes with lumens (Figs. 7 and 8). This shows that the lateral XNkx2.5/heart field at stage 22 has the potential to form either myocardial or nonmyocardial tissue. Since the lateral field readily

FIG. 7. Examples of heart gene expression in isolated explants. Examples of the most frequently observed expression patterns of explants described in Fig. 6. Note the absence of XMLC-2a and XMHCα in explants containing neural, notochordal, and somitic tissues (L_{HF}-N explants). The monoclonal antibody CT3 was used to recognize Tn-T_c.

forms beating cardiomyocytes when isolated, we propose that the entire XNkx2.5 domain is specified initially as cardiomyogenic but that suppression of myogenesis in the lateral portions of the field by surrounding tissues leads to distinct myogenic and nonmyogenic compartments. This model predicts that formation of heart tubes in isolated stage 22 lateral field tissue (L_{HF} explants) does not require *de novo* induction. Two observations are consistent with this idea. First, XNkx2.5 levels did not change as stage 22 L_{HF} explants were cultured. Second, removal of endoderm from stage 22 L_{HF} explants did not prevent heart differentiation (not shown but see Schneider and Mercola, 1999). Actual heart induction, in contrast, requires signals from underlying deep dorsal endoderm and is largely complete by stage 10.5 (Sater and Jacobson, 1989, 1990b; Nascone and Mercola, 1995).

Interestingly, genes encoding contractile proteins were differentially inhibited in our assays. Cardiac actin and XTn-I_c appeared particularly insensitive to suppression by presence of dorsal tissues, whereas XMLC-2 appeared most sensitive and XMHC-α and Tn-T_c were between these extremes. Whether this reflects a graded dose responsiveness or distinct signaling mechanisms upstream of the individual genes (suggested by differential regulation of transcription, reviewed in Chien *et al.,* 1993; Doevendans and van Bilsen, 1996; Lyons, 1996; Siddiqui and Goswami, 1997; Duroucher and Nemer, 1998) is unclear. Understanding the basis for differential responsiveness of these genes may help answer the question of whether the prospective myocardium and dorsal tissues suppress via common or distinct signaling molecules. It is clear, however, that myogenesis can be blocked at several levels. In intact embryos, the lateral heart field does not express contractile protein genes. In contrast, nonbeating explants of the lateral heart field with dorsal tissues (L_{HF}-N) formed lumens lined by endothelial cells (Fig. 8) and, occasionally, myofibrillar structures (the latter visible immunohistologically by confocal microscopy with CT3 monoclonal antibody, data not shown).

Suppression of the myogenic program downstream of XNkx2.5 may explain the data from Schultheiss *et al.* (1997) who found that application of exogenous BMP-2 and -4 induced ectopic cNkx2.5 mRNA in anterior mesoderm of chick embryos in the presence of neural plate and folds. In these experiments, ventricular MHC mRNA was induced only in the absence of neurogenic tissue. Other contractile protein genes were not examined, and it would be interesting to evaluate expression of cardiac actin or Tn-I_c, two genes we found to be refractile to suppression by dorsal tissues.

Our data are consistent with recent studies in other species in which notochord and/or neural tissue has been shown to suppress heart differentiation (Jacobson, 1960; Fautrez and Amano, 1961; Orts-Llorca, 1964; DeHaan, 1965; Rosenquist and DeHaan, 1966; Jacobson and Duncan, 1968; Sarasa and Climent, 1987; Goldstein and Fishman, 1998; Ehrman and Yutzey, 1999), insofar as suppressing signals emanate from the dorsal midline. Studies in zebrafish and chicks have attempted to discern whether notochord and/or neural tube are the source of suppressive signals. Goldstein and Fishman (1998) showed that manual and genetic ablation of the anterior notochord in zebrafish caused adjacent mesoderm to contribute to the myocardium. To our knowledge, the role of neurogenic cells has not been examined directly in zebrafish. In chicks, manual or chemical ablation of neurogenic cells causes enlarged hearts (Orts-Llorca, 1964; Sarasa and Climent, 1987), demonstrating suppression from neural plate and/or folds. Interestingly, these same studies suggested that chick notochord, in absence of myocardial or neurogenic tissues, cannot suppress. In our studies, we found that the neural tube was a potent source of inhibitory activity (Fig. 5). It is possible that amphibians are more similar to chicks than to zebrafish in this regard, although we cannot exclude the possibility that differences in experimental design account for the discrepancy.

Neural crest cells seem a likely source of suppressive signals from the neural tube since the heart field is a considerable distance from the neural tube at stage 22. Cephalic neural crest cells at stage 22 are still located just lateral to the neural tube but by stage 28 they have migrated into the heart region (Sadaghiani and Thiébaud, 1987; Ho *et al.,* 1994). Neural crest cells have been proposed to suppress heart, lens, and pronephros differentiation (for instance, see Jacobson, 1960; Von Woellwarth, 1961; Etheridge, 1972; Henry and Grainger, 1987).

Finally, it is important to note that our model is not the same as that of Goldstein and Fishman (1998). In their study, ablation of suppressing tissue in zebrafish caused a more posterior (Nkx2.5-*negative*) region to express Nkx2.5 and initiate a myocardial program. Although our studies also identified suppressive signals from the dorsal midline, we did not examine cells posterior to the XNkx2.5 domain. Rather, we examined the fate of XNkx2.5-*positive* cells that reside just dorsal (medial in chick or zebrafish terminology) to the prospective myocardium. Therefore, our model proposes a means of apportioning fates within the Nkx2.5 domain. Extended to zebrafish or chicks, it would predict that signals from the dorsal midline would suppress myogenesis in the medial portion of the Nkx2.5 domain and concomitantly specify dorsal mesocardial and dorsal pericardial identity.

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