

Visualization and functional characterization of the developing murine cardiac conduction system

Stacey Rentschler^{1,2}, Dhananjay M. Vaidya³, Houman Tamaddon³, Karl Degenhardt², David Sassoon², Gregory E. Morley^{3,*}, José Jalife³ and Glenn I. Fishman^{1,2,4,‡}

¹Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029, USA

²Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, NY 10029, USA

³Department of Pharmacology, SUNY Upstate Medical University, Syracuse, NY, USA

⁴Physiology and Biophysics, Mount Sinai School of Medicine, New York, NY 10029, USA

*Present address: Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029, USA

‡Author for correspondence (e-mail: fishmg01@doc.mssm.edu)

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SUMMARY

The cardiac conduction system is a complex network of cells that together orchestrate the rhythmic and coordinated depolarization of the heart. The molecular mechanisms regulating the specification and patterning of cells that form this conductive network are largely unknown. Studies in avian models have suggested that components of the cardiac conduction system arise from progressive recruitment of cardiomyogenic progenitors, potentially influenced by inductive effects from the neighboring coronary vasculature. However, relatively little is known about the process of conduction system development in mammalian species, especially in the mouse, where even the histological identification of the conductive network remains problematic. We have identified a line of transgenic mice where *lacZ* reporter gene expression delineates the developing and mature murine cardiac conduction system, extending proximally from the sinoatrial node to the distal Purkinje fibers. Optical mapping of cardiac electrical activity using a voltage-sensitive dye confirms that cells identified by the *lacZ* reporter gene are indeed components of the

specialized conduction system. Analysis of *lacZ* expression during sequential stages of cardiogenesis provides a detailed view of the maturation of the conductive network and demonstrates that patterning occurs surprisingly early in embryogenesis. Moreover, optical mapping studies of embryonic hearts demonstrate that a murine His-Purkinje system is functioning well before septation has completed. Thus, these studies describe a novel marker of the murine cardiac conduction system that identifies this specialized network of cells throughout cardiac development. Analysis of *lacZ* expression and optical mapping data highlight important differences between murine and avian conduction system development. Finally, this line of transgenic mice provides a novel tool for exploring the molecular circuitry controlling mammalian conduction system development and should be invaluable in studies of developmental mutants with potential structural or functional conduction system defects.

Key terms: Heart development, Conduction system, Purkinje fiber, Mouse, Optical mapping

INTRODUCTION

The rhythmic excitation of the mature heart depends upon the integrated function of the heterogeneous network of cells that together comprise the specialized cardiac conduction system (CCS). While substantial progress has been made in clarifying the molecular pathways regulating many aspects of cardiogenesis (reviewed in Srivastava and Olson, 2000), a detailed understanding of the specification, differentiation and patterning of the conduction system itself is lacking. As the number of identifiable inherited human syndromes and induced mouse mutants with defects in cardiac conduction has increased, the impetus to unravel the molecular pathways governing formation and function of this cellular network has grown.

Several fundamental questions in CCS development have been the subject of recent investigation. Perhaps foremost has been the origin of conductive cells. While conductive cells from a number of species express several genes in common with neuronal cells, an elegant series of experiments using retroviral lineage-tracing techniques has provided strong evidence that in the chick, components of the conduction system derive from bipotential cardiomyogenic cells, rather than through transdifferentiation of neural crest derivatives (Gourdie et al., 1995; Cheng et al., 1999). Indirect evidence using Cre/lox technology to mark neural crest derivatives in the developing mouse also argues against a contribution of this lineage to the CCS (Jiang et al., 2000).

Prompted by the observation that a subset of the distal elements of the avian CCS, specifically the intramural Purkinje

fibers, develop adjacent to the arterial bed, recent studies have suggested that signals from coronary arteries might locally induce cardiomyocytes to assume a conductive phenotype (Gourdie et al., 1995). This hypothesis was strengthened by *in vivo* evidence demonstrating that inhibition of coronary arterial development led to a reduction in the formation of associated Purkinje fibers, while activation of coronary arterial branching led to ectopic Purkinje fiber differentiation (Hyer et al., 1999). Additionally, *in vitro* experiments demonstrated that recombinant endothelin-1, a vascular-associated cytokine, induced embryonic chick myocytes to upregulate a number of Purkinje fiber-specific gene products (Gourdie et al., 1998). Ectopic production of biologically active endothelin-1 in embryonic chick hearts using retroviral technology further established the ability of endothelin-1 to upregulate a subset of Purkinje fiber markers *in vivo* (Takebayashi-Suzuki et al., 2000).

The timing and nature of CCS specification and patterning are also uncertain. In the chick, results from lineage-tracing analyses as well as cell birthdating experiments have led to the hypothesis that only a conduction system framework is initially specified. Elaboration of the network is thought to occur by progressive recruitment of cardiomyocytes to a conductive phenotype throughout subsequent *in ovo* maturation until just prior to hatching, rather than by outgrowth from the early framework (Gourdie et al., 1995; Cheng et al., 1999). In contrast, studies of morphology and gene expression in human embryos support the idea that specific domains present within the early embryonic heart contain precursor cells from which the entire conduction system subsequently differentiates (Wenink, 1976; reviewed in Moorman et al., 1998). Whether or not these alternative interpretations need be reconciled or in fact represent true differences between avian and mammalian CCS development remains uncertain. Indeed, there clearly are aspects of the avian conductive network that are unique. For example, in contrast to the chick, the peripheral Purkinje fiber network of most mammals is largely confined to the subendocardial lining of the ventricles, and peri-arterial Purkinje fibers have not been well documented. Thus, a patterning role for the coronary arterial system in mammalian CCS development is unresolved. It has recently been proposed that another extra-cardiac cell population, the epicardial-derived cells (EPDC), which in the chick preferentially migrates to the subendocardium and AV cushions as well as the coronary arteries, may provide the patterning function in both chick and mammalian CCS development (Gittenberger-de Groot et al., 1998).

Impulse propagation through the rapidly conducting His-Purkinje system in the mature heart is essential for activation of the ventricles from apex to base, causing efficient ejection of blood from the ventricles into the outflow tracts at the base of the heart. However, in both the chick and rat heart, it has been shown that the sequence of activation of the primitive heart tube is from inflow to outflow, and impulses are propagated slowly and sequentially through the atrium, ventricle and outflow tract (reviewed in Kamino, 1991). This peristaltic-like activation pattern, which results in activation of the common ventricle from base to apex, must eventually give way to the mature activation pattern in the more developed heart, where activation of both ventricles occurs from apex to base. This change in activation pattern is due to the development of a functional His-Purkinje system, and the stage

of development when this occurs in the chick heart is disputed. De Jong et al., reported that at H/H stages 23 and onward, corresponding to the looped heart stage, the apex of the ventricle is activated earlier than ventricular myocardium closer to the atrioventricular canal (de Jong et al., 1992). However, using similar extracellular recording techniques, Chuck et al., found this transition to occur between H/H stages 29 and 31 of chick development, the period just after interventricular septation is completed (Chuck et al., 1997a). Similar studies to determine the stage when the His-Purkinje system becomes functional in the mammalian heart have not yet been reported.

In larger mammals, components of the entire conductive network can be identified based upon morphological or molecular criteria, but to date, discrimination of the most distal aspects of the CCS in the mouse using these approaches has not been possible. In the murine heart, expression of markers including specific connexins, as well as a *lacZ* gene 'knocked-in' to either the *minK* or *HF-1b* loci, delineate the bundle branches and proximal Purkinje fibers, but none appear to delineate the full extent of the conductive network along the ventricular free walls (Delorme et al., 1995; Coppen et al., 1998; Coppen et al., 1999; Kupersmidt et al., 1999; Nguyen-Tran et al., 2000). Accordingly, developmental studies that take advantage of mouse molecular genetics have been hindered.

In this report, we describe the expression of a *lacZ* reporter gene in cells of the murine CCS, including the distal Purkinje fiber network. By following the expression pattern of the reporter gene, we have for the first time been able to visualize the progressive maturation of the entire CCS and its interconnections in a mammalian heart. In addition, this study represents the first detailed characterization of ventricular activation patterns throughout murine heart development. These optical mapping results demonstrate that a functional His-Purkinje system exists in the mouse heart well before septation has completed.

MATERIALS AND METHODS

Transgenic mice and isolation of embryos

The generation of a stable transgenic line from the MC4 *Engrailed-2/lacZ* fusion construct was described previously (Logan et al., 1993) and hereafter in this study this strain is referred to as CCS-*lacZ* transgenic mice. Mice were maintained on a CD-1 outbred background according to institutional and NIH guidelines. CD-1 outbred female mice were mated with CCS-*lacZ* homozygous male mice and the morning of the vaginal plug was designated as 0.5 d.p.c.. Female mice were sacrificed and embryos were dissected from the uterus in ice-cold phosphate-buffered saline (PBS).

Whole-mount staining for β -galactosidase activity

Tissues were collected in ice-cold PBS and fixed for 5-15 minutes, depending on size, at room temperature in fix solution (2% formaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P-40 (NP-40), 0.01% sodium desoxycholate in PBS). After fixation, tissues were rinsed several times in PBS and then stained overnight at 37°C in the dark with stain solution [5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), 2 mM $MgCl_2$, 0.02% NP-40, 0.01% sodium desoxycholate in PBS]. Hearts of older embryos and neonatal mice were then dehydrated through a methanol series and treated with benzyl alcohol:benzyl benzoate (1:2) to make the tissue transparent.

Optical mapping of adult right bundle branch

Hearts were surgically removed via a thoracotomy and placed in a custom-built perfusion/superfusion apparatus. While the heart was fully immersed in Tyrode's solution, the aorta was cannulated and Langendorff perfused at a constant pressure of 68-74 mm Hg (1-2 ml/minute). High-resolution optical mapping studies of the right bundle branch (RBB) were performed on an upright microscope equipped with a cooled CCD camera. Images were acquired at 912 frames/second from a 64×64-pixel array in the absence of any motion reduction techniques, as previously described (Tamaddon et al., 2000).

Optical mapping of embryos

Timed-pregnant dams were sacrificed, embryos dissected free from the uterus and the embryonic hearts exposed by thoracotomy. Carotid and jugular vessels were cut to exsanguinate the heart, and the whole embryo was pinned to a dish in which mapping was performed. The optical mapping apparatus has been described previously (Tamaddon et al., 2000). Embryonic hearts were stained by superfusion of 8 μ M di-4-ANEPPS (Molecular Probes) for 5 minutes. The heart preparations were placed on the stage of an upright microscope (BX50WI Olympus), and mapped using a 10× water immersion objective while beating in sinus rhythm. The spatial resolution achieved was 20 μ m. No pharmacological or mechanical motion reduction techniques were used during any of the experimental protocols. 10-15 beats were averaged to obtain a representative activation sequence.

RESULTS

Reporter gene expression in the hearts of transgenic mice

Previous studies have delineated regulatory elements from the *Engrailed-2* locus that direct *lacZ* reporter gene expression to

the embryonic mid/hindbrain region and mandibular myoblasts of transgenic mice in a pattern consistent with endogenous *Engrailed-2* transcription (Logan et al., 1993). A single line of transgenic mice harboring this reporter construct (previously termed MC4) also expressed *lacZ* in the heart. Indeed, after chemical clearing of a neonatal heart to allow for better visualization of the overall pattern of *lacZ* expression, the reporter gene appeared to delineate the cardiac conduction system (Fig. 1A-C). We therefore proceeded with a systematic analysis of the spatial profile of transgene expression in this line of mice.

Robust β -galactosidase activity was detected in the neonatal right atrium (RA); however, the level of expression was heterogeneous, and not all atrial myocytes expressed the transgene. The most discrete expression was seen within the sinoatrial (SA) node, atrioventricular (AV) node and ring, and in the left and right venous valves (Fig. 1D-F). The SA node could be visualized as a cylinder in the subepicardial region of the right atrium, near the junction of the medial wall of the superior vena cava, with the conspicuous SA nodal artery running through the node, as shown in Fig. 1D (Lev and Thamer, 1973; Viragh and Challice, 1980). Right and left prolongations of the SA node were also seen as it coursed posteriorly through the right atrium (data not shown) (Viragh and Challice, 1982). The AV node was discernible in the inferior portion of the interatrial septum to the right and above the mitral annulus, as seen in Fig. 1E (Lev and Thamer, 1973). Transgene expression also delineated fibers originating within the right atrium and coursing toward the left atrium (Fig. 1A). These fibers presumably are components of Bachmann's bundle, which is responsible for bringing the depolarizing impulse into left atrial tissue.

Fig. 1. *LacZ* expression in the CCS of neonatal hearts. (A) Low magnification view of *lacZ* expression within the heart, which delineates components of the entire cardiac conduction system. (B) Higher magnification of the region of the His bundle (H) and bundle branches. Several fibers split off from the His bundle and travel along the right side of the interventricular septum (IVS) giving rise to the right bundle branch (RBB), which is out of the plane of focus. The termination of the His bundle gives rise to the fibers of the left bundle branch (LBB), which has a characteristic fan-shaped appearance. Fibers coursing directly from left atrium (LA) to left ventricle (LV) are indicated (arrowhead). (C) Higher magnification of the extensive Purkinje fiber network within the LV. (D-F) Analysis of Eosin-stained sections demonstrates preferential transgene expression within specific regions of the right atrium (RA), including the SA node (SAN), the right (R) and left (L) venous valves and the AV node (AVN). Ventricular transgene expression delineated the His bundle, located beneath the tricuspid annulus, and the bundle branches. M, mitral valve; T, tricuspid valve. Scale bar for D-F is shown.

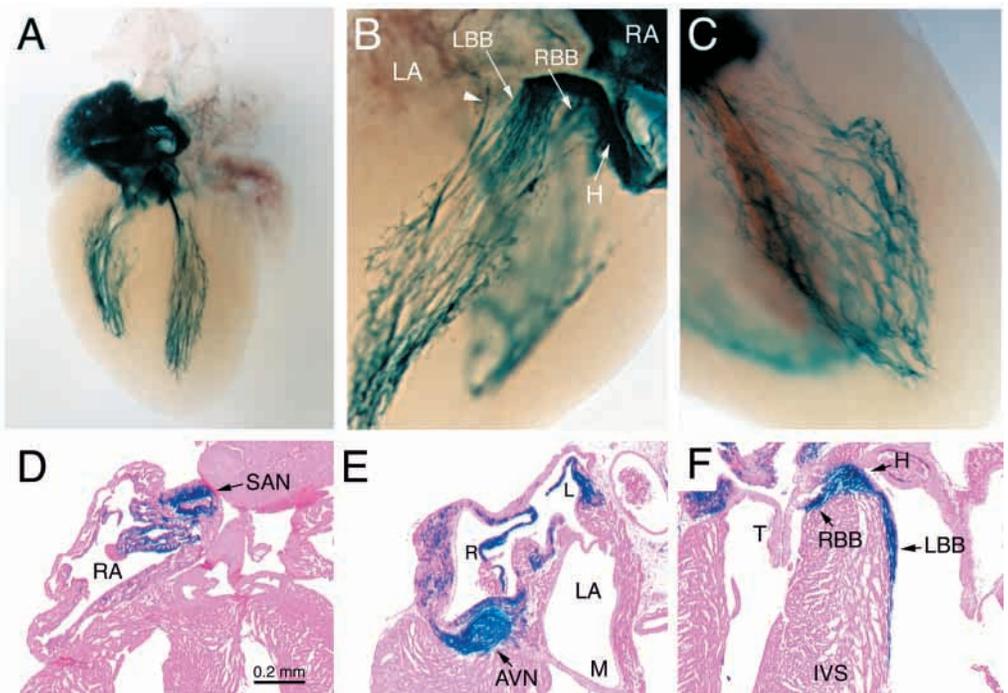
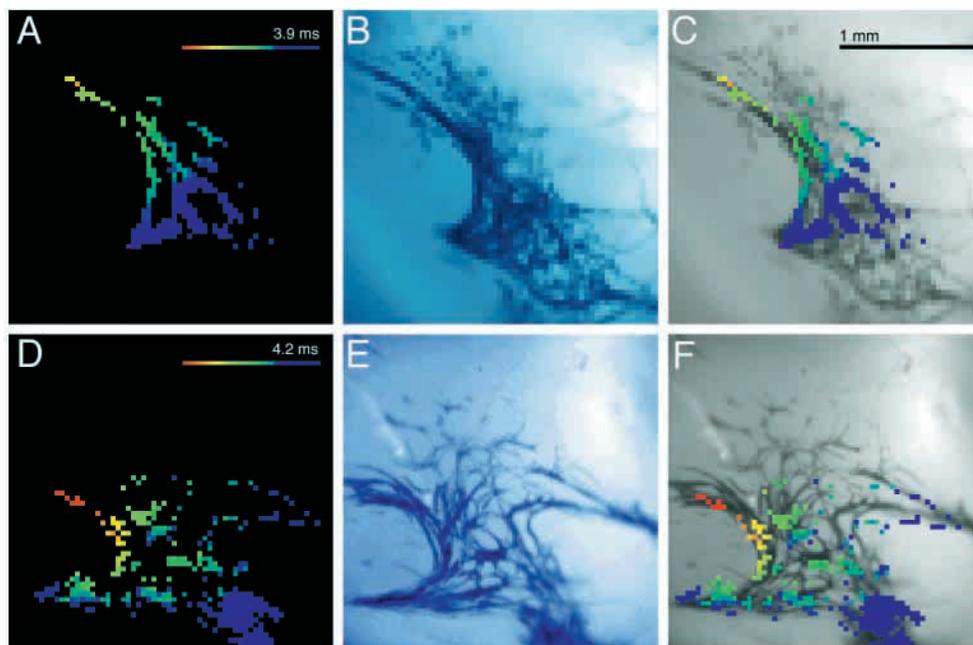


Fig. 2. Dual imaging of electrical activity and *lacZ* expression in the RBB. Maps of electrical activity along the right septal surface that correspond to activation of the RBB were recorded in two *CCS-lacZ* transgenic hearts. The color-coded scheme in each panel describes progressive activation of the RBB, red cells depolarizing first and purple cells depolarizing either (A) 3.9 milliseconds or (D) 4.2 milliseconds later. The general direction of impulse propagation in both panels is from the AV nodal region (upper left corner, out of the field of view) towards the apex of the heart (lower right corner, out of the field of view). Additionally in D, branches can be seen coursing posteriorly toward a papillary muscle (to the left of the panel), as well as towards the anterior surface of the septum. (B,E) Following optical mapping, hearts were reacted with X-gal to reveal the pattern of *lacZ* expressing cells. (C,F) Merged image of electrical activity and *lacZ* expression in individuals hearts reveals overlapping patterns.



Expression of the transgene in the neonatal heart was also found within the ventricular conduction system (Fig. 1). The transgene demarcated the AV (or His) bundle, the left (LBB) and right (RBB) bundle branches, and highlighted the extensive ramification of the Purkinje fiber network along the ventricular free walls, which has not previously been visualized in the murine heart.

Optical mapping confirms *lacZ*-expressing cells are components of the CCS

To rigorously demonstrate that *lacZ*-expressing cells are components of the CCS, we obtained high-resolution optical maps of electrical activity along the right side of the interventricular septum (IVS), reflecting activation of the RBB (Tamaddon et al., 2000). The activation maps were then compared with *lacZ* transgene expression within the same hearts (Fig. 2). Significantly, the patterns of RBB activation in all cases correlated extremely well with the patterns of *lacZ* reporter expression, supporting the assignment of these cells to the CCS.

CCS-*lacZ* transgene expression in the embryonic heart delineates progressive maturation of the cardiac conduction system

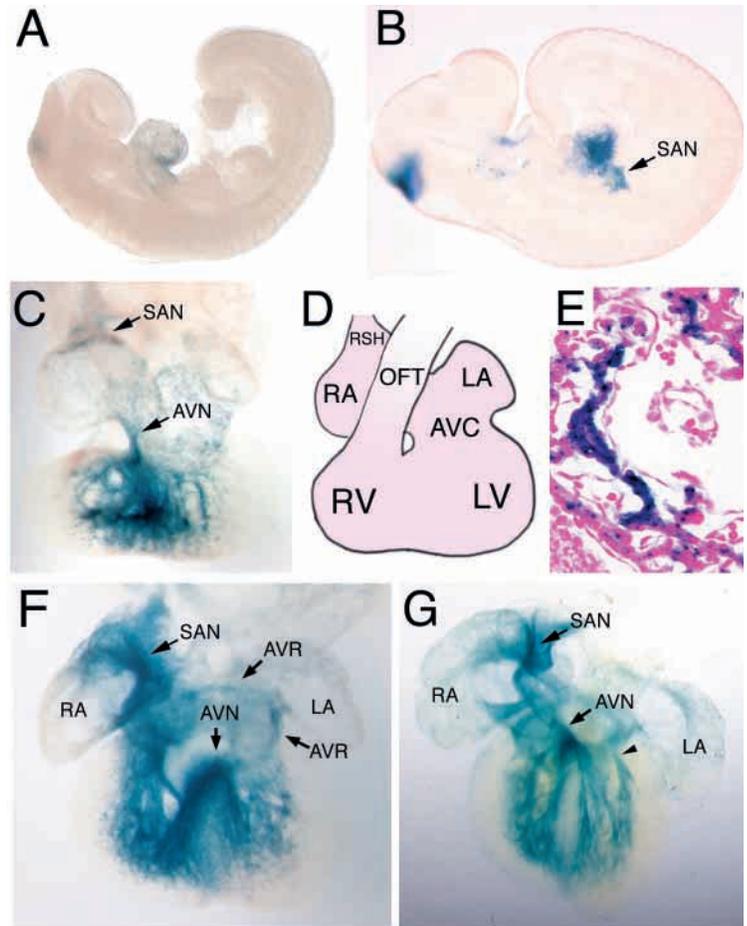
We next utilized this line of mice, hereafter referred to as *CCS-lacZ*, to examine formation of the presumptive CCS within the embryo heart at several developmental stages, as visualized by β -galactosidase activity. The first detectable transgene expression was observed at approximately 8.5 d.p.c. in both mid/hindbrain region precursors, as previously shown (Logan et al., 1993), and in the tubular heart (Fig. 3A). Expression in the heart at this stage was widespread, but was highest along the dorsal wall, a location consistent with previous morphological studies that identified the first specialized cells of the AV conduction pathway here 12 hours later (Viragh and

Challice, 1977a). By 9-9.5 d.p.c. the murine heart shows regular contractions arising from a site in the right sinus horn. Indeed, at this stage we observed a patch of cells within the right sinus horn that strongly expressed the transgene, likely demarcating the site where the SA node will become morphologically evident within the next 24 hours (Fig. 3B). Transgene expression was also seen in the interventricular region in a pattern reminiscent of, but broader than, the 'primary interventricular ring', which is thought to give rise to components of the conduction system such as the His bundle and bundle branches (reviewed in Moorman et al., 1998).

To more easily examine the pattern of *lacZ* positive cells in hearts of older embryos, the hearts were removed from the embryos and the tissue was chemically cleared. At 10.5 d.p.c. transgene expression in the heart was similar to that at 9.5 d.p.c., with highest expression in the SA nodal region, the interventricular region and in discrete fibers within both primitive ventricles (Fig. 3C,D). In addition, transgene expression could now be seen along the right side of the AV canal, where glycogen-rich cells thought to give rise to the AV node have previously been described (Viragh and Challice, 1977b), as well as in the septum primum. Histologic sections of the tubular heart at this stage demonstrated that the majority of *lacZ* positive cells within the right and left ventricles of the heart were subendocardial (Fig. 3E). However, within the left ventricle and IVS, *lacZ* positive cells could also be seen throughout a region of the compact myocardium (data not shown).

In the 12.5 d.p.c. heart, the atria are more distinct structures and the budding IVS could clearly be seen between the primitive ventricles. *CCS-lacZ* transgene expression delineated a near continuous pathway from SA node to AV node and bundle, which was in turn contiguous with the developing bundle branches located on top and astride the IVS (Fig. 3F). In addition, the trabecular component of both right and left

Fig. 3. CCS-*lacZ* expression delineates progressive maturation of the conduction network within the embryonic heart. To visualize formation and patterning of the CCS, β -galactosidase activity in CCS-*lacZ* transgenic embryos was analyzed at several developmental stages. (A) Initiation of expression within the heart occurred at 8.5 d.p.c. predominantly in CCS precursors along the dorsal wall of the AV canal. (B) By 9.5 d.p.c., expression could now be seen in precursors of the SA node (SAN) within the right sinus horn (RSH) as well as in precursors of the ventricular CCS located in the region between the undivided left and right ventricles. At later stages, hearts were dissected from the embryos following the X-gal reaction and chemically treated to better visualize the pattern of conduction cells. The labeled illustration in (D) corresponds to the 10.5 d.p.c. heart shown in (C), where the transparent outflow tract (OFT) cannot be visualized. At 10.5 d.p.c., the location of the CCS is similar to 9.5 d.p.c.; however, discrete fibers within the ventricles can now clearly be seen, as well as a group of cells along the right AV canal (AVC), where the developing AV node (AVN) is located. (E) An Eosin-stained section through the ventricular region of a 10.5 d.p.c. embryo revealed that the location of trabecular CCS cells is predominantly subendocardial. (F) In the 12.5 d.p.c. heart, the SA node and the presumptive SA ring, a bundle in the posterior right atrial wall leading towards the AV node, were detected. The AV node, located in the posterior AV canal, was continuous with the His bundle and bundle branches developing on top and astride the budding IVS. Components of the AV ring (AVR) were also detected. (G) The CCS of the 13.5 d.p.c. heart appears nearly mature, including the distal Purkinje networks within right and left ventricles. Fibers coursing from LA to LV are indicated (arrowhead). RA, right atrium; LA, left atrium.



ventricles from which the peripheral Purkinje fibers are derived also expressed the transgene. One day later at 13.5 d.p.c., the IVS was almost fully developed and it separated the networks of *lacZ* positive cells within the two ventricular chambers. At this stage, the appearance of the conduction system assumed a pattern quite similar to the neonatal configuration (Fig. 3G).

Optical mapping of embryo hearts defines the functional maturation of the CCS

We next proceeded to map electrical activity in embryonic hearts at various stages to determine whether a population of functionally 'specialized' cells exists in these hearts. Electrical activity was recorded from the hearts of 10.5 d.p.c. embryos, and the site of earliest activation of the ventricular epicardial surface that propagated radially, defined as a breakthrough, was identified. The resulting activation maps demonstrated that, in all hearts ($n=10$), breakthrough activation of the ventricular epicardial surface occurred on the anteroapical surface near the apex (Fig. 4A). Thus, even at this early stage when ventricular septation is just commencing, the developing ventricles appear to be activated through 'specialized' cells delivering the impulse to the apex and functioning as an embryonic His-Purkinje network. X-gal staining of these hearts after the mapping study demonstrated that areas of highest *lacZ* expression correlated with the breakthrough areas.

In 11.5 d.p.c. embryos the majority of hearts (13 of 20) also showed a single breakthrough near the apex of the heart,

similar to the pattern 1 day earlier (Fig. 4B). However, in the remaining hearts (7 of 20), two distinct breakthroughs were apparent on the outer curvature, one overlying each of the developing ventricles (Fig. 4C), suggesting that the bundle branches have now become functional. In addition, the ventricular activation time in hearts with two breakthroughs was markedly shorter. By 12.5 d.p.c., all hearts ($n=7$) showed two distinct breakthroughs (Fig. 4D), which correlated extremely well with the appearance of discrete bundle branches as visualized by X-gal staining (Fig. 3F). This same dual activation pattern was maintained at 15.5 d.p.c. (Fig. 4E) and throughout all subsequent stages of development (data not shown).

DISCUSSION

In this report, we describe the expression of a *lacZ* reporter gene in cells of the developing and mature murine cardiac conduction system and correlate its expression with patterns of ventricular activation. Somewhat surprisingly, in view of the phenotypic diversity of cells from distinct components of the CCS, we find a single molecular marker that appears to identify virtually all components of the conductive system network. This assignment to the CCS is based not only upon the striking appearance of *lacZ* positive cells, but is also supported by direct correlation of *lacZ* expression with functional maps of electrical activity.

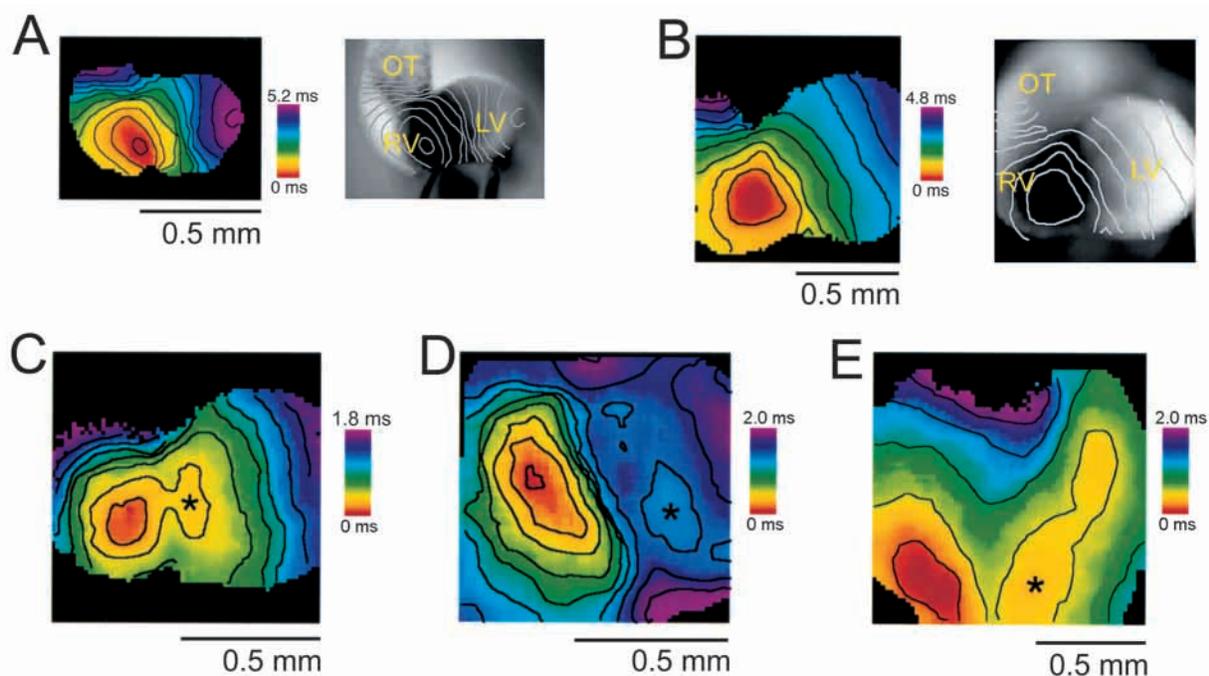


Fig. 4. Functional maturation of the murine CCS. Maps of electrical activity viewed from the anteroapical surface of developing hearts were recorded. Representative activation maps and X-gal stained images from (A) 10.5 d.p.c. and (B) 11.5 d.p.c. embryos are shown, with isochrones drawn every 0.5 millisecond. Activation maps from hearts with double breakthroughs, with the right ventricle always preceding the left ventricle, are shown at (C) 11.5 d.p.c., (D) 12.5 d.p.c. and (E) 15.5 d.p.c. The second breakthrough is indicated (asterisk). Isochrones are drawn every 0.25 millisecond.

Several classic studies have described the process of murine CCS development using structural criteria such as cellular shape, appearance of intercellular junctions, presence of glycogen granules, myofibrillar arrangements and nuclear morphology (Viragh and Challice, 1977a; Viragh and Challice, 1977b; Viragh and Challice, 1980; Viragh and Challice, 1982). While the locations of CCS precursors determined by *lacZ* expression correspond remarkably well with these early reports, our analysis of CCS development using a molecular marker allowed us for the first time to directly visualize formation of the entire CCS as an interconnected unit. Moreover, the molecular approach identified CCS cells significantly earlier than methods relying upon purely morphological criteria. For example, CCS-*lacZ* transgene expression along the dorsal wall of the 8.5 d.p.c. heart preceded morphologic differentiation of these cells into the first embryonic AV conduction pathway. Similarly, transgene expression within the right sinus horn in the 9.5 d.p.c. heart preceded the morphologic transition of sinus musculature into a compact SA node. Since the heart at this stage contracts regularly and discrete CCS-*lacZ* expression was visualized within the SA node throughout subsequent developmental stages, its expression in the 9.5 d.p.c. sinus region likely reflects the 'physiologic node', which will eventually become the compact node. Additionally, expression within the interventricular region at 9.5 d.p.c. demarcates the earliest stages of ventricular conduction system development.

Our study provides some insight into the nature of CCS development in the mammalian heart, and highlights important differences between species. In the chick heart, the AV node is

thought to develop independently from the bundle branches and Purkinje fibers, and it has been proposed that a linkage between these two components must occur, likely at the completion of ventricular septation (Gourdie et al., 1995; Chuck, 1997a; Chuck, 1997b). In contrast, *lacZ*-expressing cells in the 12.5 d.p.c. murine heart delineated a near continuous network of cells within the embryonic heart. This network extended from the region of the SA node to an area of the AV node and ring, which was in turn in direct continuity with the AV bundle and bundle branches. This observation agrees with previous murine studies suggesting that the primordial AV node and bundle develop simultaneously and in continuity with each other (Viragh and Challice, 1977b). In addition, the appearance of two ventricular breakthroughs in hearts at 12.5 d.p.c., prior to the completion of septation, suggests that impulses from the right atrium are conducted through the region of the AV node and bundle to reach the functioning bundle branches. Interestingly, connexin 40 protein expression, which has been proposed to be the marker of the early differentiating murine conduction system, has not been detected in the bundle branches until 13.5 d.p.c. (Delorme et al., 1995; Delorme et al., 1997).

The prevailing hypothesis of avian CCS development also postulates that the conductive cell network grows and is patterned throughout development, including stages much later than the end of cardiac septation, by progressive recruitment of bipotential cardiomyocytes upon an initial conductive framework (Gourdie et al., 1995; Cheng et al., 1999). Inductive signals arising from the perfused coronary vasculature are thought to be responsible for conversion of cardiomyocytes into

conduction cells and for the organization of the distal conduction network (Gourdie et al., 1998; Gourdie et al., 1999). Our optical mapping data demonstrate the appearance of a functional His-Purkinje network in the 10.5 d.p.c. murine heart, a stage when only the earliest signs of coronary vessel formation are present. By 13 d.p.c., just before the completion of ventricular septation, high pressure blood from the aorta begins to flow through the vascular bed (Viragh and Challice, 1981). At this stage, a highly patterned and functional network of conductive cells, similar to that in the adult heart, is already present in the murine heart. Together this evidence suggests that, in contrast to the chick, hemodynamic factors from the coronary arterial system may not be responsible for the induction and/or organization of the murine Purkinje network. However, whether or not some population of bipotential cardiomyocytes is continuously recruited to expand the conductive cell network will require more complex lineage analyses.

In the 13.5 d.p.c. heart, we observed a bundle of fibers coursing from the LA toward the LV along its free wall (Fig. 3G). This arrangement of fibers could also be seen in subsequent developmental stages, including the neonatal heart (Fig. 1B). The persistence of such tracts along the LV free wall that directly connect atrial and ventricular myocardium and bypass the more slowly conducting AV node may in fact contribute to ventricular pre-excitation, i.e. Wolff-Parkinson-White syndrome, which interestingly is most commonly mapped to this site (Oren IV et al., 1993).

In the embryo heart, a direct connection between the SA node and AV node, namely the SA ring, has been proposed (Wenink, 1976). Interestingly, transgene expression delineated a half-ring, which extended from the SA node along the dorsal atrial wall to the AV nodal region, and may correspond to the SA ring. Also, the proposed remnants of the embryonic SA ring in the fully developed heart, namely the right and left venous valves (Wenink, 1976), highly express the transgene (Fig. 1E).

In several regions, particularly in atrial tissue, we observed *lacZ* expression in areas that were broader than those conventionally thought to comprise the conduction system. Interestingly, the existence of 'specialized' cells within the fully developed right atrium, which form an atrial, or internodal, conductive network, has been proposed (James and Sherf, 1971). Additional studies will be required to determine whether such *lacZ* expressing cells have electrophysiological properties distinct from working atrial myocytes, or if in fact the specificity of transgene expression in atrial tissue is less stringent than in distal components of the CCS.

Finally, it is worth noting that our studies to determine whether endogenous *Engrailed-2* is expressed in the heart have been unrevealing. In addition, CCS expression was not seen in a number of additional lines of mice harboring the same transgene construct described in this study (Logan et al., 1993). Thus, it appears that the site of integration in this particular line of mice is likely to control transcription of the reporter gene, and identification of this locus will clearly be of interest.

In summary, we have identified a line of transgenic mice in which a *lacZ* reporter gene is expressed in the developing and mature murine cardiac conduction system. Moreover, we have correlated *lacZ* expression with measures of CCS function and found that formation and function of the murine CCS occurs well before the completion of ventricular septation. The CCS-

lacZ transgenic mice, which reveal the temporospatial profile of CCS formation, should be of considerable utility in studies of murine cardiovascular development. Indeed, as the number of induced mouse mutants with defects in cardiovascular development grows, including those with abnormalities in impulse conduction, the ability to visualize the full extent of the conductive system network within the developing heart should help discriminate between global defects in myocardial morphogenesis and specific perturbations of CCS formation and function.

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