Evidence for a Distinct Gap-Junctional Phenotype in Ventricular Conduction Tissues of the Developing and Mature Avian Heart


The gap-junctional proteins connexin43 and connexin42 have been shown to be expressed in the developing and mature avian heart, but their respective spatiotemporal distributions are unknown. In this study, we have immunolabeled connexin42 in the conduction tissues of the adult avian heart (nonbranching bundle, bundle branches, and Purkinje fibers) and vascular endothelial cells. Connexin43 immunolabeling was confined to vascular smooth muscle. A novel microwave-based method was used to label connexin42 and connexin43 in the same tissue section. Neither connexin42 nor connexin43 was immunolocalized in working myocardium, atrioventricular node, and atroventricular ring tissue of the bird heart. Although connexin42 first appeared in periartrial conduction myocytes and vascular endothelium at 9–10 embryonic days, the central conduction tissues, including the nonbranching bundle and proximal branches, remained immunonegative for connexin42 up until hatching (~20 embryonic days). During the early postnatal period (1–14 days), connexin42 immunolabeling progressively spread up the bundle branches toward the nonbranching bundle. Connexin42 appeared uniformly distributed along the left bundle branch by 14 postnatal days. The distribution and spread of connexin42 immunoactivity suggest that the emergence of specialized junctional contacts along ventricular fascicles occurs relatively late in heart development and coincides with the emergence of the chick from incubation within the egg.

(Key Words: heart • development • gap junctions • connexin • conduction tissue)

Gap junctions are aggregates of intercellular membrane channels that, in the myocardium, are specialized for electrical coupling and the cell-to-cell transfer of the action potential. Traditionally, the heart has been viewed as an electrical syncytium, with the characteristics of the cardiac action potential being explained largely in terms of the properties of nonjunctional membrane channels. However, evidence is emerging that heterogeneities in the distribution and type of intercellular channel may also have key roles in coordinating the function of the heart.

The protein subunits (termed connexins) that compose gap junctions are encoded by a family of related genes and demonstrate a degree of tissue specification and differential expression during development. The principal connexin polypeptide of mammalian cardiac myocytes is thought to have a molecular mass of 43 kDa (i.e., connexin43 [Cx43]), although other protein isoforms have recently been identified in heart tissues. Immunohistochemical studies on developing and adult mammalian hearts have shown that Cx43 is distributed in a highly regionalized pattern evident from early embryogenesis. The protein is principally distributed in the cells of the working myocardium, with lower amounts occurring in specialized nodal tissues. Although Cx3 is present in the atrioventricular node, it is immunologically undetectable in other parts of the central conduction axis, including the distal nonbranching bundle and bundle branches. The lack of this cardiac connexin protein in these tissues is puzzling, given the electrophysiological property of rapid conduction of the bundle and its branches and the presence of ultrastructurally detectable gap junctions within these anatomic structures. This discrepancy raises the possibility of differences in expression of connexin type between the specialized tissues of the myocardium. In a study of developing chick heart, Beyer identified expression of Cx43 as well as two previously uncharacterized connexins, connexin42 (Cx42) and connexin45 (Cx45). It was determined that expression of chick Cx42 and Cx43 persisted at significant levels throughout development and in the mature avian heart. Homologues of chick Cx42 and Cx45 have now been reported in studies of isolated canine myocytes. Canine connexin40 (Cx40) and Cx45 have a
70% and 85% amino acid identity to chick Cx42 and Cx45, respectively.

The distributions of the different connexins in the avian heart are presently unknown. To investigate possible differences in the type of intercellular channel between specialized cardiac tissues, specific antisera against Cx42 and Cx43 were used to map and quantify the distribution of immunolabeled gap junctions in the mature and developing chicken heart. By precisely defining the spatial distribution of connexin protein isoforms in the adult avian heart and examining the temporal sequence by which these patterns arise during development, further insight has been gained into how intercellular communicating channels might coordinate function in the vertebrate heart.

Materials and Methods

Antibodies

The production and characterization of anti-HJ antisera, raised against a synthetic peptide immunogen matching part of the “cytoplasmic loop” domain of Cx43 (residues 131–142), and its specificity against cardiac gap junction protein(s) have been detailed elsewhere.9,11,18 A second antisera raised against a synthetic peptide immunogen termed Cx43c, made to match residues 252–271 of Cx43, was also used. This sequence does not overlap with the HJ sequence and is located on the exposed cytoplasmic C-terminal “tail” region of the Cx43 molecule.4,13 Details of the production and characterization of this second antisera, with activity against Cx43, in both rat and chicken tissues, have been given previously.19,20 For studies of Cx42, an antisera raised against a synthetic peptide immunogen matching residues 260–279 of the 369–amino acid 42-kd molecule was used.17 This domain occupies a location in the native molecule similar to the 252–271 peptide sequence on the Cx43 molecule, although their respective amino acid sequences show little similarity. In enzyme-linked immunosorbent assays, anti-Cx43c (252–271) and anti-Cx42 (260–279) antisera reacted strongly with the synthetic peptides to which they were raised, with no evidence of cross-reactivity between antisera and unrelated peptides. In immunostaining studies using the two antisera against cultured cells expressing only connexin43, cross-reaction by the anti-Cx42 antisera was not observed (Dr. E.C. Beyer, personal communication). The anti-Cx42 antisera strongly immunostained gap junctions on myocytes isolated from canine ventricle, as assessed by light and electron microscopy.12 This reactivity of anti-Cx42 with gap junctions was abolished by preincubation with the 260–279 Cx42 peptide but was unaffected by incubation with an unrelated peptide.12

Animals

Fertilized white Leghorn (Shaver Starcross) eggs (PeeDee Hatchery, Hartsville, S.C.) were incubated at 37°C in humidified air and sampled at 6, 7, 8, 9, 10, 11, 12, 14, and 20 days of development. Chicks at 1, 7, and 14 days after hatching and adult (6- and 65-week-old) chickens were also used. Hearts from three to four chickens were sampled at each time point.

Preparation of Tissues

Embryos or hearts from neonates and adults were removed, placed in warmed Tyrode’s solution, staged,21 dissected, and perfused with 0.1 M phosphate-buffered saline (pH 7.2) through the venous pole by mouth pipette to flush out blood cells and expand the hearts before perfusion and immersion in modified (4% paraformaldehyde, 10 mM picric acid) Zamboni’s fixative.22 Perfusion with phosphate-buffered saline and subsequent perfusion/immersion in fixative were also performed in chicken hearts after hatching and in adult chicken hearts. After fixation for 4–6 hours, thoracic segments (6–9 days of incubation) or intact hearts (from older animals) were washed, dehydrated, and embedded in paraffin as described previously.11 Hearts were oriented so that frontal, sagittal, and transverse planes were cut with respect to the major axes of the heart. Serial 10-μm sections were mounted on poly-L-lysine-coated slides and dried for 1–2 hours at 65°C.

Immunohistochemistry and Light Microscopy

Immunofluorescent histochemistry of tissue sections using antisera against Cx43 (anti-HJ and anti-Cx43c) and Cx42 (anti-Cx42) and control nonimmune serum was carried out as detailed previously.11 Tissue sections were also double-stained using antisera against both Cx43 and Cx42. This was done by immunostaining with anti-Cx43 antibodies and secondary labeling with swine anti-rabbit fluorescein-conjugated antibodies. The immunostained tissue under 1 ml buffer was then microwave-fixed (10 seconds at full power in a 650-W microwave oven), and a subsequent second immunostaining with antisera against Cx42 was performed. Immunofluorescent localization of this second primary antibody was done using rhodamine-conjugated anti-rabbit secondary antibodies. Controls demonstrated that the microwave fixation step 1) caused only minor damage to the histological appearance of the tissue section, 2) did not quench fluorescence, 3) did not affect the normal reaction of the antisera with its peptide antigens, but 4) did result in abolition of the reaction between tissue-immunolocalized rabbit antibodies and swine anti-rabbit antibodies, thus preventing cross-reaction between the two different fluorochrome-conjugated rabbit secondary antibodies. This was a convenient and reliable improvement on a multiple antibody–labeling protocol described by Kołodziejczyk and Baertschi.23 Tissue sections were examined using Lasersharp MRC-500 and MRC-600 laser-scanning confocal microscopes (Bio-Rad Microscience Ltd., Hemel Hempstead, UK) with filter blocks appropriate for the fluorochrome inspected. Recording of all confocal images in this study always began immediately after immunostaining. Alcian blue–PAS staining was performed on sister sections of those used for immunohistochemistry and examined using bright-field optics. Standard light microscope and laser-scanning confocal microscope images were photographed using Kodak TMX-400 negative film.

Quantitative Analysis of Bundle Branch Myocytes

Detailed analysis of gap junction size, numerical density, and total area per nucleus was carried out on tissue sections of the developing nonbranching bundle and left bundle branch. Individual fluorescein-tagged
by dividing total spot area by the number of nuclei observed within immunostained tissues. This was done to provide an index of the amount of Cx42 immunolabeled per cell that should be independent of changes in cell volume. Propidium iodide (red) staining of nuclei in anti-Cx42 immunolabeled tissue sections was done using the technique of Ockleford et al. Twenty-four Inspection of a number of sequential confocal optical sections through bundle tissues revealed that between 0% and 15% (mean, 4%) of myocytes were multinucleated. No relation was observed between the occurrence of multinucleated bundle myocytes and postnatal age; hence, this factor did not affect relative comparisons of Cx42 immunolabeling levels.

The design of the experiment for quantifying the spread of Cx42 immunolabeling along the left bundle branch was as follows. Three animals were used at each of the following developmental time points: 1 day before hatching and 1, 7, 14, and 42 days after hatching. Four-chamber views of intact chicken myocardia were selected from hearts serially sectioned in frontal orientation. Tissue sections were always taken from the same relative plane within hearts and showed the course of the left bundle branch down through the interventricular septum from or near its divergence from the nonbranching bundle (e.g., see Figure 1). For the purpose of sampling, the left bundle branch was divided into three segments. The first was the segment of the left bundle branch nearest the divergence, the second was the midsegment, and the third was the most distal segment, where the bundle approached the endocardium of the interventricular septum. For each chick, 1 volume of immunolabeled tissue was optically sectioned in each of the three bundle branch segments. This was done in two 1-μm focus steps using a Nikon ×60–1.4 numerical aperture Planapomat objective (Kalman averaging, zoom 2, confocal aperture 35% open). After collection, the two optical sections were combined into a projection, the number of nuclei within the immunolabeled tissues was counted, and the projection was then stored on disk. Given the confocal aperture setting used and the 1-μm z-focus step between optical sections, the projections used for quantification will have an effective z-width of approximately 2 μm.

Image analyses and processing were performed on projections to give gap junction numbers, lengths, and areas, using PC-IMAGE (Foster Findlay Associates Ltd.) software as described previously. Total immunolabeled gap-junctional areas within images were calculated by summing individual gap-junctional areas. This variate was normalized by dividing by the number of nuclei counted within anti-Cx42-immunolabeled myocytes.

Two-way analyses (chick age×bundle segment location) of total gap junction area per nucleus and mean gap junction area were performed using MINITAB (Minitab Corp.). Because variance due to differences between chickens was not significant for both variates (p>0.05), data from individual animals were treated as replicates within bundle segment locations. Owing to a significant correlation between sample means and standard deviations of gap junction area per nucleus and a nonnormal distribution of gap junction mean area, the respective analyses of variance for these two variates were performed on logE-transformed data.25

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**Figure 1.** Connexin42 immunolocalization within ventricular conduction tissues in the adult avian heart. Panel a: A low-power view of the interventricular septum (ivs), frontally sectioned in the plane of the aortic valve (aov) and stained with Alcian blue–PAS. The nonbranching bundle (nb) and its fascicular left limb (lbb) descend through the muscular septum. A large artery (a) is observed in transverse section to the right of the nb. Scale bar, 1,500 μm. Panel b: A confocally imaged sister section of panel a that has been immunolabeled with connexin42 antiserum. The nb and left branching bundle show high levels of punctate immunofluorescence. The working myocardium of the ivs surrounding these specialized conduction tissues is immunonegative. Fluorescent red blood cells are observed in the sinusoidal spaces of the ivs. These cells are seen to be nucleated when viewed at high magnification and nonspecifically fluoresce when treated with either immune or preimmune serum. Scale bar, 200 μm.

(green) spots were assumed to correspond to discrete junctions within labeled tissues, and the area and the longest axis dimension of the spot were taken as measurements of gap junction size. All immunolabeled gap junctions were counted, including those occurring in intercalated disks and those occurring at regions of lateral contact between specialized myocytes. Normalized levels of anti-Cx42 immunolabeling were estimated.
Results

Cx42 Is Immunolocalized in the Conduction Tissues of the Avian Heart

In the adult ventricle, the nonbranching bundle and its branches demonstrated high levels of immunolabeling with antiserum against Cx42 (Figures 1 and 2). No Cx42 immunolabeling was seen in working myocardial tissues surrounding the bundle fascicles or, indeed, any other working myocardial tissues of the avian atria and ventricles. Immunopositive fluorescence was absent from the bundle fascicles when preimmune serum was used in place of antiserum during immunolabeling. Similarly, bundle fascicles and working myocardium did not react with Cx43 antisera. In the bird heart, Cx43 was immunolocalized solely within the smooth muscle of coronary arteries (Figures 2b and 3c). This distribution is in marked contrast to the situation in mammalian species, in which large amounts of Cx43 are expressed in working myocardial tissues.9

Consistent with the location of gap junctions at regions of cell-to-cell contact, confocal optical sectioning revealed punctate anti-Cx42 immunostaining in zones of intercellular contact between fascicular conduction myocytes; such staining was not observed at cell borders demonstrably free from contact with other cells, e.g., along the margins of the bundle fascicles (Figures 2d and 3a). In reconstructed volumes of longitudinally aligned fascicular myocytes, immunostaining demarcated classical intercalated disks at the end-to-end abutments between the conduction cells (Figures 3a and 3b) as well as prominent linear chains of spots along regions of lateral apposition (Figures 2d and 3a). When viewed en face, those gap junctions immunolabeled by Cx42 antisera within intercalated disks were organized as a rim of bright punctate fluorescence circumscribing...
the disk perimeter. This is shown at high magnification in Figure 3b, in which a bowl-shaped disk between two fascicular myocytes is reconstructed in a three-dimensional anaglyph. The pattern of Cx42 immunolabeling revealed here in the conduction tissues of the bird heart resembles the three-dimensional geometry of Cx43 distribution at intercalated disks between working (i.e., nonconduction) myocytes of mammalian species.9

Figure 4 shows the frequency distribution of the size of anti-Cx42–immunolabeled gap junctions in adult left bundle branch. In accord with other quantitative studies of cardiac gap junction morphology,9,26–29 this distribution is highly asymmetric, with 93% of immunolabeled gap junctions having areas of 0.5 μm² or less and accounting for 68% of cumulative gap-junctional area. Less than 1% of gap-junctional plaques exceed 1.5 μm² in area (7% of the cumulative area of gap junction), and these larger junctions rarely exceed 1 μm in longest axial dimension. The mean area of individual gap junctions in the adult avian bundle
fascicles is 0.18 $\mu m^2$ (standard deviation, 0.27 $\mu m^2$). There is an average of 28.3 junctional spots per nucleus (standard deviation, 21.8) and a total of 5.2 $\mu m^2$ (standard deviation, 0.489 $\mu m^2$) of gap-junctional immunofluorescence per nucleus.

No immunolabeling of gap junctions with anti-Cx42 antiserum was observed in the other elements of the avian central conduction axis upstream of the non-branching bundle, including the atrioventricular node and atrioventricular ring tissue. However, high levels of anti-Cx42 immunolabeling occurred in the subendocardial Purkinje fibers (Figure 5) and in the network of periartrial conduction myocytes (Figure 3c). The pattern of Cx42 immunolabeling in Purkinje fibers and periartrial conduction myocytes is similar to that observed for myocytes of the bundle fascicles, occurring at lateral and intercalated disk abutments between the cells.

**Connexin43 Immunolocalizes in the Coronary Arteries**

In the mammalian heart, Cx43 is principally localized within the gap junctions of working myocytes. Paradoxically, this was not the case in the avian heart, where no Cx43 immunolabeling of working myocytes was detected (Figures 2 and 3). Similarly, the specialized conduction myocytes of the bundle branch were also immunonegative for Cx43 (Figure 2c). This absence of immunohistochemically detectable Cx43 in avian working and specialized conduction myocardium held for both the anti-Cx43 peptide antibodies used (i.e., anti-HJ and anti-Cx43c). Positive Cx43 signal was absent also from adult avian myocardial tissues when prepared as unfixed, paraformaldehyde-fixed, and methanol-fixed 10-$\mu m$ frozen sections (data not shown).

Although Cx43-positive gap junctions were not observed in avian myocardium (i.e., working and conduc-


The frequency distribution and cumulative frequency distribution of sizes (µm²) of immunolabeled (anti-connexin42) gap junctions in the adult left bundle branch. The plots demonstrate that 95% of gap junctions in this tissue are less than 1.5 µm² in area.

Connexin42 is detected in the nonbranching bundle and branches in the posthatching period but not during embryonic and fetal growth.

The pattern of appearance of Cx42 immunolabeling in ventricular conduction tissues is summarized in Table 1. During incubation in ovo, central elements of the ventricular conduction system, including the nonbranching bundle and bundle branches, remained immunonegative for Cx42 (Figure 5). Cx42 immunolabeling was first observed in periartrial conduction myocytes and in vascular endothelial cells at 9–10 embryonic days (Figure 6). Later in fetal development (14–20 embryonic days), subendocardial Purkinje fibers, including some adjacent to distal segments of the immunonegative bundle branches (Figure 5e), became immunolabeled by anti-Cx42 antiserum (Figure 5f).

Only after hatching did Cx42-positive gap junctions become evident within the formerly unlabeled nonbranching bundle and bundle branch tissues. During the early posthatching period (1 and 7 embryonic days), a gradient of Cx42 immunolabeling was observed along the bundle fascicles (Figure 5), with proximal nonbranching bundle cells demonstrating lower levels of immunoreactivity (Figure 5b) than myocytes comprising distal segments of the bundle branches (Figure 5c).

Quantification confirmed that the level of immunopositive fluorescence occurred as a gradient along the left bundle branch up to 14 postnatal days, with higher expression in distal subendocardial segments of the bundle fascicles. Table 2 and Figure 7 summarize the statistics from analysis of variance (developmental stage×bundle segment location) of total area of anti-Cx42 immunolabeling per nucleus during the posthatching period. At 1 and 7 days after hatching, there were significant (p < 0.05) decreases in the total area of Cx42 immunolabeling along the left bundle branch. In the 14- and 42-day-old chicken, this variation was no longer significant (p > 0.05), with total areas of gap junction being relatively consistent along the bundle branch. Figure 8 shows a histogram of the overall Cx42 immunolabeling levels over the period from 1 day before hatching up to 6 weeks after hatching. This plot indicates that the level of immunolabeling increases rapidly after hatching, peaking at 14 days and then decreasing slightly in older chickens.

There was a correlation between the morphology of the bundle branches and the shape of myocytes that matched the posthatching distribution of Cx42 immunolabeling. Although the unlabeled fetal bundle was easily discriminated, its overall morphology was amorphous, and it was difficult to discriminate the boundaries of its densely packed cells (Figures 5d and 5e). On expression of immunologically detectable levels of Cx42, single conduction myocytes became more readily identifiable as spindle-shaped cells (compare Figures 5c and 5e), and the bundle branches assumed their characteristic fascicular morphology (Figure 2).

Although total Cx42 levels became relatively uniform along the left bundle branch beyond 14 postnatal days (Figure 7), individual immunolabeled gap junctions demonstrated systematic variations in their mean areas (p < 0.01) according to location on the bundle branch. Gap junctions more proximal to the nonbranching bundle were smaller on average than those occurring in more distal segments of the bundle branch (Table 2 and Figure 9). This variation occurred at all the posthatching stages examined, including mature 42-day-old birds.

Discussion

In this study, we have demonstrated that Cx42 is specifically immunolocalized in the gap junctions of the nonbranching bundle, bundle branches, and Purkinje fibers of the avian cardiac conduction system. A gradient of immunoreactivity was observed along the bundle branches during embryonic and neonatal development; earliest Cx42 immunolabeling was observed in subendocardial Purkinje fibers, spreading in the first week after hatching up the branch arms to include the nonbranching bundle. Cx42-rich gap junctions were not found in the working myocardium or in other components of the impulse generation and conduction system (e.g., the atrioventricular node and atrioventricular ring). In contrast to the distribution of Cx42, Cx43 was found in abundance within coronary vascular smooth muscle, but it was not detected in any of the myocardial tissues of the avian heart. This is an important distinc-
FIGURE 5. The developmental immunolabeling patterns of connexin42 in the left bundle branch (lbb). Panel a: A confocal survey view of a sectioned interventricular septum (ivs) from a chicken at 7 days after hatching is shown. The regions marked with arrows labeled 5b and 5c are shown in panels b and c, respectively. a, Large artery. Panel b: Punctate connexin42 immunolabeling is observed within the circular nonbranching bundle (nb) in the upper region of the fascicle at 7 days. Panel c: A region located lower on the lbb demonstrates higher levels of connexin42 immunolabeling (on a per cell basis) than that shown in panel b. Panel d: A confocal survey view of the ivs from a chick on the 20th fetal day. The septum has been sectioned in a plane comparable to that shown in panel a. The regions marked with arrows labeled 5e and 5f are shown in panels e and f, respectively. v, Valve. Panel e: The bundle branches and working myocardium are immunonegative for connexin42 1 day before hatching. The large (≈5 μm in diameter) bright spots to the left are autofluorescing red blood cells. Panel f: Subendocardial Purkinje fibers are immunolabeled after treatment with connexin42 antisera. These cells are located adjacent to the lower regions of the bundle branch where immunopositive signal will spread up the bundle branch during the posthatching period. p, Periarterial cells. Panels a and d and panels b, c, e, and f are at the same magnifications. Scale bars, 500 μm for panel d and 25 μm for panel f.
myocytes of the conduction system is consistent with the distribution of gap junctions characterized by ultrastructural and immunohistochemical methods in vertebrate myocardial tissues.\(^{9}\) In conclusion, there is reasonable evidence to suggest that the antiserum raised against the Cx42-based immunogen (residues 260–279 on the primary Cx42 protein sequence) is specifically immunolabeling gap-junctional structures within a subset of specialized tissues in the avian heart.

A mammalian analogue of chick Cx42 has recently been described in studies of mammalian genomic DNA.\(^{12,31}\) This gene product has been called Cx40 and demonstrates 70% amino acid homology with its chick counterpart. Connexin-specific antiserum to this protein was shown to immunolocalize in isolated canine ventricular cardiomyocytes, although the anatomic location(s) from which these cells were derived is unclear.\(^{12}\) With our present results showing that avian Cx42 immunolocalizes primarily to ventricular conduction tissues, the possibility that mammalian Cx40 may show an analogous distribution in mammalian conduction tissues would seem worthy of further investigation.

The specialized myocytes of the avian sinus node, atrioventricular node, and atrioventricular ring tissue do not show detectable levels of Cx42 and hence demonstrate a cell-to-cell communication character distinct from the conducting cells of the ventricular myocardium. Low levels of immunolabeling for Cx43 are detectable between myocytes within the mammalian atrioventricular node.\(^{11,15}\) Electron microscopy of the mammalian node reveals small and rare gap junctions, a finding consistent with the spatial distribution of Cx43 described within this tissue.\(^{16,32–34}\) The apparent lack of Cx43 immunoreactivity in avian myocardial tissues at present complicates direct comparison with the mammalian heart. Freeze-fracture studies of avian myocardium indicate that its gap junctions are tiny and infrequent,\(^{27,35}\) This, coupled with the diffuse and poorly defined nature of avian nodal tissues,\(^{36}\) probably restricts our ability to detect Cx43, even when using techniques as sensitive as laser-scanning confocal microscopy. Nonetheless, it is clear from this and from previous work\(^{10,11,13,15}\) that well-defined heterogeneities exist in the pattern of distribution and type of intercellular communicating channels between working and specialized myocardium and within the tissues of the impulse generation and conduction system of the heart. Moreover, these results are in accord with those who have proposed that the phenotypes of the proximal (e.g., atrioventricular node and proximal nonbranching bundle) and peripheral (distal bundle, bundle branches, and Purkinje fibers) conduction tissues are distinct and may be composed of cells with different embryological origins.\(^{22,37–39}\)

A lack of immunologically identifiable Cx43 in the working myocardium of avian hearts has been reported previously by El Aoumari and coworkers,\(^{40}\) who suggested that the observed immunonegativity might be accounted for by slight differences in Cx43 isoform between birds and mammals. However, sequencing of Cx43 cloned from avian hearts has since shown it to be 97% homologous in amino acid sequence to its mam-

**Table 1. Summary of the Presence or Absence of Connexin42 Immunolabeling in Ventricular Conduction Tissues With Development in Embryonic and Postnatal Chickens**

<table>
<thead>
<tr>
<th>Age</th>
<th>Periarterial Purkinje fibers</th>
<th>Subendocardial Purkinje fibers</th>
<th>Bundle branches</th>
<th>Nonbranching bundle</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;8E</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>9E–11E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12E–20E</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neonate–adult</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

E, embryonic days; –, absence of connexin42 immunolabeling; +, presence of connexin42 immunolabeling.

**Figure 6.** Connexin42 immunolabeling in the avian heart in periarterial (p) cells and vascular endothelium (e) at 10 embryonic days. Scale bar, 10 μm.
malian counterpart. Furthermore, the HJ domain used here as a peptide immunogen is identical in rat and chick, and the 26-residue Cx43c domain differs by only one amino acid. Therefore, if Cx43 were present in significant quantity in the working myocardium of the chick, it should have been detectable in the present study. A possible explanation for the Cx43 reported from Western and Northern blotting studies of avian heart might be the presence of Cx43-containing gap junctions between vascular smooth muscle cells rather than myocytes. Our results from avian arterial smooth muscle are also in accord with evidence of expression of Cx43 by mammalian vascular smooth muscle cells.

Cx42 signal reveals a distinctive temporal and spatial distribution during development of the myocardium. Although we first observe Cx42 in periarterial Purkinje fibers, which differentiate at around days 10–12 of the embryonic period, Cx42 cannot be detected within the nonbranching bundle and its branches before hatching. The embryonic bundle tissues are distinctive anatomic structures although, as pointed out by Vassal-Adams, they have an amorphous nonfascicular appearance compared with their analogues in the mature heart. To date, there are no data indicating whether fascicular myocytes are electrically coupled by gap junctions during embryogenesis. If Cx42-containing channels are the first to electrically couple these cells, then it would unexpectedly indicate that this part of the central conduction axis may not function as it does in

![Image](http://circres.ahajournals.org/)

**FIGURE 7.** Three-dimensional graph showing differences in immunolabeled (anti-cx42) gap junction area per conduction myocyte nucleus along the left bundle branch during development. In the week after hatching (i.e., at 1 and 7 days), levels of connexin42-positive gap junctions were significantly (p<0.01) higher in the lower regions of the left bundle branch than in upper regions of the bundle branch, indicating a centrifugal spread of connexin42-rich gap junctions up the bundle branch over this period. At 14 days and 42 days after hatching, there was no significant variation (p>0.05) in connexin42 immunolabeling levels along the three regions of the bundle branch.

**FIGURE 8.** Bar graph showing the overall immunolabeled (anti-cx42) areas of gap junction per nucleus along the left bundle branch during development. The capped lines represent bar standard errors. Embryological bundle branch tissues were immunonegative for connexin42 up to and including the 20th embryological day (20E). During postnatal life, immunolabeling levels of connexin42 rapidly increase, peaking at 14 days after hatching and then showing a small nonsignificant decrease (p>0.05) in 42-day-old animals.

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**Table 2.** Summary of Analysis of Variance Statistics for the Experiment Examining the Variations of Total Gap-Junctional Area per Nucleus and Mean Gap-Junctional Size With Chick Developmental Stage and Position Along the Left Bundle Branch

<table>
<thead>
<tr>
<th>Factor</th>
<th>Total area per nucleus (μm²/nuclei)</th>
<th>Mean size (μm²)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MSE</td>
<td>F</td>
</tr>
<tr>
<td>Development stage</td>
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</tr>
<tr>
<td>Bundle location</td>
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<tr>
<td>Stage×location</td>
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<td>3.5</td>
</tr>
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</table>

MSE, mean square error of the analysis of variance (ANOVA) factor; F, ANOVA variance ratio. The ANOVAs for both variates were performed using a two-way model on log E–transformed data.

*Significant at p<0.01.
†Significant at p<0.1.
the adult until incubation in ovo is complete. Electrophysiological studies of isolated cell pairs from developing avian hearts have noted a change in the regulation of gap junctions between 4 and 18 embryonic days. Such an ontogenic sequence agrees loosely with the developmental distribution of the Cx42 phenotype that we observe in ventricular conduction tissues, if it is assumed that electrical detection of gap junctions precedes morphological identification. However, until the anatomic origin of the cell pairs used can be identified, the relation between the present results and the electrophysiological data remains speculative.

The unexpected and perhaps most intriguing result of this study is that distally located Purkinje fibers demonstrate immunologically detectable Cx42 much earlier than do proximal branch fascicles. Immunoreactivity is detected in the bundle branches only later in the early postnatal period, when Cx42-rich gap junctions are observed spreading up the bundle branches toward the nonbranching bundle. This developmental gradient opposes the direction that action potential energetically propagates along the conduction axis of the mature heart and suggests possible functional differences between the specialized myocardial tissues in the avian heart before and after hatching.

Immunolocalization of conduction myocardium by Cx42 antisera is not observed before embryonic days 9–10. However, mRNAs encoding Cx42 appear at detectable levels in Northern blots17 at day 6. These findings are not inconsistent, bearing in mind the high sensitivity of Northern blotting17 compared with immunocytochemistry, and expression of mRNA would precede that of the corresponding protein. Furthermore, the newly translated Cx42 may not immediately be assembled into aggregates large enough to be detected by confocal microscopy, and/or significant expression of connexin42 may occur in tissues other than specialized myocardium (e.g., vascular precursors; Figures 3c and 6). In our ongoing work, we are addressing the question of how differential patterns of connexin expression arise in the heterogeneous tissues of the developing heart, as it is likely that the insights gained will elucidate not only the function of the ventricular conduction system but also the developmental origins and interactions of its component tissues as well.

Acknowledgments

We are indebted to Dr. Eric C. Beyer, Washington University School of Medicine, for his provision of antisera against connexin42 and connexin43 and details concerning their characterization. We are grateful to Drs. Tom Borg and Lou Terracio, University of South Carolina, for allowing us to use their laboratory facilities. The expert assistance of Stephen Rothery (photography), Pat Germroth (histology), and Tim Robson (histology) is acknowledged with gratitude.

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Circ Res. 1993;72:278-289
doi: 10.1161/01.RES.72.2.278

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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