Transitions in Cardiac Isomyosin Expression During Differentiation of the Embryonic Chick Heart

Lauren J. Sweeney, Radovan Zak, and Francis J. Manasek

The expression of different isofoms of the contractile protein myosin plays a major role in determining contractile characteristics in both cardiac and skeletal muscle in the adult. There is little evidence pertaining to putative changes in myosin phenotype during cardiac embryogenesis or if such changes could play a role in modulating the contractile characteristics of the developing heart. We examined isomyosin expression during cardiogenesis in the chick by indirect immunofluorescence microscopy with monoclonal antibodies to adult ventricular and atrial myosin heavy chains. Antibody specificity was characterized in the adult on the basis of immunofluorescence localization, ELISA, and protein blot immunoassay. Results show that the early embryonic chick heart has a different myosin phenotype than the later embryonic or adult heart. Both the embryonic ventricular and atrial myocardia initially expressed a myosin heavy chain that was recognized by antibody specific (in the adult) for ventricular myosin heavy chain. The ventricles remained reactive throughout life with the ventricular antibody, but reactivity of the atrial myocardium was confined to the initial 6 days of embryonic development. On the other hand, reactivity of the embryonic heart with multiple antibodies specific (in the adult) for atrial myosin was confined to the atrial myocardium throughout development. Thus, the distribution of myosin isoforms became similar to that of the adult myocardium by the time the embryonic heart achieved a 4-chambered configuration at 6 days in ovo. (Circulation Research 1987;61:287-295)
myosin HC are expressed early in cardiac development, and it suggests that their distribution is limited to their respective cardiac chambers. On the other hand, there is evidence on both the protein and DNA levels suggesting that transitions in myosin gene expression do occur early in avian cardiac development. Masaki and Yoshizaki\(^{17}\) found immunologic distinctions between the myosin composition of the early embryonic myocardium and that of the newly hatched chicken. More recently, Kulikowski\(^{18,19}\) has reported that there are structural differences between the types of myosin HC and myosin mRNAs present in early embryonic chick hearts (2.5-day) and those in later embryonic or adult ventricular and atrial myocardium. In addition, Zadeh et al\(^{20}\) have reported that both embryonic atrial and ventricular myocytes cease expressing their tissue-specific myosin HCs in vitro and begin to express an immunologically distinct myosin phenotype.

In this study, an immunofluorescent approach was used to investigate these questions by tissue- and stage-specific localization of myosin isoforms from the onset of myofibrillogenesis. By utilizing monoclonal antibodies that react specifically with adult atrial and ventricular myosin HC isoforms as probes, both chambers in the embryonic chick heart were found to initially express a myosin HC with ventricular antigenic properties. No transition in myosin antigenicity took place in the ventricular myocardium during development, while expression of an isomyosin with ventricular antigenic properties was repressed by 6 days of embryonic development in the atrial myocardium. In contrast, atrial antibodies reacted only with future atrial myocardial cells, beginning at 1.5 days incubation. These results provide strong evidence that there are transitions in the myofibrillar myosin gene program of at least the atrial myocardium during early cardiogenesis in the chick. The factors that influence expression of this program and its modulation have yet to be determined.

**Materials and Methods**

**Myosin Extraction and Proteolytic Digestion**

Myosin was extracted from adult (2 months or older) White Leghorn chicken atrial and ventricular muscle according to published procedures.\(^{21,22}\) Homogenized muscles were suspended in low salt wash buffer (5 mM NaPO\(_4\) buffer, pH 7.0; 40 mM NaCl, 1 mM MgCl\(_2\), 0.1 mM DTT, 0.1 mM EGTA), and myosin extracted by bringing the suspension to 0.6 M NaCl, 20 mM MgSO\(_4\), 10 mM ATP. Myosin was precipitated by the addition of ammonium sulfate and the 39–45% saturated fraction containing the myosin saved.\(^{22}\) Partial proteolytic cleavage of myosin into peptide fragments was achieved according to the method of Cleveland et al.\(^{23}\) Myosin solutions were diluted to a final concentration of 0.6 mg/ml in digestion buffer (0.125 M Tris-HCl, pH 6.8, 0.5% SDS, 10% glycerol) and heated for 3 minutes at 100° C. Proteolysis was performed by incubating chymotrypsin and myosin at a ratio of 1:8 for 30 minutes at 37° C, which was previously determined to be the optimum time course.\(^{22}\) The reaction was terminated by heating for 3 minutes at 100° C in the presence of 2% SDS and 6% mercaptoethanol.

**Gel Electrophoresis**

Myosin peptide fragments were separated on the basis of molecular weight by one-dimensional gel electrophoresis under denaturing conditions (in the presence of SDS) according to published procedures.\(^{22}\) Ten to twenty micrograms of myosin digest per lane were loaded on 15% polyacrylamide slab gels (15% acrylamide, 0.4% bisacrylamide) using the Laemmli discontinuous buffer system.\(^{24}\) Electrophoresis was carried out at 4 watts constant power for 17 hours.

**Protein Immunoblot Analysis**

Reactivity of myosin peptide fragments with antibody was determined after electrophoretic transfer of protein from gels to nitrocellulose strips, utilizing the method of Towbin et al.\(^{25}\) Each nitrocellulose strip was reacted with antibody for 1 hour, washed, and incubated for 1 hour with the appropriate peroxidase-conjugated IgG secondary antibody (Miles Laboratories, Naperville, Ill.) in Tris-buffered saline (20 mM Tris, 500 mM NaCl, 0.05% Tween, pH 7.5) and blocking solution (1% bovine serum albumin, BSA). Reactivity was determined by incubating the strips with H\(_2\)O\(_2\) and the colorimetric substrate 4-chloro-1-napthol in methanol (Polysciences Inc., Warrington, Penn.). To demonstrate all peptide fragments, separate gel strips were stained with silver. Gels were first fixed for 30 minutes in 50% methanol and 10% acetic acid (3 changes, 10 minutes each), washed overnight in water, incubated in 65 μM DTT (30 minutes), stained with silver nitrate for 30 minutes (100 mg/ml), and de-epaned in 3% sodium carbonate.

**ELISA Analysis**

Affinities of antibodies for adult chicken atrial and ventricular myosins were analyzed by solid-phase enzyme-linked imnunoassay. Alternate rows of 96-well microtiter plates were coated with atrial or ventricular myosin (0.5 μg per well) with 1% BSA in the control wells. BSA was also added to the myosin wells to prevent nonspecific binding. Serial dilutions (1:1) of each antibody were incubated in separate rows for 1 hour at 37° C. Plates were washed with phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2), and bound antibody was detected by the colorimetric β-galactosidase reaction (BRL murine Hybridoma Screening Kit, Bethesda Research Labs, Inc., Gaithersburg, Md.), which specifically detects IgG antibodies. The amount of bound antibody was measured as absorption at 450 nm in a Dynatech microtiter plate spectrophotometer (Dynatech Laboratories, Inc., Chantilly, Va.). Results are reported as absorption above background levels on rows coated only with BSA. Data points on dilution curves are the averages of duplicate tests.
Monoclonal Antibody Production and Characterization

Our library of monoclonal antibodies was screened, and new antibodies generated, to acquire probes that would detect specific cardiac myosin HCs in the adult chicken. CCM 52 was selected for its strong reactivity with myosins expressed in all cardiac myocytes, CCM 31A for its specificity for adult ventricular myosin, and CA 83 for its specificity for adult atrial myosin. CCM 31A and CCM 52 have been used by us in a previous study. Both antibodies were generated against chick cardiac myosin (CCM) from 12–18-day embryonic hearts. CCM 31A reacts with an epitope in the HMM region of the myosin HC of the chicken. CCM 52 reacts with an epitope in the LMM region of myosin HCs expressed in all chicken cardiac striated muscle cell types and in HC\_\text{P} of the myosin expressed in hearts of all mammals examined.

Six antiatrial myosin antibodies were generated by immunizing Lewis rats with 150-μg injections of adult chicken atrial (CA) myosin. Following established protocols, rat splenic lymphocytes were isolated and fused with the P3-X63-Ag8 mouse myeloma line using polyethylene glycol and the fused cells cultured in HAT medium for selection of hybridomas. Media supernatant from hybridomas was screened for atrial myosin specificity by ELISA. Specificity for the HC subunit was determined by reactivity with denatured myosin electrophoretically transferred to nitrocellulose strips from 5% Weber-Osborn SDS polyacrylamide slab gels. Results reported here with one cloned line, CA 83, are identical to results obtained with the other five lines.

Immunofluorescence Analysis

Previously established protocols for tissue preparation and immunofluorescence were followed with some modifications. The hearts from White Leghorn adult chickens and older chick embryos (Hamburger-Hamilton\textsuperscript{29} Stage 30 through hatching) were frozen at -70°C in OCT (Miles Laboratories). Entire embryos from H-H Stages 9–29 were frozen in OCT after brief fixation (10 minutes) in 4% buffered formaldehyde and sucrose infiltration (final concentration 20%). These steps enhanced structural integrity without altering antigenicity characteristics. Tissue was sectioned at 10 μm in a -20°C cryostat. Slides with adjacent serial sections were labelled with one of the battery of primary antibodies (antimyosin) and appropriate secondary antibodies (rhodamine-conjugated IgGs, Miles Laboratories) as before, but incubations were for 60 minutes each at room temperature. Sections were photographed on Kodak Plus-X film immediately after slides were coverslipped.

Results

Adult Cardiac Myosin Phenotype

The differences in heavy chain structure of isomyosins from adult chicken atrial and ventricular myocardium are reflected in the distinctive differences in the peptide fragments produced by chymotryptic digestion of these myosins (silver-stain, Figure 1A) and in the different antibody reactivities of these fragments in protein immunoblot analysis (electrophoretic transfer of fragments to nitrocellulose, Figure 1B–D). CCM 52 showed strong reactivity with myosin fragments from both adult atria and ventricles (Figure 1B), CCM 31A showed strong reactivity with the ventricular myosin digest only (Figure 1C), and CA 83 showed strong reactivity with the atrial myosin digest only (Figure 1D). ELISA analysis demonstrated the same antibody specificities (Figure 2). Immunofluorescence results demonstrated cellular localization consistent with the biochemical characterizations of these antibodies. CCM 52 reacted with all striated muscle cells in the atria and ventricles of the adult chicken heart (Figure 3A), CCM 31A reactivity was localized to ventricular myocardium (Figure 3B), and CA 83 reactivity was localized to atrial myocardium (Figure 3C). In addition, there was strong reactivity of CCM 31A and CCM 52 with myosin expressed in a small population of cells within the atrial (and ventricular) subendocardium (Figure 4A–C). These cells were confined to the location of the conduction system and its subendocardial termination as Purkinje fibers.\textsuperscript{30,31} Consistent with this, there were
FIGURE 2. ELISA analysis of affinity of antibodies CCM 52, CCM 31A, and CA 83 for adult chicken atrial (---) and ventricular (— — —) myosin coated on microtiter plates. CCM 52 (○) showed strong affinity for both atrial and ventricular myosins. CCM 31A (△) showed strong affinity only for ventricular myosin, and CA 83 (X) showed an affinity only for atrial myosin.

a few peptide fragments in the atrial digest that reacted with CCM 31A (Figure 1C). The antiatrial antibodies did not react with these cells in either the atrium or ventricle (CA 83, Figure 4D).

Thus, antibody CCM 52 is a probe that recognizes an epitope present in myosin HCs expressed in all striated muscle cell types of the adult chicken heart, CCM 31A is a probe specific for an epitope present in the ventricular myosin HC, and CA 83 is a probe specific for an epitope in the atrial myosin HC. The results obtained with CA 83 are duplicated on all tests by the other 5 hybridoma lines obtained from the same fusion (results not shown in the adult).

Embryonic Cardiac Myosin Phenotype

All myocytes that are incorporated into the heart tube until early Stage 12 (1.5 days of incubation) are destined to be part of the mature ventricular chambers (Figure 5). Between Stages 12 and 14 (second day), the formation of two chambers begins: the prospective atrium is incorporated into the heart tube, and atrial myocardial differentiation is initiated. Consistent with this developmental sequence, we found that at Stage 10 (1.25 days) all myocytes were reactive with the anticardiac antibody CCM 52 and with the ventricular-specific antibody CCM 31A, but there was no reactivity with any of the antiatrial antibodies (Figure 5). The first reactivity with antiatrial antibody (CA 83) occurred at Stage 12 in a few myocytes in the caudal end of the heart tube (asterisk, Figure 5). These cells also were labelled with CCM 52 and CCM 31A. By Stage 14, the entire, rapidly differentiating, atrial primordium reacted with all antibodies (Figure 6A–C). The intensity of this reactivity increased as atrial myofibrillogenesis became more advanced (compare Stage 14, Figure 6A–C with Stage 18, Figure 6D–F).

Through the fourth day of incubation (Stage 24), the entire atrial and ventricular portions of the heart reacted

FIGURE 3. Immunofluorescence microscopy of adjacent frozen serial sections of adult chicken heart labelled with anticardiac myosin antibodies. (A) CCM 52 reacted with all atrial and ventricular myocardium. (B) CCM 31A reacted only with ventricular myocardium. (C) CA 83 reacted only with atrial myocardium. A, atrial myocardium; V, ventricular myocardium; CT, connective tissue of the atrioventricular annulus. Bar is 100 μm.
strongly with the ventricular-myosin specific antibody CCM 31A (Stage 18, Figure 6D–E; Stage 24, Figure 7B) in the same pattern as that for the anti-cardiac antibody (CCM 52, Figures 6A–B and 7A). Reactivity with all 6 antiatrial antibodies was confined to the atrial myocardium (Figure 7C–H) and demonstrated a sharp demarcation in myosin phenotype between adjacent myocytes at the atrial-ventricular boundary (asterisks, Figure 7) at stages prior to their separation by the connective tissue of the atrioventricular annulus.

By 6 days (Stage 28), the patterns of antibody reactivities had changed so that atrial and ventricular myocardial myosin expression was similar to that of the adult heart (compare Figure 6G–I with Figure 3B–D), with the exception that a clear pattern of conduction tissue cells could not yet be detected with CCM 31A in the embryonic atrial (or ventricular) subendocardium. The loss of atrial reactivity to CCM 31A occurred over the 48-hour period from Stages 24–28, beginning in the developing atrial appendages. This is consistent with a half-life of myosin of several days. Thus, the working myocardium of the embryonic chick atrium expresses a myosin reactive with CCM 31A from the onset of its differentiation at Stage 12, but it ceases expression of this myosin by the sixth day of embryonic development.
FIGURE 6. Immunofluorescence microscopy of frozen serial sections at 3 stages of embryonic chick heart development labelled with the same antibodies as in Figure 3. A-C: 2-day heart (Stage 14) sectioned through primitive atrium (Atr.), atrioventricular canal (AVC), ventricle (Vent.), and sinus venosus (SV). D-F: 3-day heart (Stage 18) sectioned through the 2 cardiac chambers in the atrioventricular canal region. G-I: 6-day heart (Stage 28) sectioned through left L., chambers of 4-chambered structure. Anticardiac antibody CCM 52 always reacted with the myocardium of the future ventricular and atrial regions (A,D,G). Antibody specific to adult ventricle (CCM 31A) reacted with the atrial and ventricular myocardium at 2 and 3 days (B,E) but only with the ventricle by 6 days (H). Antiatrial antibody CA 83 reacted only with the future atrial region of the heart at all stages (C,F,I). The 6-day embryonic chick heart showed patterns of atrial and ventricular reactivity comparable to adult patterns (compare Figure 3). The same antibody dilutions were used at all embryonic and adult stages.

Discussion

Our results provide immunohistochemical evidence for developmental regulation of myosin expression during cardiac embryogenesis in the chick. They show that a myosin HC isoform is expressed in the myocardium of at least one cardiac chamber at the onset of cardiogenesis that is not part of the adult myosin gene program for that chamber. They also show that the transition to the adult myosin phenotype occurs early in cardiogenesis (by the sixth day). This is in agreement with Kulikowski’s evidence that there are differences in the peptide maps and mRNAs for the total myosin expressed in the early (2.5-day) embryonic heart on the one hand and the 6-day embryonic and...
adult atria and ventricles on the other. We were able, by using an immunologic approach, to demonstrate that such differences exist because of the expression of a distinct isomyosin in the developing atrial myocardium. This approach also provides the first demonstration that the working myocardium of the bird simultaneously expresses more than one myosin HC within a single myocyte, as is the case in the mammal. In addition to expression of this distinct isomyosin, we have found, in agreement with the original observations of Gonzalez-Sanchez and Bader,\textsuperscript{15} that the embryonic atrium expresses a myosin with adult atrial antigenic characteristics. Since we found the same specific reactivity with 6 different antibody lines to adult atrial myosin in the developing atrial myocardium, the embryonic atrium probably expresses the same myosin HC gene product as the adult atrium and not simply a myosin with antigenic characteristics of adult myosin HC. Furthermore, since atrial antibody reactivity was observed from Stage 12, as contrasted with earliest detection at Stage 15 in the previous study, it is now clear that future atrial myocytes express the atrial-specific isoforms as they become incorporated into the heart tube and begin to differentiate.

The interesting question raised by these results concerns the identity of the myosin HC that reacts with antibody CCM 31A in the embryonic heart. Cross-reactivity of embryonic myocytes with an antibody specific for ventricular myosin in the adult represents either the detection of ventricular myosin expression itself or the detection of one (or more) unique myosin HC gene products, which share antigenic determinants with ventricular myosin. Since we did not have multiple ventricular-specific antibodies at our disposal, it is not possible to discriminate between these two possibilities on the basis of CCM 31A reactivity alone.

In addition to expression of this distinct isomyosin, not only the atrial myocytes but also the ventricular myocardium expresses a myosin HC distinct from either of the known cardiac isoforms. These results all suggest that the myosin HC is a uniquely primordial-stage myosin HC isoform. This is more consistent with the findings of Kulikowski\textsuperscript{18,19} and Masaki and Yoshizaki,\textsuperscript{20} who observed differences in vivo between early embryonic cardiac myosin (presumably largely from ventricular myocardium) and later embryonic or adult cardiac myosin isoforms (atrial and ventricular). Such a conclusion is also consistent with the recent observations of Zadeh et al\textsuperscript{21} that both atrial and ventricular myocytes in culture express a myosin HC distinct from either of the known cardiac isoforms. These results all suggest that not only the atrial myocytes but also the ventricular myocytes could initially express a distinct, primordial-stage myosin that shares an epitope with ventricular myosin, a fact we would not have detected because of cross-reactivity of our antibody CCM 31A with epitopes present in both myosin types. The fact that Obintza\textsuperscript{22} detected only one myosin isoform in the embryonic ventricle on the basis of native gel electrophoresis could reflect the fact that the different isomyosins are not sufficiently different in charge or MW to result in resolvable differences in migration rates under the conditions employed. As an example, the conduction tissue myosin of the heart of the adult chicken has not yet been resolved as a separate band on either native

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Immunofluorescence microscopy of the AV canal region of 4-day embryonic chick heart labelled with anticardiac antibody CCM 32 (A), antiventricular antibody CCM 31A (B), and all six antiatrial antibodies (C-H). Antibodies CA 23 and CA 83 are cloned lines, while the rest are hybridoma media supernatants. All atrial antibodies showed identical localization to the atrial myocardium (A). Their reactivity stopped at the atrio-ventricular junction (asterisk), which still demonstrated myocardial continuity between chambers. White lines marked unlabelled ventricular myocardium (V) in sections stained with atrial antibodies.}
\end{figure}
or denaturing gel electrophoresis, even though a unique HC is present in quantities sufficient to be extracted by affinity chromatography. The current limitations of gel electrophoresis in resolving differences in all HC isoforms means that this approach may sometimes fail to confirm the distinctions observed by immunologic techniques or by peptide mapping of the HC subunit. If the myosin detected by CCM 31A is indeed a unique HC isoform, this would be the first evidence in any animal of expression of a myosin HC specific to embryonic stages of development in cardiac muscle.

Developmental Implications of Transitions in Myosin Gene Expression

The fact that myosin transitions may occur in the embryonic heart of the chick is of particular interest since the myosin program of the myocardium of the neonatal and adult chicken has been considered immutable. This is in contrast to the program in mammalian myocardium, which responds to a number of functional, hormonal, and nutritional challenges by transitions in myosin expression and which undergoes dramatic transitions in myosin composition at birth. Transitions are also an important factor in avian and mammalian skeletal muscle in which developmental and functional regulation of myosin expression occurs throughout life, including the expression of isoforms specific to embryonic stages. Just as one cannot assume that the myosin phenotype of late fetal skeletal muscle represents the phenotype of early embryonic muscle, these results also demonstrate that one cannot assume that the phenotype of late fetal (or neonatal) cardiac muscle represents that of the cardiac chambers during their initial development.

Since the heart functions from the onset of its morphologic development, it should not be surprising if developmentally regulated myosin transitions are programmed during early cardiogenesis. Transition to the adult myosin phenotype coincides with a period of dramatic maturation of the embryonic chick heart. During the first 6 days, the myocardium undergoes extensive accumulation and alignment of myofibrillar material, coincident with a dramatic increase in cardiac work capacity and electrogenic capacities for regulation of membrane potential. Further, as the four cardiac chambers, valves, and septa are formed, differences are established between atrial and ventricular contractile characteristics due to different functional status, myocardial morphology, and electrophysiologic properties. The challenge is to determine which of these factors, if any, are either required or permissive cues to the transition in myosin expression. One development that bears further examination in light of our findings is the relation between autonomic innervation and myosin expression. While functional autonomic regulation of contractility is not established until the second half of embryonic development, it is entirely possible that important developmental cues are provided by the initial cardiac contact with ingrowing autonomic nerves during the period of observed atrial isomyosin transitions (4-6 days). This contact may be instrumental in modulating myosin gene expression so that mature contractile responses appropriate to each chamber are developed. Hemodynamic challenges also may play a role in regulating isomyosin expression since there is a dramatic rise in ventricular blood pressure and work load over those of the atria and extensive growth and trabeculation of the ventricular walls during this period. It is tempting to speculate that increasing pressures are another possible condition needed to continue expression of the CCM-31A reactive myosin and that this stimulus is provided to the ventricular myocytes but not to the atrial myocytes during this period. If the ventricles, as well as the atria, express a distinct (CCM-31A reactive) myosin during early stages of development, as has been suggested, increasing pressures could be just one of several conditions that must be maintained for continued expression.

Acknowledgments

We would like to thank Grace Wu and Gwen Prior for their invaluable technical assistance.

References

14. Dalla Libera L, Sartore S, Schiaffino S: Comparative analysis
of chicken atrial and ventricular myosins. Biochim Biophys Acta 1979;581:283–294
15. Gonzalez-Sanchez A, Bader D: Immunochemical analysis of myosin heavy chains in the developing chicken heart. Dev Biol 1984;103:151–158
32. de la Cruz MV, Munoz-Castellanos L: Development of the Chick Heart. Baltimore, Md, Johns Hopkins University Press, 1972, pp 7–22
38. Paterson BM, Eldridge JD: α-Cardiac actin is the major sarcomeric isoform expressed in embryonic avian skeletal muscle. Science 1984;224:1436–1438
42. Forsgren S, Streher E, Thornell LE: Differentiation of Purkinje fibers and ordinary ventricular and atrial myocytes in the bovine heart: an immuno and enzyme histochemical study. Histochem J 1982;14:929–942

KEY WORDS • embryonic heart • immunofluorescence • monoclonal antibody • myocardium • myosin