Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart

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SUMMARY

A unique myosin heavy chain cDNA (AMHC1), which is expressed exclusively in the atria of the developing chicken heart, was isolated and used to study the generation of diversified cardiac myocyte cell lineages. The pattern of AMHC1 gene expression during heart formation was determined by whole-mount in situ hybridization. AMHC1 is first activated in the posterior segment of the heart when these myocytes initially differentiate (Hamburger and Hamilton stage 9+). The anterior segment of the heart at this stage does not express AMHC1 although the ventricular myosin heavy chain isoform is strongly expressed beginning at stage 8+. Throughout chicken development, AMHC1 continues to be expressed in the posterior heart tube as it develops into the diversified atria. The early activation of AMHC1 expression in the posterior cardiac myocytes suggests that the heart cells are diversified when they differentiate initially and that the anterior heart progenitors differ from the posterior heart progenitors in their myosin isoform gene expression. The expression domain of AMHC1 can be expanded anteriorly within the heart tube by treating embryos with retinoic acid as the heart primordia fuse. Embryos treated with retinoic acid prior to the initiation of fusion of the heart primordia express AMHC1 throughout the entire heart-forming region and fusion of the heart primordia is inhibited. These data indicate that retinoic acid treatment produces an expansion of the posterior (atrial) domain of the heart and suggests that diversified fates of cardiomyogenic progenitors can be altered.

Key words: heart formation, myosin heavy chain, chick embryo, retinoic acid, anteroposterior polarity

INTRODUCTION

The heart is one of the first organs to function in the developing embryos of higher vertebrates. In the chicken, a beating heart is formed within 40 hours of development (Hamburger and Hamilton, 1951; stage 10) from the fusion of bilateral heart primordia arising in the splanchnic lateral plate mesoderm. The resultant primitive heart tube, which lies along the anterior/posterior axis of the embryo, expresses cardiac contractile protein genes (reviewed in Litvin et al., 1992). Later in development, the heart tube is subdivided into atria and ventricles that are generated from the posterior and anterior segments of the heart tube, respectively. The diversified atrial and ventricular myocytes express distinct subsets of contractile protein genes and have different conductive and contractile properties (DeHaan, 1965). Major questions in the study of heart development concern the timing of atrial and ventricular diversification and the mechanisms by which the two lineages arise. One possibility is that cells are first committed to the heart lineage and then diversify into distinct atrial and ventricular lineages from a population of committed undifferentiated cardiac progenitors based on their position in the lateral plate mesoderm. Alternatively, the atrial and ventricular lineages could arise later from a common population of differentiated myocytes in the primitive heart tube. At present, studies are not available to support either model of diversification during the primary stages of cardiogenesis.

To examine the mechanisms of cardiac diversification, it is essential to have available lineage-specific markers. Myosin isoforms seem to be the most promising chamber-specific markers since, in the chicken, numerous isoforms are differentially expressed depending on the contractile properties of distinct muscle fiber types (Robbins et al., 1986; Moore et al., 1992). One myosin heavy chain gene (VMHC1) is expressed specifically in the ventricle after embryonic day 6 in the chick, but prior to that is expressed in the atria and in differentiating skeletal muscle (Bisaha and Bader, 1991). O’Brien et al. (1993) have shown that a cardiac myosin light chain 2 is expressed specifically in the ventricle after embryonic day 6 in the chick, but prior to that is expressed in the atria and in differentiating skeletal muscle (Bisaha and Bader, 1991). O’Brien et al. (1993) have shown that a cardiac myosin light chain 2 is expressed specifically in the ventricles of the primitive mouse heart. In the chick, antibodies directed against atrial myosin heavy chain isoforms have been used to distinguish between atrial and ventricular myocytes as early as stage 12, when the heart is already beating and chambers are starting to form (Sweeney et al., 1987; Gonzalez-Sanchez and Bader, 1984). Others have suggested that diversification of cardiac lineages occurs later, during valve formation, after the establishment of a uniform phenotype throughout the primitive heart tube (DeJong et al., 1990). No evidence is available that would indicate whether the atrial and ventricular lineages are distinct when cardiac myocytes first differentiate or if all myocytes are
phenotypically identical when the primitive heart tube forms. To distinguish between heart lineages during the earliest stages of chicken heart development and elucidate polarity in potential of progenitors, it is necessary that more markers be available to characterize cardiac diversification during all stages of cardiogenesis.

The diversification of atra and ventricles is a relatively simple system in which to examine the establishment of an anterior-posterior axis. Prior to chick stage 12, no differences have been detected along the anterior/posterior axis of the differentiated heart tube. Differentiation, which can be defined as the activation of cardiac-specific structural genes such as myosins and troponin I, proceeds in an anterior-to-posterior fashion within the cardiogenic mesoderm between stages 7 and 12 (Gonzalez-Sanchez and Bader, 1990; Bisaha and Bader, 1991; Han et al., 1992). Previous work has shown that the progenitors of the cardiogenic mesoderm remain as a true epithelium between stages 4 and 12 and do not undergo a mesenchymal transformation (Manasek, 1968; Stalsberg and DeHaan, 1969; Gonzalez-Sanchez and Bader, 1990; Peng et al., 1990). Thus, cardiac progenitors are fixed relative to each other and mixing of progenitors between the predifferentiated and postdifferentiated stages of development does not occur. The atrial and ventricular precursors arise within this cardiogenic mesoderm with ventricular cells anterior and atrial cells posterior. Previous studies of the limb (Tickle et al., 1985; Thaller and Eichele, 1987) and the embryo as a whole (Ruiz i Altaba and Jessell, 1991; Conlan and Rossant, 1992) have suggested that retinoic acid is an agent that influences anterior-posterior determination. The formation of distinct atria and ventricles during early heart development has been shown to be affected by retinoic acid treatment in chickens and zebrafish (Osmond et al., 1991; Stanier and Fishman, 1992). In the zebrafish, retinoic acid treatment at the blastula stage appears to produce a loss in anterior structures during heart formation with possible deletion of ventricular (anterior) cells. Osmond et al. (1991) observed changes in chick heart structures after retinoic acid treatment but did not distinguish possible variations in atrial and ventricular phenotypes along the anterior-posterior axis. Thus there may be plasticity in the diversified phenotypes of the cardiomyogenic cell lineage affected by retinoic acid treatment. This hypothesis is supported by transplantation studies in which presumptive atrial (posterior) cells take on ventricular (anterior) properties as measured by beat rate when placed in the prospective ventricular domain prior to stage 8 (Satin et al., 1988). These studies were hindered by the lack of molecular markers of specific myogenic heart lineages.

In this study, we report the isolation of a chicken atrial-specific myosin heavy chain cDNA (AMHC1). The amino acid sequence of the hinge and rod region of AMHC1 is more similar to rat cardiac myosin heavy chain isoforms than to chicken skeletal myosin heavy chains, but AMHC1 is similarly diverged from both α and β isoforms of rat cardiac myosin heavy chains (Moore et al., 1992; McNally et al., 1989). AMHC1 is expressed only in the posterior region of the forming heart tube as early as stage 9+, which is at least 24 hours earlier than any other reported heart lineage-specific contractile protein gene in the chicken. Its expression is confined to the posterior (atrial) domain of the heart throughout development. Comparison of the expression of AMHC1 with VMHC1, which is present in all differentiating cardiac myocytes, reveals that AMHC1 expression is localized to a subset of posterior cardiac myocytes at the time they initiate the expression of cardiac-specific structural genes. Anterior populations of cardiac myocytes never express AMHC1. These results demonstrate that separate populations of differentiating progenitors are diversified from the start of differentiation and suggest that a differential potential of progenitors exists along the anterior-posterior axis of the precardiac mesoderm. Still, the fate of these cells can be altered by retinoic acid. Embryos grown in vitro in the presence of retinoic acid have increased atrial domains and the expression of AMHC1 is expanded anteriorly. This expansion of the posterior compartment of the heart was observed when the overall heart morphology was structurally maintained and also when heart morphogenesis was disrupted. Taken together our results suggest that atrial and ventricular cells are diversified at least from the point of initial differentiation but that the diversified fate can be altered.

**MATERIALS AND METHODS**

**Animals and tissues**

Fertilized White Leghorn chicken eggs were obtained from Truslow farms (Chestertown, MD) or Spafas (Norwich, CT) and incubated under high humidity in a 37°C incubator. Embryos were staged according to Hamburger and Hamilton (1951). For RNA isolation, embryos were dissected and excised tissue immediately frozen in liquid nitrogen. Tissues were stored at −70°C prior to RNA preparation. For in situ analysis of RNA expression, stage 4-20 embryos were removed from the egg on paper rings, rinsed in sterile PBS [150 mM NaCl, 10 mM phosphate, (pH 7.4)], and fixed in 4% purified paraformaldehyde (‘Fixil’) (Electron Microscopy Sciences, Fort Washington, PA) in PBS at 4°C. Embryos on the rings were then transferred to 70% ethanol and stored at −20°C. Hearts of older embryos were isolated and treated similarly.

**RNA isolation**

For the atrial cDNA library, total RNA was prepared from frozen tissue by LiCl-urea-ethanol precipitation (Auffray and Rougeon, 1980). Atria isolated from embryonic day 13 (E13) chicken embryos were first homogenized in an Omnimixer (DuPont) in the presence of the LiCl-urea solution. Poly(A)+ RNA was enriched using oligo(dT)-cellulose (Pharmacia) chromatography as described by Maniatis et al. (1982).

Total RNA for northern analysis was isolated from tissues of indicated ages using guanidine thiocyanate/phenol/chloroform extraction and isopropanol precipitations as described by Chomczynski and Sacchi (1987). Frozen tissues from embryos older than E7 were pulverized in liquid nitrogen using a mortar and pestle prior to the addition of the guanidium isothiocyanate solution. RNA was quantitated by determination of the absorbance at 260 nm and stored at −70°C.

**cDNA library construction and screening**

E13 heart cDNA libraries were prepared as described in Bisaha and Bader (1991). Briefly, double-stranded cDNA was synthesized from 1 µg poly(A)+ RNA isolated from E13 chicken atria using the Boehringer Mannheim cDNA synthesis system. The cDNA was packaged in the λgt10 vector using the Amersham cDNA cloning system according to the method of Huynh et al. (1985). Approximately 4×10⁶ recombinants were present in this atrial cDNA library. The amplified E13 atrial cDNA library was screened at approxi-
mately 10,000 plaques per 150 mm diameter culture dish. Approximately 2.5×10^6 plaques were transferred to nitrocellulose filters (Shleicher and Schuell) for low stringency hybridization with a myosin heavy chain cDNA probe. A 2.2 kb neonatal pectoralis myosin heavy chain cDNA (HC-1) (Einhaber and Fischman, unpublished) was radiolabeled [32P] using the random priming method of Feinberg and Vogelstein (1983). Membranes were prehydrated in a solution containing 50% deionized formamide, 5× SSPE [1× SSPE is 0.18 M NaCl, 1 mM EDTA, 10 mM sodium phosphate (pH 7.5)], 1× Denhardt’s solution (Maniatis et al., 1982), 0.1% SDS, 100 μg/ml denatured salmon testis DNA, and 1% dextran sulfate for 1 hour at 42°C and then hybridized overnight after the addition of radiolabeled probe. The membranes were rinsed twice in 2× SSPE/0.1% SDS at room temperature for 10 minutes, transferred to 1× SSPE/0.1% SDS at 37°C for 30 minutes, and then washed twice in 1× SSPE/0.1% SDS at 42°C for 30 minutes. Positive plaques were purified by secondary screening using the same hybridization and washing conditions. Recombinant clones were identified following restriction enzyme digestion and sequence analysis. One of the six positive isolates was characterized as Atrial Myosin Heavy Chain 1 (AMHC1). This clone contains the 2.4 kb 3’ sequence of the AMHC1 mRNA. Subfragments of AMHC1 were isolated using existing PsiI restriction endonuclease sites, and two PsiI fragments were further restricted with HindIII or SacI restriction endonucleases to produce fragments less than 500 bp in length. All subfragments were inserted into pGEM3Z and the nucleotide sequence of both DNA strands were determined using the dyeoxy sequencing method (Chen and Seeberg, 1985) with the modified T7 DNA polymerase, Sequenase (U.S. Biochemicals). Additional oligonucleotides were synthesized in order to obtain the complete sequence of AMHC1.

**Northern analysis**

Equivalent amounts of RNA (15 μg) were loaded in a 1% agarose/formaldehyde denaturing gel (Lehrach et al., 1977). After electrophoresis, RNA was transferred to a Nytran filter (Shleicher and Scheull) by passive transfer blot in 10× SSC [1× SSC is 150 mM NaCl, 15 mM sodium citrate (pH 7.0)]. Hybridizations were performed in 50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt’s solution, and 100 μg/ml denatured salmon testis DNA at 42°C overnight. Membranes were hybridized to 32P-labelled random-primed cDNA probes (specific activity approx. 1×10^8 cts/minute/μg). Probes used included the 3’ PsiI/EcoRI fragment of AMHC1 and the quail glyceraldehyde-3-dehydrogenase (GAPD) plasmid (Pownall and Emerson, 1992). Hybridized blots were rinsed in 2× SSC, 0.2% SDS for 20 minutes at room temperature and washed in 0.2× SSC, 0.2% SDS for one hour at 65°C and exposed to Kodak XAR film.

**Plasmids and probes**

The Ventricular Myosin Heavy Chain (VMHC1) clones pVMHC1 and pVMHC1A were constructed as described in Bisaha and Bader (1992). The GAPD plasmid used for RNA blots was as described in Pownall and Emerson (1992). Plasmid pBSAMHC1 was constructed from the AMHC1 insert (2.4 kb) excised from the λGT10 phage and inserted into pBluescript KS+ (Strategene, LaJolla, CA). pGEMAMHC1 was constructed by inserting the EcoRI fragment into the EcoRI site of pGEM3Z (Promega, Madison, WI), pGEMAMHC1A was constructed by isolating the 3’ PsiI/EcoRI fragment (314 bp) from AMHC1 and inserting it into pGEM3Z.

**In situ analysis of RNA expression**

In situ hybridizations of AMHC1 and VMHC1 mRNAs were conducted essentially as described in Coutinho et al. (1992). Chicken embryos (stage 4-20) were removed from the egg on filter paper rings, rinsed in PBS and fixed in 4% paraformaldehyde in PBS at 4°C overnight. Embryos in the rings were then transferred to 70% ethanol and stored at −20°C. E4 hearts were removed and fixed as above for in situ hybridization. In vitro cultures of embryos

Embryos to be cultured by the New method (1955) were removed from the egg at stage 4-10 on paper rings, rinsed in sterile PBS and placed on 35 mm dishes coated in egg albumin. Medium containing 50% albumin/50%high glucose DMEM (GIBCO, Gaithersburg, MD), 10% fetal bovine serum (Sigma, St. Louis, MO), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and penicillin (100 U/ml)/streptomycin (100 μg/ml) was added. All trans-retinoic acid (Sigma) was added to culture medium at 10^-4 to 10^-7 M in 1% DMSO as described by Osmond et al. (1991). Control experiments were treated with regular culture medium containing 1% DMSO. Embryos were incubated at 37°C in 5% CO2 with high humidity for 24 hours and fixed as above for in situ hybridization.

**Quantification of myosin gene expression in situ**

AMHC1- and VMHC1-positive areas were calculated based on the mass of camera lucida cutouts of in situ hybridization from multiple experiments. Embryos were photographed from the ventral surface and cardiac cells were aligned with reference to somites, notochord and anterior intestinal portal. Embryos in these experiments did not develop beyond stage 11 so that the entire heart tube when present was visible in a ventral view. For each experiment, embryos were hybridized with either AMHC1 or VMHC1 RNA probes. Camera lucida drawings were made from slides projected of similarly magnified embryos. Heart sizes were normalized after alignment of the somites, notochord and anterior intestinal portal. The total area of the heart was calculated based on the area of VMHC1 expression, which was apparent in all differentiated cardiac myocytes. The proportion of AMHC1-positive myocytes was calculated by comparing the area of the heart that was AMHC1-positive with the total area of the heart determined by VMHC1 staining in parallel embryos. Statistical analysis of data obtained in this manner revealed little variability in calculated areas for each experimental group.

**RESULTS**

AMHC1 identifies atrial myocytes in the differentiated heart

In order to study the differentiation and diversification of cardiac lineages, an atrial-specific myosin heavy chain cDNA (AMHC1) was isolated. The identity of AMHC1 as a myosin heavy chain was confirmed after restriction enzyme and sequence analysis. An incomplete restriction map was determined for AMHC1, which revealed the presence of several...
**Fig. 1.** Sequence analysis of the AMHC1 cDNA. (A) Nucleotide and derived amino acid sequence of AMHC1, an atrial myosin heavy chain cDNA. The translational stop codon and polyadenylation addition site are indicated. The variable LMM/S2 hinge region is underlined (a.a. 78-107). (B) Comparison of the amino acid homology between AMHC1 and other myosin heavy chain hinge region sequences. Amino acid similarities with the rod hinge segments of a chicken fast skeletal embryonic (Cemb1) (Moore et al., 1992) and rat α- and β-myosin heavy chains (McNally et al., 1989) are indicated. Sequence identity is represented as (+) and divergence by the relevant amino acid. These sequences were used to determine the relatedness of myosin heavy chain isoforms.

PsI sites conserved in many myosin heavy chain genes (Saenz and Leinwand, 1986). The PsI sites were utilized to subclone subfragments AMHC1 for further sequence analysis (Fig. 1). A single open reading frame is present which encodes 765 amino acids and ends in a stop codon that leaves 90 bases of 3’ untranslated sequence. The sequence of the 3’ end of AMHC1 contains 26 adenine residues of the poly(A) tail and a canonical polyadenylation addition site. The untranslated region of AMHC1 is unique as is characteristic for distinct myosin heavy chain genes. The nucleotide sequence of AMHC1, specifically in the 3’ untranslated region, is not identical to any reported myosin heavy chain, and the amino acid sequence is divergent from other myosin sequences.

AMHC1 contains the coding sequence for the LMM and S2 segments of the myosin heavy chain rod. Comparison with other myosin heavy chain protein sequences reveals overall 73% amino acid identity with divergent amino acids clustered in the S2-LMM junction and in other characteristic regions of divergence. The deduced amino acid sequence of AMHC1 is 78% identical to the chicken embryonic fast skeletal isoform and is 89% identical to rat cardiac myosin heavy chain sequences (Moore et al., 1992; McNally et al., 1989). In the hinge region (amino acids 78 to 107), AMHC1 is 64% identical to rat α MHC and 68% identical to rat β MHC with an equal distribution of amino acids characteristic for each isoform. While AMHC1 is 82% identical to either of the cardiac isoforms, it shares only an 46% identity with the chicken skeletal MHC isoform (Fig. 1B). In addition, characteristic amino acids for fast skeletal isoforms are not absolutely conserved in amino acid positions 180, 184, 228 and 232 (Moore et al., 1992). Therefore AMHC1 does not belong to the chicken fast isoform family and cannot be classified as either α or β myosin heavy chain based on equal divergence from the rat cardiac isoforms. The intermediate homology observed between AMHC1 and rat α and β myosins suggests that avian cardiac myosin heavy chains may not be strictly homologous to those of mammals and that AMHC1 may represent a new type of cardiac myosin heavy chain.

AMHC1 expression was analyzed by northern blot of RNA isolated from differentiated hearts of chicken embryos from day 7 to hatching (Fig. 2). RNA also was isolated from brain and skeletal muscle tissue. RNA blots were hybridized with the 314 bp PsI/EcoRI fragment of AMHC1, which contains the unique 3’ untranslated sequence. Blots were rehybridized with the GAPD probe to ensure equal RNA loading in each lane (data not shown). These experiments demonstrate that AMHC1 is expressed only in the atria of embryos at each stage. AMHC1 expression is most easily detected in day 7 embryos and the level of detection decreases with age suggesting that AMHC1 expression diminishes during development. Using northern blot analysis, AMHC1 expression is never detected in the noncardiac tissues. This pattern of AMHC1 expression is in contrast to VMHC1, which is detectable in both chambers of the heart early in development and is expressed at significant levels in the somites and in pectoralis and limb muscles when they initially differentiate at days 7 and 11, respectively (Bisaha and Bader, 1991). Thus, AMHC1 represents a chamber-specific isoform of myosin heavy chain that is expressed only in the developing heart at all embryonic stages tested.

**AMHC1 is confined to the differentiating posterior progenitors during heart formation**

Since AMHC1 expression is atrial-specific after definitive chamber formation, this cDNA was used to determine when atriogenic diversification occurs. Whole-mount in situ hybridization was used to localize myosin heavy chain gene expression in developing embryos. Probes were complementary to the 3’ 2.4 kb of AMHC1 or VMHC1 mRNAs and labelled with digoxigenin UTP. At least three embryos of each stage were tested and observed, and representative embryos are shown in Fig. 3. Control sense orientation probes did not hybridize to embryos at any stage. As expected, VMHC1 was expressed in all cardiac myocytes from the initiation of differentiation (Bisaha and Bader, 1991). At stage 8, VMHC1...
Fig. 3. Whole-mount in situ hybridization of VMHC1 and AMHC1 RNA probes with early chicken embryos. VMHC1 (A,C,E,G) and AMHC1 (B,D,F,H) digoxigenin RNA probes derived from the 3' 2-2.5 kb of each mRNA were hybridized with embryos of indicated stages (see
Materials and Methods). Embryos of Hamburger and Hamilton (1951) stage 8 (A,B), stage 9 (C,D), stage 10 (E, F), stage 12 (G) and stage 14 (H) are represented. Note that at stage 14 (H) an AMHC1-negative portion of the heart has looped over the atriogenic segment of the heart tube.
expression was first detected in the two heart primordia (Fig. 3A). No VMHC1 expression was detected prior to stage 8. Higher levels of VMHC1 expression were detected in the fusing heart tubes (stage 9, Fig. 3C), and the expression domain of VMHC1 continued to expand caudally with the progression of heart differentiation from stages 8 to 12. In the stage 10-12 heart, VMHC1 expression extended from the most anterior fused heart (aortic roots) to the differentiated outflow tract. Thus, VMHC1 expression was activated in an anterior-to-posterior fashion within the fusing heart primordia in both ventriculogenic and atrioogenic myocyte populations.

In contrast, AMHC1 was expressed in only a posterior subset of cardiac myocytes as they differentiated. Unlike VMHC1, AMHC1 expression was not detected in stage 8 embryos (Fig. 3B). When AMHC1 was first detected at stage 9, it was expressed only in a population of cells in the lateral part of the posterior heart primordia (Fig. 3D). The fused heart anterior to the AMHC1-positive cells, which were AMHC1-negative, expressed high levels of VMHC1 in parallel embryos (compare Fig. 3C and D). As the heart primordia continued to fuse at stages 10 and 11, AMHC1 expression was confined to the posterior fused heart and the remainder of the fused primordia (Fig. 3F). The proportion of AMHC1-expressing cells in the heart was determined by comparing the total myogenic region of the heart, as indicated by VMHC1 expression, with the area of the heart that was AMHC1-positive. In similarly staged embryos, the region of AMHC1 expression was confined to the region associated with the anterior intestinal portal, posterior to the curvature of the heart. Comparison of AMHC1- and VMHC1-staining patterns indicated that AMHC1-positive cells occupied 27.2% of the total myogenic area and that this reactivity was confined to the posterior-most portion of the heart tube (Fig. 3; Table 2).

In situ hybridization studies determined that AMHC1 was activated only in the newly fused posterior primordia corresponding to the caudal progression of differentiation and was never expressed in the anterior heart tube. In contrast, VMHC1 was expressed in all differentiatated cardiac myocytes. While the border between atrial and ventricular cells is ill-defined during early cardiogenesis, AMHC1-positive and -negative cells appeared to be colocalized only in the border region. At later stages of heart development, AMHC1 mRNA continued to be expressed only in the atria. The border defined by AMHC1 and VMHC1 expression was consistent with the atrial/ventricular junction as defined by valve formation at stage 18 demonstrating that AMHC1-positive cells are coincident with atrial myocytes. In situ hybridization of whole hearts from 4-day-old embryos (E4) showed that high levels of AMHC1 expression was atrial specific (Fig. 4). At this late stage, VMHC1 was inactive in the atria and its expression was ventricle-specific in the heart. These in situ expression analyses demonstrate that, while VMHC1 is expressed in all differentiating cardiac myocytes, AMHC1 expression is localized to the posterior differentiated heart tube at the earliest stages of cardiogenesis.

Retinoic acid alters the domain of atrial-specific gene expression

Retinoic acid has been reported to affect heart development by altering heart morphology (Osmond et al., 1991; Stanier and Fishman, 1992). Since AMHC1 expression is confined to the posterior heart tube and is the earliest marker reported for atrial diversification, we set out to study the effects of retinoic acid on myogenic diversification using molecular markers for the atrial myogenic lineage diversification. Retinoic acid was added to whole embryo cultures (New, 1955) grown from stages 4-9 to stages 11-13 in vitro (Fig. 5). All trans-retinoic acid was added in medium containing 1% DMSO at 10\(^{-4}\) M. As a control, selected embryos were grown in 1% DMSO. The overall structure of the hearts treated with DMSO was similar to those of nontreated embryos with the exception that some stage 4 to 5 embryos exhibited partial bifida (Table 1). The pattern of myogenic differentiation was similar to that seen in nontreated embryos (Table 1; Fig. 5A) and the differential expression of AMHC1 in posterior myocytes was unaffected when compared with the control group (Table 2; Fig. 5B). When retinoic acid was added to the culture medium, a dose- and age-dependent effect on heart development was observed. In general, more severe morphological changes were observed with early (stage 4-5) treatments or higher concentrations (10\(^{-4}\) M) of retinoic acid. Full or partial cardiac bifida caused by the inhibition of fusion of bilaterally positioned cardiac primordia were the predominant morphologically abnormal phenotypes (Table 2). In the most extreme cases, severe embryonic malformations included the absence of a heart. With 10\(^{-5}\) M retinoic acid, heart abnormalities were observed but tubular hearts formed in many cases. Beating

| Table 1. Dose-dependent and age-dependent effects of retinoic acid treatment on chicken embryos grown in culture |
|---------------------------------------------------------------|---|---|---|---|
| **Heart tube** | **Partial bifida** | **Full bifida** | **Heart absent** |
| DMSO-treated* | | | |
| st. 4-5 | 50 (5) | 50 (5) | 0 (0) | 0 (0) |
| st. 6-7 | 100 (4) | 0 (0) | 0 (0) | 0 (0) |
| st. 8-9 | 100 (13) | 0 (0) | 0 (0) | 0 (0) |
| 10\(^{-4}\) M retinoic acid | | | |
| st. 4-5 | 0 (0) | 0 (0) | 56 (5) | 44 (4) |
| st. 6-7 | 0 (0) | 25 (2) | 63 (5) | 12 (1) |
| st. 8-9 | 0 (0) | 58 (7) | 42 (5) | 0 (0) |
| 10\(^{-5}\) M retinoic acid | | | |
| st. 4-5 | 37 (3) | 25 (2) | 37 (3) | 0 (0) |
| st. 6-7 | 28 (2) | 57 (4) | 14 (1) | 0 (0) |
| st. 8-9 | 67 (6) | 33 (3) | 0 (0) | 0 (0) |

*Culture medium plus or minus retinoic acid in 1% DMSO was added to embryos of the stages indicated and incubated overnight prior to fixation.
* The percentage of embryos with each phenotype with (n) are indicated.

| Table 2. Expansion of AMHC1 gene expression in retinoic acid-treated chicken embryos |
|---------------------|---------|---------|---------|
| %AMHC1-positive* |
| Normal (st. 10-11) | 27.2 ± 3.2 |
| DMSO-treated | 34.4 ± 6.2 |
| Retinoic acid-treated |
| heart tube | 58.2 ± 4.6 |
| partial bifida | 88.3 ± 5.1 |
| full bifida | 100 ± 0 |

*Means were calculated for at least 5 embryos in each group based on the area of AMHC1-positive myocytes relative to the area of VMHC1-positive myocytes in parallel experiments. Retinoic acid concentration was 10\(^{-5}\) M in 1% DMSO in all cases. Values are expressed ± s.e.m.
hearts were observed only in embryos treated during stages 8-9. In contrast, beating hearts were observed in DMSO-treated embryos for all stages treated. More dramatic morphological abnormalities were observed with 10^{-4} M retinoic acid treatment, often resulting in the absence of a heart tube. Lower doses of retinoic acid (10^{-6} or 10^{-7} M) had less severe effects on heart morphology. The resulting mild inhibition of heart primordia fusion or apparently normal hearts demonstrated that the age-related effects of retinoic acid treatment were less severe than the effects with higher doses. The distribution of heart abnormalities in Table 1 is in general agreement with those of Osmond et al. (1990). Like Osmond et al. (1990) we observed a variety of phenotypes produced with each embryonic stage and retinoic acid concentration tested with the distribution of abnormalities shifted in an age- and dose-dependent manner.

The diversification of cardiomyogenic cells in retinoic acid-treated embryos was determined by assaying for myosin heavy chain isoform expression (Fig. 5). Control DMSO-treated embryos were assayed for VMHC1 and AMHC1 gene expression and showed normal lineage diversification with activation of AMHC1 expression in only the posterior region of the heart (Fig. 5B; Table 2). While addition of retinoic acid at 10^{-5} M often produced morphologically normal fused hearts, the atrial domain as measured by in situ hybridization with the AMHC1 probe was expanded (Fig. 5D). The area of AMHC1 expression for retinoic acid-treated embryos with normally fused heart tubes was 58% of the whole heart. This is compared with the 34% AMHC1-positive area in embryos grown in the absence of retinoic acid (Table 2). The sizes of the hearts in nontreated and in retinoic acid-treated hearts with normal morphology were not statistically different as determined by the area of VMHC1 gene expression. In addition, the anterior and posterior boundaries of the heart, as revealed by VMHC1 hybridization, appeared to be unaffected (Fig. 5C). These data indicate that morphologically normal hearts from retinoic acid-treated embryos exhibit expanded atrial (posterior) domains but that the overall myogenic compartment of the heart is unchanged.

Morphologically abnormal hearts exhibiting full or partial cardia bifida were often produced with the addition of 10^{-5} M retinoic acid (Fig. 5C-H; Table 1). Analysis of AMHC1 gene expression revealed an expansion of AMHC1-expressing cells that became coincident with VMHC1-positive cells. In addition, retinoic acid treatment prevented the heart primordia from forming a fully fused heart tube (Fig. 5E,F). In the partially fused hearts, 88% of the heart-forming region expressed AMHC1 (Table 2). With earlier retinoic acid treatments (stage 4-7), many embryos exhibited complete inhibition of heart formation. Still, when these embryos were assayed for cardiomyogenic differentiation, differentiated cardiac myocytes expressing both VMHC1 and AMHC1 were present in the fully bifid cardiogenic regions (Fig. 5G,H). Cardiac myocytes expressing both myosin isoforms extended over the anterior intestinal portal in the unfused heart primordia. In these embryos, 100% of the heart-forming region expressed AMHC1 demonstrating an expansion of this diversified cell type to include the entire cardiomyogenic population. While the morphologies of partially and fully bifid hearts were very abnormal, cardiogenic commitment and differentiation took place as measured by VMHC1 expression. However, a progressive increase in the area of AMHC1-positive cells relative to the VMHC1-positive region was apparent. These results suggest that retinoic acid affects the diversification of the cardiac myocytes within the cardiogenic mesoderm with a resultant expansion of the posterior (atrial) lineage in all embryos tested.

DISCUSSION

Analysis of cardiomyogenic diversification has been inhibited by the lack of appropriate markers of atrial and ventricular cardiac lineages. Using a new molecular marker directed against an atrial-specific myosin heavy chain, we demonstrate that atrial and ventricular progenitors are distinct at the time that they first differentiate (i.e. express cardiac structural genes). In situ hybridization with RNA probes demonstrated that AMHC1-positive cells are confined to the posterior components of the heart from stage 9+. In contrast, VMHC1 is expressed in both anterior and posterior cardiac myocytes and is activated in the most anterior cardiac myocytes at stage 8+, at least 6 hours earlier than AMHC1 appears in the posterior heart. These data demonstrate that cardiogenic diversification is an early and patterned feature of the initial phases of heart development. Still the diversification of the progenitors along the anterior/posterior axis can be altered by retinoic acid.
Fig. 5. Retinoic acid treatment of chicken embryos grown in vitro. Whole-mount in situ hybridization of control embryos (no retinoic acid; A,B) or embryos treated at different developmental stages with $10^{-5}$ M retinoic acid are represented (C-H). Probes used hybridized to VMHC1
(A,C,E,G) and AMHC1 (B,D,F,G) mRNA. Embryos in C, D were treated at stage 8-9, embryos in E,F were treated at stage 7-8 and embryos in G,H were treated at stage 5-7. After 24 hours of treatment, embryos were fixed for in situ hybridization.
AMHC1 is expressed in an atrial-specific manner

The isolation of an atrial-specific myosin heavy chain cDNA has facilitated the detection of newly differentiated atrial progenitors during heart formation. The AMHC1 cDNA contains a unique 3′ untranslated sequence and includes the coding sequences for the S2 and LMM regions of the myosin rod segment. Previous studies have identified myosin gene families based on the composition of the rod sequence. The amino acid sequence of AMHC1 is more similar to the rat cardiac myosin heavy chain isoforms than to chicken fast skeletal isoforms. In the isoform-specific LMM-S2 border region, AMHC1 is equally divergent from rat α and β cardiac myosin isoforms (McNally et al., 1989; Moore et al., 1993). Thus AMHC1 represents a new myosin isoform which does not clearly fall into an α- or β-like class. AMHC1 is unlike any other cardiac myosin heavy chain isoform that has been characterized in the highly divergent hinge region, and therefore may represent a new classification of myosin heavy chain. In situ and northern blot analyses demonstrated that AMHC1 expression is atrial-specific. From the initiation of its expression and throughout development, AMHC1 is expressed only in the atrial myocardic compartment of the heart, and AMHC1 is never expressed in myogenic cells anterior to the developing atrial/ventricular valves. While previous studies using monoclonal antibodies against atrial myosin heavy chains have placed the time of atrial diversification (i.e. expression of atrial-specific gene products) at stage 12-15, it is clear from the present study that atrigenic diversification occurs before the definitive heart is formed.

Atrigenic diversification occurs during the initial phases of cardiomyogenesis

There has been disagreement as to whether the initial phases of heart differentiation include diversification of atrial and ventricular lineages in the chicken as visualized by differential chamber-specific myosin gene expression (Gonzalez-Sanchez and Bader, 1984; Sweeney et al., 1987; Evans et al., 1988; De Jong et al., 1990). DeJong et al. (1990) have suggested that all cardiac myocytes exhibit a single phenotype during early embryogenesis and that atrial and ventricular diversification occur later in development, as late as stage 15 (DeJong et al., 1990). Other studies indicated the existence of a distinct atrial phenotype based on antibody reactivity at stages 12-15 (Gonzalez-Sanchez and Bader, 1984; Sweeney et al., 1987). In the present study, in situ hybridization analysis of AMHC1 and VMHC1 gene expression demonstrated that cardiac myocytes exhibit diversified phenotypes at stages 8-12, much earlier than has been demonstrated previously. VMHC1 expression was detected in the anterior progenitors at stage 8. In contrast, AMHC1 is activated only in a subset of the posterior cardiogenic progenitors at the time they differentiate (stages 9+ to 12). Thus early diversification is evident in the population of AMHC1-positive cells localized in the posterior heart primordia before fusion into the heart tube. These cells are distinct from anterior ventriculogenic cells which are AMHC1-negative. AMHC1 continues to be confined to the posterior atrigenic region of the heart during looping and valve formation and is atrial-specific in the four-chambered heart. Thus, the expression pattern of AMHC1 in a subset of cardiac myocytes in the posterior region of the heart demonstrates that cardiac diversification has occurred when cardiac myocytes differentiate before the heart is formed. These data and those of O’Brien et al. (1993), which describe a ventricular-specific myosin light chain gene expressed in the mouse primitive heart tube, suggest that cardiac diversification occurs during early heart formation. In addition, fate mapping studies in the zebrafish have determined that cardiogenic mesoderm cells are restricted to the atrial or ventricular lineage during the mid-blastula stage (Stanier et al., 1993). Therefore lineage diversification appears to be a very early event in vertebrate cardiogenesis.

Retinoic acid treatment expands the domain of AMHC1 gene expression

Differentiation of the cardiogenic mesoderm occurs in anterior-posterior fashion as the laterally placed cardiac primordia fuse to form the primitive heart tube (Han et al., 1991; Litvin et al., 1992). The splanchnic mesoderm in which the cardiac progenitors arise is maintained as a true epithelium from gastrulation throughout the formation of the primitive heart tube (Peng et al., 1990). Therefore, the anterior-posterior position of cardiac progenitors relative to each other is maintained in this epithelium during early cardiogenesis. Studies of DeHaan and others have shown very little or no mixing of progenitors along the anterior-posterior axis during heart formation (Manasek, 1968; Stalsberg and DeHaan, 1969; Gonzalez-Sanchez and Bader, 1990; Peng et al., 1990). Thus the relative positions of undifferentiated progenitors in the lateral plate are maintained in the positions of differentiated myocytes in the heart tube. It follows that ventriculogenic and atrigenic populations of cardiogenic mesoderm are fixed within this anterior-posterior positional context.

In the normal embryo, atrigenic differentiation begins 6 hours after the initiation of cardiomyogenesis and is confined to a posterior subset of cells that occupies 27% of the stage 10-11 heart. Treatment of early embryos with retinoic acid expands the posterior (atrial) domain of the heart as revealed by the expression pattern of AMHC1. In the retinoic acid-treated embryos in which heart morphogenesis was normal, the AMHC1 domain was expanded to occupy 58% of the heart. The relative increase in the percentage of atrial myogenic cells in the retinoic acid-treated hearts does not come as a result of deletion of anterior progenitors or the addition of previously uncommitted progenitors as the overall size of the myogenic component of the heart remains constant (Table 2; Fig. 5). Instead, the data suggest a conversion of myocytes from the anterior (ventricular) lineage to the posterior (atrial) lineage as the relative positions of the progenitors in the epithelium of the cardiogenic plate are unchanged. This hypothesis is further supported by data from embryos with more severe retinoic acid-induced heart abnormalities. In these partially bifid hearts, the AMHC1-positive myocytes occupy 88% of the heart and, in unfused full bifid hearts, the entire heart-forming region expresses AMHC1. Lack of fusion or inhibition of the morphogenetic movements of the bilaterally positioned cardiogenic mesoderm are not directly responsible for the expansion of the lineage since embryos with surgically induced cardia bifida diversify into atrial and ventricular lineages and do not display an expansion of the atrial domain (Yutzey, unpublished).
Polarity in heart formation

Taken together, these data indicate that retinoic acid, which has been shown to affect anterior-posterior positional information during embryogenesis (Tickle et al., 1985; Thaller and Eichele, 1987; Ruiz i Altaba and Jessell, 1991; Conlan and Rossant, 1992), can influence the diversification of cardiac myocytes. Retinoic acid treatments reported in this study and transplantation experiments of Satin et al. (1988) demonstrate that the diversified fate of cardiac myocytes can be altered as late as stage 9. Thus, the diversified phenotype of cardiac myocytes is not immutable and may be influenced by positional effects.

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