Atrial Chamber-specific Expression of the Slow Myosin Heavy Chain 3 Gene in the Embryonic Heart*

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The quail slow myosin heavy chain 3 (slow MyHC 3) gene is expressed in the developing heart and in slow muscles of the developing limb. It is first expressed in the pulsatile cardiac tube in the embryo, and as the heart chamberizes its expression becomes restricted to the atria. To identify regulatory elements responsible for atrial-specific expression, the 5′ upstream region of slow MyHC 3 gene was investigated. An atrial regulatory domain (ARD1) between −840 and −680 acts as an atrial cell-specific enhancer in primary cardiocyte cultures. ARD1 also specifies atrial-specific expression in vivo when the ARD1/heterologous promoter was introduced into developing chick embryos by a replication-competent retroviral vector. ARD1 is the first atrial cell-specific enhancer to be identified. Fine deletion and mutation analysis within ARD1 defined a 40-base pair vitamin D3 receptor (VDR)–like element that controls atrial cell-specific expression of the slow MyHC 3 gene by inhibiting its expression in ventricular cardiocytes.

One of the earliest organs to form, the vertebrate heart develops as a pulsatile tube by the ventral fusion of two cardiac mesoderm regions of the early embryo (1). The beating tubular heart rapidly folds to form chambers (the atria and ventricles) that differ in morphology, physiology, and in the muscle contractile protein genes each expresses (2–5). Molecular mechanisms for cardiogenesis (6–11) and for atrial and ventricular chamber specification are not well understood. Based on the coexpression of α- and β-MyHC1 genes throughout the tubular heart in mammals, it has been suggested that atrial and ventricular chamber specification occurs only when the tubular heart begins to chamberize (12). More recently, it has been proposed that diversification of atrial and ventricular cells may occur earlier because an atrial-specific MyHC gene, AMHC1, is first expressed in the posterior region of the fusing cardiac tube, the future atrial compartment of the heart (13).

To understand cardiac development, several genes expressed in the developing heart such as MLCs (14, 15), α-MyHC (16–19), β-MyHC (20–22), cardiac troponin T (23, 24), muscle creatine kinase (25), ANF (26, 27), and cardiac α-actin (28, 29) have been studied to delineate cis-elements that interact with transcription factors to control gene expression in the cardiac compartments. While cis-elements and transcription factors have been identified that regulate these genes in the heart (30–39), distribution of the identified transcription factors in atrial and ventricular chambers of the heart has shed little light on the molecular mechanisms generating atrial and ventricular cell types.

The ventricular chamber-specific contractile protein gene, MLC-2v, has served as a model system to identify cis-elements and trans-acting factors that restrict the expression of genes to the ventricular chamber. Both positive (HF-1a and HF-1b) and negative (HF-3) cis-elements have been shown to regulate restricted expression of the MLC-2v gene in adult transgenic mouse (36). However, cis-elements have not been reported for atrial-specific genes either during embryonic development or in the adult.

We have reported the identification and characterization of a new slow myosin heavy chain gene, slow MyHC 3, that is expressed in the developing quail heart and embryonic slow skeletal muscles (40) and that is closely related (if not homologous) to the chicken AMHC1 (13). Initially the slow MyHC 3 gene is expressed throughout the tubular heart, but as the heart chamberizes, expression of the slow MyHC 3 gene in the ventricles is down-regulated, whereas expression in the atria is maintained. By delineating cis-elements within the regulatory region of the gene, we can identify a transcription factor or factors that are important for specification of the atrial chamber. We have identified four regions within the slow MyHC 3 promoter that contain either positive or negative cis-elements. One of these regions, 160 bp of the 5′ flanking sequence between −840 and −680 designated as atrial regulatory domain 1 (ARD1), functions as an atrial-specific enhancer in primary cardiocyte cultures as well as in the embryo. The function of the ARD1 in vivo was investigated by using an avian retroviral vector, RCAN/PCAT/F (41), to deliver an ARD1/SV40 promoter/CAT construct into embryos. In developing embryos ARD1 increased expression of the CAT reporter 12.3-fold in the atria compared with other non-cardiac tissues including skeletal and smooth muscle. Deletion and mutation of the ARD1, coupled with transient transfection of atrial and ventricular cardiocyte cultures, identified a 40-base pair vitamin D3 receptor (VDR)-like element essential for atrial-specific expression. The VDR-like element residing within the ARD1 was found to regulate atrial-specific expression by inhibiting reporter expression in ventricular cardiocytes.

**EXPERIMENTAL PROCEDURES**

Heart Cell Cultures—Quail eggs were purchased from Strickland Quail Farm, Pooler, GA. Embryonic atrial and ventricular cells were prepared as described by Barry et al. (42). The atria and ventricles from ED6 quail hearts were dissociated at 37 °C with constant agitation

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* The abbreviations used are: MyHC, myosin heavy chain; ARD1, atrial regulatory domain 1; VDR, vitamin D3 receptor; bp, base pairs; CAT, chloramphenicol acetyltransferase; CEF, chicken embryonic fibroblast; SV40, simian virus 40; MLC, myosin light chain; ANF, atrial natriuretic factor; AMHC, atrial-specific myosin heavy chain gene; mAb, monoclonal antibody; ED, embryonic day; PAGE, polyacrylamide gel electrophoresis; UTR, untranslated region.
during six successive 8-min incubations in 0.025% trypsin (Difco) in the Ca\(^{2+}\)/Mg\(^{2+}\)-free Hanks' solution. For transfection, cells were plated at densities of 2.5 \(\times\) 10\(^5\) on 60-mm collagen-coated dish (Collagen, bovine tendon, Worthington). For immunostaining, 5 \(\times\) 10\(^5\) cells were plated on 35-mm collagen coated dishes. The atrial and ventricular cardiomyocytes were initially plated in heart serum-containing medium consisting of 40% M-199, 53% basic salts solution, 6% fetal calf serum (HyClone), 1% glutamine, and 0.1% penicillin/streptomycin (42). On day 2 of incubation the medium on all dishes was changed to heart serum-free medium composed of Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (HyClone), 1% glutamine, 0.3% penicillin/streptomycin according to Libby (43). The cultures were fed daily after day 2 with heart serum-free medium.

Immunocytochemistry—Immunostaining for MyHC in culture was described previously (44, 45). mAb F59 reacts with fast isoforms of MyHC in striated muscles, and mAb S58 reacts with slow isoforms of MyHC. A series of 5 flanking sequence of the slow MyHC 3 gene was cloned into the pCAT-promoter vector containing a SV40 promoter without enhancer. A deletion in this sequence it contains. Injection sites were localized in pilot experiments by using a dye but unincubated chicken eggs at Hamburger and Hamilton stage 1 on approximately 10\(^6\) virus-producing CEFs was injected into fertilized but unincubated chicken eggs at Hamburger and Hamilton stage 1 on the day the eggs were set (41, 52). A small hole that would allow a hypodermic needle to pass was made by drilling with a small scissors but unincubated chicken eggs at Hamburger and Hamilton stage 1 on the day the eggs were set (41, 52). A small hole that would allow a hypodermic needle to pass was made by drilling with a small scissors near the center of the large end of eggs. Injections were targeted to the surface of egg yolk close to the blastodisc but not into the blastodisc itself. Injection sites were localized in pilot experiments by using a dye solution. The injection holes were sealed with paraffin. Infected eggs were incubated until the embryos completed fetal development at ED18. Blood was collected from these fetuses prior to sacrifice and tested for the presence of the viral capsid antigen p27 by enzyme-linked immunosorbent assay (ELISA). Tissues were collected and stored at \(-80^\circ\)C until analysis. All CAT assays were performed with extracts of these tissue samples.

Whole Mount Staining—Whole mount immunostaining was performed according to Page et al. (46) to identify embryos infected with the retrovirus. The RCAN retroviral infected embryos were incubated overnight at 4°C with a 1:2500 dilution of rabbit antiviral capsid protein p27 (SPAFAST, Inc.). The RCAN retroviral infected embryos were incubated overnight at 4°C with a 1:2500 dilution of rabbit antiviral capsid protein p27 (SPAFAST, Inc.). The RCAN retroviral infected embryos were incubated overnight at 4°C with a 1:2500 dilution of rabbit antiviral capsid protein p27 (SPAFAST, Inc.). The RCAN retroviral infected embryos were incubated overnight at 4°C with a 1:2500 dilution of rabbit antiviral capsid protein p27 (SPAFAST, Inc.).
protein p27 antibody (anti-p27) and visualized with horseradish peroxidase-goat anti-rabbit IgG (Sigma).

Deletion and Mutation Constructs—Three genomic sequences between −840 and +18, −808 and +18, −768 and +18 were amplified from the SM3CAT:840 template DNA by PCR (49). The PCR products were directionally cloned into the HindIII-XbaI site of the pCAT-basic vector. These three deletion SM3CAT constructs were designated SM3CAT:840D, SM3CAT:808D, and SM3CAT:768D, respectively.

Mutations were made in two sites within the SM3CAT:840D by PCR mutagenesis (49). The AGGAC half-site in the VDR-like element from −780 to −775 was replaced by a SalI restriction site, GTCGAC. Sequences from −840 to −796 were replaced by a Sall restriction site, GTCGAC. Sequences from −840 to −796 and −801 to +18 were PCR-amplified, respectively. The PCR products were digested by HindIII + XbaI and directionally cloned into the pCAT-basic vector. The mutant construct was designated mut-VDR. Mutations were also introduced between −780 and −775 in the SM3CAT:840D in which sequence 5'-GGCGGA-3' was replaced by SalI site, 5'-GTCGAC-3'. This mutant construct served as a control for mut-VDR and was designated mut-Ctrl. All constructs were sequenced using the dsDNA Cycle Sequencing System (Life Technologies, Inc.).

RESULTS

Characterization of Atrial and Ventricular Cardiocytes in Primary Culture—Embryonic atrial and ventricular cardiocytes isolated from ED6 quail hearts express myosin heavy chains when cultured on collagen-coated dishes. These cultures initially contained 90% atrial or ventricular cardiocytes and 10% non-cardiocytes as distinguished by immunostaining for fast MyHC isoforms using the mAb F59. Although both cardiocytes and non-cardiocytes increased in number in the culture period, the percentage of cardiocytes in the culture decreased to about 75% by culture day 3 and remained at this level for at least 14 days of culture. Changing to serum-free medium on the 2nd day of culture promoted the differentiation of atrial and ventricular cardiocytes and avoided overgrowth of non-cardiocyte cells.

As determined by cell morphology, beating rates, and staining for MyHC expression, atrial and ventricular cardiocyte cultures were healthy for up to 2 weeks of incubation. All cardiocytes, whether ventricular or atrial, were identified by the expression of fast MyHC isoforms staining with mAb F59 (Fig. 1, B and E). At day 3 in culture, both atrial and ventricular cardiocytes were beating synchronously. Atrial and ventricular cardiocytes could be distinguished by differences in morphology and slow MyHC expression. Atrial cardiocytes had a less flattened morphology on the collagen substrate than ventricular cardiocytes (compare Fig. 1, A and D), and atrial, but not ventricular, cardiocytes stained with mAb S58 which recognizes slow MyHC 3 (Fig. 1, C and F).

The amount of slow MyHC 3 transcripts in the chambers of embryonic hearts and in atrial and ventricular cultures were quantitated by RNA slot blots. Total cellular RNA were extracted from ED6 atria, ventricles, and from atrial and ventricular cultures maintained for 2, 5, and 7 days. RNA samples were probed with a 3'UTR oligonucleotide specific for slow MyHC 3. At ED6, slow MyHC 3 was expressed in vivo in the atrium (Fig. 2, A and B, lane A) at a 6.7-fold higher level than the ventricle (Fig. 2, A and B, lane F). Expression of the slow MyHC 3 RNA in atrial cardiocyte cultures was sustained for the culture period at a constant level (Fig. 2, A and B, lanes B-D). In contrast, by 2 days the level of slow MyHC 3 transcripts in the ventricular cardiocyte cultures (Fig. 2, A and B, lanes G-I) was similar to control levels seen in RNAs from liver (Fig. 2, A and B, lane J) or from ED18 pectoralis major skeletal muscle (Fig. 2, A and B, lane E), a skeletal muscle that in the adult expresses fast, but no slow, MyHCs. Extending the time in culture up to 7 days did not lead to expression of slow MyHC 3 RNA in ventricular cardiocyte cultures (Fig. 2, A and B, lane I). These atrial cardiocyte cultures expressed 9-fold more slow MyHC 3 than ventricular cardiocyte cultures.

Expression of slow MyHC 3 protein was examined by Western blotting using extracts from atrial and ventricular cultures at days 2, 5, and 7 of incubation (Fig. 2C). In the cultures of atrial cardiocytes, slow MyHC 3 protein expression remained constant at high levels (Fig. 2C, lanes C, E, and G), similar to that present in ED6 atria in vivo (Fig. 2C, lane A). In contrast, slow MyHC 3 was undetectable in extracts of cultured ventricular cardiocytes at days 2, 5, or 7 (Fig. 2C, lanes D, F, and H). We conclude that atrial cardiocytes express slow MyHC 3 protein at constant high levels for at least a week in culture, whereas ventricular cardiocytes do not express slow MyHC 3 at any point during the culture period. Therefore, cultures of atrial and ventricular cardiocytes comprise a model system to investigate the mechanism of atrial-specific expression of the slow MyHC 3 gene.

Localizing of an Atrial-specific cis-Element(s) in the Slow MyHC 3 Gene—To define the atrial-specific cis-element(s) of the slow MyHC 3 gene, a series of reporter constructs were made from two genomic clones, QSM4 and QSM6 (40). The slow MyHC 3 regulatory regions ranging from 290 to 8500 bp upstream from transcription start site and including intragenic sequences of exon I, intron I, and a portion of exon II were fused to the bacterial chloramphenicol acetyltransferase (CAT) gene.
were cotransfected with psv-Convenient restriction sites, shown in Fig. 3, and ventricular cultures. In the atrial cardiocyte cultures, the SM3CAT:8500 construct was expressed at a high level in the CEFs, largely due to increased expression in atrial cultures. SM3CAT:680 expression is not cardiac chamber-specific. The SM3CAT:680 construct is not expressed at significant levels in either cardiocytes or fibroblasts, whereas constructs containing 840 bp or more of upstream sequence all showed atrial cardiocyte-specific expression. Expression of the SM3CAT:840 in atrial cardiocytes was 7-fold higher than that of ventricular cardiocytes and 14-fold higher than that of CEFs (Table I). Inclusion of additional 5′ flanking sequence to −3200 had a modest increase in the atrial/CEF ratio and no effect on the relative expression between atrial and ventricular cardiocytes. With the addition of slow MyHC 3 sequence between −3200 and −4500, the level of expression in atrial cultures increased to 15-fold higher than in ventricular cultures and 2 orders of magnitude higher than in CEFs, largely due to increased expression in atrial cultures. Transfection studies have mapped four regulatory regions associated with differences in levels of reporter expression (Fig. 4). First, the 290 bp upstream from the transcription start site directs high levels of non-chamber-specific expression. Second, the upstream region between positions −680 and −290 contains a negative cis-element(s) which strongly inhibits the reporter activity in all three cell types. Third, the region between −840 and −680 contains an atrial cardiocyte-specific cis-element(s), permitting expression in atrial cardiocytes but not in ventricular cardiocytes or CEFs. Fourth, the region between −4500 and −3200 further increased the level of CAT expression by 3-fold in atrial cardiocytes but had no effect on ventric-
ular cardiocytes or CEFs. Therefore, the expression of slow MyHC 3 gene in cardiocytes is regulated by both positive and negative cis-elements.

Primary atrial or ventricular cell cultures contain a heterogeneous population of cells. As determined by immunostaining (Fig. 1), about 75% of the cells from either chamber are cardiocytes and about 25% are non-cardiocytes from day 3 in culture onward. To determine which cells in the cardiac cultures express slow MyHC 3 CAT constructs, CAT protein was localized immunologically. Cultures were transfected with the SM3CAT:4500 construct, which directs the highest level of expression among atrial cardiocytes (filled bars), ventricular cardiocytes (open bars), and CEFs (stippled bars). Expression from each of the slow MyHC 3 CAT constructs is reported relative to the SV40 CAT control plasmid. Constructs containing 840 bp or more of upstream sequence showed atrial cell-specific expression.

| Constructs | Activity ratio | A/V | A/C |
|------------|----------------|-----|-----|
| −290       | 1              | 4.7 |     |
| −680       | 1              | 1.1 |     |
| −840       | 7              | 14  |     |
| −1600      | 8              | 8   |     |
| −2600      | 8              | 31  |     |
| −3200      | 7              | 28  |     |
| −4500      | 15             | 99  |     |
| −6000      | 18             | 90  |     |
| −8500      | 21             | 56  |     |

Fig. 4. Identification of regulatory cis-elements in the slow MyHC 3 gene that direct atrial cell-specific transcription. Atrial and ventricular cardiocytes derived from ED6 hearts as well as CEFs were transfected with chimeric CAT reporter plasmids described in the legend to Fig. 3. Within each experiment, the effect of differences in transfection efficiency between plates was minimized by expressing the activity of each slow MyHC 3:CAT construct relative to β-Gal activity. Because transfection efficiencies of CEFs are greater than atrial or ventricular cardiocytes, a separate culture dish was used for transfection with a SV40:CAT control plasmid (Promega) in every experiment to allow comparisons of the levels of expression among atrial cardiocytes (filled bars), ventricular cardiocytes (open bars), and CEFs (stippled bars). Expression from each of the slow MyHC 3:CAT constructs is reported relative to the SV40:CAT control plasmid. Constructs containing 840 bp or more of upstream sequence showed atrial cell-specific expression.

Table 1

The ARD1 of the Slow MyHC 3 Gene Regulates Atrial-specific Expression in the Embryo—To characterize the cis-element that conferred atrial chamber-specific expression on the slow MyHC 3 gene in vivo, we employed a replication-competent retroviral vector, RCAN, for gene transfer to early developing chick embryos. The RCAN vector (41) was specifically designed to express DNA inserts from an internal promoter and to separate the expression of the insert from viral gene expression. A heterologous promoter, which contained ARD1 fused in the forward orientation to a heterologous promoter, the pCAT-promoter in which CAT is driven by the SV40 promoter without an enhancer. The two heterologous promoter constructs named ARD1/SV/F (forward) and ARD1/SV/R (reverse), as well as a control, pCAT-promoter, were transfected into atrial cardiocyte, ventricular cardiocyte, or CEF cultures (Fig. 6). Both constructs, with a single copy of either the forward or the reverse orientation of ARD1, increased CAT expression by 3-fold in atrial cardiocytes whereas their expression was unchanged in ventricular cardiocytes and CEFs compared with the control pCAT-promoter plasmid. Thus, sequence in the ARD1 region has attributes of an atrial cardiocyte-specific enhancer.

The ARD1 of the Slow MyHC 3 Gene Regulates Atrial-specific Expression in Cardiocyte Cultures—Inclusion of the slow MyHC 3 sequence located between −840 and −680, designated ARD1, in reporter constructs increased expression 7-fold in atrial cardiocyte cultures relative to ventricular cardiocyte cultures (Table I). The 160 bp of ARD1 were PCR-amplified and fused in both the forward and the reverse orientation to a heterologous promoter, the pCAT-promoter in which CAT is driven by the SV40 promoter without an enhancer. The two heterologous promoter constructs named ARD1/SV/F (forward) and ARD1/SV/R (reverse), as well as a control pCAT-promoter, were transfected into atrial cardiocytes and CEFs (Fig. 6). Of the 15 embryos harvested at ED5 and were stained with an antibody against the viral capsid protein p27 (Fig. 7A). Of the 15 em-
bryos that formed after infection, 8 (53%) were infected and 7 (47%) were not. All tissues of the eight positive embryos were ubiquitously stained (Fig. 7A), demonstrating a global infection by the viral construct. No tissues were stained in the seven p27 negative embryos, suggesting that there were no mosaic infections. A control ED5 embryo that was not injected with the virus was stained with anti-capsid protein p27 (Fig. 7B). There was no viral protein present in the control embryo.

Six chicken embryos infected with RCAN/ARD1-PCAT/F were permitted to develop to fetal stage ED18. These six fetuses were shown to be infected by using an enzyme-linked immunosorbent assay to analyze blood samples for the presence of viral capsid antigen p27. Extracts of brain, kidney, intestine, liver, lung, gizzard, stomach, atrium, ventricle, medial adductor (a slow skeletal muscle), and pectoralis major (a fast skeletal muscle) from each embryo were used in an enzymatic CAT assay (Fig. 8A). High levels of CAT activity were found only in the atria of all embryos. Levels in the atria were 12.3-fold higher than those observed in the non-cardiac tissues and 4.6-fold higher in the atria than that in the ventricles. A 2.7-fold increase was observed in the amount of CAT present in the ventricles relative to non-cardiac tissues, including skeletal and smooth muscles. Viral gene expression in each tissue was also measured directly by detecting p27 protein through Western blot analysis. Fig. 8B showed p27 expression from one viral infected embryo. Results from all six embryos showed equivalent levels of viral p27 protein expression in all tissues, confirming the global nature of infection and indicating that viral gene expression is not specifically enhanced in cardiac tissues.

These results demonstrate that in vivo ARD1 can restrict transcription of a reporter gene to cardiac muscle in ED18 fetuses with preferential expression in atria and indicate that regulatory elements residing within ARD1 play a pivotal role in conferring atrial-specific expression of the slow MyHC 3 gene.
Fig. 8. The ARD1 regulates atrial chamber-specific transcription in the embryo. A, Homogenates were prepared from 11 tissues of 6 transgenic ED18 chicken fetuses that formed eggs infected with the retrovirus RCAN/ARD1-PCAT/F prior to incubation. Extracts were adjusted to an equivalent protein concentration, and CAT assays were performed under identical conditions. The error bars represent the standard error of the mean. Levels in the atria were 12.3-fold higher than those observed in the non-cardiac tissues and 4.6-fold higher than that in the ventricles. B, extract (20 μg of proteins) of each of the tissues used in the CAT assay in A above were electrophoresed and transferred to nitrocellulose membranes. The viral capsid protein p27 was detected with a polyclonal rabbit anti-p27 antibody. Results from one embryo are shown and each tissue from all six fetuses showed equivalent amounts of the viral protein expression.

A Vitamin D$_2$ Receptor-like Sequence is an Essential Element for Atrial-specific Expression in Cardiocyte Cultures—Sequence motifs with homology to previously identified regulatory motifs were identified in ARD1, including HF-1A (54), M-CAT (55), E-box (28), vitamin D$_2$ receptor (VDR), or retinoic acid receptor-like element (56), and GATA (37, 39) (Fig. 9A). To further define the cis-elements that regulate atrial-specific expression, deletions were made to remove portions of ARD1 in the context of the SM3CAT:840 construct (Fig. 9B). First, deletion of intron 1 and exon 2 sequences, to form the construct SM3CAT:840D, had no effect on the level of atrial-specific expression of the reporter (Fig. 9C). Second, primers were designed which deleted slow MyHC 3 promoter sequences from the 5' end of SM3CAT:840D, removing HF-1A, M-CAT, and E-box motifs (SM3CAT:808D), or HF-1A, M-CAT, E-box, and VDR motifs (SM3CAT:768D). Deletion of the sequences between −840 and −808 (SM3CAT:808D) had no effect on the level of expression in atrial cardiocytes, ventricular cardiocytes, and CEFs. However, further deletion to −768 (SM3CAT:768D) resulted in an increase of the CAT expression in ventricular cardiocytes to a level equal to that observed in atrial cardiocytes, whereas expression in atrial cardiocytes and CEFs remained unchanged compared with SM3CAT:840D (Fig. 9C). These results suggest that the HF1A, M-CAT, and E-box motifs within ARD1 are not essential for atrial-specific expression of slow MyHC 3 but that the 40 bp between −808 and −768, including the VDR-like element, act as a negative element in ventricular cardiocytes and have no role in atrial cardiocytes and CEFs.

To demonstrate that the VDR-like element specifies atrial-specific expression of slow MyHC 3 gene, the 5' copy of the AGGACA direct repeat was mutated to GTCCGAC in the context of SM3CAT:840D (mut-VDR, Fig. 10A). As observed with deletion construct SM3CAT:768D, CAT expression in mut-VDR increased 6-fold in ventricular cardiocytes, whereas expression in atrial cardiocytes and CEFs remained unchanged compared with SM3CAT:840D (Fig. 10B). In contrast, no effect on chamber-specific expression was observed when a comparable 6-bp sequence between the VDR-like element and the GATA motifs was mutated (mut-Ctrl, Fig. 10, A and B). These results demonstrate the VDR-like cis-element as an essential component of slow MyHC 3 atrial-specific expression.

DISCUSSION

We have found that the quail slow MyHC 3 gene is initially expressed in both atrial and ventricular chambers but that it is rapidly down-regulated in the ventricle by ED6. A 160-bp enhancer, ARD1, appears to be the primary element that restricts slow MyHC 3 expression to the atria both in vitro and in vivo. Deletion or mutation of a VDR-like element within ARD1 resulted in loss of the atrial-specific expression of the reporter, implicating the VDR-like motif as a key element in the atrial-specific expression of the slow MyHC 3 gene.

Primary Heart Cultures Provided a Model System in Vitro to Characterize Atrial or Ventricular-specific cis-Elements of
Chamber-specific Genes—We have used an avian atrial and ventricular primary cell culture system to study cardiac chamber-specific gene regulation. The defined heart serum-free medium we employed (43) greatly limits overgrowth of cultures by fibroblasts, ensuring that cardiocytes are the predominant cell type in the cultures. Cardiocytes retain characteristics found in vivo. In this culture system the endogenous slow MyHC 3 gene is continuously expressed in atrial cardiocytes but disappears by 2 days of incubation of ventricular cardiocyte cultures (Fig. 2). Thus, we have a model culture system with which to dissect the transcriptional control cassettes of the slow MyHC 3 gene. This system may also be useful for studying the regulatory pathways of other chamber-specific markers.

Identification of Atrial-specific cis-sequences—ARD1, a 160-bp fragment of the quail slow MyHC 3 promoter between -840 and -680, is competent to drive expression of a heterologous CAT reporter gene in an atrial-specific fashion in primary cell cultures and embryos. This region has properties of an atrial-specific enhancer. In contrast, sequence lying between -680 and -290 acts as a silencer in cardiac and fibroblast cells, effectively repressing the high level of CAT reporter expression in these cell types seen when only 290 bp of proximal promoter sequence is present (Figs. 3 and 4). Interestingly, the SM3CAT:680 construct is expressed at a high level in embryonic skeletal muscle cultures (40), identifying a difference in the regulatory elements affecting expression of this gene in the two types of striated muscle cells. Thus, cis-sequences controlling expression of the slow MyHC 3 gene in embryonic atrial cells and embryonic slow skeletal muscle cells are segregated, as shown previously by other groups who compared cardiac and skeletal muscle specific regulatory elements within the same gene (60–62).

We further analyzed the ARD1 region in the developing embryo via the RCAN/ARD1-PCAT/F retrovirus. While infected embryos showed global viral infection throughout all tissues (Figs. 7A and 8B), reporter expression was markedly enhanced by ARD1 in atria, and to a less extent in ventricle, but not at all in skeletal or smooth muscles nor non-muscle cells by ED18 of development (Fig. 8A). To our knowledge, this report constitutes the first characterization of cis-regulatory sequences located in a relatively small region (160 bp) that confines transcription preferentially to the atrial compartment in vivo. A number of laboratories have initiated studies in transgenic mice to identify in vivo regulatory regions in genes expressed in the atria. Among genes expressed in the atria, the best characterized regulatory regions were those of $\alpha$-cardiac MyHC, MLC3f, and ANF. A 2-kb upstream region of the $\alpha$-cardiac MyHC gene was shown to direct tissue-specific expression of the transgene (16), and a thyroid hormone-responsive element, TRE2, was shown to be important for reporter activity in both the atrium and ventricle (19). Recently, a 2-kb promoter region and 260-bp enhancer sequence of the MLC3f gene was shown to be sufficient to direct atrial- and left ventricular-specific expression of a reporter in adult mice (15), while a 2.4-kb regulatory region of the ANF gene was shown to direct the reporter expression equally well in both atrial and ventricular cells (26). As the cis-elements regulating atrial expression of these genes become more defined, it will be possible to determine to what extent divergent and overlapping pathways regulate atrial expression of slow MyHC 3 gene and other cardiac-expressed genes.

Within ARD1, sequences homologous to five known cis-elements were identified (Fig. 9A). From 5' to 3' they are as follows: a HF-1A element, a M-CAT element, an E box, a vitamin D$_3$ receptor (VDR) or retinoic acid receptor-like element, and a GATA element. The HF-1A is an element that contributes to cardiac-specific expression of the MLC-2v gene in the mouse (54). The M-CAT, initially described as a crucial
element for cardiac troponin T gene expression in cardiac cells (55), has been shown to regulate a number of contractile protein genes in both cardiac and skeletal muscles (60). Some evidence suggests an E box-dependent pathway in the regulation of some cardiac genes including α-cardiac actin (28), muscle creatine kinase (25), and α-MyHC (63). The recent discovery of new bHLH proteins, eHAND and dHAND, supports this notion (8). However, deletion results (Fig. 9C) suggest that the E box, as well as HF-1A, M-CAT motif within ARD1 is not essential for atrial-specific expression of the slow MyHC 3 gene.

Both deletion (Fig. 9) and mutation (Fig. 10) of the VDR-like element within the ARD1 of the slow MyHC 3 gene led to up-regulation of the reporter expression in ventricular cardiocytes, suggesting that the VDR-like element controls atrial-specific expression of the slow MyHC 3 gene in embryonic heart by negative regulation. It would be extremely interesting to know how the VDR-like element inhibits expression of slow MyHC 3 in ventricular cardiocytes. Members of the steroid and thyroid hormone (T3) receptor superfamily of transcriptional regulators have been implicated in many critical aspects of vertebrate development, including heart morphogenesis (64). Retinoic acid receptors (RXRs and RARs), vitamin D receptors, and thyroid hormone receptors bind similar cis-elements (56). T3 has been shown to regulate both α- and β-MyHC expression during cardiac development (19, 62), whereas vitamin D, negatively regulates the expression of ANF in cardiac cells (31).

Also located within ARD1 is a GATA element (Fig. 9A). Recently a GATA element has been shown to regulate cardiac muscle-specific expression of the α-MyHC gene (37) and cardiac troponin C (57). Inclusion of the GATA element (SM3CAT: 768D) drives reporter expression in ventricular cells to a level equivalent to that in atrial cells. Thus, the GATA motif and/or its sequence between 768 and 680 is able to relieve the inhibition observed in the SM3CAT:680 construct. The ARD1 does not contain a CARG box, which was shown to be important in expression of the ANF gene in both atrial and ventricular cells (27), or a MEF2 site, which is important in control of the expression of a number of contractile protein genes in cardiac, skeletal, and smooth muscle (9, 10, 65).

The Slow MyHC 3 Gene Can Serve as a Molecular Marker of Atrial Specification—Several myosin heavy and light chain genes have been used as markers to study atrial and ventricular chamber specification. In mice, two cardiac MyHC isoforms, α-MyHC and β-MyHC, are initially coexpressed in the atrium and ventricle. β-MyHC showed preferential expression in the ventricles by day 9.5 postcoitum, while α-MyHC showed preferential expression in the atrium and ventricle.

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