Irx4 Forms an Inhibitory Complex with the Vitamin D and Retinoic X Receptors to Regulate Cardiac Chamber-specific slow MyHC3 Expression*

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The slow myosin heavy chain 3 gene (slow MyHC3) is restricted in its expression to the atrial chambers of the heart. Understanding its regulation provides a basis for determination of the mechanisms controlling chamber-specific gene expression in heart development. The observed chamber distribution results from repression of slow MyHC3 gene expression in the ventricles. A binding site, the vitamin D response element (VDRE), for a heterodimer of vitamin D receptor (VDR) and retinoic X receptor α (RXRα) within the slow MyHC3 promoter mediates chamber-specific expression of the gene. Irx4, an Iroquois family homeobox gene whose expression is restricted to the ventricular chambers at all stages of development, inhibits AMHC1, the chick homolog of quail slow MyHC3, gene expression within developing ventricles. Repression of the slow MyHC3 gene in ventricular cardiomyocytes by Irx4 requires the VDRE. Unlike VDR and RXRα, Irx4 does not bind directly to the VDRE. Instead two-hybrid and co-immunoprecipitation assays show that Irx4 interacts with the RXRα component of the VDR/RXRα heterodimer and that the amino terminus of the Irx4 protein is required for its inhibitory action. These observations indicate that the mechanism of atrial chamber-specific expression requires the formation of an inhibitory protein complex composed of VDR, RXRα, and Irx4 that binds at the VDRE inhibiting slow MyHC3 expression in the ventricles.

Although a molecular description of cardiogenesis is being defined, the molecular mechanisms that control cardiomyocyte differentiation into either an atrial or ventricular phenotype remain unclear (1–10). Several chamber-restricted genes have been used as markers to investigate atrial and ventricular lineage diversification (11–21). One of the earliest atrial chamber-specific genes expressed during cardiogenesis, the quail slow MyHC3 gene, serves as a model system to identify molecular pathways governing chamber-specific expression (22–25). The slow MyHC3 gene, a homologue of chick AMHC1 (20, 26, 27) is most closely homologous to the cardiac α-MyHC and β-MyHC in mammals (28). Slow MyHC3 is initially expressed throughout the tubular heart, and atrial chamber-restricted expression is subsequently established by down-regulation in the ventricles during chamber formation (25).

Using a combination of transient transfection assays and transgenic approaches, a 840-base pair fragment of the slow MyHC3 promoter was identified that controls atrial-specific expression (23, 29). Atrial-specific expression is achieved by the positive effects of a GATA factor-binding element in the atria and by inhibition through a nuclear hormone-binding element, vitamin D response element (VDRE),1 in the ventricles (25). Mutational analysis of the slow MyHC3 promoter revealed that the VDRE alone is required for ventricular inhibition (25). The VDRE binds a heterodimer of retinoic X receptor α (RXRα) and vitamin D receptor (VDR), which suppresses slow MyHC3 expression within ventricular but not atrial cardiomyocytes (25). Because both RXRα and VDR are expressed within atrial and ventricular cardiomyocytes, it was postulated that an unknown ventricular-specific inhibitor, in addition to VDR and RXRα, must act through the negative VDRE element (24).

Recently, Bao and co-workers (30) identified an Iroquois family homeobox gene, Irx4, the expression of which is confined to the ventricles throughout heart development in birds and in mammals (30, 31). In mice with targeted disruption of the Irx4 gene there is inappropriate expression of atrial genes within the ventricle (32). Irx4 expression is down stream of Nkx2–5 and dHand and activates the expression of the ventricle myosin heavy chain 1 gene (VMHC1) and suppresses the expression of the AMHC1/slow MyHC3 gene in the ventricles in the chicken (30, 31). However, the molecular mechanism by which Irx4 regulates the inhibition of slow MyHC3/AMHC1 gene expression and activation of VMHC1 gene expression is not known. Here we report that down-regulation of the slow MyHC3 gene by Irx4 is through the VDRE element. Furthermore, Irx4 and the RXRα subunit of the VDR/RXRα heteroduplex form a protein complex, which inhibits slow MyHC3 gene expression in the ventricles.

EXPERIMENTAL PROCEDURES

Transfections and Plasmids—Primary cultures of embryonic day six atrial and ventricular quail cardiomyocytes were cultured and transfected as described previously (25). Typical transfections included 3 μg of CAT reporter plasmid, 3 μg of the Irx4 or 3 μg of the Irx4 dominant-negative H+ en’ expression plasmids, and 1 μg of pSV-β-gal as a reference plasmid to control for transfection efficiency. The CAT constructs used were described previously (23, 25). Briefly, SM3CAT:840D, SM3CAT:808D, and SM3CAT:768D extend from −840, −808, and −768 bp respectively, of the slow MyHC3 promoter through the first exon of non-coding sequence (+18 bp) fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter. CAT reporter constructs ARD1:CAT (previously designated SVARDUP230), contain a 160-bp fragment of the slow MyHC3 promoter between positions −840 and −680, VDRE-1

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1 The abbreviations used are: VDRE, vitamin D response element; RXRα, retinoic X receptor α; VDR, vitamin D receptor; bp, base pair(s); VMHC1, ventricle myosin heavy chain 1 gene; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.
GATA-CAT contains both the VDR and the GATA element between positions −808 and −741, and GATA-CAT contains the GATA element between positions −775 and −741. All control elements were positioned upstream of the minimal SV40 promoter. The SV40:CAT construct contained only the minimal SV40 promoter fused to CAT. Irx4 was cloned into a RCAS viral vector (30). Transfection efficiencies were normalized to β-galactosidase expression, and CAT assays were performed as described previously (23).

**Two-hybrid Assay**—To detect Irx4 interactions with RXRα or VDR, we employed the mammalian MATCHMAKER two-hybrid assay kit (CLONTECH) with slight modifications. The RXRα or VDR genes were inserted in frame with a Gal4 DNA-binding domain in the pM expression vector (CLONTECH), to create pM-RXRα or pM-VDR, respectively. Embryonic day six quail atrial cardiomyocytes were transfected with the Gal4 reporter plasmid pG5CAT, pM-RXRα or pM-VDR to mediate binding to the Gal4 sites in the reporter plasmid with or without the RCAS-Irx4 expression vector. Three μg of each construct was transfected/60-mm culture. In experiments where the Irx4 expression vector was not transfected along with the reporter, 3 μg of empty expression vector was co-transfected such that in each transfection, 9 μg of plasmid DNA was used.

**In Vitro Translation, Co-immunoprecipitation, and Western Blot Analysis**—Irx4 (30), RXRα (33), and VDR (4) were cloned into a pBlue-script KSII vector. In vitro transcription with Large Scale RNA Production Systems was performed according to the manufacturer (Promega). A rabbit reticulocyte lysate kit from Promega was used for *in vitro* translation. Interactions between Irx4 and RXRα or VDR were assessed by co-immunoprecipitation of *in vitro* translated RXRα or VDR with Irx4. Irx4 contained a HA tag immediately preceding the stop codon. Monoclonal anti-VDR antibody was purchased from Biomol, and anti-RXRα antibody was provided by Dr. Elizabeth Allegretto of Ligand Pharmaceuticals, Inc. Co-immunoprecipitation was performed by mixing 5 μl of each *in vitro* translated protein with the antibody against one of the proteins in 0.5 ml immunoprecipitation buffer according to Elion (34). Protein G-agarose beads (Sigma) were added to precipitate the antibody-antigen complex, the precipitate was subjected to SDS-PAGE and then transferred to nitrocellulose. Anti-VDR (rat IgG), anti-RXRα (rabbit IgG), or anti-HA to HA-tagged Irx4 (rabbit IgG) were incubated with the blots to determine which proteins co-precipitated. Western blots were performed as reported previously (23). horseradish peroxidase-conjugated goat against rabbit IgG or rat IgG secondary antibodies were used for detection.

**Irx4 Deletion Constructs**—Deletions of the RCAS-Irx4 vector were made via site-directed polymerase chain reaction cloning. Primers with unique restriction site sequences were used to amplify selected regions of the RCAS-Irx4 vector, which were cloned into the pcDNA3.1/His C expression vector (Invitrogen) using standard protocols (25). The Δ5′ construct lacks 5′ sequence encoding amino acids 2–134 of Irx4 but retains the remaining 3′ sequence including the homeodomain; the Δ3′ construct lacks 3′ sequence encoding amino acids 269–515 but contains the remaining 5′ sequence including the homeodomain of Irx4; the ΔHD construct lacks sequence encoding amino acids 135–268 containing the homeodomain while retaining 5′ and 3′ sequence as a fused product; and the Δ5′Δ3′ construct contains sequence encoding amino acids 135–268, containing primarily the homeodomain. Care was taken to ligate Irx4 cDNA sequences into the expression vector such that in-frame protein products were formed.

**RESULTS**

The 840 bp immediately upstream of the *slow MyHC3* transcriptional initiation site is sufficient to drive correct atrial-specific expression of a reporter gene both in mammals and in birds (23, 29). We have previously demonstrated that a VDRE within this SM3CAT:840D construct is the essential element required for ventricular-specific down-regulation in *in vitro* and in vivo (23, 25). Recently, an *Iroquois* family homoeobox gene, Irx4, was shown to regulate chamber-specific expression of myosin isoforms by suppressing the expression of the AMHC1/*slow MyHC3* gene and activating the expression of the VMHC1 in the ventricles (30).

**Irx4 Acts at the VDRE to Inhibit Expression in Atrial Cardiomyocytes**—That Irx4 acts through the VDRE was demonstrated by a series of co-transfections into atrial cardiomyocytes of an Irx4 expression vector, RCAS-Irx4, along with SM3CAT:840D and deletion derivatives. Expression of Irx4 reduced expression of the CAT reporter from co-transfected SM3CAT:840D by nearly 100% (Fig. 1). A similar reduction in reporter expression was observed following deletion of *slow MyHC3* sequence upstream of the VDRE (SM3CAT:808D).

However, when the VDRE sequence was removed (SM3CAT: 768D), Irx4 expression no longer reduced CAT expression in atrial cardiomyocytes (Fig. 1). These results identify a 40-bp region of the *slow MyHC3* promoter, denoted as the site of Irx4 action.

These results were refined by expression of Irx4 in atrial cardiomyocytes co-transfected with shorter regions of the *slow MyHC3* promoter, ARD1 (23). We have previously shown that the sequence between positions −840 and −680, designated as ARD1 (see lower panel in Fig. 2) serves as an atrial-specific enhancer both in *in vitro* and in *ovo* (23). Irx4 suppressed reporter expression from both ARD1:CAT and VDRE-GATA:CAT (sequence containing only the VDRE and GATA binding site elements up-stream of a minimal SV40 promoter) (Fig. 2). However, when the VDRE was removed (GATA:CAT) expression of Irx4 had no effect on reporter expression. A ubiquitously expressed SV40:CAT was unaffected by Irx4 expression. These observations suggest that a functional inhibitory complex can form in atrial cardiomyocytes at the VDRE if Irx4 is present.

**RCAS-H + e′ Acts at the VDRE to Relieve Inhibition in Ventricular Cardiomyocytes**—To investigate the role of Irx4 in ventricular cardiomyocytes, Bao and co-workers (30) infected avian embryos prior to heart formation with a putative dominant-negative form of Irx4, designated RCAS-H + e′, a construct that encodes a fusion protein composed of the chick Irx4 homeodomain and the repressor domain of the *Drosophila* Engrailed protein. RCAS-H + e′ prevented the normally occurring down-regulation of *slow MyHC3* in the ventricles of infected embryos as cardiac chamber formation proceeded (30).

To identify the promoter element required for the action of the
RCAS-H\(^{1}\)enr fusion protein, it was co-transfected with the SM3CAT:840D construct into ventricular cardiomyocytes (Fig. 3). RCAS-H\(^{1}\)enr significantly increased CAT expression from SM3CAT:840D in ventricular cardiomyocytes, indicating that Irx4 has a target site(s) within the 840-bp promoter. Co-transfection of RCAS-H\(^{1}\)enr also up-regulated CAT expression from the SM3CAT:808D construct. However, when the VDRE element was deleted, but the GATA site retained (SM3CAT:768D), inhibition was lost in ventricular cardiomyocytes whether RCAS-H\(^{1}\)enr was present or not (Fig. 3). These results demonstrate that the element(s) required for Irx4 action is located between positions \(-808\) and \(-768\), a region that contains the VDRE element.

The site of Irx4 action in ventricular cardiomyocytes was refined by co-transfection of RCAS-H\(^{1}\)enr with ARD1:CAT and deletions derived from it. Co-transfection of ARD1:CAT with RCAS-H\(^{1}\)enr into ventricular cardiomyocytes resulted in a 3-fold increase in reporter expression (Fig. 4). Co-transfection of VDRE-GATA:CAT (containing only the VDRE and the GATA elements) with RCAS-H\(^{1}\)enr also increased CAT expression in ventricular cardiomyocytes (Fig. 4). However, expression of the GATA:CAT construct, in which the VDRE sequence had been removed, was not affected by RCAS-H\(^{1}\)enr expression (Fig. 4). The ubiquitously expressed SV40:CAT control was unaffected by co-transfection with RCAS-H\(^{1}\)enr. These results, together with the results of RCAS-Irx4 transfection of atrial cardiomyocytes, localize the Irx4 site of action to the VDRE element, the previously identified inhibitory sequence (23, 24), and indicate that in either atrial or ventricular cardiomyocytes an inhibitory complex can form if Irx4 is present.

Association of Irx4 and RXRa Proteins—Previous studies have shown that both RXRa and VDR proteins bind to the VDRE to influence slow MyHC3 expression (23). However, because both classes of nuclear hormone receptors are expressed in both atria and ventricles we postulated that a ventricle-specific transcriptional repressor, acting at the VDRE, was responsible for inhibition of slow MyHC3 expression (23, 24). The demonstration that Irx4 was required for down-regulation of slow MyHC3 expression (30) made it the likely candidate to act at this site. Because Irx4 is a homeobox-containing transcription factor, and these factors are known DNA-binding proteins, binding of Irx4 directly to the VDRE was tested. In vitro translated Irx4 consistently failed to bind the VDRE in mobility shift assays (negative data not shown) while the same preparation was active in a co-immunoprecipitation assay (see below). This suggests that Irx4 does not exert its effect through directly binding to the VDRE.
Immunoprecipitation experiments. A characteristic doublet of Irx4 was detected in the precipitate using an anti-HA antibody (Fig. 6B, lane 1). As a control, a rabbit IgG antibody added to the Irx4 and RXRα protein mix did not result in a precipitate that contained Irx4 (Fig. 6B, lane 2). Conversely, when the HA-tagged Irx4 and RXRα proteins were combined and immunoprecipitated with the anti-HA antibody, RXRα was detected in the precipitates by the anti-RXRα antibody in the Western blot (Fig. 6B, lane 3). Immunoprecipitation of the Irx4 protein complex by anti-HA was specific because a control rabbit IgG antibody that does not recognize Irx4 did not result in a precipitation of RXRα when added to the Irx4 and RXRα protein mix (Fig. 6B, lane 4). These results confirm that Irx4 and RXRα proteins can physically interact. In contrast an antibody against the HA-tag of Irx4 failed to precipitate VDR, and an antibody against VDR did not co-immunoprecipitate Irx4 (Fig. 6B, lanes 5 and 6). These experiments support the idea that a protein-protein interaction between Irx4 and RXRα, rather than between Irx4 and VDR, mediates the inhibition of slow MyHC3 via the VDRE.

The Amino-terminal of Irx4 Mediates Inhibition—A structure-function analysis was performed to identify the region(s) of the Irx4 protein required for its inhibitory function. Deletions of the Irx4 cDNA were made, inserted in frame in a eucaryotic expression vector, and co-transfected with the SM3CAT:840D reporter into atrial or ventricular cardiomyocytes (Fig. 7). Atrial cardiomyocytes have no endogenous Irx4, and no significant inhibition of reporter expression was observed from amino-terminal-truncated Irx4 (Δ5’), homeodomain-truncated Irx4 (ΔHD), or the Irx4 homeodomain alone (Δ3’–Δ5’). However, a significant (8- to 10-fold) inhibition of reporter expression was observed from a carboxyl-terminal-truncated Irx4 (Δ3’). These results demonstrate that the amino-terminal of Irx4 is required for inhibition (Fig. 7).

The importance of the amino-terminal was reinforced by co-transfection of SM3CAT:840D and the amino-terminal-truncated Irx4 (Δ5’) into ventricular cardiomyocytes. Against a background of endogenous Irx4, this construct was able to partially relieve inhibition of the reporter (Fig. 7). In contrast, no significant relief from inhibition was observed in ventricular cardiomyocytes expressing carboxyl-terminal-truncated Irx4 (Δ3’), homeodomain-truncated Irx4 (ΔHD), or the Irx4 homeodomain alone (Δ3’–Δ5’), indicating that the inhibition of slow MyHC3 expression in the ventricle by Irx4 is mediated via the VDRE. Three pieces of evidence suggest that Irx4 acts through the VDRE element rather than the positive regulatory region.

**DISCUSSION**

We have previously shown that atrial-specific expression of the slow MyHC3 gene is positively regulated by a GATA factor-binding element, essential for cardiac-specific expression, and a negative-acting VDRE element, required to prevent expression in the ventricle (23–25, 29). These two elements are sufficient to drive atrial-specific expression of a reporter construct in primary cardiomyocyte cultures in vitro and in transgenic mice (23, 29). Subsequently, a ventricle-restricted homeodomain protein, Irx4, was discovered, that was capable of regulating cardiac chamber-restricted expression of myosin heavy chain genes including the chicken homologue of slow MyHC3, AMHC1 (30–32). In the current study we demonstrate that the inhibition of slow MyHC3 expression in the ventricle by Irx4 is mediated via the VDRE. Three pieces of evidence suggest that Irx4 acts through the VDRE element rather than the positive GATA element. First, misexpression of Irx4 in atrial cardiomyocytes down-regulates expression of a reporter construct containing the VDRE (VDRE-GATA:CAT) but has no effect on a reporter construct with only the GATA element (GATA:CAT). Second, a dominant-negative form of Irx4, previously shown to up-regulate expression of AMHC1/slow MyHC3 in the ventri-
cles in vivo (30), relieved inhibition of the VDRE-GATA:CAT reporter in ventricular cardiomyocytes but had no effect on expression of the GATA:CAT, which lacks the VDRE element.

Third, Irx4 interacts with the RXRα subunit of the heterodimeric complex of VDR/RXRα that binds at the VDRE element of the slow MyHC3 promoter. Homeobox-containing proteins found in cardiac tissue have been viewed as DNA-binding transcription factors (8, 35–37); accordingly, we investigated whether Irx4 could directly bind the VDRE sequence of slow MyHC3. Electrophoretic mobility shift assays consistently failed to detect an Irx4 binding activity to the VDRE and surrounding sequence (negative data not shown), under conditions where the VDR/RXRα clearly bound (25). A DNA motif, TTAATTAA, to which Iroquois family members are likely to bind, has been reported (38). No sequence similar to this motif is present in or around the VDRE nor within the 840-bp promoter fragment capable of directing atrial-specific expression of the slow MyHC3 gene. Although no high affinity binding of Irx4 to the VDRE site was detected, the possibility can not be excluded that binding of a RXRα/VDR heterodimer to the VDRE element facilitates binding of Irx4 to a weak affinity DNA site somewhere in the 40-bp fragment of the slow MyHC3 promoter between −808 and −768. The absence of an Irx4 binding motif in the slow MyHC3 promoter has prompted others to suggest that the mechanism of Irx4 action is via protein-protein interaction (32).

Our previous results indicated that binding of a VDR/RXRα heterodimer to the VDRE is required for down-regulation of slow MyHC3 expression in the ventricle (23). Because no consistent difference in protein expression levels of VDR or RXRα was detectable between atrial and ventricular nuclear extracts (23), it is unlikely that Irx4 acts directly or indirectly to up-regulate the levels of VDR or RXRα proteins in the ventricles. Two-hybrid and co-immunoprecipitation assays, demonstrated that Irx4 can physically interact with RXRα but not VDR. Co-immunoprecipitation further demonstrated that this interaction is strong enough to withstand the isolation process. An antibody against RXRα co-immunoprecipitated Irx4 when both proteins were expressed in vitro, while an anti-VDR antibody did not co-immunoprecipitate Irx4 when VDR and Irx4 were co-expressed. Conversely, an antibody against the HA-tag of Irx4 co-immunoprecipitated RXRα but not VDR. Thus, the

![Figure 6](https://www.jbc.org/content/288/11/28839/f6.large.jpg)

**FIG. 6.** There is a physical association between Irx4 and RXRα in vitro. A, in vitro translated Irx4 (HA-tagged), RXRα, or VDR proteins were resolved by SDS-PAGE and detected on immunoblot by anti-HA, anti-RXRα, or anti-VDR antibodies, respectively. B, in vitro translated Irx4 (HA-tagged) and RXRα were mixed and immunoprecipitated (IP) by anti-RXRα or anti-HA antibody. The immune complexes were resolved by SDS-PAGE and analyzed by immunoblot (IB), showing that Irx4 co-precipitates with RXRα. In vitro translated Irx4 (HA-tagged) and VDR were mixed and immunoprecipitated by anti-HA or anti-VDR antibody (Lanes 1 and 3). Specificity was demonstrated by the failure of a rabbit IgG to immunoprecipitate Irx4 (Lane 2) or RXRα (Lane 4). In contrast, VDR did not co-immunoprecipitate with Irx4 (Lanes 5 and 6). The immunoprecipitating antibodies (anti-RXRα and anti-HA) separated from antigen by SDS-PAGE are rabbit IgGs and are visible as the lower band in Lanes 1–4. Molecular masses in kDa are shown to the right of the blot.

![Figure 7](https://www.jbc.org/content/288/11/28839/f7.large.jpg)

**FIG. 7.** The amino end of Irx4 is essential for inhibition. The slow MyHC3 reporter construct, SM3CAT:840D, was transfected alone (no Irx4 construct) or co-transfected with the Irx4 deletion constructs Δ5', Δ3', ΔHD, and Δ3' Δ5' into atrial or ventricular cardiomyocytes. It should be noted that endogenous Irx4 is present in the ventricular but not the atrial cardiomyocytes. In atrial cardiomyocytes, the Δ3' construct inhibited reporter expression while none of the other Irx4 deletion constructs had an effect. In ventricular cardiomyocytes, only deletion of the 5' end of Irx4 (Δ5') affected SM3CAT:840D expression, resulting in a partial relief of inhibition of reporter expression.
inhibitory action of Irx4 is likely to be mediated via a protein-protein interaction of Irx4 with RXRα.

A region of the Irx4 protein encompassing a portion of the amino terminus and part of the homeodomain is important in the inhibition. An amino-terminal truncation of the Irx4 protein, (Δ5′), relieved inhibition in ventricular cardiomyocytes. Because ventricular cardiomyocytes express the native Irx4 protein, the relief of inhibition afforded by Δ5′ was minimal (2-fold). While expression of Irx4 proteins with a deletion of either the amino-terminal (Δ5′) or the homeodomain (ΔHD) had no effect in atrial cardiomyocytes, a deletion of the carboxyl-terminal (Δ3'), which retains the amino-terminal and the homeodomain, significantly inhibited expression of a co-transfected slow MyHC3 reporter construct. That the DNA-binding homeodomain by itself (Δ3′Δ5′) or with the carboxyl portion of the protein present (Δ5′) did not inhibit reporter expression in atrial cells provides additional evidence that Irx4 works via protein-protein interaction rather than by binding the slow MyHC promoter at, or near, the VDRE.

Fusion of the engrafted repressor to Irx4 created a protein that relieved inhibition of the chicken slow MyHC3 homologue (Ref. 30 and this study), making it likely that the homeodomain also contributes to the inhibitory action of Irx4. Exactly how this dominant negative construct functions in the assays presented here or in vivo is not clear. Bao and co-workers (30) postulated that fusion of the DNA homeodomain with the repressor domain of Engrailed created a protein that interfered with transcriptional activation by the wild-type protein, thus producing a dominant negative effect as well as a potential gain-of-function effect due to active repression. On the one hand, the engrafted-derived moiety may be acting by sterically interfering with the normally occurring Irx4/RXRα protein-protein interaction. Although we showed that Irx4 and RXRα interact, it remains possible that, in vivo, Irx4 acts indirectly to up-regulate transcription of a repressor at an as yet unidentified locus. Clearly, if there were such a repressor, it must act through the VDRE of the slow MyHC3 gene in the ventricle to regulate slow MyHC3 gene expression. Were this the case, then it must also be postulated that the dominant negative Irx4-engrafted protein inhibits expression from the unidentified locus. Although this hypothesis is a possibility, based on the data here, a more parsimonious explanation favors a model where Irx4 interacts directly with RXRαs to down-regulate slow MyHC3 gene expression in the ventricle.

Precedence for inhibitory co-factors in cardiomyocytes is found in the literature. A co-factor that regulates ventricle-specific expression of MLC-2v has been reported by Zou and co-workers (39). A 28-bp sequence containing HF-1a and MEF-2 elements within the MLC-2v promoter region confers ventricular chamber-specific gene expression in transgenic mouse (40). A nuclear ankyrin-like repeat protein, CARP (cardiac ankyrin repeat protein), which acts as a cofactor of the HF-1a-binding protein, YB-1, displays a transcriptional inhibitory activity in cardiomyocytes (39, 41). Interestingly, upstream of the VDRE, the slow MyHC3 promoter contains a HF-1a-like motif. However, this region of the slow MyHC3 promoter is not essential for chamber-specific expression of the gene (Ref. 25, and this study).

Homeobox-containing proteins often rely on co-factors for DNA binding and for regulation of transcription (42–45). Cooperative protein-protein interaction between a homeodomain protein and a nuclear hormone receptor to regulate gene expression has been reported in Drosophila embryos (43). Ftz-F1, a member of the nuclear hormone-receptor superfamily, serves as a co-factor to facilitate the binding of the homeodomain protein, Fushi tarazu (Ftz), to weak affinity DNA sites (43).

However, few other systems demonstrate that protein-protein interactions mediate the action of homeodomain proteins in the absence of DNA binding.

Mechanisms for chamber-preferential expression of α-MyHC, β-MyHC, and ANF during embryonic development are not clear. Interestingly, another member of the nuclear hormone receptor superfamily, thyroid hormone receptor, is also implicated in the regulation of myosin heavy chains. Expression of α-MyHC and β-MyHC, the two major isoforms of myosin heavy chains in the rodent myocardium, changes in the ventricles in response to developmental changes in thyroid hormone (46, 47). Whether Irx4 binds cooperatively with thyroid hormone receptor to regulate expression of α-MyHC or β-MyHC is not known. Although an inhibitory element is found in the ANF promoter, the mechanism by which the inhibitory element suppresses ANF expression in the ventricle is unclear (36).

Previously we proposed an activation model and an inhibition model to depict regulation of chamber-specific expression of cardiac genes during embryonic development (24). Ventricle-specific expression and inhibitory function of Irx4 fit well with the inhibition model for atrial-specific expression. However, Irx4 is not only an inhibitor of slow MyHC3/AMHC1 but also an activator of VMHC1 in the ventricles (30). The activation model fits ventricular chamber-specific activation of VMHC1 by Irx4. How Irx4 acts as an inhibitor of one gene and an activator of another within the same cell type is not known. Because different homeobox-containing proteins have similar DNA-binding functions in vitro and require cofactors to achieve their biological functions (42), cooperative interaction between Irx4 and a cofactor or direct binding of Irx4 to a target site in the VMHC1 promoter could activate VMHC1 expression in ventricular cardiomyocytes. Investigation of how Irx4 regulates cardiac genes will continue to broaden our understanding of diversification between atrial and ventricular cardiomyocytes during development.

Acknowledgments—We are grateful to Dr. Elizabeth Allegruito for providing the rabbit polyclonal anti-RXRα antibody. Sandra Conlon provided excellent technical assistance, and Gloria Garcia provided excellent assistance in preparation of the manuscript. Dr. Gordon Cann provided helpful discussions.
Irx4 Inhibits Ventricular Expression of Slow MyHC3

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J. Biol. Chem. 2001, 276:28835-28841.
doi: 10.1074/jbc.M103716200 originally published online May 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103716200

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