CARP, a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway

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SUMMARY

To identify the molecular pathways that guide cardiac ventricular chamber specification, maturation and morphogenesis, we have sought to characterize factors that regulate the expression of the ventricular myosin light chain-2 gene, one of the earliest markers of ventricular regionalization during mammalian cardiogenesis. Previously, our laboratory identified a 28 bp HF-1a/MEF-2 element in the MLC-2v promoter region, which confers cardiac ventricular chamber-specific gene expression during murine cardiogenesis, and showed that the ubiquitous transcription factor YB-1 binds to the HF-1a site in conjunction with a co-factor. In a search for interacting co-factors, a nuclear ankyrin-like repeat protein CARP (cardiac ankyrin repeat protein) was isolated from a rat neonatal heart cDNA library by yeast two-hybrid screening, using YB-1 as the bait. CARP is localized in the cardiac myocyte nucleus. Co-transfection assays indicate that CARP can negatively regulate an HF-1-TK minimal promoter in an HF-1 sequence-dependent manner in cardiac myocytes, and CARP displays a transcriptional inhibitory activity when fused to a GAL4 DNA-binding domain in both cardiac and noncardiac cell context. Northern analysis revealed that carp mRNA is highly enriched in the adult heart, with only trace levels in skeletal muscle. During murine embryogenesis, endogenous carp expression was first clearly detected as early as E9.5 specifically in heart and is regulated temporally and spatially in the myocardium. Nkx2-5, the murine homologue of Drosophila gene tinman was previously shown to be required for heart tube looping morphogenesis and ventricular chamber-specific myosin light chain-2 expression during mammalian heart development. In Nkx2-5−/− embryos, carp expression was found to be significantly and selectively reduced as assessed by both whole-mount in situ hybridizations and RNase protection assays, suggesting that carp is downstream of the homeobox gene Nkx2-5 in the cardiac regulatory network. Co-transfection assays using a dominant negative mutant Nkx2-5 construct with CARP promoter-luciferase reporter constructs in cardiac myocytes confirms that Nkx2-5 either directly or indirectly regulates carp at the transcriptional level. Finally, a carp promoter-lacZ transgene, which displays cardiac-specific expression in wild-type and Nkx2-5−/− background, was also significantly reduced in Nkx2-5−/− embryos, indicating that Nkx2-5 either directly or indirectly regulates carp promoter activity during in vivo cardiogenesis as well as in cultured cardiac myocytes. Thus, CARP is a YB-1 associated factor and represents the first identified cardiac-restricted downstream regulatory gene in the homeobox gene Nkx2-5 pathway and may serve as a negative regulator of HF-1 dependent pathways for ventricular muscle gene expression.

Key words: mouse, heart development, ankyrin repeats, homeobox gene, tinman, Nkx2-5, YB-1, transcription regulation, myosin light chain-2v

INTRODUCTION

Cardiac muscle progenitor cells originate from the lateral splanchnic mesoderm in vertebrates, migrate anterolaterally, fuse to form a single cardiac tube at the ventral midline and then undergo an elaborate series of steps of differentiation and morphogenesis to form a functional heart (Garcia-Martinez and Schoenwolf, 1993; Rosenquist and DeHaan, 1996; Sater and Jacobson, 1990; Wilens, 1995). Despite the extensive knowledge at the embryological level, the molecular mechanisms of the complex process of cardiogenesis is relatively unclear. In this regard, the specification of distinct atrial and ventricular chambers is one of the critical steps during vertebrate cardiogenesis. To identify molecular pathways that guide this process, we have utilized the ventricular specific myosin light chain-2 (MLC-2v) gene as a model system, which is the earliest ventricular chamber-specific gene expressed during mammalian cardiogenesis (O'Brien et al., 1993). Using
a combination of transient assays and transgenic approaches, we have recently identified a 28-bp minimal cis-element (HF-1), which contains two adjacent (HF-a and MEF-2) sites, which confer cardiac and ventricular chamber-specific expression in an anterior-posterior gradient (Zhu et al., 1991; Navankasuttras et al., 1992, 1994; Ross et al., 1996). The ubiquitous transcription factor YB-1 has been shown to bind to the HF-a site and can regulate MLC-2v promoter activity in co-transfection assays (Zou and Chien, 1995). Biochemical studies have demonstrated that YB-1 has a cofactor and it is this complex that occupies the HF-a site (Zou and Chien, 1995). As such, the identification of YB-1-associated proteins becomes critical in elucidating the pathways of ventricular specification.

In the current study, we report the isolation and initial characterization of a YB-1-associated protein that is cardiac restricted early during murine cardiogenesis and which contains ankyrin repeats. CARP is a nuclear factor and has negative transactivation activity. In situ analysis reveals that carp is expressed abundantly and specifically in the early embryonic heart, supporting an important role during cardiogenesis. Using both in situ and RNase protection analyses, carp expression was found to be dramatically and selectively reduced in embryos that carry a targeted interruption of the tinman homologue required for ven-tricular chamber specification and morphogenesis. Co-transfection assays using an Nkx2-5 dominant-negative cDNA construct suggest that Nkx2-5 can regulate the carp gene activity at the transcriptional level. A carp promoter-lacZ transgene, which recapitulates the cardiac-specific expression of the endogenous gene, is significantly reduced in the Nkx2-5−/− background, providing additional evidence that Nkx2-5 either directly or indirectly regulates the carp promoter during in vivo cardiogenesis. Thus, carp is downstream in the Nkx2-5 homeobox regulatory network and may act as a negative regulator of HF-1-dependent pathways for ventricular muscle gene expression. As such, these studies suggest that Nkx2-5 may simultaneously activate positive and negative regulators to titrate the level of expression of specific subsets of cardiac muscle genes.

**MATERIALS AND METHODS**

**Isolation of YB-1-binding proteins by the yeast two-hybrid system**

To isolate YB-1-associated proteins, we utilized the two-hybrid interaction trap system developed by the laboratory of Dr Roger Brent (Gyuris et al., 1993). Briefly, poly(A) RNA was isolated from a total RNA preparation from rat neonatal ventricular chamber and the first strand cDNA was synthesized using the Moloney virus reverse transcriptase with an oligo(dT) primer including an XhoI site. After second strand cDNA synthesizes, the 5′ ends were ligated to an EcoRI adapter and the cDNA inserts were ligated into the vector pGEX-4T-1 yielding 1.3 million independent clones. 92% of the cDNA clones from the library contained inserts with an average of 1.0 kb and ranged from 500 base pair to several Kbs. 1.3 million clones were screened using a YB-1-LexA fusion protein as the bait construct, following previously described procedures (Gyuris et al., 1993). 400 positive clones were obtained during leucine minus selection and 26 positive clones displayed blue color during the lacZ selection. Four of these positive clones were identical and encoded a c-terminal region of carp. To assess tissue distribution of CARP expression, northern blot analysis was performed (Clontech) and exposed for 4 days with double intensifying screens and at −80°C. The full-length rat carp cDNA was cloned by screening a rat neonatal heart λgt11 cDNA library with the c-terminal region of carp as a probe.

**Expression and purification of a GST-CARP fusion protein, generation of CARP antibodies and immunoprecipitation analyses**

The carp cDNA was subcloned into the pGEX vector (Pharmacia) and expressed in the BL21 strain. After IPTG induction, the bacterial cells were harvested and lysed by several freeze-thaw cycles, followed by sonication. The GST-CARP fusion protein was purified from the bacterial lysate by glutathione beads and injected into rabbits to generate polyclonal antisera. Bleeds were tested at various dilution by western blotting. Immunoprecipitation was performed as previously described (Zou and Chien, 1995). 10 μg of either the GST protein alone or the GST-CARP protein were added to 1 ml of the cardiac myocyte whole-cell extracts and incubated at 4°C for 2 hours with gentle shaking. Glutathione-sepharose beads (Pharmacia) were added to the tube and incubated for 1 hour. The beads were washed three times with lysis buffer and the GST or GST-CARP fusion proteins were eluted with 10 mM glutathione under native conditions, followed by SDS-PAGE and western blotting.

**In situ hybridization analyses**

The nucleotide sequence of the mouse and rat carp cDNA was 97% identical, including the 5′ and 3′ noncoding region. The 1700 base pair rat carp fragment was subcloned into pRC/CMV via a NotI site in the sense and antisense orientations downstream of the T7 promoter. The template constructs were linearized with XhoI. Digoxigenin labeling was performed according to Boehringer Mannheim. Alkaline hydrolysis was carried out to reduce the probe size to an average of 250 base pairs. Analogous studies were performed with the MLC-2a probes, prepared as previously described (Kubalak et al., 1994). A fragment of rat YB-1 cDNA (500 bp at the C-terminal cDNA coding region) was amplified by PCR subcloned into pRc/CMV via NotI and sequence verified. The rat and mouse YB-1 cDNA display 99% sequence identity.

To generate the 35S-labeled riboprobe, in vitro transcription assays were carried out using 35S-UTP, instead of the Dig-UTP, with 10 mM DTT. cRNA probes were alkaline hydrolysed in a 20 mM NaHCO3/Na2CO3 (2:3) buffer with 10 mM DTT for 60 minutes at 60°C. Free nucleotides were removed by running through the neutralized hydrolysis reaction through a Sephadex G-50 spin column.

Timed pregnant mice were purchased from Harlan Sprague Dawley, Inc. Mouse embryos were isolated under a dissecting microscope and submerged in 1x PBS. The whole-mount in situ hybridization closely followed previously published methods (Wilkinson, 1992). For in situ with the embryonic sections, the timed mouse embryo paraffin-embedded sections were purchased from Novagen. For the 35S-labeled cRNA probe, we followed the protocol kindly provided by Dr Gary Lyons (Lyons et al., 1990, 1991a,b). For in situ with the Dig-labeled probe, we followed previously published methods (Becker et al., 1992; O’Donnell et al., 1989).

**RNase protection assays**

A 260 bp cDNA fragment of the rat carp was used for a RNase protection probe. In this region, the rat and mouse carp have 99% sequence identity. The E9.0 heart tube was dissected out to RNase protection assays using ‘Direct Protect’ kit from Ambion. After lysis of heart tube samples, they were divided equally to two tubes. One was hybridized to a carp probe and an EF1α probe, which serves as internal control; the other was hybridized to MLC-2a probe and EF1α probe as internal control. The MLC-2a and EF1α probes were prepared according to previously published information (Kubalak et al., 1994).
Cardiac myocytes were prepared by a percoll gradient technique and plated in gelatin-coated 6 cm plates at 10^5 cell/ml at plating medium (Sheng et al., 1996). After overnight incubation, cardiaic myocytes attached to the plates and the medium was switched to 4% horse serum for 3-5 hours prior to transfection. Co-transfection in cardiac myocytes was performed by calcium-phosphate precipitation, as initially described (Chen and Okajima, 1987) and subsequently modified (Zou and Chien, 1995). The luciferase activity was normalized to the β-gal activity to account for variations in the efficiency of transfection, as previously described (Zou and Chien, 1995). For the GAL4-CARP fusion construct, the reporter was a GAL4 DNA-binding site and CAT reporter (Clontech). CAT assays were carried out according to the method described by Promega, using the liquid scintillation counting.

Transgenic mice
A mouse carp genomic DNA clone was isolated using the rat carp cDNA as the probe and various lengths of the promoter were tested for cardiac-specific expression in early cardiac development (Chen, Kuo, and Zou, unpublished data). A 2.5 kb fragment can confer cardiac-specific expression in co-transfection assays. Transgenic lines were established using a 2.5 kb data. A 2.5 kb fragment can confer cardiac-specific activity (Chen, Kuo, Zou and Chien, unpublished results). Fluorescent microscopy.

Immunostaining
Primary cardiac myocytes were isolated from neonatal rat heart ventricular chambers and plated on the chamber slides precoated with 1% gelatin and laminin (Sigma). After culturing for 24 hours, the cells were rinsed in 1x PBS for 15 minutes, fixed in 4% paraformaldehyde for 10 minutes and neutralized in 50 mM NH4Cl (in PBS) for 5 minutes each. Cells were then washed twice with PBS, permeabilized and blocked in PBS, 1% BSA, 0.3% Triton X-100 for 15 minutes each. Cells were then washed twice with PBS, permeabilized and blocked in PBS, 1% BSA, 0.3% Triton X-100 for 15 minutes each. The slides were subsequently subjected to immunostaining.

RESULTS

Isolation, primary structure and tissue distribution of CARP
Using YB-1 as the bait in the yeast two-hybrid system, 1,3...
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Fig. 2. CARP is a YB-1-interacting protein. Lanes 1 and 2 are a co-immunoprecipitation of the radiolabeled cardiac myocyte extracts with a YB-1 antibody and its preimmune. The cardiac myocyte extracts were immunoprecipitated with 5 μl anti-YB-1 antiserum (Zou and Chien, 1993). The immunoprecipitated complex were run on an SDS-PAGE gel and exposed to X-ray film. Lane 1 is the preimmune control. Lanes 4, 5 are a western blot (with CARP antibody) of co-immunoprecipitated (with YB-1 antibody) unlabeled cardiac myocyte extracts. Lane 3 is the western blot of the cardiac whole-cell extracts, showing the endogenous CARP protein (40×10^3 M_r) and documenting the specificity of the antiserum. Lane 4 is the preimmune control of immunoprecipitation. CARP is found to be in the same complex as YB-1 (Lane 5). IgG is immunoglobulin visualized by the secondary antibody. Lanes 6 and 7 are co-immunoprecipitation of radiolabeled cardiac myocyte extracts with CARP antibody (2 μl). Lane 6 is the preimmune control. Lanes 9, 10 are western blot (with YB-1 antibody) of a GST-CARP pull down elutes. Lane 8 is the western blot of the whole-cell extracts, showing the size of the endogenous YB-1 protein and the specificity of the antibody. Lane 9 is a control with the GST protein only. Lane 10 shows YB-1 protein was pulled down by the GST-CARP fusion protein from cardiac myocyte extracts.

Fig. 3. Endogenous CARP is a nuclear protein. (A) Immunostaining of cardiac myocytes cultured in serum-containing medium using an anti-CARP antibody. (B) Immunostaining of cardiac myocytes cultured in serum-free medium using anti-CARP antibody. (C) Immunostaining of cardiac myocytes cultured in phenylephrine-containing medium using anti-CARP antibody. (D) Immunostaining of COS1 cells cultured in serum-containing medium using anti-CARP antibody. (E) Immunostaining of cardiac myocytes cultured in serum-containing medium using anti-YB-1 antibody. (F) Immunostaining of the same cardiac myocytes as E using monoclonal anti-myomesin antibody. (G) Immunostaining of cardiac myocytes cultured in serum-free medium using anti-YB-1 antibody. (H) Immunostaining of cardiac myocytes cultured in phenylephrine-containing medium using anti-YB-1 antibody.
northern blotting revealed that it is highly enriched in the adult heart (Fig. 1C), with a trace amount detected in skeletal muscle. As a control, the same northern blot was hybridized with the human β-actin probe, which detects an equal amount of signal in all tissues assayed (Fig. 1D). The expression of carp in murine lung tissue reflects the presence of atrial tissue, which invaginates the pulmonary vein in the adult mouse (Jones et al., 1994). Further evidence for the lack of expression in lung tissue has been revealed by analysis of carp knockout mice which harbor a knock-in of lacZ into the endogenous carp locus (Chen and Chien, unpublished results).

CARP is a YB-1-associated nuclear protein in cardiac myocytes

To independently confirm that CARP is a YB-1-binding protein, we performed in situ hybridization experiments using carp antisense probes. (A) Whole-mount in situ hybridization using the antisense carp probe. +/+ and −/−, wild-type littermate; −/−, carp knockout mutant embryos at the Nkx2-5 locus. (B) Whole-mount in situ hybridization using the antismyosin light chain 2 probe. (C) A higher magnification of the mutant embryo showing that the CARP message at the anterior portion of the heart tube is reduced even more than that at the posterior portion of the heart tube. (D) A higher magnification of a mutant embryo from a different litter, showing the same anterior-posterior gradient of CARP message in the mutant heart tube. ct: conotruncus.

Fig. 5. Endogenous carp expression is dependent on a cardiac-enriched homeobox gene Nkx2-5, the murine homologue of the Drosophila tinman gene. (A) Whole-mount in situ hybridization using the antisense carp probe. +/+ and −/−, wild-type littermate; −/−, carp knockout mutant embryos at the Nkx2-5 locus. (B) Whole-mount in situ hybridization using the atrial myosin light chain 2 probe. (C) A higher magnification of the mutant embryo showing that the CARP message at the anterior portion of the heart tube is reduced even more than that at the posterior portion of the heart tube. (D) A higher magnification of a mutant embryo from a different litter, showing the same anterior-posterior gradient of CARP message in the mutant heart tube. ct: conotruncus.
protein in native cardiac myocytes, we employed biochemical analyses using immunoprecipitation. As assessed by co-immunoprecipitation and GST-CARP pulldown assays, YB-1 and CARP formed a stable complex in rat cardiac myocytes, suggesting that CARP is an endogenous YB-1-binding protein. Using an anti-YB-1 antibody (Fig. 2, lane 2), we detected a 40×10^3 M_r band (the size of CARP by in vitro translation, data not shown), in addition to the YB-1 band, in the co-immunoprecipitation complexes from [35S]methionine-labeled cardiac myocyte extracts. In the preimmune control, the 40×10^3 M_r band was not detected, documenting the specificity of the antibody (lane 1). Using the anti-CARP antibody, CARP protein was detected in the co-immunoprecipitation complex by the anti-YB-1 antibody (lane 5). As shown in Fig. 2 (lane 7), a band corresponding to the expected size of YB-1 was observed, in addition to the CARP band, in the co-immunoprecipitation complex from the [35S]methionine-labeled cardiac myocyte extracts, using the anti-CARP antibody. A strong band with a relative molecular mass of 65-68×10^3 M_r was observed in the co-immunoprecipitation complex (lane 7). The identification of this protein is currently in progress. Since the YB-1 protein has a similar migration rate to the IgG, the purified GST-CARP fusion protein was added to the cardiac myocyte extracts to pull down interacting proteins. The anti-YB-1 antibody detected the YB-1 band in the interacting complex by western blotting (lane 10). It should be noted that the conditions to assess YB-1-CARP protein-protein interactions were performed at near physiological conditions to represent the endogenous cell context. Thus, these data indicate that CARP forms a stable physical complex with the YB-1 protein within cardiac myocytes. Yeast two hybrid assays revealed that the ankyrin repeat domain of CARP was sufficient to mediate the interaction with YB-1, thereby providing further support for the specificity of the YB-1/CARP interaction (data not shown).

As noted above, the deduced amino acid sequence of CARP contained a consensus nuclear localization sequence. To determine the subcellular localization of the CARP protein, we performed immunolocalization studies in cardiac myocytes. The anti-CARP antibody revealed that the endogenous CARP protein was localized in the nucleus of cardiac myocytes (Fig. 3A). A nuclear signal was not detected in a noncardiac cell line COS1 (Fig. 3D) and after staining of cardiac myocytes with preimmune serum control (data not shown). In addition, a strong signal enriched in the nucleus was not detected in cardiac myocytes in serum-free medium (Fig. 3B). The CARP protein levels in cells cultured in serum-free medium or serum-containing medium did not show any significant difference when equal amount of whole-cell extracts were analyzed by western analysis (Zou and Chien, unpublished data). In contrast, the YB-1 protein was always found in the nucleus in cardiac myocytes in serum-free medium (Fig. 3C). To test whether YB-1 was also expressed in the heart at early stages, we performed in situ hybridization with a YB-1 probe. Fig. 4F shows that YB-1 was highly expressed in the myocardium of the cardiac tube at E8.0 with a much lower level in pericardium and the neural tube (except the floorplate). At E10.0, YB-1 was also strongly expressed in the trabeculated myocardium. In fact, heart is one of the tissues that displays the highest level of YB-1 expression at E10 (Fig. 4H). YB-1 was also expressed highly in the E9.0 heart and, interestingly, shows a strong signal in the dermamyotome and a much weaker signal in the sclerotome suggesting that YB-1 might be important to the skeletal muscle development as well (Fig. 4G). Thus, YB-1 is expressed at a high level in embryonic heart, thereby documenting co-localization of CARP and YB-1 during cardiogenesis.

carp is significantly and selectively reduced in the Nkx2.5<sup>−/−</sup> background

In Drosophila, cardiac mesoderm forms a simple tube containing myocytes within the dorsal vessel (Bodmer, 1995). The homeobox gene tinman is expressed in the dorsal vessel, and a tinman null mutation results in the loss of the dorsal vessel and its progenitors (Bodmer, 1993). Vertebrate homologues of tinman have been identified in mouse (Lints et al., 1993; Komuro and Izumo, 1993) Xenopus (Tonissen et al., 1993; Evans et al., 1995) zebrafish (Lee et al., 1996) and chick.
(Schultheiss et al., 1995), all of which display highly restricted expression in early cardiac mesoderm. A knockout of the mouse homologue Nkx2-5 reveals a specific loss of the ventricular myosin light chain-2 gene expression and results in an arrest of cardiac tube looping morphogenesis, with embryonic lethality around E9.5-E10.0 (Lyons et al., 1995). In contrast to MLC2v, expression of several other cardiac muscle genes was found to be unaffected: α myosin heavy chain, β myosin heavy chain, myosin light chain-2a, cardiac actin, myosin light chain-1a, myosin light chain-1v and troponin I, documenting the specific relationship with MLC-2v (Lyons et al., 1995). Since CARP interacts with a component of the HF-1a/MEF-2 pathway of ventricular specificity and displays an early cardiac-restricted pattern of expression during early stages of cardiogenesis, it became of interest to determine if CARP expression would require Nkx2-5. As displayed in Fig. 5A, carp expression was dramatically reduced in the Nkx2-5−/− embryos, as indicated by whole-mount in situ hybridization. As a control, whole-mount in situ hybridization with the riboprobe of myosin light chain-2a was performed in parallel. As previously reported, (Lyons et al., 1995), the level of MLC-2a expression was unchanged (Fig. 5B), indicating that the reduction of carp signal is not simply due to a generalized loss of cardiac tissue in the Nkx2-5−/− background. RNase protection assays using a carp probe confirmed the specific decrease of expression using RNA derived from either the wild-type or the Nkx2-5−/− heart tubes at E9.0 (Fig. 6). It is noted that carp expression in the anterior portion of the Nkx2-5−/− heart tube, probably corresponding to the conotruncal portion of the outflow tract, displayed the lowest level of carp signal (below the level of detection) compared to the more posterior portions of the heart tube (Fig. 5C,D), which also display dramatically reduced carp expression compared to the wild-type heart tube. This suggests that carp expression at the rostral end of heart tube was particularly dependent on Nkx2-5. This alteration in the uniform expression of carp at E9.0 supported the notion that the loss of carp expression at the anterior region of the heart tube might contribute to the defect of cardiac looping morphogenesis.

**Fig. 6.** RNase protection assays confirm that carp mRNA is dramatically and specifically reduced in the Nkx2-5 knockout background. Lane 1 is free probes containing carp and EF1α. Lane 2 is wild-type E9 heart tube with carp and EF1α probes. Lane 3 is mutant E9 heart tube with carp and EF1α probes. Lane 4 is the same wild-type E9 heart tube as lane 2 with MLC-2a and EF1α probes. Lane 5 is the same mutant E9 heart tube as lane 3 with MLC-2a and EF1α probes. Lane 6 is free probes of MLC-2a and EF1α.

**Fig. 7.** Nkx2-5 regulates the carp promoter in cardiac myocytes. (A) Co-transfection assays in cardiac myocytes using a dominant negative Nkx2-5 mutant and the carp promoters. The cotransfection assays were carried out using 4 μg of carp promoter-luciferase reporter, 4 μg of Nkx2-5 expression construct and 1 μg SV40-lacZ internal control by calcium phosphate precipitation. (B) Nkx-2-5-Engrailed fusion protein represses the 2.5 kb carp promoter at various concentrations.

**Nkx2-5 can regulate carp at the transcriptional level**

To confirm that carp is downstream of Nkx2-5 and to test whether Nkx2-5 can regulate the carp gene at the transcriptional level, we utilized a CARP promoter construct in cotransfection assays employing a dominant negative-Nkx2-5 mutant construct. A 10 kb fragment of carp genomic DNA contained sequences which can confer cardiac-specific expression to a luciferase reporter in cultured cells (Chen, Kuo, Zou and Chien, unpublished results). Deletion studies also demonstrate that a 2.5 kb fragment still retains cardiac specificity in cultured cells, although the promoter activity decreases with larger deletions (Chen, Kuo, Zou and Chien, unpublished results). A fusion protein of Nkx2-5 with the inhibitory domain of Engrailed can inhibit the 10 kb and the 2.5 kb promoter activities over 80-85% in cardiac myocytes (Fig. 7A,B). The dominant negative Nkx2-5 mutant can functionally block the endogenous Nkx2-5 thus mimicking the loss of Nkx2-5 function in cardiac myocytes. The engrailed inhibitory domain alone displayed moderate inhibitory activity on the carp promoter constructs, although to a significantly less extent than the specific inhibition caused by the Nkx2-5-Engrailed fusion protein. Both the Engrailed-Nkx2-5 and the Engrailed only constructs contained the nuclear localization signal. All transfections were normalized by co-transfecting an unrelated SV40-LacZ control reporter. Taken together, these studies support the concept that carp is
downstream of Nkx2-5 and that the regulation is at the transcriptional level. Defining the precise Nkx2-5-responsive element in the carp promoter and defining the mechanism by which Nkx2-5 regulates the CARP promoter is currently in progress.

The carp promoter activity depends on Nkx2-5 during in vivo cardiogenesis

To independently test whether Nkx2-5 regulates the carp promoter at the transcription level during in vivo cardiogenesis, we employed a transgenic approach by generating transgenic lines with carp promoter-lacZ reporter genes. Transgenic mice lines harboring the 2.5 kb CARP promoter driving lacZ displayed cardiac-specific β-gal staining of the reporter, thereby mimicking the pattern of endogenous carp expression (Chen, Kuo, Zou and Chien, unpublished results). We subsequently crossed these transgenic lines into the Nkx2-5−/− background. As displayed in Fig. 8A, the carp promoter transgenes recapitulated the specific reduction of CARP message in the Nkx2-5−/− background, thereby providing further direct support for the conclusion that carp is downstream of Nkx2-5 in the heart regulatory network, as shown in the in vitro co-transfection assays. Moreover, while a residual amount of carp transgene expression can be detected in Nkx2-5 mutant embryos, the conotruncus region of the mutant heart was not expressing the transgene (Fig. 8B,C), which again recapitulates the specific loss of carp mRNA in the conotruncus region as assessed by whole-mount in situ (Fig. 5C,D). The β-gal-staining was clearly negative in the endocardium, either in wild-type or mutant embryos, which also agrees with the results that were obtained by in situ hybridization studies (Fig. 4). Fig. 8D-F displays the sectioning of the β-gal-stained embryos.

CARP functions as a negative transcriptional regulator in co-transfection assays

To test whether CARP has a positive or negative role in the regulation of HF-1-dependent pathways of transcriptional activation, we employed co-transfection assays with a CARP expression vector and a luciferase reporter gene driven by an HF-1 element fused to a minimal TK promoter, identical to the HF-1 element utilized in our previous studies that documented ventricular chamber-specific expression in transgenic embryos (Ross et al, 1996). As shown in a titration experiment (Fig. 9A), increasing the amounts of CARP expression vector resulted in a reduction of the HF-1-TK promoter activity in cardiac myocytes, as assessed by the luciferase reporter levels. In these studies, the activity has been normalized to β-galactosidase activity driven by an SV40 promoter as internal control. In a similar titration assay employing a CARP expression vector, CARP did not affect the minimal TK promoter itself, supporting the notion that the negative regulation by CARP was dependent on the HF-1 element (Fig. 9B).

To assess whether CARP contains an inhibitory domain, a construct encoding a GAL4 DNA-binding domain GAL4(DBD)-CARP fusion protein was expressed in both cardiac myocytes and noncardiac cells, COS1 with a GAL4 DNA-binding site-CAT reporter construct. In titration experiments (Fig. 9C,D), in both cell types, GAL4DBD-CARP fusion protein inhibited the Gal 4 reporter activity down to 20-25% of the initial reporter activity, suggesting that CARP contains a transcriptional inhibitory domain. These results are consistent with the negative function manifested by co-transfection assays with the HF-1 reporter noted previously. Taken together, these results suggest that CARP may serve as a negative regulator of HF-1-dependent pathways for ventricular muscle gene expression.
Fig. 9. CARP functions as a negative transcriptional regulator in co-transfection assays. (A) Co-transfection with CARP expression vector and the HF-1-TK minimal promoter construct in cardiac myocytes. Same amount of total DNA were used in each reaction, by including CMV expression vector without the CARP insert. (B) Co-transfection with CARP expression vector and the TK minimal promoter. (C) Co-transfection using GAL4 DNA-binding domain-CARP fusion protein expression vector and Gal4-CAT reporter in cardiac myocytes. Equal amounts of total DNA were used in each reaction, by including the GAL4 DNA-binding domain only expression vector. (D) Co-transfection using GAL4 DNA-binding domain CARP fusion protein expression vector and the Gal4-CAT reporter in COS1 cells.

**DISCUSSION**

**CARP is a YB-1-associated protein**

The formation of distinct atrial and ventricular chambers is a critical step during cardiogenesis. Currently, relatively little is known regarding the molecular and positional cues that dictate individual steps of cardiac chamber specification, maturation and morphogenesis. Studies employing ventricular muscle cell-fibroblast heterokaryons suggest that a combinatorial pathway may exist for activation of ventricular muscle genes (Evans et al., 1994). To address this question, we have utilized a variety of transgenic and gene targeting approaches in mouse model systems to identify molecular pathways that control these critical steps during cardiogenesis. To define the molecular cues that guide early stages of ventricular chamber specification, our laboratory has employed the molecular cues that guide early stages of ventricular chamber morphogenesis, providing a model system in which to investigate the cues that dictate anterior/posterior (right ventricle/left ventricle) gradients during mammalian heart development.

The ubiquitous transcription factor YB-1 binds to the HF-1a site in the MLC-2v promoter in conjunction with a co-factor and positively regulates the MLC-2v promoter (Zou et al., 1995). In a search for interacting co-factors, we now report the isolation and characterization of a nuclear ankyrin-like repeat protein (CARP) (cardiac ankyrin repeat protein) isolated from a rat neonatal heart cDNA library via two-hybrid screening, using YB-1 as bait. Northern analysis revealed that CARP mRNA is highly enriched in the adult heart with only trace levels in skeletal muscle.

A number of independent approaches, including co-immunoprecipitation and GST CARP pulldown studies, have revealed that CARP forms a physical complex with YB-1 in cardiac myocytes. To our knowledge, CARP is the first described tissue-specific co-factor for YB-1 and raises the question as to the potential role of this tissue-restricted co-factor in the regulation of YB-1 function. In situ analyses in the current study demonstrate that, although YB-1 is widely expressed, it is preferentially expressed in cardiac muscle during early heart development and comprises one of the major tissues that expresses YB-1 at high levels. In addition, YB-1 also appears to be expressed preferentially in the dermamyotome compartment versus the sclerotome compartment in somites during somitogenesis, implicating a role for YB-1 in the control of the skeletal muscle gene program. The interaction of YB-1 with tissue-specific partners, such as CARP, might identify YB-1 as playing a more specialized role in the control of tissue-specific gene programs, which has been suggested by transient transfection assays in a wide variety of
other cell types (Grant and Deeley, 1993; Ruiz-Lozano et al., 1994; Wolfe et al., 1992).

Although CARP interacts with YB-1, a number of distinct criteria suggest that CARP is not a component of the major endogenous HF-1a-binding activity. Previous studies have identified the size of the major interacting co-factor to be $30 \times 10^3 \text{ M}_r$, while the size of CARP is approximately $40 \times 10^3 \text{ M}_r$. In addition, antibodies directed against CARP do not supershift the major endogenous HF-1a-binding activity (Zou and Chien, unpublished data) and CARP has a negative transcriptional regulatory effect on an HF-1-dependent promoter activity, as opposed to the positive regulatory effect identified for the endogenous HF-1a activity. Finally, GAL4 assays indicate that CARP contains domains that can act as negative transcriptional regulators, supporting a potential role of CARP in the negative regulation of a subset of ventricular muscle genes. In this manner, one might propose that CARP may act to antagonize the actions of p30 via interaction with YB-1, serving as a negative regulator of HF-1a-dependent genes by sequestration of YB-1. The analysis of CARP-deficient murine embryos should be valuable in identifying the precise role of CARP in the control of ventricular chamber specification, maturation and adaptation. Studies in the initial two hybrid screening identified other positive clones that interact with YB-1, which are currently the subject of analyses to determine their relationship to the p30 YB-1 co-factor (Zou et al., 1995).

**CARP is an early marker of cardiac muscle lineages**

Recent studies have underscored that combinatorial pathways may be responsible for the activation of the cardiac muscle gene program (Evans et al., 1994; Ross et al., 1996). In addition, an increasing body of evidence suggests that discrete programs may exist for various regions of the heart: right ventricular chamber, left ventricular chamber, atrial and outflow tract (Ross et al., 1996). A number of nuclear factors have been implicated in the control of the cardiac muscle gene program and are expressed early during cardiogenesis, including MEF-2 (Edmondson et al., 1994; Lilly et al., 1995), GATA-4 (Greepin et al., 1995; Jiang and Evans, 1996) and Nkx2-5 (Lints et al., 1993), dHAND and eHAND (Cserjesi et al., 1995; Olson and Srivastava, 1996). Each of these transcription factors are markedly enriched in heart, but are also expressed in a variety of other tissues. In fact, none of these genes appear to be expressed in an exclusively muscle-restricted fashion. The nuclear regulatory factors that might influence the cardiac muscle gene program and that would themselves be more restricted in their pattern of expression might serve as downstream target genes or cofactors that work to restrict the subsequent program of gene expression in the myocardium during the course of cardiogenesis. In this regard, CARP expression occurs specifically in myocardium at a relatively early stage during cardiogenesis (at least E8.5). In addition, recent studies analyzing embryos that harbor a knock-in of the lacZ reporter gene into the endogenous CARP locus also document early restricted expression of CARP in the primitive heart tube before the completion of heart tube fusion at early E8.0 (Chen, Kuo, Zou and Chien, unpublished results). At later points in time (after E9.5), trace detection of CARP promoter activity can be observed in skeletal muscle shown by the knock-in of lacZ. However, throughout embryogenesis, CARP expression is found primarily in heart. Trace levels in skeletal muscle, which can be revealed by lacZ, are not detected by in situ hybridizations. These studies suggest that CARP may be an excellent marker for early stages of the process of cardiac specification and elucidating the mechanisms that mediate the cardiac-restricted expression of CARP could be instrumental in identifying hierarchical signaling pathways that activate expression of the muscle gene program. In addition, it could become of interest to determine whether the expression of CARP in various regions of the heart tube, i.e., outflow tract, right ventricle, left ventricle and atrial, is under the control of separate genetic mechanisms. As noted in the current study, a 2.5 kb promoter fragment of the CARP promoter can direct a high level of cardiac-restricted expression of a lacZ reporter gene early during cardiogenesis. Thus, the CARP promoter may have utility for directing the early, high level cardiac-specific expression of genes for rescue strategies in various embryos harboring the loss of function of ubiquitously expressed genes that produce embryonic lethality due to cardiac defects, such as the RXRα (Sucov et al., 1994; Dyson et al., 1995; Gruber et al, 1996), βARK-1 (Jaber et al., 1996), erb B2 (Lee et al., 1995), erb B4 (Gassmann et al., 1995) and neuregulin (Meyer and Birchmeier, 1995)-deficient embryos.

**CARP is downstream in the Nkx2-5 homeobox gene pathway**

The absence of the MyoD family of myogenic factors in cardiac myocytes has led to the view of cardiac muscle as an exciting model system to study the genetic mechanisms of how these two closely related, but distinct mesodermal cell lineages achieve the differentiated myogenic phenotype. To date, the only candidate that may be capable of specifying the cardiac muscle cell lineage is the Drosophila homeobox gene tinman. tinman is expressed in the two major cell types of the Drosophila heart, the cardiac and pericardial cells. In tinman mutants, no visceral mesoderm of the midgut or cardiac mesoderm forms, causing the loss of the dorsal vessel and its progenitors. tinman has been shown to regulate several genes that are important for visceral mesoderm development, including a related NK-homeodomain gene, bagpipe/NK-3 (Aspiazu and Frasch, 1993). Vertebrate homologues of tinman are highly conserved across species and have been identified in mouse (Komuro and Izumo, 1993; Lints et al., 1993), Xenopus (Tonissen et al., 1994; Evans et al., 1995), zebrafish and chick (Schultheiss et al., 1995). The mammalian homolog of the Drosophila tinman homeobox gene, Nkx2-5, is specifically required for ventricular chamber-specific myosin light chain-2 (MLC-2v) expression and looping morphogenesis during mammalian heart development.

While it is apparent that the Nkx2-5 gene is required for normal cardiogenesis, it is less clear how this homeobox genes triggers or influences downstream cardiac muscle gene programs. Nkx2-5, as with many other homeobox genes, is expressed in a number of tissues (heart, pharyngeal endoderm, thyroid, tongue, spleen) and must interact with other regulatory circuits that control tissue-specific transcription. The current study suggests that one of the mechanisms by which Nkx2-5 may establish cell-specific gene programs is via the transcriptional activation of tissue-restricted nuclear cofactors, such as CARP, which in turn may participate in the regulation of differentiated gene programs.

In Nkx2-5-deficient embryos, carp expression was found to be sig-
significantly and selectively reduced by in situ hybridization and RNase protection studies. In addition, transient co-transfection assays have provided evidence that CARP promoter activity can be regulated by Nkx2-5 in the cardiac muscle cell. Finally, transgenic mice that harbor a 2.5 kb carp promoter-lacZ reporter gene display cardiac-specific expression of the transgene early during cardiogenesis. Breeding these carp transgenic mice into the Nkx2-5−/− background has resulted in a significant reduction of the Carp-lacZ reporter gene in the Nkx2-5−/− embryos, providing further direct evidence that the carp gene lies downstream of Nkx2-5. Interestingly, both the endogenous carp gene and the 2.5 kb carp-lacZ transgene display a preferential loss of expression in the conotruncal region of the heart tube. The carp expression in the conotruncus region is more sensitive to Nkx2-5 mutation. This study provided an Nkx2-5 downstream regulatory gene in the cardiac regulatory network that is highly restricted to heart. It should be noted that previously our laboratory has reported that a 250 bp MLC2v-lacZ transgene, which displays high levels of expression in the conotruncus and bulbus cordis region, was not affected by the Nkx2-5 mutation (Ross et al., 1996). While it is clear that Nkx2-5 regulates carp at the transcriptional level, it is currently unclear whether carp plays a role to shape the cardiac muscle-specific gene expression or activates another factor(s) which in turn regulates the carp expression in the conotruncus region. The dominant-negative Nkx2-5 mutation (Ross et al., 1996). This study has independently cloned an identical rat carp gene via a differential display approach and has documented cardiac restricted expression during murine embryogenesis.

REFERENCES


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