Control of segmental expression of the cardiac-restricted ankyrin repeat protein gene by distinct regulatory pathways in murine cardiogenesis

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SUMMARY

Although accumulating evidence suggests that the heart develops in a segmental fashion, the molecular mechanisms that control regional specification of cardiomyocytes in the developing heart remain largely unknown. In this study, we have used the mouse cardiac-restricted ankyrin repeat protein (CARP) gene as a model system to study these mechanisms. The CARP gene encodes a nuclear co-regulator for cardiac gene expression, which lies downstream of the cardiac homeobox gene, Nkx 2.5, and is an early marker of the cardiac muscle cell lineage. We have demonstrated that the expression of the gene is developmentally down regulated and dramatically induced as part of the embryonic gene program during cardiac hypertrophy. Using a lacZ/knock-in mouse and three lines of transgenic mouse harboring various CARP promoter/lacZ reporters, we have identified distinct 5' cis regulatory elements of the gene that can direct heart segment-specific transgene expression, such as atrial versus ventricular and left versus right. Most interestingly, a 213 base pair sequence element of the gene was found to confer conotruncal segment-specific transgene expression. Using the transgene as a conotruncal segment-specific marker, we were able to document the developmental fate of a subset of cardiomyocytes in the conotruncus during cardiogenesis. In addition, we have identified an essential GATA-4 binding site in the proximal upstream regulatory region of the gene and cooperative transcriptional regulation mediated by Nkx2.5 and GATA-4. We have shown that this cooperative regulation is dependent on binding of GATA-4 to its cognate DNA sequence in the promoter, which suggests that Nkx2.5 controls CARP expression, at least in part, through GATA-4.

Key words: CARP, Cardiac hypertrophy, Transcriptional regulation, Nkx2.5, GATA-4, Heart segment specificity, Cardiogenesis, Mouse

INTRODUCTION

Cardiogenesis is among the earliest and most important steps during vertebrate development, which arises through a complex series of morphogenetic events (reviewed by Fishman and Chien, 1997; Olson and Srivastava, 1996). Early during embryogenesis, cardiac progenitor cells within the anterior lateral plate mesoderm are induced to form the bilaterally symmetrical cardiac primordia. The paired primordia first fuse anteriorly to give rise to a distinct cardiac crescent, which condenses further to form a single, linear heart tube at the ventral midline. It has been shown that the linear heart tube is organized along the anterior-posterior axis into segments that are destined to form the aortic sac, conotruncus (outflow tract), right ventricle, left ventricle and atria. The straight heart tube soon undergoes rightward and ventrocaudal looping. Following looping morphogenesis, the atrial, ventricular and conotruncal segments of the heart become morphologically identifiable and these regions of the heart ultimately develop into four distinct chambers and the great vessels.

The formation of the four distinct cardiac chambers and their accompanying great vessels is essential to the functional operation of both the embryonic and adult hearts. The regulatory mechanisms that control cardiomyocyte specification and diversification into different lineages in various compartments of the developing heart have been a topic of extensive study. It is known that atrial and ventricular cardiomyocytes display contractile, electrophysiological, morphological, and biochemical properties that are unique to each chamber. These distinct regional properties are presumably conferred by the selective activation of subsets of cardiac muscle gene programs in the atrial and ventricular segments during cardiac development. For example, the myosin light chain-2 ventricular (MLC-2v) gene is expressed only in the ventricular segment, whereas the myosin light chain-2 atrial (MLC-2a) gene is initially expressed in both atrial and ventricular segments but becomes restricted to the atria later during cardiogenesis (Kubalak et al., 1994; O’Brien et al., 1993).

To determine if distinct regulatory pathways control the regional-specific expression of the cardiac muscle gene
program during cardiogenesis, we have used the mouse cardiac-restricted ankyrin repeat protein (CARP) gene as a molecular model system. CARP expression is restricted mainly to the heart, and to a lesser extent, skeletal muscle, and it is expressed in cardiomyocytes throughout the whole heart. CARP is one of the earliest markers of the cardiac muscle cell lineage in the bilateral cardiac primordia, and it is downstream in the Nkx2.5 pathway that defines the early heart field (Zou et al., 1997). The CARP gene encodes a nuclear protein which contains four ankyrin repeats within its carboxyl terminal end, and CARP has been previously demonstrated to negatively regulate the expression of cardiac genes, including the MLC-2v and atrial naturetic factor (ANF) genes, in vitro (Zou et al., 1997; Jeyaseelan et al., 1997).

In this study, we have examined whether the regional expression of CARP in various cardiac segments is under the control of distinct regulatory pathways. In addition, we have examined the dynamic regulation of these pathways in the postnatal heart, and have evidence that the expression of the CARP gene is developmentally down-regulated in the adult heart and the expression is dramatically increased, as part of the induction of an embryonic gene program, during cardiac hypertrophy. Based on the studies of the 5’ flanking sequence of the gene in vitro, we created three lines of transgenic mouse harboring various CARP promoter/b-galactosidase (lacZ) reporter genes as well as a lacZ/ knock-in mouse as a control. Our data indicated that distinct regulatory elements within the 5’ flanking sequence of the CARP gene were capable of directing region-specific (atrial versus ventricular, and left versus right) transgene expression in the heart. Moreover, we have identified a 213-base pair (bp) sequence element that is sufficient to confer conotruncal-specific transgene expression. Taken together, our studies indicate that the uniform expression of CARP in the heart is due to the presence of distinct cis regulatory elements in the CARP gene that confer specificity for the atrial, left ventricular, right ventricular, and conotruncal segments of the heart.

As we have reported previously, CARP is downstream in the Nkx2.5 pathway and Nkx2.5 can regulate CARP expression at a transcriptional level (Zou et al., 1997). In the current studies, we have identified an essential GA TA-4 binding site in the CARP gene and cooperative transcriptional regulation mediated by Nkx2.5 and CARP has been previously demonstrated to negatively regulate the expression of cardiac genes, including the MLC-2v and atrial naturetic factor (ANF) genes, in vitro (Zou et al., 1997; Jeyaseelan et al., 1997).

MATERIALS AND METHODS

Microsurgical techniques

Transverse aortic constriction (TAC) was performed as previously described (Rockman et al., 1991). Sham-operated mice underwent a similar open-chest operation but without TAC.

RNA isolation and northern blot analysis

Total RNA was isolated from the left ventricle or cultured cells using RNAzol B (Tel-Test). For northern blot analysis, 10 µg of the total RNA was electrophoresed on a 1% agarose gel, blotted, and hybridized with [α-32P]dATP-labelled CARP and GAPDH cDNA probes in the QuickHyb solution (Stratagene) as described by suggested protocols.

Isolation of the CARP genomic DNA

A probe containing the entire coding region of the CARP cDNA was used to screen the mouse 129 sv phage genomic DNA library (Stratagene), as described by the manufacturer’s protocol. Four overlapping clones were isolated from the screening and a restriction map of the gene was constructed from these overlapping clones.

Primer extension analysis

An oligonucleotide primer (5’-GTATACGCTTTTCTTCTCACTCAG-TACCATC-3’) complementary to the 5’ end of the mouse CARP cDNA was 32P-labelled with T4 polynucleotide kinase and annealed to 20 µg of total RNA from the mouse heart. The primer extension assay was performed as previously described (Zhu et al., 1991).

Cell cultures and transfection assays

Neonatal rat ventricular cardiomyocytes were isolated and cultured as previously described (Zhu et al., 1991). COS1 and CV1 cells were grown in DMEM with 10% fetal bovine serum. Calcium phosphate transfection, luciferase, and cellular β-gal assays were performed as previously described (Zhu et al., 1991).

Construction of plasmids

To construct p0.176Luc, the CARP genomic DNA was used as a template with primers JA2 (~176 to ~153) and JA11 (~47 to +25) in a polymerase chain reaction (PCR). The BamHI/Xhol-digested PCR fragment was subsequently subcloned into pXP2, which contains a copy of the luciferase gene. To construct p0.295Luc, the PCR fragment of primers JA11 and JA7 (~295 to ~274) was subcloned into the HincII site of p7/318 (Ambion). A HindIII-Xhol fragment was subsequently isolated and subcloned into pXP2 to make the p0.295Luc. To construct p0.54Luc, p0.796Luc, and p2.5Luc, a 364 bp PstI-SacII, a 620 bp HindIII-SacII, and a 2.4 kb BamHI-SacII fragments were isolated from the CARP genomic DNA and subcloned into p0.295Luc to make the respective constructs. To construct p10Luc, a 10 kb KpnI-SacII fragment and a 223 bp SacI-Xhol fragment were isolated from the CARP genomic DNA and the p0.295Luc, respectively, which were subsequently subcloned into the KpnI/Xhol sites in pXP2. To construct p2x0.128TATALuc, the PCR fragment (~166 to ~39) of primers 1 and 2 and the PCR fragment (~166 to ~47) of primers 3 and JA11 were digested with BamHI/EcoRI and EcoRI/Xhol, respectively, and ligated into pXP2. To make p0.295GATAmP, the PCR fragment (~295 to ~52) of primers 4 and 5 and the PCR fragment (~52 to ~47) of primers 6 and JA11 were digested with BamHI/PstI and PstI/Xhol, respectively, and ligated into pXP2. The PCR fragment (~295 to ~261) of primers 4 and 7 the PCR fragment (~261 to ~47) of primers 8 and JA11 were subjected to the same treatment to make p0.295GATAmD. A SacII/Xhol fragment from p0.295GATAmP was isolated and ligated to a SacII/Xhol digested p0.295GATAmD to make p0.295GATAmP&D. To construct p2x5lacZ, a 2.5 kb BamHI-Xhol fragment from the p2.5Luc and a 3.3 kb Xhol-SmaI fragment from pSDKlacZ (A gift from Janet Rossant) were ligated into the BamHI/SmaI sites in p7/318. To construct p0.295lacZ, a 342-bp HindIII-Xhol fragment from p0.295Luc and a 3.3 kb Xhol-BamHI fragment from pSDKlacZ were ligated into p7/318. To construct p2x0.128TATAlacZ, a BamHI-Xhol fragment was isolated from p2x0.128TATAluc, blunt-ended, and subcloned into the XhoI site in pSDKlacZ. The sequences of primers 6 and 8, which contain mutations in the GATA sites, are shown below. The mutated sequences are indicated by underlines.

| Primer 6: 5’-AAACCTGCAGCACCCACCCACTAAGCTATAAAC-3’ |
| Primer 8: 5’-AAAGTTCAGGGAAGCCAGGGGACACGTGCC-3’ |

Whole-mount and histological analysis of transgenic embryos

Embryos or postnatal hearts were fixed and stained using previously
的发展和诱导调节CARP基因在胚胎和成年心脏中的表达

在本研究中，我们首次探讨了CARP是否是胚胎期心肌肥大基因程序中的成员。我们研究了CARP基因在一种左心室肥大模型（如胚胎性动脉收缩术）中胚胎性心脏和成年心脏中表达的变化。Northern印迹分析显示了CARP表达的显著提高，这发生在TAC手术后7天（Fig. 1A）。在4天和7天时，接受TAC的试验组相比对照组（数据未显示）出现了左心室重量和体重比显著增加的现象，这表明了左心室肥大的发展。这种左心室肥大的发展进一步通过ANF基因，一种已知的肥大标志，得到确认（Fig. 1A）。综上所述，我们的数据表明CARP的诱导表达与心肌肥大的发展相关。

结果

胚胎性及诱导性调节CARP基因在胚胎性及成年心脏中的表达

在这个研究中，我们首次探讨了CARP是否是胚胎期心肌肥大基因程序中的成员。我们研究了CARP基因在一种左心室肥大模型（如胚胎性动脉收缩术）中胚胎性心脏和成年心脏中表达的变化。Northern印迹分析显示了CARP表达的显著提高，这发生在TAC手术后7天（Fig. 1A）。在4天和7天时，接受TAC的试验组相比对照组（数据未显示）出现了左心室重量和体重比显著增加的现象，这表明了左心室肥大的发展。这种左心室肥大的发展进一步通过ANF基因，一种已知的肥大标志，得到确认（Fig. 1A）。综上所述，我们的数据表明CARP的诱导表达与心肌肥大的发展相关。

电泳图的移动性转变分析

这些探针被标记[γ-32P]ATP，使用T4核苷酸激酶。5 μl的哺乳动物肝脏裂解液使用poly(dI·dC)作为模板被孵育0.2 ng的探针和2 μg的poly(dI·dC)在20 μl的缓冲液中，该缓冲液条件为：10 mM Tris-Cl(pH: 7.4), 50 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol and 0.05% Nonidet P-40，在室温下孵育1小时。探针与蛋白质复合物被5%的聚丙烯酰胺凝胶（在0.5x TBE缓冲液中）分离。杂交后的凝胶被冲洗2小时低温（4°C），然后置于与未标记探针竞争的条件下。

转录表达实验以确定上游cis调节元件

为了确定5’ cis调节元件，我们制作了一组 Luciferase报告基因的载体，其中包含CARP基因的5’端。这些CARP报告基因载体在COS1细胞中被成功转染，且在原始的neo抗性筛选中表现出良好的转染效率。通过Western印迹分析证实CARP基因在胚胎性及成年心脏中表达的变化。Northern印迹分析显示了CARP表达的显著提高，这发生在TAC手术后7天（Fig. 1A）。在4天和7天时，接受TAC的试验组相比对照组（数据未显示）出现了左心室重量和体重比显著增加的现象，这表明了左心室肥大的发展。这种左心室肥大的发展进一步通过ANF基因，一种已知的肥大标志，得到确认（Fig. 1A）。综上所述，我们的数据表明CARP的诱导表达与心肌肥大的发展相关。

在光的潜在重要性的情况下，我们进一步研究了CARP基因在胚胎性及成年心脏中的表达。Northern印迹分析显示了CARP表达的显著提高，这发生在TAC手术后7天（Fig. 1A）。在4天和7天时，接受TAC的试验组相比对照组（数据未显示）出现了左心室重量和体重比显著增加的现象，这表明了左心室肥大的发展。这种左心室肥大的发展进一步通过ANF基因，一种已知的肥大标志，得到确认（Fig. 1A）。综上所述，我们的数据表明CARP的诱导表达与心肌肥大的发展相关。

Isolation and characterization of the CARP gene

In the light of the potential importance of developmental control of CARP expression in the heart, we characterized the structure of the gene and systematically studied the CARP promoter. The CARP genomic DNA was isolated from the 129sv mouse genomic DNA library. A restriction map of the gene and sequences for all of the exons and their immediately adjacent intron regions were determined (Fig. 2A,B). Our sequence analyses revealed that the gene was divided into 9 exons, and interestingly, each of the exons 5, 6, 7 and 8 encoded one ankyrin repeat which contained 33 amino acids. The transcription initiation site in the heart was identified by primer extension (Fig. 2C). Sequence analysis of the 5’ flanking region also indicated the presence of a canonical TATA box located 31 bp upstream of the defined transcription initiation site (Fig. 2D).

Transient expression assays to identify upstream cis regulatory elements of the CARP gene that confer cardiac specificity

To identify 5’ cis regulatory elements that control the cardiac specificity of the CARP gene, we made a series of luciferase reporter constructs whose expression were driven by 5’ nested deletions of the CARP promoter (Fig. 3A). These CARP promoter/luciferase reporter constructs were transfected into primary neonatal rat ventricular cardiomyocytes as well as COS1 cells to test the relative cardiac specificity of the various truncated CARP promoters. Primary cardiomyocytes displayed a high level of endogenous CARP expression, whereas COS1 cells exhibited a barely detectable level of expression of the gene, which allowed the cells to be used as a negative control in transient expression assays (Fig. 3B). Both cells were also co-transfected with a CMV promoter/lacZ reporter construct (pON2) to control the efficiency of transfection. Our data indicated a gradual decrease in luciferase activity with progressive 5’ deletion of the CARP promoter in cardiomyocytes. In contrast, in COS1 cells, all of the CARP constructs displayed a similar background level of luciferase activity.

Figure 1. CARP is a marker for heart hypertrophy and the expression of CARP is developmentally down-regulated. (A) Northern blot analyses showed induction of CARP expression in heart hypertrophy. Total RNA was isolated from the left ventricle of mice that underwent various lengths of time of TAC procedure (TAC) or sham-operation (Sham) as indicated at the top. Hr, hour; d, day. The level of expression of the glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as a control. (B) Northern blot analyses showed developmental down-regulation of CARP expression. Total RNA was isolated from the ventricle of adult mouse (Adult) as well as pooled ventricles from mice of embryonic day 13-15 (Embryo).
one copy of the sequence element spanning from +47 to −38, including the TATA box, exhibited a much higher activity (Fig. 3C). Taken together, these in vitro data suggest that a 213-bp CARP sequence spanning from +47 to −166 is sufficient to confer cardiac-specific gene expression.

**Distinct regulatory elements of the CARP gene direct segment-specific gene expression in the heart**

Based on the in vitro results, we created three lines of transgenic mice harboring various lacZ reporter genes, p2.5lacZ, p0.295lacZ and p2x0.128TA TAlacZ, whose expression were directed by the CARP promoters derived from p2.5Luc, p0.295Luc, and p2x0.128TA TALuc, respectively. In addition to these transgenic lines, a lacZ/knock-in mouse in which the expression of the lacZ gene was under the control of the endogenous CARP promoter was also included in the analyses (Chen, J. and K. R. Chien, unpublished data). The pattern of the lacZ gene expression in the knock-in mouse was used as a control for the endogenous CARP promoter activity.

For the p2.5lacZ transgenic mice, we analyzed six lines for the pattern of expression of the lacZ gene by whole-mount staining with X-gal. Among the six independent lines, five displayed cardiac and skeletal muscle-specific transgene expression, and one showed no detectable level of transgene expression. For the p0.295lacZ transgenic mice, six lines were analyzed for X-gal expression. Five lines were positive for cardiac and skeletal muscle-specific transgene expression, and one showed no detectable level of transgene expression. For the p2x0.128TA TAlacZ transgenic mice, three lines were analyzed.

**Fig. 2.** Structural organization of the CARP genomic DNA. (A) Structure and restriction map of the CARP genomic DNA. All the nine exons are shown and indicated by the numbers below the genomic DNA. All the nine exons are shown below the corresponding exon sequence. (B) Sequence of the nine exons and their immediately adjacent introns. The exons and introns are shown in the upper and lower cases, respectively. Deduced amino acid sequence is shown below the corresponding exon sequence. (C) Identification of the transcription initiation site by primer extension. H, heart; L, liver. Lanes A, C, G, and T show sequencing ladder of the CARP genomic DNA using the same primer. (D) Sequence of the 5′ flanking region (−299 to +62) of the CARP gene. The putative TATA box, two potential GATA sites (the proximal GATA site is in a reverse orientation), an E-box, and an AT-rich sequence are underlined and labelled. The translational initiation site is also underlined. The arrow indicates the transcriptional initiation site identified in the primer extension analysis. (GenBank access no. AF041847.)
Two lines exhibited cardiac-specific expression of the transgene, and one line displayed an ectopic expression of the transgene in the forebrain with no expression in the heart, presumably due to the site of integration (Table 1).

As all of the positive lines from a specific transgenic construct displayed a similar pattern of transgene expression in the initial studies, we selected one representative line from each of the three transgenic constructs for more detailed analyses. Line 22 from the p2.5lacZ mice was selected for further study, and this transgenic line displayed a pattern of β-galactosidase expression similar to that of the lacZ/knock-in mice during early stages of cardiac development. Expression of the transgene in the p2.5lacZ mice was detected as early as around embryonic day (E) 8 in the cardiac crescent (Fig. 4A). Prior to E16, the transgene was expressed throughout the myocardium as well as in the somites, similar to the pattern of transgene expression in the knock-in mice (Figs 4B,C,F,G, 5A,B). After E16, while the knock-in mice displayed a uniform β-galactosidase expression throughout the myocardium, ventricular β-galactosidase expression in the p2.5lacZ mice began to diminish. In neonates of the p2.5lacZ transgenic mice, cardiac expression of the transgene was restricted to both atria and the right ventricle (Fig. 6). In adult mice, while the transgene was still expressed in the atria, its expression became undetectable in both ventricles (data not shown).

Line 37 from the p0.295lacZ transgenic construct and line 49 from the p2x0.128kbTA TαlacZ transgenic construct were selected for detailed analyses of transgene expression. For the p0.295lacZ transgenic embryos, expression of the transgene was specific to both the myocardium of the heart and the somites, and cardiac expression was first detected at around E9-9.5. Cardiac expression of the transgene was restricted to the conotruncal and right ventricular segments of the primitive heart (Figs 4D,H, 5C). In neonates, transgene expression was further restricted to the base of the great arteries and the dorsal upper region of the right ventricle, and the expression became completely undetectable in adult hearts (Fig. 6). In the p2x0.128kbTA TαlacZ transgenic mice, transgene expression was detected only in the heart, not in the somites, and cardiac expression of the transgene was specific to cardiomyocytes in the conotruncal segment of the primitive heart (Figs 4E,I,J, 5D). In neonates, transgene expression was found to be restricted to a thin band of cardiomyocytes situated at the junction of the pulmonary artery and the right ventricle (Fig. 6). In adult hearts, transgene expression became undetectable (data not shown). The pattern of cardiac transgene expression for all three lines of transgenic and knock-in mice are summarized in Table 1.

Studies of the developmental fate of conotruncal cardiomyocytes in the p2x0.128kbTA TαlacZ mice using the transgene as a conotruncal-specific marker

Taking advantage of the conotruncal-specific transgene expression in the p2x0.128kbTA TαlacZ mice, we followed the developmental fate of a subset of cardiomyocytes using the transgene as a conotruncal-specific segmental marker. When examined at E8-8.5, the expression of the transgene was not...
detectable (data not shown). At approximately E9-9.5, transgene expression was detected in the conotruncal segment of the primitive heart (Figs 4E, 7A), and the expression appeared to be all around the conotruncus (Fig. 7B). A similar pattern persisted at E11-11.5 (Fig. 4I,J). At E14.5, after the septation of the outflow tract into the aorta and the pulmonary artery was completed and the valvular primordia of these great arteries were formed, transgene expression was detected at the junction of the great arteries and the ventricles with a gradient of expression, higher in the cardiomyocytes surrounding the base of the pulmonary artery and lower in those surrounding the base of the aorta (Fig. 7C-F). Histological sections of the heart of an embryo at E14.5 showed that the transgene was expressed in cardiomyocytes located right below the semilunar valve (Fig. 7G). In 2-day-old neonates, transgene expression was restricted to a thin band of cardiomyocytes located at the junction of the pulmonary artery and the right ventricle, immediately below the semilunar valve, and there was no detectable transgene expression in cardiomyocytes surrounding the base of the aorta (Fig. 7H).

Identification of an essential GATA-4 binding site in the proximal CARP promoter: Nkx2.5 may control CARP expression indirectly through GATA-4

We have previously reported that CARP is downstream of the Nkx2.5 gene in the cardiac regulatory network, and Nkx2.5 can regulate CARP expression at a transcriptional level. It has been shown that endogenous CARP expression is dramatically reduced in Nkx2.5−/− embryos at E9, with more reduction at the anterior portion than at the posterior portion of the heart tube (Zou et al., 1997). Since the regions exhibiting the preferential loss of CARP expression in the Nkx2.5−/− embryos coincide with the regions of the transgene expression displayed in the p0.295lacZ mice, it became of particular interest to determine whether the CARP sequence in p0.295lacZ contained the Nkx2.5-responsive element. To address this question, we crossed the p0.295lacZ transgenic mice into the Nkx2.5−/− background. At E9-9.5, while the p0.295lacZ/Nkx2.5−/− mice displayed uniform transgene expression in both the conotruncus and bulbar cordis, the p0.295lacZ/Nkx2.5−/− mice exhibited a dramatic loss of transgene expression in these segments of the heart (Fig. 8A-D).

These results suggest that the CARP sequence in the p0.295lacZ construct (from -295 to +47) contains at least one direct or indirect Nkx2.5-responsive element. We have analyzed the sequence within this region and found no apparent binding site for Nkx2.5. Instead, sequence analysis indicated the presence of two potential GATA sites within this region (Fig. 2D). To determine the roles of these potential GATA sites in controlling the cardiac specificity of the CARP promoter, we generated three luciferase reporter constructs, p0.295GATAmp, p0.295GATAmD and p0.295GATAmp&D, which contain...
mutations in the proximal, distal, and both the proximal and distal GATA sequences, respectively (Fig. 9A). Transfection studies of p0.295GATAmP, p0.295GATAmD, and p0.295GATAmP&D showed an 82%, 15%, and 90% decrease in cardiac-specific transcription, respectively, when compared to p0.295Luc. These results indicate that the proximal GATA site is essential in controlling the cardiac-specific transcription of the CARP promoter.

GATA-4 is a cardiac-restricted transcription factor, which has been shown to control the expression of many cardiac genes. To further determine whether GATA-4 could bind to the identified GATA sites, electrophoretic mobility shift assays were performed. 32P-labelled oligonucleotide probes corresponding to either the proximal or the distal GATA sites were tested, but only the proximal GATA probe could form a specific complex with GATA-4 (Fig. 9B). A probe containing mutations in the core sequence of the proximal GATA site was unable to form the specific complex with GATA-4. In addition, the complex could be competed away by the addition of a 100-fold excess of the unlabelled wild-type oligonucleotide, but not by the mutant oligonucleotide. Furthermore, the specific complex was supershifted when incubated with an anti-GATA-4 antibody (Fig. 9B). These data indicate that the identified proximal GATA site is a bona fide GATA-4 binding site.

Taken together, we have shown that the Nkx2.5-responsiveness is retained within the sequence spanning from −295 to +47, which contains no apparent Nkx2.5 binding site, but has an essential GATA-4 binding site. Since Nkx2.5 and GATA-4 have been shown to work cooperatively to regulate the expression of several cardiac genes (Durocher et al., 1997; Lee et al., 1998; Sepulveda et al., 1998), it is of particular interest to determine whether Nkx2.5 may exert its control on the CARP promoter indirectly through GATA-4. To study the potential interaction between Nkx2.5 and GATA-4 in controlling the activity of the CARP promoter, p0.295Luc and p0.295GATAmP were co-transfected separately with either control vectors, pcDNA3/GATA-4, pCGN/Nkx2.5, or both of the expression vectors in CV-1 cells. The transactivation studies of p0.295Luc indicated that neither GATA-4 nor Nkx2.5 alone was sufficient to activate the CARP promoter in CV-1 cells (Fig. 9C). However, when both GATA-4 and Nkx2.5
were present, an approximately 3.2-fold activation was observed. In contrast, when p0.295GATAmP was tested, this cooperative transcriptional activation mediated by GATA-4 and Nkx2.5 was not observed. These data demonstrate that cooperative regulation of the CARP promoter mediated by GATA-4 and Nkx2.5 is dependent on the binding of GATA-4 to its cognate sequence in the promoter. Our results suggest that Nkx2.5 may control the CARP promoter, at least in part, through GATA-4.

**DISCUSSION**

**CARP is a true marker for cardiac hypertrophy and a member of the cardiac embryonic gene program**

Our studies have demonstrated that CARP expression is dramatically increased in a mouse model of concentric heart hypertrophy induced by pressure overload. It has been shown previously that in the muscle LIM protein (MLP) null mouse, which exhibits a phenotype of dilated cardiomyopathy with eccentric hypertrophy, CARP expression is also significantly increased (Arber et al., 1997). Induction of CARP expression in two different mouse models of cardiac hypertrophy, which demonstrate different pathophysiology, establishes CARP as a true marker for cardiac hypertrophy. It is known that an embryonic gene program, which is constitutively expressed during embryonic heart development and is down-regulated in adult hearts, becomes reactivated during cardiac hypertrophy. This fetal gene program includes ANF, β-myosin heavy chain, and skeletal α-actin (Izumo et al., 1987; Lee et al., 1988; Schwartz et al., 1986). We have shown that CARP expression is developmentally down-regulated in adult hearts, indicating that CARP is a new addition to the embryonic gene program.

Interestingly, CARP expression is also induced during denervation of skeletal muscle in the mouse (Baumeister et al., 1997). The induction of CARP expression in response to stress in both cardiac and skeletal muscles suggests that CARP may play a role in mediating cellular responses to various stresses in the striated muscle. This putative in vivo role of CARP is not inconsistent with previous studies, which have demonstrated that CARP may function as a co-regulator for cardiac gene expression in vitro.

**Distinct regulatory pathways confer atrial, left ventricular, right ventricular, and conotruncal segment-specificity for CARP expression**

During early cardiac development, while the knock-in and the p2.5lacZ transgenic mice showed transgene expression in cardiomyocytes throughout the whole heart, transgene expression in the p0.295lacZ mice was restricted to part of the right ventricle and the conotruncus. These results indicate the presence of distinct cis regulatory elements within the region between –295 and –2500 that may direct atrial and left ventricular-specific gene expression. In addition, the p2x0.128TATAlacZ mice exhibited a pattern of transgene expression that was restricted to the conotruncal segment of the heart, indicating that the 213 bp sequence (from –166 to +47) contains regulatory elements that can confer conotruncal-specific gene expression. We showed that after dimerization of the 128 bp sequence, the in vitro activity of p2x0.128TATALuc was dramatically increased compared to that of p0.179Luc. It could be argued that this dramatically increased activity was due to an artifactual synergy between factors or the creation of a new factor-binding site at the dimer junction, and either case might skew the real expression pattern in vivo. While this alternative explanation is possible, we think it is unlikely in this case as the conotruncal-specific transgene expression exhibited by the p2x0.128TATAlacZ mice does reflect a subset of patterns of transgene expression shown in other transgenic mice harboring longer CARP regulatory sequence.

The absence of transgene expression in the right ventricle and skeletal muscle in the p2x0.128TATAlacZ mice also suggests that the sequences between –166 and –295 contain regulatory elements that may direct part of the right ventricle and skeletal muscle-specific gene expression. Moreover, developmental studies of the three lines of transgenic mouse demonstrated developmental down-regulation of transgene expression in various compartments of the heart, indicating the

### Table 1. Founder lines and the relative level of transgene expression in lacZ/Knock-in, 2.5kb/lacZ, 0.295kb/lacZ, and 2x0.128kbTATA/lacZ mouse embryos at approximately embryonic day 11.5

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>Cono-truncus</th>
<th>Right ventricle</th>
<th>Left ventricle</th>
<th>Right atrium</th>
<th>Left atrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>lacZ/ Knock-in</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>6</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>0.295kb/ 21</td>
<td>+++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2x0.128</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

An arbitrary scale was assigned by the intensity of X-gal staining with ++++ being the most intense staining, – having no detectable staining.
presence of distinct cis regulatory elements that may control heart segment-specific expression of the CARP gene at different developmental stages. Our studies have demonstrated that distinct 5’ regulatory elements of the CARP gene can direct heart segment-specific gene expression, suggesting that the pan-cardiac expression of

Fig. 7. The pattern of transgene expression during conotruncal development in the p2x0.128TATAlacZ transgenic mice. Hearts from various developmental stages of the p2x0.128TATAlacZ transgenic mice were stained for β-galactosidase activity in whole mount (A-E), followed by histological sectioning and eosin staining (F-H). (A,B) The ventral (A) and top (B) view of the heart from an embryo at E9.5. (C) The ventral view of the heart from an embryo at E14.5. (D) The ventral and dorsal views of the heart from an embryo at E14.5 in which the atria of the heart were removed for a better view of the X-gal staining. V, ventral; D, dorsal. Note that the transgene expression is absent on the dorsal side of the aorta. (E) The top view of the heart shown in D. Ao, aorta; P, pulmonary artery. (F) Two representative transverse sections at the junction of the great arteries and the ventricles of the heart shown in C. A, atrium. (G) A sagittal section of the heart from an embryo at E14.5 showing that the transgene is expressed in cardiomyocytes located right below the semilunar valve of the pulmonary artery (indicated by the asterisk). (H) A sagittal section of the heart from a 2-day-old neonate.

Fig. 8. Down regulation of transgene expression in p0.295lacZ/Nkx2.5+/+ mice. The p0.295lacZ/Nkx2.5+/+ and p0.295lacZ/Nkx2.5−/− mice were stained for β-galactosidase activity in whole mount at E9.5 (A,B), followed by histological sectioning and eosin staining (C,D). (A) A p0.295lacZ/Nkx2.5+/+ mouse. CT, conotruncus; BC, bulbar cordis; LV, left ventricle. (B) A p0.295lacZ/Nkx2.5−/− mouse. A, atrium. (C,D) Sections of (C) a p0.295lacZ/Nkx2.5+/+ and (D) a p0.295lacZ/Nkx2.5−/−embryo. AS, aortic sac; EC, endocardium.
the endogenous CARP gene is due to the combination of segmental-specific expression of the gene in various compartments of the heart. Previous studies of the MLC-2v and desmin promoters have also identified cis regulatory elements that can direct a left-right gradient of ventricular transgene expression as those seen in our present study (Ross et al., 1996; Kuisk et al., 1996). Additionally, studies of the GATA-6 and the MLC-5f promoters have demonstrated the presence of discrete regulatory elements that can direct regionalized expression of the transgene in the atrioventricular (AV) canal as well as in the right atrial/left ventricular chambers and the AV canal, respectively (Franco et al., 1997; He and Burch, 1997). Taken together, these data indicate that distinct regulatory programs are operative in different compartments of the heart. It suggests that cardiac myocytes of the conotruncus, AV canal, and each of the four chambers are intrinsically different in their molecular properties. Instead of using a common set of transcriptional factors to drive the expression of a gene throughout the whole heart, distinct cardiac segments may direct their region-specific gene expression with an individually unique combinatorial set of factors.

**Nkx2.5 and GATA-4 cooperatively regulate CARP expression**

Emerging evidence is now suggesting that, as opposed to the master-regulatory mechanism present during skeletal myogenesis, cardiac myogenic specification might occur through a combinatorial pathway (Evans et al., 1994; Ross et al., 1996). In our studies, we have demonstrated the essential roles of two cardiac-restricted transcription factors, Nkx2.5 and GATA-4, in controlling the cardiac specificity of the CARP promoter. Nkx2.5 is the mouse homologue of the Drosophila homebox gene tinman, which specifies the cardiac muscle cell lineage in Drosophila, and it has also been shown to be required for heart tube looping morphogenesis during murine heart development (Lyons et al., 1995). Like Nkx2.5, GATA-4 is also among the earliest markers of the cardiac muscle cell lineage, and this zinc finger transcription factor has been shown to be required for ventral

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**Fig. 9.** Identification of an essential GATA-4 binding site in the proximal CARP promoter and cooperative transcriptional regulation mediated by Nkx2.5 and GATA-4. (A) Schematic diagrams of p0.295Luc, p0.295GATAmP, p0.295GATAmD, and p0.295GATAmP&D. The sequences at both the proximal and distal GATA sites are shown, and the mutations are in lower case. (B) Electrophoretic mobility shift assay with in vitro-translated GATA-4 protein. The sequences of the wild-type and mutant oligonucleotide probes for the proximal GATA site are shown in A. Lanes 1 and 2: The wild-type probe was incubated with non-programmed or GATA-4 programmed reticulocyte lysates, respectively. Lane 3: The mutant probe was incubated with the GATA-4 programmed lysate. Lanes 4-6: The wild-type probe was incubated with GATA-4 only (lane 4), GATA-4 plus a 100-fold excess of the unlabelled wild-type oligonucleotide competitor (lane 5), or GATA-4 plus a 100-fold excess of the unlabelled mutant oligonucleotide competitor (lane 6). Lanes 7-9: The wild-type probe was incubated with control lysate (lane 7), GATA-4 (lane 8), or GATA-4 in the presence of an anti-GATA-4 antibody (lane 9). C, the specific complex; S, the supershifted complex. (C) Transactivation of the CARP promoter by Nkx2.5 and GATA-4 in CV1 cells. 4 μg of the reporter construct, 2 μg of pcDNA3/GATA-4, 2 μg of pCGN/Nkx2.5, and 1 μg of pON2 were used in the transfection assay. The fold of transactivation was calculated by using the luciferase activity of p0.295Luc in the absence of both pcDNA3/GATA-4 and pcGN/Nkx2.5 as a standard.
morphogenesis and heart tube formation (Kuo et al., 1997; Molkentin et al., 1997).

Many studies have previously demonstrated that Nkx2.5 and GATA-4 can work cooperatively to transactivate the promoters of several cardiac genes, including the ANF and the cardiac α-actin genes (Durocher et al., 1997; Lee et al., 1998; Sepulveda et al., 1998). For the CARP promoter, we have shown that, although required, individually, neither Nkx2.5 nor GATA-4 is sufficient to activate the promoter in a heterologous system. However, when both are present, Nkx2.5 and GATA-4 can work in concert to activate the promoter, and this positive cooperation is dependent on the binding of GATA-4 to its cognate DNA sequence in the promoter. These results suggest that Nkx2.5 may exert its control on the CARP promoter, at least in part, through GATA-4. Several studies have previously demonstrated a physical association between Nkx2.5 and GATA-4 in vivo as well as in vitro (Durocher et al., 1997; Sepulveda et al., 1998). It is possible that our observed cooperation between Nkx2.5 and GATA-4 is due to direct binding of Nkx2.5 to GATA-4. However, it cannot be excluded that Nkx2.5 may also act upstream of other GATA-4 interacting factors to control the activity of the CARP promoter.

It is possible that there may be other Nkx2.5-responsive elements present in sequences upstream of -295, which may be involved in direct binding of Nkx2.5 to the DNA or interaction of Nkx2.5 with other factors, such as serum response factor (SRF), which has been shown to cooperate with Nkx2.5 to regulate the cardiac α-actin promoter (Chen et al., 1996). A recent study has indicated that the cooperative transcriptional regulation mediated by Nkx2.5 and GATA-4 can have either a positive or negative effect on promoter activity depending on the context of the promoter (Shiojima et al., 1999). In addition, Nkx2.5 and GATA-4 have been shown to act in combination in an autoregulatory feedback loop supporting Nkx2.5 expression (Reecy et al., 1999). Taken together, these data indicate the complex nature of the cardiac regulatory network, in which the expression of cardiac genes are fine-tuned by complex combinatorial interactions among cardiac-restricted as well as ubiquitous transcription factors.

It has been shown that GATA-4 can interact with API and NF-AT3 to synergistically activate cardiac transcription in different models of cardiac hypertrophy (Herzig et al., 1997; Molkentin et al., 1998). In this study, we have shown that CARP is a cardiac hypertrophic marker, and like many other hypertrophic markers, such as ANF, BNP, and β-MHC, CARP also contains an essential GATA-4 binding site in its upstream regulatory region. It is interesting to speculate, but remains to be determined, whether GATA-4 may be also involved in mediating the dramatic induction of CARP expression in cardiac hypertrophy.

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