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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/β-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 GATA-4 binding site in the proximal upstream regulatory region of the *CARP* gene and cooperative transcriptional regulation mediated by Nkx2.5 and GATA-4. We have also shown that this cooperative regulation is dependent on the binding of GATA-4 to its cognate DNA sequence in the promoter, which suggests that Nkx2.5 may exert its control on the *CARP* promoter, at least in part, through GATA-4.

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-^{32}P]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′-GTTACCAGCTCCTCTACTCTCAG-TACCATC-3′) complementary to the 5′ end of the mouse *CARP* cDNA was ^{32}P-labelled with T_4 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 *Bam*HI/*Xho*I-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 *Hin*cII site of p7/318 (Ambion). A *Hin*dIII-*Xho*I 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 *Pst*I-*Sac*II, a 620 bp *Hin*dIII-*Sac*II, and a 2.4 kb *Bam*HI-*Sac*II fragments were isolated from the *CARP* genomic DNA and subcloned into p0.295/Luc to make the respective constructs. To construct p10Luc, a 10 kb *Kpn*I-*Sac*II fragment and a 223 bp *Sac*II-*Xho*I fragment were isolated from the *CARP* genomic DNA and the p0.295Luc, respectively, which were subsequently subcloned into the *Kpn*I/*Xho*I 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 *Bam*HI/*Eco*RI and *Eco*RI/*Xho*I, 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 *Bam*HI/*Pst*I and *Pst*I/*Xho*I, 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 *Sac*II/*Xho*I fragment from p0.295GATAmP was isolated and ligated to a *Sac*II/*Xho*I digested p0.295GATAmD to make p0.295GATAmP&D. To construct p2.5lacZ, a 2.5 kb *Bam*HI-*Xho*I fragment from the p2.5Luc and a 3.3 kb *Xho*I-*Sma*I fragment from pSDlacZpA (A gift from Janet Rossant) were ligated into the *Bam*HI/*Sma*I sites in p7/318. To construct p0.295lacZ, a 342-bp *Hin*dIII-*Xho*I fragment from p0.295Luc and a 3.3 kb *Xho*I-*Bam*HI fragment from pSDKlacZpA were ligated into p7/318. To construct p2x0.128TATAlacZ, a *Bam*HI-*Xho*I fragment was isolated from p2x0.128TATALuc, blunt-ended, and subcloned into the *Xho*I site in pSDKlacZPA. 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′-AAAACTGCAGCACCCAGCCCTAGCTATATAAC-3′
Primer 8: 5′-AAAACTGCAGGGAAGCCAGGGGACAGCTGCC-3′

Whole-mount and histological analysis of transgenic embryos

Embryos or postnatal hearts were fixed and stained using previously

described techniques (Ross et al., 1996). For paraffin-based histological analysis, specimens were postfixed in 4% formaldehyde, dehydrated and embedded in paraffin. Sections were cut at 5-8 μm and counter-stained with 1% eosin.

Electrophoretic mobility shift assay

The annealed oligonucleotide probes were labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. 5 μl of the programmed rabbit reticulocyte lysate using the pcDNA3/GATA-4 as a template was incubated with 0.2 ng of the probe and 2 μg of polydI.dC in 20 μl of binding buffer (10 mM Tris-HCl/pH: 7.4, 50 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol and 0.05% Nonidet P-40) at room temperature for 1 hour. The nucleoprotein complexes were separated by 5% polyacrilamide gel in 0.5× Tris borate/EDTA buffer at 4°C for 2 hours at 200 V.

RESULTS

Developmental and inducible regulation of CARP expression in the embryonic and adult hearts

In this study, we first explored whether CARP is a member of the inducible fetal gene program in the heart. We examined the expression of the *CARP* gene in a mouse model of left ventricular hypertrophy induced by pressure overload using a microsurgical technique involving transverse aortic constriction (TAC). Northern blot analyses indicated that CARP expression was dramatically induced 4 days after the TAC procedure, and this increased level of CARP expression persisted to day 7 post-TAC procedure (Fig. 1A). At days 4 and 7, the mice subjected to TAC also showed a significant increase in the ratio of the left ventricle weight to the body weight compared to the sham control (data not shown), indicating the development of left ventricular hypertrophy. This development of ventricular hypertrophy was further confirmed by the induction of the *ANF* gene, which is a well-established marker for cardiac hypertrophy (Fig. 1A). Taken together, our data indicated that the induction of CARP expression was correlated with the development of heart hypertrophy.

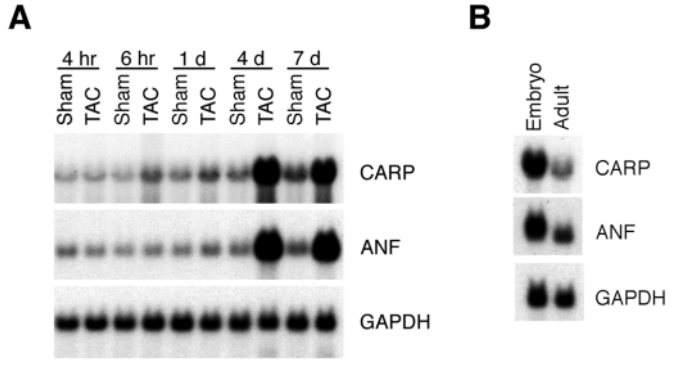

Fig. 1. CARP is a marker for heart hypertrophy and the expression of CARP is developmentally down-regulated. (A) Northern blot analyses showed induction of CARP expression in heart hypertrophy. Total RNA was isolated from the left ventricle of mice that underwent various lengths of time of TAC procedure (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).

During cardiac hypertrophy, it is known that an embryonic gene program becomes reactivated. We asked whether CARP, as a hypertrophic marker, also belongs to this fetal gene program. Accordingly, we examined CARP expression in both embryonic and adult ventricles. Northern blot analyses showed a high level of ventricular CARP expression in embryos, which became down-regulated in adult ventricles, similar to developmental regulation of the *ANF* gene in the ventricle (Fig. 1B). This developmental down regulation of CARP expression in the heart indicates that CARP, like other known hypertrophic markers, also belongs to the fetal gene program.

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. Using the luciferase activity of p10Luc, which contained approximately 10 kilo base pairs (kb) of the 5′ flanking sequence of the *CARP* gene, as a relative standard, p2.5Luc, p0.796Luc, p0.54Luc, p0.295Luc and p0.176Luc retained approximately 98%, 56%, 37%, 20% and 0.2% activity, respectively (Fig. 3C). While p0.176Luc displayed only background level of activity, an additional construct, p2x0.128TATALuc, which contained two tandem repeats of the 0.128 kb 5′ flanking sequence spanning from −39 to −166 and

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 transgene 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 p2x0.128TATAlacZ, whose expression were directed by the *CARP* promoters derived from p2.5Luc, p0.295Luc, and p2x0.128TATALuc, 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 β-gal expression. Five lines were positive for cardiac and skeletal muscle-specific transgene expression and one line was negative. For the p2x0.128kbTATAlacZ transgenic mice, three lines were analyzed.

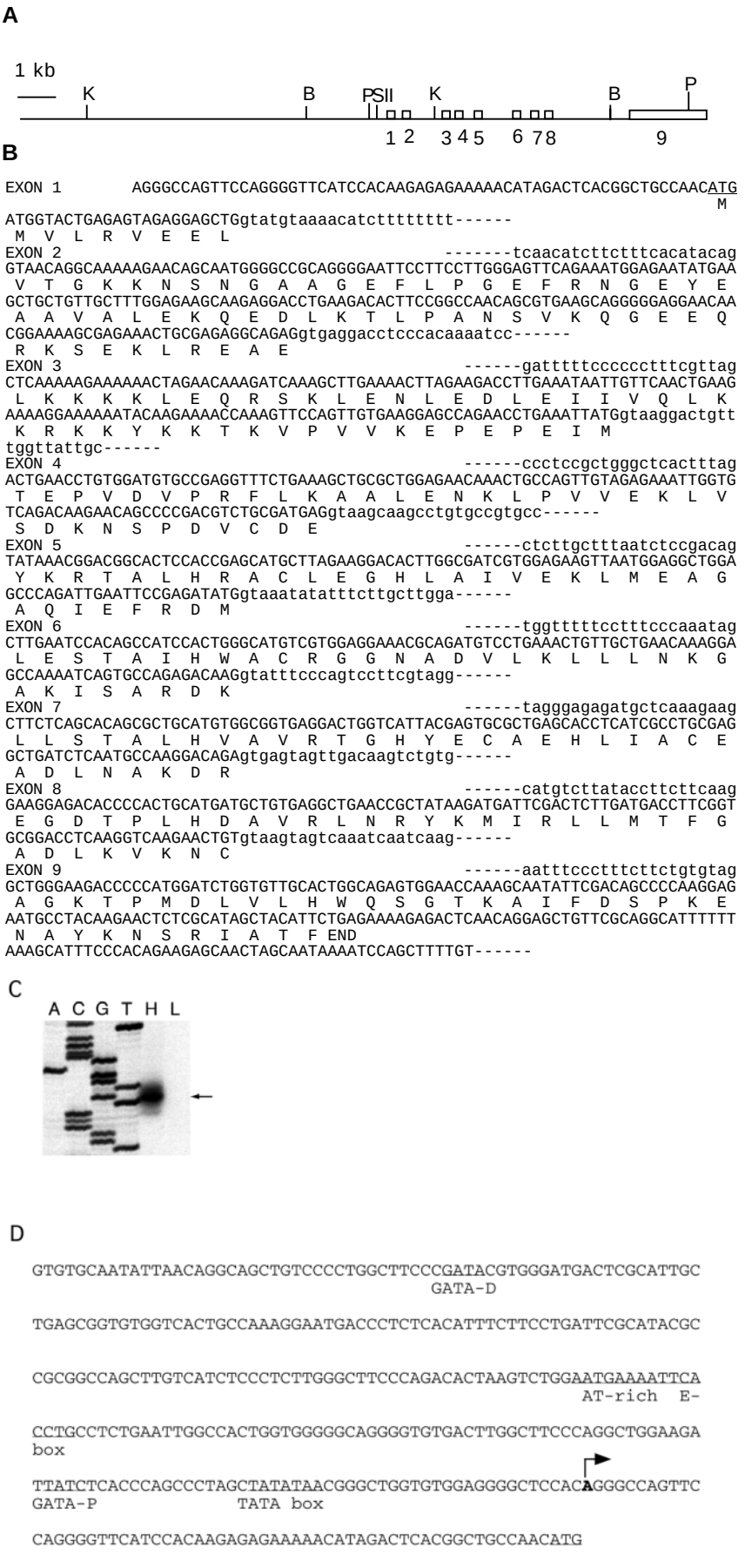

Fig. 2. Structural organization of the *CARP* genomic DNA. (A) Structure and restriction map of the *CARP* genomic DNA. All the nine exons are shown and indicated by the numbers below the boxes. B, *Bam*HI; K, *Kpn*I, P, *Pst*I; and SII, *Sst*II. (B) Sequence of the nine exons and their immediately adjacent 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 (from –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 accession 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 p2x0.128kbTATAlacZ 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 p2x0.128kbTATAlacZ 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 p2x0.128kbTATAlacZ mice using the transgene as a conotruncal-specific marker

Taking advantage of the conotruncal-specific transgene expression in the p2x0.128kbTATlacZ 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

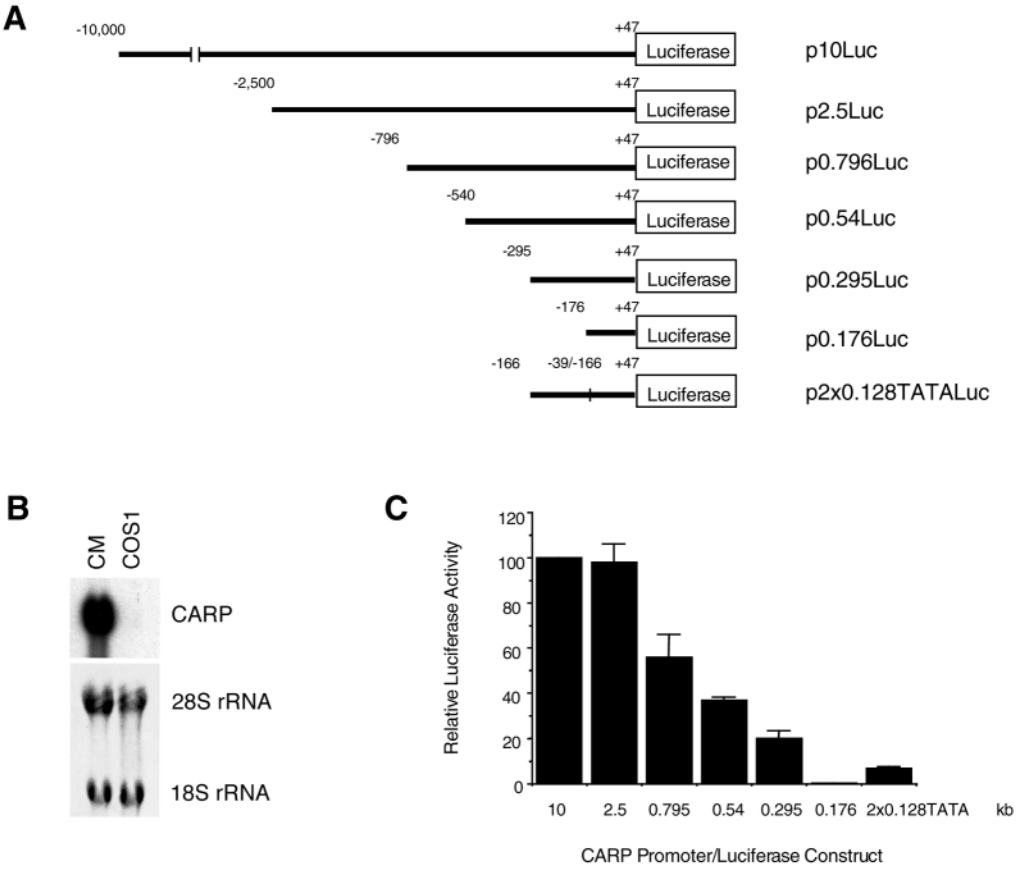

Fig. 3. Transient expression assay to identify regulatory *cis* elements that confer cardiac specificity. (A) Schematic diagrams of a series of luciferase constructs containing 5′ nested deletions of the *CARP* promoter. The names of the constructs are shown on the right. For convenience, the constructs were named according to the 5′ end of the truncated *CARP* promoters (the numbers indicate the number of kilobases). Note that in addition to the corresponding 5′ flanking sequences, all of the constructs contain 47 base pairs of the 5′ untranslated region. (B) Northern blot analyses showed specific expression of the *CARP* gene in cultured primary cardiomyocytes (CM), but not in COS1 cells (COS1). The 28S and 18S rRNAs were used as the loading control. (C) A histogram showing relative promoter activities of the series of luciferase constructs in cardiomyocytes using the luciferase activity of p10Luc as the relative standard. All transfections were performed in duplicate, and the data were derived from two transient expression assays.

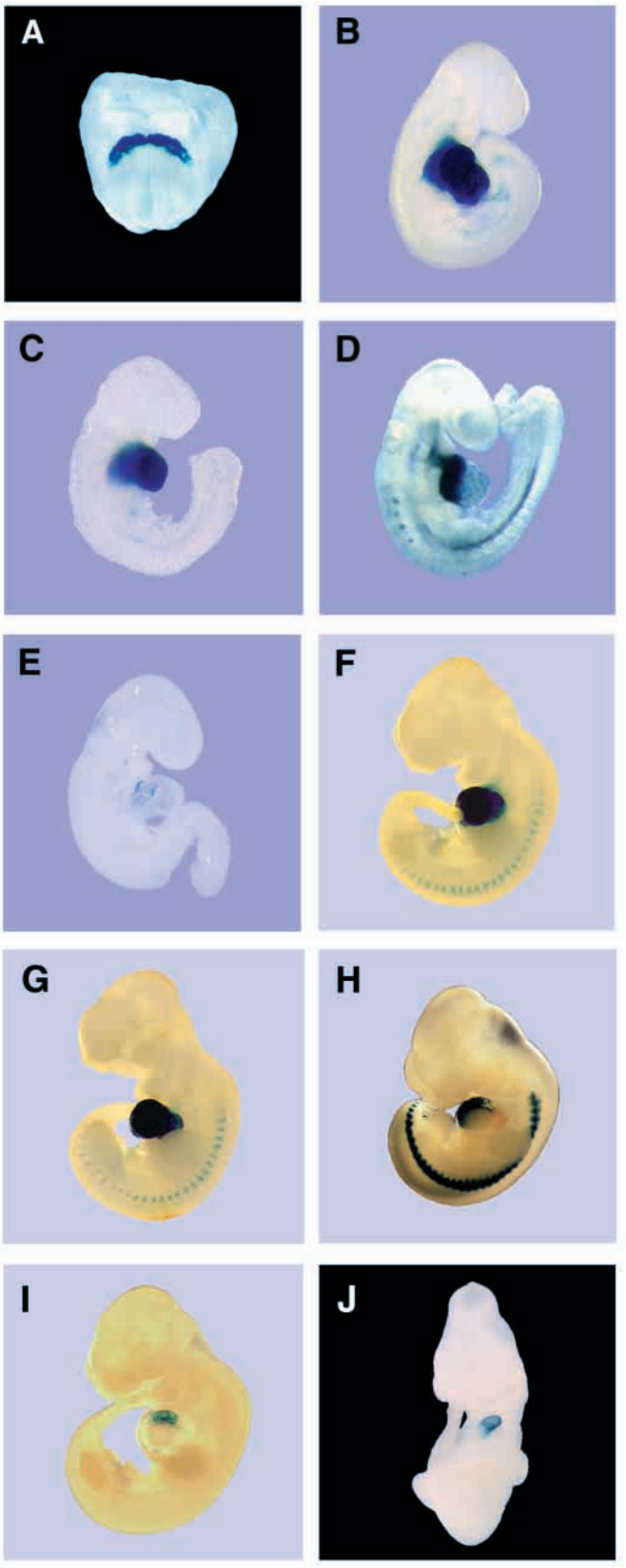

Fig. 4. Distinct regulatory elements direct region-specific transgene expression in mice. Embryos of the *lacZ*/knock-in, p2.5lacZ, p0.295lacZ, and p2x0.128TATlacZ transgenic mice were stained in whole mount for β-galactosidase activity. (A) A p2.5lacZ transgenic embryo at E8-8.5. (B-E) Embryos stained at E9-9.5. (B) A *lacZ*/knock-in embryo. (C) A p2.5lac transgenic embryo. (D) A p0.295lacZ transgenic embryo. (E) A p2x0.128TATlacZ transgenic embryo. (F-J) Embryos stained at E11-11.5. (F) A *lacZ*/knock-in embryo. (G) A p2.5lac transgenic embryo. (H) A p0.295lacZ transgenic embryo. (I) A p2x0.128TATlacZ transgenic embryo. (J) The frontal view of the same embryo shown in E.

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

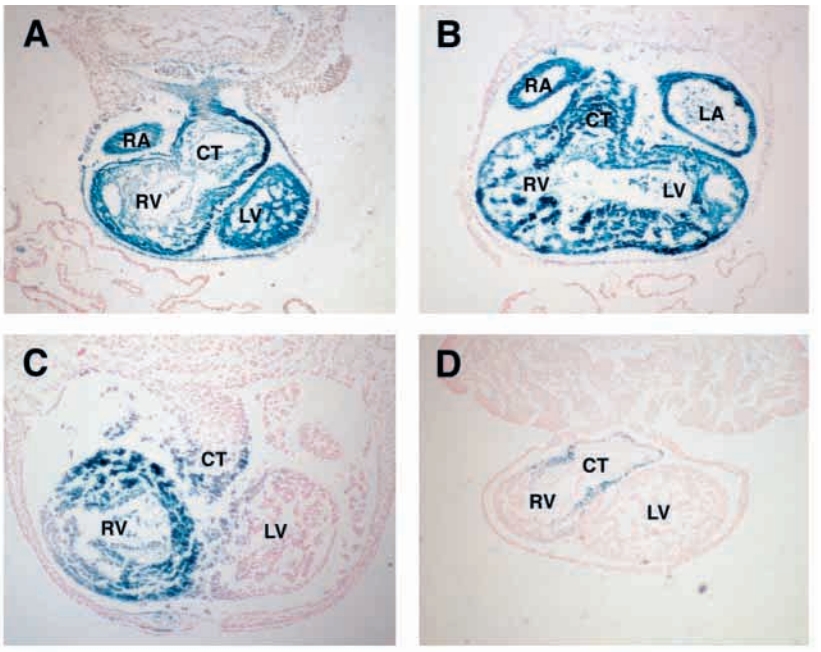

Fig. 5. Representative histological sections demonstrate that distinct regulatory elements confer heart segment-specific transgene expression. Transverse sections of X-gal-stained transgenic embryos at E11-11.5. A A *lacZ*/knock-in embryo. B A p2.5lacZ transgenic embryo. (C) A p0.295lacZ transgenic embryo. (D) A p2x0.128TATlacZ transgenic embryo. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; CT, conotruncus.

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. ^{32}P-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

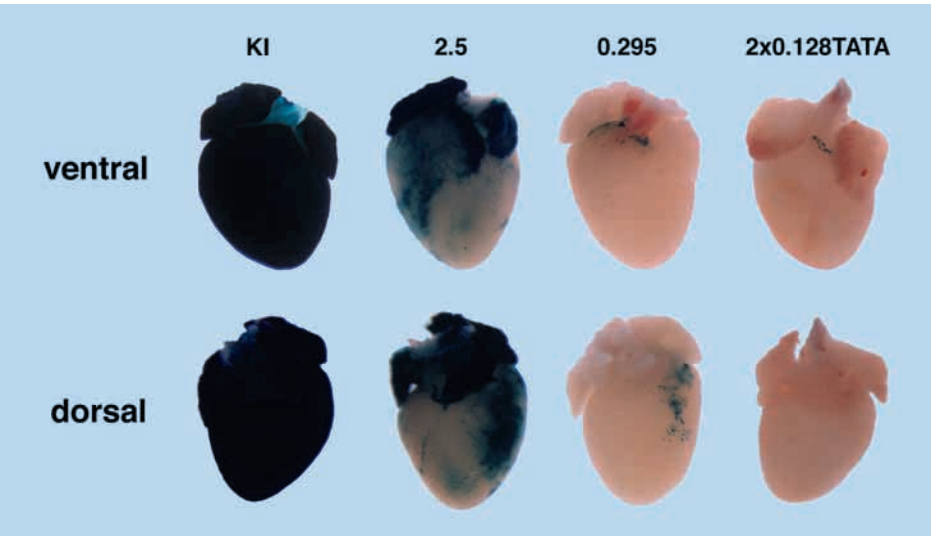

Fig. 6. Developmental regulation of transgene expression in the heart. Hearts of day 2-3 neonates from (left to right) the *lacZ*/knock-in, p2.5lacZ, p0.295lacZ, and p2x0.128TATlacZ transgenic mice were stained for β-galactosidase activity in whole mount.

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

Transgenic lines		Cono-truncus	Right ventricle	Left ventricle	Right atrium	Left atrium
lacZ/ Knock-in		++++	++++	++++	++++	++++
	1	+++++	+++++	+++++	+++++	+++++
	6	+++++	+++++	+++++	+++++	+++++
2.5 kb/	8	+++++	+++++	+++++	+++++	+++++
lacZ	10	++++	++++	++++	++++	++++
	22	++++	++++	++++	++++	++++
	14	++	++	–	–	–
0.295kb/	21	+++	+++	–	–	–
lacZ	23	++	++	–	–	–
	37	+++	+++	–	–	–
	47	++	++	–	–	–
2x0.128	8	+	–	–	–	–
kbTATA/ *lacZ*	49	+	–	–	–	–

An arbitrary scale was assigned by the intensity of X-gal staining with +++++ being the most intense staining, – having no detectable staining.

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

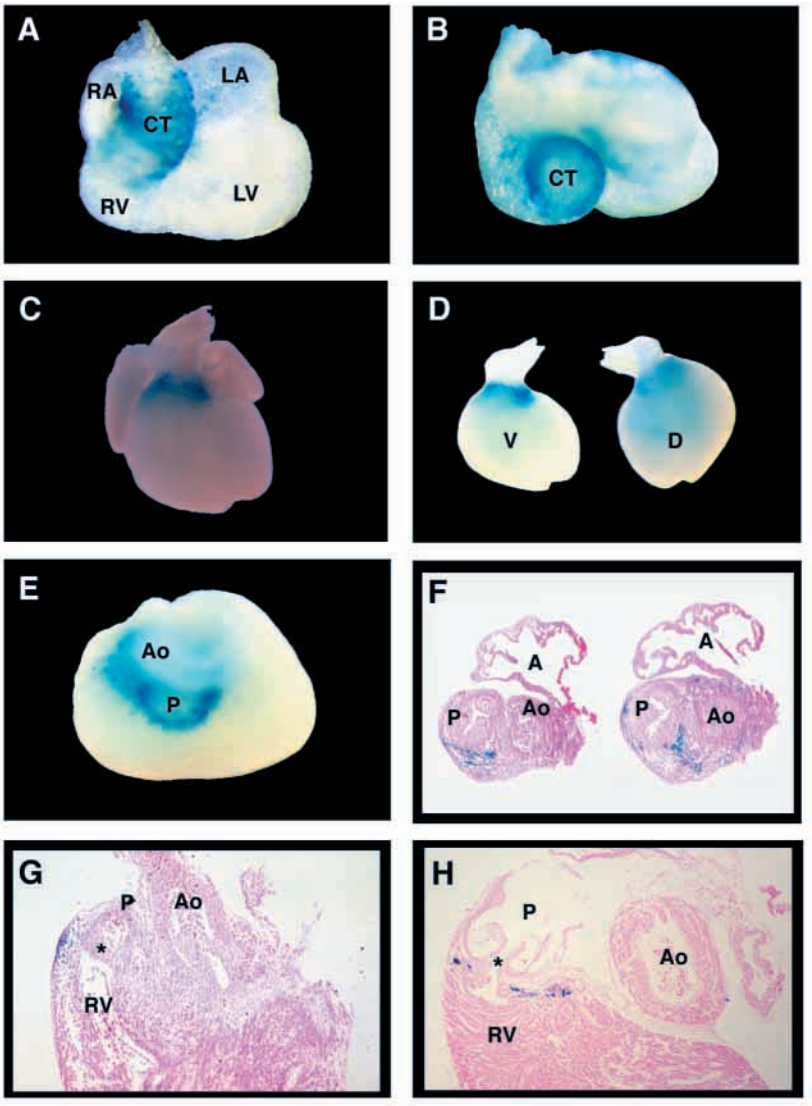

Fig. 7. The pattern of transgene expression during conotruncal development in the p2x0.128TATAlacZ transgenic mice. Hearts from various developmental stages of the p2x0.128TATAlacZ transgenic mice were stained for β-galactosidase activity in whole mount (A-E), followed by histological sectioning and eosin staining (F-H). (A,B) The ventral (A) and top (B) view of the heart from an embryo at E9-9.5. (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.

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

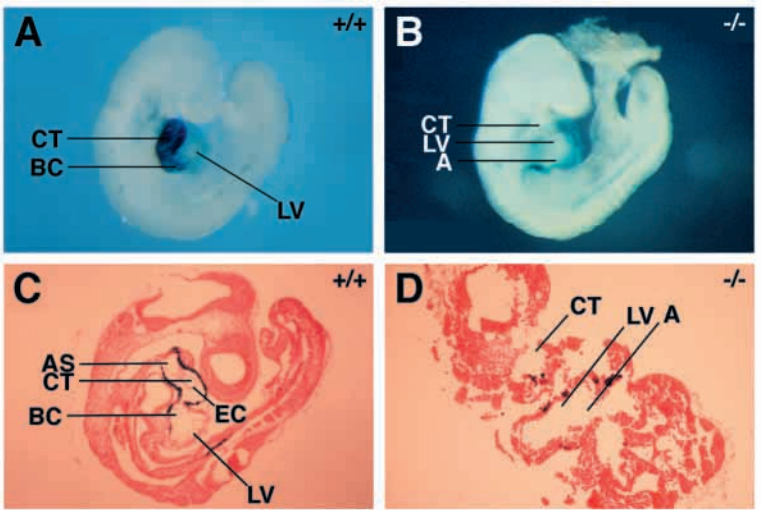

Fig. 8. Down regulation of transgene expression in p0.295lacZ/*Nkx2.5*$^{-/-}$ mice. The p0.295lacZ/*Nkx2.5*$^{+/+}$ and p0.295lacZ/*Nkx2.5*$^{-/-}$ mice were stained for β-galactosidase activity in whole mount at E9.5 (A,B), followed by histological sectioning and eosin staining (C,D). (A) A p0.295lacZ/*Nkx2.5*$^{+/+}$ 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-3f* 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 homeobox 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

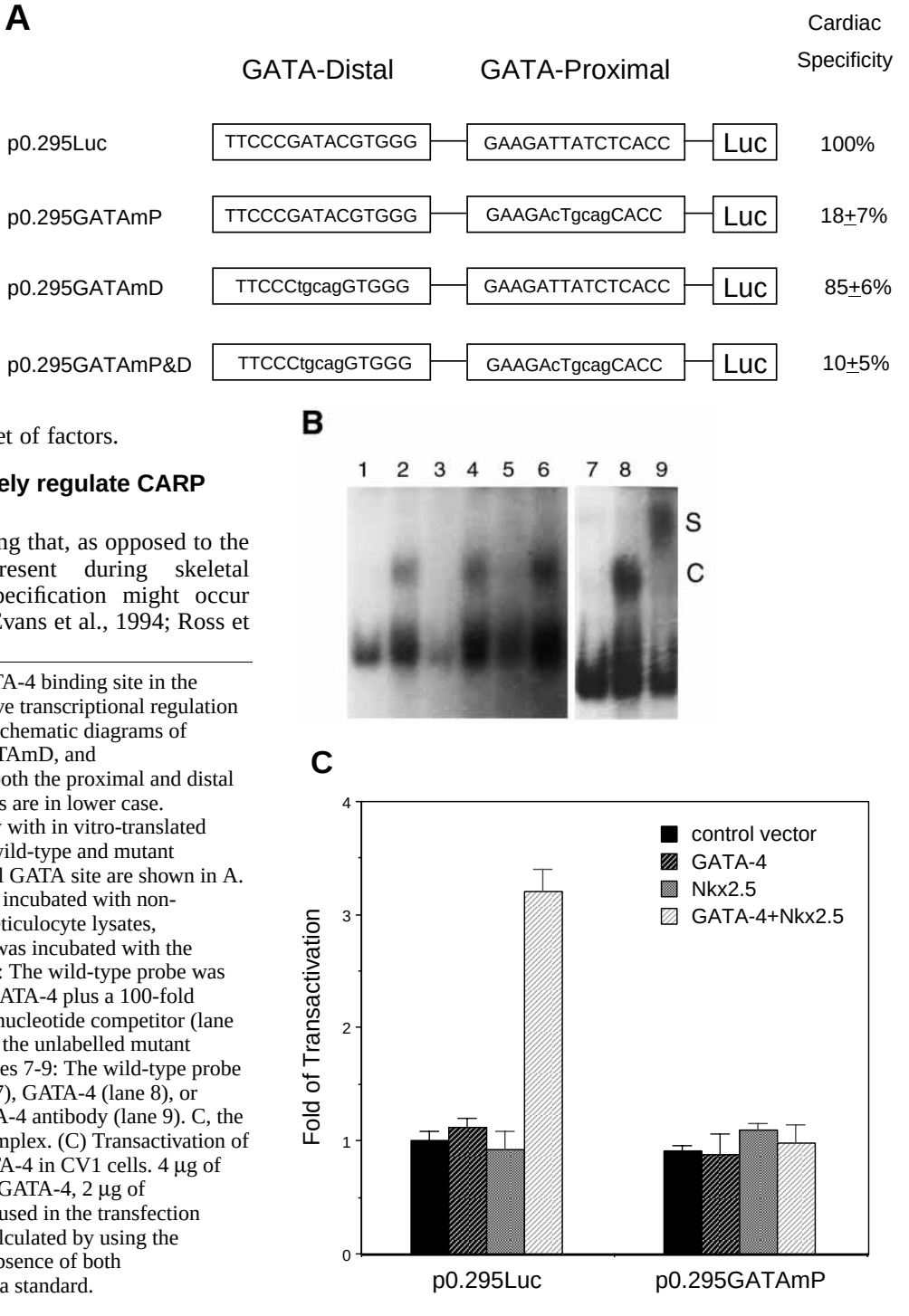

Fig. 9. Identification of an essential GATA-4 binding site in the proximal *CARP* promoter and cooperative transcriptional regulation mediated by Nkx2.5 and GATA-4. (A) Schematic diagrams of p0.295Luc, p0.295GATAmP, p0.295GATAmD, and p0.295GATAmP&D. The sequences at both the proximal and distal GATA sites are shown, and the mutations are in lower case. (B) Electrophorectic mobility shift assay with in vitro-translated GATA-4 protein. The sequences of the wild-type and mutant oligonucleotoide 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 AP1 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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