A Novel Cardiac-Restricted Target for Doxorubicin

CARP, A NUCLEAR MODULATOR OF GENE EXPRESSION IN CARDIAC PROGENITOR CELLS AND CARDIOMYOCYTES

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Doxorubicin (Dox), a cardiotoxic antineoplastic drug, disrupts the cardiac-specific program of gene expression (Kurabayashi, M., Dutta, S., Jeyaseelan, R., and Kedes, L. (1995) Mol. Cell. Biol. 15, 6386–6397; Jeya-

The widely used, but cardiotoxic, chemotherapeutic agent adriamycin (doxorubicin; Dox)1 alters transcriptional events specific to the myocardium (1–4). Initially we demonstrated that Dox selectively inhibits the accumulation of mRNA of heart-specific genes in cardiomyocytes (5) including transcripts of nuclear genes encoding enzymes critical in the production and maintenance of ATP in cardiomyocytes (1). Dox also inhib-

We report here the identification of a cardiac-restricted nuclear protein that is constitutively expressed and whose mRNA level is exquisitely sensitive to Dox. Hence we have named this protein cardiac adriamycin-responsive protein (CARP). CARP mRNA is present at the earliest stages of cardiac morphogenesis. It was detected by in situ hybridization within the cardiogenic plate of 7.5-day post coitum (p.c.) embryos, and in 8.5-day p.c. em-

CARDIOMYOCYTES*

The cloned full-length 1749 nucleotide CARP cDNA encodes a 319-amino acid 40-kDa polypeptide containing five tandem ankyrin repeats. CARP appears to be the rat homolog of the Drosophila melanogaster gene responsible for cardiac development. The cloned full-length 1749 nucleotide CARP cDNA encodes a 319-amino acid 40-kDa polypeptide containing five tandem ankyrin repeats. CARP appears to be the rat homolog of previously reported human single-copy gene (C-193; Chu, W., Burns, D. K., Sweirlick, R. A., and Presky, D. H. (1995) J. Biol. Chem. 270, 10236–10245), whose mRNA is inducible by cytokines only in human endothelial cells. CARP appears to function as a nega-

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1 The abbreviations used are: Dox, doxorubicin; PCR, polymerase chain reaction; ANF, atrial natriuretic factor; bp, base pair(s); CAT, chloramphenicol acetyltransferase; cTnC, cardiac troponin C; Dn, daunomycin; RACE, rapid amplification of cDNA ends; UTR, untranslated region; PBS, phosphate-buffered saline; CMV, cytomegalovirus; aa, amino acids; TNF-α, tumor necrosis factor α; LPS, lipopolysaccha-

ride; p.c., post coitum.

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involved in commitment and maintenance of the vertebrate cardiac phenotype remain unclear. Here, we provide evidence that CARP plays a negative role in cardiac-specific transcription and speculate that it may be important in the maintenance of the cardiac phenotype. Moreover, its rapid and sensitive elimination by adriamycin exposure may well contribute to the cardiac-specific toxicity of adriamycin and the development of cardiomyopathy.

MATERIALS AND METHODS

Plasmide—A cDNA of CARP fused with a FLAG peptide at its carboxyl terminus was synthesized by PCR. The sense primer with a BamHI site at its 5′ end was complimentary to the amino-terminal codons of CARP, starting from the ATG. The antisense primer spanned the carboxyl-terminal codons with the antisense FLAG amino acids and an XbaI site at the 5′ end. The PCR product was digested with BamHI and XbaI and cloned in the BamHI and XbaI sites of pAC-CMV (13). The resulting construct is pACCMV-CARP-FL.

The cardiac atrial natriuretic factor (ANF) promoter construct, which contains the first 700 bp of the promoter, was generously provided by Dr. Heikki Ruskoaho (University of Oulu, Oulu, Finland). β-Actin promoter spanned the carboxyl-terminal codons with the antisense FLAG amino acids and an XbaI site at the 5′ end. The PCR product was digested with BamHI and XbaI and cloned in the BamHI/XbaI sites of pAC-CMV (13). The resulting construct is pACCMV-CARP-FL.

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To generate plasmid CMV-NXX2.5, plasmid cMlx1.2 (kindly provided by Dr. Richard Harvey, Victoria, Australia) was digested with EcoRI restriction endonuclease. The resulting fragment was ligated into EcoRI site of the pcDNA3 vector (Invitrogen). The orientation of the plasmid was confirmed by DNA sequencing. To generate the GAL4 DNA-binding domain-CARP fusion construct, CARP cDNA was PCR-amplified using Pfu polymerase and subcloned into plasmid GALO (gift from G. F. Tomaselli), which contains four GAL4 DNA-binding sites. The resulting plasmid was named GAL-CARP, and to ascertain that the plasmid was in a correct reading frame, CARP was sequenced in both orientations. To create plasmid CMV-CARP, CARP cDNA was excised from the TA cloning vector by XbaI digestion and was then filled in with phage T4 DNA polymerase, followed by BamHI cleavage. CARP cDNA was ligated into HindIII/blunt/BamHI-digested pcDNA3 vector (Invitrogen). UAS 4-tk luciferase was a gift from Dr. Ron Evans (Salk Institute, La Jolla, CA).

Primary Neonatal Rat Cardiac Myocyte Culture and Dox Treatment—Neonatal rat cardiomyocytes from 2–4-day-old Sprague-Dawley rats were prepared using a previously described protocol (16). The cells were grown at 37 °C with 5% CO2 in modified Eagle’s medium containing 5% fetal calf serum, 2 mM glutamine, 1% penicillin/streptomycin, and 1% 5-bromodeoxyuridine. After the cells start beating 24 h post-culture, they were treated either with 1 μM Dox or 1 μM daunomycin (Dn) for 24 h.

RNA Isolation and Northern Blotting—Total RNA was isolated using Trizol reagent (Life Technologies, Inc.) from drug-treated and untreated cardiomyocytes. The concentrations of the RNA were measured by spectrophotometry. The integrity of the RNA was ensured by analysis in 1.2% formaldehyde-agarose gels. After electrophoresis RNA was transferred to Hybond N+ nylon membranes (Amerham Corp.). The cDNA inserts excised from clones were labeled with [α-32P]dCTP by a random primer DNA labeling kit (Boehringer Mannheim). The membranes were prehybridized and hybridized as described earlier (2), washed in 1% SDS, 50 mM NaCl, and 1 mM EDTA three times at 55 °C for 15 min each, and exposed to Kodak XRR film at −80 °C.

mRNA Differential Display—Differential display was carried out using the RNA map® kit (GeneHunter Corporation) following the supplied protocol (14). Briefly 200 ng of total RNA from neonatal rat cardiomyocytes was used for one reverse transcription reaction containing 100 units of Moloney murine leukemia virus reverse transcriptase, 2 μl of four primers in one reaction (10 μM T12MA, T12MC, T12MG, or T12MT) in a 20-μl reaction volume in the presence of 20 μM dNTPs and the reverse transcription buffer (25 mM Tris-Cl, pH 8.3, 37.6 mM KCl, 1.5 mM MgCl2, 5 mM dithiothreitol). The reverse transcription reaction product (2 μl) was used for one PCR reaction in a volume of 20 μl containing 2 μl of the same four primers, 10 mM Tris, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, 2 μl dNTP, 2 μl of 20 μM arbitrary primer, 1 μl of [α-35S]dATP (1200 Ci/mmol), and 0.2 μl of AmpliTaq (Perkin-Elmer). The PCR reactions were performed for 40 cycles as follows: 94 °C for 30 s, 40 °C for 2 min, 72 °C for 30 s, followed by 5 min at 72 °C for the elongation step. The PCR products were analyzed on a 6% sequencing gel. The differentially expressed bands were excised from the dried gel and eluted. The eluted DNAs were amplified using the same primers used for initial amplification using the same PCR conditions except that the dNTP concentration was increased (20 μM). The PCR products were cloned using the TA cloning vector (Invitrogen), and the inserts from the obtained colonies were used as probes in Northern blot analysis.

FIG. 1. A, differential screening for Dox- or Dn-responsive mRNAs. Differential display was carried out using total RNA extracted from rat neonatal cardiomyocytes left untreated (lane C) or treated with 1 μM Dox (lane Dx) or 1 μM Dn (lane DN) for 24 h. Radiolabeled PCR products were analyzed in a 6% polyacrylamide gel. The sequencing gel shows differentially regulated genes. B, Northern blot analysis of CARP expression in control cardiomyocytes (lane C) and cardiomyocytes treated with Dox (lane Dx) or Dn (lane DN). CARP partial cDNA was recovered from the differential display sequencing gel, PCR-amplified, and radiolabeled to probe 10 μg of total RNA made from untreated cardiomyocytes or cardiocytes grown in the presence of 1 μM Dox or 1 μM Dn for 24 h. The methylene blue-stained pattern of the blot shows that an equal amount of RNA was loaded in each lane.

FIG. 2. A, CARP and troponin I (Tnl) mRNA levels in cardiomyocytes exposed to Dox or Dn for 2–48 h. 10 μg of total RNA was extracted from control (lane C) and Dox-treated cardiomyocytes at time 2, 4, 8, 16, 24, and 48 h and probed with CARP and tropo-
Cloning of CARP cDNA by 5' RACE—Based on a 3'-UTR sequence, an antisense primer was synthesized. 5' RACE was performed using poly(A)⁺ mRNA isolated from rat neonatal cardiomyocytes plus the antisense primer following the company protocol supplied with the Marathon cDNA Amplification kit (Clonetech Laboratories, Inc.). The RACE product was cloned in the TA vector (Invitrogen), and the entire cDNA was sequenced. Double stranded sequencing of the positive clones were carried out using a Sequenase 2.0 kit (U. S. Biochemical Corp.). Nucleotide and predicted amino acid sequence searches were performed using BLAST by e-mail.

Immunostaining—Primary rat neonatal cardiomyocytes isolated as described previously were cultured on coverslips. 5' RACE product was cloned in the TA vector (Invitrogen), and the entire cDNA corresponding to the 3'UTR sequence, the predicted 5'UTR sequence, and the 3'UTR sequence were sequenced. The cDNA was cloned in the TA vector (Invitrogen), and the entire cDNA was sequenced. Double stranded sequencing of the positive clones were carried out using a Sequenase 2.0 kit (U. S. Biochemical Corp.). Nucleotide and predicted amino acid sequence searches were performed using BLAST by e-mail.

In Situ Hybridization—The protocol that was used to fix and embed C57BL/6xDBA/2 embryos and post-natal tissues is described in detail in Lyons et al. (18). Briefly, embryos were fixed in 4% paraformaldehyde in PBS overnight, dehydrated and infiltrated with paraffin. 5-7-μm serial sections were mounted on gelatinized slides. The sections were digested with proteinase K, post-fixed, treated with triethanolamine/acetic anhydride, washed, and dehydrated. A 640-bp cDNA corresponding to the 3’-UTR of CARP was subcloned into PCRII (Invitrogen). This plasmid was linearized with XhoI and transfected with S6P polymerase to generate the antisense probe. This plasmid was linearized with BamHI and transcribed with T7 polymerase to generate the sense control probe. The cRNA transcripts were synthesized using a Maxiscript kit (Ambion) and labeled with 32P-UTP (>1000 Ci/mmol; Amerham). Sections were hybridized overnight at 52 °C in 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 10 mM NaPO₄, 10% dextran sulfate, 1 × Denhardt’s solution, 50 mg/ml total yeast RNA, and 50,000 cpm/ml 32P-labeled cRNA probe. The tissue was subjected to stringent washing at 65 °C in 50% formamide, 2 × SSC, 10 mM dithiothreitol and washed in PBS before treatment with 20 mg/ml RNase A at 37 °C for 30 min. Following washes in 2 × SSC and 0.1 × SSC for 10 min at 37 °C, the slides were dehydrated and dipped in Kodak NTB-2 nuclear track emulsion and exposed for 1 or 2 weeks in light-tight boxes with desiccant at 4 °C. Photographic development was carried out in Kodak D-19. Slides were counterstained lightly with toluidine blue and analyzed using both lightfield and darkfield optics of a Zeiss Axioshot microscope. The sense control cRNA probe (identical to the mRNAs) always gave background levels of hybridization signal (data not shown). Embryonic structures were identified with the help of the following atlases: Rugh (19) and Kaufman (20).

Cell Culture and DNA Transfection—Primary neonatal rat cardiomyocytes from 2-day-old Sprague-Dawley rats were prepared as described previously (16). The cells were grown at 37 °C with 5% CO₂ in modified Eagle’s medium supplemented with 5% fetal calf serum, 2 mM glutamine, 1% penicillin/streptomycin, and 1% 5-bromodeoxyuridine. 24 h after plating, transfections were carried out by calcium phosphate precipitation. The cells were harvested 30 h post-transfection, and luciferase activity was measured with a Monolight 2000 luminometer. For CAT reporter constructs, the cardiomyocytes were harvested 48 h after transfection. CAT assays (21) were quantitated on an AMBI Dual Radioanalytic Imaging System for the acetylated products. In all the experiments, protein concentration was measured by the Bradford method (22). The amount of reporter gene was kept constant at 2 μg/dish, and various concentrations of CMV-CARP or CMV backbone construct were cotransfected. Unless otherwise mentioned, the results were obtained from duplicate or triplicate transfections of at least two independent cardiacocyte preparations, using at least two independent DNA plasmid preparations.

10T1/2 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. DNA transfection was performed as described previously by calcium phosphate precipitation. Each culture dish was incubated for 20 h with a calcium phosphate-DNA precipitate containing 2 μg of reporter plasmid and increasing concentrations of a GAL-CARP fusion construct. 3 μg of Nkx2.5 construct and 2 μg of CARP expression vector were cotransfected with 2 μg of ANP promoter. After removal of excess DNA, the cells were kept in fresh 10% calf serum. 24 h later, cell extracts were prepared and analyzed for reporter activity, which was normalized to the protein content. These transfections were performed in triplicate and repeated twice with different preparations of each plasmid.

RESULTS

Identification of CARP mRNA by Differential Display—To identify a cardiac-specific target of Dox, we analyzed the differential expression patterns of genes in cultured neonatal rat cardiomyocytes by a differential display technique (23). We compared the effects of Dox with a Dox-derivative Dn to confirm the results. The differential display was carried out using total RNA extracted from treated and control cells of primary neonatal rat cardiomyocytes by growing the cells in media containing 1 μM Dox or Dn for 24 h. To identify the most sensitive transcripts, the concentrations of Dox or Dn used were reduced to half that usually required for a complete cessation of tissue-specific gene expression in cardiomyocytes (2 μM). The reverse transcription was done using oligo(dT) primers followed by PCR with arbitrary oligos containing 10 nucleotides. Several different combinations of oligo(dT) and arbitrary primers were used to cover a larger number of messenger RNAs. We identified 20 differentially expressed bands, of which 8 bands were repressed in their expression during both Dox and Dn treatment. The DNA bands were counterstained lightly with toluidine blue and subjected to Northern blotting. Out of eight clones, seven were positives only in Northern blots of RNA from the drug-treated cells. One among the seven clones, a 640-bp partial cDNA, had characteristics (described below) that made it the subject of intensified analysis. The differential display band
from which it was cloned is shown in Fig. 1A, and the Northern blot against RNA from untreated and Dox- and Dn-treated cardiocytes is shown in Fig. 1B. The mRNA band detected in untreated cardiomyocytes is drastically reduced in the presence of Dox or Dn in both the differential display pattern (Fig. 1A) as well as in the Northern blot (Fig. 1B). The size of the mRNA in Northern blot is estimated as 2.0 kilobases. We named the protein encoded by this clone as CARP for cardiac adriamycin-responsive protein.

**CARP Is Extremely Sensitive to Dox and Dn**—Because Dox affects the levels of mRNA for cardiac housekeeping genes, the rate at which tissue-specific mRNAs disappear following Dox exposure may be a reflection of turnover of those transcripts. The effects of Dox on the time for half-decay of mRNA for a number of sarcomeric protein is in the order of 12–20 h (2, 3). In sharp contrast, CARP mRNA levels in primary neonatal rat cardiomyocytes exposed to 1 μM Dox or Dn falls by half by 2 h and is almost undetectable by 12 h (Fig. 2A). The mRNA for a representative sarcomeric protein, cardiac troponin I, which falls steadily in response to Dox or Dn, has an apparent t1/2 of 16 h and is still detectable at 36 h. As expected, the expression of the housekeeping gene cyclophilin is minimally affected by the drugs. The relative levels of CARP and cardiac troponin I mRNAs following Dox exposure are compared in Fig. 2B. These results suggest that in the presence of Dox, CARP mRNA levels are very labile.

**CARP Expression Is Constitutive and Highly Restricted to Cardiomyocytes**—Radiolabeled CARP cDNA was used to probe Northern blots of rat RNA derived from various organs. CARP transcripts were easily detected in myocardium but not in RNA extracted from adult rat brain, kidney, testis, liver, or skeletal muscle (Fig. 3A). These results suggest that CARP mRNA is tissue-specific and is detectable only in heart. We also wanted to analyze whether the expression of CARP is restricted to cardiomyocytes or is expressed also in the plentiful fibroblastic noncardiomyocytes that populate myocardium including vascular endothelium. We cultured pure populations of such nonmyocytes and carried out Northern blots with these extracted RNAs and RNAs from nonmuscle cells (10T1/2 and HeLa). The results (Fig. 3B) show that high level expression is detected in cardiac myocytes but not in cardiac fibroblasts or 10T1/2 or HeLa cells. We also examined RNA from both the myoblast and myotube stages of C2C12 cell differentiation and detected the presence of low levels of CARP transcripts (Fig. 3C) at both stages of differentiation but far below the levels found in heart. When normalized for differences in RNA transfer to the membrane, densitometry scans of the autoradiographs demonstrate a 10–12-fold increase of CARP mRNA levels from myoblasts to myotubes (not shown).

**Cloning and Sequence Analysis of CARP cDNA**—Disappointingly, after a determination of the nucleotide sequence of...
the 640-bp cDNA obtained from differential display cloning, the sequence remained unidentified after internet searches using BLAST and TIGR for data base analysis. We then cloned 1.7 kilobases of the cDNA by 5’ RACE using the Marathon cDNA amplification kit (see “Materials and Methods”), rat cardiomyocyte poly(A)+ mRNA, and the antisense primer of the 3’-UTR. The entire cDNA was sequenced (Fig. 4A), and the single open reading frame was analyzed. The 1749-bp cDNA encodes a protein of 319 aa starting with an ATG at nucleotide position 61 and ending with a TGA at nucleotide 1018. There is a polyadenylation signal (AAUAAA) starting at base 1731. The 3′-UTR contains a stretch of five mRNA destabilization signals in tandem and two other AU-rich regions.

The full-length sequence was used to search for similarity with known sequences using BLAST. One match was found for a human cDNA that has 64% DNA sequence identity and 91% amino acid identity. The encoded protein (C-193) (20) is inducible by cytokines in human endothelial cells and is most likely the human homolog of CARP. The 3’-UTR of C-193 is not similar to the 3’-UTR of rat CARP, although mRNA degradation signals are present in both the UTRs. However, the protein coding regions of C-193 and CARP share 85% DNA sequence homology. The alignment of the proteins is shown in the Fig. 4C. Both proteins have ankyrin-like repeats and nuclear localization signals. The open reading frame of the cDNA was transcribed in vitro using SP6, and the products of a reticulocyte lysate translation reaction were analyzed by SDS-polyacrylamide gel electrophoresis (not shown). The synthesized polypeptide appears to be 40 kDa, as compared with the computer predicted molecular mass of 36.2 kDa (pl 8.8).

Analysis of the CARP polypeptide revealed a number of putative protein motifs. There are five tandem 33-amino acid long ankyrin-like repeats (Fig. 4, B and D); four of these had been identified at homologous locations in C-193, but what appears to be a fifth ankyrin repeat (Anky1) in CARP is also present in C-193. The CARP polypeptide sequence contains a number of other putative protein motifs (Fig. 4D). There are three 4-residue potential nuclear localization signals: KKKK (at residue 70), KRRK (residue 93), and KRKK (residue 94); there is another potential nuclear localization signal, RK-SEKVREAEKKKKLE, starting at residue 59. CARP has five putative protein kinase C phosphorylation sites at the following locations: TGK (11–13 aa), SEK (62–64 aa), SDK (141–143 aa), SAR (213–215 aa), and SLK (305–307 aa). There is one putative cAMP-dependent protein kinase phosphorylation site, KEKNS (13–16 aa). CARP also has four presumed casein kinase II phosphorylation sites: SSGE (19–22 aa), SKLE (79–82 aa), SAR (213–216 aa), and SLKE (305–308 aa). There are two N-glycosylation sites, NGSS (17–20 aa) and NGTK (297–300 aa), and two N-myristoylation sites, GAKISA (209–214 aa) and GADLNV (275–280 aa). There is one amidation site, TGKK (11–14 aa). No transmembrane domain homolog was found.

C-193 has been shown to localize to nuclei in endothelial cells (20). To confirm that CARP has the same characteristic in cardiomycocytes, we constructed a CARP eukaryotic expression vector driven by a cytomegalovirus promoter producing a fusion protein of CARP with the 8-amino acid FLAG peptide at the carboxyl-terminal of the CARP protein. We transfected cardiomycocytes and 30 h later examined the transfected cells by immunofluorescence using FLAG monoclonal antibody and tracked down the protein predominantly in nuclei. The photomicrographs in Fig. 5 demonstrate the localization of CARP in a typical binucleate cardiomycocyte. No significant staining was observed in cells transfected with an unrelated cDNA cloned in the same vector.

We tried unsuccessfully to induce or superinduce endogeneous CARP mRNA levels in cardiac fibroblasts or cardiomycocytes with interleukin-1, TNF-α, and LPS. In control experiments these agents were able to induce early message for interleukin-6 in brain endothelial cells (data not shown). In addition, continuous exposure of cardiomycocytes to either TNF-α or LPS had no effect on the extent of CARP mRNA sensitivity to Dox.

Localization of CARP mRNAs in Mouse Embryos and Postnatal Hearts—The timing and pattern of CARP expression was examined by in situ hybridization using highly sensitive 35S-UTP-labeled probes on a developmental series of mouse embryo sections beginning at 7.5 days post coitum (p.c.). At least five embryos were examined at each stage between 10.5 and 14.5 days. CARP transcripts were detected in the heart at every 12-h time point between 7.5 days p.c. and birth (Fig. 6). There were no stages at which CARP mRNA could not be detected. Transcript levels persisted but gradually decreased in neonatal, 2-week-old, and adult hearts. CARP transcripts were initially detected within the cardiogenic plate of 7.5-day p.c. embryos prior to formation of the heart tube (data not shown). In 8.5-day p.c. embryos, CARP transcripts were present in uniformly high levels in the myocardium (Fig. 6A). As the heart looped to form common atrial and ventricular chambers, CARP appeared to be nonuniformly distributed in the myocardium (Fig. 6B). Throughout cardiac development, CARP expression was specific for the myocardium; endocardial cushions and valves exhibited only background levels of signal (Fig. 6, C and D). In general, the CARP hybridization signal was slightly stronger in the atrium than in the ventricle. Although CARP transcripts were uniformly distributed within the ventricle during early heart morphogenesis, by day 15.5 the signal was stronger along the inside of the myocardial wall (Fig. 6E), and later was more concentrated in the trabeculae than within the outer compact layer (Fig. 6, G and H). Similarly, expression appeared to be more prevalent within inner cellular layers of the atrium, including cristae and septa (data not shown). The inner layers of the ventricle and atrium may reflect regions of myocyte proliferation, because the networks of cristae and trabeculae exhibit a considerable degree of expansion during these stages of development (for review see Ref. 24). Alternatively, the transmural gradient of CARP expression in fetal and neonatal hearts may represent its role in the transcriptional reg-
FIG. 6. Localization of CARP gene transcripts to the developing myocardium. In situ hybridizations for CARP transcripts are shown at various stages of embryonic development in the mouse. All sections were photographed under darkfield illumination. A, 8.5-day p.c. embryo, frontal section. CARP transcripts are localized to the early heart. B, 10.5-day p.c. embryo, parasagittal section. CARP transcripts are distributed nonuniformly throughout the myocardium and are absent in valve tissue (arrow). D, 13.5-day p.c. embryo, parasagittal section. CARP transcripts are detected specifically within the myocardium but are absent in endocardial cushions (arrow). E and F, sections of 15.5-day p.c. embryo, parasagittal section. One section (E) was hybridized to an antisense CARP cRNA probe, whereas the other (F) was hybridized to a sense probe as a negative control. CARP transcripts are detected only in the myocardium and are most highly concentrated along the inner layer of the ventricular wall (arrow in E). Only the background level of signal is observed in the section hybridized to the sense probe (F; the position of the arrow corresponds to that in E). Red blood cells within the atrial and ventricular chambers (E and F) are refractile under darkfield illumination. G and H, sections of heart and lung dissected from 16.5-day p.c. embryo (G) and neonate (H). Although present throughout the myocardium, CARP is most strongly expressed in the atrium (not shown in H) and in the innermost trabeculations of the ventricle. The abbreviations are: a, atrium; b, brain; ba, branchial arches; d, deciduum; f, forelimb bud; h, heart; hf, headfold; i, intestine; j, jaw; l, lung; lv, liver, n, neural tube; s, spinal cord; v, ventricle. Scale bar, 250 μm for A–C; 500 μm for E–H; 1 mm for D.

ulation of cardiac muscle genes that follow a similar pattern of temporal and spatial expression during heart development (for review see Refs. 12 and 25).

Throughout development CARP is transcribed exclusively in the heart, with the exception that CARP signal appears weakly in some skeletal muscles. CARP is expressed in the tongue at 14.5 days p.c. (not shown) and falls below the level of detection of the in situ hybridization technique by 15.5 days p.c. Similarly there is an even lower level of expression in 10.5-day p.c. myotomes and in 14.5-day p.c. pectoral and intercostal muscles. The pattern of CARP expression is reminiscent of the previously reported pattern of expression of Nkx 2.5 (9) (the putative homolog of tinman (7)).

CARP Functions as a Repressor of Muscle-Specific Promoters in Cardiomyocytes—To investigate whether CARP has a functional role in regulating cardiac-specific gene expression, we performed transient transfection experiments using a variety of cardiac-specific and control promoter reporter constructs and a CARP expression vector. As shown in Fig. 7, CARP negatively regulates transcription of at least two cardiac promoters in cardiac cells. Cotransfection of CARP resulted in a dose-dependent inhibition of ANF and CTnC promoter activities (Fig. 7, A and B). Importantly, CARP had no significant effect on the activity of the noncardiac β-actin promoter (Fig. 7C).

Recently, Durocher et al. (26) reported that the cardiac ANF promoter is a target for the product of the homeobox gene, Nkx2.5. Because the pattern of CARP expression parallels the developmental expression of Nkx2.5 in the myocardium (see above), we chose to investigate the potential involvement of CARP on ANF transcriptional activity. As expected, the expression of ANF promoter in HeLa cells was markedly increased by Nkx2.5. However, cotransfection of CARP resulted in a strong inhibition of this transactivation (Fig. 7D). Similar results were observed when cotransfections were performed in 10T1/2 cells (data not shown).

To test whether the repressor activity of CARP is independent of any cardiac-specific factors such as Nkx 2.5, we cotransfected the ANF promoter-reporter construct and CARP expression vector in the nonmuscle cell line 10T1/2. As seen in Fig. 7E, ANF promoter was inhibited by CARP in a dose-dependent manner. The fold inhibition was the same as observed in cardiac cells.

To determine whether the CARP protein itself contains an inhibitory domain, CARP was fused to a GAL4 DNA-binding domain (GAL-CARP) and was cotransfected with a GAL4 DNA-binding site-luciferase reporter construct (UAS4tk-luciferase) in 10T1/2 cells. Cotransfection of GAL-CARP resulted in a 76% inhibition of UAS4tk-luciferase activity (Fig. 7F). A 50% inhibition of G4tk-CAT transcription was also observed in 10T1/2 cells (data not shown).

These results confirm that
FIG. 7. Inhibition of muscle-specific promoters by CARP in cardiomyocytes. Transient transfections were carried out in primary neonatal rat cardiomyocytes. Cotransfections were performed using 2 μg of reporter gene and increasing amounts of CMV-CARP or just CMV backbone. Luciferase and CAT activities were measured. The results are expressed relative to the activity of the tested promoter in the absence of CARP, which was taken as 100%, and are corrected for protein content. The same amount of total DNA was used in each transfection. CARP down-regulates ANF promoter (A) and cTnC promoter (B) and does not significantly effect on β-actin promoter (C). The results represent the means ± S.D. of two or three independent experiments carried out in duplicate. D, CARP abolishes Nkx2.5 inducibility. Transient cotransfections were done in HeLa cells using 2 μg of ANF promoter construct, 3 μg of Nkx2.5 expression vector, and 2 μg of CARP expression vector. 24 h after transfection, the cells were harvested and luciferase activity was measured. The results are expressed relative to the activity of ANF promoter in the absence of CARP, which was taken as 100%. The data represent the averages ± S.D. of three independent experiments. E, CARP inhibits ANF promoter in nonmuscle cells. Transient transfections were carried out in 10T1/2 cells using 2 μg of reporter gene and increasing concentrations of CARP expression vector. 30 h after transfection, luciferase activity was measured. The results are expressed relative to the activity of the promoter in the absence of CARP, which taken as 100%, and are corrected for protein content. The same amount of total DNA and of vector backbone were
CARP contains domains that act as negative transcriptional regulators.

**DISCUSSION**

**CARP Is an Early Target for Dox—**Adriamycin (Dox) leads to degenerative cardiomyopathy and selectively inhibits the expression of muscle-specific genes such as α-actin, troponin I, myosin light chain 2, and muscle creatine kinase in cardiomyocytes (5). In muscle Dox blocks the differentiation program by inhibiting the activity of the myogenic regulatory gene family members (myoD), myogenin, MRF4, and myf-5; reviewed in Ref. 27). The disruption of muscle transcription regulatory pathways by Dox is at least in part due to the induction of the negative helix-loop-helix regulator Id (3). Although there is no apparent homolog for myoD in the heart, we have used Dox sensitivity as a criterion in an attempt to search for factors performing similar functions in regulating cardiac-specific genes. We cloned CARP by a differential display screening procedure designed to find transcripts in myocardiocytes that are highly sensitive to both doxorubicin and daunomycin.

The expression of CARP fits this criterion very well. When analyzed by Northern blotting, CARP expression is abolished or undetectable within several hours following Dox treatment of primary rat neonatal cardiomyocytes. The disappearance of CARP mRNA following Dox treatment is much more rapid than the effects on tissue-specific gene expression in both cardiac and skeletal muscle cells (2). Its rapid and sensitive elimination by Dox exposure might well contribute to the cardiac-specific toxicity of Dox and the development of cardiomyopathy. CARP expression is highly tissue-specific. Our results show that it is expressed constitutively and vigorously only in cardiomyocytes and only transiently and very weakly in a few skeletal muscles or in myogenic C2C12 cells.

CARP appears to be the homolog of C-193, a recently cloned human cytokine-inducible protein (28). C-193 and CARP are both nuclear proteins, and C-193 appears to bind DNA. It is inducible in endothelial cells by cytokines including interleukin-1α, TNF-α, and LPS (28). Several early response genes are also inducible by cytokines and LPS (28). A major characteristic of the mRNAs of early response genes is their short half-life and the presence of a destabilization signals in the mRNA sequence (28). Similarly CARP and C-193 both have mRNA destabilization signals in their 3’-UTRs. Both CARP and C-193 also have ankyrin-like repeats and nuclear localization signals. CARP has five ankyrin-like repeats in tandem and C-193 has four, but one ankyrin-like repeat may have been missed during the computer analysis of C-193 (28). Ankyrin-like repeats found in many polypeptides appear to be involved in protein-protein interactions (29, 30). Several well characterized transcription factors, such as NF-kB (31–33), IκB (29, 34), bcl3 (35), and GABPβ (30) have both ankyrin repeats and nuclear localization signals.

The characteristics of CARP are consistent with the notion that it has a nuclear regulatory function. These include the rapid depletion of CARP mRNA in the presence of Dox, its early expression in cardiac progenitor cells, its rapid transcription following exposure to cytokines in endothelial cells, and its nuclear localization.

Because C-193 mRNA is inducible with cytokines in endothelial cells, we tried unsuccessfully to superinduce CARP mRNA levels in cardiomyocytes with TNF-α and LPS. In control experiments these agents did induce CARP mRNA in bicipital vein endothelial cells (data not shown). However, the effect of Dox could not be rescued by treating the cardiomyocytes with TNF-α and LPS cytokine induction. Cytokine and LPS also failed to induce CARP expression in cardiac fibroblasts (data not shown). This is not surprising because C-193 was found to be inducible only in endothelial cells and not in other cell lines tested (28).

**CARP Has a Repressor Activity—**The onset of CARP expression in the cardiogenic plate and early heart tube coincides with that of several known transcription factors, including Nkx2.5/Csx (8, 9), Me2C (36), GATA-4 (37, 38), and dHAND (11). To investigate whether CARP has a functional role in regulating cardiac-specific gene expression, we tested the effect of CARP overexpression on several naturally occurring cardiac-specific promoter-reporter constructs in primary rat neonatal cardiomyocytes. The cardiac troponin-C and the atrial natural factor promoters were both significantly inhibited by CARP.

Interestingly, both Nkx2.5 and CARP are expressed in the heart throughout embryonic development and also in tongue muscle at 14.5 days p.c. (9), suggesting that their products might functionally interact. We performed transient transfection experiments using ANF promoter-reporter constructs and a CARP expression vector in the presence or the absence of an Nkx2.5. Because CARP suppressed ANF promoter expression even in the absence of Nkx 2.5, we cannot establish direct interaction with Nkx 2.5 activity by these assays. However, the ability of CARP protein tethered to a promoter to suppress transcription does establish the ability of CARP to directly inhibit transcriptional activation.

While this manuscript was in preparation, Kenneth Chien and collaborators reported the identification of a cardiac restricted cDNA from rat heart using a yeast two-hybrid approach (39). By coincidence their clone was named CARP for cardiac ankyrin repeat protein. Exchange of sequence information by our laboratories prior to publication revealed that the two clones are identical. The Chien group isolated CARP through a two-hybrid screen designed to detect mRNAs in cardiomyocytes that encode peptides interacting with the transcription factor YB-1. Those observations further strengthen our notion that CARP is a nuclear regulatory molecule.

**CARP Is Cardiac-restricted and Expressed Early during Cardiac Development—**The transcription of CARP in the heart both during development and after birth suggests that it plays a role in the growth and/or morphogenesis of the myocardium. CARP transcripts are uniformly distributed in the myocardium during early heart morphogenesis, although at later stages they appear to be more prevalent within inner cellular layers of the myocardium, including cristae of the atrium and trabeculae of the ventricle. These inner layers may reflect regions of concentrated myocyte division, because the networks of cristae and trabeculae exhibit a considerable degree of expansion during these stages of development (for review see Ref. 24). Alternatively, the transmural gradient of CARP expression in fetal and neonatal hearts may represent its role in the transcriptional regulation of cardiac muscle genes that follow a similar pattern of temporal and spatial expression during heart development (for review see Ref. 25). In either case, CARP might...
play a key role in the maintenance of the cardiac phenotype. Perhaps one of the most interesting features emerging from this study is the discovery that CARP is restricted to the myocardium. Other factors involved in the control of the cardiac program such as *Mcf2*, *GATA4*, *Nkx2.5*, *dHand*, and *eHand* are expressed at high levels in the heart but are also present in a variety of other tissues. Because CARP is highly sensitive to Dox we continue to ask whether its rapid elimination plays a role in Dox cardiotoxicity. Dox selectively depletes cardiac-specific mRNAs including those encoding structural proteins (2, 5) and those involved in ATP generation (1), but these effects appear much later than the rapid effects on CARP mRNA. Alternatively, the normal role of CARP may be to modulate gene expression in a cell whose function and microanatomy are exquisitely sensitive to fluctuations of structural protein concentration.

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