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CHAMP, A Novel Cardiac-Specific Helicase Regulated by MEF2C

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MEF2C is a MADS-box transcription factor required for cardiac myogenesis and morphogenesis. In *MEF2C* mutant mouse embryos, heart development arrests at the looping stage (embryonic day 9.0), the future right ventricular chamber fails to form, and cardiomyocyte differentiation is disrupted. To identify genes regulated by MEF2C in the developing heart, we performed differential array analysis coupled with subtractive cloning using RNA from heart tubes of wild-type and *MEF2C*-null embryos. Here, we describe a novel MEF2C-dependent gene that encodes a cardiac-restricted protein, called CHAMP (*c*ardiac *h*elicase *a*ctivated by *M*EF2 *p*rotein), that contains seven conserved motifs characteristic of helicases involved in RNA processing, DNA replication, and transcription. During mouse embryogenesis, CHAMP expression commences in the linear heart tube at embryonic day 8.0, shortly after initiation of MEF2C expression in the cardiogenic region. Thereafter, CHAMP is expressed specifically in embryonic and postnatal cardiomyocytes. At the trabeculation stage of heart development, CHAMP expression is highest in the trabecular region in which cardiomyocytes have exited the cell cycle and is lowest in the proliferative compact zone. These findings suggest that CHAMP acts downstream of MEF2C in a cardiac-specific regulatory pathway for RNA processing and/or transcriptional control. © 2001 Academic Press

INTRODUCTION

The heart is the first organ to form during mammalian embryogenesis (reviewed in Srivastava and Olson, 2000; Fishman and Chien, 1997). Formation of the heart involves commitment of cells from the anterior lateral mesoderm to a cardiogenic fate in response to inductive cues from adjacent endoderm. Cardiac precursor cells localized to a region known as the cardiac crescent, which spans the anterior ventral midline of the embryo, migrate ventrolaterally to form a linear heart tube at embryonic day (E) 8.0 in the mouse. The linear heart tube is patterned from anterior to posterior into segments that give rise to the outflow tract, right ventricle, left ventricle, and atria. Rightward looping of the heart tube is essential for orientation of the right and left ventricular chambers and alignment of the heart with the inflow and outflow tracts. Later events of chamber maturation, septation, endocardial development, and valvulogenesis give rise to the mature multichambered heart.

Several mouse and zebrafish mutants exhibit specific defects in cardiac looping, ventricular morphogenesis, and chamber maturation (reviewed in Fishman and Olson, 1997; Stainier, 2000). The phenotypes of these mutants, which often result in ablation of specific segments of the heart, have led to the notion that distinct transcriptional networks control formation of different cardiac compartments. Many of the genes shown to be required for these morphogenetic events encode transcription factors, but the target genes that mediate the actions of these factors are largely unknown.

The basic helix–loop–helix (bHLH) transcription factors, dHAND and eHAND, are expressed preferentially in the developing right and left ventricular chambers, respectively, and have been implicated in formation of these cardiac segments (Srivastava *et al.,* 1995, 1997; Biben and Harvey, 1997; Firulli *et al.,* 1998; Yelon *et al.,* 2000; Riley *et al.,* 2000). The cardiac homeodomain protein Nkx2.5 is

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required for looping morphogenesis (Lyons, 1995) and is a regulator of eHAND expression (Biben and Harvey, 1997). Irx4, a homeodomain protein of a different class, is expressed specifically in the ventricular chambers and appears to be both necessary and sufficient for ventricular gene expression (Bao *et al.,* 1999). The zinc-finger transcription factors GATA-4 and GATA-5 have also been shown to be required for ventral morphogenesis and formation of the linear heart tube in mice and zebrafish, respectively (Kuo *et al.,* 1997; Molkentin *et al.,* 1997; Reiter *et al.,* 1999).

The MADS-box transcription factor MEF2C is expressed at the late cardiac crescent stage (E7.75) and subsequently throughout the linear, looping, and multichambered heart (Edmondson *et al.,* 1994). At E8.0–8.5 (the linear heart tube stage), *MEF2C* mutant and wild-type embryos are indistinguishable, whereas by E9.0, when the heart tube should undergo rightward looping to form the right ventricular chamber, the heart tube of *MEF2C* mutant embryos remains linear, with a single hypoplastic ventricular chamber fused directly to an enlarged atrial chamber (Lin *et al.,* 1997). Cardiomyocytes within the mutant myocardial wall become disorganized at this stage and the heartbeat becomes sluggish and irregular. Mutant embryos also develop pericardial effusion, indicative of hemodynamic insufficiency and heart failure.

There are four *MEF2* genes in vertebrates, *MEF2A*, *-B*, *-C*, and *-D*, which are expressed in overlapping patterns in developing muscle and neural cell lineages, and at lower levels in other cell types (reviewed in Black and Olson, 1998). MEF2 factors bind an A/T-rich sequence in the control regions of numerous skeletal, cardiac, and smooth muscle-specific genes (Gossett *et al.,* 1989). Functional redundancy among the vertebrate *MEF2* genes has precluded a complete analysis of MEF2 function in the mouse. However, in *Drosophila*, there is only one *MEF2* gene, which is expressed in developing muscle cell lineages (Lilly *et al.,* 1994; Nguyen *et al.,* 1994). In *Drosophila* embryos lacking MEF2, skeletal, cardiac, and visceral myoblasts are properly specified and positioned, but they cannot differentiate, and there are severe abnormalities in morphogenesis of the visceral musculature (Lilly *et al.,* 1995; Ranganayakulu *et al.,* 1995; Bour *et al.*, 1995). This severe muscle phenotype suggests that MEF2 acts in myoblasts to activate downstream muscle-specific genes involved in differentiation and morphogenesis.

To further understand the functions of MEF2C in the developing heart, we attempted to identify MEF2C-dependent genes by differential array analysis coupled with subtractive hybridization using RNA isolated from heart tubes of wild-type and *MEF2C*-null mouse embryos. Among several MEF2C-dependent genes down-regulated in the heart tube of *MEF2C* mutants, we discovered a cardiac-restricted gene encoding a putative helicase, termed CHAMP (*c*ardiac *h*elicase *a*ctivated by *M*EF2 *p*rotein), with homology to RNA helicase superfamily I. Our results suggest that CHAMP acts downstream of MEF2C in a cardiac-specific regulatory pathway for RNA processing and/or transcriptional control.

MATERIALS AND METHODS

Breeding of Mice and Genotyping

Mice heterozygous for a *MEF2C*-null mutation were generated as previously described (Lin *et al.,* 1997). Intercrosses of *MEF2C* heterozygous mice in the mixed 129SVEV/C57BL6 background were performed to obtain homozygous-null embryos between E9.0–9.5. Hearts were excised from homozygous embryos and wild-type littermates and stored frozen at −80°C. Care was taken to make sure that only viable embryos with beating hearts were used. Genotypes of individual embryos were determined by PCR analysis of yolk sac DNA as previously described (Lin *et al.,* 1997).

RNA Preparation, cDNA Synthesis, and Subtractive Hybridization

Total RNA was prepared by using Trizol reagent (Gibco) from 40 hearts of *MEF2C*-null and the wild-type littermates, respectively. Five hundred nanograms each of total RNA were subjected to reverse-transcription and PCR amplification by using the SMART cDNA synthesis system (Clontech). Reactions were terminated at 18 cycles in the linear-increase range of PCR amplification. cDNA larger than 1 kb was enriched by size-fractionation and was digested with *Rsa*I.

Subtractive hybridization was performed by using wild-type heart cDNA as a tester and *MEF2C*-null heart cDNA as a driver (forward subtraction, WT-KO) by the PCR-Select system (Clontech). Briefly, wild-type heart cDNA was ligated separately with two different adaptors, and each sample was hybridized with an excess amount of *MEF2C*-null heart cDNA. These samples were combined and hybridized to form double-stranded cDNA with different adaptors at the ends. cDNA clones representing transcripts specifically expressed in the wild-type heart were preferentially amplified by PCR using the primers specific to the adaptors. Simultaneously, the reverse subtractive hybridization (KO-WT) was also performed using *MEF2C*-null heart cDNA as a tester and wild-type heart cDNA as a driver to enrich for cDNA representing transcripts highly expressed in *MEF2C*-null hearts.

Differential Array Analysis

Subtracted PCR fragments were subcloned into pCRII-TOPO plasmids (Invitrogen), and 1000 bacterial clones were recovered and cultured for 5 h. cDNA inserts of the plasmid clones were amplified by PCR using adapter-specific primers and were arrayed in duplicate onto replica nylon membranes. Subtracted PCR fragments from the forward (WT-KO) and reverse (KO-WT) subtractions were labeled with [^{32}P]dCTP. Each membrane was hybridized with either forward or reverse probes in Rapid-hyb buffer (Amersham) at 65°C and washed serially, with a final wash in 0.1× SSC, 0.1% SDS at 65°C. Autoradiography was performed by using PhosphorImaging (Molecular Dynamics). After stripping and prehybridization, one of the replica membranes was hybridized with ^{32}P-labeled cDNA probes prepared from whole embryo without heart tissues.

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Southern Blot Analysis of PCR-Amplified cDNA

To examine the expression patterns of isolated genes in *MEF2C*-null hearts and wild-type hearts, we performed Southern blot analysis of PCR fragments obtained by SMART cDNA synthesis ("virtual" Northern analysis). Approximately the same amount of cDNA mixtures for the *MEF2C*-null hearts, wild-type hearts, and whole embryo minus heart tissues was electrophoresed on a 1.5% agarose/TAE gel and transferred onto nylon membranes. The membranes were hybridized with the PCR fragments of individual clones in Rapid-hyb buffer at 65°C and washed serially, with a final wash in 0.1× SSC, 0.1% SDS at 65°C. Signals were visualized by autoradiography.

Isolation and Characterization of CHAMP

The original 0.6-kb cDNA clone R15-C5 isolated from subtractive cloning was used to screen a mouse E10.5 heart cDNA library (Stratagene) and an adult heart cDNA library (Clontech). The longest cDNA obtained from these libraries was 1.6 kb in length. The remaining 0.4 kb of sequence at the 5′ end was obtained by 5′-RACE, using gene-specific primers from the 5′ end of the 1.6-kb clone. The final 2.0-kb cDNA clone contained no 5′-UTR. Extensive efforts have failed to identify additional 5′ sequence for the CHAMP cDNA. The sequence of the 2-kb cDNA has been deposited in GenBank with accession number AF340211.

Bioinformatics

Using BLAST search with CHAMP, significant homologies were obtained with the helicase Mov-10 (Mooslehner *et al.,* 1991), Upf1p (Leeds *et al.,* 1991), Smubp-2 (Chen *et al.,* 1997), and Hcs1p (Biswas *et al.,* 1997) (E value $< e^{-25}$). All of these helicases are from the helicase superfamily I (de la Cruz *et al.,* 1999). The sequence alignments of CHAMP with Mov-10, Upf1p, Smubp-2, and Hcs1p were performed with ClustalW alignment and manually adjusted.

An EST (accession no. AL133068) that appears to encode portions of the testis isoform of human CHAMP was obtained by using tblastn with CHAMP. The open reading frame of the EST AL133068 (678 amino acids) was further used in a BLAST search, and a larger protein sequence (1315 amino acids) conceptually translated from human chromosome 22q13 was obtained (accession no. 10880095). The C-terminal portion of this sequence overlaps with the open reading frame of EST AL133068 and has 690 more residues at its N terminus. A putative testis isoform of human CHAMP was deduced by using sequences of EST AL1033068 and 10880095. The genomic structure of *CHAMP* was obtained by BLASTing the human genome database with the sequence of putative human testis CHAMP.

In Situ Hybridization

Whole-mount *in situ* hybridization and radioactive section *in situ* hybridization were performed as previously described (Nakagawa *et al.,* 1999) on mouse embryos from E7.75 to E15.5 and on adult mouse heart. Plasmids containing nucleotides 589–994 and 1420–2020 of the CHAMP cDNA (See Fig. 3) were used as the templates for making ^{35}S-UTP-labeled and digoxigenin-labeled riboprobes for section and whole-mount *in situ* hybridization, respectively. cDNA probes corresponding to these two fragments yielded the same results on Northern blot analysis (see below).

Northern Blot Analysis

Northern blot analysis was performed on a mouse adult tissue poly(A)+ RNA blot (Clontech) by using ^{32}P-labeled *CHAMP* cDNA fragments corresponding to nucleotides 589–994 and 1420–2020 as probes. The membrane was prehybridized and hybridized in Rapid-hyb buffer at 65°C and washed serially, with a final wash in 0.2× SSC, 0.1% SDS at 65°C. Autoradiography was performed at −80°C for 15 h with an intensifying screen.

Preparation of Anti-CHAMP Antibody and Immunostaining

Rabbit polyclonal anti-CHAMP antibody was raised against residues 531–550 near the carboxyl terminus. The antibody was affinity-purified with peptide antigen using a Sufolink kit (Pierce). For immunoblot analysis of CHAMP protein, cell extracts were separated by SDS–PAGE, transferred to PVDF membranes, and probed with affinity-purified anti-CHAMP antibody at 1/1000 dilution. Antibody–antigen complexes were visualized by a chemiluminescence detection system (Tropix). Indirect immunoflourescence staining of N-terminal FLAG-tagged CHAMP in HeLa cells was performed following a protocol with anti-CHAMP antibody at 1/50 dilution and monoclonal anti-FLAG antibody M2 (McKinsey *et al.,* 2000). Immunohistochemistry was performed by using anti-CHAMP antibody on paraffin-embedded tissue sections, as described (Cianga *et al.,* 1999).

RESULTS

Identification of MEF2C-Dependent Genes

To identify potential MEF2C-dependent genes in the early heart tube, we performed differential array analysis using cDNA derived from subtractive hybridization of RNA isolated from heart tubes of wild-type and *MEF2C* mutant embryos at E9.0 to E9.5. At this stage, homozygous mutants were viable and were visually identifiable by cardiac malformation. The genotypes of individual embryos were confirmed by PCR analysis of yolk sac DNA.

One thousand cDNA clones obtained from subtractive hybridization of wild-type and *MEF2C*-null heart tubes were arrayed in duplicate onto replica nylon membranes. The arrayed membranes were then probed with cDNA from the forward and reverse subtractions as described in Materials and Methods. To identify clones that were potentially cardiac-specific, one of the arrays was subsequently stripped and hybridized with ^{32}P-labeled cDNA prepared from wild-type embryos without the heart.

Approximately 169 of 1000 arrayed clones, obtained from the initial subtraction, showed higher expression in wild-type compared to *MEF2C* mutant heart tubes. Of these 169 potential MEF2-dependent clones, 54 appeared to be cardiac-specific, based on their lack of hybridization to cDNA from embryos without the heart. The clones that showed more than 3-fold differences in expression between wild-type and mutant heart tubes are listed in Table 1. Based on sequence analysis, we were able to categorize

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TABLE 1
Classification of MEF2C-Dependent cDNA Clones

Genes	Numbers of clones
Muscle genes	
Myosin light chain-2	2
Myosin light chain-3	3
Titin	3
Vascular smooth muscle α-actin	1
Cardiac troponin T	1
Calsequestrin	1
SERCA	1
Na^+/Ca^{2+} exchanger	1
Muscle LIM protein	2
Slow skeletal muscle troponin I	1
Stress-response and growth-related genes	
αB-crystallin	2
Cripto	3
Pituitary tumor transforming gene (PTTG)	1
Hsp60	2
Proteasome subunit RC10-11	1
Ubiquitin protein ligase Nedd-4	1
Electron transport and energy production genes	
ATPase subunit 6	10
NADH dehydrogenase subunit 1	2
Cytochrome oxidase subunit 1 (COX1)	3
Cytochrome oxidase subunit 2 (COX2)	1
ATP synthase α subunit	1
ATP synthase γ subunit	1
Novel genes	10

MEF2C-dependent genes into four major classes: (1) muscle genes, (2) stress- and growth-related genes, (3) genes encoding enzymes involved in electron transport and ATP synthesis, and (4) novel genes. The expression patterns of representative genes from each class were confirmed by "virtual" Northern analysis, in which RNA from wild-type and *MEF2C* mutant heart tubes, and from E9.25 embryos without the heart, was converted to cDNA and probed by Southern blot (data not shown).

As expected, muscle-specific genes represented a significant proportion of the MEF2C-dependent genes (Table 1). Multiple contractile protein genes and genes encoding proteins involved in calcium handling [calsequestrin, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and Na^+/Ca^{2+} exchanger (NCX1)] were specifically down-regulated in mutant hearts. Among muscle genes found to be dependent on MEF2C, *cardiac troponin T*, *myosin light chain (MLC)-2*, and *MLC-3* have been shown to contain essential MEF2 sites in their promoters (Ross *et al.,* 1996; McGrew *et al.,* 1996; Lin *et al.,* 1997), suggesting that they are direct targets for MEF2C.

Several genes encoding proteins involved in protein degradation (ubiquitin protein ligase Nedd-4) and protein folding (Hsp60 and αB-crystallin) were also identified from the subtraction screen (Table 1). Among these, αB-crystallin is expressed in the primitive heart tube at E8.5 and subsequently throughout the developing heart, as well as skeletal muscle lineages (Benjamin *et al.,* 1997), in a pattern that overlaps that of MEF2C during embryogenesis (Edmondson *et al.,* 1994).

We also identified two genes that are cell growth-related, *pituitary tumor transforming gene* (*PTTG*) and *Cripto*. *PTTG* has been demonstrated to be capable of inducing fibroblast growth factor expression *in vitro*. Its expression strongly correlates with pituitary tumorigenesis (McCabe and Gittoes, 1999). The promoter region of rat *PTTG* contains two MEF2C consensus binding sites at −909 to −900 (CTAAAAATAA) and at −780 to −771 (CTTTAATTAA) (Pei, 1998), suggesting that it may be a direct target of MEF2C. Cripto, a member of the EGF-CFC protein family (Shen and Schier, 2000), is expressed in the forming heart at E7.5–8.0, concomitant with MEF2C expression, and is required for cardiac development (Dono *et al.,* 1993; Johnson *et al.,* 1994; Minchiotti *et al.,* 2000).

The largest class of genes down-regulated in *MEF2C* mutants encoded proteins involved in energy metabolism and electron transport (Table 1). Since several of these genes are encoded by the mitochondrial genome, we believe that their down-regulation may be a secondary pathophysiological consequence of heart failure in mutant embryos.

We also identified numerous novel clones representing MEF2C-dependent genes. Some of these sequences had no matches in EST databases, whereas others matched ESTs with no known functions. Since the cDNAs isolated by this technique are biased toward 3′-untranslated regions, it is possible that some of these sequences represent unidentified 3′ portions of known genes. Confirmation of the uniqueness of these clones will require isolation of full-length cDNAs (see below).

CHAMP, A Cardiac Helicase Regulated by MEF2C

We chose to focus on one of the novel MEF2C-dependent cDNAs, clone R15-C5, which was found by virtual Northern blot to represent a cardiac-restricted transcript expressed at levels about 5-fold higher in heart tubes from wild-type embryos compared with *MEF2C* mutants (Fig. 1A). This differential expression pattern was confirmed by whole-mount *in situ* hybridization with E8.0 embryos (Fig. 1B). R15-C5 transcripts were expressed throughout the heart tube of wild-type embryos, whereas they were undetectable in *MEF2C* mutants at this stage.

The initial R15-C5 cDNA was 600 nucleotides in length and contained only a short open reading frame followed by a noncoding region and a poly(A) stretch, suggesting that it represented the 3′ end of a transcript. At the time it was first identified, there was no match for this sequence in the database, but subsequently, we found ESTs from mouse testis, ovary, and heart. Screening of mouse E10.5 embryo and adult heart cDNA libraries and 5′-RACE (see Materials

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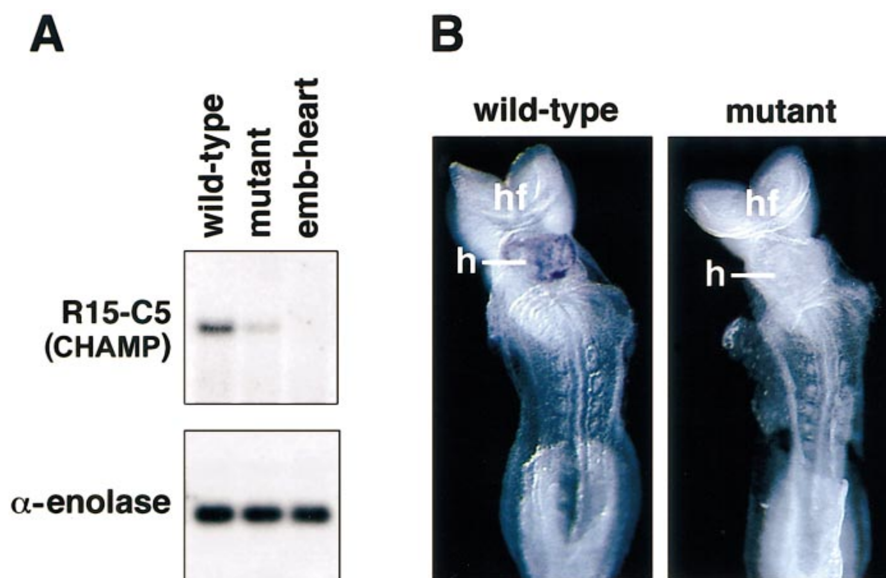

FIG. 1. RC15–5 expression in wild-type and MEF2C mutant embryos. (A) Transcripts for R15-C5 were detected by "virtual" Northern analysis by using cDNA prepared from heart tubes of wild-type and MEF2C mutant embryos at E9.0–E9.5 and whole E9.25 embryos without heart, as described in Materials and Methods. α-Enolase, which is expressed ubiquitously and is independent of MEF2C, was included as a control. (B) Transcripts for R15-C5 were detected by whole-mount *in situ* hybridization with E8.0 wild-type and *MEF2C* mutant embryos. R15-C5 transcripts were specifically expressed in the heart tube of wild-type embryos. Expression was not detected in the mutant. h, heart; hf, headfold.

and Methods) yielded a cDNA clone of about 2 kb. Using cDNA fragments derived from this clone as probes in Northern blots of adult mouse tissues, we detected a single 2-kb transcript only in the heart and lower levels of a 4.4-kb transcript in testis (Fig. 2).

Sequencing of the 2-kb cDNA clone revealed an ATG codon at the beginning of a 550-amino acid open reading frame with the potential to encode a putative protein with a predicted molecular weight of 62 kDa, p*I* 9.2 (Fig. 3). This novel protein contains seven conserved motifs characteristic of RNA helicase superfamily I (de la Cruz *et al.,* 1999). Based on its cardiac-specific expression and homology to other helicases, we refer to this protein as *c*ardiac *h*elicase *a*ctivated by *M*EF2 *p*rotein, CHAMP. CHAMP is most closely related to Mov-10 (Fig. 3C), a protein of unknown function first identified from a random proviral integration in a transgenic mouse strain (Mooslehner *et al.,* 1991). In contrast to CHAMP, Mov-10 is expressed ubiquitously. Other related helicases include Upf1, which mediates the degradation of nonsense mRNAs (Leeds *et al.,* 1991; Cui *et al.,* 1995), smupb2, which acts as a transcriptional activator or repressor (Chen *et al.,* 1997; Fukita *et al.,* 1993; Shieh *et al.,* 1995), and Hcs1p, a DNA helicase required for DNA replication (Biswas *et al.,* 1997) (Fig. 3C).

Members of RNA helicase superfamily I are related through a common central region, containing the seven conserved motifs flanked by divergent sequences at both ends (Fig. 3D). This central region is essential and sufficient for helicase activity, which unwinds RNA and/or DNA duplexes with energy derived from ATP hydrolysis (de la Cruz, 1999). Mutational analyses have revealed that motifs I, Ia, and II are involved in ATP binding and hydrolysis (Weng *et al.,* 1996). Motifs III and VI are involved in unwinding activity and RNA/DNA binding, respectively. The variable N- and C-terminal regions of superfamily I helicases have been postulated to participate in recognition and subcellular localization of substrates. The sequences of the seven helicase motifs, as well as their spacing, in CHAMP were highly conserved relative to other members of this helicase superfamily. CHAMP also contains five repeated motifs of the sequence TRNDXQSITNV/I near the amino terminus (Fig. 3B). These motifs do not correspond to the consensus for any protein kinases nor are they found in any other proteins we have been able to identify.

We also characterized the CHAMP transcript expressed in testis by isolating corresponding cDNAs and by analysis of genomic sequence conceptually translated from human chromosome 22q13 by the NCBI annotation project (accession no. 10880095). Based on this information, we found that the open reading frame of the human testis transcript encodes at least 826 additional amino acids at the 5′ end of the cardiac sequence (data not shown; sequence is available

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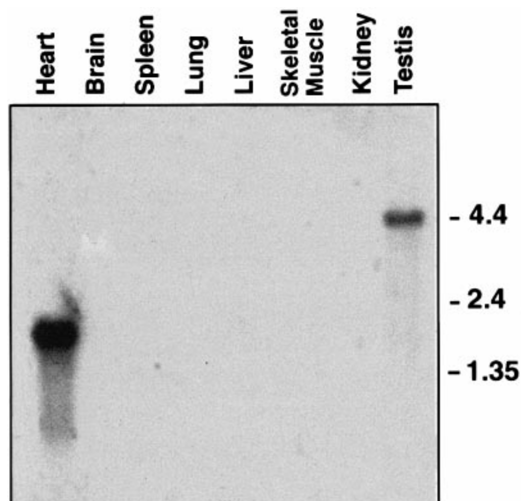

FIG. 2. Northern analysis of CHAMP RNA expression. CHAMP transcripts were detected by Northern analysis of RNA from the indicated adult mouse tissues. A single transcript of about 2 kb was detected in the adult heart and a larger and less abundant transcript of about 4.4 kb was detected in testis. Equivalent quantities of RNA were loaded in each lane.

upon request.). Comparison of cDNA and genomic sequence indicates that the cardiac transcript of human *CHAMP* is encoded by 13 exons (data not shown). The extended sequence predicted for the testis form of CHAMP is contained in 15 additional exons. Thus, the entire human gene is predicted to contain 28 exons, spanning 73 kb of DNA. These findings suggest that the cardiac and testis isoforms are generated from different promoters.

Embryonic Expression Pattern of CHAMP

The expression pattern of CHAMP during mouse embryogenesis was determined by *in situ* hybridization. CHAMP transcripts were not detected in the cardiac crescent at E7.75 by whole-mount *in situ* hybridization (data not shown). CHAMP expression was first observed in the linear heart tube at E8.0 (Fig. 4A). The highest expression of CHAMP was in the anterior region of the primitive heart tube that is fated to form the ventricular segments. CHAMP expression was not detected at the most posterior branches of the forming heart tube at this stage (Fig. 4A). These branches, known as the sinus venosae, later form the atrial chambers (DeHaan, 1965). The onset of CHAMP expression is about a half-day later than the initial expression of MEF2C (Edmondson *et al.,* 1994), which is consistent with *CHAMP* being a downstream target gene of MEF2C. The ventricular expression of CHAMP was maintained in the looped heart tube at E9.5 (Fig. 4B). At this stage, a low level of CHAMP expression was also detectable in atrial precursor cells. Subsequently, CHAMP expression was seen exclusively in the heart throughout development and into adulthood (Figs. 4D–4G). Radioactive section *in situ* hybridization at E13.5 and E15.5 indicated that CHAMP was specifically expressed within myocardial cells (Fig. 4D). No CHAMP expression was detected in the embryonic vasculature or outflow tract.

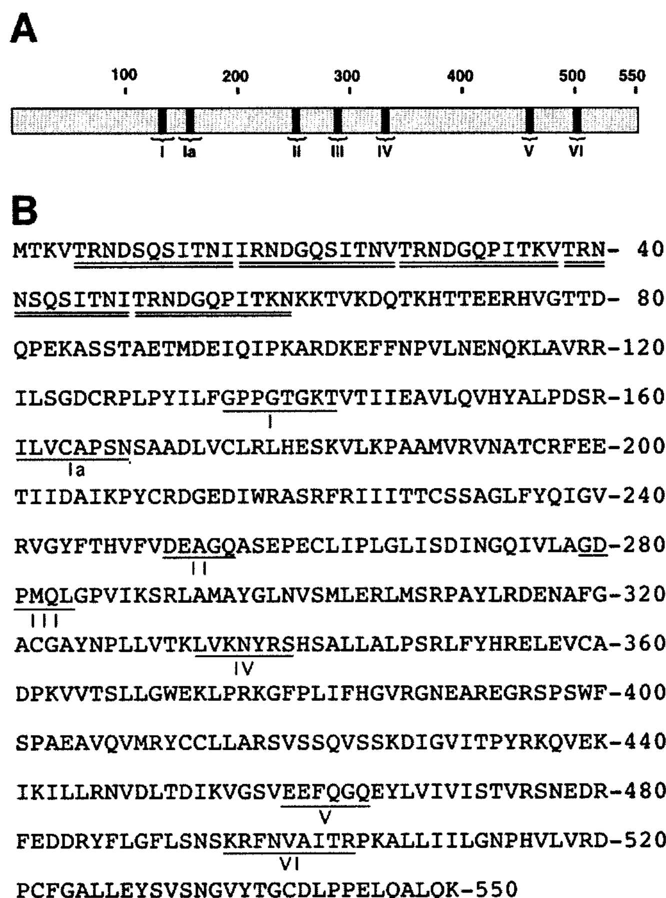

FIG. 3. Deduced amino acid sequence of CHAMP and its alignment to other helicases. (A) Schematic diagram of the CHAMP protein. The positions of the seven conserved motifs characteristic of RNA/DNA helicases are indicated. (B) The mouse CHAMP cDNA contained an open reading frame of 550 amino acids, a 370-bp 3′-UTR, and a putative polyadenylation signal not shown. The seven conserved helicase motifs are underlined. The TRNDXQSITNV/I repeats near the amino-terminus are designated by double underlines. (C) Sequence alignment of CHAMP with members of RNA helicase superfamily I: mouse Mov-10 (Genpept accession no. 127560), human UPF1 (GI 1633577), yeast Upf1p (GI 83347), mouse Smubp-2 (GI 730752), and yeast Hcs1p (GI 6322835). (D) The seven conserved helicase motifs of CHAMP are shown aligned with similar motifs in the aforementioned RNA helicase superfamily members. The number of amino acids that flank the helicase motifs are shown in parentheses. The conserved functional motifs include an ATPase motif (I, Ia and II), helicase motif (III), and RNA binding motif (VI).

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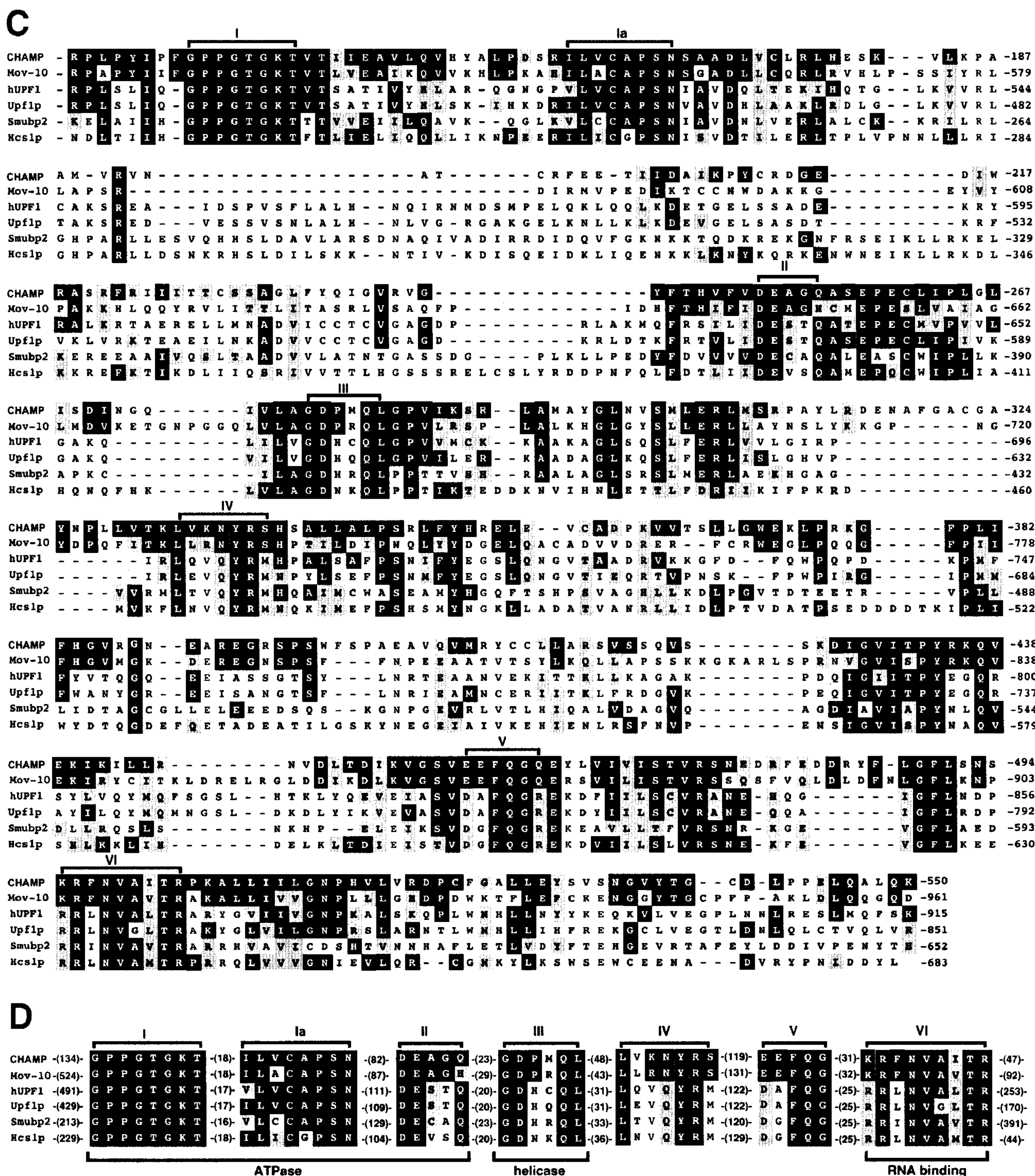

FIG. 3—*Continued*

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Subcellular Localization of CHAMP

Using an antibody raised against residues 531–550 of CHAMP, we examined the size and subcellular distribution of the protein. Western blot analysis showed that FLAG-tagged CHAMP from transfected HeLa cells migrated as a single band of 62 kDa (Fig. 5A, lanes 2 and 4) that comigrated with the endogenous protein from adult mouse heart extracts (Fig. 5A, lane 5). The bands from transfected HeLa cells and heart extracts were effectively competed when the antibody was preadsorbed with the CHAMP antigen used to generate the antibody (Fig. 5A, lanes 7 and 8). We conclude from these results that the cloned cDNA encodes the form of CHAMP protein expressed in the heart. The anti-CHAMP antibody also detected a band of about 170 kDa in heart extracts that was competed by the cognate peptide. We are uncertain of the identity of this protein, but it seems unlikely to be a larger form of CHAMP because the 2-kb CHAMP transcript detected in heart is not large enough to encode a protein of this size.

The subcellular localization of the CHAMP protein was examined by immunofluorescent staining of HeLa cells transfected with an expression vector encoding FLAG-tagged CHAMP (Fig. 5B). CHAMP was localized predominantly to the cytoplasm of transfected HeLa cells. Immunofluorescent staining of sections of embryonic mouse hearts with anti-CHAMP antibody also showed that endogenous CHAMP protein was localized primarily to the cytoplasm of cardiomyocytes. At E10.5, CHAMP expression was restricted specifically to cardiac myocytes, with no detectable staining in epicardial or endocardial cells (Figs. 6A and 6B). By E15.5, when the ventricular wall consists of a well-defined proliferating compact zone and less mitotically active trabeculations, expression of CHAMP appeared to be graded, with highest levels in myocytes within the trabeculations (Fig. 6E) and lowest levels in the proliferating compact zone (Fig. 6D).

DISCUSSION

Through an analysis of cDNA clones representing MEF2C-dependent genes in the mouse heart tube, we identified a novel cardiac-restricted helicase, CHAMP, in addition to numerous muscle-specific and more widely expressed genes that appear to require MEF2C for expression. CHAMP expression is restricted to embryonic and postnatal cardiomyocytes, although an alternative transcript is expressed at a low level in testis. Consistent with the conclusion that CHAMP expression is dependent on MEF2C, CHAMP transcripts are not detected until E8.0, the linear heart tube stage, a half-day after MEF2C is first expressed in the cardiac crescent (Edmondson *et al.,* 1994). CHAMP appears to be expressed in an anterior–posterior gradient along the heart tube at E8.0, an expression pattern similar to that of a *lacZ* reporter gene controlled by tandem copies of the MEF2 consensus sequence (Naya *et al.,* 1999) and transgenes controlled by the *MLC-2v* and *desmin* promoters, which require MEF2 binding sites for expression (Ross *et al.,* 1996; Kuisk *et al.,* 1996).

The expression of CHAMP at the time of cardiac looping and trabeculation is similar to that of neurotrophin-3 (NT-3) and its receptor Trk C (Lin *et al.,* 2000). The NT-3:trk C signaling pathway regulates cardiac myocyte proliferation at this stage of heart development (Lin *et al.,* 2000). Whether the preferential expression of CHAMP in the trabecular region of the developing heart, where the proliferative rate is diminished, reflects a role for CHAMP as a negative regulator of cardiomyocyte proliferation and growth remains to be determined.

Functions of RNA Helicase Superfamily I

CHAMP is most closely related to members of RNA helicase superfamily I, which includes yeast Upf1p, Sen1p, Hcs1p, and murine Smubp-2. Members of this helicase superfamily play diverse roles in DNA and RNA metabolism (de la Cruz *et al.,* 1999). Upf1p is required for nonsense mRNA decay to limit the accumulation of aberrant proteins that arise through inefficient splicing and premature termination of translation (Leeds *et al.,* 1991; Cui *et al.,* 1995). Upf proteins are also required to control the accumulation of a large number of mRNAs (Lelivelt *et al.,* 1999). Yeast Upf1p has been shown to possess helicase activities that unwind both RNA and DNA duplexes unidirectionally from 5′ to 3′ ends (Czaplinski, 1995; Kim *et al.,* 1999). Sen1p is required for tRNA splicing and has been postulated to be involved in biosynthesis and processing of other RNAs, such as rRNA and small nuclear and nucleolar RNAs (DeMarini *et al.,* 1992; Kim *et al.,* 1999). Hcs1p is a DNA helicase required for DNA replication (Biswas *et al.,* 1997), and Smubp-2 is a transcription factor (Chen *et al.,* 1997; Sebastiani *et al.,* 1995). Smubp-2 has been shown to bind two 12-*o*-tetracanoylphorbol-13-acetate-responsive elements in the Epstein–Barr virus immediate-early *BZLF1* promoter (Gulley *et al.,* 1997), which is regulated by MEF2 (Liu *et al.,* 1997). Overexpression of Smubp-2 in B lymphocytes represses the *BZLF1* gene promoter, possibly by disruption of a functional TBP-TFIIA-TATA box complex (Zhang *et al.,* 1999). The Rat homolog of Smubp-2 (cardiac transcription factor-1) was proposed to transactivate the *atrial natriuretic factor* (*ANF*) promoter through interaction with a *cis*-acting myocyte-specific element (Sebastiani *et al.,* 1995). RNA helicases have also been implicated in transcriptional coregulation during development (Nakajima, *et al.*, 1997). We have tested the potential responsiveness of several cardiac gene promoters, including the *ANF* promoter, to CHAMP, but have observed no effect (data not shown).

CHAMP protein appears to be localized primarily to the cytoplasm of cardiomyocytes, suggesting that its biochemical substrate may be RNA in nature. Human Upf1 and Upf2 are also cytoplasmic proteins and function in translational

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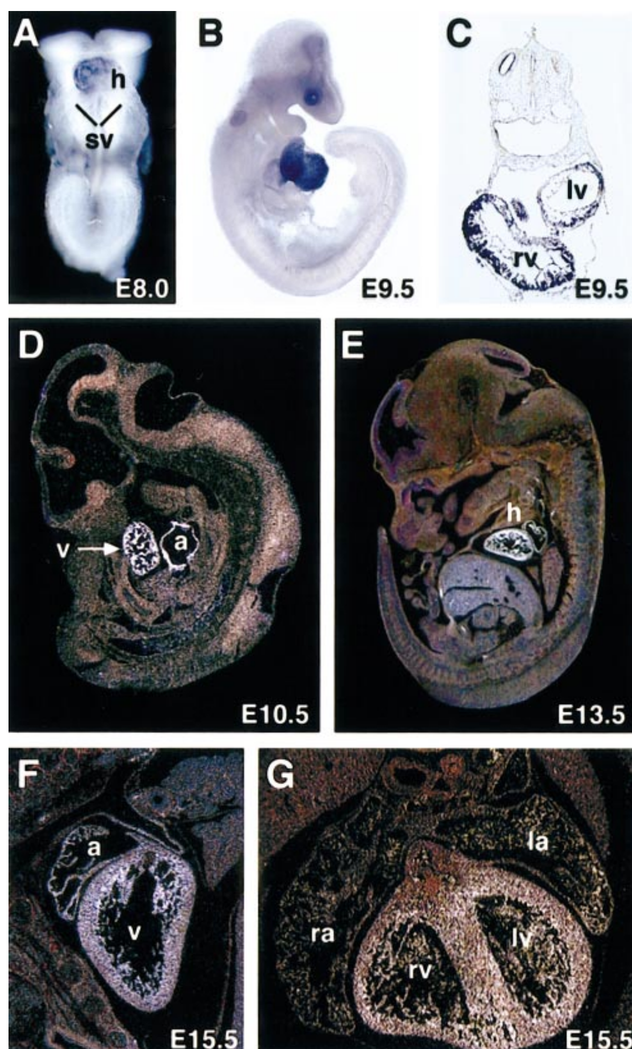

FIG. 4. *CHAMP* expression during mouse embryogenesis detected by whole-mount and radioactive section *in situ* hybridization. CHAMP expression was detected by whole-mount *in situ* hybridization with mouse embryos at E8.0 (A) and E9.5 (B). (C) A transverse vibratome section of the embryo shown in (B). (D–G) Radioactive *in situ* hybridizations of CHAMP transcripts. (D, E) Sagittal sections of E10.5 and 13.5 embryos, respectively. (F) A sagittal section of the heart of an E15.5 embryo. (G) A transverse section of the heart of an E15.5 embryo. *CHAMP* transcripts were detected only in myocardial cells. Staining in the head in (B) is nonspecific background. a, atrium; h, heart; la, left atrium; lr, left ventricle; ra, right atrium; rv, right ventricle; sv, sinus venosae; v, ventricle.

termination and in monitoring translational fidelity (Serin *et al.,* 2001). Other cytoplasmic RNA helicases include eiF-4A and the *Drosophila* protein VASA. eiF-4A is thought to function in unwinding mRNA during translational initiation (Rozen *et al.,* 1990). VASA is required for the formation of posterior pole plasm (Lasko and Ashburner, 1998). VASA acts as positive regulator of *oskar* RNA translation (Webster *et al.,* 1997) and is required for the proper localization and translation of *nanos* RNA (Gavis *et al.,* 1996). It is possible that CHAMP may control the proliferation of cardiomyocytes by regulating the mRNAs of proteins involved in cell cycle progression. Interestingly, a new RNA helicase, *carpel factory* (CAF), has been identified in *Arabidopsis thaliana* (Jacobson *et al.,* 2000). CAF acts epistatically downstream of a MADS-box type transcription factor Agamous. Disruption of *CAF* causes unregulated cell division.

Other MEF2C-Dependent Genes

In addition to CHAMP, we identified numerous muscle structural genes, as well as genes encoding growth and stress-responsive proteins, that appear to be regulated by MEF2C. Several of the muscle genes identified have been shown to be direct targets for MEF2. Whether the others also contain MEF2-binding sites in their control regions, or whether they are regulated indirectly by MEF2, remains to be determined. It should be pointed out that there are cardiac genes, such as *ANF*, *cardiac α-actin*, *α-myosin heavy chain*, and *MLC-1A*, that were shown previously to be down-regulated in the hearts of MEF2C mutants (Lin *et al.,* 1997), but were not identified in our screen. Thus, the screen was not completely saturating, despite the fact that several differentially expressed genes were identified multiple times.

Unexpectedly, the largest number of MEF2C-dependent sequences identified in our screen encoded proteins involved in energy metabolism and electron transport. Some of these genes, such as those encoding ATPase subunit 6 and ATP synthase α and γ subunits, are nuclear, whereas others are encoded by the mitochondrial genome. A similar subtractive scheme to identify genes dependent on the retinoic acid X receptor (RXR)-α in the heart at mid (E10.5) and late (E13.5) stages identified five metabolic genes, including the gene encoding the 14.5-kb subunit of the NADH-ubiquinone oxidoreductase complex, as being RXRα-dependent (Ruiz-Lozano *et al.,* 1998). It is interesting to note that nuclear-encoded muscle-specific forms of some cytochrome c oxidase genes contain a number of striated muscle-specific regulatory motifs, including MEF2 sites in their proximal promoter regions (see review by Lenka *et al.,* 1998).

Cardiac contractions are first observed as a peristaltic wave at E8.0 in the mouse, and contractions become stronger and more rhythmic as the heart undergoes looping and the chambers develop at E9.5. At this stage, the heart depends on oxidative phosphorylation to meet its high energy demands. The down-regulation of the above genes would be expected to result in depletion of cellular ATP stores with resulting energy deprivation and heart failure, as is observed in *MEF2C* mutant embryos.

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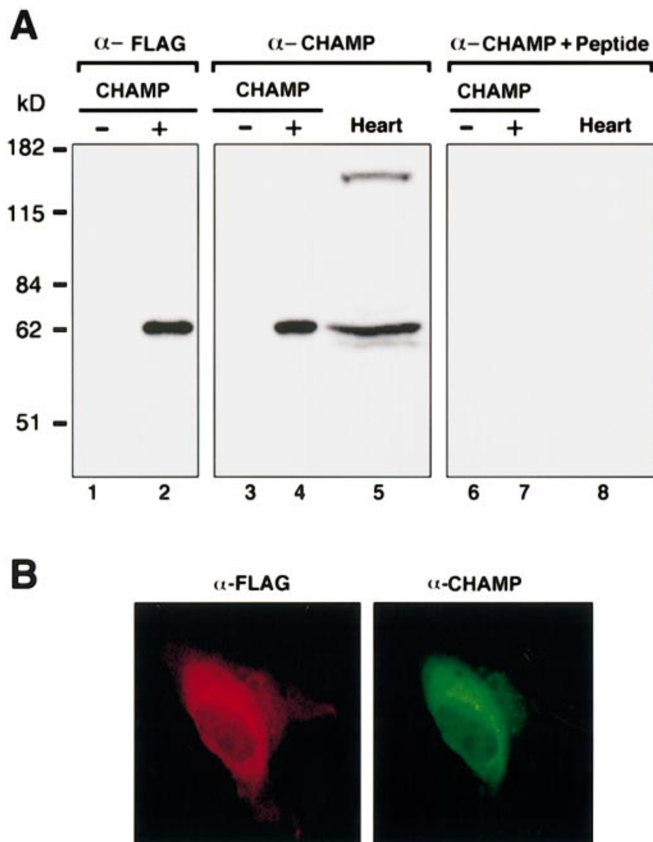

FIG. 5. Detection of CHAMP protein by immunoblot and immunofluorescence. (A) HeLa cells were transfected with pcDNA-FLAG-CHAMP (+) or pcDNA vector alone (−) as a negative control (lanes 1–4). Twenty-four hours later, cell extracts were prepared and CHAMP protein was detected by Western blot with anti-FLAG (lanes 1 and 2) or anti-CHAMP (lanes 3 and 4) antibody. In lanes 6–8, anti-CHAMP antibody was preadsorbed with peptide antigen prior to performing the Western blot. Cell extracts from adult mouse heart were immunoblotted with anti-CHAMP antibody in lanes 5 and 8. (B) HeLa cells transfected with pcDNA-FLAG-CHAMP were stained with anti-FLAG antibody (left) and with anti-CHAMP antibody (right), using Texas-Red conjugated with anti-mouse IgG and FITC conjugated with anti-rabbit IgG as the secondary antibody, respectively.

Roles for MEF2 in Development and Disease

In addition to the essential role of MEF2C in cardiac development, recent studies have revealed a potential role for MEF2 factors in cardiac hypertrophy in response to pressure overload and other stimuli (Passier *et al.,* 2000; Kolodziejczyk *et al.,* 1999; reviewed in Naya and Olson, 1999). Signal-dependent activation of MEF2-dependent genes has been shown to involve mitogen-activated protein kinase (Zhao *et al.,* 1999), calcium, calmodulin-dependent protein kinase (Passier *et al.,* 2000; Lu *et al.,* 2000), and calcineurin (Chin *et al.,* 1998; Mao *et al.,* 1999; Wu *et al.,* 2000), as well as the Notch signaling pathway (Wilson-Rawls *et al.,* 1999). In this regard, it is notable that many of the genes that were found to be down-regulated in *MEF2C*-null heart tubes are up-regulated during cardiac hypertrophy (Hwang *et al.,* 1997). Thus, it will be especially interesting to determine whether MEF2 factors regulate the same sets of genes in the adult myocardium as in the early heart tube and whether genes that are regulated by MEF2 in the embryonic heart are involved in reactivation of the "fetal" gene program during hypertrophic growth of the adult myocardium. It will also be interesting to determine

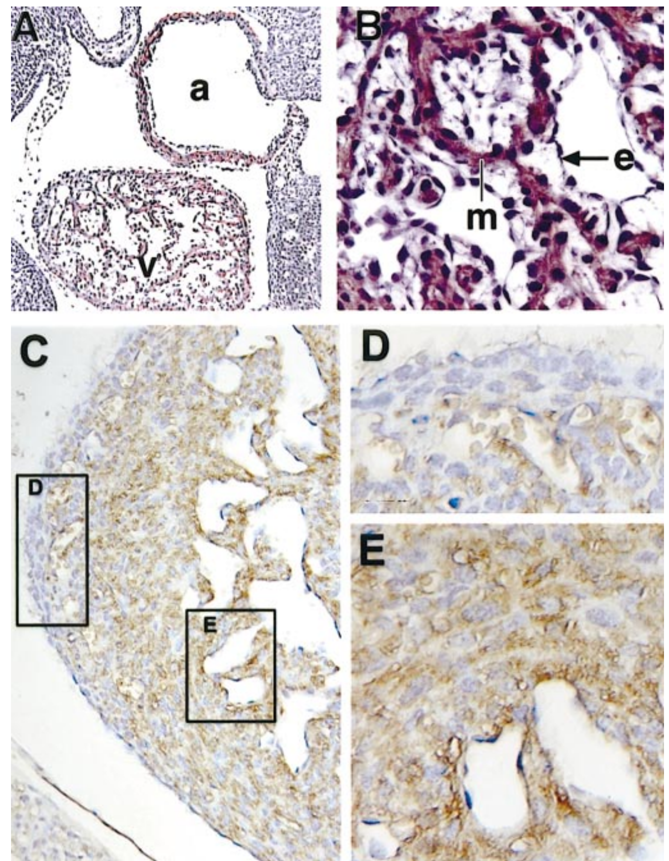

FIG. 6. Immunostaining of CHAMP in the heart of an E10.5 mouse embryo. Paraffin-embedded sagittal sections of a mouse E10.5 (A and B) and E15.5 (C–E) embryos were deparaffinized and probed with anti-CHAMP antibody. The bound anti-CHAMP antibody was detected by sequential incubation with biotinylated secondary antibody, followed by horseradish peroxidase-conjugated streptavidin, and visualized after addition of diaminobenzidine. Sections were counterstained with hematoxylin. CHAMP protein was restricted to myocardial cells (m) and was not detected in endocardial (e) or epicardial cells. a, atrium; v, ventricle. At E15.5, CHAMP staining was highest in the trabecular region (E) and was much reduced in the proliferative compact zone (D).

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whether regulation of CHAMP enzyme activity affects cardiac function.

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