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The MADS-Box Factor CeMEF2 Is Not Essential for *Caenorhabditis elegans* Myogenesis and Development

Daryl Dichoso,* Thomas Brodigan,* Kyu Yeong Chwoe,† Jin Sook Lee,† Reymond Llacer,* Morgan Park,* Ann K. Corsi,* Stephen A. Kostas,‡ Andrew Fire,‡ Joohong Ahnn,† and Michael Krause*

**Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-0510; †Department of Life Sciences, Kwangju Institute of Science and Technology, Kwangju, Korea; and ‡Department of Embryology, Carnegie Institute of Washington, Baltimore, Maryland 21210*

MEF2 is an evolutionarily conserved MADS (MCM1, Agamous, Deficiens, and serum response factor) box-type transcription factor that plays a critical role in vertebrate and *Drosophila melanogaster* myogenesis. We have addressed the developmental role of the single MEF2-like factor, CeMEF2, in *Caenorhabditis elegans.* Using expression assays and two *mef-2* deletion alleles, we show that CeMEF2 is not required for proper myogenesis or development. Moreover, a putative null *mef-2* allele fails to enhance or suppress the phenotypes of mutants in CeMyoD or CeTwist. Our results suggest that despite its evolutionary conservation of sequence and DNA binding properties, CeMEF2 has adopted a divergent role in development in the nematode compared with *Drosophila* and vertebrates.

Key Words: **MEF2; MADS box; myogenesis; *C. elegans;* SRF.**

INTRODUCTION

Several transcription factors are known to regulate muscle gene expression and are required for proper myogenesis. Among these factors is the evolutionarily conserved myocyte enhancer factor-2 (MEF2) group that appears to be present in most, if not all, types of vertebrate muscle tissues. MEF2 was initially purified from muscle cells as a DNA binding activity that recognized a *cis*-acting regulatory sequence in the promoter of the muscle creatine kinase (MCK) gene (Gossett *et al.*, 1989). MEF2 was identified independently in vertebrates in a search for genes related to serum response factor (SRF; originally called RSRF factors) and DNA binding studies demonstrated that the RSRFs were components of MEF2 DNA binding activity (Pollock and Treisman, 1991). Subsequent studies have identified MEF2 sites upstream of numerous muscle-specific genes, and there is now a large volume of evidence supporting its role as a positively acting transcription factor required for high level expression of muscle-specific genes (reviewed in Black and Olson, 1998). MEF2 has also been shown to be important in the activation of nonmuscle genes in neurons, B cells, and T cells, helping to explain, at least in part, its broad tissue expression profile in vertebrates (Dodou *et al.*, 1995; Black *et al.*, 1996; Schulz *et al.*, 1996; Swanson *et al.*, 1998; Satyaraj and Storb, 1998; Rao *et al.*, 1998; Naya *et al.*, 1999). Recent reports suggest that MEF2 can also respond to calcium fluxes to regulate neuron survival (Mao *et al.*, 1999) and T cell apoptosis (Youn *et al.*, 1999). For both muscle-specific and nonmuscle gene expression, MEF2 often acts combinatorially with other transcription factors to result in full transcriptional activation of target genes (Molkentin *et al.*, 1995; Shore and Sharrocks, 1995; Black *et al.*, 1996; Sartorelli *et al.*, 1997).

MEF2 factors are a subgroup of the larger MADS family of transcription factors. The MADS family members all share a highly conserved amino-terminal domain spanning approximately 60 amino acids known as the MADS box, as first noted in the original four members of the family, MCM1, Agamous, Deficiens, and serum response factor (Schwarz-Sommer *et al.*, 1990). The MADS-box domain is involved in DNA binding and dimerization, and these factors typically act as homodimers or heterodimers (Pollock and Treisman, 1991; Molkentin *et al.*, 1996). The

members of the MEF2 group of MADS factors have an extended region of 29 conserved amino acids, known as the MEF box, that is a signature domain for this subgroup. The MEF box is involved in interactions with other proteins and also influences DNA binding. The preferred MEF2 DNA binding site ($CTA(A/T)_4TAG/A$) is distinct from the binding site of other MADS-box family members.

There are four MEF2 genes in vertebrates (*mef2a, mef2b, mef2c, mef2d*) that are differentially spliced to generate multiple MEF2 isoforms (Pollock and Treisman, 1991; Yu *et al.*, 1992; Breitbart *et al.*, 1993). This isoform complexity, and presumed redundancy of function, has complicated the analysis of the roles of MEF2 in the mouse. The clearest result to date comes from the elimination of *mef2c* in mice, which causes embryonic death due to severe cardiovascular defects (Lin *et al.*, 1998; Bi *et al.*, 1999).

In *Drosophila melanogaster* there is a single *mef2* gene that is expressed in all developing muscle tissues and specific regions of the brain (Lilly *et al.*, 1994, 1995; Bour *et al.*, 1995; Taylor *et al.*, 1995). Elimination of DMEF2 activity results in the complete absence of all muscle differentiation in *Drosophila*, providing the cleanest and most dramatic evidence for the myogenic role of MEF2 (Lilly *et al.*, 1995; Bour *et al.*, 1995). Although muscle differentiation is blocked, muscle cell precursors are detected in homozygous D-*mef2* null mutant animals, demonstrating that muscle fate determination can occur in the absence of DMEF2 activity.

Promoter analysis of the D-*mef2* gene has placed D-*mef2* downstream of other transcription factors important for regulating *Drosophila* myogenesis. For example, in somatic muscle D-*mef2* is a direct target of activation by the basic helix-loop-helix (bHLH) factor Twist (Cripps *et al.*, 1998; Nguyen and Xu, 1998), and Twist has been shown to be essential for mesoderm formation and myogenesis in *Drosophila* (Nüsslein-Volhard *et al.*, 1984; Simpson, 1983; Baylies and Bate, 1996). In cardiac muscle, D-*mef2* is activated by the homeodomain factor Tinman (Gajewski *et al.*, 1997; Nguyen and Xu, 1998), and Tinman is required for heart formation and differentiation (Azpiazu and Frasch, 1993; Bodmer, 1993).

As part of our studies of myogenesis in the nematode, we were interested to determine if a MEF2-like factor(s) was important for muscle formation in *Caenorhabditis elegans*. We have identified a single gene in *C. elegans* encoding a protein related to MEF2; no additional MEF2-like factors have been identified in the nearly complete (>99%) genome sequence (*C. elegans* Sequencing Consortium, 1998). We isolated two deletion mutants of *mef-2*, at least one of which is likely to be a null allele. Surprisingly, loss of *mef-2* activity had little or no effect on *C. elegans* development and myogenesis. Double mutants of the putative null *mef-2* mutant and either CeMyoD or CeTwist mutants showed no enhancement of the single-mutant phenotypes. These results suggest that despite a high level of protein sequence conservation, MEF2 factors have adopted evolutionarily divergent roles with respect to myogenesis.

MATERIALS AND METHODS

Cloning

The *C. elegans mef2*-like gene was originally identified by PCR of genomic DNA using degenerate primers to regions of the conserved MADS and MEF box domains based on vertebrate and *Drosophila* sequences. The gene was cloned from a genomic *C. elegans* library (kindly provided by C. Link) and the gene and flanking regions were sequenced (GenBank Accession No. U36199). The genomic region was mapped to a YAC grid by Coulson and colleagues and the corresponding cosmids (W10D5 and F27D1) were subsequently sequenced by the Genome Sequencing Consortium (Accession No. Z79758). A single cDNA clone was isolated from a mixed-stage *C. elegans* library (kindly provided by B. Barstead) and was sequenced in its entirety (Accession No. U36198). No expressed sequence tags (ESTs) corresponding to this gene have been identified to date.

Reporter constructs were derived from genomic phage clones. The largest genomic fragment used was an ~12-kb *Pst*I restriction fragment covering approximately 9.1 kb of the 5′ flanking region of the gene, exons I and II and part of exon III. This genomic fragment was fused in-frame to the green fluorescent protein (GFP) reporter vectors TU63 and pPD95.73 to yield the plasmids pKM3000 and pKM3001, respectively (Chalfie *et al.*, 1994; A. Fire, G. Seydoux, J. Ahnn, and S. Q. Xu, personal communication), or to *lacZ* using the vector pPD22.11 (Fire *et al.*, 1990). For the TU63 and pPD22.11 constructs an in-frame stop codon within the multiple cloning site was eliminated. Derivatives of these reporters included a truncation of the 5′ end of the clone to a *Hin*dIII restriction site located at −3660 bp relative to the ATG (pMF3) or a *Hin*dIII site within intron I 269 bp downstream of the putative ATG (pMF2, pKM2007).

To inhibit the D1081.2 gene by RNA-mediated interference (RNAi) we cloned an ~500-bp segment of the locus using the primers MWK 356 (CGCGAATTCCTTCACTCCATTTCTCGCCCCATCAATGGC) and MWK 357 (CGCGGATCCGATGCAATGGCTGCTGATTCGATGAGCTG) into the vector pVZ-1 and synthesized RNA *in vitro* from T3 and T7 RNA polymerase promoters. Double-stranded RNA was prepared and injected into early adult hermaphrodites as described (Fire *et al.*, 1998).

Strains Used

Two deletion alleles of *mef-2* were isolated, KM129 *mef-2(gv1)* and KM130 *mef-2(gv2)*, from a mutant library using the method of Barstead and Moulder as described in Dernburg *et al.* (1998). Deletion strains were backcrossed to wild-type N2 animals at least three times prior to use in subsequent experiments or genetic crosses. Double-mutant combinations were made between *mef-2(gv1)* and the CeMyoD null mutant allele *hlh-1(cc450)*, the CeMyoD temperature-sensitive allele *hlh-1(cc561)*, the CeTwist mutant allele *hlh-8(nr2061)*, or the temperature-sensitive *pha-1*(*e2123ts*) allele. The *mef-2(gv1)* allele was assayed for muscle defects by direct observation, by antibody staining, and by introducing the integrated reporters that expressed in either the postembryonic mesoderm (*hlh-8::gfp;* Harfe *et al.*, 1998a) or the vulval muscles (*egl-15::gfp;* Harfe *et al.*, 1998b).

Gel Shifts

The *mef2* cDNA clone (pKM1027) was used to synthesize RNA that was translated in a rabbit reticulocyte lysate (Promega) and

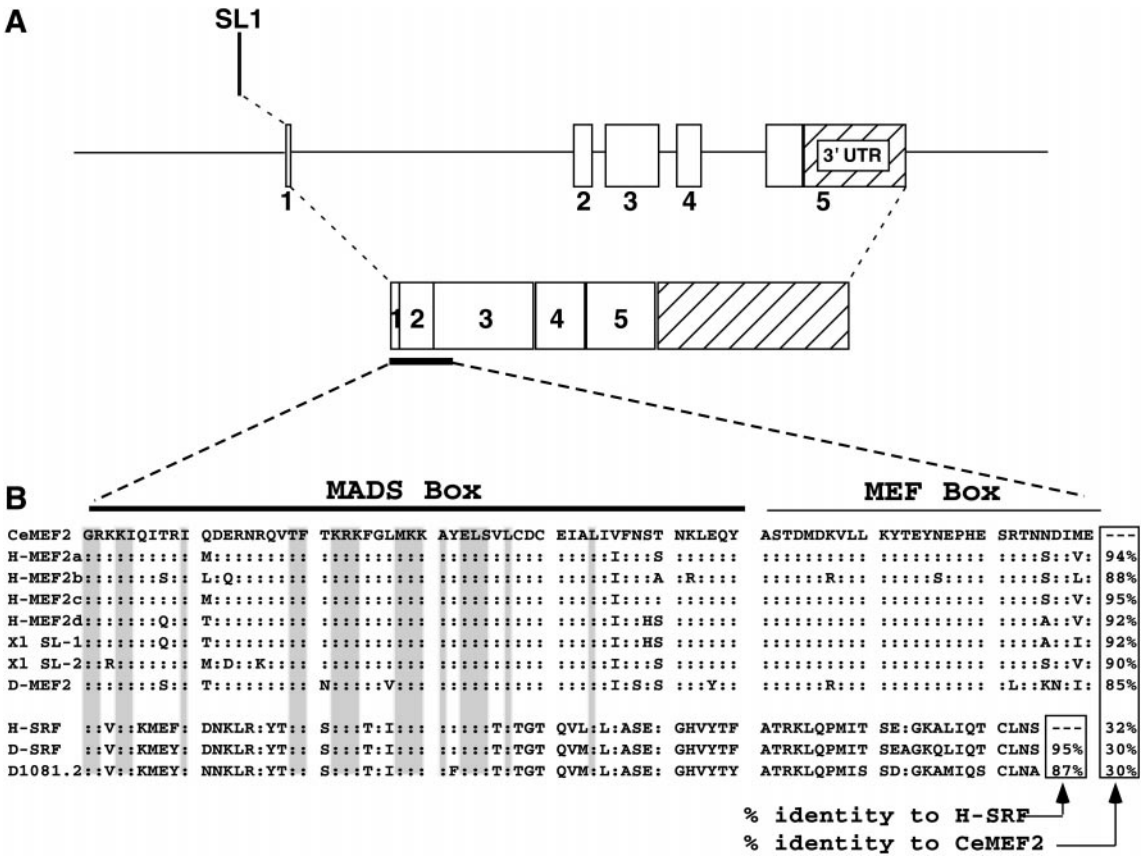

FIG. 1. CeMEF2 gene structure and sequence comparison. (A) The exon/intron structure is shown schematically for *mef-2* with the cDNA structure shown below. SL1 *trans*-splicing to the first exon is indicated by the labeled vertical black bar above the gene. Coding regions corresponding to the MADS and MEF domains are underlined by a heavy black line under the cDNA structure. (B) Amino acid sequence comparisons of MEF2 and SRF factors. The MADS and MEF domains of several factors are compared with CeMEF2 with the percentage identity shown at right. Sequences include human H-MEF2a, b, c, and d (Pollock and Treisman, 1991; Yu *et al.*, 1992; Breitbart *et al.*, 1993; McDermott *et al.*, 1993); *Xenopus laevis* SL-1 and SL-2 (Chambers *et al.*, 1992); *D. melanogaster* D-MEF2 (Lilly *et al.*, 1994); and CeMEF2. The lower portion of the comparison shows SRF from human (H-SRF; Norman *et al.*, 1988) and *Drosophila* (D-SRF; Affolter *et al.*, 1994) compared to the gene D1081.2 encoding a second MADS factor in *C. elegans*. Shaded residues are identical in both MEF and SRF. Note that D1081.2 is more similar to SRF than to MEF2 within the MADS domain and lacks similarity to CeMEF2 within the MEF domain.

used in gel shifts as previously described (Krause *et al.*, 1997). Double-stranded oligonucleotide probes used were either a canonical MEF2 binding site (MWK216 CGCTCTAAAAATAACCCT and its complement) or a mutated MEF2 site (MWK 217 CGCTCTA-AggcTAACCCT and its complement).

RESULTS

C. elegans Has a Single MEF2-Related Factor

Using a degenerate PCR-based strategy, we identified a single gene (*mef-2*) that encodes a protein (CeMEF2) related to MEF2 from other organisms. The nearly completed (>99%) genome sequence of *C. elegans* has failed to reveal any additional MEF2-like factors (*C. elegans* Sequencing Consortium, 1998). Comparison of the sequence from the genomic region of *mef-2* with our cDNA sequence information reveals a gene structure composed of five exons and four introns spanning approximately 5.2 kb (Fig. 1A). Structural features of interest in *mef-2* are a potential *trans*-spliced leader sequence splice site 55 bp upstream of the start codon, a large first intron (2376 bp), a relatively long (811 bp) 3′ untranslated region (UTR), and a canonical poly(A) signal sequence (AATAAA) located upstream of the poly(A) tail addition site (Krause, 1995). The initial 5′-end sequence of a *mef-2* cDNA suggested that the SL1 *trans*-spliced leader might be present on the RNA (Krause, 1987). We have confirmed by reverse transcriptase-polymerase chain reactions that SL1, but not the alternately spliced leader SL2, is *trans*-spliced to a canonical 3′ intron splice acceptor sequence located 55 bp upstream of the putative ATG.

The predicted CeMEF-2 protein is 340 amino acids in length with a predicted molecular weight of 36.5 kDa. A canonical 56-residue MADS domain followed immediately by a 29-residue MEF domain is found at the amino terminus of the coding region. A comparison of these MADS and MEF domains to MEF2 proteins identified in other species shows a remarkable degree of evolutionary conservation of these domains (Fig. 1B). CeMEF2 is 94% identical to human MEF2a across these two domains with only five residue differences, two of which are conservative changes. The MADS and MEF domains of CeMEF2 are quite distinct from the amino-terminal domains of the only other known *C. elegans* MADS box factor encoded by the gene D1081.2. Sequence comparisons show that D1081.2 lacks a MEF box and is more similar to SRF in the MADS domain than to MEF2 homologs, making the distinction between these two *C. elegans* MADS-box factors unambiguous (Fig. 1B).

CeMEF2 Has Conserved Binding Specificity

The MEF2 DNA binding site was initially defined by the segment of the MCK gene used to purify the binding activity (Gossett *et al.*, 1989). Subsequent DNA binding studies have determined a consensus MEF2 site to be YTA(A/T)$_4$TAR (Pollock and Treisman, 1991). The conservation of CeMEF2 with vertebrate and *Drosophila* MEF2 factors throughout the MADS and MEF DNA binding domains suggested that CeMEF2 could bind a typical MEF2 binding site. We tested this hypothesis using rabbit reticulocyte-translated protein in gel mobility shift assays. CeMEF2 binds strongly to the canonical MEF2 sequence and fails to bind when bases in the core of the site are changed to nonconsensus residues (Fig. 2).

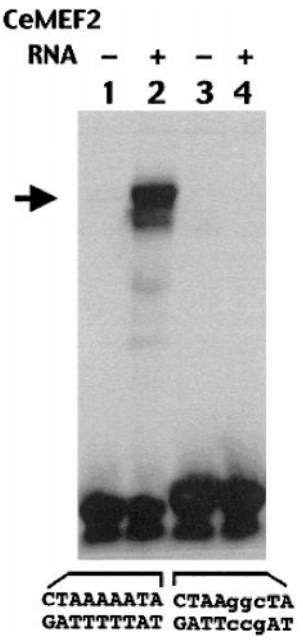

FIG. 2. CeMEF2 binds a consensus MEF2 binding site. *In vitro* translation products from reaction with (+) or without (−) *mef-2* RNA were used in gel shift experiments. Two probes were used, one with the consensus MEF2 binding site (CTAAAAATA) and the other in which core residues of the MEF2 binding site were altered (in lowercase: CTAAggcTA). A single strong band (arrow) is seen with *mef-2* RNA using the consensus oligonucleotide probe. Use of large amounts of CeMEF-2 translation product resulted in two strongly shifted species, presumably reflecting homodimer and oligomeric complexes (data not shown).

mef-2 Is Expressed Ubiquitously throughout Most of Development

We have assayed the developmental profile of *mef-2* expression using a combination of Northern blot analysis and reporter gene fusions in transgenic *C. elegans.* A *mef-2* cDNA probe detects one prominent and two minor mRNAs (1.9, 1.6, and 1.3 kb) by Northern analysis of either total or poly(A)$^+$ RNA (Fig. 3). These messages are detected at all stages of development. The longest message (1.9 kb) is consistent with the size expected from our single cDNA clone. Given the long 3′ UTR, differences in transcriptional termination or poly(A) addition sites within this UTR might be responsible for the other two *mef-2* mRNA size classes (although we have been unable to confirm this with cDNA or EST clones).

We have used *gfp* and *lacZ* reporter gene fusions to assay temporal and spatial patterns of *mef-2* promoter activity. Using a genomic segment extending ~9.3 kb upstream of the predicted ATG, we observed initial embryonic expression in neurons and later expression in all tissues. This expression pattern begins late in embryogenesis (two-fold stage) and continues throughout postembryonic development (Fig. 4). A similar pattern was observed with reporter constructs containing only ~3.6 kb of upstream genomic sequences, although the intensity of expression was reduced compared with the longer construct. Truncation of the 5′ end of the reporter genes to a restriction site within the first intron eliminated expression of the transgenic reporter genes. We have raised antibodies against a bacterially expressed CeMEF2 fusion protein. The polyclonal antibodies recognized the fusion protein by Western blot analysis, but we have been unable to detect the endogenous CeMEF2 protein by either Western blot or *in situ* immunolocalization.

mef-2 Is a Nonessential Gene

Using the method of Barstead and Moulder (Dernburg *et al.*, 1998), we screened a mutagenized population for individual animals harboring deletions within the *C. elegans mef-2* gene. The deletion screen was done by PCR using nested primers located in intron I and the untranslated region of exon V that bracketed an approximately 2-kb segment of the gene. Two independent deletion events were identified in a screen of a library representing 4.8×10^5

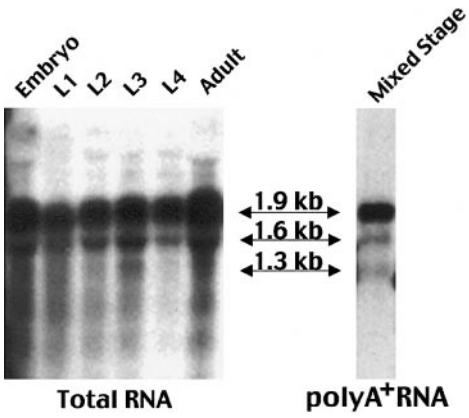

FIG. 3. Northern analysis of *mef-2* expression in *C. elegans.* To the left is an autoradiograph of a developmental Northern blot of total RNA (15 μg/lane) isolated from staged populations of animals as indicated at top. Ethidium bromide staining of the gel prior to transfer confirmed near-equal loading of RNA in all lanes of the gel. To the right is a Northern blot of poly(A)$^+$ RNA (1.5 μg per lane) isolated from a mixed-stage population of animals. Three messages are detected using a full-length *mef-2* cDNA probe. A prominent band of 1.9 kb corresponding in size to a single cDNA clone is present throughout development and weaker bands of 1.6 and 1.3 kb are also visible.

mutagenized chromosomes. Animals with *Cemef-2* deletions were identified and culled by sib selection through successively smaller pools until single animals homozygous for the each deletion allele were isolated.

One of the deletions, *mef-2(gv1),* removes 1376 bp beginning in intron I and extending through intron IV (Fig. 5A). This deletion is predicted to be a null allele because the deletion removes most of the MADS and MEF domains that are required for MEF2 function (Pollock and Treisman, 1991). The second deletion, *mef-2(gv2),* removes 760 bp beginning in exon III and ending in exon V. This deletion leaves the MADS and MEF domains intact but results in a truncation of the C-terminal 167 residues of the protein and a frame shift in the coding region after the deletion break point. The rearrangements in both deletion alleles have been confirmed by Southern blot analysis of genomic restriction fragment patterns (data not shown). Northern blot analysis of the two deletion alleles reveals that stable, albeit expectedly smaller, mRNAs can still be detected in these animals. A prominent mRNA band of 1.2 kb was observed in *mef-2(gv1)* and of 1.6 kb in *mef-2(gv2)* compared with a wild-type size of 1.9 kb (Fig. 5B).

We have not detected any strong visible phenotype in animals homozygous for either *mef-2* deletion allele although *mef-2(gv1*) animals are slightly short and fat, or Dumpy (Dpy), as adults. Both *mef-2(gv1)* and *mef-2(gv2)* homozygous mutant animals develop, move, and reproduce at rates indistinguishable from those of wild-type animals. Staining with a muscle-specific monoclonal antibody to myosin heavy chain or the filamentous actin binding protein phalloidin failed to reveal any overt defects in myogenesis. We have also assayed specific muscle groups to look for subtle defects in myogenesis during postembryonic development corresponding to the period of highest *mef-2::gfp* reporter gene expression. Two GFP reporter genes that are expressed postembryonically provide a sensitive assay for mesodermal patterning (*hlh-8::gfp*) and vulval muscle formation (*egl-15::gfp*) (Harfe *et al.,* 1998a,b). No defects in reporter gene expression or pattern were observed in the *mef-2(gv1)* strain containing either reporter gene. We have also tested the ability of a *mef-2::gfp* reporter construct to be expressed in the *mef-2(gv1)* mutant background to look for autoregulatory requirements and perhaps reveal a molecular phenotype for the mutant. Expression of this reporter is indistinguishable from that observed in a wild-type genetic background. It is possible that CeMEF2 is required for more subtle aspects of development such as

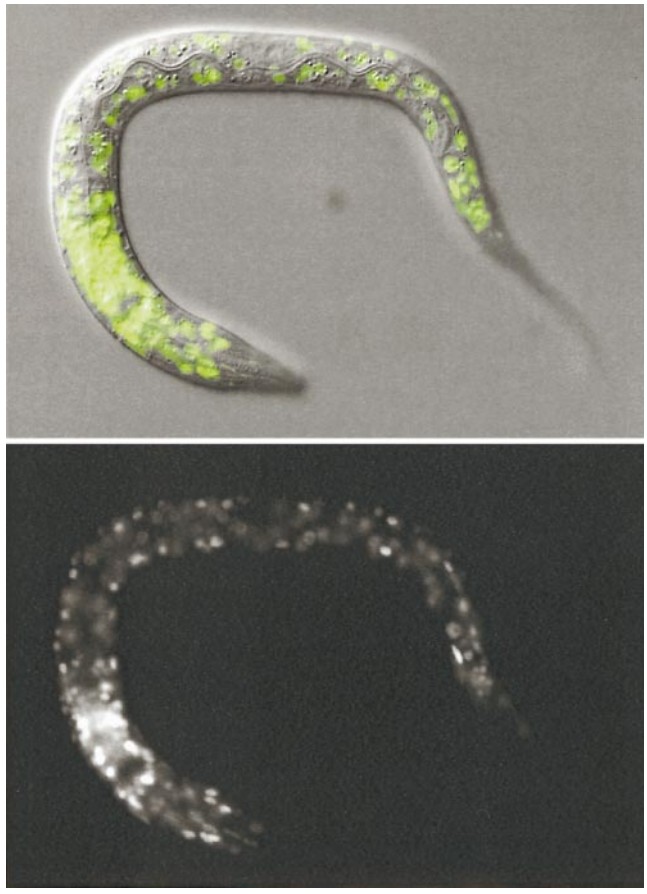

FIG. 4. Expression pattern of a *mef-2::gfp* reporter gene. Nearly ubiquitous somatic cell expression is shown in this L1 larva harboring a reporter gene containing ~12 kb of genomic *mef-2* sequence. The top shows a merged bright-field and GFP image; below is the fluorescent image alone.

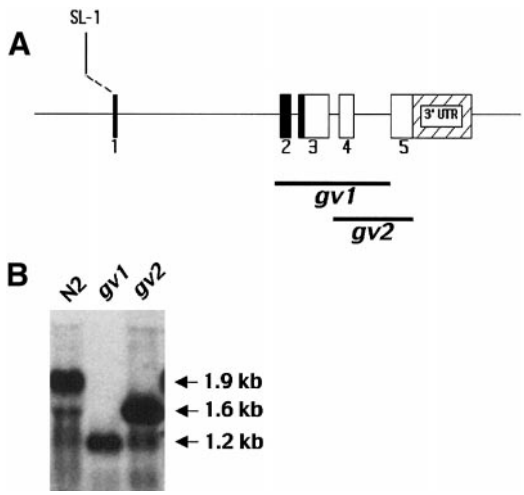

FIG. 5. *mef-2* deletion alleles and RNA expression. (A) A schematic of the *mef-2* gene with exons shown as boxes and the MADS/MEF box regions shaded in black. Heavy black lines below the gene indicate the genomic DNA missing in the *gv1* and *gv2* deletion alleles. (B) Northern blot analysis of total RNA isolated from mixed populations of wild-type (N2) and *mef-2* mutant alleles (*gv1* and *gv2*).

neuronal function and animal behavior; we have not tested directly for these traits.

Cemef-2 Mutants Failed to Enhance Other Muscle Mutants

Redundancy among myogenic factors acting in concert could mask the effects of the loss of any one factor. MEF2 has been shown to interact with myogenic bHLH factors (Molkentin *et al.*, 1995; Molkentin and Olson, 1996) and to be a direct target of Twist inhibition in other organisms (Spicer *et al.*, 1996). We have generated strains with double mutant combinations between *mef-2(gv1)* and predicted null alleles of *hlh-1* (encoding CeMyoD), *hlh-8* (encoding CeTwist), or *pha-1* (encoding a bZIP-related transcription factor) to see if redundancy of these other myogenic factors and CeMEF2 might be revealed. We assayed all double mutants for obvious enhancement of single-mutant phenotypes with respect to myogenesis. Loss of CeMyoD activity results in morphological defects during embryogenesis and loss of muscle contractility (Chen *et al.*, 1992). Homozygous *hlh-1* mutant animals hatch out as malformed larvae that are unable to move and that usually die during the L1 stage. We tested both the null *hlh-1(cc450)* and the temperature-sensitive *hlh-1(cc561)* alleles for phenotypic changes when placed in combination with the putative null allele *mef-2(gv1)*. Double-homozygous *mef-2(gv1); hlh-1(cc450)* and *mef-2(gv1); hlh-1(cc561)* animals were indistinguishable from *hlh-1* mutant homozygotes alone (Fig. 6). Loss of CeTwist activity affects only a subset of mesoderm, causing a loss of some of the enteric and egg-laying muscles in the hermaphrodite (Corsi *et al.*, 2000). Double-homozygous *mef-2(gv-1); hlh-8(nr2061)* animals are indistinguishable from homozygous *hlh-8(nr2061)* mutants with no obvious defects in muscle groups other than those affected by CeTwist mutations alone (data not shown). Finally, we tested double mutants of *mef-2(gv1)* and the gene *pha-1*, which encodes a transcription factor required for complete differentiation of the pharynx, a muscular organ used to pump food into the animal (Schnabel and Schnabel, 1990; Granato *et al.*, 1994). Double-homozygous *mef-2(gv1); pha-1(e2123ts)* were phenotypically identical to *pha-1(e2123ts)* alone when raised at the restrictive temperature based on pharyngeal morphology using Nomarski optics and staining with the monoclonal antibody 3NB12, which recognizes the pharyngeal muscles (Okamato and Thompson, 1985).

Although no other MEF2-like sequences have been identified in the genome, a related SRF-like MADS box factor is encoded by the D1081.2 locus. In vertebrates, SRFs are important for proper regulation of muscle-specific and non-muscle genes (reviewed in Treisman 1990, 1994). To test the possibility that D1081.2 provided redundant function in animals lacking CeMEF2, we inhibited D1081.2 function by RNAi (Fire *et al.*, 1998) in wild-type and *mef-2(gv1)* mutant animals. *D1081.2(RNAi)* in a wild-type background caused a progressive larval uncoordinated (Unc) phenotype. Although many progeny of *D1081.2(RNAi)*-injected her-

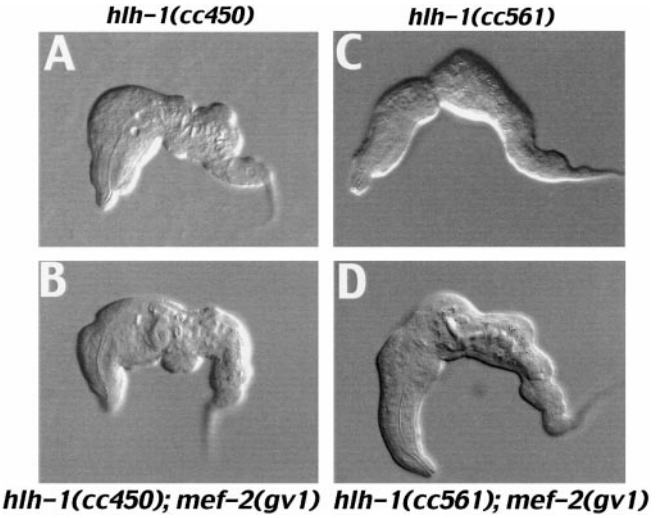

FIG. 6. *mef-2* mutations fail to enhance or suppress the phenotype in animals deficient for CeMyoD activity. The *hlh-1(cc450)* null (A) and *hlh-1(cc561)* temperature-sensitive (C) CeMyoD L1 mutant phenotypes are compared with *hlh-1; mef-2(gv1)* double-homozygous mutants (B and D, respectively). Double-mutant animals are indistinguishable from the single *hlh-1* mutant animals; most of the animals progress to the L1 stage, arrest development, and die.

maphrodites became Unc, this phenotype was not fully penetrant. Because the onset of the Unc phenotype was progressive and variable it is difficult to quantitate precisely the fraction of affected progeny. The *mef-2(gv1); D1081.2(RNAi)* phenotype was qualitatively similar to that of *D1081.2(RNAi)* in a wild-type background. In order to observe the quantitative similarity of RNAi in affected wild-type versus *mef-2(gv1)* animals, we scored day 2 progeny (maximal RNAi effect) from individual adult animals injected with D1081.2 dsRNA for the Unc phenotype. Of injected animals giving rise to Unc progeny, an average of 81% of the progeny were affected (n = 393, range 45–96%) in the wild-type background and an average of 68% (n = 431, range 8–97%) were affected in the *mef-2(gv1)* background. We considered these percentages to be similar given the difficulty of scoring affected progeny accurately.

DISCUSSION

At the amino acid level, there is an extremely high evolutionary conservation of MEF2 factors throughout the amino-terminal MADS and MEF domains, implying conservation of function across these regions. Indeed, we find that CeMEF2 strongly binds a canonical MEF2 site and fails to bind DNA in which core residues in the binding site have been altered. In *Drosophila*, D-MEF2 is essential for all types of myogenesis (Lilly *et al.*, 1995; Bour *et al.*, 1995; Ranganayakulu *et al.*, 1995) and in mammals at least two of the MEF2 isoforms are essential for viability (Lin *et al.*, 1998; Bi *et al.*, 1999; E. Olson, personal communication). We have shown here that despite the conservation in protein sequence and DNA binding activity, CeMEF2 is not essential for *C. elegans* myogenesis and development.

One explanation for the lack of muscle defects associated with loss of CeMEF2 is redundancy of function via another MEF2 isoform or related factor. Although the essentially complete genomic sequence has failed to reveal additional MEF2-like factors, *C. elegans* does have one additional MADS box transcription factor (D1081.2) that is most closely related to serum response factors. SRF is generally considered to be a transcriptional mediator of extracellular signals (e.g., growth factors) and is known to be important in many vertebrate tissues (reviewed in Treisman, 1990, 1994). In addition to regulating *c-fos* in many cell types, SRF has also been demonstrated to be important for cell-type-specific transcription in muscle cells. For example, SRF binding sites have been identified upstream of numerous smooth muscle-specific promoters (Browning *et al.*, 1998), and recently SRF has been shown to be required for coronary smooth muscle cell development (Landerholm *et al.*, 1999). SRF null mice are embryonic lethal with severe gastrulation defects and fail to form mesoderm, demonstrating a general requirement for SRF during development (Arsenian *et al.*, 1998). In *Drosophila*, the only known SRF-like factor is encoded by the *pruned* gene, which is required for terminal outgrowth and branching of the tracheal system (Affolter *et al.*, 1994; Guillemin *et al.*, 1996). Our RNAi results with the SRF-like factor encoded by the D1081.2 locus in *C. elegans* suggest that this factor is required postembryonically for proper locomotion of the animal. The RNAi results do not distinguish between defects associated with nerve versus muscle function so we cannot assume that D1081.2 is required for proper muscle function. We have, however, tested directly for redundancy between D1081.2 and *mef-2* using D1081.2 RNAi in a *mef-2(gv1)* mutant background. Our results provide no evidence of redundant function for these two factors.

C. elegans appears to have evolved a developmental program of myogenesis that is independent of MEF2 functions. In *Drosophila*, all myogenesis is dependent on MEF2, and mutant studies in the mouse suggest an essential role for MEF2 in at least some aspects of muscle development. Apparently *C. elegans* myogenesis has diverged during evolution. Several other unique aspects of *C. elegans* myogenesis have been described previously. In vertebrates, heterodimers of members of the bHLH transcription factor families MyoD and E are required for muscle cell fate determination and differentiation. The activity of these heterodimeric complexes is negatively regulated by HLH Id factors that are capable of competing for binding to E factors but fail to bind DNA as heterodimeric complexes (reviewed in Weintraub *et al.*, 1991). In contrast, CeMyoD is not required for muscle cell fate identity in *C. elegans* and is not required for expression of most muscle-specific genes (Chen *et al.*, 1992). Moreover, the *C. elegans* E/Daughterless-related factor CeE/DA is not coexpressed with CeMyoD during embryogenesis in differentiating striated muscles and fails to heterodimerize efficiently with CeMyoD *in vitro* (Krause *et al.*, 1997; Zhang *et al.*, 1999). No Id-like gene has been identified among the 24 known HLH genes present in the *C. elegans* genomic sequence. Although CeMEF2, CeMyoD, and CeE/DA all display a high level of evolutionary conservation across the core functional domains, the precise roles in nematode muscle development are apparently divergent from those in other systems. These divergent roles presumably reflect functions and protein–protein interactions that are mediated by the substantial regions of these proteins that are not conserved.

Why are the MADS and MEF domains of CeMEF2 so highly related to those of other species if the factor itself is not essential in *C. elegans*? Conservation across the MADS and MEF domains reflects DNA binding and dimerization constraints, aspects of CeMEF2 function that our present studies confirm to be conserved. These properties must be evolutionarily selected for by conferring some advantages to *C. elegans*, although these advantages are not evident under laboratory culture conditions. Like SRF, MEF2 factors function as part of a complex of factors present at the promoters of target genes. MEF2 has been reported to interact directly with both tissue-restricted bHLH factors (Molkentin *et al.*, 1995; reviewed in Yun and Wold, 1996) and more general transcription factors such as p300 (Sar-

torelli *et al.*, 1997) and SP1 (Grayson *et al.*, 1998). Perhaps a major function of MADS box factors is to stabilize or maintain activated transcription complexes to ensure full-level expression of target genes in response to extracellular cues. During evolution, one can imagine that a common ancestral MADS box factor duplicated, allowing MEF2 and SRF-related factors to evolve more specialized functions. During this specialization, *C. elegans* myogenesis may have become more dependent on the SRF-related factor, whereas *Drosophila* myogenesis became dependent on the MEF2-related factor. In vertebrates, both MEF2 and SRF are important myogenic factors, reflecting perhaps a middle evolutionary road relative to *Drosophila* and *C. elegans*.

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