

Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis

Diane G. Edmondson¹, Gary E. Lyons², James F. Martin¹ and Eric N. Olson^{1,*}

¹Department of Biochemistry and Molecular Biology, Box 117, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030, USA

²Department of Anatomy, The University of Wisconsin Medical School, Madison, WI 53706, USA

*Author for correspondence

SUMMARY

Members of the MEF2 family of transcription factors bind a conserved A/T-rich sequence in the control regions of many skeletal and cardiac muscle genes. To begin to assess the roles of the different *Mef2* genes in the control of muscle gene expression *in vivo*, we analyzed by *in situ* hybridization the expression patterns of the *Mef2a*, *Mef2c* and *Mef2d* genes during mouse embryogenesis. We first detected MEF2C expression at day 7.5 postcoitum (p.c.) in cells of the cardiac mesoderm that give rise to the primitive heart tube, making MEF2C one of the earliest markers for the cardiac muscle lineage yet described. By day 8.5, MEF2A, MEF2C and MEF2D mRNAs are all detected in the myocardium. By day 9.0, MEF2C is expressed in rostral myotomes, where its expression lags by about a day behind that of *myf5* and several hours behind that of myogenin. MEF2A and MEF2D are expressed at a lower level than MEF2C in the myotome at day 9.5 and are detected in more embryonic tissues than MEF2C. Expression of each of the MEF2 transcripts is observed in

muscle-forming regions within the limbs at day 11.5 p.c. and within muscle fibers throughout the embryo at later developmental stages. The expression of MEF2C in the somites and fetal muscle is distinct from that of MEF2A, MEF2D and the myogenic bHLH regulatory genes, suggesting that it may represent a distinct myogenic cell type. Neural crest cells also express high levels of MEF2 mRNAs between days 8.5 and 10.5 of gestation. After day 12.5 p.c., MEF2 transcripts are detected at high levels in specific regions of the brain and ultimately in a wide range of tissues. The distinct patterns of expression of the different *Mef2* genes during mouse embryogenesis suggest that these genes respond to unique developmental cues and support the notion that their products play roles in the regulation of muscle-specific transcription during establishment of the cardiac and skeletal muscle lineages.

Key words: myogenesis, muscle gene expression, MEF2, mouse

INTRODUCTION

The activation of tissue-specific transcription during embryogenesis is controlled by networks of regulatory factors that modulate activity of their target genes. Activation of skeletal muscle gene expression is controlled by a small family of muscle-specific transcription factors that share homology within a basic-helix-loop-helix (bHLH) motif. Four members of this muscle regulatory gene family, MyoD, myogenin, *myf5* and MRF4, have been identified in vertebrate species; each can activate the program for skeletal muscle differentiation when introduced into a variety of nonmuscle cell types (reviewed in Emerson, 1990; Olson, 1990; Weintraub et al., 1991). During embryogenesis, these myogenic regulators are expressed in myogenic cells within the somites and limb buds, consistent with their roles as regulators of skeletal muscle cell fate (reviewed in Lyons and Buckingham, 1992).

Myogenic bHLH proteins are expressed exclusively in skeletal muscle and have not been detected in the heart, despite the fact that many of the genes that they regulate are expressed

in both skeletal and cardiac muscle (Olson, 1993). It is unclear whether the regulatory programs governing cardiac and skeletal muscle transcription are entirely divergent or whether these two striated muscle cell types may express common myogenic regulatory factors. Insight into this question will require the identification of transcriptional regulators that are expressed in cardiac muscle precursors during early embryogenesis.

Myogenic bHLH proteins activate muscle transcription by binding to the E-box DNA consensus sequence (CANNTG) in the control regions of muscle-specific genes (Emerson, 1990; Olson, 1990; Weintraub et al., 1991). Although most skeletal muscle genes contain E-boxes in their control regions, several that are activated by the MyoD family do not. This has led to the proposal that myogenic bHLH proteins act through intermediate myogenic regulators to activate some muscle-specific genes. One such regulator is myocyte enhancer factor (MEF)-2, which was originally defined as a muscle-specific DNA-binding activity that recognizes a conserved A/T-rich element associated with numerous muscle-specific genes (Gossett et al.,

1989). The importance of the MEF2 site for muscle-specific transcription has been demonstrated by mutations of this site, which impair transcription of muscle genes, and by the observation that tandem copies of the MEF2 site can direct muscle-specific transcription from basal promoters (Braun et al., 1989; Cheng et al., 1993; Edmondson et al., 1992; Gossett et al., 1989; Nakatsuji et al., 1992; Yu et al., 1992).

MEF2 DNA-binding activity is upregulated during differentiation of established muscle cell lines and can be induced in nonmuscle cells by myogenin and MyoD (Cserjesi et al., 1991; Lassar et al., 1991), consistent with the notion that MEF2 lies in a regulatory pathway 'downstream' of these myogenic factors. However, there is also evidence that MEF2 participates in the regulation of the myogenic bHLH genes. A MEF2 site in promoter of the *myogenin* gene, for example, is essential for *myogenin* transcription in cultured skeletal muscle cells (Edmondson et al., 1992); mutation of this site also alters the expression of reporter genes linked to the *myogenin* promoter in the somites and in the limb buds of transgenic mouse embryos (Cheng et al., 1993; Yee and Rigby, 1993). The TATA box of the *Xenopus MyoDa* gene also binds MEF2 and mediates MEF2-dependent transcription (Leibham et al., 1994). Thus, members of the MEF2 and myogenic bHLH regulatory gene families appear to participate in a complex regulatory network involving direct and indirect positive feedback loops.

MEF2 DNA-binding activity is also present in neonatal cardiac myocytes, indicating that MEF2 expression can be controlled independently of the skeletal muscle bHLH proteins (Zhu et al., 1991; Yu et al., 1992; Navankasattusas et al., 1992; Ianello et al., 1991). The majority of cardiac gene control regions described have MEF2 sites and where its function has been analyzed, this site is essential for cardiac muscle transcription (Zhu et al., 1991; Bassel-Duby et al., 1992; Amacher et al., 1993; Navankasattusas et al., 1992).

The recent cloning of four *Mef2* genes from mice (Martin et al., 1993, 1994), humans (Pollock and Treisman, 1991; Yu et al., 1992; McDermott et al., 1993; Leifer et al., 1993; Breitbart et al., 1993) and frogs (Chambers et al., 1992) revealed that MEF2 proteins belong to the MADS family of transcription factors, named for *MCMI*, which regulates mating type-specific genes in yeast, *Agamous* and *Deficiens*, which have a homeotic function in plants, and *Serum Response Factor* (SRF), which mediates serum-inducible transcription (reviewed in Pollock and Treisman, 1991). The MADS domain is a 56-amino acid motif that mediates DNA binding and dimerization. The four MEF2 gene products are highly homologous within the MADS domain and an adjacent region, known as the MEF2 domain, but they are divergent in their carboxyl termini. Additional complexity of this family of regulators arises from alternative splicing of MEF2 transcripts, yielding isoforms with common DNA-binding domains and unique carboxyl terminal regions. MEF2A, MEF2B, and MEF2D are expressed in a wide range of adult tissues (Pollock and Treisman, 1991; Yu et al., 1992; Breitbart et al., 1993; Martin et al., 1994), while MEF2C expression is restricted to skeletal muscle, brain, and spleen (McDermott et al., 1993; Martin et al., 1993). MEF2C transcripts are upregulated during myogenesis in tissue culture and are induced in 10T1/2 fibroblasts by myogenin, suggesting that the *Mef2c* gene is a target for myogenic bHLH proteins (Martin et al., 1993).

To define further the roles of MEF2 proteins in skeletal and cardiac muscle development, we analyzed the patterns of expression of the *Mef2a*, *Mef2c*, and *Mef2d* genes (the only mouse *Mef2* gene products cloned to date) by in situ hybridization during mouse embryogenesis. *Mef2* gene transcripts were detected at high levels in myogenic cells of the myotome and the embryonic heart prior to day 11.5 p.c. MEF2A and MEF2D were also expressed at low levels in a wide range of tissues. The expression of MEF2 in the early heart and somites suggests that members of this regulatory gene family play important roles in activation of muscle-specific transcription in both cardiac and skeletal muscle and raises the possibility that MEF2 could account for the overlapping pattern of gene expression in these two muscle cell types.

MATERIALS AND METHODS

Preparation and prehybridization of tissue sections

The protocol used to fix and embed mouse embryos is described in detail in Lyons et al. (1990). Sections of at least three different embryos were examined at each stage of development. Briefly, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated and infiltrated with paraffin; 5-7 µm thick serial sections were mounted on gelatinized slides; 1-3 sections were mounted per slide, deparaffinized in xylene and rehydrated. The sections were digested with proteinase K, post-fixed, treated with triethanolamine/acetic anhydride, washed and dehydrated.

Probe preparation

To distinguish between transcripts within the *Mef2* multigene family, we used probes derived from the 3' coding and noncoding regions of the mRNAs that are not conserved between the different genes. Under the hybridization conditions used, the individual MEF2 probes did not cross-hybridize with other MEF2 transcripts. The MEF2A probe was transcribed from a mouse MEF2A cDNA that contains codons 120-507 (J. Martin and E. Olson, unpublished data), cloned into the vector pAMP1 (Bethesda Research Laboratories). This mouse cDNA corresponds to nucleotides 774-1935 of the human sequence (Yu et al., 1992). Two probes, which gave identical in situ hybridization patterns, were used for MEF2C. One probe encompassed the first 91 codons of the MEF2C open reading frame plus 139 nucleotides of 5' untranslated region (Martin et al., 1993). The other encompassed nucleotides 673-1046. Both cDNAs were cloned into pBluescript SKII (Stratagene). The MEF2D probe encompassed nucleotides 641-1355 (codons 136-374) of mouse MEF2D (Martin et al., 1994), cloned into pBluescript SKII. Under the experimental conditions used, no cross-hybridization of probes was detected.

The myf-5 cDNA (kindly provided by Hans Arnold and Eva Bober) in pBluescript is 311 bp in length and corresponds to nucleotides 15 to 326 relative to the putative transcriptional start site of the mouse gene (Ott et al., 1991). The MyoD cDNA was kindly provided by Harold Weintraub and Andrew Lassar. For this probe, corresponding to nucleotides 751 to 1785 of the MyoD mRNA (Davis et al., 1987), the Henikoff-modified Bluescript plasmid was linearized with *MluI*, and T3 RNA polymerase was used. The myogenin probe represents the full-length cDNA (Edmondson and Olson, 1989), cloned into pBluescript. The α-cardiac actin probe was described previously (Sassoon et al., 1988; Lyons et al., 1990).

The cRNA transcripts were synthesized according to manufacturer's conditions (Stratagene) and labeled with [³⁵S]UTP (>1000 Ci/mmol; Amersham). cRNA transcripts larger than 100 nucleotides were subjected to alkali hydrolysis to give a mean size of 70 bases for efficient hybridization. For whole-mount in situ hybridization a standard RNA synthesis was carried out using a nucleotide mix con-

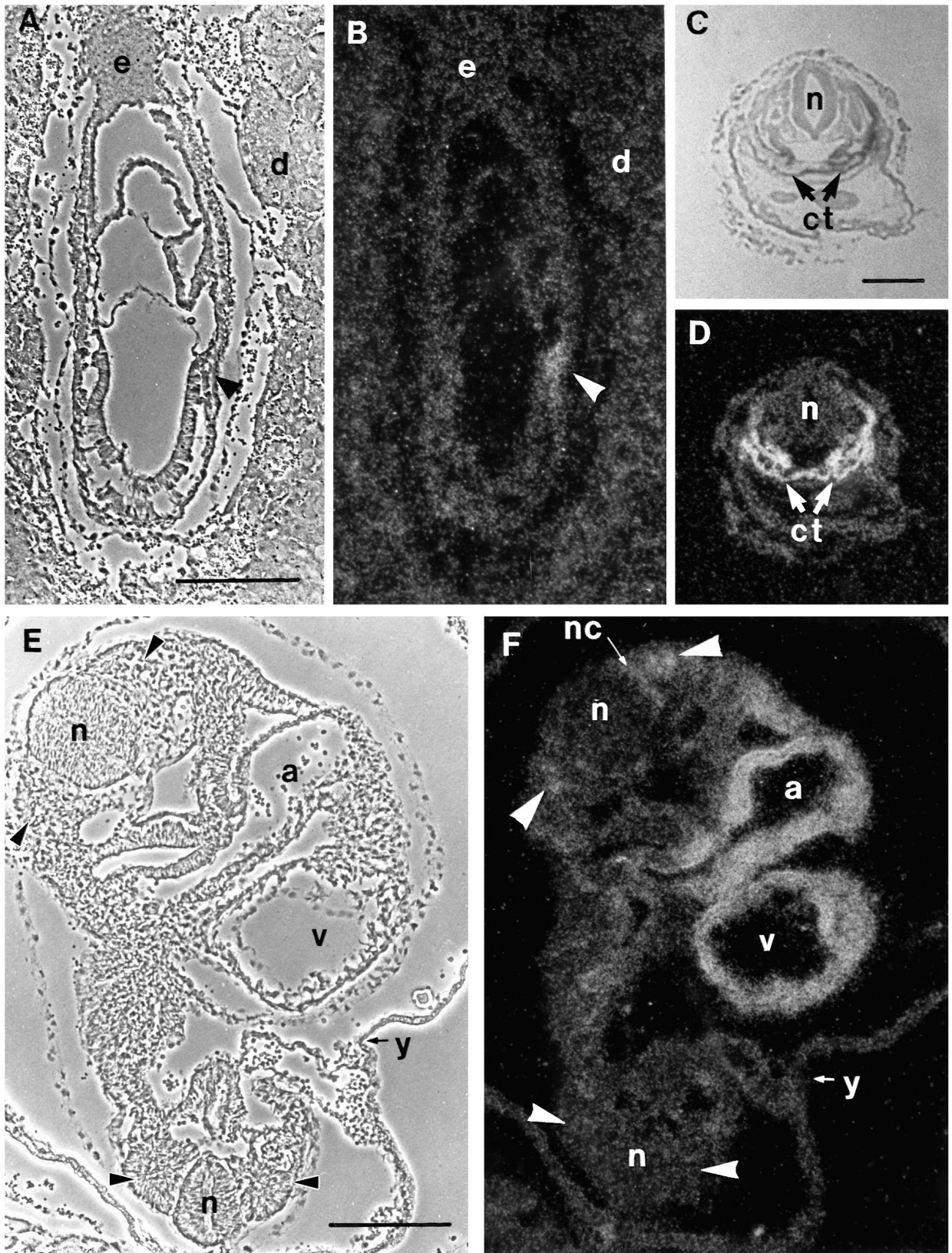


Fig. 1. Detection of MEF2C transcripts in the developing heart. Arrowheads in E and F indicate the location of somites. ct, cardiac tube; a, atrium; d, decidua; e, ectoplacental cone; n, neural tube; nc, neural crest; v, ventricle; y, yolk sack. Bar, 200 μ m. (A) Phase-contrast micrograph corresponding to B. (B) Parasagittal section through a day 7.5 p.c. mouse embryo. Arrowheads indicate the location of the cardiac anlage. (C) Phase-contrast micrograph corresponding to D. (D) Transverse section through a day 8.0 mouse embryo. (E) Phase-contrast corresponding to F. (F) Transverse section of an day 8.5 mouse embryo.

taining digoxigenin-substituted UTP (Boehringer). Hydrolyzed digoxigenin probes were quantitated by filter detection and the final concentration of probe was adjusted to 0.5 µg/ml.

Hybridization and washing procedures

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, 50 µg/ml total yeast RNA and 50-75,000 cts/minute/µl ³⁵S-labeled cRNA probe. The tissue was subjected to stringent washing at 65°C in 50% formamide, 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 10 mM DTT, and washed in PBS before treatment with 20 µg/ml RNase A at 37°C for 30 minutes. Following washes in 2× SSC and 0.1× SSC for 15 minutes at 37°C, the slides were dehydrated and dipped in Kodak NTB-2 nuclear track emulsion and exposed for one week in light-tight boxes with desiccant at 4°C. Photographic development was carried out in Kodak D-19. Slides were analyzed using both light- and dark-field optics of a Zeiss Axiophot microscope.

Whole-mount in situ hybridization

In situ hybridization of embryos was performed similarly to previous methods for mouse (Conlon and Rossant, 1992) and for *Xenopus* (Hemmati-Brivanolu et al., 1990). After fixation in 4% paraformaldehyde in PBS for 2 hours to overnight, embryos were immersed in methanol and stored at -20°C until they were ready to be used. Embryos were warmed to room temperature through a graded series of methanol:PBS + 0.1% Tween-20 (PBT), and then treated with 50 µg/ml proteinase K for 5-15 minutes at room temperature. After two 5-minute washes in PBT with 2 mg/ml glycine, embryos were refixed in 0.2% glutaraldehyde, 4% paraformaldehyde in PBS for 30 minutes. Embryos were then washed with three changes of PBT and treated with 0.1% sodium borohydride in PBT for 20 minutes. Following another 3 washes through PBT over 15 minutes, embryos were washed with hybridization buffer containing 50% formamide, 5× SSC, 1 mg/ml tRNA, 100 µg/ml heparin, 1× Denhardt's, 0.1% Tween-20, 0.1% CHAPS (Sigma) and 5 mM EDTA. Once the embryos had settled to the bottom of the hybridization vial, the buffer was replaced with fresh hybridization buffer and the embryos were prehybridized overnight at 55°C. In the morning the embryos were transferred to Eppendorf tubes containing hybridization buffer with digoxigenin-labeled probe and hybridized overnight at 50-60°C; the exact temperature was chosen depending upon the A-T content of the probe.

Following hybridization, the embryos were washed in glass scintillation vials with fresh hybridization buffer, 50-60°C, 10 minutes; 50% hybridization buffer: 50% 2× SSC, 0.3% CHAPS, 50-60°C, 10 minutes; 25% hybridization buffer:75% 2× SSC, 0.3% CHAPS, 50-60°C, 10 minutes; 2× SSC, 0.3% CHAPS, 50-60°C, two times 10 minutes. Embryos were then treated with 20 µg/ml RNase A and 10 units/ml RNase T1 in 2× SSC, 0.3% CHAPS for 30 minutes at 37°C. After one more 10-minute wash at room temperature, a high stringency wash was done (0.2× SSC, 0.3% CHAPS at 60°C for 30 minutes, two times). Embryos were equilibrated for at least 15 minutes in 500 mM NaCl, 10 mM Pipes, pH 6.8, 1 mM EDTA, 0.1% Tween-20, and then placed in a heat block at 70°C for 20 minutes. After cooling on ice, embryos were blocked by incubation for 1 hour at room temperature in BBT (PBT with 2 mg/ml BSA) containing 2 mM levamisole and 20% freshly heat-inactivated goat serum and then rocked overnight at 4°C in a 1/5000 dilution of anti-digoxigenin antibody (Boehringer) in blocking buffer. The antibody had been pre-adsorbed with mouse embryo extract. The following day, embryos were washed with six changes of BBT (Tween-20 concentration increased to 1%) with 2 mM levamisole over a period of 24 hours. For chromogenic reactions, three 20-minute washes with alkaline phosphatase buffer (100 mM Tris, pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween-20, 5 mM levamisole) were performed, and then the embryos were stained for 1-3 hours in alkaline phosphatase buffer

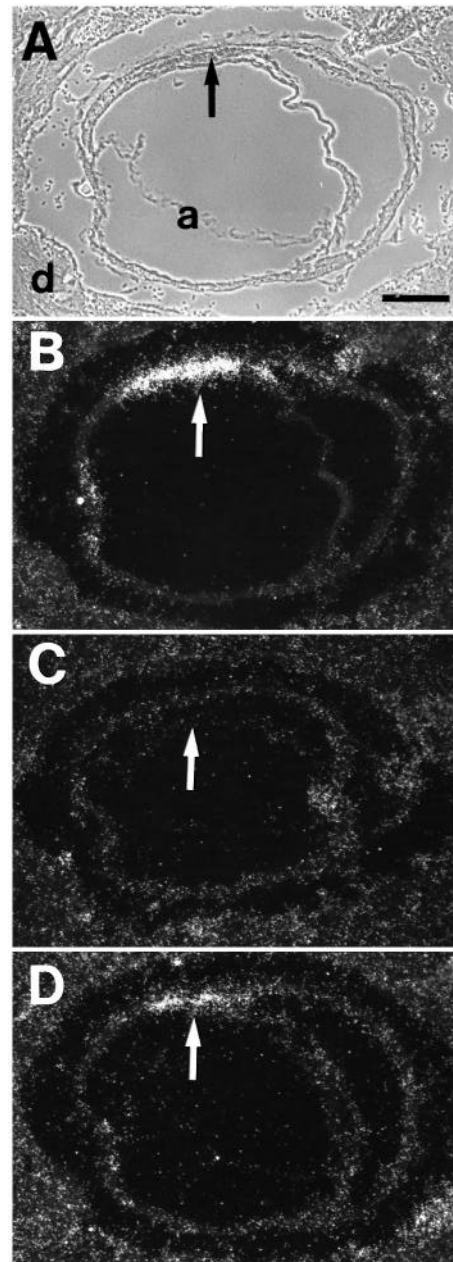


Fig. 2. MEF2C is expressed in cardiac mesoderm prior to cardiac α -actin. Transverse serial sections of a day 7.5 p.c. embryo. Arrows show the location of the cardiogenic plate. a, amnion; d, decidua. Bar, 100 µm. (A) Phase-contrast micrograph; (B) MEF2C probe; (C) cardiac α -actin probe; (D) MEF2C probe.

containing 337 µg/ml NBT and 175 µg/ml BCIP (Boehringer). The reaction was stopped and the embryos stored in 4% paraformaldehyde in PBS.

RESULTS

MEF2C expression is an early marker for the cardiac muscle lineage

To begin to assess the roles of *Mef2* genes in the regulation of muscle gene expression during mouse embryogenesis, we

analyzed the patterns of expression of transcripts for MEF2A, MEF2C and MEF2D by in situ hybridization of 5 μ m sections of postimplantation mouse embryos beginning at day 7.5 p.c. (early headfold stage). MEF2C transcripts were detected at day 7.5 p.c. in a localized region of thickened mesothelial cells in the anterior region of the embryo, representing the cardiac mesoderm (Figs 1B, 2B,D). Determination of cardiac muscle precursors has occurred by this stage, but the primitive heart tube has not yet formed (Rugh, 1990). At day 7.0 p.c., there was no detectable expression of MEF2 (data not shown). To determine whether the localized expression of MEF2C in myocardiogenic progenitor cells preceded the expression of genes encoding cardiac sarcomeric proteins (Sassoon et al., 1988; Lyons et al., 1990), we hybridized serial sections with probes for MEF2C (Fig. 2B,D) and a cardiac actin (Fig. 2C). MEF2C was clearly expressed at high levels prior to the appearance of α -cardiac actin transcripts. Elsewhere in the embryo, MEF2C mRNA was not detected above background levels at this stage. MEF2C transcripts were detected at high levels by day 8.0 p.c. in the primitive cardiac tube and in the sinus venosus (Figs 1D, 3A) and by day 8.5 p.c. in the newly formed common atrium and ventricle (Fig. 1F). Expression of MEF2C in the heart continued through days 9.5 (Figs 3B, 4B), 10.5 (Figs 5B, 6B,E) and 11.5 p.c. (data not shown), after which time it began to decline, ultimately reaching background levels.

Transcripts for MEF2A and MEF2D were first detected at high levels in the heart at day 8.5 p.c. (data not shown), one day after MEF2C transcripts were first observed in the cardiac primordium. Expression of MEF2A and MEF2D was maintained in the heart through day 11.5 p.c. (Figs 4C, 5C,D and data not shown).

MEF2 gene expression marks myogenic cells in the somite

Skeletal muscle is derived from the somites, which mature in a rostrocaudal progression beginning at day 8.0 p.c. of mouse development (reviewed in Lyons and Buckingham, 1992). *Myf5* transcripts are initially detected at day 8.0 p.c. in the dermamyotome of the rostral somites, making *myf5* the first bHLH myogenic regulator to be expressed (Ott et al., 1991). At the time *myf5* is first expressed, the somites appear as a sphere of epithelial-like cells and have not yet become compartmentalized into the myotome, sclerotome and dermatome. Myogenin expression begins in the rostral myotomes at day 8.5 p.c. (Wright et al., 1989; Sassoon et al., 1989), whereas MRF4 and *MyoD* are not expressed in the myotome until day

9.0 and 10.5 p.c., respectively (Bober et al., 1991; Hinterberger et al., 1991; Sassoon et al., 1989).

To define further the regulatory relationship between the myogenic bHLH and MEF2 families, we compared the expression patterns of these genes during somite maturation. As in the cardiac lineage, MEF2C was the first member of the MEF2 family to be expressed in the skeletal muscle lineage. MEF2C transcripts first became detectable in the myotomes of the rostral-most somites at days 8.5-9.0 p.c. By day 9.5 p.c., they were present at high levels in the rostral somites (Figs 3B, 4B). The temporal pattern of MEF2C expression in the somites relative to that of *myf5* can be seen by comparing these transcripts in rostral and caudal somites of a day 9.5 p.c. embryo. Whereas *myf5* transcripts were present in the compartmentalized rostral somites, as well as in the less mature caudal somites, MEF2C expression was restricted to the more rostral

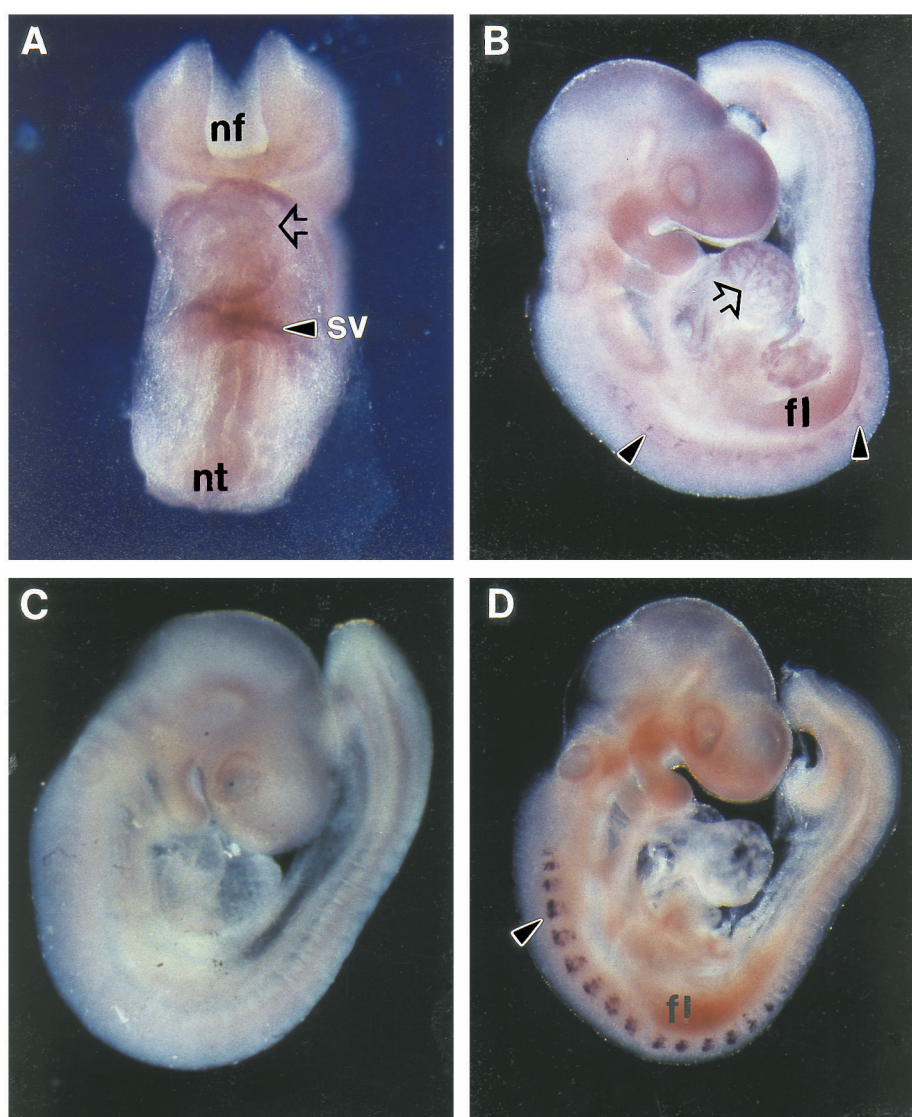


Fig. 3. Whole-mount in situ hybridization of day 8.0 and 9.5 p.c. embryos showing MEF2C and myogenin expression. Arrowheads point to somites and arrows to the heart. nf, neural folds; nt, neural tube; fl, forelimb bud; sv, sinus venosus. (A) Day 8.0 p.c. embryo hybridized with the MEF2C antisense probe; (B) day 9.5 p.c. embryo hybridized with the MEF2C antisense probe; (C) day 9.5 p.c. embryo hybridized with a MEF2C sense probe; (D) day 9.5 p.c. embryo hybridized with a myogenin antisense probe.

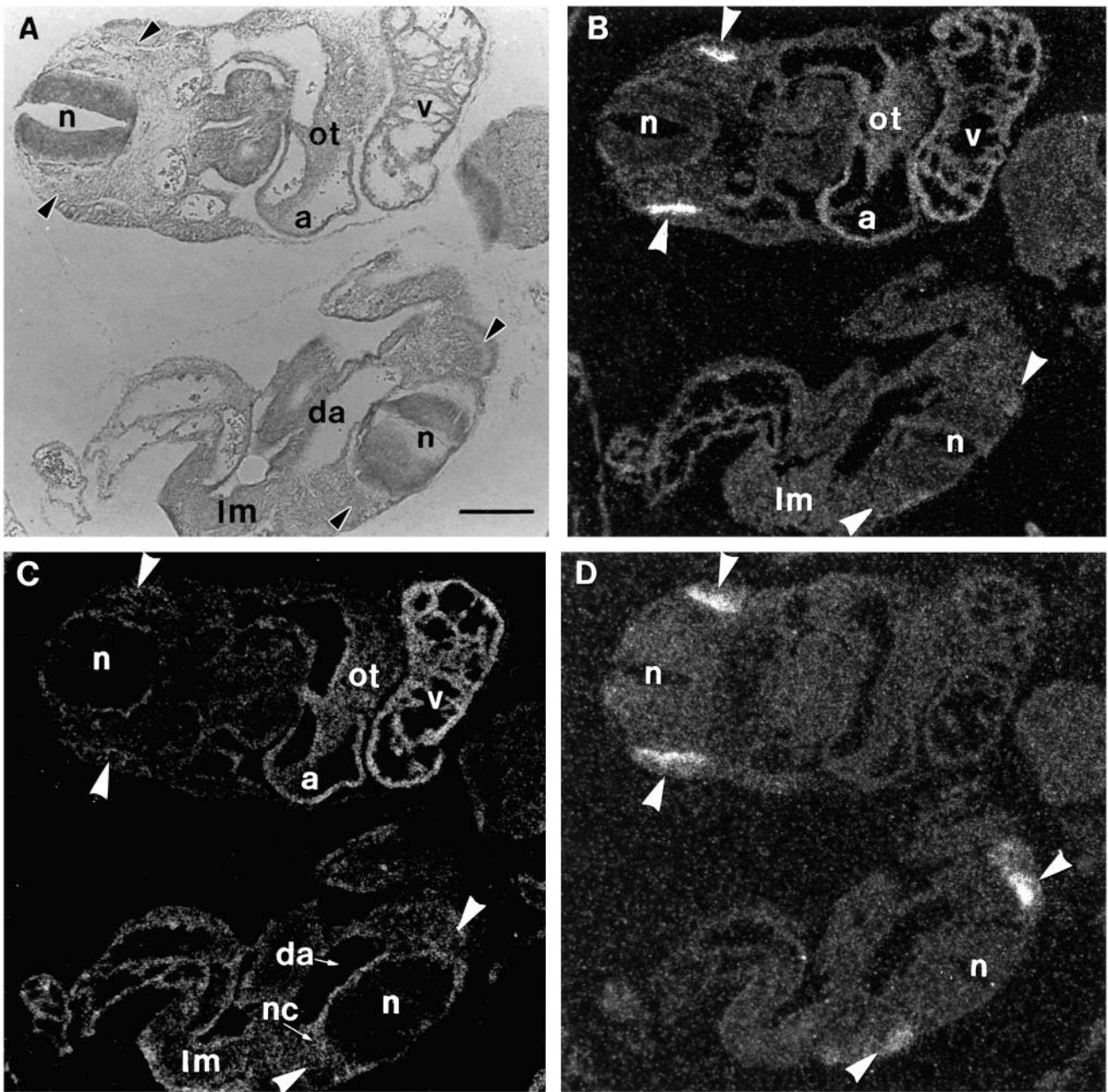


Fig. 4. Detection of MEF2 and myf5 transcripts in serial transverse sections of a day 9.5 p.c. mouse embryo. Arrowheads indicate rostral (at upper left) and caudal somites (at lower right). a, atrium; da, dorsal aorta; lm, lateral mesoderm; n, neural tube; nc, neural crest cells; ot, outflow tract of developing heart; v, ventricle. Bar, 100 μ m. (A) Phase-contrast micrograph. Serial slides were hybridized with probes for (B) MEF2C; (C) MEF2A; (D) myf5.

somites at this stage (Fig. 4, compare B and D). We estimate that the appearance of MEF2C in the somites lags by about 18 hours that of myf5.

MEF2C expression in the somites progressed caudally between days 9.0 and 11.5 p.c., in parallel with somite maturation. Within the somite, MEF2C expression was initially localized to a group of myocytes in the medial region of the myotome (Fig. 6E) and subsequently appeared throughout the myotome. MEF2A transcripts were detected at day 9.5 p.c. Expression of MEF2A initially lags behind that of MEF2C (Fig. 4; compare B and C), but by day 10.5 p.c. MEF2A was expressed in myotomes more caudal than was MEF2C (data

not shown). MEF2A expression was also detected at low levels throughout the lateral mesoderm and in neural crest cells migrating away from the neural tube beginning at day 9.5 p.c. (Fig. 4C). MEF2D expression was also detectable in myotomes at days 9.5 p.c. (data not shown), 10.5 p.c. (Fig. 5D), and 11.5 p.c. (data not shown). At these and at all stages examined, MEF2D mRNAs appeared in a wide range of cell types throughout the embryo, but their levels were highest in the somites and heart (Fig. 5D).

Given the importance of a MEF2-binding site in the *myogenin* gene promoter for *myogenin* transcription in myogenic cells from somites and limb buds (Cheng et al.,

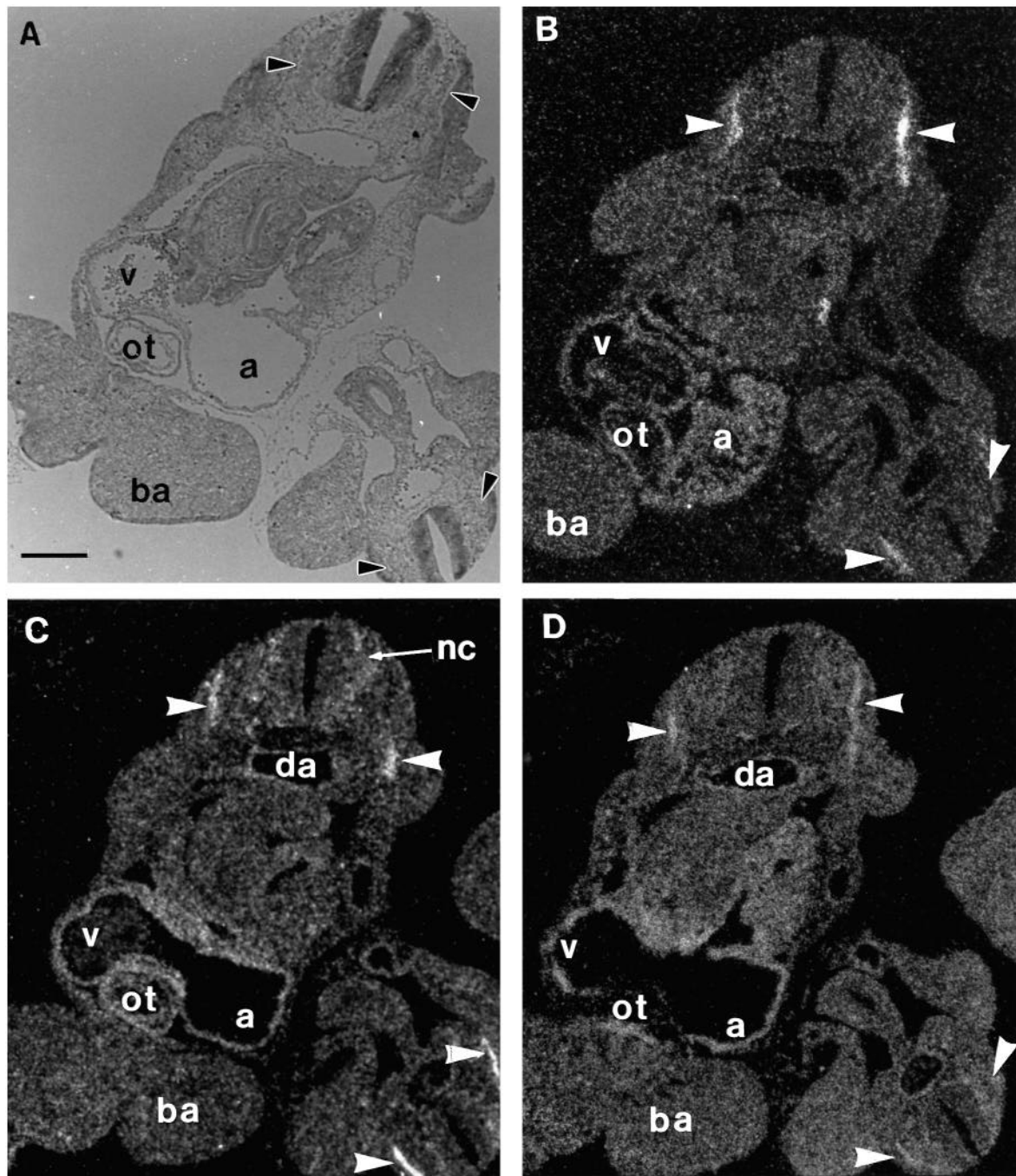


Fig. 5. Detection of MEF2 transcripts in serial transverse sections of a day 10.5 p.c. mouse embryo. Arrowheads indicate rostral (upper) and caudal myotomes (lower). a, atrium; ba, branchial arch; da, dorsal aorta; nc, neural crest; ot, outflow tract of developing heart; v, ventricle. Bar, 50 μ m. (A) Phase-contrast micrograph; (B) MEF2C; (C) MEF2A; (D) MEF2D.

1993; Yee and Rigby, 1993), we compared the temporal pattern of expression of myogenin and MEF2C. At day 9.5 p.c., myogenin and MEF2C transcripts were detected in the myotomes (Fig. 3B,D). Comparison of six different embryos revealed that myogenin was not expressed in the 9 most caudal (youngest) somites while MEF2C was not expressed in the 11 most caudal somites (Fig. 3B,D). This indicates a lag of approximately 6 hours between the activation of myogenin and MEF2C expression. Comparison of myogenin and MEF2C mRNA expression in thin sections of day 10.5 embryos also

confirmed that myogenin expression preceded that of MEF2C (data not shown). By day 11.5 p.c., MEF2C expression began to decline in the rostral somites, whereas myogenin expression was maintained at high levels (Fig. 6F,G).

Within the somitic region, the spatial patterns of myogenin and MEF2C expression were overlapping, but distinct. Myogenin transcripts showed sharp boundaries of expression in the central region of the somites (Figs 3D, 6A,D,F). In contrast, MEF2C transcripts appeared to be distributed homogeneously throughout the more caudal, less mature somites

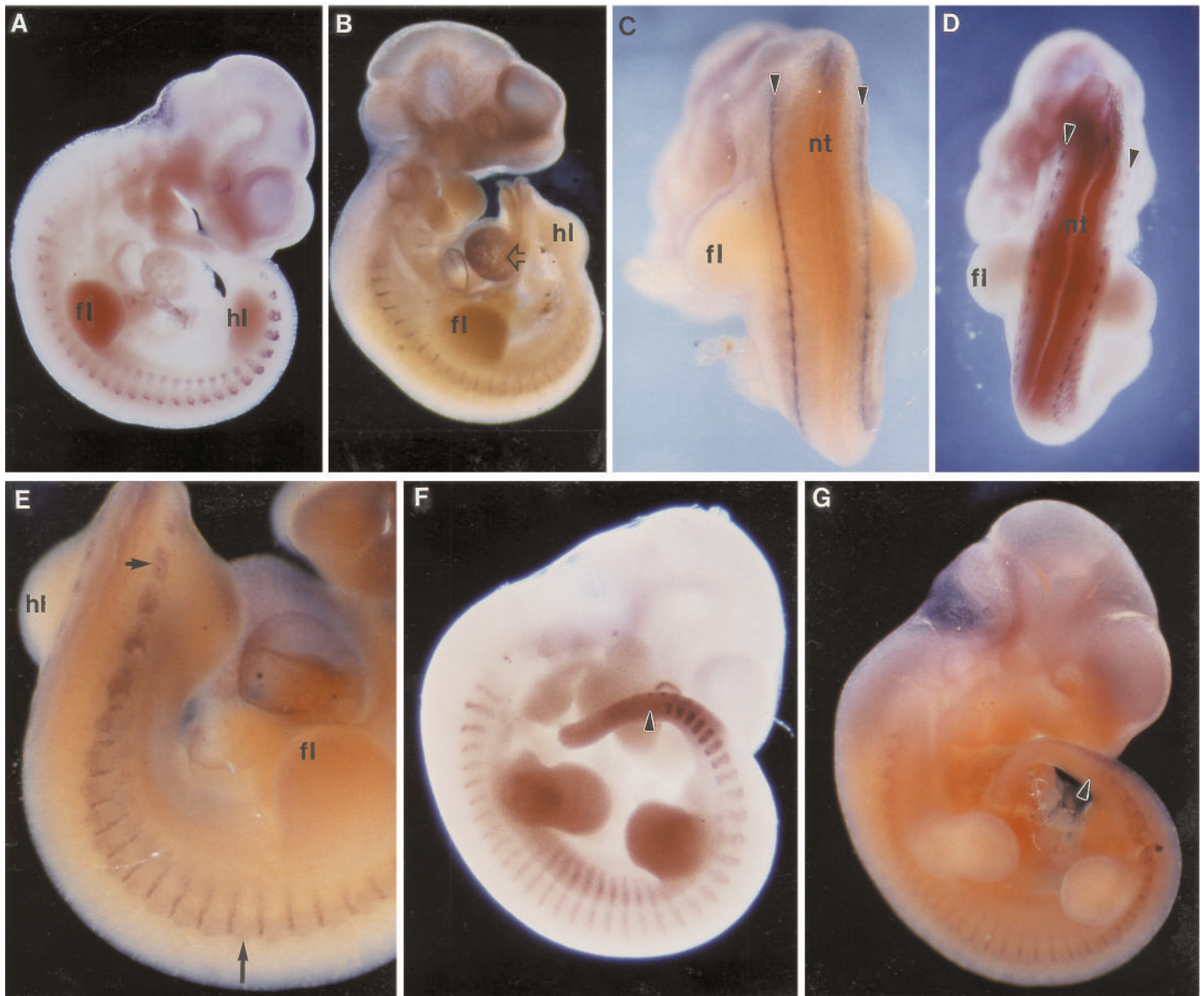


Fig. 6. Detection of MEF2C and myogenin transcripts in day 10.5 and 11.5 p.c. mouse embryos by whole-mount in situ hybridization. fl, forelimb; hl, hindlimb; nt, neural tube. (A) Myogenin, day 10.5 p.c. (B) MEF2C, day 10.5 p.c. The arrow indicates the heart. (C) MEF2C, day 10.5 p.c., dorsal view. Arrowheads in C and D indicate MEF2C and myogenin transcripts in dorsal regions of somites. (D) myogenin, day 10.5 p.c., dorsal view. (E) MEF2C, day 10.5, high magnification. The large and small arrows indicate old and young somites, respectively, which show distinct expression patterns of MEF2C. (F) myogenin, day 11.5 p.c. Arrowheads in F and G indicate the last somite that shows expression of myogenin or MEF2C transcripts. (G) MEF2C, day 11.5 p.c.

(Figs 3B, 6E), whereas in older somites MEF2C transcripts were enriched in the regions between or at the edges of the myotomes (Figs 3B, 6B,C,E,G). MEF2C transcripts also were expressed predominantly in the dorsal regions of the more caudal somites, whereas myogenin transcripts extended more ventrally and did not show the preferential localization between the somites observed with MEF2C. By day 10.5, columns of MEF2C-expressing cells appeared in the ventral regions between the somites (Fig. 6B,E); sectioning of embryos stained by whole-mount hybridization for MEF2C revealed that these cells were within the myotome of the somite. Expression of MEF2C transcripts at the edges of somites was especially evident in tangential sections through

the somites, which showed segmental expression of myogenin but continuous expression of MEF2C (data not shown). By comparison, MEF2A and MEF2D expression was restricted to the myotomal regions of the somites and did not extend into the intersomitic regions. The patterns of expression of MEF2A and MEF2D were similar to myogenin.

Expression of MEF2 transcripts in limb buds and skeletal muscle fibers

In the forelimb buds, *myf5* expression is first observed at about day 10.5 p.c., with myogenin and *MyoD* expression beginning about a half-day later (Ott et al., 1991). Expression of these genes in the hindlimb buds lags about a half-day behind the

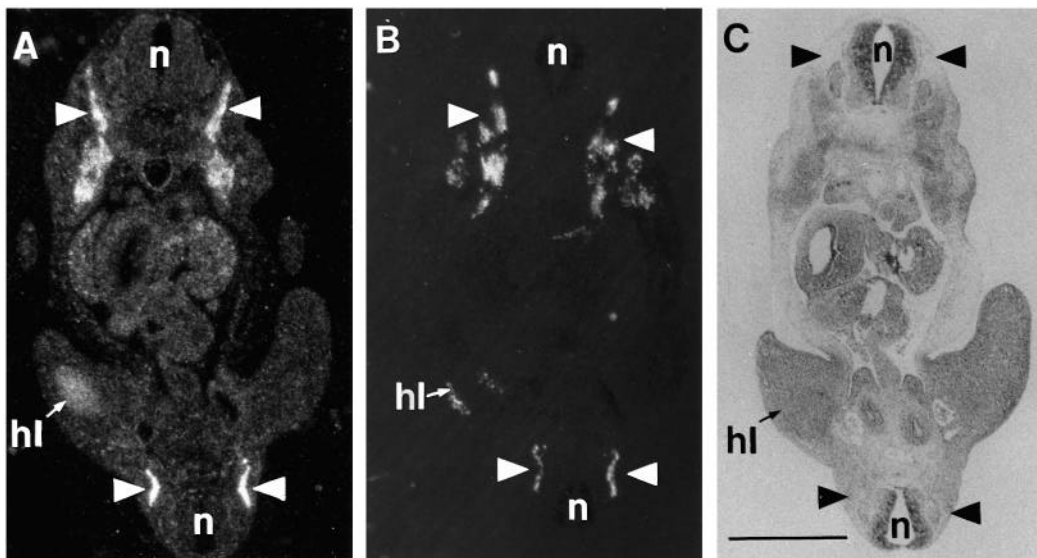


Fig. 7. Detection of myogenin and MEF2 transcripts in limb buds of day 11.5 p.c. mouse embryos. Serial sections through a day 11.5 p.c. embryo were hybridized with the indicated probes. Arrowheads indicate rostral (upper) and caudal (lower) somites. hl, hindlimb; n, neural tube. Bar, 800 μ m. (A) MEF2C; (B) myogenin; (C) phase-contrast micrograph.

forelimb buds, reflecting the rostrocaudal progression of maturation of the somites, from which myogenic cells of the limbs are derived (reviewed in Lyons and Buckingham, 1992).

MEF2C transcripts were detected within the forelimb and hindlimb buds at day 11.5 (Fig. 7A), at about the same time as transcripts for myogenin (Fig. 7B) and MyoD (data not shown). However, the MEF2C transcripts appeared to be localized to different regions of the developing limbs (compare Fig. 7A and B). MEF2C transcripts continued to be expressed in differentiating skeletal muscle fibers throughout the limbs and trunk. MEF2A and MEF2D transcripts were also detected in the premuscle masses of the limb buds, but at a lower level than those of MEF2C (data not shown). MEF2A and MEF2D were also expressed in a wider range of tissues and cells than was MEF2C at this stage.

By day 13.5 p.c., MEF2C transcripts were present at high levels in skeletal muscles throughout the embryo (Fig. 8B). MEF2A was also expressed at the highest levels in skeletal muscle at this stage, but its level of expression was lower than that of MEF2C and there was expression in nonmuscle cell types (Fig. 8C). The expression patterns of MEF2C and myogenin overlapped, but again were distinct within developing skeletal muscle fibers (Fig. 8, compare B and D). MEF2C was expressed at the highest levels near the terminal regions of the muscle fibers, whereas myogenin transcripts were distributed homogeneously throughout the muscle fibers. Whether MEF2C defines a specific muscle nucleus type or state of muscle maturation remains to be determined. By day 16.5 p.c., expression of MEF2A had declined in skeletal muscle, while MEF2C and MEF2D expression was maintained at a high level (data not shown).

MEF2 expression in smooth muscle cells

MEF2C transcripts also appear to be expressed in vascular smooth muscle cells throughout development. MEF2C showed high levels of expression in the dorsal aorta of later stage embryos and coronary vessels in hearts of newborns, suggesting it may participate in smooth muscle cell differentiation. We also noted several areas of MEF2 expression that are consistent with a role in developing blood vessels. Between days 8.5

and 10.5 p.c., each of the three *Mef2* genes was expressed in neural crest cells (Figs 1F, 4B,C, 5C,D) migrating through and around somites. Since MEF2 transcripts are not detected in neural crest derivatives such as dorsal root ganglia or Schwann cells in motor neurons, the population of crest cells that is MEF2-positive may be, in part, destined to form vascular smooth muscle. This conclusion is supported by the observation that MEF2 mRNAs were detected in the dorsal aorta and other vessels in the embryo (Fig. 5B-D) and in the tunica media of the aorta and other vessels at later stages (data not shown). At day 10.5 p.c., MEF2A transcripts were present at higher levels than MEF2C in what appeared to be blood vessels forming around the neural tube (Fig. 5B-D). Cells that appear to represent blood vessels within the midbrain at day 10.5 p.c. also contain transcripts for MEF2C, detected by whole-mount in situ hybridization (Fig. 6B). Sectioning of day 11.5 whole-mounts also revealed columns of stained cells that appear to be developing intercostal arteries (data not shown).

MEF2 gene expression in nonmuscle cell types

While MEF2 expression appears to be confined predominantly to myogenic lineages during early embryogenesis, MEF2 expression becomes more widespread as development proceeds. MEF2 mRNAs were detected in neural crest derivatives in the frontonasal region of the day 13.5 embryo (Fig. 8B,C). MEF2D gene transcripts were also detected, in cartilaginous prevertebrae and in developing ribs at a low level (data not shown). MEF2A mRNAs were detected in the condensing cartilage in limb digits (Fig. 8C). MEF2 transcripts were also detected in the developing gut. At day 11.5 p.c., MEF2C and MEF2A were expressed at low levels and MEF2D at higher levels in cells that will contribute to the smooth muscle of the gut (Fig. 7A and data not shown). Finally, each of the MEF2 genes was expressed in the developing brain in a complex pattern. Some regions of the brain expressed all three genes at different levels whereas other regions expressed only a single member of this gene family (Fig. 8 compare B and C). The developmental pattern of MEF2 gene expression in the brain will be described in detail elsewhere (Lyons and Olson, unpublished data).

DISCUSSION

Our results demonstrate that the *Mef2a*, *Mef2c* and *Mef2d* genes show restricted and sometimes overlapping patterns of expression during early mouse embryogenesis. *Mef2d* was the most widely expressed member of this gene family; *Mef2a* was more restricted and *Mef2c* was the most restricted, largely to the striated muscle lineages. A summary of the expression patterns of these *Mef2* genes compared to other markers of the skeletal and cardiac muscle lineages is presented in Fig. 9. The *Mef2* genes are first expressed in cardiac muscle precursors and subsequently in differentiating skeletal muscle cells, and represent the only transcription factors described to date that mark both of these early striated muscle lineages. In light of the potent transcriptional activity of MEF2 proteins and the presence of MEF2 sites in many skeletal and cardiac muscle genes, it is likely that the early activation of *Mef2* gene expression in the skeletal and cardiac muscle lineages is important for establishment of these specific programs of muscle gene expression.

Mef2 gene expression is an early marker for the cardiac muscle lineage

The cardiac muscle lineage in vertebrates is derived from the anterior lateral plate mesoderm, which gives rise to cardiogenic progenitor cells soon after gastrulation (about day 7.5 p.c. in the mouse) (Rugh, 1990). MEF2C is expressed within the cardiac mesoderm by day 7.5 p.c., which is well before the formation of the primitive heart tube. The early expression of MEF2C in the heart precedes that of cardiac muscle structural genes such as α -cardiac actin and myosin heavy chain (Lyons et al., 1990), raising the possibility that it is involved in establishing the transcriptional program associated with cardiac determination or differentiation or both. In this regard, the β -myosin heavy chain gene contains a MEF2 site in its promoter that is important for

expression in cardiac muscle (Yu et al., 1992). MEF2A and MEF2D were also expressed in the developing heart, but they were not detected until about day 8.5 p.c. and their expression was not as restricted as that of MEF2C.

In *Drosophila*, the formation of cardiac muscle is dependent on the homeobox gene *tinman*, which is expressed in the meso-

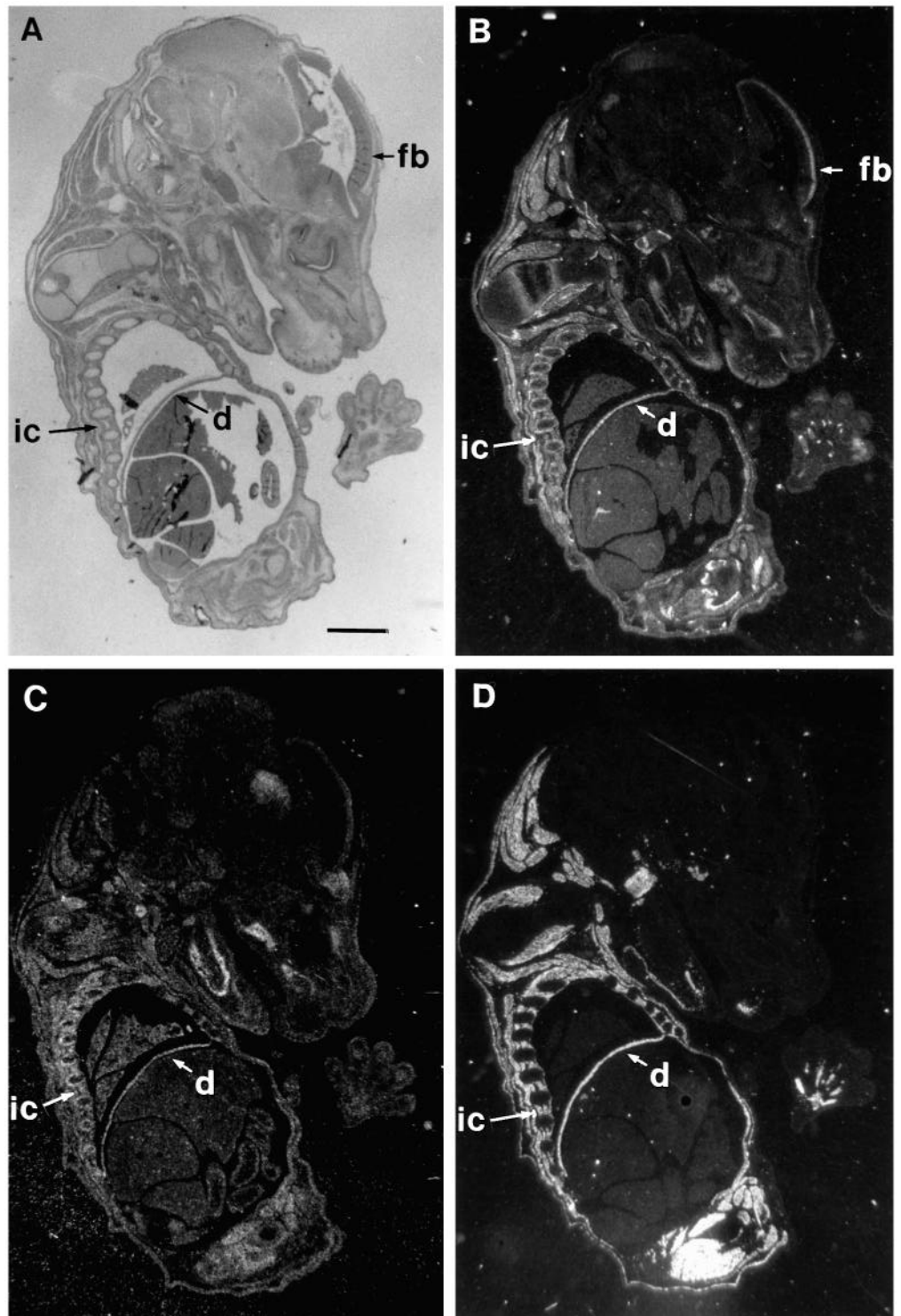


Fig. 8. MEF2 mRNAs are detected in a number of different tissues at 13.5 days p.c. Serial slides were hybridized with the indicated probes. d, diaphragm; fb, forebrain; ic, intercostal muscle. Bar, 1 mm. (A) Phase-contrast micrograph; (B) MEF2C; (C) MEF2A; (D) myogenin.

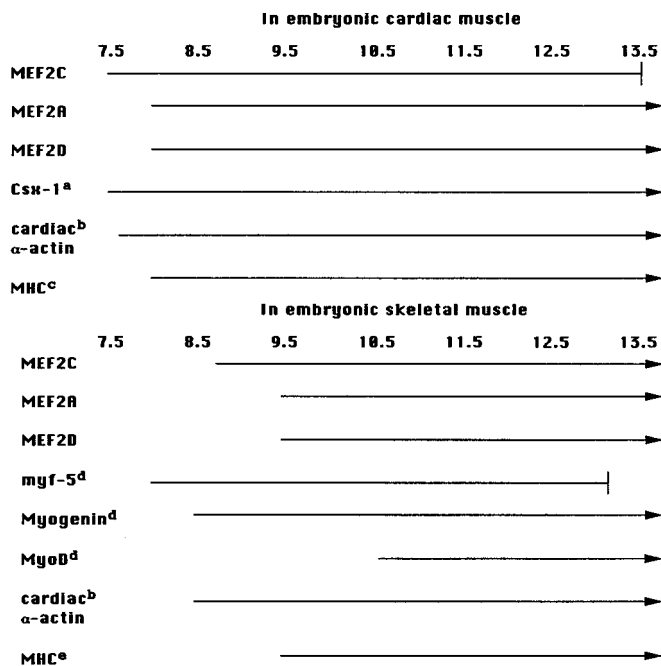


Fig. 9. Temporal patterns of expression of *Mef2* genes relative to other muscle markers in developing skeletal and cardiac muscle. Expression patterns of each gene during mouse embryogenesis are indicated by the lines. Sources for data not shown in this study were: ^aLints et al., 1993; ^bKomuro and Izumo, 1993; ^cSassoon et al., 1988; ^dLyons et al., 1990; ^eOtt et al., 1991; ^fLyons et al., 1990.

dermal progenitors of the heart (Bodmer, 1993; Azpiazu and Frasch, 1993). Recently, a vertebrate homologue of *tinman*, termed *csx/Nkx-2.5*, was cloned and shown to be expressed in early heart progenitor cells and their descendants (Komuro and Izumo, 1993; Lints et al., 1993). The initial appearance of MEF2C transcripts in the cardiac mesoderm appears to coincide with the expression of *csx/Nkx-2.5*. Given the well-documented interactions among MADS and homeodomain proteins (Keleher et al., 1989; Grueneberg et al., 1992; see also Herskowitz, 1989), it will be interesting to determine whether MEF2C and *csx/Nkx-2.5* collaborate to regulate gene expression in the developing heart.

The ability of myogenin and MyoD to induce MEF2 DNA-binding activity (Cserjesi et al., 1991; Lassar et al., 1991) and MEF2C mRNA (Martin et al., 1993) in transfected fibroblasts has led to the suggestion that MEF2 lies within a regulatory pathway downstream of myogenic bHLH proteins. This raises the obvious question of how MEF2 expression becomes activated in the cardiac muscle lineage where the skeletal muscle bHLH proteins are not expressed (Olson, 1993). Perhaps MEF2 expression in the heart is controlled by regulatory pathways independent of cell-specific bHLH proteins. Alternatively, other cell-specific bHLH proteins could regulate MEF2 expression in the cardiac primordium. In this regard, DNA-binding assays with cardiac muscle nuclear extracts have suggested the existence of specific bHLH proteins in the heart (Litvin et al., 1993; Sartorelli et al., 1992). Moreover, using the yeast two-hybrid system, we have recently isolated cDNA clones encoding two novel bHLH proteins that are expressed in the developing heart (Cserjesi, Lyons and Olson, unpublished data).

Expression of MEF2A, MEF2C and MEF2D lags behind that of *myf5* in the somites and limb buds

In contrast to the cardiac muscle lineage, in which *Mef2* gene expression precedes expression of muscle structural genes, in the skeletal muscle lineage *Mef2* gene expression appears to be activated during differentiation. MEF2C transcripts began to appear in myocytes within the somite myotome between days 8.5 and 9 p.c. and MEF2A and MEF2D were expressed soon thereafter. MEF2C expression in the myotome begins about one day later than the initial appearance of *myf5* transcripts and several hours later than the appearance of myogenin transcripts. MRF4 is transiently expressed in the somite myotome between embryonic days 9.0 and 13.5 (Hinterberger et al., 1991; Bober et al., 1991). Recently, it has been reported that there is a 2-day lag between the appearance of myogenin transcripts and myogenin protein in the somite myotome (Cusella-DeAngelis et al., 1992). Thus, if MEF2C expression is regulated by a myogenic bHLH protein in the somites, *myf5* would be the most likely regulator. Since MyoD is not expressed in the somites until after transcripts for MEF2C, it is unlikely to participate in activation of MEF2 expression.

The expression of MEF2C in the somites is distinct from that of MEF2A, MEF2D and the myogenic bHLH genes. While the latter are expressed centrally in the somite, MEF2C is expressed at the highest levels on the peripheral edges of the somites. This suggests that cells expressing MEF2C may represent a distinct myogenic cell type.

The lag between expression of mRNAs encoding myogenic bHLH proteins and MEF2 gene products during mouse embryogenesis is similar to the expression patterns of these gene products in *Xenopus* in which MEF2D(SL1) and MEF2A(SL2) are expressed in the somitic mesoderm of early embryos and in myotomes of tadpoles, slightly later than the expression of MyoD (Chambers et al., 1992). The restriction of *Mef2* gene expression to the early cardiac and skeletal muscle lineages is also observed in *Drosophila*, which contains only a single *Mef2* gene (Lilly et al., 1994; H. Nguyen and B. Nadal-Ginard, personal communication).

Within the limb buds, MEF2C gene transcripts were first detected between days 11 and 11.5 p.c., at about the same time as myogenin and MyoD mRNAs. *Myf5* is expressed in the limb buds beginning at days 10.5-11.0 p.c. and would therefore also be the most likely myogenic bHLH protein to regulate MEF2C expression in these myogenic cells (Ott et al., 1991). MRF4 mRNAs are not detected by *in situ* hybridization in the premerger masses of limb buds (Bober et al., 1991; Hinterberger et al., 1991) at 11.5 days, but these transcripts have been detected by PCR (Hannon et al., 1992) so we cannot rule out that this myogenic regulatory factor is also involved in the upregulation of MEF2 gene expression.

Mutations within the *myogenin* promoter have led to the conclusion that MEF2 is required for appropriate regulation of *myogenin* gene expression in the somites and limb buds (Cheng et al., 1993; Yee and Rigby, 1993). Based on its temporal pattern of expression, it seems unlikely that MEF2C is required for the initial activation of *myogenin* expression in the somite myotomes and limb buds. We therefore favor the possibility that MEF2C, which is expressed only slightly later than myogenin in the somites and simultaneously with myogenin expression in the limb buds, may be involved in amplifying *myogenin* transcription through an indirect positive feedback loop.

Recently, Breitbart et al. (1993) reported that MEF2D protein expression immediately precedes myogenin expression during differentiation of C2 muscle cells. This led to the suggestion that MEF2D might initially activate *myogenin* transcription. The expression pattern of MEF2D transcripts, which follow those of myogenin, argue against a role for MEF2D in activation of *myogenin* transcription in the embryo, but it remains possible that it participates in *myogenin* gene activation in culture.

The control regions for the *MRF4* and *MyoD* genes have not yet been defined in detail, but the finding that these genes are expressed concurrently or after MEF2C, respectively, in the myotome raises the possibility that they are regulated by a MEF2 protein. In this regard, the promoter of the mouse *MRF4* gene contains a MEF2 site that can mediate trans-activation by MEF2 in tissue culture (J. Martin and E. Olson, unpublished data).

The expression patterns of the *Mef2* genes suggest that MEF2 proteins, especially MEF2C, participate in early transcriptional events associated with establishment of myogenic lineages and that, later in development, these proteins acquire broader and somewhat divergent regulatory roles in multiple cell types. It should be pointed out that there is evidence for post-transcriptional regulation of MEF2 expression such that the pattern of expression of MEF2 mRNAs does not correspond to the pattern of expression of the proteins (Yu et al., 1992; Breitbart et al., 1993). Thus, we cannot yet be certain that the pattern of expression of MEF2 mRNAs precisely reflects the pattern of expression of MEF2 proteins in the embryo.

MEF2 as a potential regulator of muscle gene expression in the cardiac and skeletal muscle lineages

The expression of many of the same muscle-specific genes in cardiac and skeletal muscle (Lyons et al., 1990) could, in principle, be controlled by regulatory factors unique to each lineage or by factors common to both muscle lineages. The presence of MEF2 sites in the control regions of most skeletal and cardiac muscle genes (Gossett et al., 1989; Yu et al., 1992; Edmondson et al., 1992; Breitbart et al., 1993), and the appearance of MEF2 transcripts in differentiating skeletal and cardiac muscle cells during embryogenesis, suggests that MEF2 may regulate muscle genes in both striated muscle lineages. However, it should be pointed out that many members of the MADS family act in conjunction with other factors, which may be widely expressed or cell type-restricted (Grueneberg et al., 1992; Keleher et al., 1989). Thus, expression of MEF2 alone may not be sufficient for activation of skeletal and cardiac muscle gene expression. The dependence of MEF2 on other factors for activity is illustrated by the regulation of the *MCK* gene, which is controlled by a distal upstream enhancer that contains a MEF2 site, two E-boxes, and a binding site for the mesodermal homeodomain protein MHox (Cserjesi et al., 1992). Although MEF2 is expressed in cardiac and skeletal muscle cells by days 7.5 and 9.0 p.c., respectively, *MCK* gene expression is not activated in these cell types until several days later (Lyons et al., 1991).

Finally, the identification of factors that regulate gene expression in the cardiac muscle lineage has been difficult, due in part to the absence of established cardiac muscle cell lines.

The finding that members of the *Mef2* gene family are expressed in committed cardiac progenitors prior to expression of cardiac muscle structural genes provides an opportunity to dissect molecularly the earliest events of cardiac lineage determination and differentiation. Targeted inactivation of the *Mef2* genes will allow the precise roles of these transcription factors in regulating cardiac as well as skeletal muscle gene expression to be tested.

We are especially grateful to P. Cserjesi for assistance in initial experiments. G. E. L. would like to thank B. Micales for excellent technical assistance. We also thank K. Tucker for editorial assistance. Supported by grants from NIH, the Muscular Dystrophy Association and The Robert A. Welch Foundation to E. N. O.; G. E. L. was supported by grants from the Muscular Dystrophy Association and the American Heart Association of Wisconsin.

REFERENCES

- Amacher, S. L., Buskin, J. N. and Hauschka, S. D. (1993). Multiple regulatory elements contribute differentially to muscle creatine kinase enhancer activity in skeletal and cardiac muscle. *Mol. Cell. Biol.* **13**, 2753-2764.
- Azpiazu, N. and Frasch, M. (1993). *tinman* and *bagpipe*: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev.* **7**, 1325-1340.
- Bassel-Duby, R., Hernandez, M. D., Gonzalez, M. A., Krueger, J. K. and Williams, R. S. (1992). A 40-kilodalton protein binds specifically to an upstream sequence element essential for muscle-specific transcription of the human myoglobin promoter. *Mol. Cell. Biol.* **12**, 5024-5032.
- Bober, E., Lyons, G., Braun, T., Cossu, G., Buckingham, M. and Arnold, H. (1991). The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. *J. Cell Biol.* **112**, 1255-1265.
- Bodmer, R. (1993). The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* **118**, 719-729.
- Braun, T., Tannich, E., Buschhausen-Denker, G. and Arnold, H. H. (1989). Promoter upstream elements of the chicken cardiac myosin light-chain 2-A gene interact with *trans*-acting regulatory factors for muscle-specific transcription. *Mol. Cell. Biol.* **9**, 2513-2525.
- Breitbart, R., Liang, C., Smoot, L., Laheru, D., Mahdavi, V. and Nadal-Ginard, B. (1993). A fourth human MEF-2 transcription factor, hMEF2D, is an early marker of the myogenic lineage. *Development* **118**, 1095-1106.
- Chambers, A. E., Kotecha, S., Towers, N. and Mohun, T. J. (1992). Muscle-specific expression of SRF-related genes in the early embryo of *Xenopus laevis*. *EMBO J.* **11**, 4981-4991.
- Cheng, T.-C., Wallace, M., Merlie, J. P. and Olson, E. N. (1993). Separable regulatory elements govern *myogenin* transcription in embryonic somites and limb buds. *Science* **261**, 215-218.
- Conlon, R. A. and Rossant, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. *Development* **116**, 357-368.
- Cserjesi, P. and Olson, E. N. (1991). Myogenin induces the muscle-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. *Mol. Cell. Biol.* **11**, 4854-4862.
- Cserjesi, P., Lilly, B., Bryson, L., Wang, Y., Sassoon, D. and Olson, E. N. (1992). MHox: a mesodermally restricted homeodomain protein that binds an essential site in the muscle creatine kinase enhancer. *Development* **115**, 1087-1101.
- Cusella-De Angelis, M., Lyons, G., Sonnino, C., De Angelis, L., Vivarelli, E., Farmer, K., Wright, W., Molinaro, M., Bouchè, M., Buckingham, M. and Cossu, G. (1992). MyoD1, myogenin independent differentiation of primordial myoblasts in mouse somites. *J. Cell Biol.* **116**, 1243-1255.
- Davis, R. L., Weintraub, H. and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987-1000.
- Edmondson, D. G. and Olson, E. N. (1989). A gene with homology to the *myc* similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev.* **3**, 628-640.
- Edmondson, D. G., Cheng, T.-C., Cserjesi, P., Chakraborty, T. and Olson, E. N. (1992) Analysis of the myogenin promoter reveals an indirect pathway

- for positive autoregulation mediated by MEF2. *Mol. Cell. Biol.* **12**, 3665-3677.
- Emerson, C. P.** (1990). Myogenesis and developmental control genes. *Curr. Opin. Cell Biol.* **2**, 1065-1075.
- Gossett, L., Kelvin, D., Sternberg, E. and Olson, E.** (1989). A new myocyte-specific enhancer binding factor that recognizes a conserved element associated with multiple muscle specific genes. *Mol. Cell. Biol.* **9**, 5022-5033.
- Gruneberg, D. A., Natesan, S., Alexandre, C. and Gilman, M. Z.** (1992). Human and Drosophila homeodomain proteins that enhance the DNA-binding activity of the serum response factor. *Science* **257**, 1089-1095.
- Hannon, K., Smith, C., Bales, K. and Santerre, R.** (1992). Temporal and quantitative analysis of myogenic regulatory and growth factor gene expression in the developing mouse embryo. *Dev. Biol.* **151**, 137-144.
- Hemmati-Brivanlou, A., Frank, D., Bolce, M. E., Brown, B. D., Sive, H. L. and Harland, R. M.** (1990). Localization of specific mRNAs in Xenopus embryos by whole-mount in situ hybridization. *Development* **110**, 325-330.
- Herskowitz, I.** (1989). A regulatory hierarchy for cell specialization in yeast. *Nature* **324**, 749-757.
- Hinterberger, T., Sassoon, D., Rhodes, S. and Konieczny, S.** (1991). Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Dev. Biol.* **147**, 144-156.
- Iannello, R. C., Mar, J. H. and Ordahl, C. P.** (1991). Characterization of a promoter element required for transcription in myocardial cells. *J. Biol. Chem.* **266**, 3309-3316.
- Keleher, C. A., Passmore, S. and Johnson, A. D.** (1989). Yeast repressor $\alpha 2$ binds to its operator with yeast protein MCM1. *Mol. Cell. Biol.* **9**, 5228-5230.
- Komuro, I. and Izumo, S.** (1993). *Csx*: A murine homeobox-containing gene specifically expressed in the developing heart. *Proc. Natl. Acad. Sci. USA* **90**, 8145-8149.
- Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H.** (1991). Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* **66**, 305-315.
- Leifer, D., Krainc, D., Yu, Y.-T., McDermott, J., Breitbart, R. E., Heng, J., Neve, R. L., Kosofsky, B., Nadal-Ginard, B. and Lipton, S. A.** (1993). MEF2C, a MADS/MEF2-family transcription factor expressed in a laminar distribution in cerebral cortex. *Proc. Natl. Acad. Sci. USA* **90**, 1546-1550.
- Leibham, D., Wong, M.-W., Cheng, T.-C., Schroeder, S., Weil, P. A., Olson, E. N. and Perry, M.** (1994). Binding of TFIID and MEF2 to the TATA element activates transcription of the *Xenopus MyoDa* promoter. *Mol. Cell. Biol.* **14**, 686-699.
- Lilly, B., Galewsky, S., Firulli, A. B., Schulz, R. A. and Olson, E. N.** (1994). *mef2*: A MADS box transcription factor expressed in differentiating mesoderm and muscle cell lineages during *Drosophila* embryogenesis. *Proc. Natl. Acad. Sci. USA*, in press.
- Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I. and Harvey, R. P.** (1993). *Nkx-2.5*: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* **119**, 419-431.
- Litvin, J., Montgomery, M., Goldhamer, D., Emerson, C. and Bader, D.** (1993). Identification of DNA-binding protein(s) in the developing heart. *Dev. Biol.* **156**, 409-417.
- Lyons, G. E., Schiaffino, S., Barton, P., Sassoon, D. and Buckingham, M.** (1990). Developmental regulation of myosin gene expression in mouse cardiac muscle. *J. Cell Biol.* **111**, 2427-2436.
- Lyons, G. E., Muhlebach, S., Moser, A., Masood, R., Paterson, B., Buckingham, M., Perriard, J. C.** (1991). Developmental regulation of creatine kinase gene expression by myogenic factors in mouse and chick embryos. *Development* **113**, 1017-1029.
- Lyons, G. E. and Buckingham, M. E.** (1992). Developmental regulation of myogenesis in the mouse. *Semin. Dev. Biol.* **3**, 243-253.
- Martin, J. F., Schwarz, J. J. and Olson, E. N.** (1993). Myocyte enhancer factor (MEF) 2C: A tissue-restricted member of the MEF-2 family of transcription factors. *Proc. Natl. Acad. Sci. USA* **90**, 5282-5286.
- Martin, J. F., Miano, J. M., Hustad, C. M., Copeland, N. G., Jenkins, N. A. and Olson, E. N.** (1994). A *Mef2* gene that generates a muscle-specific isoform via alternative mRNA splicing. *Mol. Cell. Biol.* **14**, 1647-1656.
- McDermott, J., Cardoso, M., Yu, Y., Andres, V., Leifer, D., Krainc, D., Lipton, S. and Nadal-Ginard, B.** (1993). hMEF2C gene encodes skeletal muscle- and brain-specific transcription factors. *Mol. Cell. Biol.* **13**, 2564-2577.
- Nakatsuji, Y., Hidaka, K., Tsujino, S., Yamamoto, Y., Mukai, T., Yanagihari, T., Kishimoto, T. and Sakoda, S.** (1992). A single MEF-2 site is a major positive regulatory element required for transcription of the muscle-specific subunit of the human phosphoglycerate mutase gene in skeletal and cardiac muscle cells. *Mol. Cell. Biol.* **12**, 4384-43909.
- Navankasattusas, S., Zhu, H., Garcia, A. V., Evans, S. M. and Chien, K.** (1992). A ubiquitous factor (HF-1) and a distinct muscle factor (HB-1b/MEF2) form an E-box-independent pathway for cardiac muscle gene expression. *Mol. Cell. Biol.* **12**, 1469-1479.
- Olson, E. N.** (1990). MyoD family: a paradigm for development? *Genes Dev.* **4**, 1454-1461.
- Olson, E. N.** (1993). Regulation of muscle transcription by the MyoD family: the heart of the matter. *Circ. Res.* **72**, 1-6.
- Ott, M.-O., Bober, E., Lyons, G., Arnold, H. and Buckingham, M.** (1991). Early expression of the myogenic regulatory gene, *myf-5*, in precursor cells of skeletal muscle in the mouse embryo. *Development* **111**, 1097-1107.
- Pollock, R. and Treisman, R.** (1991). Human SRF-related proteins: DNA-binding properties and potential regulatory targets. *Genes Dev.* **5**, 2327-2341.
- Rugh, R.** (1990) *The Mouse: Its Reproduction and Development*, Oxford University Press, Oxford, UK.
- Sartorelli, V., Hong, N., Bishopric, N., and Kedes, L.** (1992). Myocardial activation of the human cardiac α -actin promoter by helix-loop-helix proteins. *Proc. Natl. Acad. Sci. USA* **89**, 111-115.
- Sassoon, D., Garner, I. and Buckingham, M.** (1988). Transcripts of α -cardiac and α -skeletal actins are early markers for myogenesis in the mouse embryo. *Development* **104**, 155-164.
- Sassoon, D., Lyons, G., Wright, W., Lin, V., Lassar, A., Weintraub, H. and Buckingham, M.** (1989). Expression of two myogenic regulatory factors: myogenin and MyoD1 during mouse embryogenesis. *Nature* **341**, 303-307.
- Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y. and Lassar, A.** (1991). The *myoD* gene family: nodal point during specification of the muscle cell lineage. *Science* **251**, 761-766.
- Wright W., Sassoon D, and Lin V.** (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD1. *Cell* **56**, 607-617.
- Yee, S.-P. and Rigby, W. J.** (1993). The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes Dev.* **7**, 1277-1289.
- Yu, Y., Breitbart, R., Smoot, L., Lee, Y., Mahdavi, V. and Nadal-Ginard, B.** (1992). Human myocyte specific enhancer factor 2 (MEF2) comprises a group of tissue restricted MADS box transcription factors. *Genes Dev.* **6**, 1783-1798.
- Zhu, H., Garcia, A., Ross, R., Evans, S. and Chien, K.** (1991). A conserved 28-base pair element (HF-1) in the rat cardiac myosin light chain 2 gene confers cardiac-specific and α -adrenergic inducible expression in cultured neonatal rat myocardial cells. *Mol. Cell. Biol.* **11**, 2273-2281.