

Transcriptional activity of MEF2 during mouse embryogenesis monitored with a MEF2-dependent transgene

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SUMMARY

The four members of the MEF2 family of MADS-box transcription factors, MEF2-A, MEF2-B, MEF2-C and MEF2-D, are expressed in overlapping patterns in developing muscle and neural cell lineages during embryogenesis. However, during late fetal development and postnatally, MEF2 transcripts are also expressed in a wide range of cell types. Because MEF2 expression is controlled by translational and post-translational mechanisms, it has been unclear whether the presence of MEF2 transcripts in the embryo reflects transcriptionally active MEF2 proteins. To define the temporospatial expression pattern of transcriptionally active MEF2 proteins during mouse embryogenesis, we generated transgenic mice harboring a *lacZ* reporter gene controlled by three tandem copies of the MEF2 site and flanking sequences from the *desmin* enhancer, which is active in cardiac, skeletal and smooth muscle cells. Expression of this MEF2-dependent transgene paralleled expression of MEF2 mRNAs in developing myogenic lineages and regions of the adult brain. However,

it was not expressed in other cell types that express MEF2 transcripts. Tandem copies of the MEF2 site from the *c-jun* promoter directed expression in a similar pattern to the *desmin* MEF2 site, suggesting that transgene expression reflects the presence of transcriptionally active MEF2 proteins, rather than other factors specific for DNA sequences flanking the MEF2 site. These results demonstrate the presence of transcriptionally active MEF2 proteins in the early muscle and neural cell lineages during embryogenesis and argue against the existence of lineage-restricted MEF2 cofactors that discriminate between MEF2 sites with different immediate flanking sequences. The discordance between MEF2 mRNA expression and MEF2 transcriptional activity in nonmuscle cell types of embryos and adults also supports the notion that post-transcriptional mechanisms regulate the expression of MEF2 proteins.

Key words: MEF2, Transcriptional activity, Transgenic mouse

INTRODUCTION

The Myocyte Enhancer Factor-2 (MEF2) family of transcription factors is encoded by four genes, *mef2-a*, *mef2-b*, *mef2-c* and *mef2-d*, each of which gives rise to multiple proteins via alternative mRNA splicing (Pollock and Treisman, 1991; Yu et al., 1992; Chambers et al., 1992; Leifer et al., 1993; McDermott et al., 1993; Breitbart et al., 1993; Martin et al., 1993, 1994). MEF2 proteins, also referred to as RSRFs (Pollock and Treisman, 1991) (for Related to Serum Response Factors), share about 80% amino acid identity within a MADS (MCM1, Agamous, Deficiens, Serum Response Factor) domain that mediates DNA binding and dimerization, and an adjacent region, called the MEF2 domain, that influences DNA-binding affinity and cofactor interactions (reviewed in Black and Olson, 1998). MEF2 proteins bind the DNA sequence CTA(A/T)₄TAG/A as homodimers and heterodimers and are potent transactivators through that sequence (Gossett et al., 1989; Cserjesi and Olson, 1991). Whereas a single MEF2-binding site is transcriptionally inactive, when the

MEF2 site is multimerized or combined with binding sites for heterologous transcription factors, it can direct high levels of transcription (Gossett et al., 1989; Kaushal et al., 1994).

MEF2 was originally defined as a muscle-specific DNA-binding activity that recognized an A/T-rich sequence in the control regions of numerous skeletal and cardiac muscle genes (Gossett et al., 1989). During mouse embryogenesis, MEF2 transcripts are expressed in the cardiac, skeletal and smooth muscle cell lineages, as well as in neural crest cells and specific regions of the brain (Edmondson et al., 1994; Lyons et al., 1995; Subramanian and Nadal-Ginard, 1996; Molkenin et al., 1996; Lin et al., 1998). MEF2B and MEF2C are initially expressed in the cardiac mesoderm at about embryonic day (E) 7.5, followed about a day later by expression of MEF2A and MEF2D. MEF2C transcripts are detected in the somites at E8.5, concomitant with the onset of myocyte differentiation in the myotome. Mice lacking MEF2C die at embryonic day 9.5 due to severe cardiovascular defects (Lin et al., 1997). Whereas many MEF2-dependent genes fail to be upregulated in the hearts of MEF2C null embryos, some cardiac genes known to

be regulated by MEF2 are expressed in the absence of MEF2C. These results suggest that another MEF2 factor, probably MEF2B, can substitute for MEF2C to support expression of these genes.

While it is clear that MEF2 factors play important roles in muscle differentiation, there is also evidence for the involvement of MEF2 in the control of nonmuscle genes. In a majority of these cases, the MEF2 family has been shown to activate gene expression in response to mitogenic signaling. A MEF2 site has been identified, for example, in the *c-jun* promoter, where it mediates transcriptional activation via a MAP kinase signaling cascade (Han et al., 1992; Han and Prywes, 1995; Kato et al., 1997). Similarly, the orphan steroid receptor gene, *Nur77*, and the Epstein-Barr Virus lytic switch gene, *BZLF1*, both contain functional MEF2 sites within their promoters that respond to activated calcium signaling pathways (Woronicz et al., 1995; Liu et al., 1997).

MEF2 mRNAs are abundantly expressed in the developing and adult central nervous system (Leifer et al., 1993; Martin et al., 1993; McDermott et al., 1993; Lyons et al., 1995; Lin et al., 1996) arguing for the existence of downstream neuronal target genes. Recently, a functional MEF2-binding site was identified in the promoter of the NMDA (N-methyl-D-aspartate) receptor subunit 1 (NR1) gene (Kraicn et al., 1998). MEF2C binds this site and synergistically interacts with Sp1 to activate *NR1* expression in cerebrocortical neurons. A putative MEF2 site has also been identified in the promoter of the neurofilament light chain (NF-L) gene (Yaworsky et al., 1997). Moreover, studies have demonstrated that MEF2 proteins activate transcription in collaboration with neurogenic basic helix-loop-helix transcription factors (Black et al., 1996; Mao and Nadal-Ginard, 1996), suggesting an important role for the MEF2 family in neuronal differentiation.

The cell type distribution of MEF2 activity has been controversial. Some studies have reported that MEF2 DNA-binding activity is restricted to muscle cells (Gossett et al., 1989; Cserjesi et al., 1991; Yu et al., 1992; Breitbart et al., 1993), whereas other studies have concluded that MEF2 factors are expressed at comparable levels in muscle and nonmuscle cells (Pollock and Treisman, 1991; Braun et al., 1994; Dodou et al., 1995). This confusion may be due in part to the complexity of MEF2 regulation. For example, it is clear that MEF2 mRNAs are widely expressed and there is evidence for translational regulation of MEF2 expression (Black et al., 1997). In addition, phosphorylation of the DNA-binding and transcription activation domains of MEF2 factors influence DNA binding and transcriptional activity, respectively (Molkentin et al., 1996; Han et al., 1997; Kato et al., 1997; Zhao et al., 1999).

In light of the discordance between MEF2 DNA-binding activity and MEF2 mRNA expression, we sought to determine the pattern of expression of transcriptionally active MEF2 proteins during mouse embryogenesis. Using a *lacZ* reporter gene under the control of tandem copies of the *desmin* MEF2 DNA-binding site in transgenic mice, we show MEF2 activity in the developing heart, skeletal and smooth muscle and specific regions of the brain. The pattern of expression of the MEF2-dependent transgene suggests that MEF2 proteins are transcriptionally active in early myogenic and neuronal cell lineages where they regulate tissue-specific gene expression.

MATERIALS AND METHODS

Construction of *mef2-lacZ* reporters

Complementary oligonucleotides encompassing the MEF2 site from the mouse *desmin* enhancer (Kuisik et al., 1996) and human *c-jun* promoter (Han and Prywes, 1995), as well as a mutant MEF2 site with *HindIII* sites on both ends were synthesized and annealed. The sequences of the top strands (MEF2 site underlined, mutant MEF2 site in bold) were:

desmin: GGCCTTTCCTTCTCCTCTATAAAATACCAGCTCTGG-TATTTC

mutant MEF2: GGCCTTTCCTTCTCCTCTATAGCGACCAGC-TCTGGTATTTC

c-jun: GGGGTGACATCATGGGCTATTTTATAGGATTGACTG-GTAGCA

The oligonucleotides were ligated and cloned into the *HindIII* site of the vector *hsp68-lacZ*, which contains a *lacZ* reporter gene linked to the *heat shock protein (hsp) 68* promoter (Kothary et al., 1989). The number of inserts in the resulting plasmids was determined by DNA sequencing. Transgenic mice were generated using constructs containing three tandem copies of each MEF2 site in a head-to-tail orientation. These constructs were referred to as *des-MEF2-lacZ*, *mut-MEF2-lacZ* and *jun-MEF2-lacZ*.

Creation of transgenic mice

Transgenic mice were created by injection of transgenes into the male pronuclei of fertilized mouse oocytes, as described previously (Cheng et al., 1992, 1993). Briefly, DNA was gel purified and eluted using a QIAEXII kit (QIAGEN). Fertilized eggs from B6C3F1 or FVB female mice were collected for pronuclear injections. *lacZ* expression was assayed in F₀ embryos or in F₁ offspring that were shown by Southern analysis to carry the transgene.

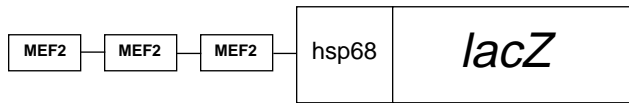
β-galactosidase staining and histology

Embryos were fixed in 2% paraformaldehyde/0.2% glutaraldehyde in PBS on ice for 30 to 90 minutes followed by washing twice in PBS. Embryos were then immersed in X-gal stain (5 mM ferrocyanide, 5 mM ferricyanide, 2 mM MgCl₂, 1 mg/ml X-gal in dimethylformamide) overnight at room temperature. Transgenic embryos were subsequently processed for histology. Brain sections were prepared by perfusing adult mice with 4% paraformaldehyde. Coronal sections were obtained by embedding dissected brains in a 50% chicken egg albumin/2.5% glutaraldehyde protein matrix (Herzog and Brosamle, 1997) and sectioned with a vibratome at 400 μm prior to staining. For detailed localization of β-galactosidase expression, brain slices were dehydrated, embedded in paraffin and sectioned at 5 μm.

RESULTS

Expression of a MEF2-dependent transgene in cardiac muscle precursors

To monitor the expression of functional MEF2 factors during mouse embryogenesis, we created transgenic mouse lines harboring a *lacZ* reporter gene linked to the *hsp 68* gene promoter and three tandem copies of the MEF2 site from the *desmin* enhancer in a head-to-tail orientation (*des-MEF2-lacZ*) (Fig. 1). Of 20 independent transgenic lines that carried the integrated reporter gene, 3 expressed *lacZ*. Similar expression patterns were observed in these lines, except that two of the lines expressed *lacZ* in the neural tube, whereas one did not. Several independent F₀ founder transgenic mice showed the same expression pattern in muscle lineages as these stable lines. Subtle variations in expression among the lines



desmin GGCCTTTCTCTCTCTATAAATACCAGCTCTGGTATTCA

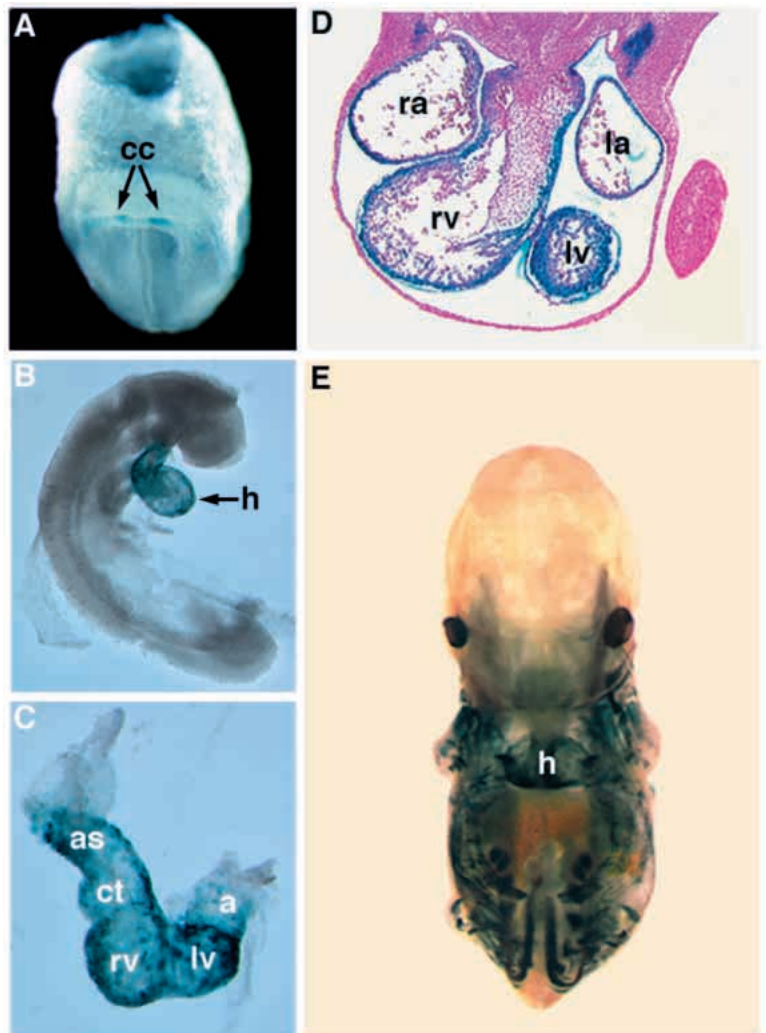
c-jun GGGGTGACATCATGGGCTATTTTTAGGGATTGACTGGTAGCA

Fig. 1. Schematic representation of the multimerized MEF2 site *lacZ* reporter. (A) Three copies of the MEF2 site with flanking sequences were cloned upstream of *hsp68-lacZ*. Multimers are in the same orientation relative to the transcription start site. (B) Sequences of the MEF2 sites and flanking sequences from the *desmin* and *c-jun* genes.

were observed at certain developmental stages (not shown), which probably reflect position effects that depend on the sites of transgene integration. Consistent with previous results, the *hsp-lacZ* transgene lacking MEF2 sites was not expressed at detectable levels in embryos, neonates or adult mice (Kothary et al., 1989; data not shown). Moreover, two independent F₀ founder transgenic mice that carried the *hsp-lacZ* reporter gene harboring a mutant MEF2 site (*mut-MEF2-lacZ*) expressed *lacZ* in a non-specific fashion, demonstrating that *des-MEF2-lacZ* detects MEF2 activity.

MEF2B and MEF2C are the first members of the MEF2 family to be expressed during mouse embryogenesis, with transcripts appearing in cardiac progenitor cells of the anterior lateral plate mesoderm by day 7.5 p.c. (Edmondson et al., 1994; Molkenin et al., 1996). Expression of the *des-MEF2-lacZ* transgene was first detected at day 7.5 p.c. in these cardiogenic precursors (Fig. 2A), suggesting that MEF2B or MEF2C proteins are present in these cells and are responsible for activating expression of the transgene. Expression of the transgene was not homogeneous throughout the cardiac crescent, but rather, localized to clusters of cells near the medial region of the cardiac crescent. By day 8.5 p.c., *des-MEF2-lacZ* was expressed throughout the looping heart tube (Fig. 2B), consistent with the homogeneous expression of the four *mef2* genes. Staining was evident in the aortic sac, conotruncus, the future right and left ventricles, and the developing atria (Fig. 2C). The expression pattern of the transgene continued to be superimposable over that of MEF2 mRNAs at this stage.

Fig. 2. Expression of *des-MEF2-lacZ* in the cardiac mesoderm and heart tube during mouse embryogenesis. (A) At E7.5, *lacZ* expression is evident in a subset of cardiac progenitor cells in the medial region of the cardiac crescent (cc, arrows). (B) By E8.5, transgene expression is detected uniformly throughout the looping cardiac tube (h). (C) Specific expression is observed in the aortic sac (as), conotruncus (ct), future right (rv) and left (lv) ventricles, and the developing atria (a). (D) Transverse section through an E11.5 heart reveals expression in the right (rv) and left (lv) ventricles and the right (ra) and left (la) atria. (E) Whole-mount E14.5 embryo demonstrates expression throughout the heart (h) and skeletal musculature.

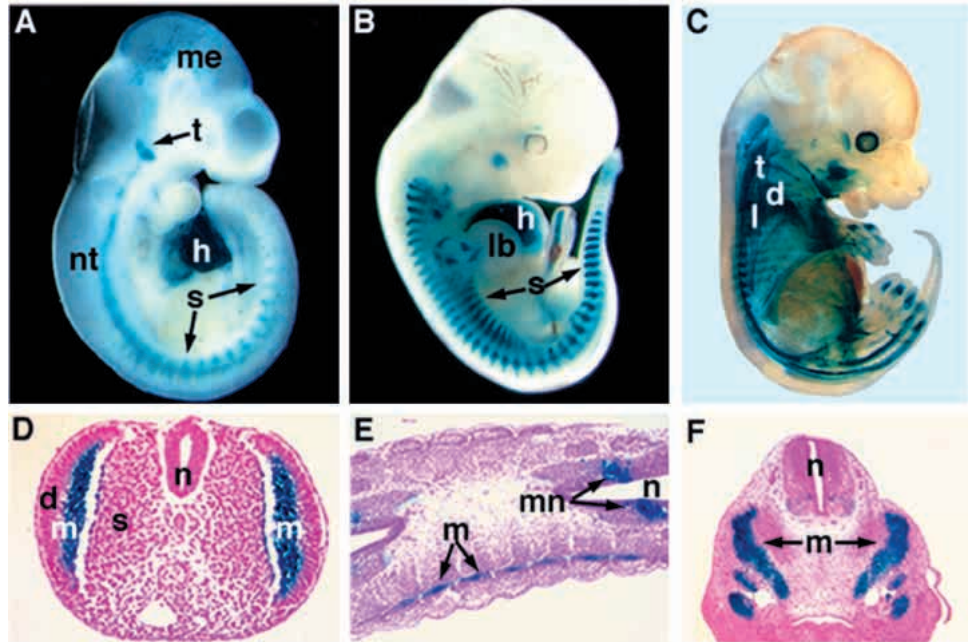


At day 9.5 p.c., the *des-MEF2-lacZ* transgene continued to be expressed uniformly throughout the developing heart (Fig. 3A) and, by day 11.5 p.c., expression was detected in both atria and ventricles of the heart. Transverse sections through X-gal-stained embryos showed restricted expression in the myocardium of all four chambers of the heart (Fig. 2D), with no expression in the endocardium. Expression in the heart continued through day 14.5 p.c. (Fig. 2E), after which time it began to decline to low but detectable levels into adulthood (not shown). Expression of the endogenous MEF2 genes also declines in the heart by this stage (Edmondson et al., 1994).

Expression of MEF2-dependent transgene in the somites

Expression of the MEF2-dependent *lacZ* transgene was detected in the rostral somites beginning at day 9.0-9.5 p.c. (Fig. 3A), which corresponds to the time in which MEF2C transcripts appear in these cells (Edmondson et al., 1994). The metameric expression pattern progressed caudally through day 11.5 p.c., contiguous with somite maturation (Fig. 3B). Transverse sections of E11.5 embryos showed specific *lacZ* expression throughout the myotomal compartment within the somite (Fig. 3D,E). As the somites matured, β -gal staining was

Fig. 3. Expression of *des-MEF2-lacZ* in the somites and skeletal muscle during mouse embryogenesis. (A) Whole-mount E9.5 embryo expresses the *lacZ* reporter in the most rostral somites (s) as well as in the heart (h), neural tube (nt), mesencephalon (me) and trigeminal ganglia (t). (B) An E11.5 whole-mount embryo demonstrating the metameric pattern of expression in the somites (s) and in cells within the limb bud (lb). (C) At E14.5, transgene expression is observed throughout the embryonic musculature. (D) Transverse section through the caudal somites (s) shows transgene expression in the myotome (m) and (E) in the ventral region of the neural tube (n) which gives rise to motor neurons (mn). (F) Transverse section through the rostral somites of an E11.5 embryo demonstrates transgene expression within differentiated myocytes (m) that extend longitudinally through the myotome. Trapezius (t), latissimus dorsi (l), deltoid (d).



detected in differentiated myocytes that extended longitudinally through the myotomes (Fig. 3F). There was no expression of *lacZ* in the sclerotomes or dermatomes (Fig. 3D). A similar expression pattern has been observed for MEF2C mRNA.

At day 11.5 p.c., *lacZ* expression was also observed in cells throughout the limb buds (see Fig. 3B). We are uncertain whether these cells represent a specific lineage. However, by day 12.5 p.c., expression of *lacZ* in the limb buds was restricted to differentiating skeletal muscle fibers, which suggests that the earlier expression in the limb buds marks skeletal muscle precursor cells that migrate from the ventrolateral region of the somite.

Expression of the transgene was demonstrable in mature skeletal muscle fibers throughout the embryo at day 14.5 p.c. MEF2 mRNAs are expressed at high levels in skeletal muscle at this stage (Edmondson et al., 1994). As shown in Fig. 3C, transgene expression could be readily detected in the trapezius, latissimus dorsi and deltoid embryonic muscles. Sagittal sections through an embryo at day 15.5 p.c. revealed staining distributed homogeneously along the entire length of all muscle fibers (Fig. 4A). Expression persisted in neonatal (Fig. 4B) and adult skeletal muscle (not shown). Interestingly, staining of adult hindlimb muscle from a 2-month-old transgenic animal showed localized expression in a subset of

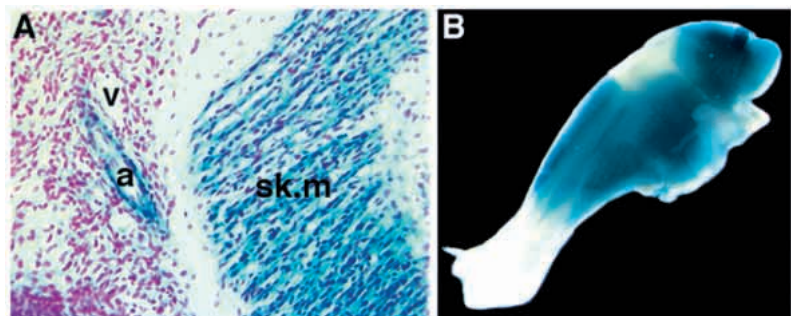
muscle fibers. Whether, this transgene is being expressed in a fiber-type-specific manner is currently under investigation.

MEF2C transcripts are also expressed in vascular smooth muscle cells throughout development (Edmondson et al., 1994; Lin et al., 1998). Expression of *des-MEF2-lacZ* was detected in a subset of vessels in sectioned day 15.5 p.c. embryos (Fig. 4A) and in the adult (not shown). Specific staining was observed in arteries but not veins of *des-MEF2-lacZ* transgenic animals. The reason for this restricted expression in the vasculature is unclear. Finally, we failed to detect transgene expression in the vascular plexus of E9.5 yolk sacs (not shown) in contrast to previously reported expression of MEF2C mRNA in this region (Lin et al., 1998).

The *c-jun* and *desmin* MEF2 sites direct similar patterns of expression

Numerous studies have documented the importance of DNA sequences flanking MEF2 sites for muscle gene activation (Yu et al., 1992; Black and Olson, 1998). DNA-binding site selection experiments have demonstrated that MEF2 proteins in neuronal nuclear extracts preferentially bind sequences immediately adjacent to and including the core MEF2 site whereas MEF2 DNA-binding activity from muscle extracts does not exhibit these sequence constraints (Andres et al., 1995). The activity of other MADS-box proteins is also

Fig. 4. Expression of *des-MEF2-lacZ* transgene in skeletal and vascular smooth muscle. (A) Sagittal section of E15.5 embryo demonstrating expression distributed homogeneously along the entire length of muscle fibers (sk.m). Expression is also clearly evident in the vasculature of this transgenic embryo. Staining is localized to an artery (a) but not a vein (v). (B) β -gal staining is evident in all hindlimb muscle of a 3-day-old neonate.



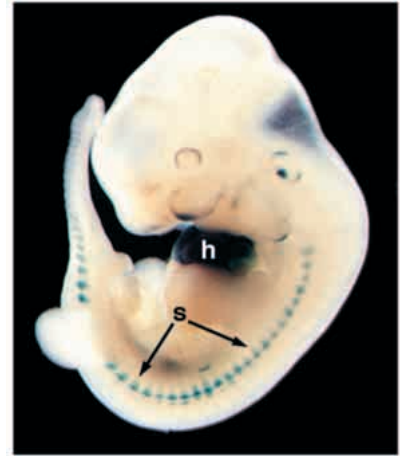
dependent on sequences adjacent to their binding sites. To begin to determine whether the specific expression pattern of *des-MEF2-lacZ* was dependent solely on MEF2 factors or, alternatively, was also influenced by other factors that bound surrounding DNA sequences contained in the multimerized MEF2 site, we created a transgene, called *jun-MEF2-lacZ*. This transgene contained three tandem copies of the MEF2 site from the *c-jun* promoter, along with surrounding DNA sequence. When this transgene was used to generate F₀ transgenic mice, it showed the same expression pattern as *des-MEF2-lacZ* (compare Fig. 5 with Fig. 3B). Since *desmin* and *c-jun* are normally expressed in entirely different patterns, these results suggest that the expression of these transgenes is controlled by the levels of transcriptionally active MEF2 protein, not by gene-specific cofactors that recognize sequences flanking the MEF2 sites.

Expression of *mef2-lacZ* in the brain

Beginning at about 11.5 p.c., MEF2 transcripts begin to be expressed in highly specific patterns throughout differentiating neurons in the brain (Leifer et al., 1993; Lyons et al., 1995). The temporospatial pattern of MEF2 gene expression is coincident with neuronal maturation and suggests an important role in development of the central nervous system. The identification of a functional MEF2 site in the *NR1* gene (Kraic et al., 1998) supports the notion that MEF2 proteins are indeed involved in neuron-specific gene expression. A putative MEF2 site has also been identified in the promoter of the *NF-L* gene (Yaworsky et al., 1997). Furthermore, MEF2 has been shown to synergistically interact with members of the neurogenic family of basic helix-loop-helix transcription factors (Black et al., 1996; Mao and Nadal-Ginard, 1996). Consistent with the proposed role of MEF2 proteins in neuronal differentiation, the *des-MEF2-lacZ* transgene was expressed in a pattern correlating with that of endogenous MEF2 gene expression in the nervous system.

Coronal sections through the brain of a 2-month-old *des-MEF2-lacZ* transgenic animal revealed a striking and highly complex pattern of *lacZ* expression. Prominent expression

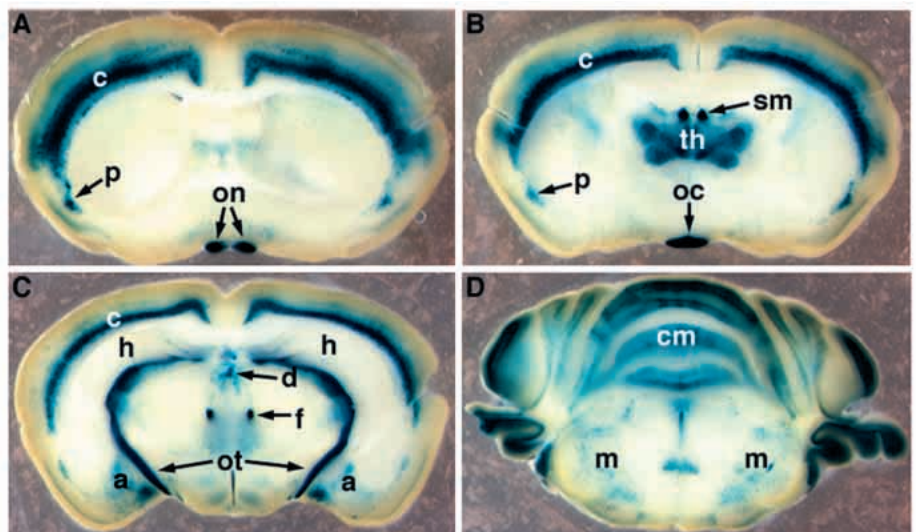
Fig. 5. Expression of *c-jun-MEF2-lacZ* in day 11.5 embryos. Transgene is expressed exclusively in muscle lineages at this time point. Expression is evident throughout the heart (h) and in the somites (s).



was observed in the internal pyramidal cell layers V and VI of the cerebral cortex (Fig. 6A-C). In the postnatal murine brain, all four MEF2 mRNAs are expressed throughout the cerebral cortex with MEF2C gene transcripts being more abundant in layers V and VI (Lyons et al., 1995). Staining was also detected in the piriform cortex (Fig. 6A,B), as well as the optic nerve (Fig. 6A), optic chiasm (Fig. 6B) and optic tracts (Fig. 6C). Consistent with expression in optic neurons, the transgene was also expressed in the developing neural retina (not shown). Progressing caudally, expression became clearly evident in the dorsal and medial thalamic nuclei and the stria medularis (Fig. 6B). Cells within the amygdala, fasciculus retroflexus and the habenular nuclei of the diencephalon (Fig. 6C) also expressed the transgene. The most caudal region of the brain demonstrated expression in the vestibular nuclei of the lateral medulla and in cells within the granular cell layer of the cerebellum (Fig. 6D). No staining was evident in the molecular or Purkinje cell layers of the cerebellum.

Additional regions of expression in the adult brain included the olfactory bulbs, superior colliculus, raphe nuclei and pontine nuclei (data not shown). However, we failed to detect

Fig. 6. Expression of *des-MEF2-lacZ* in specific regions of the brain of adult mice. (A) Coronal section of a β -gal-stained adult brain demonstrates substantial expression in the cerebral cortex (c). Upon thin sectioning expression is localized to the internal pyramidal layers V and VI. Expression is also detected in the piriform cortex (p) and optic nerve (on). (B) Dorsal and medial thalamic nuclei (th) as well as the stria medularis (sm) are staining in a more caudal region of the adult brain. (C) Staining of the optic tracts (ot), amygdala (a), fasciculus retroflexus (f) and habenular nuclei of the diencephalon (d) are clearly evident, but not in the hippocampus (h). (D) Distinct expression of the *des-MEF2-lacZ* transgene in the cerebellum (cm) and vestibular nuclei of the lateral medulla (m). Thin sections revealed restricted expression in a subpopulation of cells within the granular cell layer of the cerebellum.



any transgene expression in the hippocampus or dentate gyrus (Fig. 6B,C), in contrast to previously reported MEF2 mRNA expression in these regions of the adult brain (Lyons et al., 1995). At earlier developmental stages, we observed expression in scattered neurons within the mesencephalon/midbrain (Fig. 3A), in the trigeminal ganglia (Fig. 3A,B) and within the ventral region of the neural tube that gives rise to motor neurons (Fig. 3E), demonstrating that MEF2 is transcriptionally active even at the earliest time points of neuronal differentiation. In conclusion, our results illustrate that MEF2 factors are transcriptionally active in the developing and adult brain and are likely performing important functions in neuronal maintenance.

Expression of *mef2-lacZ* in adult tissues

We stained a variety of adult tissues from *des-MEF2-lacZ* transgenic mice for *lacZ* expression. In contrast to the high level of *lacZ* expression seen in developing and adult skeletal muscle and brain, there was no appreciable β -gal staining in other adult tissues, including the heart (data not shown).

DISCUSSION

The results of this study demonstrate that a multimerized MEF2 site linked to *lacZ* is a sensitive and specific indicator of transcriptionally active MEF2 factors during embryogenesis and provide several insights into the regulation of MEF2 expression and function. (1) In the embryo, expression of the MEF2-dependent transgene and, by inference, transcriptionally active MEF2 protein, is restricted primarily to developing muscle cell lineages and the brain, correlating with sites of highest MEF2 mRNA expression (Edmondson et al., 1994; Lyons et al. 1995). However, during late fetal development, when MEF2 mRNAs become widely expressed, the MEF2-dependent transgene continues to show a restricted expression pattern, suggesting that post-transcriptional mechanisms limit the expression of transcriptionally active MEF2 protein to specific lineages during embryogenesis. (2) A MEF2 site from the *desmin* gene, which is expressed only in muscle cells, can, when multimerized, direct expression in developing brain as well as muscle and a MEF2 site from the *c-jun* promoter can direct expression in a similar fashion. Thus, these MEF2 sites and immediately adjacent sequences do not discriminate between different cell types that express MEF2 factors. (3) Since the expression patterns of the transgenes do not correlate with any specific MEF2 isoform, our results indicate that the *desmin* and *c-jun* MEF2 sites do not discriminate between the various MEF2 proteins and are equally capable of serving as platforms for transcriptional activation by all MEF2 family members in vivo.

MEF2 sites are contained in the promoters of muscle-specific genes that show lineage-restricted and temporal-specific expression patterns (Black and Olson, 1998). For example, *desmin* is expressed in cardiac, skeletal and smooth muscle cells. Expression of *desmin* in skeletal and cardiac muscle is dependent on a muscle-specific enhancer located at about -1.0 kb, which contains a single, essential MEF2-binding site (Li et al., 1993; Kuisk et al., 1996). Desmin expression in smooth muscle cells is controlled by a separate enhancer that does not contain a MEF2 site. Myogenin is

expressed exclusively in the skeletal muscle lineage beginning at E8.5. The *myogenin* promoter contains an essential MEF2 site shown to mediate a positive feedback loop between myogenin and MEF2 (Edmondson et al., 1992; Cheng et al., 1993; Yee and Rigby, 1993). The *MRF4* gene is also expressed specifically in skeletal muscle and contains a MEF2 site in its promoter (Black et al., 1995; Naidu et al., 1995). MRF4 is expressed late relative to myogenin. Transcription of the *MCK* and *MLC2v* genes in skeletal and cardiac muscle, respectively, is controlled by muscle-specific enhancers that also contain MEF2 sites (Navankasattusas et al., 1992; Amacher et al., 1993; Lee et al., 1994). Thus, MEF2 binding alone cannot account for different expression patterns of muscle genes.

There are numerous examples of MADS-box proteins that interact with cofactors to control specific patterns of gene expression. One of the best characterized examples is serum response factor (SRF), which recruits several cofactors to the *c-fos* promoter through protein-protein interactions (Dalton and Treisman, 1992; Grueneberg et al., 1992). In many cases, these cofactors bind sequences immediately adjacent to the CARG box. Because the sequences surrounding the MEF2 sites in the above muscle genes are distinct, we investigated whether these sequences in collaboration with MEF2 might interact with unique sets of cofactors that conferred the distinct expression patterns to these genes. Our results show that tandem copies of MEF2 sites from muscle and non-muscle genes with different expression patterns direct the same expression pattern of a linked *lacZ* transgene. Our interpretation of these findings is that these reporters monitor the levels of transcriptionally active MEF2 factors in cells and do not rely on tissue-specific DNA-binding cofactors for expression. However, we cannot formally rule out the possibility that MEF2 proteins interact with non-DNA-binding cofactors to augment and perhaps restrict transcriptional activity in muscle cells and in neurons. The behavior of these transgenes differs from that of native muscle gene control regions, which contain single MEF2 sites. In those cases, MEF2 is important for expression, but other factors are also required.

Interestingly, the expression patterns of our transgenes differ from that reported by Ross et al. (1996) in which two copies of the MEF2 site from the *MLC2v* gene along with flanking sequences were reported to direct expression of a *lacZ* reporter to the ventricles of the developing heart. The flanking sequences described in that study contain binding sites for widely expressed positive and negative *trans*-acting factors (Navankassattusas et al., 1992, 1994; Lee et al., 1994). Furthermore, the core MEF2 site may also harbor sequences that bind an additional tissue-restricted nuclear factor (Zhu et al., 1993). It is likely that combinatorial interactions between these broadly expressed factors and MEF2 proteins influence the overall expression pattern of their transgene. It is noteworthy that the sequences flanking the MEF2 sites from the *desmin* and *c-jun* promoters do not show any substantial homology with each other or with sequences surrounding the *MLC2v* MEF2 site.

Previous studies have demonstrated that the four *mef2* genes are abundantly expressed in the developing and adult nervous system (Leifer et al., 1993; Martin et al., 1993; McDermott et al., 1993; Lyons et al., 1995; Lin et al., 1996). We have extended those observations by showing that the *des-MEF2*-

lacZ transgene is expressed in a highly complex fashion in the developing and adult brain. These results demonstrate for the first time that MEF2 factors are indeed transcriptionally active in mature neuronal cells in vivo. Cooperative interaction between MEF2 proteins and members of the neurogenic family of basic helix-loop-helix transcription factors suggests that MEF2 is a regulator of neuron-specific gene expression (Black et al., 1996; Mao and Nadal-Ginard, 1996). The identification of a functional MEF2 site in the promoter of the *NR1* gene (Krainc et al., 1998) validates the involvement of MEF2 in neuron-specific gene expression. A putative MEF2 site has also been identified in the promoter of the *NF-L* gene (Yaworsky et al., 1997). However, the biological significance of that site remains to be determined. Thus, it is becoming increasingly apparent that MEF2 proteins are important components in neuronal differentiation. Our results reinforce the notion that members of the MEF2 family of transcription factors are likely performing critical functions in neuronal development and maintenance; hence, the role of MEF2 in the brain warrants further investigation.

While the expression of our MEF2-dependent transgenes reveals high levels of transcriptionally active MEF2 protein in developing muscle and neural cell lineages, it is well established that MEF2 factors are expressed in nonmuscle cell types, where they mediate gene activation in response to MAP kinase signaling (Han and Prywes, 1995; Kato et al., 1997; Zhao et al., 1999). As a consequence, our MEF2-dependent transgenes reveal cell types with high levels of MEF2 activity, but the sensitivity of the assay and/or transgene reporter does not detect those cell types that express lower, basal levels of MEF2. It will be of particular interest to use these MEF2-*lacZ* mice to monitor post-translational regulation of MEF2 activity in vivo in response to various stimuli.

There are numerous examples in which transcription factors are rendered inactive by post-translational mechanisms. Thus, the expression of a transcription factor in a particular cell does not necessarily indicate that the factor is transcriptionally active. The use of transgenes linked to multimerized binding sites such as those reported here may be generally useful for detecting transcription factors that are transcriptionally active in the embryo.

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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.
- Andres, V., Cervera, M. and Mahdavi, V. (1995). Determination of the consensus binding site for MEF2 expressed in muscle and brain reveals tissue-specific sequence constraints. *J. Biol. Chem.* **270**, 23246-23249.
- Black, B. L., Martin, J. F. and Olson, E. N. (1995). The mouse MRF4 promoter is transactivated directly and indirectly by muscle specific transcription factors. *J. Biol. Chem.* **270**, 2889-2892.
- Black, B. L., Ligon, K. L., Zhang, Y. and Olson, E. N. (1996). Cooperative transcriptional activation by the neurogenic basic helix-loop-helix protein MASH1 and members of the myocyte enhancer factor-2 (MEF2) family. *J. Biol. Chem.* **271**, 26659-26663.
- Black, B. L., Lu, J. and Olson, E. N. (1997). The MEF2A 3' untranslated region functions as a cis-acting translational repressor. *Mol. Cell. Biol.* **17**, 2756-2763.
- Black, B. L. and Olson, E. N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu. Rev. Cell Dev. Biol.* **14**, 167-196.
- Braun, T., Bober, E., Rudnicki, M. A., Jaenisch, R. and Arnold, H. H. (1994). MyoD expression marks the onset of skeletal myogenesis in *Myf-5* mutant mice. *Development* **120**, 3083-3092.
- Breitbart, R. E., Liang, C., Smoot, L. B., Laheru, D. A., Mahdavi, V. and Nadal-Ginard, B. (1993). A fourth human MEF2 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., Hanley, T. A., Mudd, J., Merlie, J. P. and Olson, E. N. (1992). Mapping of myogenin transcription during embryogenesis using transgenes linked to the myogenin control region. *J. Cell Biol.* **119**, 1649-1656.
- 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.
- Cserjesi, P. and Olson, E. N. (1991). Myogenin induces muscle-specific enhancer factor MEF-2 independently of other muscle-specific gene products. *Mol. Cell. Biol.* **11**, 4854-4862.
- Dalton, S. and Treisman, R. (1992). Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. *Cell* **68**, 597-612.
- Dodou, E., Sparrow, D. B., Mohun, T. and Treisman, R. (1995). MEF2 proteins, including MEF2A, are expressed in both muscle and non-muscle cells. *Nucleic Acids Res.* **23**, 4267-4274.
- Edmondson, D. G., Lyons, G. E., Martin, J. F. and Olson, E. N. (1994). *Mef2* gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* **120**, 1251-1263.
- Gossett, L. A., Kelvin, D. J., Sternberg, E. A. and Olson, E. N. (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.
- Grueneberg, D., Natesan, S., Alexandre, C. and Gilman, M. Z. (1992). Human and Drosophila homeodomain proteins that enhance the DNA-binding activity of serum response factor. *Science* **257**, 1089-1095.
- Han, J., Jiang, Y., Li, Z., Kravchenko, V. V. and Ulevitch, R. J. (1997). Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature* **386**, 296-299.
- Han, T. H., Lamph, W. W. and Prywes, R. (1992). Mapping of epidermal growth factor-, serum-, and phorbol ester-responsive sequence elements in the c-jun promoter. *Mol. Cell. Biol.* **12**, 4472-4477.
- Han, T. H. and Prywes, R. (1995). Regulatory role of MEF2D in serum induction of the c-jun promoter. *Mol. Cell. Biol.* **15**, 2907-2915.
- Herzog, A. and Brosamle, C. (1997). Semi free-floating treatment: a simple and fast method to process consecutive sections for immunohistochemistry and neuronal tracing. *J. Neuroscience Methods* **72**, 57-61.
- Kato, Y., Kravchenko, V. V., Tapping, R. I., Han, J., Ulevitch, R. J. and Lee, J. D. (1997). BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J.* **16**, 7054-7066.
- Kaushal, S., Schneider, J. W., Nadal-Ginard, B. and Mahdavi, V. (1994). Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. *Science* **266**, 1236-1240.
- Kothary, R., Clapoff, S., Darling, S., Perry, M. D., Moran, L. A. and Rossant, J. (1989). Inducible expression of an hsp68-*lacZ* hybrid gene in transgenic mice. *Development* **105**, 707-714.
- Krainc, D., Bai, G., Okamoto, S., Carles, M., Kusiak, J. W., Brent, R. N. and Lipton, S. A. (1998). Synergistic activation of the N-methyl-D-aspartate receptor subunit 1 promoter by myocyte enhancer factor 2C and Sp1. *J. Biol. Chem.* **273**, 26218-26224.
- Kuisk, I. R., Li, H., Tran, D. and Capetanaki, Y. (1996). A single MEF2 site governs desmin transcription in both heart and skeletal muscle during mouse embryogenesis. *Dev. Biol.* **174**, 1-13.
- Lee, K. J., Hickey, R., Zhu, H. and Chien, K. R. (1994). Positive regulatory

- elements (HF-1a and HF-1b) and a novel negative regulatory element (HF-3) mediate ventricular muscle-specific expression of myosin light-chain 2-luciferase fusion genes in transgenic mice. *Mol. Cell. Biol.* **14**, 1220-1229.
- Leifer, D., Krainc, D., Yu, Y. -T., McDermott, J. C., 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.
- Li, H. and Capetanaki, Y.** (1994). An E box in the desmin promoter cooperates with the E box and MEF-2 sites of a distal enhancer to direct muscle-specific transcriptions. *EMBO J.* **13**, 3580-3589.
- Liu, S., Liu, P., Borrás, A., Chatila, T. and Speck, S. H.** (1997). Cyclosporin A-sensitive induction of the Epstein-Barr virus lytic switch is mediated via a novel pathway involving a MEF2 family member. *EMBO J.* **16**, 143-153.
- Lin, Q., Schwarz, J., Bucana, C. and Olson, E. N.** (1997). Control of cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276**, 1404-1407.
- Lin, Q., Lu, J., Yanagisawa, H., Webb, R., Lyons, G. E., Richardson, J. A. and Olson, E. N.** (1998). Requirement of the MADS box transcription factor MEF2C for vascular development. *Development* **125**, 4565-4574.
- Lin, X., Shah, S. and Bulleit, R. F.** (1996). The expression of MEF2 genes is implicated in CNS neuronal differentiation. *Brain Res. Mol. Brain Res.* **42**, 307-316.
- Lyons, G. E., Micales, B. K., Schwarz, J., Martín, J. F. and Olson, E. N.** (1995). Expression of *mef2* genes in the mouse central nervous system suggests a role in neuronal maturation. *J. Neurosci.* **15**, 5727-5728.
- Mao, Z. and Nadal-Ginard, B.** (1996). Functional and physical interactions between mammalian achaete-scute homolog 1 and myocyte enhancer factor 2A. *J. Biol. Chem.* **271**, 14371-14375.
- 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. C., Cardoso, M. C., Yu, Y. -T., Andres, V., Leifer, D., Krainc, D., Lipton, S. A. and Nadal-Ginard, B.** (1993). hMEF2C gene encodes skeletal muscle- and brain-specific transcription factors. *Mol. Cell. Biol.* **13**, 2564-2577.
- Molkentin, J. D., Li, L. and Olson, E. N.** (1996). Phosphorylation of the MADS-box transcription factor MEF2C enhances its DNA binding activity. *J. Biol. Chem.* **271**, 17199-17204.
- Naidu, P. S., Ludolph, D. C., To, R. Q., Hinterberger, T. J. and Konieczny, S. F.** (1995). Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. *Mol. Cell. Biol.* **15**, 2707-2718.
- Navankasattusas, S., Zhu, H., Garcia, A. V., Evans, S. M. and Chien K. R.** (1992). A ubiquitous factor (HF-1a) and a distinct muscle factor (HF-1b/MEF2) form an E-box independent pathway for cardiac muscle gene expression. *Mol. Cell. Biol.* **12**, 1469-1479.
- Navankasattusas, S., Sawadogo, M., vanBilsen, M., Dang, C. V. and Chien, K. R.** (1994). The helix-loop-helix protein upstream stimulating factor regulates the cardiac ventricular myosin light-chain 2 gene via independent cis regulatory elements. *Mol. Cell. Biol.* **14**, 7331-7339.
- Pollock, R. and Treisman, R.** (1991). Human SRF-related proteins: DNA-binding properties and potential regulatory targets. *Genes Dev.* **5**, 2327-2341.
- Ross, R. S., Navankasattusas, S., Harvey, R. P. and Chien, K. R.** (1996). An HF-1a/HF-1b/MEF-2 combinatorial element confers cardiac ventricular specificity and establishes an anterior-posterior gradient of expression. *Development* **122**, 1799-1809.
- Subramanian, S. V. and Nadal-Ginard, B.** (1996). Early expression of the different isoforms of the myocyte enhancer factor-2 (MEF2) protein in myogenic as well as non-myogenic cell lineages during mouse embryogenesis. *Mech Dev.* **57**, 103-112.
- Woronicz, J. D., Lina, A., Calnan, B. J., Szychowski, S., Cheng, L. and Winoto, A.** (1995). Regulation of Nur77 orphan steroid receptor in activation-induced apoptosis. *Mol. Cell. Biol.* **15**, 6364-6376.
- Yaworsky, P. J., Gardner, D. P. and Klappen, C.** (1997). Transgenic analyses reveal developmentally regulated neuron- and muscle-specific elements in the murine neurofilament light chain gene promoter. *J. Biol. Chem.* **272**, 25112-25120.
- Yee, S. P. and Rigby, P. W.** (1993). The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes Dev.* **7**, 1277-1289.
- Yu, Y.-T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V. and Nadal-Ginard, B.** (1992). Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. *Genes Dev.* **6**, 1783-1798.
- Zhao, M., New, L., Kravchenko, V. V., Kato, Y., Gram, H. M., Padova, F. D., Olson, E. N., Ulevitch, R. J. and Han, J.** (1999). Regulation of the MEF2 family of transcription factors by p38. *Mol. Cell. Biol.* **19**, 21-30.
- Zhu, H., Nguyen, V. T. B., Brown, A. B., Pourhossieni, A., Garcia, A. V., van Bilsen, M. and Chien, K. R.** (1993). A novel, tissue-restricted zinc finger protein (HF-1b) binds to the cardiac regulatory element (HF-1b/MEF2) within the rat myosin light chain-2 gene. *Mol. Cell. Biol.* **13**, 4432-4444.