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Different *MRF4* Knockout Alleles Differentially Disrupt Myf-5 Expression: *cis*-Regulatory Interactions at the *MRF4/Myf-5* Locus

Jeong Kyo Yoon,* Eric N. Olson,† Hans-Henning Arnold,‡ and Barbara J. Wold*,[1]

**Division of Biology, 156-29, California Institute of Technology, Pasadena, California 91125;* †*Hamon Center for Basic Cancer Research, University of Texas Southwestern Medical School at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9148; and* ‡*Department of Cell and Molecular Biology, University of Braunschweig Spielmannstrasse 7, 38106 Braunschweig, Germany*

Three different null alleles of the myogenic bHLH gene *MRF4/herculin/Myf-6* were created recently. The three alleles were similar in design but were surprisingly different in the intensity of their phenotypes, which ranged from complete viability of homozygotes to complete lethality. One possible explanation for these differences is that each mutation altered expression from the nearby *Myf-5* gene to a different extent. This possibility was first raised by the observation that the most severe *MRF4* knockout allele expresses no Myf-5 RNA and is a developmental phenocopy of the *Myf-5* null mutation. Furthermore, initial studies of the two weaker alleles had shown that their differences in viability correlate with the intensity of rib skeletal defects, and the most extreme version of this rib defect is the hallmark phenotype of *Myf-5* null animals. In the present study we tested this hypothesis for the two milder *MRF4* alleles. By analyzing compound heterozygous animals carrying either the intermediate or the weakest *MRF4* knockout allele on one chromosome 10 and a *Myf-5* knockout allele on the other chromosome, we found that both of these *MRF4* alleles apparently downregulate Myf-5 expression by a *cis*-acting mechanism. Compound heterozygotes showed increased mortality of the normally viable *MRF4* allele, together with intensified rib defects for both *MRF4* alleles and increased deficits in myotomal Myf-5 expression. The allele-specific gradation in phenotypes also suggested that rib morphogenesis is profoundly sensitive to quantitative differences in Myf-5 function if Myf-5 products drop below hemizygous levels. The mechanistic basis for *cis* interactions at the *MRF4/Myf-5* locus was further examined by fusing a DNA segment containing the entire *MRF4* structural gene, including all sequences deleted in the three *MRF* knockout alleles, with a basal promoter and a *lacZ* reporter. Transgenic embryos showed specific LacZ expression in myotomes in a pattern that closely resembles the expression of Myf-5 RNA. *cis*-acting interactions between *Myf-5* and *MRF4* may therefore play a significant role in regulating expression of these genes in the early myotomes of wildtype embryos. © 1997 Academic Press

INTRODUCTION

Muscle regulatory factors (MRFs) are transcription factors of the bHLH class. In the mouse, they are collectively essential for proper skeletal muscle determination and differentiation (reviewed in Weintraub, 1993; Arnold and Braun, 1996; Molkentin and Olson, 1996; Yun and Wold, 1996). Genes encoding MRF4 (herculin/Myf-6) and Myf-5 are closely linked, with only 8.5 kb separating their coding sequences on mouse chromosome 10/human chromosome 12 (Braun *et al.*, 1989; 1990; Miner and Wold, 1990), while *MyoD* and *myogenin*, the other genes in this four-member family, are unlinked (Braun *et al.*, 1989). One knockout allele of *Myf-5* (Braun *et al.*, 1992) and three knockout alleles of *MRF4* (Braun and Arnold, 1995; Patapoutian *et al.*, 1995; Zhang *et al.*, 1995) have been made in the mouse germline. The three *MRF4* alleles were substantially similar in design, but the resulting phenotypes differed unexpectedly from each other in viability and in skeletal defects (reviewed in Olson *et al.*, 1996). In particular, the three *MRF4* knockouts vary in the severity of rib defects which

[1] To whom correspondence should be addressed.

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range from mere rib stubs coupled with lethality at birth in the strongest allele (Braun and Arnold, 1995) to the presence of a few misshapen bifurcated ribs and full viability in the weakest allele (Zhang *et al.*, 1995). The third allele falls between the first two in rib phenotype and produces rare homozygous viable animals (Patapoutian *et al.*, 1995; reviewed in Olson *et al.*, 1996). Within the *MRF* family, rib abnormalities have also been described for *Myf-5* and *myogenin* knockouts. This defect is most extreme for *Myf-5* in which null homozygotes develop only residual rib stubs, a phenotype essentially identical to the strongest *MRF4* allele (Braun *et al.*, 1992; Braun and Arnold, 1995). *Myf-5* null embryos also fail to develop an early myotomal compartment, and it has been postulated that there is an essential inductive interaction between the early myotome and the portion of the sclerotome that produces ribs (Braun *et al.*, 1994; Grass *et al.*, 1996).

What is the regulatory basis for the differences in the three *MRF4* alleles? One hypothesis is that the *MRF4* alleles vary in the extent to which expression of the adjacent *Myf-5* gene is inhibited, since different levels of Myf-5 expression are observed in the three *MRF4* nulls. Thus Myf-5 RNA is undetectable in the somites of the most severe *MRF4* allele (Braun and Arnold, 1995) and is significantly reduced in the somites of the intermediate *MRF4* allele (Patapoutian *et al.*, 1995). On the other hand, Myf-5 expression was not much affected in the weakest allele (Zhang *et al.*, 1995). Mechanistically, *cis*- or *trans*-acting interactions between *MRF4* and *Myf-5* could be responsible, and these possibilities are not mutually exclusive. Because MRFs are sequence-specific DNA binding transcription factors, *trans*-acting interactions between MRF4 protein and *Myf-5* regulatory elements are plausible. Moreover, cross-regulatory interactions among members of the MRF family have been detected previously in tissue culture model systems (Braun *et al.*, 1989; Thayer *et al.*, 1989; Hollenberg *et al.*, 1993; Naidu *et al.*, 1995), although no specific evidence for an MRF4 effect on *Myf-5* gene expression has been reported. *cis*-acting interactions between *MRF4* and *Myf-5* are possible because they are adjacent to each other in the chromosome. For the most severe of the three *MRF4* alleles, there is already substantial evidence suggesting that *cis* disruption explains the extreme Myf-5 expression deficit and hence the rib phenotype (Floss *et al.*, 1996). Thus, compound heterozygous mice carrying null *Myf-5* and wildtype *MRF4* on one chromosome and null *MRF4* and wildtype *Myf-5* on the other did not ameliorate the phenotypic deficits seen in this strong *MRF4* null homozygote, but instead were phenocopies of *Myf-5* null homozygotes (Floss *et al.*, 1996).

To better understand the phenotypic properties of either *MRF4* or *Myf-5* nulls and to gain a clearer picture of how these genes are regulated we generated and characterized the phenotypes of *Myf-5*$^{+/m1}$; *MRF4*$^{+/-}$ compound heterozygotes for the two milder *MRF4* alleles. These were produced from intercrosses between the *Myf-5*^{m1} knockout allele (Braun *et al.*, 1992) and either the intermediate (Patapoutian *et al.*, 1995) or weak (Zhang *et al.*, 1995) *MRF4* knockout allele (Fig. 1). Evidence from molecular, cellular, and anatomical phenotypes all suggested that *cis*-acting effects from each *MRF4* allele reduced expression of the adjacent *Myf-5* gene, as was the case for compound heterozygotes with the strongest *MRF4* allele (Floss *et al.*, 1996). Reduction of Myf-5 below haploid levels in compound heterozygotes correlated with increasingly severe skeletal deficiencies compared with the corresponding *MRF4* homozygotes. In the case of the fully viable *MRF4* allele, this also correlated to elevated lethality in the compound heterozygotes. For each cross, the severity of early myotome deficiency predicted the intensity of later rib skeletal defects. Further experiments showed that a DNA segment that contains the *MRF4* structural gene can drive expression of a reporter gene in the somites of transgenic embryos at Day 8.5 postcoitum (e8.5) in a pattern that resembles Myf-5 RNA. Implications of the genetic and molecular results for regulation of MRF4 and Myf-5 expression are discussed.

MATERIALS AND METHODS

Mouse Strains and Genotyping

Mouse embryos were obtained from the intercrosses between *Myf-5*^{m1} heterozygotes (Braun *et al.*, 1992) and either *MRF4*^{bh1} heterozygous (Patapoutian *et al.*, 1995) or *MRF4*$^{-/-}$ mutant strains (Zhang *et al.*, 1995; Fig. 1). The age of embryos was determined by considering noon on the day of copulation as 0.5 days postcoitum (e0.5) and was verified by counting somite number. Genotype of progeny was determined by genomic PCR on DNA from embryos or newborn animals as described (Patapoutian *et al.*, 1995). A primer set for genotyping a gene consists of one forward primer and two reverse primers that detect both wildtype and mutant alleles simultaneously. For the *Myf-5*^{m1} allele (Braun *et al.*, 1992; Fig. 1), PCR was performed with the following three primers; Myf-5-forward, 5′-CAGAGAGACAGTCCCAAACTCCGG-3′ (from *Myf-5* promoter); Myf-5-reverse, 5′-CCAAACTCATCCTCTGGTGAGGGG-3′ (from *Myf-5* exon1); PGK-reverse, 5′-GCGCTACCGGTGGATGTGGAATC-3′ (from *PGK* promoter). This primer set produced a 202-bp PCR product for wildtype and about a 290-bp for the mutant allele (data not shown). For the *MRF4* mutant allele (Zhang *et al.*, 1995; Fig. 1), the following three primers were used; MRF4-forward, 5′-GGGAGACTGATGCTCCATGACAGC-3′ (from *MRF4* promoter); MRF4-reverse2, 5′-TTCTCTTGCAAGTCTTGCAAGCCC-3′ (from *MRF4* exon1); neo-reverse, 5′-CGCCTTCTTGACGAGTTCTTCTGA-3′ (from the *neo* gene). This primer set generated a 461-bp wildtype PCR product and about a 700-bp mutant product (data not shown). For the *MRF4*^{bh1} allele (Patapoutian *et al.*, 1995; Fig. 1), MRF4-forward, MRF4-reverse (5′-GTGTTGCTCTCCACTGCTGTCGCT-3′) and PGK-reverse were used as described previously (Patapoutian *et al.*, 1995).

Total RNA Preparation and RT–PCR

Embryos were harvested at e10.5 and head and limbs were removed. Total RNA was then prepared from the trunk (Chomczynski and Sacchi, 1987) and semiquantitative RT–PCR was performed using 100 ng of total RNA as described previously (Robinson and Simon, 1991; Patapoutian, 1995). Primers for GAPDH and MRF4

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were described elsewhere (Patapoutian *et al.*, 1995). For measuring Myf-5 expression, the following primers were used: Myf-5-forward2, 5'-GACGGACGGCTGCAGTTCTCCCC-3' (from *Myf-5* exon1); Myf-5-reverse2, 5'-CGGGACCAGACAGGGCTGTTACATTCAGGC-3' (from *Myf-5* exon 2). PCR reactions were performed for 19 cycles for GAPDH; 27 cycles for MRF4; and 26 cycles for Myf-5, as described previously (Patapoutian *et al.*, 1995).

Whole-Mount in Situ Hybridization and Immunohistochemical Analysis of Embryos

Whole-mount *in situ* hybridization was performed as described previously (Conlon and Rossant, 1992). Mouse *Myf-5* cDNA (1.2 kb) and rat *MRF4* cDNA (1.4 kb) were used as templates to synthesize anti-sense and sense digoxigenin-conjugated RNA probes. The rat MRF4 RNA probe is specific enough to detect mouse MRF4 RNA, since the rat cDNA sequence is highly homologous to the mouse cDNA sequence (24 different nucleotides in 1.4 kb). Both sense probes failed to generate any specific signal in embryos (data not shown).

Immunohistochemical staining of embryo frozen sections was performed as described previously (Patapoutian *et al.*, 1995). Anti-α-actinin antibodies (Sigma, MO) and anti-myogenin antibodies (conditioned medium of F5D hybridoma culture, Wright *et al.*, 1991) were used in 1:400 and 1:5 dilutions, respectively. The primary antibodies were detected by fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG antibodies (Jackson Immuno Research Inc., PA).

Whole-Mount Skeletal Staining

Skeletons of newborn mice or e18.5 fetuses were prepared according to Hogan *et al.* (1994). In brief, carcasses were fixed in 100% EtOH followed by staining with Alcian blue for cartilage and alizarin red for bone. Skeletons were visualized following incubation in 2% KOH solution.

Transgene Construction and Production of Transgenic Mice

A 56-bp human *β-globin* promoter (Lawn *et al.*, 1980) that is flanked by *Sal*I and *Xho*I restriction enzyme sites was synthesized and inserted into the *Sal*I site of pnlacZF plasmid to obtain the pglobin-lacZ plasmid. This human *β-globin* promoter contains only a TATA box and a transcription initiation site and has been previously shown to produce specific LacZ expression derived by inserted upstream enhancers in transgenic animals (Yee and Rigby, 1993). A 2.9-kb *Sal*I–*Bam*HI DNA fragment from *MRF4* genomic DNA was inserted in front of the human *β-globin* promoter of pglobin-lacZ to create SBgZ transgene (Fig. 7A). Transgenic animals were primarily generated according to Hogan *et al.* (1994). Fertilized eggs were obtained from matings of C57BL/6J X DBA/2J F_1 hybrid ($B_6D_2F_1$) and DNA-injected eggs were implanted into the oviduct of CD-1 foster females. Transgenic embryos were harvested at e8.5 to e10.5 and LacZ expression was monitored by histochemical assay for β-galactosidase activity, as described elsewhere (Hogan *et al.*, 1994). Stable transgenic lines were also established and LacZ expression was monitored in the progeny at different embryonic stages. Embryos were then embedded into paraffin and sectioned at 7 μm thickness. The sections were counterstained with 0.5% eosin.

RESULTS

Intercrosses of $MRF4^{bh1}$ and $Myf\text{-}5^{m1}$ Heterozygous Mice

In the process of analyzing $MRF4^{bh1}$ homozygous animals, we had previously found that *Myf-5* gene expression at e10.0 and e11.0 was severely reduced compared with either heterozygous or wildtype littermates, even though Myf-5 RNA expression initiated normally at e8.0 (6–7 somites) (Patapoutian *et al.*, 1995). In that study we could not distinguish between *cis*-acting and *trans*-acting mechanisms for the effects of the *MRF4* mutation on *Myf-5*. For a *trans*-regulatory contribution, MRF4 protein would be needed to regulate Myf-5 expression, either directly or indirectly, in myotomes during the specific time window in which MRF4 is expressed (early MRF4 expression begins at about e9.0 and ends at around e11.5; Smith *et al.*, 1994). A similar spatiotemporal restriction would have to apply to any *cis* effects from the mutated *MRF4* locus, since Myf-5 expression at an earlier time (around e8.0) in these embryos initiated normally (Patapoutian *et al.*, 1995).

$MRF4^{bh1}$ heterozygous mice were intercrossed with $Myf\text{-}5^{m1}$ heterozygous mice to produce compound heterozygous animals (for gene structures of these alleles, see Fig. 1). If reduced Myf-5 expression in $MRF4^{bh1}$ homozygous animals is due to a *trans*-acting effect (either direct or indirect) of MRF4 on *Myf-5*, a straightforward prediction is that one wildtype *MRF4* allele in a compound heterozygous mouse would partly or entirely complement the deficiency by acting in *trans* on the intact *Myf-5* allele on the other chromosome. The idea that a single wildtype *MRF4* gene could be sufficient to partially or fully complement is supported by the observation that $MRF4^{bh1}$ heterozygotes appear to be wildtype with respect to skeletal development and early myotome formation (Patapoutian *et al.*, 1995). The same is true for simple *Myf-5* heterozygotes which are fully viable and have normal skeletal development (Braun *et al.*, 1992). On the other hand, if the effect of *MRF4* mutation on *Myf-5* is due mainly to a *cis*-acting mechanism, the prediction is that the skeletal phenotype and Myf-5 expression deficit would be similar to that in $MRF4^{bh1}$ homozygotes or would become even more severe. Genotyping of offspring from the intercrosses was done by PCR on genomic DNA (see Materials and Methods) and Mendelian segregation was observed in embryos and fetuses harvested before birth. Thus, we obtained 23 wildtype (28.7%), 18 $MRF4^{bh1}$ heterozygous (22.5%), 20 $Myf\text{-}5^{m1}$ heterozygous (25.0%), and 19 compound heterozygous (23.8%) offspring. No complementation of lethality was observed since all newborn compound heterozygous mice died at birth, apparently due to respiratory distress. This phenotype contrasts dramatically with simple $Myf\text{-}5^{m1}$ or $MRF4^{bh1}$ heterozygotes which had normal viability (Braun *et al.*, 1992; Patapoutian *et al.*, 1995). These initial viability results suggested that the skeletal deficiency thought to cause respiratory insufficiency in $Myf\text{-}5^{m1}$ and most $MRF4^{bh1}$ homozygotes also affects the compound heterozygous animals.

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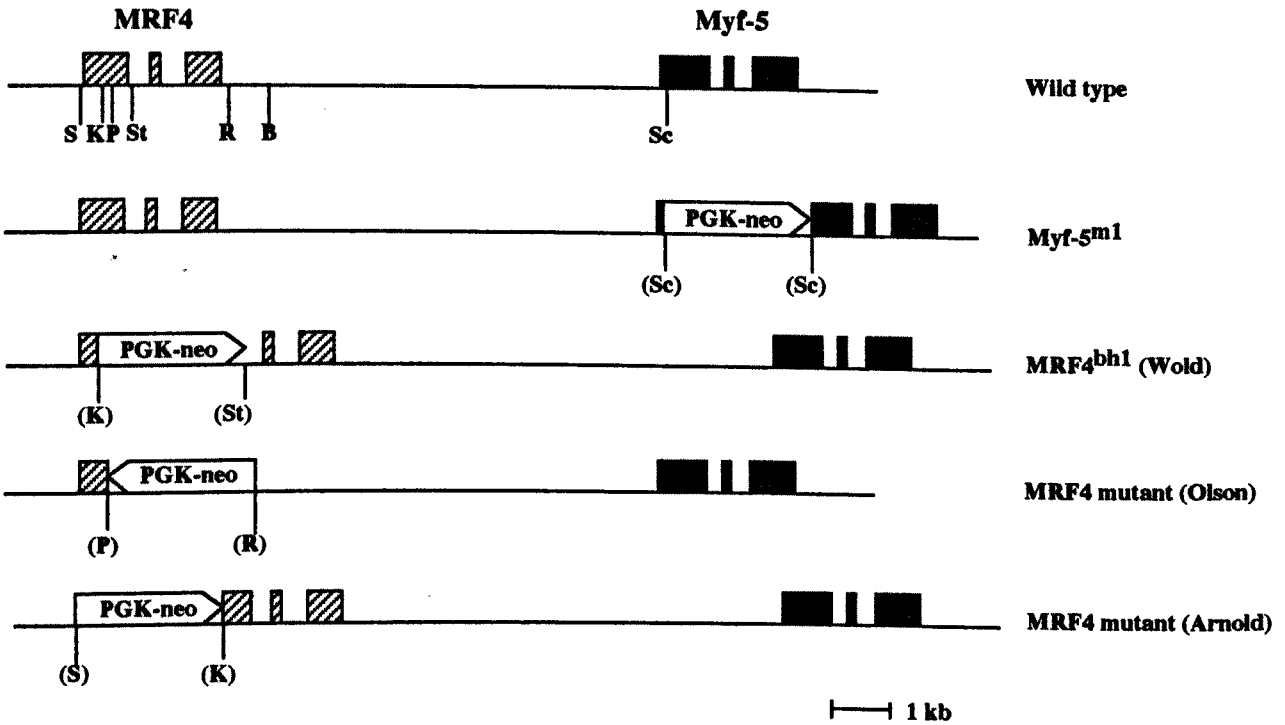

FIG. 1. Gene structures of *MRF4* and *Myf-5* mutant alleles. Schematic structures of the *Myf-5* null allele (Braun *et al.*, 1992) and three *MRF4* null alleles (Patapoutian *et al.*, 1995; Braun and Arnold, 1995; Zhang *et al.*, 1995) are shown. Abbreviations for restriction enzymes are as follows; B, *Bam*HI; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sc, *Sca*I; St, *Stu*I. Restriction enzyme sites in parentheses represent the sites destroyed.

Rib Abnormalities in Compound Heterozygous Mice

Skeletons of newborn mice or e18.5 fetuses were examined and compared with *Myf-5*^{m1} and *MRF4*^{bh1} homozygotes. In the compound heterozygous mice, severe rib abnormalities were observed and these deficiencies were more extensive than those in *MRF4*^{bh1} homozygotes. They were not, however, as severe as those in *Myf-5*^{m1} homozygotes (Fig. 2). Thus, in the compound heterozygous animals, none of the first seven ribs was attached to the sternum and the sternum itself showed irregular ossification of sternebrae. In addition, the length of each rib in the compound heterozygotes was generally shorter than the corresponding rib from either wildtype or *MRF4*^{bh1} homozygous animals but substantially longer than the rib stubs of *Myf-5*^{m1} homozygotes (Fig. 2, compare E and F with G and H). Finally, bifurcation of ribs and fusion of adjacent ribs, features common in *MRF4*^{bh1} homozygotes, were also observed (Fig. 2H). The most straightforward explanation for failure of the *Myf-5*^{m1} chromosome to complement the *MRF4*^{bh1} mutant allele is that the *MRF4*^{bh1} mutation downregulates Myf-5 in *cis* and that the rib phenotype is mainly the result of the Myf-5 deficiency (see below and Discussion).

Myotomal Phenotype

We next examined the levels of Myf-5 and MRF4 expression in the compound heterozygous embryos relative to singly heterozygous and wildtype littermates. Embryos were harvested at e10.5 and total RNA was prepared from the trunk (minus limbs and head in order to focus on the myotomal compartment where MRF4 and Myf-5 are both expressed). Relative Myf-5 and MRF4 expression levels were measured by RT–PCR (see Materials and Methods). RNA samples from the e10.5 embryos homozygous for *MRF4*^{bh1} and *Myf-5*^{m1} were also included as controls. No Myf-5 expression was detected in *Myf5*^{m1} homozygotes, as expected (Fig. 3). At this time in development, Myf-5 expression in *MRF4*^{bh1} homozygotes was severely reduced relative to wildtype or heterozygous *MRF4*^{bh1} embryos (Patapoutian *et al.*, 1995, and Fig. 3), and Myf-5 expression was even more profoundly reduced in the *MRF4*$^{+/bh1}$; *Myf-5*$^{+/m1}$ compound heterozygous embryos (Fig. 3). Previous studies had shown that myotomal MRF4 RNA was not detected in *Myf-5*^{m1} homozygous embryos (Braun *et al.*, 1992) and it was also not detectable at this stage in the compound heterozygous embryos (Fig. 3). These RNA expression measurements confirm that the compound heterozygous embryo is profoundly deficient in Myf-5 RNA expression and that this effect is

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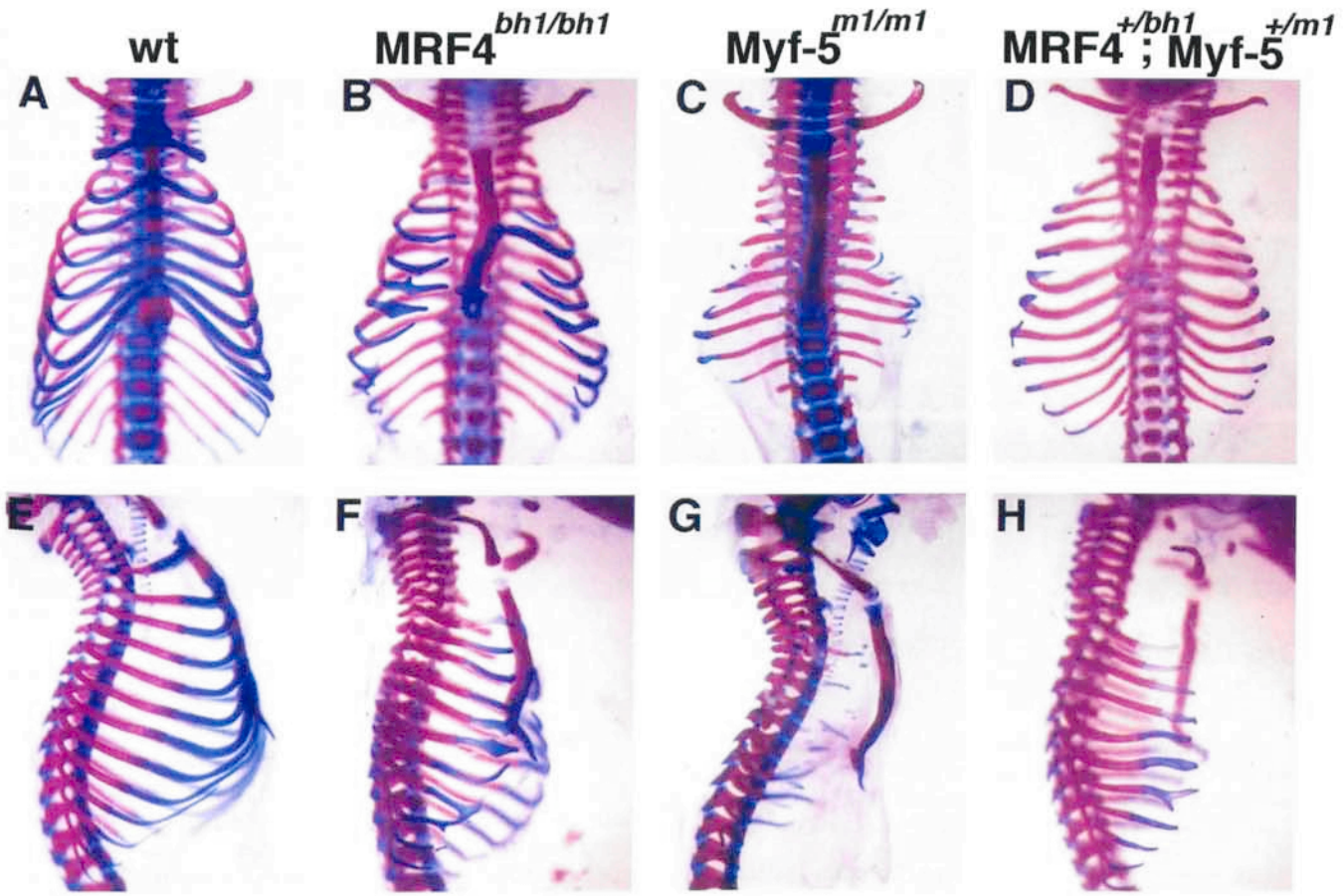

FIG. 2. Whole-mount skeletal analysis of newborn mice. Compound heterozygous animals were obtained from intercrosses between *MRF4*^{bh1} heterozygotes and *Myf-5*^{m1} heterozygotes. Skeletons of newborn wildtype (A and E) and the compound heterozygous animals (D and H) were prepared as described under Materials and Methods. For comparison, skeletons of *MRF4*^{bh1} homozygous (B and F) and *Myf-5*^{m1} homozygous mice (C and G) were also analyzed. To better view the rib cage, forelimbs were removed. Top panels (A–D) are frontal views and bottom panels (E–H) are lateral views of rib skeletons.

discernibly more severe in the compound heterozygote than it is in *MRF4* homozygotes.

Myotome formation at e10.5 was compared in wildtype, *MRF4*^{bh1} homozygous, *Myf-5*^{m1} homozygous, and *MRF4*$^{+/bh1}$; *Myf-5*$^{+/m1}$ compound heterozygous embryos by immunostaining with antibodies directed against α-actinin and myogenin. α-Actinin, a muscle filament protein, is useful for visualizing individual myocytes and for monitoring their organization into the myotomal unit. At the forelimb level, α-actinin was detected in the entire myotome of wildtype embryos, but was essentially undetectable in both *Myf-5* homozygous embryos and the compound heterozygous embryos (Figs. 4A, 4C, and 4D). In *MRF4*^{bh1} homozygous embryos, the myotome consists of very few myocytes, though they are clearly detectable (Fig. 4B; Patapoutian *et al.*, 1995). This differs from *Myf-5*^{m1} homozygotes in which no myocytes are detected at this stage (Fig. 4C). Moreover, the myocytes that are present in *MRF4*^{bh1} homozygotes are poorly organized relative to each other, and the resulting myotomes are misshapen as well as small in size (Fig. 4B). This *MRF4*^{bh1} myotomal phenotype was also observed at the interlimb level, which had not been examined extensively in the first study but may be especially pertinent to the developing ribs (Figs. 4E–4H). Myogenin is the MRF family member most intimately associated with myocyte differentiation *in vivo* (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993; Olson and Klein, 1994), and its expression is thought to mark most, and possibly all, somitic cell nuclei that are committed to differentiate as skeletal muscle or that have already differentiated (Smith *et al.*, 1994). Myogenin normally precedes α-actinin expression, but it was also undetectable in the compound heterozygous embryos (Fig. 4L). This failure of myogenin expression of the early myotome is a phenocopy of *Myf-5*^{m1} homozygous embryos. Taken together, these results show that in the *MRF4*$^{+/bh1}$; *Myf-5*$^{+/m1}$ compound heterozygotes there has been an intensifica-

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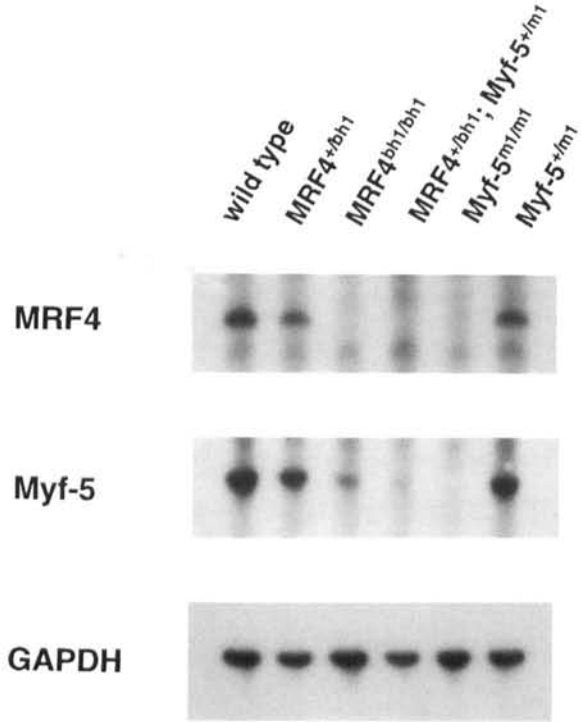

FIG. 3. Expression of MRF4 and Myf-5 in the compound heterozygous embryo. Total RNA was prepared from e10.5 embryos and their genotypes were determined by genomic PCR using yolk sac DNA. Expression levels of MRF4 and Myf-5 were examined by RT–PCR. GAPDH expression was used as a control.

tion of myotomal deficit at the molecular level. This mirrors the increased deficit in Myf-5 RNA expression, compared with *MRF4*^{bh1} homozygous embryos. The deficit in myotome formation also appears to correlate with the intensity of the rib phenotype.

The Mildest MRF4 Null Allele Is Also Intensified in MRF4$^{+/-}$*; Myf-5*$^{+/m1}$ *Compound Heterozygotes*

The Olson group *MRF4* allele (see Fig. 1) is milder than the *MRF4*^{bh1} allele and homozygotes are fully viable. However, these animals have a rib malformation phenotype that is similar in character to the defect in the lethal *MRF4*^{bh1} allele described above (Zhang *et al.*, 1995). It was therefore uncertain whether the MRF4 deficiency in these animals was solely and directly responsible for the skeletal phenotype or whether a *cis* effect on Myf-5 expression might also occur in these mutants. Prior studies had shown that homozygotes of this *MRF4* knockout express apparently normal levels of Myf-5 RNA at e11.5, but it remained possible that Myf-5 expression at earlier developmental stages which were not examined in that study was affected.

MRF4$^{+/-}$; *Myf-5*$^{+/m1}$ compound heterozygous mice were generated by intercrossing a *MRF4* homozygous mouse with a *Myf-5* heterozygous mouse (see Materials and Methods). The resulting offspring were either *MRF4* heterozygotes or compound heterozygotes with a Mendelian segregation ratio (51 and 49%, respectively). Of newborn compound heterozygotes, 63% (10 of 16) died immediately at birth, but the remainder survived. We examined the skeletons of viable and inviable compound heterozygotes and their littermates. Control *MRF4*$^{+/-}$ or *Myf-5*$^{+/-}$ simple heterozygotes had normal rib skeletons (Fig. 5A; Braun *et al.*, 1992; Zhang *et al.*, 1995), but the ribs of the compound heterozygotes were abnormal. The defects resembled those observed in the *MRF4* homozygotes from this allele (Zhang *et al.*, 1995), but the rib/sternum defect was discernibly more severe in some compound heterozygous animals with respect to proper joining of ribs to the sternum. Attachment failure of at least one rib to the sternum was observed in most of these compound heterozygotes, although the identity of the affected rib(s) and the number of ribs affected varied from one individual to the next (Figs. 5B, 5C, 5E, and 5F). Mice homozygous for this allele also display rib defects, but attachment to the sternum appears to be normal (Zhang *et al.*, 1995). It also appeared that the intensity of the rib defect (in particular, the location and number of ribs that fail to join the sternum) may correlate with the degree of lethality at birth (compare Figs. 5B and 5E for a typical live animal phenotype and Figs. 5C and 5F for a lethal phenotype).

Compound heterozygous embryos were examined at e10.5 to evaluate myotome development. Using anti-α-actinin antibodies to visualize the developing myotomes, we found that myocytes were detectable, but that their number was very much reduced. Moreover, the conformation of the myotome was severely defective in the compound heterozygous embryos (Fig. 6). Only a small dorsal myotomal compartment was found to express α-actinin. This myotomal phenotype is, however, less severe than that of *MRF4*$^{+/bh1}$; *Myf-5*$^{+/m1}$ compound heterozygotes described earlier (Figs. 4D, 4H, and 4L). Taken together, these results suggest that even the mildest of the *MRF4* alleles exerts a negative *cis*-regulatory effect on its neighboring *Myf-5* gene.

DNA Containing the MRF4 Structural Gene Can Drive Myotomal Expression of LacZ in a Myf-5-like Pattern

Disruption of the *MRF4* locus in all three cases has apparently interfered with *cis*-regulation of transcription from the *Myf-5* gene, though the extent of disruption varied greatly among the alleles. The molecular basis for such *cis* effects could be interference by the inserted foreign *PGK-neo* sequences, or the disruption of endogenous regulatory elements resident in *MRF4* that are needed for proper Myf-5 expression, or a combination of both mechanisms. To assay for endogenous *cis*-regulatory elements, a transgene called SBgZ was constructed in which a 2.9-kb DNA region from the *MRF4* locus was inserted in front of a basal promoter (human *β-globin* promoter) which was linked to the *lacZ* sequence (Fig. 7A). This *MRF4* DNA segment includes

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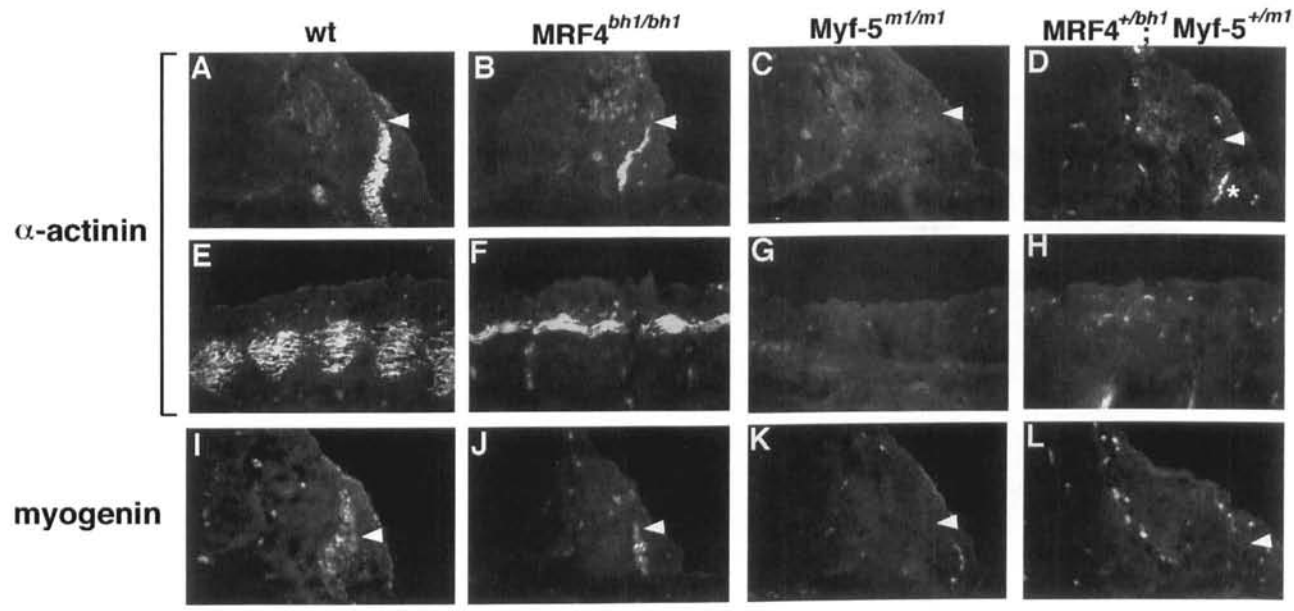

FIG. 4. Immunostaining of myotomes of e10.5 embryos. Wildtype (A, E, and I) and compound heterozygotes (D, H, and L) were harvested from intercrosses between *MRF4*^{bh1} and *Myf-5*^{m1} heterozygous mice. *MRF4*^{bh1} (B, F, and J) and *Myf-5*^{m1} homozygous embryos (C, G, and K) were also harvested. Frozen e10.5 embryos were sectioned at 10 μm thickness and the sections were incubated with anti-α-actinin antibody (A–H) or anti-myogenin monoclonal antibody (I–L) followed by incubation with FITC-conjugated secondary antibodies. Forelimb level myotomes (A–D and I–L) and interlimb myotomes (E–H) were examined. The positions of the arrowheads in A to D indicate the dorsal lip of dermomyotome. The arrowheads in I to L point to the position of the medial myotome. The asterisk in D indicatess nonspecific autofluorescent signals from red blood cells.

all sequences that had been deleted from the three *MRF4* null alleles (Figs. 1 and 7A), and it directly abuts (but does not overlap) the intergenic DNA segment previously shown to direct reporter gene expression in a typical Myf-5 pattern for head muscles and their precursors as well as some later intercostal muscle expression (Fig. 8; Patapoutian *et al.*, 1993). That intergenic segment, however, notably lacked the ability to drive expression in myotomes before e12 (Patapoutian *et al.*, 1993). The SBgZ transgene was injected into fertilized eggs that were implanted into foster mothers, embryos were harvested either transiently or from stable transgenic lines at different embryonic stages, and LacZ expression was monitored. This transgene gave robust LacZ expression in somites at e8.5, and the pattern strongly resembled that for Myf-5 *in situ* RNA hybridization in wildtype embryos (Figs. 7B and 7C). Consistent with prior studies, at this age, no MRF4 RNA was detected in the somites (Fig. 7D). At e9.5, both Myf-5 RNA and LacZ expression were deteced in all but the few most caudal somites, while MRF4 RNA has begun to accumulate in somites of stage IX to XV (Figs. 7E–7G; Table 1). This pattern of SBgZ expression contrasts strongly with that from the transgene carrying the adjacent intergenic DNA segment, which failed to direct any early myotomal expression (Patapoutian *et al.*, 1993). We also noted that expression of SBgLacZ was stronger in old (rostral) somites, where a large triangular domain was positive, than in young (caudal) somites, where a smaller domain was positive (Fig. 7F). This pattern appears to reflect the characteristic rostrocaudal gradient pattern of somite formation and maturation.

The domain of LacZ expression in SBgZ embryos has the triangular shape previously noted for a *lacZ* gene "knockin" allele at the *Myf-5* locus in which LacZ coding sequences were introduced directly into the *Myf-5* gene (Cossu *et al.*, 1996; Tajbakhsh *et al.*, 1996). This same triangular shape was also seen in age-matched somites of the companion whole-mount *in situ* hybridization for Myf-5 RNA (Fig. 7E). For comparison, MRF4 RNA expression was also monitored. The domain of myotomal expression for MRF4 RNA substantially overlaps that of Myf-5 RNA, but appears to be more restricted spatially and has a less pronounced triangular shape, and MRF4 RNA could not be detected in very young somites (Figs. 7D and 7G; Table 1). These spatiotemporal relationships between Myf-5 and MRF4 are in agreement with prior RNA and protein expression studies (Bober *et al.*, 1991; Hinterberger *et al.*, 1991; Ott *et al.*, 1991; Smith *et al.*, 1994). Thus, the earliest Myf-5 transcripts were observed by *in situ* hybridization in somites III and IV (third and fourth from the segmental plate), but the youngest somites detectably expressing MRF4 transcripts in parallel hybridizations were 9th to 15th somites from the segmental plate (Fig. 7G; Table 1). *LacZ* transgene expression was first detected in somites III to VI (third to sixth from the segmental plate). We conclude that this expression pattern resem-

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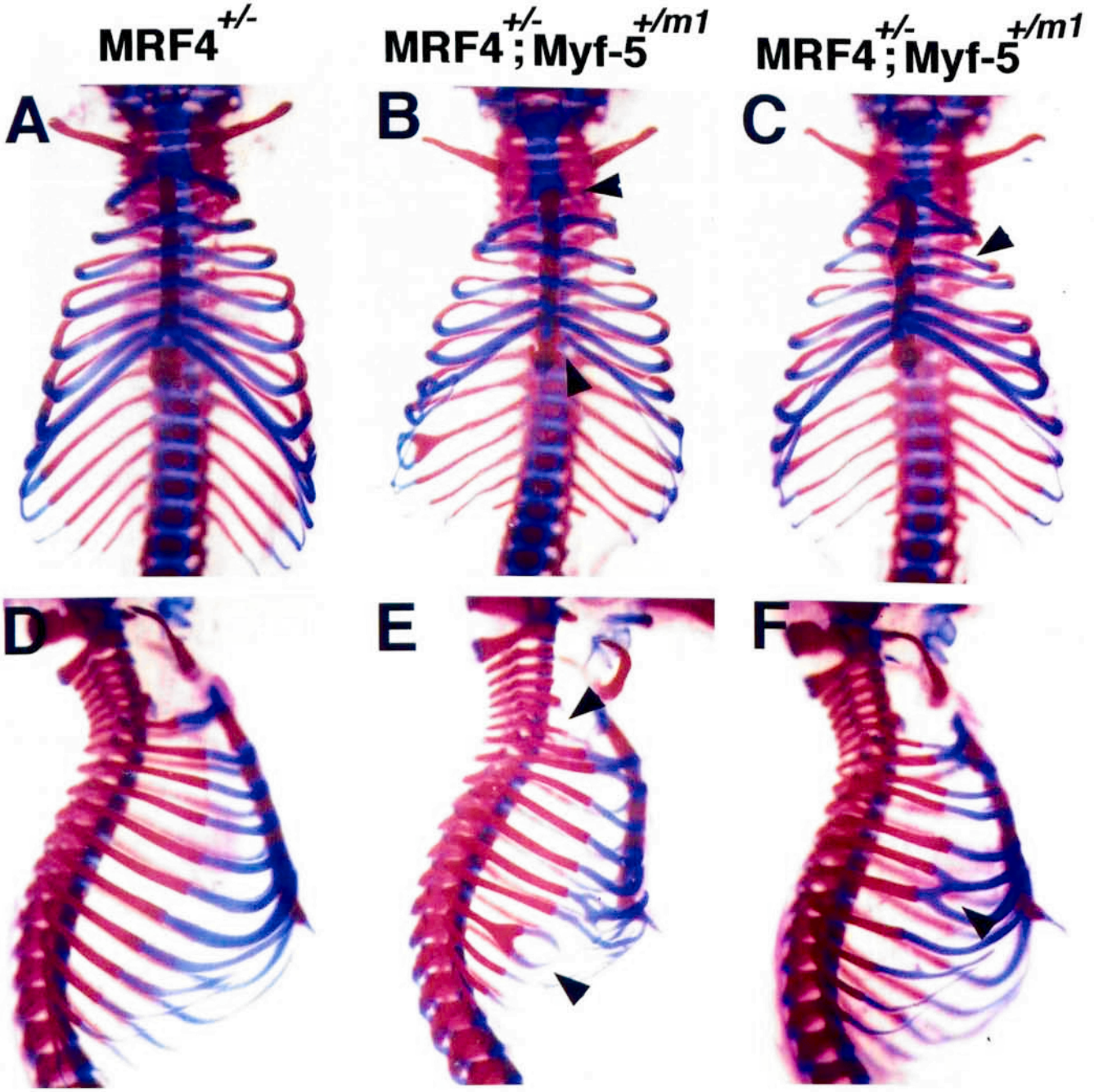

FIG. 5. Enhanced rib deficit in compound heterozygous mice from intercrosses between *Myf-5*$^{+/m1}$ and the mildest *MRF4*$^{+/-}$ (Olson allele) animals. Skeletons of newborn mice were prepared as described. Skeletons of wildtype (A and D), a dead compound heterozygote (B and E), and a live compound heterozygote (C and F) were compared. The forelimbs of animals were removed for better viewing of the rib skeleton. Frontal views of rib cage were shown in top panels (A to C) and bottom panels (D to F) show lateral views. The arrowheads indicate ribs that failed to attach to the sternum and showed bifurcations and fusions.

bles either Myf-5 or a combination of Myf-5 and MRF4 expression domains in both spatial and temporal aspects.

Transverse paraffin sections of a e9.5 SBgZ embryos at the forelimb level showed that LacZ staining is most prominently localized in a dorsal domain of the dermamyotome and the dorsal tip of newly formed myotome (Fig. 7H). In

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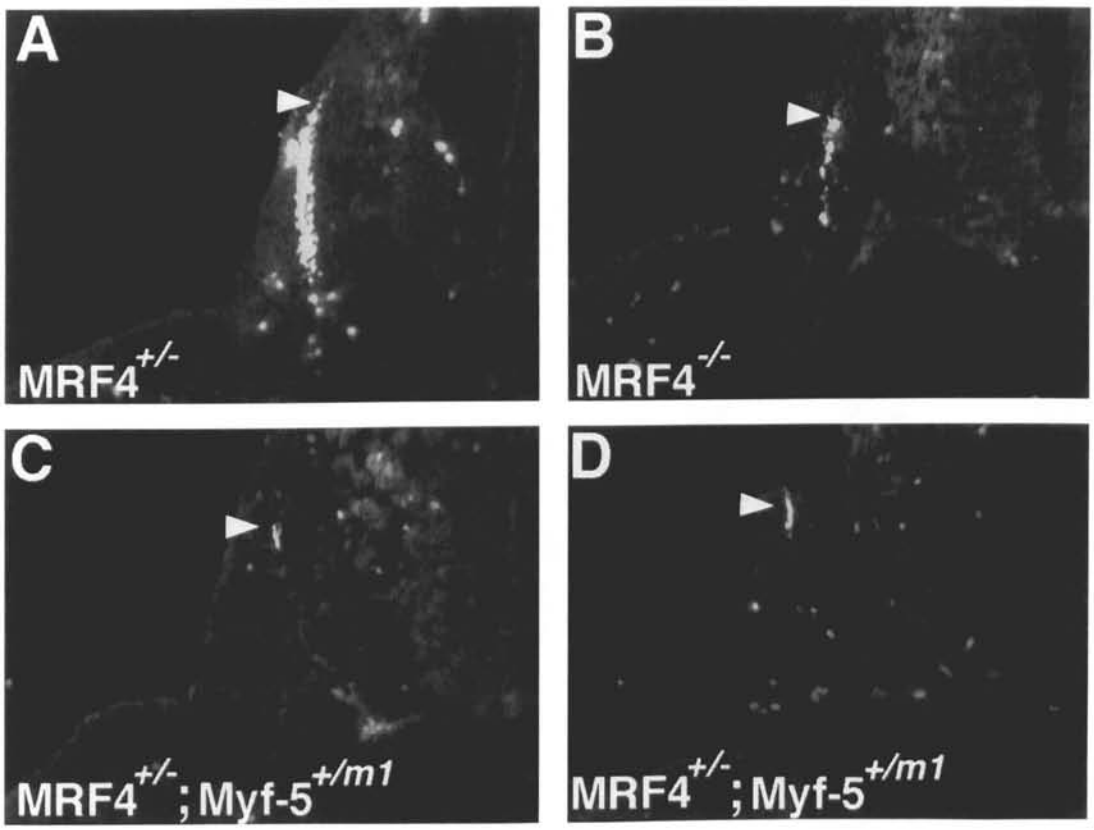

FIG. 6. Reduced expression of α-actinin in the compound heterozygous embryo. e10.5 $MRF4^{+/-}$ (A) and compound heterozygous embryos (C and D) were obtained from the intercrosses between $Myf\text{-}5^{+/m1}$ and the $MRF4^{-}$ (Olson allele). For comparison, an e10.5 $MRF4^{-/-}$ embryo (B) was harvested from $MRF4^{-/-}$ and $MRF4^{-/-}$ intercrosses. Sections 10 μm thick were prepared from frozen embryos and sections containing forelimb level myotomes were immunostained with anti-α-actinin antibodies followed by FITC-conjugated secondary antibodies.

newer (more caudal) undifferentiated somites, LacZ expression was detected in the dorsorostral region, as previously reported for Myf-5 RNA and protein (Fig. 7I; Ott *et al.*, 1991; Smith *et al.*, 1994). We conclude that this segment of the *MRF4* locus, which includes the full region deleted in the combined *MRF4* knockouts, contains one or more *cis*-acting regulatory elements that confer upon a neutral reporter gene regulation typical of early somitic and myotomal Myf-5 and Myf-5 plus MRF4 expression. Differential disruption of these functional elements may contribute to the differing degrees of Myf-5 depression observed in each of *MRF4* null alleles.

DISCUSSION

We have investigated the basis for differences among *MRF4/herculin/Myf-6* knockouts. By studying compound heterozygous mice we found evidence for *cis*-acting negative effects on Myf-5 expression caused by disruption of the adjacent *MRF4* locus. In both Wold and Olson group *MRF4* null alleles, rib skeletal defects were intensified in *MRF4/Myf-5* compound heterozygotes compared with the corresponding $MRF4^{-/-}$ phenotypes. The severity of rib defects evident in compound heterozygous mice correlated directly with the extent of defective myotomal development around e10.5 and with the diminution of Myf-5 RNA. These findings are consistent with prior studies of the most severe *MRF4* allele which produces phenocopies of the $Myf\text{-}5^{m1}$ knockout in either $MRF4^{-/-}$ homozygotes (Braun and Arnold, 1995) or in $MRF4^{+/-}$; $Myf\text{-}5^{+/m1}$ compound heterozygotes (Floss *et al.*, 1996). Taken together, all results point to *cis*-acting effects of each of the *MRF4* mutations on *Myf-5* gene expression, and the degree of damage to normal Myf-5 expression appears to correlate with the severity of each allele. Deficits in Myf-5 activity could also readily explain differences in lethality among the alleles, since the magnitude of rib malformation (especially rib joining to the sternum) appears to be closely related to death from respiratory insufficiency.

Origins and Significance of Variation among the MRF4 Alleles

What is the source of variation among the *MRF4* knockout alleles in Myf-5 expression? The causes are likely to be

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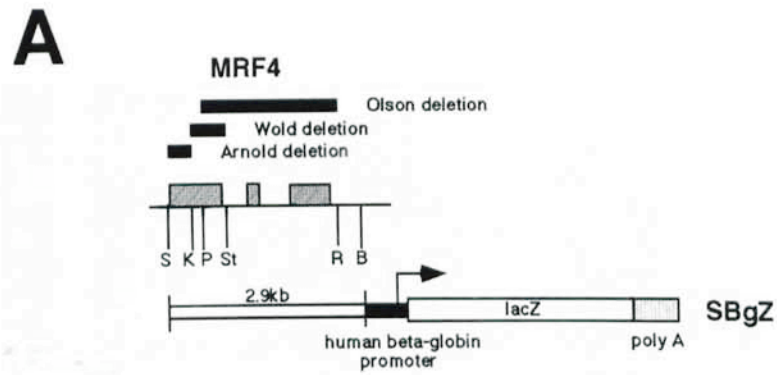
A
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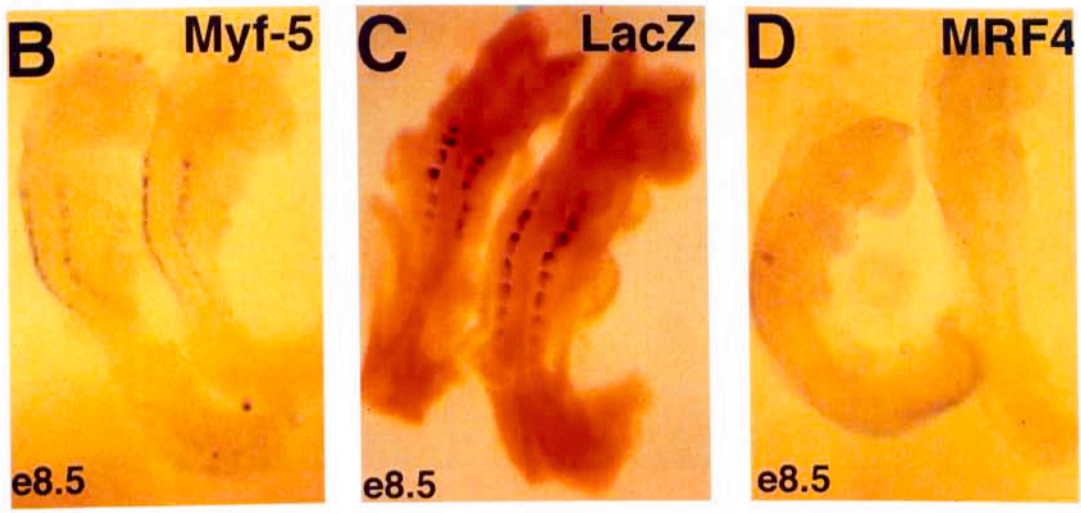
Olson deletion
Wold deletion
Arnold deletion
S K P St
R B
2.9kb
lacZ
SBgZ
human beta-globin promoter
poly A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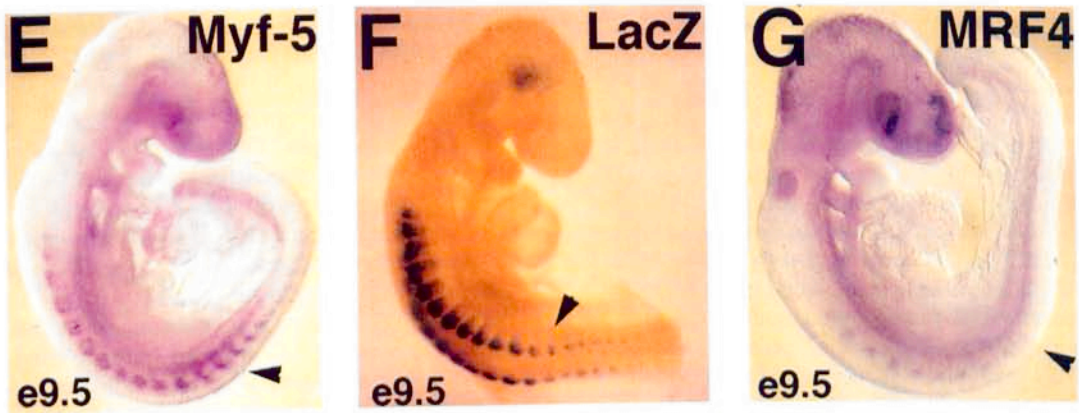
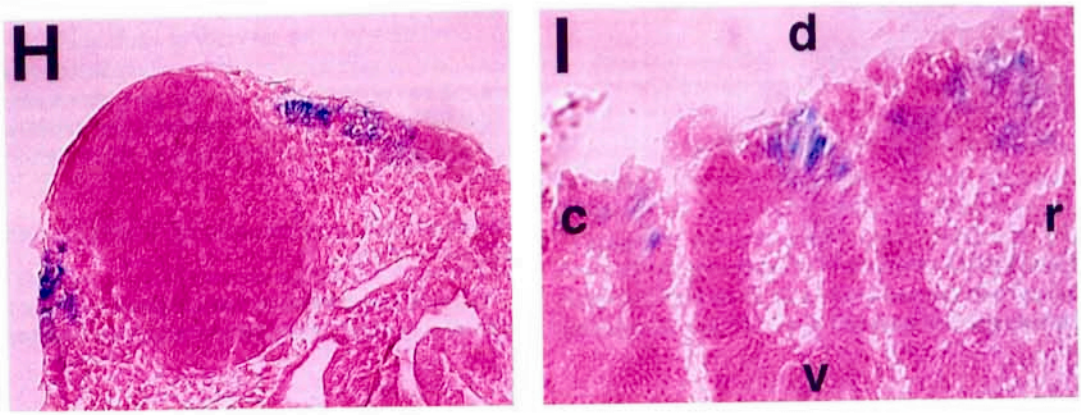
e8.5
C LacZ
e8.5
D MRF4
e8.5


E Myf-5
e9.5
F LacZ
e9.5
G MRF4
e9.5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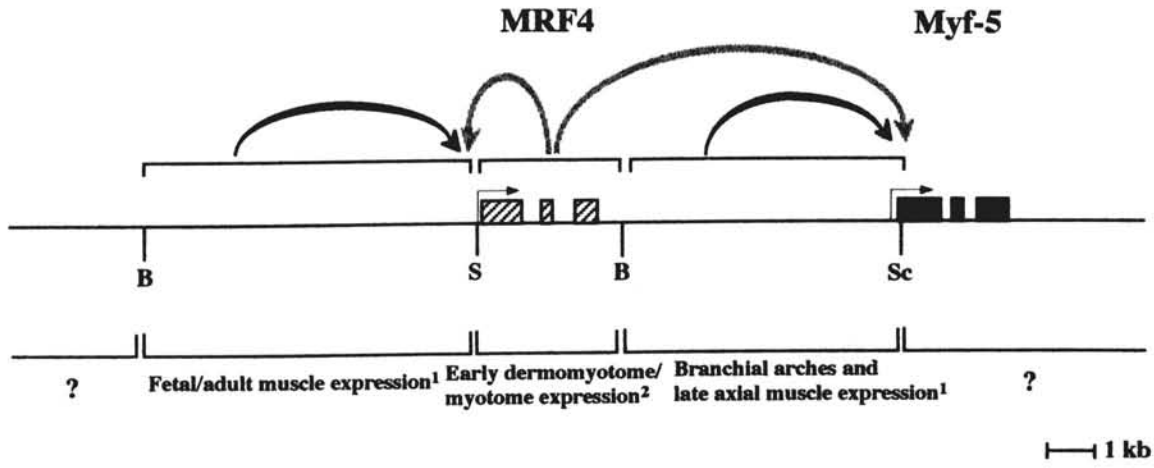

FIG. 8. Model for *cis*-regulatory interactions at *MRF4/Myf-5* locus. *cis*-regulatory domains in the *MRF4/Myf-5* locus are summarized from the study by Patapoutian *et al.* (1993)[1] and from this study.[2] Solid arrows indicate verified regulatory interactions and gray arrows indicate possible interactions revealed in this study that may affect expression of either *MRF4* or *Myf-5* or both, as discussed in the text. The domain of myotomal expression for MRF4 appears, at the current level of resolution, to be a subdomain of myotomal Myf-5 B, *Bam*HI; S, *Sal*I; Sc, *Sca*I.

differences in specific DNA sequences deleted from *MRF4* and/or differences in the position and orientation of *PGK-neo* sequences that were inserted at the *MRF4* locus which then cause different degrees of downregultion from the neighboring *Myf-5* gene (Olson *et al.*, 1996; Fig. 7A). The first possibility is the more interesting one because it would suggest that during normal development of wildtype embryos there are biologically significant *cis*-regulatory interactions between *MRF4* and *Myf-5*. Data showing that *cis*-acting regulatory information from the wildtype *MRF4* locus can direct LacZ expression in a Myf-5-like early somitic pattern (Fig. 7) provided direct support for this possibility. Thus deletion of regulatory elements resident in *MRF4* may be partly or even entirely responsible for the Myf-5 phenotypes of *MRF4* alleles. However, it is important to note that the presence of detectable *cis*-regulatory elements does not rule out *cis*-acting disruption caused by the residual *PGK-neo* cassettes. In other gene systems, including the *globin* and *Hox* clusters, such interactions have been unequivocally demonstrated, and they specifically involved *PGK-neo* cassettes like those used in the *MRF4* knockouts (reviewed in Olson *et al.*, 1996). Moreover, this kind of artifactual position effect caused by an insertion could affect other *cis*-regulatory elements positioned upstream of *MRF4* or downstream within *Myf-5* and beyond. Therefore, the relative contributions coming from the deletion of wildtype *MRF4* sequences versus the positional effects of *PGK-neo* DNA will need to be resolved by new *MRF4* mutations that lack insertional residue.

While this study and Floss *et al.* (1996) both presented evidence that argues in a straightforward way that there are *cis*-acting interactions of *MRF4* on *Myf-5*, a pertinent question is whether other mechanisms could also be contributing. The answer is clearly yes. Neither study could entirely rule out some *trans*-acting interaction between *MRF4* and *Myf-5*, although the mildest of the *MRF4* null

FIG. 7. Expression of a *lacZ* transgene driven by *MRF4* DNA mimics the Myf-5 RNA expression pattern. (A) Structure of the *lacZ* transgene, SBgZ. The regions from *MRF4* locus deleted in three *MRF4* alleles are shown as thick lines with corresponding boundary restriction enzyme sites. S, *Sal*I; K, *Kpn*I; P, *Pst*I; St, *Stu*I; R, *Eco*RI; B, *Bam*HI. (B and E) Whole-mount *in situ* hybridization of Myf-5 RNA in e8.5 (B) and e9.5 (E) embryos. (D and G) Whole-mount *in situ* hybridization of MRF4 RNA in e8.5 (D) and e9.5 (G) embryos. (C and F) Expression of LacZ in e8.5 (C) and e9.5 (F) transgenic embryos. While both Myf-5 RNA and LacZ expression are readily detectable in somites of e8.5 embryos, MRF4 RNA expression is not detected at this stage (B–D). Arrowheads in E, F, and G indicate stage XIII somites. Both Myf-5 RNA and LacZ expression are readily detectable in more caudal (younger) somites related to somite XIII (E and F). In contrast, somite XIII is the youngest somite expressing MRF4 RNA (G). Head mesenchyme expression was noted in SBgZ embryos and not reflect any currently known domain of endogenous Myf-5 or MRF4 expression. (H) Transverse paraffin section of a e9.5 transgenic embryo at the forelimb level shows LacZ-positive cells in dorsomedial region of dermamyotome and in the emerging myotome. (I) Parasagittal paraffin section of caudal somites from a e9.5 transgenic embryo reveals LacZ-positive cells in the dorsorostral domain of undifferentiated somites. Sections were counterstained with 0.5% eosin. v, ventral; d, dorsal; c, caudal; r, rostral.

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TABLE 1
Expression of Marker Genes in 20- to 31-Somite Mouse Embryos

Gene	Position of first somite expressing marker gene[a]
lacZ	III to VI ($n = 9$)[b]
Myf-5	III to IV ($n = 8$)
MRF4	IX to XV ($n = 8$)

[a] Somites are numbered consecutively from caudal to rostral as Roman numerals so that somite I indicates the youngest somite.
[b] Nine embryos include three transgenic embryos from transient determination and six embryos from a stable transgenic line.

homozygotes places rather stringent upper limits on what that phenotype might be. Nor did either study eliminate the possibility that there are relatively subtle embryonic, fetal, or adult phenotypes attributable to MRF4 alone. For example, modest deficiencies in intercostal muscle and deep muscles of the back in the late fetus, reduced expression of embryonic MHC RNA, and upregulation of myogenin in surviving adult mice are phenotypes that may be due to MRF4 deficiency alone (Braun *et al.*, 1995; Patapoutian *et al.*, 1995; Zhang *et al.*, 1995). This can only be resolved unequivocally by generating subtle alleles of *MRF4* that would, for example, introduce a stop codon and leave no inserted sequence residue. Until that has been done, an uncertain portion of the phenotype in the weakest of the three alleles may be due to MRF4 protein and its function. Finally, a single null allele of *Myf-5-* has been used in these studies, and it may be important that it also left behind residue from the inserted gene segment and the selection cassette (Braun *et al.*, 1992). It is presently uncertain if there are any *cis* effects of this *Myf-5* mutation on MRF4 expression, but myotomal MRF4 expression was undetectable in *Myf-5^{m1}* homozygotes and the reason for this is unknown. A *cis*-regulatory effect of the *Myf-5* mutation on MRF4 expression could explain those data and raises the prospect that *cis*-regulatory interactions between *Myf-5* and *MRF4* are reciprocal.

cis-Acting Regulatory Elements from the MRF4 Locus and Their Regulatory Targets

Early and substantial myotome-specific regulatory elements were detected within the 2.9-kb *MRF4* segment that includes the full *MRF4* structural gene and also encompasses the full combined region of deletions made in the three *MRF4* alleles. It is not clear, however, whether in wildtype animals these elements act exclusively on Myf-5 expression, on MRF4 expression, or on both, since their somitic domains of expression overlap significantly. The Myf-5 pattern is more extensive and two lines of evidence favor the view that genuine *cis* elements for a Myf-5 pattern are present. Thus the expression patterns of both Myf-5 and MRF4, as determined by companion whole-mount *in situ* hybridization experiments, showed that Myf-5 is expressed in most somites including very caudal (new) ones, while MRF4 expression is restricted to older, more rostral somites (i.e., appears later in somite maturation than Myf-5) (Figs. 7B–7G; Table 1). This is in agreement with previous reports for RNA (Bober *et al.*, 1991; Hinterberger *et al.*, 1991; Ott *et al.*, 1991) and for protein (Smith *et al.*, 1994). LacZ expression from this transgene was detected in most somites of both e8.5 and e9.5 embryos (Figs. 7C and 7F) and clearly appeared in undifferentiated somites where no MRF4 RNA has been detected (Figs. 7C and 7I). It is, however, important to point out that the sensitivity of the β-galactosidase assay is very high, so that small amounts of MRF4 transcripts in early somites that have not been detected by *in situ* hybridization could conceivably exist and generate a LacZ-positive domain. But a second aspect of the transgene expression pattern is also more reminiscent of Myf-5 (or Myf-5 plus MRF4) than of MRF4 alone. This is the strong dorsal-most signal (and later triangular domain) that typifies Myf-5 RNA (Figs. 7E and 7H; Ott *et al.*, 1991) or *Myf-5-lacZ* knock-in alleles (Cossu *et al.*, 1996; Tajbakhsh *et al.*, 1996) and is also seen in these transgenic embryos (Fig. 7F). As this DNA segment is large enough to contain more than one independent enhancer, further dissection will be needed to see whether the MRF4 pattern can be physically separated from the elements driving the more inclusive Myf-5 domain. It is also formally possible that the MRF4 expression domain within the myotome is the result of a combination of positive-acting *Myf-5* regulatory elements and negative *cis*-acting elements that limit MRF4 expression to a subdomain. While this might seem a baroque possibility, there is strong precedent for regulation of spatial domains in this manner in other embryo systems (reviewed in Gray and Levine, 1996; Kirchhamer *et al.*, 1996). What is certain, however, is that regulatory elements within this DNA segment direct significant early myotomal expression.

Among the four MRFs, *cis*-regulatory DNA sequences that direct embryonic and fetal expression of *myogenin* or *MyoD* have been identified, and these elements seem to act widely in both embryonic and fetal myogenesis (Goldhamer *et al.*, 1992, 1995; Cheng *et al.*, 1993; Yee and Rigby, 1993; Faermen *et al.*, 1995). The picture for *MRF4* and *Myf-5 cis*-acting regulatory machinery has emphasized from the beginning that distinct *cis*-regulatory domains are needed to drive expression within each of several spatiotemporal subdomains. Those known for *MRF4* and *Myf-5* are summarized in Fig. 8. A 5.5-kb intergenic region between *MRF4* and *Myf-5* directs LacZ expression in branchial arches (later giving rise to head muscles) as well as expression later in development in some intercostal muscles (Patapoutian *et al.*, 1993; Fig. 8). But this DNA lacks information needed for early axial expression or for limb expression, and the implication was that other *cis*-acting regulatory elements must be located elsewhere. Sequences 5′ of *MRF4* were also previously shown to contain elements that direct fetal/adult MRF4 expression but not myotomal MRF4 expression

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(Patapoutian *et al.*, 1993; Fig. 8). The DNA domain tested in the present study begins to fill these gaps for MRF4 and Myf-5 patterns. It remains to be determined if the 2.9-kb DNA segment from *MRF4* contains independent elements that separately drive expression of MRF4 or Myf-5 or whether further spatiotemporal subdivisions of myotomal domains exist. It was also noted by Patapoutian *et al.* (1995) that embryos homozygous for the $MRF4^{bh1}$ allele initiated Myf-5 RNA expression normally around e8.0 (6–7 somites), although Myf-5 RNA expression was severely reduced by e10–e11. It is presently not known whether the *cis*-regulatory element(s) responsible for expression at e8.0 is included within the *MRF4* segment tested here. Finally, there may be additional regulatory elements needed for full and proper myotomal expression of either MRF4 or Myf-5 located elsewhere within or 3′ of *Myf-5* or 5′ of *MRF4 cis*-acting sequences identified previously.

For the developmental problem of myogenic precursor specification, the initiation of early Myf-5 expression is of special interest because Myf-5 plays a crucial role in the determination of early myotomal myoblasts (Tajbakhsh *et al.*, 1996). Multiple distinct *cis*-acting regulatory elements associated with specific spatiotemporal domains fit nicely with the emerging understanding of extracellular signals that induce the myotomal, axial, and head muscle lineages in which Myf-5 is expressed. Thus, considerable recent evidence suggests that multiple signals, both positive and negative, are needed to specify individual subdomains of the axial musculature (such as the early dorsal myotome or the later ventrolateral myotome) or the appendicular muscle progenitors derived from the somite (reviewed in Lassar and Munsterberg, 1994; Cossu *et al.*, 1996; Molkentin and Olson, 1996; Yun and Wold, 1996). These signals come from different tissue sources within the developing embryo such as the dorsal neural tube, the dorsal ectoderm, the ventral neural tube, and the lateral mesoderm. The challenge will be to connect specific signals with individual *cis* elements and to identify the transcriptional regulators of Myf-5 and MRF4 that specify activation and/or repression in each spatiotemporal domain.

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