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# A conserved *MRF4* promoter drives transgenic expression in *Xenopus* embryonic somites and adult muscle

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**ABSTRACT** **The muscle regulatory factor MRF4 is expressed in both embryonic and adult vertebrate skeletal muscle cells. In mammals the *MRF4* gene has a complex *cis*-regulatory structure, with many kilobases (kb) of upstream sequence required for embryonic expression in transgenic mice. Here, initial functional comparison between *Xenopus* and mammalian *MRF4* genes revealed that 610 base pairs (bp) of the *XMRF4a* proximal promoter drove substantial transgenic expression in *X. laevis* myogenic cells, from somites of neurula embryos through adult myofibers, and as little as 180 bp gave detectable expression. Over 300 bp of *XMRF4a* proximal promoter sequence is highly conserved among three *X. laevis* and *X. tropicalis MRF4* genes, but only about 150 bp shows significant identity to mammalian *MRF4* genes. This most-conserved *XMRF4a* region contains a putative MEF2 binding site essential for expression both in transgenic embryos and in transfected mouse muscle cells. A rat *MRF4* minimal promoter including the conserved region also was active in transgenic *X. laevis* embryos, demonstrating a striking difference between the mouse and *Xenopus* transgenic systems. The longest *XMRF4a* promoter construct tested, with 9.5 kb of 5'-flanking sequence, produced significantly greater expression in transfected mouse cells than did promoters 4.3-kb or shorter, suggesting that the intervening region contains an enhancer, although no increased expression was evident when this region was included in transgenic *X. laevis* embryos. Further identification and analysis of *Xenopus MRF4* transcriptional control elements will offer insights into the evolution of this gene and of the myogenic gene regulatory network.**

KEY WORDS: *Xenopus, transgenic, muscle, MRF4, promoter*

## Introduction

Within the genetic and epigenetic regulatory network that governs skeletal myogenesis in mammals, one transcription factor family, comprising MRF4, MyoD, myogenin and Myf5, occupies a central position (Pownall *et al.*, 2002; Berkes and Tapscott, 2005; Bryson-Richardson and Currie, 2008). These four closely related myogenic regulatory factors (MRFs) control gene expression during myoblast specification and myofiber differentiation, maintenance, hypertrophy, repair, and regeneration, but the MRFs' individual roles in these cellular events remain incompletely understood. Though MRF4 was for some years regarded primarily as a myofiber differentiation factor and unimportant for myoblast determination, which was thought to depend instead on MyoD and Myf5, more precise gene targeting showed that *MRF4* can function as a determination gene when *MyoD* and *Myf5* are absent (Kassar-Duchossoy *et al.* 2004). The revised

model, in which either *Myf5* or *MRF4* acts upstream of *MyoD* to direct embryonic multipotent mesodermal cells into the myogenic lineage, is more consistent with the observations that *MRF4* transcripts appear before those of *MyoD* and precede or are contemporaneous with those of *Myf5* in both mouse somites (Summerbell *et al.*, 2002) and chick limbs (Lin-Jones and Hauschka, 1996).

*In vitro* and transgenic approaches have both contributed to our current understanding of mammalian *MRF4* regulation. Transfection of cells *in vitro* showed that E-box motifs (CANNTG) in rat and mouse *MRF4* proximal promoters are required for their activation by MyoD, myogenin, or Myf5. MEF2 binding, at a site encompassing the TATA box, is also required for maximal muscle-

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specific expression (Black *et al.*, 1995; Naidu *et al.*, 1995). Although 130 base pairs (bp) of rat *MRF4* proximal promoter suffices for expression in transfected muscle cells, greater expression is seen with 5 kilobases (kb) of upstream sequence (Hinterberger *et al.*, 1992), which includes an enhancer region comprising several E-boxes and a consensus MEF2 site (Kerkvliet and Hinterberger, 1997). The proximal promoter by itself has no activity in myotomes and very little in myofibers of transgenic mice, but 8.5 kb of 5'-flanking sequence activates expression in thoracic somitic myocytes and in fetal myofibers (Pin *et al.*, 1997). When added to the proximal promoter, the enhancer at –5 kb drives increases expression chiefly in fast muscle fibers at fetal

stages but fails to activate expression in the embryonic somites (Pin and Konieczny, 2002). Similarly for the mouse *MRF4* gene, a 7.5-kb promoter fragment drives partial expression in somites (Fomin *et al.* 2004), but fuller expression depends on an enhancer located between –88 kb and –140 kb from the *MRF4* coding region (Carvajal *et al.*, 2001). Recent work (Carvajal *et al.*, 2008) shows that numerous enhancers 5' of the *MRF4* coding region interact in mutually exclusive ways with the *MRF4* promoter and the closely linked *Myf5* promoter in mouse. One particular enhancer located at –8 kb from the *MRF4* coding region was previously shown to direct temporally and spatially distinct activity from the two promoters (Chang *et al.*, 2004, 2007).
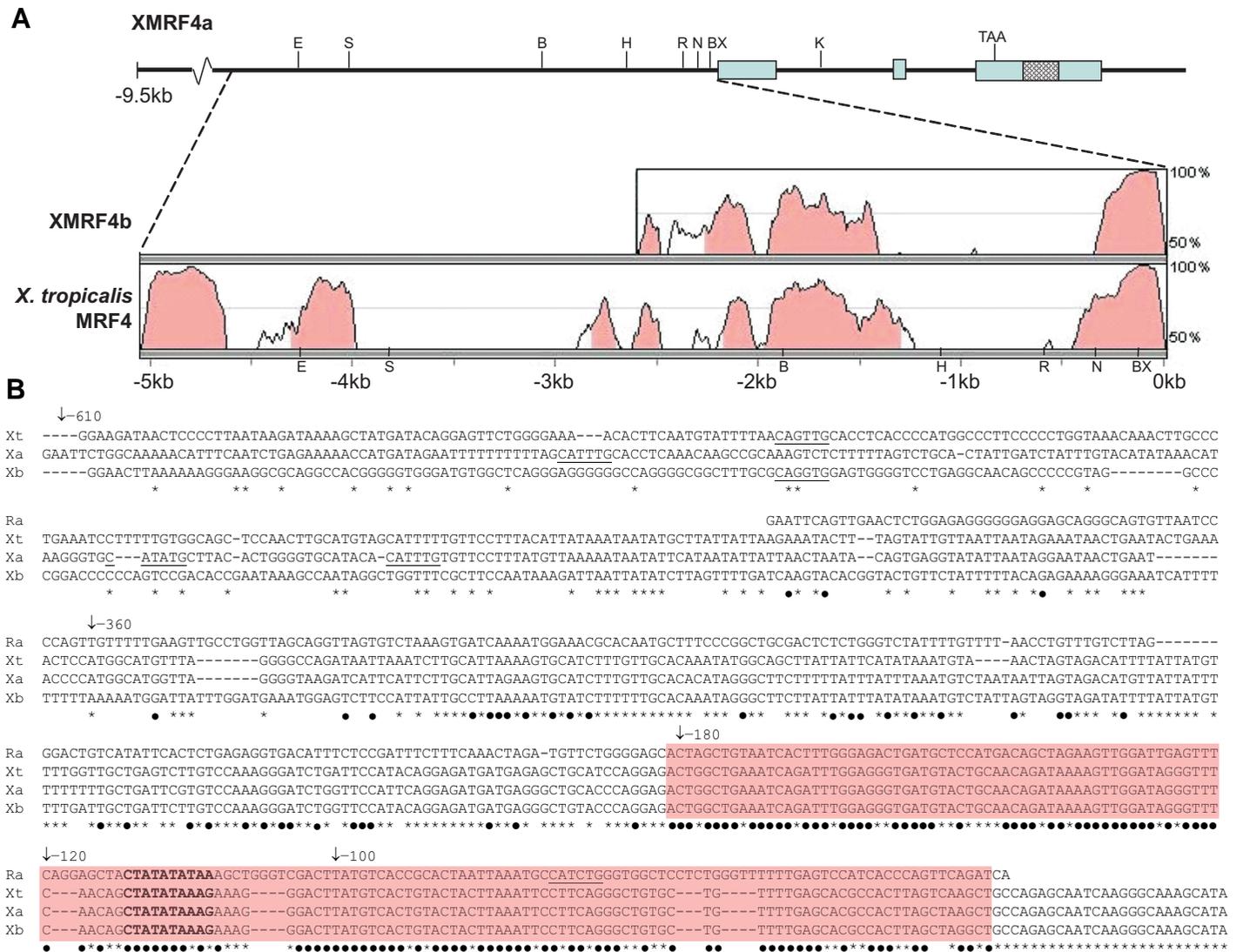


**Fig. 1. Structure of the *XMRF4a* genomic clone and comparison to other *MRF4* gene sequences. (A)** *Upper portion of the figure shows the 5'-flanking region, exons, introns, and 3'-flanking sequence of the 13-kb clone. Restriction enzyme sites mentioned in the text (B, BamH I; BX, BstX I; E, EcoR V; H, Hind III; K, Kpn I; N, Nco I; R, EcoR I; S, Sac I) and the stop codon are indicated. Cross-hatching identifies the P2X7 gene homology region (see text). Lower portion shows results of VISTA alignments of 5066 bp of XMRF4a 5'-flanking sequence versus XMRF4b and X. tropicalis MRF4 loci. Shading indicates conservation of 70% or greater. For XMRF4b, only 2325 bp of sequence was available for comparison.* **(B)** *Alignment of nucleotide sequences from rat MRF4 (Ra), X. tropicalis MRF4 (Xt), XMRF4a (Xa), and XMRF4b (Xb) promoters. Numbering refers to positions 5' from the XlMRF4a start codon. –610 indicates the EcoR I site, –360 the Nco I site, –180 the BstX I site, and –120 and –100 indicate the 5' ends of PCR-generated deletions. The MEF2 site is in bold type and E boxes (potential MRF binding sites) are underlined. Black dots beneath nucleotides indicate conservation in all four species, while asterisks indicate conservation only among the three Xenopus genes. Shading covers the 150-bp highly conserved region described in the text.*

The MRF family is conserved throughout the vertebrate classes, yet the genes' individual expression patterns differ among representatives of the classes that have been examined. Several differences between rodents and *Xenopus* exemplify this. In mice, myogenin is expressed in the myotomes, while in *Xenopus*, *Xmyogenin* mRNA reportedly appears only in the secondary myogenesis of limbs and dorsal body muscles during metamorphosis (Nicolas *et al.*, 1998). In rats (Hinterberger *et al.*, 1991) and in mice (Miner and Wold, 1990), *MRF4* mRNA is more abundant than *MyoD* mRNA in adult muscle, whereas in *Xenopus* the reverse holds true (Jennings, 1992). Muscle denervation in rats leads to increased *MRF4* transcript levels (Adams *et al.*, 1995), but denervation of *Xenopus* muscle leads to decreased levels of *XMRF4* mRNA (Jennings, 1992; Nicholas *et al.*, 2000). These observations indicate that the MRF gene regulatory network must function somewhat differently in *Xenopus* than it does in mammals.

Here, I show that *Xenopus* myogenic cells require only a short *MRF4* promoter for transgenic activity. Approximately 180 bp 5' to the start codon of an *X. laevis MRF4* gene sufficed for transgenic expression in embryonic myotomes. A rat *MRF4* minimal promoter, containing a core sequence that is conserved in all vertebrate *MRF4* genes, also drove expression in transgenic *Xenopus* embryos, clearly demonstrating a major functional difference between the mouse and frog transgenic assay systems. Postmetamorphic transgenic animals bearing either the rat or the *Xenopus* minimal promoter displayed reporter expression in trunk, limb and cranial muscles. Including additional sequence up to 610 bp 5' of the start codon resulted in greater embryonic expression. Although transgenesis assays gave no evidence of any *cis*-regulatory activity located 5' of the 610-bp promoter, sequence comparison among *Xenopus MRF4* genes showed strong conservation of several upstream regions, and transient transfection of mouse C2C12 myoblasts pointed to an enhancer between 4.3 kb and 9.5 kb 5' of the coding region.

## Results

### Gene cloning and sequence analysis

Two distinct *XMRF4* sequences, apparently corresponding to the two *MRF4* loci in the duplicated genome of *X. laevis,* were represented in multiple clones from a lambda genomic library. One clone of *XMRF4a* (gene designation consistent with Della Gaspera *et al.*, 2006) contained the full coding sequence in three exons, approximately 9.5 kb of 5'-flanking sequence, and approximately 1 kb of 3'-flanking sequence (Fig. 1A). An *XMRF4b* (Della Gaspera *et al.*, 2006) clone with all three exons was partially sequenced but the lengths of its flanking regions were not determined. The two *XMRF4* genes displayed greater than 93% identity for approximately 330 bp 5' to the start codon. This region included a TATA box that is conserved in all available vertebrate *MRF4* gene sequences. The mammalian, lizard and chicken *MRF4* genes also contain a MEF2 binding site (CTATATATAA) that overlaps the TATA box; in *X. laevis* and *X. tropicalis MRF4* genes, the corresponding site (CTATATAAAG) deviated at one nucleotide from a MEF2 consensus site [YTA(A/T$_4$)TAR (Black and Olson, 1998)]. A 150-bp region flanking the TATA box and putative MEF2 site in the *Xenopus MRF4* genes displayed 71% identity with the corresponding region of the rat *MRF4* promoter (Fig. 1B). No E boxes were present within the conserved proximal 330-bp region of any of the three *Xenopus MRF4* genes. In *XMRF4a*, three E boxes lie at −450 bp, −475 bp and −555 bp from the first codon, and one is found in a similar location in *XMRF4b* and in *X. tropcalis MRF4*.

Another region of conservation between *XMRF4a* and *-b* began in *XMRF4a* approximately 1.1 kb upstream of the end of the 330-bp proximal region and extended at least 1.2 kb upstream from there. It was conserved between *XMRF4a* and *X. tropicalis MRF4* as well. VISTA alignment with the *X. tropicalis* sequence indicated the existence of an even more strongly conserved region beginning about 4 kb upstream from the *XMRF4a* start codon and continuing to the end of the available 9673-bp *XMRF4a* sequence (Fig. 1A). No identity with the *Xenopus MRF4* conserved upstream regions was indicated in any other available vertebrate genome sequence by VISTA or BLAST analysis.

A BLASTN search of GenBank using the *XMRF4a* sequence as a query showed that the 3' untranslated region of *XMRF4a* contains a segment nearly identical to a portion of the coding sequence of an unrelated gene in *X. laevis* (Fig. 1A). A 289-bp sequence region, flanked by TA repeats, displayed 96% identity to a fragment of the cDNA for the P2X7 ATP-gated ion channel (Paukert *et al.*, 2002).

### Endogenous *MRF4* expression in *X. laevis*

*In situ* hybridizations showed *XMRF4* transcripts in the presomitic mesoderm beginning by at least stage 15 (Fig. 2A), consistent with our previous RT-PCR results (Ataian *et al.*, 2003). Expression continued in somites and myotomes at all stages



**Fig. 2. Whole mount *in situ* hybridization of *X. laevis* embryos with the full-length *XMRF4* probe or *XMyf5* probe.** *All are oriented anterior to the right.* **(A)** *Stage 14-15 early neurula, dorsal view.* XMRF4 *expression is seen in presomitic mesoderm prior to segmentation, as well as in the anterior.* **(B)** *Stage 20 neurula, lateral view.* XMRF4 *staining is most intense in the somites but also evident in the eye primordia.* **(C)** *Stage 16 neurula, dorsolateral view.* XMyf5 *expression is confined to the posterior mesoderm.* **(D)** *Stage 31-32 tailbud embryo, lateral view. In addition to the myotomal staining,* XMRF4 *expression is evident in the eyes, brain, branchial arches, otic vesicles, and head mesoderm.* **(E)** *Stage 31-32 tailbud embryo, lateral view.* XMyf5 *expression is seen in tailbud mesoderm, dorsal and ventral myotomal cells, and in primordia of some cranial muscles.*
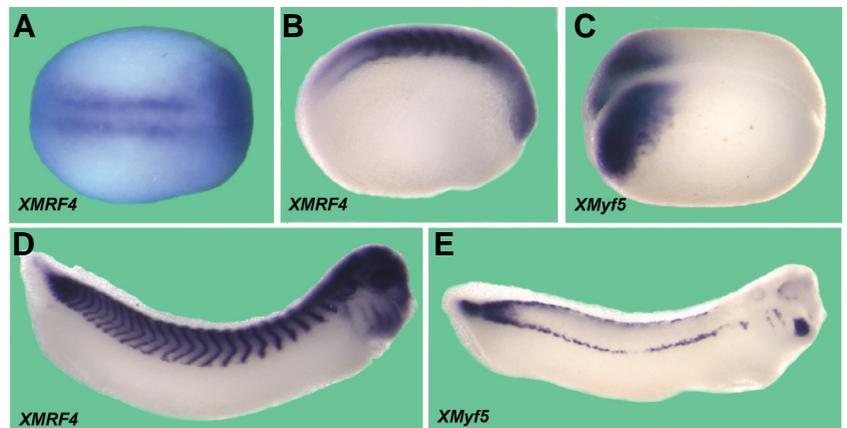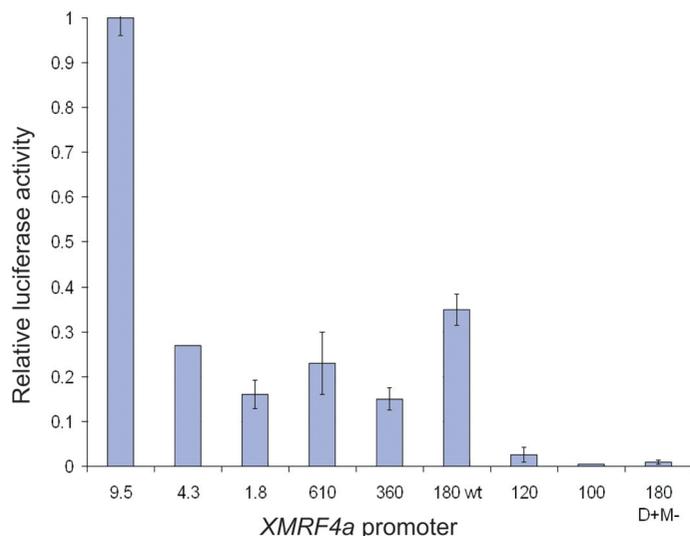
**Fig. 3**. **Relative activity of *XMRF4a* promoters in mouse myotubes.** *The graph shows averages of values ± s.e.m. from two to four independent transient transfection experiments for each* XMRF4a *construct, except for 4.3-kb which is a single datum. Each experimental value is the average of two separate C2C12 cell transfections, expressed as the ratio of test construct to RL-SV40 control, normalized to the value obtained for a positive control construct.*
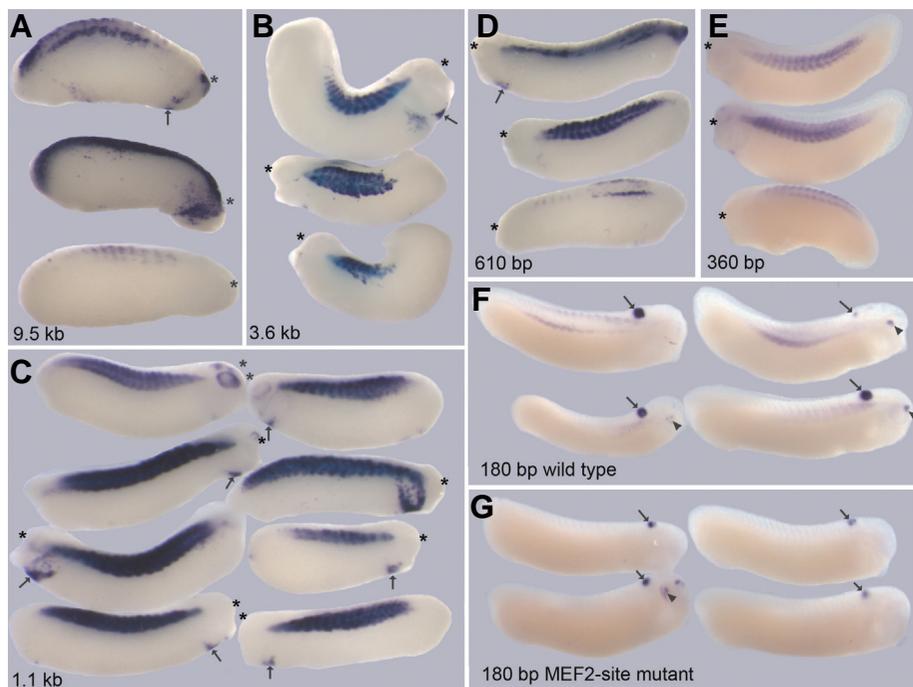


**Fig. 4**. **Relative activity of *XMRF4a* promoters in transgenic *X. laevis* embryos.** *Lengths of the promoters (described in the text) are indicated. Each panel shows lateral views of representative positive individuals from a single experiment. All specimens are oriented with dorsal sides towards the top of the figure, but because expression often was asymmetric, either the left or right side may be shown, whichever had more intense staining, and the anterior end is marked with an asterisk. In panels* **(A–D)**, *arrows indicate ectopic expression in cardiogenic regions. Embryos in* **(F,G)** *are co-transgenic for both the indicated* XMRF4a-GFP *construct and the* gamma-crystallin-GFP *construct; all are oriented with the anterior to the right side of the figure. Staining of the hindbrain (arrows) and, in some cases, the lens of the eye (arrowheads), results from* gamma-crystallin-GFP *expression.*

examined (Fig. 2 B,D) with transcripts confined to the perinuclear region of myocytes. Unexpectedly, staining was also seen in the anterior of embryos, apparently in the eyes, brain, and other structures (Fig. 2 B,D); this expression will be described more fully elsewhere.

Because *MRF4* and *Myf5* genes are closely linked in the genomes of mammals and share overlapping regulatory regions (Carvajal *et al.*, 2001, 2008), it was of interest to examine the *XMyf5* expression pattern in conjunction with that of *XMRF4*. As demonstrated by other investigators (e.g., Martin and Harland, 2001; Polli and Amaya, 2002), *XMyf5* transcripts were detected in the posterior mesoderm at neurula (Fig. 2C) and tailbud (Fig. 2E) stages, becoming restricted to dorsal and ventral lips of the myotomes. *XMyf5* expression was also seen in branchial arches and in primordia of cranial and extraocular muscles of late tailbud embryos; however, no *XMyf5* expression was detected in neural structures, and at every stage its pattern was clearly distinct from that of *XMRF4*.

### Cell transfections

Transient transfection of C2C12 mouse myoblasts with *XMRF4a* 5'-flanking sequence fragments (hereafter called "promoters" even if several kb in length) linked to a luciferase reporter demonstrated that the TATA/MEF2 site plus approximately 60 bp of additional 5' sequence (180wt) constitutes a minimal promoter in myotubes that differentiated from these cells (Fig. 3). Constructs that included only 6 bp of *XMRF4a* sequence 5' to the TATA/MEF2 site (120) had greatly reduced expression, while constructs in which the promoter was truncated 10 bp downstream of the site (100) showed no activity. When the TATA/MEF2 site was mutated to abolish MEF2 binding (180 D+M-), promoter activity also was lost. Longer constructs that included genomic sequence as far as 4.3 kb 5' from the first *XMRF4a* codon resulted in slightly less expression than did the minimal promoter. However, when the entire available 9.5 kb of *XMRF4a* 5'-flanking sequence was included, expression occurred at levels approximately threefold greater than that from the minimal promoter, implying the presence of an enhancer located between −4.3 kb and −9.5 kb that is active in mammalian myotubes.

### Transgenic animals

To study *XMRF4a* transcriptional regulation in *X. laevis* embryonic cells *in vivo*, test promoters were linked to a green fluorescent protein (GFP) cDNA and inserted as transgenes. Tailbud embryos were examined rather than earlier stages in order to allow for accumulation of detectable transcript levels from low-activity insertion sites. Constructs containing 9.5 kb, 3.6 kb, 1.1 kb, 610 bp, 360 bp, or 180 bp of wild-type *XMRF4a* promoter sequence all were expressed in myotomal muscle (Fig. 4 A-F), at frequencies ranging

TABLE 1

**FREQUENCY OF TRANSGENIC MYOTOMAL EXPRESSION BY *MRF4* PROMOTERS**

| Promoter | Experiment no. | GFP+ embryos |
|---|---|---|
| *XMRF4a* promoters | | |
| 9.5 kb | 1 | 24% (11/46) |
| | 2 | 22% (15/69) |
| | total | 23% (26/115) |
| 3.6 kb | 1 | 27% (25/94) |
| | 2 | 15% (8/53) |
| | 3 | 59% (10/17) |
| | total | 26% (43/164) |
| 1.1 kb | 1 | 36% 87/239 |
| | 2 | 8% (10/126) |
| | 3 | 55% (40/73) |
| | total | 31% (137/438) |
| 610 bp | 1 | 67% (8/12) |
| | 2 | 7% (3/44) |
| | 3 | 57% (12/21) |
| | total | 30% (23/77) |
| 360 bp | 1 | 20% (26/129) |
| | 2 | 25% (1/4) |
| | total | 20% (27/133) |
| 180 bp (wt) | 1 | 8% (13/158) |
| | 2 | 6% (9/147) |
| | 3 | 20% (5/25) |
| | total | 8% (27/330) |
| 180 bp (D+M-) | 1 | 0% (0/120) |
| | 2 | 0% (1*/228) |
| | total | 0% (1*/348) |
| rat *MRF4* promoter | | |
| 410 bp | 1 | 7% (6/87) |
| | 2 | 12% (15/123) |
| | total | 10% (21/210) |

*faint, questionable staining

from 8% to 31% (Table 1). In repeated experiments with the same construct, I obtained large variations in the percentage of positive embryos, preventing statistically valid quantitative ranking among these promoters based on their expression frequencies alone. When the *gamma-crystallin*-GFP construct was included as a control, successful transgene insertion could be inferred from GFP expression in the hindbrain and (at late tailbud stages) in the lenses of the eyes. Among these reporter-positive embryos, the 3.6-kb and 1.1-kb *XMRF4a* promoters gave myotomal expression in large majorities of cases, whereas the 180-bp promoter gave expression in less than a third of cases (Table 2). The intensity of *in situ* hybridization staining in the myotomes varied greatly within all groups of embryos carrying the same *XMRF4a*-GFP construct. However, the intensity of staining seen with the 360-bp or 180-bp promoters never reached the maximal level seen with the longer promoters. There was also a tendency for expression from the two shorter promoters to be confined to the dorsal and ventral ends of myotomes (Fig. 4 E,F). (Unlike the endogenous *XMRF4* staining, GFP reporter transcripts were not localized to the perinuclear region of myocytes, possibly because they lack a 3' untranslated region.) One other qualitative difference was that the 610-bp and longer promoters occasionally produced expression in the branchial arches, in the cardiogenic region and the vicinity of eyes (Fig. 4 A-D), a result not seen with the two shorter promoters. Transgenic embryos in which GFP was under the control of a 2.3-kb *XMRF4b* promoter showed myotomal expression identical to that seen with similar-sized *XMRF4a* constructs (data not shown).

Just as with mouse myoblasts transfected *in vitro*, mutation of the MEF2 site in the 180-bp *XMRF4a* promoter essentially abolished reporter gene expression in myotomal muscle of transgenic

TABLE 2

**FREQUENCY OF CO-EXPRESSION OF γ-CRYSTALLIN AND *XMRF4A* PROMOTERS**

| *XMRF4a* promoter length | % crys-GFP+ that were also *XMRF4a*-GFP+ |
|---|---|
| 3.6 kb | 88% (14/16) |
| 1.1 kb | 93% (67/72) |
| 180 bp (wild-type) | 30% (13/43) |
| 180 bp (D+M-) | 2% (1*/60) |

embryos. In one such experiment, inclusion of the *gamma-crystallin*-GFP control resulted in expression in brain or lens in 26% of the specimens, confirming that transgene insertion had taken place in those embryos despite the absence of myotomal expression (Fig. 4G).

To test the hypothesis that a minimal mammalian *MRF4* promoter, due to its strong identity with the *Xenopus* proximal promoters, would be active in *X. laevis* embryos, 410 bp of rat *MRF4* promoter sequence (Hinterberger *et al.*, 1992; Naidu *et al.*, 1995) was linked to the GFP cDNA. The rat promoter produced expression similar to that seen with the shorter *XMRF4a* promoters both in frequency (Table 1) and in pattern and intensity (Fig. 5).

Transgenic tadpoles carrying the *XMRF4a*-GFP constructs were raised through metamorphosis in order to determine whether their transgenes would be expressed in limb muscles. The 3.6-kb, 1.1-kb, 610-bp (Fig. 6A) and 180-bp promoters all drove GFP expression in post-metamorphic skeletal muscles in the trunk, limbs, and head (animals bearing the 360-bp promoter have not yet been fully examined). In sections of transgenic hindlimb muscle, GFP expression was apparently restricted to myofibers (Fig. 6 B,C).

In order to compare the onset of *XMRF4a*-GFP transgene expression to that of the endogenous *XMRF4* genes, strongly-expressing transgenic adults were mated with wild types to provide embryos for analysis of early stages. F1 offspring carrying the *XMRF4a* 610-bp promoter construct had detectable GFP expression in somites by at least stage 20 (Fig. 7A). F1 embryos carrying the 3.8-kb and the 1.1-kb transgenes showed similar



**Fig. 5**. **Activity of the rat *MRF4* minimal promoter in transgenic *X. laevis* embryos.** In situ *hybridization shows GFP expression in representative positive individuals, oriented with dorsal side towards the top of the figure, anterior towards the left.*
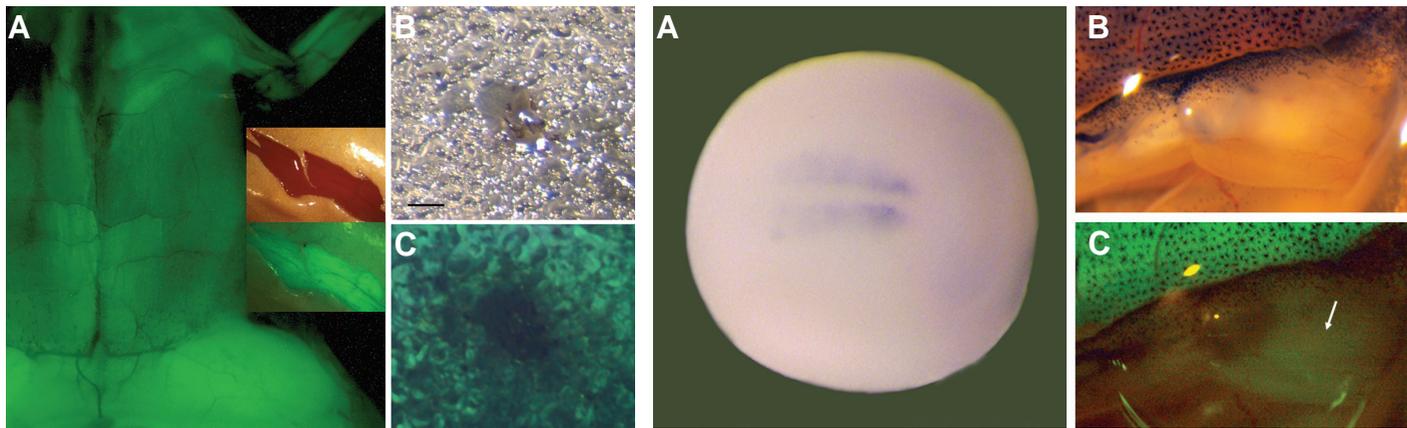
**Fig. 6 (Left). Postmetamorphic expression of *XMRF4a* transgenes. (A)** *GFP fluorescence driven by the 610-bp promoter in skeletal muscle, ventral view, anterior at top, skin removed. This is a composite of two separate exposures of the same animal. Most of the trunk and the left forelimb and proximal hindlimb are shown, but the head is out of the frame. Inset shows reflected-light (upper) and fluorescence (lower) images of a ventral skin incision in the hindlimb of a frog carrying the 1.1-kb promoter. GFP fluorescence in muscle is clearly distinguishable by color from autofluorescence in the skin.* **(B)** *Reflected-light image of a 50 μm frozen section of hindlimb muscle from an* XMRF4a–1.1-GFP *transgenic frog. Note the neurovascular bundle in the center.* **(C)** *GFP fluorescence in the same section as in (B). Scale bar, 100 μm.*

**Fig. 7 (Right). Onset of *XMRF4a* transgene expression. (A)** In situ *hybridization showing GFP expression under control of the 610-bp promoter in the early somites of an F1 individual at stage 18-20, dorsal view, anterior to the right.* **(B)** *Reflected-light image of the right hindlimb of a stage-56 F1 animal carrying the 1.1-kb promoter construct.* **(C)** *GFP fluorescence from the same limb as in (B). Note that the developing muscle (arrow) in the limb shows much lower intensity of fluorescence than do the tail myotomes at the top of the figure.*

results (data not shown). During metamorphosis of these animals, GFP fluorescence became detectable in hindlimb muscle at approximately stage 56 (Fig. 7 B,C).

## Discussion

With this study, *Xenopus laevis MRF4* becomes the second member of the *X. laevis* MRF gene family, after *XMyf5* (Yang *et al.*, 2002), subjected to transgenic analysis of its *cis*-regulatory regions. Embryonic myotomes and, later, limb muscles expressed an *XMRF4a* proximal promoter as short as –180 bp from the first codon, while a 610-bp promoter produced somewhat stronger embryonic expression.

Promoter expression in any transgenic founder reflects a balance between the inherent cell-type-specific transcriptional activity of the test sequence and the repressive properties of the chromosomal site into which it randomly inserts. In principle, a stronger promoter will show detectable activity even in relatively repressive genomic sites, and in permissive sites it will appear more active than weaker promoters. The longer promoters tested here, ranging from 610 bp up to 9.5 kb, all gave detectable expression in myotomes at similar frequencies, while the 360-bp and 180-bp wild-type promoters displayed lower expression frequencies. Large batch-to-batch variances reduced the statistical significance of these differences when test constructs alone were inserted, but inclusion of the *gamma-crystallin*-GFP construct as a marker of transgene co-insertion served to filter the data so that much larger differences were seen between the 1.1-kb and 180-bp constructs' expression frequencies, consistent with their relative intensities of *in situ* hybridization staining. Taken together, the expression frequencies and the staining intensities suggest that the 610-bp promoter contains the all regulatory elements required for *XMRF4a* expression in myotomes of tailbud embryos, al-

though its initial expression appeared somewhat delayed and more restricted than the endogenous gene (compare Figs. 2A and B with Fig. 7A).

These results stand in striking contrast to those obtained with mouse or rat *MRF4* promoters in transgenic mice, where the regulatory activity required for detectable somitic expression is found at least 5.6 kb upstream from the coding region (Fomin *et al.*, 2004). Yet as shown here, a rat *MRF4* 410-bp proximal promoter is at least minimally effective in transgenic *Xenopus*, demonstrating an important functional difference between the two transgenic systems. This conclusion differs from previous studies of mammalian promoters in transgenic *X. laevis* (e.g., Beck and Slack, 1999; Lim *et al.*, 2004) which emphasized the similarities of transgene expression in the two systems. In those studies, however, proximal promoters that lacked activity in mice were not tested in for activity *Xenopus*.

*XMRF4a* promoter fragments were examined for their efficacy driving reporter expression in mouse muscle cells *in vitro* as well as in transgenic *X. laevis.* Here too, the two types of data are not congruent. Luciferase assays of transfected mouse myotubes showed that the 9.5-kb promoter was very effective, while the 4.3-kb to 180-bp promoters were less but about equally effective. This result suggests the existence of an enhancer located between –4.3 kb and –9.5 kb from the coding region. Paradoxically, however, this *X. laevis* sequence region failed to display enhancer activity in transgenic embryos; promoters from 9.5 kb down to 610 bp in length performed indistinguishably. In addition, the *in vitro* results point more strongly to a central role for the –180 promoter than do the transgenics, where this promoter displayed minimal activity.

Within the 180-bp *XMRF4a* promoter, only the 150 bp flanking the putative MEF2 site has significant sequence identity to mammals, and truncation of the rat 0.4-kb promoter has shown that this conserved region accounts for all of its *in vitro* activity (Naidu *et al.*,

1995). Deletion of the 5' end of this region from the *XMRF4a* −180 promoter to make the −120 promoter essentially eliminated its *in vitro* activity, suggesting that these approximately 60 bp have a key role both in mammals and in *Xenopus*. As it is in the mammalian *MRF4* (Black *et al.*, 1995, Naidu *et al.*, 1995) and *Xenopus MyoDa* (Leibham *et al.*, 1994) genes, the putative MEF2 binding site in the *XMRF4a* minimal promoter was obligatory for expression. Loss of MEF2 binding to the *XMRF4a* −180D+M-promoter was not tested biochemically in this study; however, electrophoretic mobility shift assays of an identical mutant MEF2 site in *Xenopus MyoDa* showed that *in vitro* synthesized XMEF2A protein, as well as nuclear extracts from developing embryos or from C2 myotubes, bound to the wild-type but not to the mutant site (Leibham *et al.*, 1994; Wong *et al.*, 1994).

In rat and mouse *MRF4* promoters as well as in many other muscle genes, MEF2 acts synergistically with MRF proteins, which bind at E-boxes in the proximal promoter. The three E-boxes in the 610-bp *XMRF4a* promoter have not been tested functionally, and it is unknown whether they are binding sites for *Xenopus* MRF proteins, but the lower transgenic activity observed for the 360-bp promoter, which lacks these sites, is consistent with a possible role of the E-boxes in activating transcription.

Sequence alignment of *X. laevis XMRF4a* and *XMRF4b* and *X. tropicalis MRF4* genes shows conserved and non-conserved regions within their first few kb of 5'-flanking sequence, but expression differences between *XMRF4a* constructs did not coincide with the boundaries of conserved regions. The 1.1-kb promoter lacks the conserved regions found between −1 kb and −3 kb in the 3.6-kb promoter and upstream of −4 kb in the 9.5-kb promoter, yet all three constructs gave very similar patterns and intensities of reporter gene expression. While evolutionary sequence constraint is associated with an observed genome function in many cases (Hardison, 2000), other constrained regions likely have critical functions not detected by experimental assays employed thus far (de la Calle-Mustienes *et al.*, 2005; Margulies *et al.*, 2007). If the *Xenopus MRF4* and *Myf5* genes have overlapping regulatory regions as they do in mammals (Carvajal *et al.*, 2001, 2008), some of the conserved sequence 5' to their coding region will likely contain regulatory elements functionally associated with the *Myf5* promoter rather than with *MRF4*. Whatever their functions, the presence of strikingly similar patterns of sequence conservation between the *X. laevis MRF4a* and -*b* loci and between *X. laevis* and *X. tropicalis MRF4*, with no similarity to mammals, strongly suggests that the conserved regions exert control over aspects of skeletal muscle development unique to frogs. Conversely, non-conserved as well as conserved sequence elements must contribute to the regulation of *XMRF4a* and -*b* because their transcript levels differ during the course of muscle development and during regeneration (Della Gaspera *et al.*, 2006).

The 610-bp and longer *XMRF4a* promoters showed non-myotomal transgenic expression in some cases, albeit in a pattern that differed from that of endogenous *XMRF4,* which showed extrasomitic expression domains in brain, eye, branchial arches, and other anterior structures. Because it followed a consistent pattern in multiple founder embryos, transgene expression in the cardiogenic and branchial regions at tailbud stage may not represent simply random ectopic expression resulting from the transgenesis procedure (i.e., misregulated transcription of randomly inserted or extrachromosomal DNA, or aberrant cell movements due to embryo manipulation). Non-myotomal *XMRF4a* transgene expression may instead represent an abnormal manifestation, in the experimental context, of normal *XMRF4a* expression in anterior structures. This context includes the absence of potential intronic and 3' regulatory regions and the absence of a linked *Myf5* promoter, which in transgenic mice results in abnormal *MRF4* promoter activity (Carvajal *et al.*, 2008). Furthermore, sequence upstream of −9.5 kb may be required for activating a normal *XMRF4a* expression pattern in the brain, eyes, and other anterior structures.

This initial study of the *cis*-regulatory structure of the *XMRF4a* gene in comparison to mammalian *MRF4* has identified a highly-conserved core promoter that sufficed for transgenic myotomal and postmetamorphic expression in *X. laevis;* demonstrated that additional elements within −610 bp of the *XMRF4a* coding region enhanced myotomal expression; pointed to an upstream enhancer within the −9.5-kb region; and shown that use of different transgenic and *in vitro* assays can lead to very different results. Identification and analysis of the putative upstream enhancer, as well as finer-scale deletion and point-mutation analysis of the *XMRF4a* 610-bp promoter, to fully understand their activities both in mammalian cells and in transgenic *Xenopus* embryos, should provide insight into the evolution of transcriptional control of this gene. Establishment of additional breeding lines carrying these transgenic constructs will facilitate discovery of the regulatory elements involved in *XMRF4a* transcriptional responses to physiological events such as denervation and muscle injury.

## Materials and Methods

### Isolation and sequence analysis of XMRF4 promoters

A 230-bp *Not* I-*Pvu* II fragment at the 5' end of a *Xenopus laevis MRF4* cDNA (Jennings, 1992) was used to screen a genomic library (Lambda Fix II, Stratagene). Recovered inserts were subcloned into pBluescript (Stratagene). One insert of about 13 kb in length, designated *XMRF4a*, was sequenced through 9673 bp (GenBank Accession No. EU045573) including 5066 bp 5' to the start codon. From another insert, designated *XMRF4b*, a 6-kb *Eco*R I fragment was partially sequenced including 2325 bp 5' to the start codon (GenBank Accession No. EU045574). The *XMRF4a* 5' sequence was used as a query for a BLAST search of the *X. tropicalis* v4.1 sequence (Joint Genome Institute, http://www.jgi.doe.gov) that identified the orthologous region of the *X. tropicalis* genome (scaffold 5, 1883000 to 1888563). The VISTA computational tool [http://genome.lbl.gov./vista (Brudno *et al.*, 2007)], with the calculation window set to 100 bp, was used for comparisons between these 5' sequence regions. An alignment of the *X. tropicalis* region against other vertebrate genomes was inspected at the UCSC Bioinformatics Site [http://genome.ucsc.edu (Kent *et al.*, 2002)].

### XMRF4a promoter-reporter gene constructs

After subcloning a 1.9-kb *Eco*R I-*Kpn* I fragment containing 610 bp of sequence 5' to the start codon into pBluescript, a *Bam*H I site was introduced just ahead of the start codon by PCR with proofreading polymerase (Vent, New England Biolabs). This 610-bp *Eco*R I-*Bam*H I fragment was then used to generate shorter promoters, by restriction digestion at an *Nco* I site (−360 bp) or a *Bst*X I site (−180 bp), and by PCR to introduce a *Kpn* I site about 6 bp upstream of the MEF2/TATA site (−120 bp) or about 6 bp downstream of the MEF2/TATA site (−100 bp). PCR-based modifications utilized primers consisting of 18-20 bp of *XMRF4a* sequence with an additional restriction enzyme recognition site.

A mutated version of the 180-bp promoter designed to prevent binding of MEF2 to its site (D+M–), as previously demonstrated in the *Xenopus XMyoDa* promoter (Leibham *et al.*, 1994), was made by site-directed mutagenesis (MORPH kit, 5-prime-to-3-prime Inc., Boulder, CO). The mutagenic oligonucleotide (CAACAGgTATATAAgcAAAGGG) differed from the wild-type sequence at the nucleotides shown in lower-case letters. Other promoter constructs were generated from the full-length *XMRF4a* clone by using PCR with proofreading polymerase (DyNAzyme, Finnzymes Oy., Espoo, Finland) to introduce an *Xho* I site just ahead of the start codon and a *Kpn* I site at the 5' end of the clone. The resulting 9.5-kb product was inserted into a plasmid and subsequently digested with *Eco*R V, *Sac* I, *Bam*H I, or with *Hin*d III to create a 4.3-kb, a 3.6-kb, a 1.8-kb, and a 1.1-kb promoter, respectively.

Green fluorescent protein reporter constructs were made by excising the GFP5 (Siemering *et al.*, 1996):SV40 polyadenylation cassette from *XNkx2.5*-GFP5 (Sparrow *et al.*, 2000) with *Kpn* I and *Not* I and inserting it into pBluescript. *XMRF4a* promoter fragments were then inserted at the *Kpn* I site. A mammalian *MRF4*-GFP5 promoter-reporter construct was produced by inserting the -336 to +71 fragment of rat *MRF4* (Naidu *et al.*, 1995). Luciferase reporter constructs for cell transfection were made by ligating the *XMRF4a* promoter fragments into pGL2-Basic (Promega). Procedural details are available on request. All mutants and constructs were verified by sequencing.

### Generation and analysis of transgenic Xenopus laevis

*MRF4*-GFP5 and n*gamma-crystallin*-GFP3 (Offield *et al.*, 2000) plasmids were digested with restriction enzymes to release the promoter-reporter cassettes, which were then separated from the vector by agarose gel electrophoresis and extracted from the gel with clean-up mini-columns (Qiagen or Promega). For a positive control in some experiments, the *gamma-crystallin*-GFP3 construct was included along with the *XMRF4a*-GFP5 construct, as embryos incorporating one construct have a high probability of incorporating another co-injected construct as well (Hartley *et al.*, 2001). Approximately 100 ng of each DNA was used per transgenesis reaction by the method of Smith *et al.* (2006), which is based on the original REMI method (Kroll and Amaya, 1996) but omits egg extract and restriction enzyme from the pre-injection incubation of DNA with sperm nuclei. GFP mRNA was routinely visualized in embryos fixed at tailbud stage by *in situ* hybridization (Sive *et al.*, 2000) with a digoxigenin-labeled probe transcribed from the first 380 bp of the GFP5 cDNA, with BM Purple (Roche) as the substrate for alkaline phosphatase. In some experiments, living GFP+ embryos were identified by epifluorescence microscopy (Leica MZFLIII) and raised through metamorphosis. In order to examine transgene expression in early embryos, sexually mature transgenic individuals were mated with wild-type individuals. For visualization of GFP in adult tissues, post-metamorphic animals expressing GFP were anesthetized deeply with benzocaine, skinned, and photographed with epifluorescence. The animals were then sacrificed. Animal care and euthanasia followed protocols approved by University of Alaska Anchorage IACUC.

### In situ hybridization probes for endogenous *XMRF4* mRNA and for *XMyf5* mRNA

An antisense digoxigenin-labeled RNA probe was transcribed from the full-length *XMRF4a* cDNA followed by alkaline hydrolysis to produce fragments several hundred bp in length. A probe for *XMyf5* was transcribed from a 470-bp *Eco*R I–*Pst*I fragment of the cDNA (Hopwood *et al.*, 1991). *In situ* hybridization was performed as for the GFP probe.

### Cell culture and transfection

C2C12 mouse myoblasts were grown in DMEM with 20% fetal bovine serum (FBS) and were plated at approximately 50% confluence on gelatin-coated 24-well plates prior to transfection. Equal molar amounts of the different *XMRF4a*-GL2 plasmids were used, together with a constant amount of renilla luciferase plasmid RL-SV40 (Promega) as a control for transfection efficiency. Following transfection using Qiagen Superfect or GTS GenePorter2 reagent, cells were maintained in 20% FBS for 6 hours and then switched to 2% horse serum to promote differentiation for 5 days before harvest and assay (Dual-Luciferase Kit, Promega).

## References

ADAMS, L., CARLSON, B.M., HENDERSON, L. and GOLDMAN, D. (1995). Adaptation of nicotinic acetylcholine receptor, myogenin, and MRF4 gene expression to long-term muscle denervation. *J Cell Biol* 131: 1341-1349.

ATAIAN, Y., OWENS, J. and HINTERBERGER, T. (2003). MRF4 gene expression in *Xenopus* embryos and aneural myofibers. *Dev Dyn* 226: 551-554.

BECK, C.W. and SLACK, J.M. (1999). Gut specific expression using mammalian promoters in transgenic *Xenopus laevis*. *Mech Dev* 88: 221-227.

BERKES, C.A. and TAPSCOTT, S.J. (2005). Myod and the transcriptional control of myogenesis. *Semin Cell Dev Biol* 16: 585-595.

BLACK, B.L., MARTIN, J.F. and OLSON, E.N. (1995). The mouse MRF4 promoter is trans-activated directly and indirectly by muscle-specific transcription factors. *J Biol Chem* 270: 2889-2892.

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.

BRUDNO, M., POLIAKOV, A., MINOVITSKY, S., RATNERE, I. and DUBCHAK, I. (2007). Multiple whole genome alignments and novel biomedical applications at the vista portal. *Nucleic Acids Res*. 35(Web Server issue):W669-674

BRYSON-RICHARDSON, R.J. and CURRIE, P.D. (2008). The genetics of vertebrate myogenesis. *Nat Rev Genet* 9: 632-646.

CARVAJAL, J.J., COX, D., SUMMERBELL, D. and RIGBY, P.W. (2001). A BAC transgenic analysis of the MRF4/MYF5 locus reveals interdigitated elements that control activation and maintenance of gene expression during muscle development. *Development* 128: 1857-1868.

CARVAJAL, J.J., KEITH, A. and RIGBY, P.W. (2008). Global transcriptional regulation of the locus encoding the skeletal muscle determination genes MRF4 and MYF5 . *Genes Dev* 22: 265-276.

CHANG, T.H., PRIMIG, M., HADCHOUEL, J., TAJBAKHSH, S., ROCANCOURT, D., FERNANDEZ, A., KAPPLER, R., SCHERTHAN, H. and BUCKINGHAM, M. (2004). An enhancer directs differential expression of the linked MRF4 and MYF5 myogenic regulatory genes in the mouse. *Dev Biol* 269: 595-608.

CHANG, T.H., VINCENT, S.D., BUCKINGHAM, M.E. and ZAMMIT, P.S. (2007). The a17 enhancer directs expression of MYF5 to muscle satellite cells but MRF4 to myonuclei. *Dev Dyn* 236: 3419-3426.

DE LA CALLE-MUSTIENES, E., FEIJOO, C.G., MANZANARES, M., TENA, J.J., RODRIGUEZ-SEGUEL, E., LETIZIA, A., ALLENDE, M.L. and GOMEZ-SKARMETA, J.L. (2005). A functional survey of the enhancer activity of conserved non-coding sequences from vertebrate iroquois cluster gene deserts. *Genome Res* 15: 1061-1072.

DELLA GASPERA, B., SEQUEIRA, I., CHARBONNIER, F., BECKER, C., SHI, D.L. and CHANOINE, C. (2006). Spatio-temporal expression of MRF4 transcripts and protein during *Xenopus laevis* embryogenesis. *Dev Dyn* 235: 524-529.

FOMIN, M., NOMOKONOVA, N. and ARNOLD, H.H. (2004). Identification of a critical control element directing expression of the muscle-specific transcription factor MRF4 in the mouse embryo. *Dev Biol* 272: 498-509.

HARDISON, R.C. (2000). Conserved noncoding sequences are reliable guides to regulatory elements. *Trends Genet* 16: 369-372.

HARTLEY, K.O., HARDCASTLE, Z., FRIDAY, R.V., AMAYA, E. and PAPALOPULU, N. (2001). Transgenic *Xenopus* embryos reveal that anterior neural development requires continued suppression of BMP signaling after gastrulation. *Dev*

*Biol* 238: 168-184.

HINTERBERGER, T.J., MAYS, J.L. and KONIECZNY, S.F. (1992). Structure and myofiber-specific expression of the rat muscle regulatory gene MRF4. *Gene* 117: 201-207.

HINTERBERGER, T.J., SASSOON, D.A., RHODES, S.J. and KONIECZNY, S.F. (1991). Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Dev Biol* 147: 144-156.

HOPWOOD, N.D., PLUCK, A. and GURDON, J.B. (1991). *Xenopus* MRF-5 marks early muscle cells and can activate muscle genes ectopically in early embryos. *Development* 111: 551-560.

JENNINGS, C.G. (1992). Expression of the myogenic gene MRF4 during *Xenopus* development. *Dev Biol* 151: 319-332.

KASSAR-DUCHOSSOY, L., GAYRAUD-MOREL, B., GOMES, D., ROCANCOURT, D., BUCKINGHAM, M., SHININ, V. and TAJBAKHSH, S. (2004). Mrf4 determines skeletal muscle identity in myf5:Myod double-mutant mice. *Nature* 431: 466-71.

KENT, W.J., SUGNET, C.W., FUREY, T.S., ROSKIN, K.M., PRINGLE, T.H., ZAHLER, A.M. and HAUSSLER, D. (2002). The human genome browser at ucsc. *Genome Res* 12: 996-1006.

KERKVLIET, C.M. and HINTERBERGER, T.J. (1997). Distal regulatory regions of the rat MRF4 gene. *Biochem Biophys Res Commun* 237: 170-176.

KROLL, K.L. and AMAYA, E. (1996). Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* 122: 3173-3183.

LEIBHAM, D., WONG, M.W., CHENG, T.C., SCHROEDER, S., WEIL, P.A., OLSON, E.N. and PERRY, M. (1994). Binding of TFIID and MEF2 to the TATA element activates transcription of the *Xenopus* Myoda promoter. *Mol Cell Biol* 14: 686-699.

LIM, W., NEFF, E.S. and FURLOW, J.D. (2004). The mouse muscle creatine kinase promoter faithfully drives reporter gene expression in transgenic *Xenopus laevis*. *Physiol Genomics* 18: 79-86.

LIN-JONES, J. and HAUSCHKA, S.D. (1996). Myogenic determination factor expression in the developing avian limb bud: An RT-PCR analysis. *Dev Biol* 174: 407-422.

MARGULIES, E.H., COOPER, G.M., ASIMENOS, G., THOMAS, D.J., DEWEY, C.N., SIEPEL, A., BIRNEY, E., KEEFE, D., SCHWARTZ, A.S., HOU, M. *et al.* (2007). Analyses of deep mammalian sequence alignments and constraint predictions for 1% of the human genome. *Genome Res* 17: 760-774.

MARTIN, B.L. and HARLAND, R.M. (2001). Hypaxial muscle migration during primary myogenesis in *Xenopus laevis*. *Dev Biol* 239: 270-280.

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.

NICOLAS, N., GALLIEN, C.L. and CHANOINE, C. (1998). Expression of myogenic regulatory factors during muscle development of *Xenopus*: Myogenin mRNA accumulation is limited strictly to secondary myogenesis. *Dev Dyn* 213: 309-321.

OFFIELD, M.F., HIRSCH, N. and GRAINGER, R.M. (2000). The development of *Xenopus* tropicalis transgenic lines and their use in studying lens developmental timing in living embryos. *Development* 127: 1789-1797.

PAUKERT, M., HIDAYAT, S. and GRUNDER, S. (2002). The P2X(7) receptor from *Xenopus laevis*: Formation of a large pore in *Xenopus* oocytes. *FEBS Lett* 513: 253-258.

PIN, C.L. and KONIECZNY, S.F. (2002). A fast fiber enhancer exists in the muscle regulatory factor 4 gene promoter. *Biochem Biophys Res Commun* 299: 7-13.

PIN, C.L., LUDOLPH, D.C., COOPER, S.T., KLOCKE, B.J., MERLIE, J.P. and KONIECZNY, S.F. (1997). Distal regulatory elements control MRF4 gene expression in early and late myogenic cell populations. *Dev Dyn* 208: 299-312.

POLLI, M. and AMAYA, E. (2002). A study of mesoderm patterning through the analysis of the regulation of xMyf-5 expression. *Development* 129: 2917-2927.

POWNALL, M.E., GUSTAFSSON, M.K. and EMERSON, C.P., JR. (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu Rev Cell Dev Biol* 18: 747-783.

SIEMERING, K.R., GOLBIK, R., SEVER, R. and HASELOFF, J. (1996). Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr Biol* 6: 1653-1663.

SIVE, H.L., GRAINGER, R.M. and HARLAND, R.M. (2000). *Early development of Xenopus laevis: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

SMITH, S.J., FAIRCLOUGH, L., LATINKIC, B.V., SPARROW, D.B. and MOHUN, T.J. (2006). *Xenopus laevis* transgenesis by sperm nuclear injection. *Nat Protoc* 1: 2195-2203.

SPARROW, D.B., CAI, C., KOTECHA, S., LATINKIC, B., COOPER, B., TOWERS, N., EVANS, S.M. and MOHUN, T.J. (2000). Regulation of the tinman homologues in *Xenopus* embryos. *Dev Biol* 227: 65-79.

SUMMERBELL, D., HALAI, C. and RIGBY, P. (2002). Expression of the myogenic regulatory factor MRF4 precedes or is contemporaneous with that of Myf5 in the somitic bud. *Mech Dev* 117: 331-335.

WONG, M.W., PISEGNA, M., LU, M.F., LEIBHAM, D. and PERRY, M. (1994). Activation of *Xenopus* myod transcription by members of the MEF2 protein family. *Dev Biol* 166: 683-695.

YANG, J., MEI, W., OTTO, A., XIAO, L., TAO, Q., GENG, X., RUPP, R.A. and DING, X. (2002). Repression through a distal TCF-3 binding site restricts *Xenopus* myf-5 expression in gastrula mesoderm. *Mech Dev* 115: 79-89.

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