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

TIMOTHY J. HINTERBERGER*
Biomedical Program, University of Alaska Anchorage, Anchorage, AK, USA

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-

Abbreviations used in this paper: GFP, green fluorescent protein; MRF, myogenic regulatory factor.
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).

![Diagram of MRF4 enhancer and promoter elements](image)

**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, BstXI; E, EcoRI; F, 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 XMRF4a start codon. –610 indicates the EcoRI site, –360 the Nco I site, –180 the BstXI 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), XMRF4 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 XMRF4 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 XMRF4 promoter for transgenic activity. Approximately 180 bp 5’ to the start codon of an X. laevis XMRF4 gene sufficed for transgenic expression in embryonic myotomes. A rat XMRF4 minimal promoter, containing a core sequence that is conserved in all vertebrate XMRF4 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 XMRF4 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 XMRF4 gene sequences. The mammalian, lizard and chicken XMRF4 genes also contain a MEF2 binding site (CTATTTAAA) that overlaps the TATA box; in X. laevis and X. tropicalis XMRF4 genes, the corresponding site (CTATAAAA) deviated at one nucleotide from a MEF2 consensus site [YTA(A/T)nTAR (Black and Olson, 1998)]. A 150-bp region flanking the TATA box and putative MEF2 site in the Xenopus XMRF4 genes displayed 71% identity with the corresponding region of the rat XMRF4 promoter (Fig. 1B). No E boxes were present within the conserved proximal 330-bp region of any of the three Xenopus XMRF4 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. tropicalis XMRF4.

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 XMRF4 as well. VISTA alignment with the X. tropicalis XMRF4 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 9613-bp XMRF4a sequence (Fig. 1A). No identity with the Xenopus XMRF4 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 pre-somite 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...
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
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 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. 4E,F). (Unlike the endogenous gamma-crystallin expression, 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 promoter gave expression in less than a third of cases (Table 2) 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. 6B,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 expression (Fig. 7B).
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, although 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., 2004).
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 XMFE2A 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 do 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 XMRF4a 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-myohtomal transgenic expression in some cases, albeit in a pattern that differed from that of endogenous XMRF4, which showed extrasomatic 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-myohtomal 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 intrinsic and 3' regulatory regions and the absence of a linked MMy5 promoter, which in transgenic mice results in abnormal XMRF4 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 XMRF4a610-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 NotI-PvuII fragment at the 5'-end of a X. 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-bp EcoRI 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 188563). 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-bp EcoRI-KpnI fragment containing 610 bp of sequence 5' to the start codon into pBluescript, a BamHI site was introduced just ahead of the start codon by PCR with proofreading polymerase (Vent, New England Biolabs). This 610-bp EcoRI-BamHI fragment was then used to generate shorter promoters, by restriction digestion at an NcoI site (–360 bp) or a BstXI site (–180 bp), and by PCR to introduce a KpnI 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 MyoD promoter (Leibham et al., 1994), was made by site-directed mutagenesis (MORPH kit, 5-prime-to-3-prime Inc., Boulder, CO). The mutagenic oligonucleotide (CAACAGTATATAAGCAGACGGG) differed from the wild-type sequence at the nucleotides shown in lower-case letters. Other promoter constructs were generated from the full-length XMRF4a cloning by using PCR with proofreading polymerase (Dynazyme, Finnzymes Oy., Espoo, Finland) to introduce an XhoI site just ahead of the start codon and a KpnI site at the 5' end of the clone. The resulting 9.5-kb product was inserted into a plasmid and subsequently digested with EcoR V, SacI, BanHI, or with Hind 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 KpnI and NotI and inserting it into pBluescript. XMRF4apromoter fragments were then inserted at the KpnI site. A mammalian MRF4 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 mediated 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 digamma-crystallin-GFP5 (Ofield 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 (1995). Luciferase reporter constructs for cell transfection were mediated by ligating the XMRF4a promoter fragments into pGL2-Basic (Promega). Procedural details are available on request. All mutants and constructs were verified by sequencing.

In situ hybridization probes for endogenous XMRF4 mRNA and for XMyoD 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 XMyoD was transcribed from a 470-bp EcoR I–PstI 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).

Acknowledgements

I thank Sylvia Evans and Rob Grainger for providing me with opportunities to learn frog transgenesis in their laboratories and for plasmids. Many of my students at the University of Alaska Anchorage contributed to aspects of this project. This work was supported by grants from NIH (HD35326) and from the Alaska BRIN program.

References


Further Related Reading, published previously in the *Int. J. Dev. Biol.*


**Myoskeletin, a factor related to Myocardin, is expressed in somites and required for hypaxial muscle formation in Xenopus**

Hui Zhao, Martha L. Rebbert and Igor B. Dawid

**Limb muscle development.**

Bodo Christ and Beate Brand-Saberi

**Involvement of myogenic regulator factors during fusion in the cell line C2C12.**

Stéphane Dedieu, Germain Mazères, Patrick Cottin and Jean-Jacques Brustis

**Control of the expression of the Mrf4 and Myf5 genes: a BAC transgenic approach**

JJ Carvajal, D Cox, D Summerbell, PWJ Rigby
Int. J. Dev. Biol. (2001) 45: S139-S140

**Targeted inactivation of myogenic factor genes reveals their role during mouse myogenesis: a review.**

H H Arnold and T Braun

*5 yr ISI Impact Factor (2008) = 3.271*