The mouse muscle creatine kinase promoter faithfully drives reporter gene expression in transgenic *Xenopus laevis*

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Lim, Wayland, Eric S. Neff, and J. David Furlow. The mouse muscle creatine kinase promoter faithfully drives reporter gene expression in transgenic *Xenopus laevis*. *Physiol Genomics* 18: 79–86, 2004. First published March 9, 2004; 10.1152/physiolgenomics.00148.2003.—Developing *Xenopus laevis* experience two periods of muscle differentiation, once during embryogenesis and again at metamorphosis. During metamorphosis, thyroid hormone induces both muscle growth in the limbs and muscle death in the tail. In mammals, the muscle creatine kinase (MCK) gene is activated during the differentiation from myoblasts to myocytes and has served as both a marker for muscle development and to drive transgene expression in transgenic mice. Transcriptional control elements are generally highly conserved throughout evolution, potentially allowing mouse promoter use in transgenic *X. laevis*. This paper compares endogenous *X. laevis* MCK gene expression and the mouse MCK (mMCK) promoter driving a green fluorescent protein reporter in transgenic *X. laevis*. The mMCK promoter demonstrated strong skeletal muscle-specific transgene expression in both the juvenile tadpole and adult frog. Therefore, our results clearly demonstrate the functional conservation of regulatory sequences in vertebrate muscle gene promoters and illustrate the utility of using *X. laevis* transgenesis for detailed comparative study of mammalian promoter activity in vivo.

transgenesis; frog; green fluorescent protein; gene expression

DURING MID-GASTRULATION of *Xenopus laevis* development, many mesodermal cells differentiate into muscle tissue (14, 33). Several muscle-specific genes are activated during the differentiation from myoblasts into myocytes (5, 16), including a muscle-specific isoform of creatine kinase (MCK) (5, 16). Muscle differentiation occurs on two occasions in *Xenopus* development, during embryogenesis and again at metamorphosis. Thyroid hormone initiates tadpole metamorphosis, causing adult limb muscle to develop prior to juvenile tissue resorption such as tail muscle (7). Near the climax of metamorphosis, tail muscle fibers enter apoptosis and fragment into sarcolytes, which are phagocytosed as the tail resorbs (28).

The mouse MCK (mMCK) gene has been extensively studied as a model for muscle-specific trancription (5, 9, 16, 35, 36). In addition, the mMCK promoter has been used to overexpress foreign genes in rodent muscle by transgenesis, adenoviral infection, or direct muscle injection (11, 18, 21, 26). The wild-type mMCK gene is expressed in adult mouse cardiac and skeletal muscle (5, 18, 23). The mMCK promoter comprises a 358-bp proximal regulatory region and a 206-bp enhancer ~1 kb upstream of the transcription start site (1, 17, 34, 35). There are six regulatory transcription elements within the upstream enhancer: CArG, AP2, A/T rich, Left and Right E boxes, and MEF-2 (1, 4, 25). These regulatory elements and transcription factors have been highly conserved throughout mammalian evolution (15, 17, 37, 40). Cell culture studies using mutated E boxes within the MCK enhancer revealed altered expression in skeletal muscle myocytes but not cardiomyocytes (1). Conversely, mutation of the CArG site affects cardiomyocytes, but not skeletal muscle myocytes. The MCK enhancer A/T rich site is important for expression in both myocyte types. Mutation of the three E boxes decreased or abolished expression in all muscle types except fast muscles (34). Experiments with transgenic mice using the wild-type mMCK promoter show stronger expression in skeletal muscle than cardiac muscle (8). Since few mammalian promoters have been explored for use in transgenic *X. laevis*, the mMCK promoter may be useful for muscle-specific expression in *Xenopus* throughout embryonic, larval, and adult development (2, 3, 13, 24, 31).

This study compares endogenous *X. laevis* MCK (xMCK) expression with reporter gene fluorescence driven by the mMCK promoter in transgenic *X. laevis*. Both the mMCK promoter and the native xMCK gene have similar expression patterns in the embryo and metamorphosing tadpole. Neural transgene expression was only detected in early tadpole stages. The mMCK promoter had similar transgene expression in the tadpole compared with the *Xenopus* cardiac actin promoter. However, unlike the cardiac actin promoter, the mMCK promoter showed robust activity in the adult frog as well as reduced cardiac muscle transgene expression. Thus the mMCK promoter appears to be an excellent muscle-specific promoter for transgene expression throughout development into adulthood. Our studies demonstrate the functional conservation of gene regulatory sequences across vertebrate phylogeny and the potential for using the *Xenopus* system for detailed comparative studies of the mechanisms underlying developmental gene regulation.

MATERIALS AND METHODS

Cloning. 1256mMCK was excised from pJECAT 1256 MCKAT (gift of Jean Buskin and Stephen Hauschka, University of Washington, Seattle, WA) and inserted into pCS GFP3 (Gift of Enrique Amaya, Wellcome/CRC Institute, Cambridge, UK) using *HinDIII* and *EcoRI* restriction enzymes to create pCS 1256MCK GFP3. The cytomegalovirus promoter was then excised by digesting and Klenow filling *SalI* and *HindIII* restriction enzyme sites, creating plasmid pS 1256MCK GFP3. An early translation initiation site between the MCK promoter and GFP3 was removed by digesting and Klenow filling with *BstEII* and *EcoRI*, creating p1256MCK GFP3.

*Xenopus* MCK cDNA was cloned by searching GenBank using mMCK gene sequences. Two cDNA clones (4680019 and 4408570) with the highest homology in the predicted open reading frame (ORF)
were obtained from the IMAGE Consortium (Research Genetics). The two clones were sequenced in both directions by the University of California at Davis DNA sequencing facility. Clone 4408570 contains 3/MCK ORF sequence. Clone 4680019 contains the complete xMCK cDNA and provided 5/MCK ORF sequence along with the identical sequence provided by the previous clone.

Northern analysis and whole mount in situ hybridization. For Northern analysis, total RNA was extracted from stages 54 and 60 tadpole tissues using TRIzol reagent (Invitrogen) according to manufacturer’s specifications. Tissues were homogenized with a Tekmar Tissumizer and sheared with a 21-gauge needle three times. Probes were synthesized from the Xenopus MCK cDNA clone 4408570 and the Xenopus ribosomal protein L8 cDNA (rpL8) using Random Primers DNA Labeling System (Invitrogen) per the manufacturer’s instructions. Labeled probe was purified from free nucleotide using ProbeQuant G-50 Micro columns (Amersham Pharmacia). Northern analysis was performed as described previously (39).

Whole mount in situ probe synthesis and hybridization procedure was performed as previously described (12). In situ probes were synthesized from the Xenopus MCK cDNA clone 4408570 using the DIG RNA labeling kit (Roche Molecular Biochemicals) with either T7 or SP6 RNA polymerase for sense and antisense transcripts, respectively, per the manufacturer’s instructions. Probes were purified from free nucleotide as described above and were visualized by polyacrylamide gel electrophoresis.

Transgenesis. Transgenic animals were created as previously described with the exclusion of egg extract (19). Plasmid p1256mMCK GFP3 and pCarGFP2 (3.27 kb upstream cardiac actin promoter driving GFP; gift of Enrique Amaya, Wellcome/CRC Institute, Cambridge, UK) were linearized with NotI restriction enzyme (New England Biolabs). Animals were raised in 0.1/11000 Marc’s modified Ringer solution (MMR) (19). To create F1 generation transgenic X. laevis, we first determined the temporal and spatial expression of the endogenous xMCK gene by whole mount in situ hybridization. Early embryonic xMCK in situ hybridizations show somite MCK expression along the dorsal ridge (Fig. 3C). At stage 30 (tail bud), xMCK expression is

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RESULTS

X. laevis and mMCK amino acid sequences are highly conserved. Incomplete X. laevis cDNA fragments have previously been reported in the literature to have a high degree of similarity to human muscle creatine kinase (22, 29). This similarity is conserved with the full-length clone. Translation of the predicted ORF from the obtained full-length xMCK clone revealed an 85% amino acid homology and a greater than 70% nucleotide homology compared with mMCK (Fig. 1). Using the cDNA as a probe on Northern blots of total RNA from tadpole tissues before (stage 54) and at the climax (stage 60) of metamorphosis, we detected a single band of 1.6 kb in limb and tail but not in brain or intestine (Fig. 2). The size of the xMCK mRNA corresponds closely to the size of the isolated xMCK cDNA clone.

Activation of the mMCK promoter in transgenic Xenopus closely follows the expression of the endogenous xMCK gene. To determine how faithfully the mMCK promoter is regulated in transgenic X. laevis, we first determined the temporal and spatial expression of the endogenous xMCK gene by whole mount in situ hybridization. Early embryonic xMCK in situ hybridizations show somite MCK expression along the dorsal ridge (Fig. 3C). At stage 30 (tail bud), xMCK expression is

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![Fig. 1. Predicted Xenopus muscle creatine kinase (xMCK) amino acid sequence and comparison with mouse MCK (mMCK) amino acid sequence. Identical amino acids are highlighted in black; conservative amino acids are highlighted in gray.](http://physiolgenomics.physiology.org/)

![Fig. 2. Stage 54 and 60 northern analysis of xMCK and ribosomal protein L8 (rpL8). Top: blot hybridized with an xMCK cDNA probe shows similar stage 54 and 60 tail expression and strong upregulation between stage 54 and 60 in limb tissue. Bottom: blot hybridized to an rpL8 cDNA probe as a loading control.](http://physiolgenomics.physiology.org/)
specifically localized in dorsal skeletal muscle. We next created transgenic *X. laevis* embryos by integrating a fragment of the 1256mMCK promoter driving the expression of green fluorescent protein (GFP). GFP fluorescence is detected in a pattern virtually identical to the endogenous xMCK gene, in both early and late embryonic stages (Fig. 3, B and F). Over 100 positive transgenic embryos were examined at least through swimming tadpole stages during the course of multiple transgenesis experiments. All animals showed the same pattern of transgene expression, with differences observed in the level of expression but not pattern of expression as expected for the F0 generation. Therefore, animals shown in Figs. 3–8 are representative of the transgene expression patterns observed.

In addition to skeletal muscle expression, low but detectable neural GFP fluorescence is detected during embryogenesis and continues through stage 48 in transgenic animals with the 1256mMCK promoter. This promoter induces detectable GFP fluorescence in the midbrain (mesencephalon and diencephalon), low levels in the hindbrain, and no fluorescence in the forebrain (Fig. 4). The *X. laevis* cardiac actin promoter, a promoter that has been previously used to express transgenes in skeletal muscle, also shows similar neuronal transgene expression in the midbrain (Fig. 4C). Neural GFP fluorescence driven by the 1256mMCK promoter gradually decreased and was not detectable in premetamorphic tadpoles (Fig. 4D), consistent with xMCK Northern analysis at these stages (Fig. 2).

The mMCK promoter is active in tadpole skeletal muscle through metamorphosis. Examination of stage 53, premetamorphic, animals shows GFP fluorescence in all mature skeletal muscle (Fig. 5), including facial, ocular, and tail musculature. A tail cross section clearly shows GFP fluorescence restricted to muscle fibers and not in fins or notochord (Fig. 5D). mMCK induces robust lateral fast tail muscle GFP fluorescence and weak slow muscle cord fluorescence (Fig. 5E). The cardiac actin promoter shows similar fast tail muscle activity but also very robust slow muscle cord fluorescence (Fig. 4H). As the tadpole enters metamorphosis, thyroid hormone induces the growth of adult limbs. Stage 53 animals
show no detectable GFP fluorescence in the growing limbs, and Northern blot analysis shows very low levels of limb xMCK expression at stage 54 (Fig. 2). GFP fluorescence is first detected in limbs of mMCK transgenic animals at stage 55 (Fig. 5H). This view clearly shows distinct lower hindlimb muscle fluorescence in the gastrocnemius and peroneus along with the quadriceps in the upper hindlimb. The forelimbs are still differentiating beneath the opercular epithelium at this stage, although GFP fluorescence is clearly detectable in this tissue (not shown).

Tadpoles have three-chambered hearts with two atria and one ventricle. As expected, stage 47 transgenic Xenopus cardiac actin promoter animals showed robust ventricular and outflow tract GFP fluorescence (Fig. 6A). The thin walls of the atria may preclude accumulation of GFP to detectable levels. In contrast, stage 47 transgenic 1256mMCK promoter animals showed very weak ventricular GFP fluorescence and no detectable outflow tract or atrial fluorescence, even in the tadpoles with the highest level of skeletal muscle expression (Fig. 6B). Therefore, in our hands the mMCK promoter appears to be more specific to fast skeletal muscle expression than the more commonly used Xenopus cardiac actin promoter.

Upon the completion of adult limb growth during metamorphosis the tadpole begins resorbing the tail. mMCK promoter-driven tail GFP fluorescence begins early and remains high until metamorphic climax at stage 60. The distal tail of mMCK promoter driving GFP transgenic animals showed fragmented fluorescence during tail resorption as a result of intact fibers surrounded by fibers that are missing or have diminished GFP fluorescence (Fig. 7A). This fragmented pattern spreads toward the proximal tail, resulting in complete loss of tail GFP fluorescence by stage 63 (Fig. 7B). Cardiac actin promoter experiments show identical temporal and spatial fluorescence fragmentation during metamorphosis (6).

The mMCK promoter is active in juvenile frog skeletal muscle and the F1 generation. Upon completion of metamorphosis, juvenile frog skeletal muscle shows strong GFP fluorescence driven by the mMCK promoter (Fig. 8). Strong GFP fluorescence is observed in all skeletal muscle, even in adult frogs as old as 18 mo of age. To determine whether the
1256mMCK promoter is active in the F1 generation, eggs from a sexually mature 1256mMCK-positive female were fertilized with sperm from a wild-type male. Approximately 70% of the offspring showed robust GFP fluorescence in a skeletal muscle-specific manner. Since greater than 50% of offspring were positive for the inherited transgene, we infer that there was more than one chromosomal integration site in the founder animal, as is the case for other examples of transgene inheritance in *X. laevis* (24).

**DISCUSSION**

MCK gene regulation has been well conserved throughout evolution. In transgenic *X. laevis*, mMCK promoter activity closely mirrors endogenous xMCK gene expression. Therefore mMCK appears to be a candidate promoter for tracing muscle development. The mMCK promoter begins inducing transgene expression in the embryo in differentiating myoblasts. It becomes fully active in juvenile skeletal muscle with relatively weak cardiac muscle expression. Muscle differentiation occurs at two developmental stages: embryogenesis and metamorphosis. During metamorphosis, adult tissues such as the limbs grow before death of larval tissues such as the tail. In the limbs, mMCK promoter activity is first detected in stage 55 tadpoles. At this stage many muscles have either or both origins and insertions established (27). The foot musculature is also well defined at this stage. Before stage 55, little to no limb movement is detected, consistent with the lack of transgene expression. By stage 58, all the muscles except the terminal phalanges have completed development, and all limb muscles show robust GFP fluorescence.

Once the adult limbs have been fully developed for locomotion, the metamorphosing tadpole will initiate tail resorption. Myofibers expressing GFP began fragmenting at stage 60, at the climax of metamorphosis. This coincides with the beginning of observed muscle apoptosis. Muscle fiber fragmentation progresses from the distal tail toward the proximal tail. Sachs et al. (32) observed the same phenomenon of distal-to-proximal tail muscle fragmentation coincident with *Bax* induction. In contrast to our findings and those of Sachs et al. (32), Nishikawa and Hayashi (28) reported the opposite phenomenon of proximal-to-distal tail muscle death using a TUNEL assay. All tail movement and mMCK-driven GFP fluorescence is lost by stage 63. The drastic decrease in GFP fluorescence between stages 60 and 63 correlates with the greatest apoptotic cell increase in the tail and fast muscle fiber loss (10, 32). Interestingly, we observed low and variable GFP fluorescence in the dorsal tail cords described by Elinson et al. (10), indicating that the mMCK promoter is less active in
slow-type muscle fibers. Das et al. (6) visualized the loss of fast muscle GFP fluorescence in stage 63 tadpoles and persisting slow muscle fibers until stage 64 in cardiac actin promoter transgenic tadpoles. Clearly mMCK GFP fluorescence is very weak in slow muscle fiber cords, and no fluorescence is visible by stage 63 in our 1256mMCK animals.

We observed transient midbrain transgene expression from the 1256mMCK promoter in addition to strong skeletal muscle activity. Previous transgenic mouse studies with this promoter did not report neural activity, although these animals were examined postnatally. In situ hybridizations in X. laevis embryos did not confirm xMCK neural expression, but the mRNA could have been below the assay detection levels. A few papers have identified nonmuscle MCK expression in human and rat brain (18, 30, 38, 41). Xenopus cardiac actin promoter activity mirrored the mMCK promoter activity, indicating the presence of a common transient nonmuscle transcription factor activity in early development. Since all neuronal GFP expression is abolished in premetamorphic tadpoles, transient mMCK promoter activity in the mouse brain may have been missed in previous transgenic studies and may require further study.

The mMCK promoter has many potential uses for decoding gene function. X. laevis has a tetraploid genotype that limits functional analysis to overexpression of dominant negative proteins or ribozyme and RNA interference (RNAi)-based gene approaches. The strong ubiquitous cytomegalovirus promoter is the most common currently used promoter for these studies. Thus there is a need for the characterization of multiple cell- and developmental stage-specific promoters. Currently there are very few characterized Xenopus promoters. The Xenopus cardiac actin promoter is a cardiac and skeletal muscle-specific promoter that has been used in transgenic experiments. Although the cardiac and skeletal muscle regulatory components of the cardiac actin promoter have been identified, it is difficult to evaluate the degree that promoter mutations designed to reduce cardiac expression also affect

Fig. 6. Cardiac mMCK and Xenopus cardiac actin promoter activity. A: ventral stage 47 transgenic Xenopus cardiac actin GFP tadpole expressing strong expression in the ventricle (V) and outflow tract (O) (also known as conus arteriosus, conotruncus, and truncus arteriosus). B: ventral stage 49 transgenic 1256mMCK GFP tadpole with weak ventricle (V) expression. C: Light image of heart region of animal shown in B. *Light below the ventricle is an artifact of the reflective abdominal tegument.

Fig. 7. Tail resorption observed in 1256mMCK GFP transgenic tadpoles. A: lateral fluorescent image of stage 60 1256mMCK promoter driving GFP transgenic tadpole. The proximal tail is on the left. B: dorsal fluorescent image of stage 63 1256mMCK GFP transgenic animal. HL, hindlimb; T, tail.
skeletal muscle expression (20). Second, the apparently unde-
detectable cardiac actin expression in adult Xenopus compared
with the mMCK promoter compromises its utility for expres-
sion studies throughout development. Further studies may
identify cardiac actin promoter regulatory elements not present
in this 3.27-kb promoter fragment necessary for adult expres-
sion. The mMCK promoter can be used to overexpress wild-
type or mutant proteins specifically in Xenopus skeletal mus-
cle, as has been demonstrated in transgenic mice (21). The
Xenopus system also offers the advantage of providing a much
larger number of transgenic animals at a fraction of the cost of
similar studies in mice. In addition, transgenes are active in F1
and F2 generations in X. laevis (24). Although the F0 genera-
tion, as shown in this paper, shows varying absolute expression
levels due to differences in transgene number and insertion
site, one can easily determine the spatial and temporal activity
of a promoter in question.

Thus X. laevis proves to be an excellent vertebrate model
organism for examining promoter activity in vivo, including
those promoters derived from mammalian genes. Properties
of the X. laevis system, such as complete external develop-
ment and a simplified transgenesis method, make it an ideal
system for examining transgene expression during develop-
ment from egg to adult. As the genomes of more organisms
are sequenced, potential enhancer sequences may be iden-
tified by regions of sequence similarity. These regions can
be tested for tissue-specific and developmental stage-spe-
cific activity in a transgenic model such as we have explored
here. Other mammalian tissue-specific promoters are cur-
rently being evaluated by our laboratory and others. Adap-
tation of the technique to the diploid X. laevis relative X.
tropicalis also offers a much shorter generation time for
ease of creating transgenic lines and the distribution of these
lines to investigators interested in the control of muscle and

Fig. 8. 1256mMCK promoter driving GFP transgene expression in adult X. laevis and F1 generation offspring. Light (A) and
fluorescent images of dorsal (B) and ventral (C) stage 66 transgenic X. laevis are shown, just after the completion of metamorphosis.
D and E: light and fluorescent images, respectively, of F1 offspring from the animal in A–C. Note robust tail skeletal muscle
fluorescence in two of the four sibling animals shown. Autofluorescence accounts for the signal observed in the digestive tract of
the siblings lacking GFP fluorescence in tail muscle.
other tissue-specific gene expression programs common to all vertebrates.

REFERENCES


