Published in final edited form as:
J Exp Biol. 2008 July ; 211(Pt 13): 2172–2184. doi:10.1242/jeb.016592.

Expression of myogenic regulatory factors in the muscle-derived electric organ of *Sternopygus macrurus*

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SUMMARY

In most groups of electric fish, the current-producing cells of electric organs (EOs) derive from striated muscle fibers but retain some phenotypic characteristics of their precursor muscle cells. Given the role of the MyoD family of myogenic regulatory factors (MRFs) in the transcriptional activation of the muscle program in vertebrates, we examined their expression in the electrocytes of the gymnotiform *Sternopygus macrurus*. We estimated the number of MRF genes in the *S. macrurus* genome and our Southern blot analyses revealed a single *MyoD*, *myogenin*, *myf5* and *MRF4* gene. Quantitative RT-PCR showed that muscle and EO transcribe all MRF genes. With the exception of *MyoD*, the endogenous levels of *myogenin*, *myf5* and *MRF4* transcripts in electrocytes were greater than those detected in muscle fibers. These data indicate that MRF expression levels are not sufficient to predict the level to which the muscle program is manifested. Qualitative expression analysis of MRF co-regulators MEF2C, Id1 and Id2 also revealed these genes not to be unique to either muscle or EO, and detected similar expression patterns in the two tissues. Therefore, the partial muscle program of the EO is not associated with a partial expression of MRFs or with apparent distinct levels of some MRF co-factors. In addition, electrical inactivation by spinal cord transection (ST) resulted in the up-regulation of some muscle proteins in electrocytes without an accompanying increase in MRF transcript levels or notable changes in the co-factors MEF2C, Id1 and Id2. These findings suggest that the neural regulation of the skeletal muscle program *via* MRFs in *S. macrurus* might differ from that of their mammalian counterparts. Together, these data further our understanding of the molecular processes involved in the plasticity of the vertebrate skeletal muscle program that brings about the muscle-like phenotype of the non-contractile electrogenic cells in *S. macrurus*.

Keywords

electric organ; myogenic regulatory factor; electrical activity; skeletal muscle; electric fish

INTRODUCTION

The wide range in contractile, biochemical and morphological properties among muscle fibers is a common feature of skeletal muscle in virtually all animal species (Burke, 1981; Talmadge et al., 1993). During development, this muscle fiber heterogeneity is largely regulated by cell lineage (Miller and Stockdale, 1986; Stockdale, 1992). However, it has been demonstrated that, once established, differentiated muscle fibers are not fixed but are highly capable of changing their phenotype in response to changes in external factors to meet the altered

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functional demands. It is well documented that the innervating motoneurons play a key role in the maintenance and plasticity of the differentiated muscle phenotype including fiber size, metabolic profile and contractile properties (Buller et al., 1960; Buonanno et al., 1998; Schiaffino et al., 1999). Numerous studies have demonstrated that the neural influence is exerted largely through the electrical activation pattern imposed on the muscles (Gundersen, 1998; Lømo, 1974; Pette and Staron, 1990; Schiaffino and Reggiani, 1996). However, the specific signaling pathways and regulatory molecules that link electrical activity to changes in muscle-specific gene expression remain largely unknown.

Skeletal muscle gene expression is controlled by a complex group of regulators that derive from one of two major families of transcription factors. The MyoD family of myogenic regulatory factors (MRFs) consists of MyoD, myogenin, myf5 and MRF4 (Emerson, 1990; Weintraub, 1993). MRFs encode structurally related, sequence-specific transcription factors that bind to E-box consensus promoter elements (CANNTG) (Emerson, 1990; Fujisawa-Sehara et al., 1992; Weintraub, 1993) and are essential for the transcriptional activation of many muscle-specific genes during differentiation of skeletal muscle (Buckingham, 1994; Olson, 1990). Once differentiation is complete, MRFs continue to be expressed, although at very low levels (Buonanno et al., 1992; Eftimie et al., 1991; Weis et al., 2000; Wright et al., 1989). The MRF expression patterns can be up-regulated in adult muscles following changes in electrical activation patterns or complete removal of motoneuron synaptic input (Buonanno et al., 1992; Eftimie et al., 1991; Voytik et al., 1993; Weis et al., 2000). Such alterations in neural input lead to changes in MRF expression levels that often precede changes in the transcription of known target muscle genes (Buonanno and Fields, 1999; Voytik et al., 1993; Witzemann and Sakmann, 1991). Hence, these studies have implicated MRFs as key mediators in the regulation of muscle-specific proteins by neural input (Emerson, 1990). Furthermore, MRFs are known to be under the control of both positive and negative regulatory factors. Among these factors, Id proteins act as negative regulators, whereas MEF2 proteins are reported to enhance MRF induction of muscle-specific genes (for a review, see Puri and Sartorelli, 2000). The isolation of clearly defined subsets of muscle-specific proteins, however, would greatly facilitate the study of the mechanism(s) by which the network of regulation of MRFs might mediate the neural regulation of the skeletal muscle program.

To study the roles that MRFs play in regulating select protein components of the muscle program, we used a novel but simple system - the phenotypic plasticity between muscle and the muscle-derived electric organ (EO) in the electric fish *Sternopygus macrurus*. In *S. macrurus*, some skeletal muscle fibers fully differentiate only to undergo an extreme phenotypic conversion into the non-contractile, electrogenic cells of the EO. The mature electrocytes share many, but not all, cellular features with muscle fibers by maintaining the expression of only a partial myogenic program. For example, mature electrocytes are multinucleated similar to their muscle precursors, but do not have sarcomeres or T-tubules (Patterson and Zakon, 1996; Unguez and Zakon, 1998a). Electrocytes also express desmin, actin, sarcomeric α-actinin, and acetylcholine receptors, but they do not contain myosin, tropomyosin or troponin-T (Cuellar et al., 2006; Patterson and Zakon, 1996; Unguez and Zakon, 1998a). Similar to skeletal muscle fibers, the mature phenotype of electrocytes is influenced by neural input (Unguez and Zakon, 1998b). Electrocytes are innervated by electromotoneurons (EMNs), which exert a continuous activation pattern that ranges from 50 to 200 Hz depending on sex (Mills et al., 1992). Removal of this activation by spinal cord transection (ST) results in the formation of sarcomeres *de novo* and detection of sarcomeric proteins such as myosin and tropomyosin (Unguez and Zakon, 1998b). Hence, manipulation of the EO phenotype provides an excellent model to study the neural regulation of clearly defined subsets of muscle proteins.

In this study, we examined whether enhancement of the muscle program in electrocytes by electrical inactivation is due to an expression pattern of MRFs or their co-regulators that correlates with the level and number of muscle proteins that are up-regulated after ST. We show that both muscle and EO transcribe all four MRFs and that the EO contains higher levels of *myogenin*, *myf5* and *MRF4* transcripts than skeletal muscle. Further, ST resulted in the up-regulation of some muscle proteins in electrocytes without an accompanying increase in MRF transcript levels. In addition, our expression studies did not reveal expression patterns of *MEF2C* and *Id* transcripts that were tissue specific or inactivity dependent. These data contribute to our understanding of the expression of MRFs and the plasticity of the vertebrate skeletal muscle program that brings about the muscle-like phenotype of the non-contractile electrogenic cells in *S. macrurus*.

MATERIALS AND METHODS

Animal tissue preparation

S. macrurus (L.) is native to South America and was obtained commercially from Segrest Farms (Gibsonton, FL, USA). Adult fish, 20-35 cm in length, were housed individually in 57-76 l aerated aquaria maintained at 25-28°C, and fed three times a week. Fish were overdosed using 2-phenoxyethanol (1:500) and the liver, brain, ventral skeletal muscle and EO were excised. Tissues were immediately immersed in RNA*later*™ (Ambion, Austin, TX, USA) and stored at -80°C until RNA extraction. All procedures used in this study followed the American Physiological Society Animal Care Guidelines and were approved by the Animal Use Committee at New Mexico State University.

Spinal cord transection surgery

Adult fish were anesthetized with 2-phenoxyethanol (750 μl l^{-1}). An incision (~3 cm) was made on the dorsal side at the level of the pectoral fin. A partial laminectomy was performed to expose the spinal cord. The spinal cord was cut with a scalpel and complete transection was verified visually under a stereoscope. In one sham experiment, a partial laminectomy was performed, but the spinal cord was left intact. Wounds were sutured and treated with a topical antibiotic (nystatin and triamcinolone acetonide ointment USP, E. Fougeranco and Co., Melville, NY, USA). Fish were monitored until fully recovered from anesthesia in their tanks. Stress Coat® (Aquarium Pharmaceuticals, Inc., Chalfont, PA, USA) was added to the tanks as an additional anti-infection agent.

To test for functional electrical inactivation of the EO by ST, the EO discharge (EOD) was assessed three times a week. Only those fish that did not have detectable EOD from the day of ST to the end of the 5 week survival period were used for further analyses. The 5 week survival period was chosen based on previous reports that some sarcomeric proteins are re-expressed in clusters within the cytoplasm of most electrocytes (Unguez and Zakon, 1998b). Five weeks after ST, fish were re-anesthetized and the distal 5-6 cm segment of the tail was amputated (~25% of total fish length). The most proximal centimeter of the excised tail segment was frozen on cork with Tissue Freezing Medium™ (TBS, Durham, NC, USA) in isopentane chilled in liquid nitrogen and used for immunohistochemical analyses. The remaining tail segment was dissected to separate muscle from EO and each tissue was immediately submerged in RNA*later* and stored at -80°C. The EOD continued to be monitored after tail amputation to determine the time at which it reappeared and, hence, indicate regeneration of spinal axons to innervate electrocytes.

RNA isolation

Total cellular RNA was isolated from adult skeletal muscle, 5 week ST muscle and liver using a protocol described previously (Kim et al., 2004). To remove residual DNA, total RNA

isolated from each tissue used for RT-PCR experiments was treated with either DNase I, amplification grade (Invitrogen, Carlsbad, CA, USA; RT-PCR) or TURBO DNA-*free*™ (Ambion; real-time RT-PCR) and analyzed by spectrophotometry (OD_{260}/OD_{280}). On average, total RNA isolations yielded 1-3% of starting material from each of the different tissues.

Cloning and isolation of *S. macrurus myf5* and *MRF4*

Heterologous degenerate oligonucleotide primers were designed for *myf5* and *MRF4* based on the respective GenBank vertebrate sequences using CODEHOP (Rose et al., 1998). Alignments used to generate *myf5* primers corresponding to the CSDEDEHVRA (sense primer) and the QVENNYYSLPG (antisense primer) domains included protein sequences from zebrafish (accession number NP_571651.1), puffer fish (accession number CAC39208.1), mouse (accession number NP_032682.1), human (accession number NP_005584.1), striped bass (accession number AF463525_1), carp (accession number BAA33566.1), chicken (accession number S41126), red-spotted newt (accession number Q91154), *Xenopus tropicalis* (accession number AAL11024.1), *Xenopus laevis* (accession number P24700) and cow (accession number P17667). *MRF4* primers corresponding to the SSGDEHVLA (sense primer) and the HWKKTCNTW (antisense primer) domains were designed using alignments from protein sequences of zebrafish (accession number AAQ67704), and two different sequences from puffer fish (accession numbers AAR20812 and CAC39207).

To clone partial *myf5* and *MRF4* cDNAs from *S. macrurus*, we carried out reverse transcription (RT) and PCR separately. The RT reaction from 250-300 ng total RNA was performed using the SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen) following the manufacturer's specifications. PCR amplification of the newly generated cDNAs (0.5-1 μg of cDNA) was carried out using a touchdown PCR module to increase amplification specificity with either Platinum® *Taq* DNA polymerase (*MRF4*; Invitrogen) or Advantage™ 2 PCR enzyme system (*myf5*; BD Biosciences, Palo Alto, CA, USA). Following an initial 2 min denaturation at 94°C, the touchdown PCR profile included eight cycles of 30s denaturation at 94°C, 30 s primer annealing at 64-68°C (depending on the primers) and 60 s elongation at 72° C. Further PCR amplification was performed in the same reaction tube immediately following the touchdown PCR step, and this profile consisted of 20-28 cycles of 30 s denaturation at 94° C, 30 s primer annealing at 63-64°C (depending on the primers) and 60 s elongation at 72°C, and finishing with a final elongation step at 72°C for 7 min. RT and PCR conditions (primer concentrations, input RNA, cycling conditions) were initially optimized and these were identical for all samples.

RT-PCR products for *myf5* and *MRF4* were PCR purified using the QIAquick® PCR purification kit (Qiagen, Valencia, CA, USA), subcloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA), and transformed into JM109 high efficiency competent cells (Promega). Plasmids from 10-20 cDNA clones of each transcript were isolated using the QIAprep® spin miniprep kit (Qiagen), sequenced in both directions using an Applied Biosystems (Foster City, CA, USA) automated DNA sequencer (Model 3100) and analyzed using the Vector NTI suite 8.0 software (InforMax, Inc., Bethesda, MD, USA).

Upon verification of the cloned sequences obtained from the EO, homologous primers for each transcript specific to *S. macrurus* were generated from the on-line Primer3 software program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Qualitative RT-PCR experiments were performed on skeletal muscle, EO, liver and brain following similar RT and PCR parameters to those described above. RT-PCR experiments using *S. macrurus*-specific *myf5* (YGLPAES, sense; SSIVDRL, antisense) and *MRF4* (HCEGQC, sense; CSAKDHS, antisense) primers resulted in partial sequences of 209 bp and 301 bp, respectively. The cloned partial sequences from *myf5* and *MRF4* were used for 3′- and 5′-rapid amplification of cDNA

ends (RACE) experiments (see below). Qualitative RT-PCR experiments also included *MyoD* and *myogenin* using homologous primers corresponding to the cysteine-rich (WACKACK; sense primer) and serine-rich (SSPRSNC; antisense primer) domains of *S. macrurus MyoD*, and the MNPNQRL (the region between the first and second helix domains; sense primer) and MRSLTSIV (antisense primer) domain of *S. macrurus myogenin* (Kim et al., 2004). PCR products were run out on a 1-2.5% agarose gel stained with ethidium bromide (10 μl/100 ml). To monitor DNA contamination in all experiments, control reactions were performed without the cDNA template or reverse transcriptase enzyme. To ensure consistency in reaction loading and execution, qualitative RT-PCR experiments using *S. macrurus*-specific glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were carried out (Kim et al., 2004).

The full-length coding sequences of both *myf5* and *MRF4* were obtained by RACE prepared from total RNA (300 ng to 1 μg) of EO from *S. macrurus* using a protocol adapted from Chen and Soriano for amplification of the 3′ end of *myf5* (Chen and Soriano, 2003) and the SMART™ RACE cDNA amplification kit (BD Biosciences, San Jose, CA, USA) for amplifying the 5′ end of *myf5*, and the 3′ and 5′ ends of *MRF4*. RACE first-strand synthesis was performed using oligo-ligated mRNA for 3′ and 5′ amplifications. *myf5*-specific primers for 3′-RACE included 5′-CCCGACAGCCTCCACCAG-3′ and 5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTGAAGCTTTTTTTTTTTTTTTT-3′, and for 5′-RACE were 5′-GCTCTCCGCAGGCAGGCCATAGTA-3′ and 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′. *MRF4*-specific primers for 3′-RACE were 5′-GGACTTGTTGCACACCCTGGACGAG-3′ and 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′, and for 5′-RACE were 5′-TTGAGTCGTCTTCTCTCCCGCAGAGTG-3′ and 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′. Purified products from *myf5* and *MRF4* RACE experiments were subcloned into the pCR® 2.1-TOPO® vector (Invitrogen), transformed into One Shot® MACH1™ T1 phage-resistant chemically competent *E. coli* cells (Invitrogen), isolated with the QIAprep spin miniprep kit, and sequenced in both directions using either a Li-Cor-4200L Global IR2 DNA (Lincoln, NE, USA) or an Applied Biosystems automated DNA sequencer (Model 3100).

Nucleotide and deduced amino acid sequences were analyzed using the Vector NTI Suite 8.0 (InforMax, Inc.). Searches were performed using the Basic Local Alignment Search Tool (BLAST) network service from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were generated with ClustalW from the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw). The two novel sequences described here have been submitted to the DNA databases with accession numbers DQ016032 and DQ059552 for *S. macrurus myf5* and *MRF4*, respectively.

Cloning and isolation of *S. macrurus Id* and *MEF2* genes

Heterologous degenerate oligonucleotide primers for *Id1*, *Id2* and *Id4* were also generated using CODEHOP (Rose et al., 1998). *Id1* primers were designed based on alignments from human (accession number AAH00613.1), rat (accession number NP_036929.1), mouse (accession number AAH25073.1) and trout (accession number CAA69656.1), and correspond to the QQMNVFLYDMN (sense primer) and the IDYIWDLQL (antisense primer) domains. *Id2* primer alignments included trout (accession number CAA69657.1), zebrafish (accession number AAH56303.1), rat (accession number NP_037192.1), human (accession number NP_002157.2), mouse (accession number AAH06921.1) and *Xenopus laevis* (accession number AAH41527.1), and correspond to the SKTPVDDP (sense primer) and the DYILDLQI (antisense primer) domains. *Id4* primers were designed using alignments from mouse (accession number NP_112443.1), human (accession number AAH14941.1), *Xenopus laevis*

(accession number AAP34250.1), horse (accession number NP_989613.1) and rat (accession number NP_783172.1), and correspond to the ALCLQCDMN (sense primer) and the DYILDLQL (antisense primer) domains.

Partial *Id1*, *Id2* and *Id4* cDNAs from *S. macrurus* were cloned using an RT-PCR approach similar to that used to clone *myf5* and *MRF4* (see above). Upon verification of the cloned sequences obtained from adult skeletal muscle, homologous primers for *Id1* and *Id2*, but not *Id4*, specific to *S. macrurus* were generated using Primer3. Homologous primers for *MEF2C* were also generated using Primer3 based on a cDNA sequence isolated from an *S. macrurus* EO cDNA library. RT-PCR experiments using *S. macrurus*-specific *Id1* (VPTLPPNK, sense; AAASDADE, antisense) and *Id2* (LYNMNDLYS, sense; KMEILQHV, antisense) primers amplified partial sequences of 304 bp and 101 bp, respectively. *Id4* was detected only in brain tissue of *S. macrurus* using heterologous degenerate primers. RT-PCR experiments with *S. macrurus*-specific *MEF2C* primers (SWLLVS, sense; PSAIST, antisense) amplified a partial sequence of 265 bp.

Quantification of myogenic regulatory factors in skeletal muscle and EO

Primer and probe design—Primers and probes used for TaqMan real-time quantitative RT-PCR of MRFs were designed based on the *S. macrurus*-specific sequences of *MyoD* (accession number AY396566), *myogenin* (accession number AY396565) and *MRF4* (accession number DQ059552) using Primer Express software (version 1.5, Applied Biosystems; Table 1). Primers and probes for *myf5* were designed using Primer3 (Table 1). Probes for each MRF were designed with the fluorescent reporter dye FAM (6-carboxy-fluorescein) attached to the 5′ end and a quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) attached to the 3′ end.

Synthesis of standards and generation of standard curves—For each MRF, an RNA standard was generated by subcloning each amplicon behind a T7 or Sp6 RNA polymerase promoter in a plasmid vector (pGEM-T Easy, Promega). cDNAs specific to *MyoD*, *myogenin*, *myf5* and *MRF4* were generated using the One-Step RT-PCR superscript with Platinum *Taq* system (Invitrogen) according to the manufacturer's specifications. Reverse transcription from 1 μg of total RNA extracted from skeletal muscle of *S. macrurus* was reverse transcribed for 30 min at 50°C, followed by a 2 min incubation at 94°C. PCR amplification of the cDNAs corresponding to *MyoD*, *myogenin*, *myf5* and *MRF4* mRNAs was performed in the same reaction tube immediately following the RT step. The PCR profile included 30-40 cycles of 15 s denaturation at 94°C, 30 s primer annealing at 58°C, 30 s elongation at 72°C, and a final extension for 7 min at 72°C. PCR products were purified, subcloned into the pGEMT-Easy vector, transformed into JM109 frozen competent cells (Promega), and sequenced for verification as described above prior to *in vitro* transcription. MRF cDNAs were transcribed *in vitro* using the SP6/T7 transcription kit (Roche, Pleasanton, CA, USA) and their copy number was calculated {formula: [X g μl^{-1} RNA/(transcript length in nucleotides × 340)] × $6.022\times10^{23}=Y$ molecules μl^{-1}} to generate standard curves using 5-6 dilutions of known RNA amounts.

TaqMan probe RT-PCR—Real-time quantitative RT-PCR experiments were carried out in triplicate in 96-well optical plates on total RNA from skeletal muscle (N=5) and EO (N=5) using the one-step QuantiTect probe RT-PCR kit (Qiagen) and performed in a MJ research DNA engine Opticon2 system (Bio-Rad Laboratories, Richmond, CA, USA). Each reaction (25 μl) included tissue RNA (1-2 μl at 300 ng μl^{-1}) or RNA standard, 2× QuantiTect probe RT-PCR master mix (12.5 μl), sense and antisense primers (1.25 μl at 0.5 $\mu moll^{-1}$, each), TaqMan probe (1.25 μl at 0.1 $\mu moll^{-1}$), QuantiTect probe RT mix (0.25 μl) and RNase-free water (variable). PCR parameters were 48°C for 60 min, 95°C for 10 min, 45 cycles of 95°C for 15

s and 60°C for 1 min. To monitor DNA contamination in all experiments, control reactions without the RNA template were performed in triplicate and one reaction without the reverse transcriptase enzyme was carried out per tissue sample.

Data analysis—The number of PCR cycles needed to generate a fluorescence signal greater than a predefined threshold is defined as the threshold cycle value, or C_t. The C_t value for each RT-PCR reaction was extrapolated using the respective standard curve to determine the number of copies (molecules μl^{-1}) of *MyoD*, *myogenin*, *myf5* and *MRF4* mRNA molecules in skeletal muscle and EO tissue. To confirm product size, the RT-PCR products were also run on a 1.5% agarose gel stained with ethidium bromide (10 μl/100 ml).

Statistical analysis—All data are presented as means ± s.e.m. Group differences were determined using a split-plot in a completely randomized design with treatment (control *vs* ST) as the whole-plot factor, tissue type (muscle *vs* EO) as the split-plot factor, and fish as the experimental unit. All statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC, USA) with the α-level set at $P \leq 0.05$.

Southern blot analysis

Liver and brain tissues used for genomic DNA isolation were dissected and immediately immersed in liquid nitrogen. Genomic DNA was isolated from these tissues using a protocol adapted from the DNA isolation method of Sambrook et al. (Sambrook et al., 1989). To remove residual RNA, total DNA isolated from each tissue was treated with RNAse A (0.1 μg μl^{-1}; Qiagen) and analyzed by electrophoresis and spectrophotometry (OD_{260}/OD_{280}). Genomic DNA (40 μg) was singly digested with *Bam*HI, *Eco*RI and *Hin*dIII, and double digested with *Bam*HI/*Eco*RI, *Bam*HI/*Hin*dIII, and *Eco*RI/*Hin*dIII for 6 h at 37°C. Restriction enzyme digests of *S. macrurus* genomic DNA (10 μg per digest) were size fractionated on 1% agarose gels in 1× TAE buffer and blots were generated using standard methods as described by Sambrook et al. (Sambrook et al., 1989) for alkaline transfer onto nylon membranes.

Radiolabeled probes were synthesized by ^{32}P-oligolabeling of *MyoD* (640bp, nucleotides 7-646, accession number AY396566), *myogenin* (805 bp, nucleotides 588-1395, accession number AY396565), *myf5* (718bp, nucleotides 441-1156, accession number DQ016032) and *MRF4* (636bp, nucleotides 433-1092, DQ059552) specific to *S. macrurus*. Probes against *myogenin*, *myf5* and *MRF4* spanned the 3′ untranslated regions (UTRs) of the genes. Unincorporated radioactivity was removed with a Sephadex G-50 spin column. Following alkaline transfer, nylon membranes were neutralized in 0.5 $moll^{-1}$ Na_2HPO_4 (pH 7.2). Probes were boiled and added to the pre-hybridization solution (1% BSA, 1 $mmoll^{-1}$ EDTA, 0.5 $moll^{-1}$ Na_2HPO_4, 7% SDS) in the bag with the membrane. Blots were hybridized in hybridization buffer overnight and then washed in several changes of 1 $mmoll^{-1}$ EDTA, 40 $mmoll^{-1}$ Na_2HPO_4, 1% SDS. The first wash also contained 3% fish gelatin and 5% SDS. The hybridizations and washes were carried out at 65°C (high stringency, ≥85% complementarity for probe hybridization).

Immunolabeling

Serial cryosections (18 μm) mounted on glass slides and air dried at room temperature were rehydrated in 0.1 $moll^{-1}$ phosphate-buffered saline (PBS, pH 7.4), incubated in blocking solution [PBS and 2% bovine serum albumin (BSA)] for 30 min, and subsequently incubated overnight (12-15 h) in primary antibody diluted in blocking solution at room temperature. Monoclonal antibodies against all sarcomeric myosin heavy chains (MHCs; MF20, 1:100 dilution), fast MHC (A4.74; 1:50), slow MHC (N2.261; 1:100), tropomyosin (CH1; 1:50 dilution) and neurofilament-associated protein (3A10; 1:100 dilution) were purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). Monoclonal antibodies

against developmental MHC (DEV; 1:50) and troponin-T (1:100) were purchased from Novacastra Laboratories (Vector Laboratories, Burlingame, CA, USA) and Sigma (St Louis, MO, USA), respectively. Filamentous actin was visualized using rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA). Fluorescein-conjugated secondary antibodies (AlexaFluor 488 or 565; Molecular Probes; 1:200) were used to detect the antibody-antigen complex. Images of immunolabeled tissue sections were visualized and captured using a BioRad 1024 confocal microscope (Bio-Rad Laboratories).

RESULTS

S. macrurus myf5 and MRF4 proteins are highly conserved

PCR-coupled RACE generated a 1108 bp *S. macrurus myf5* cDNA (accession number DQ016032). This cDNA contains a 237 amino acid open reading frame that is flanked by a 67 bp 5′ UTR and a 327 bp 3′ UTR. Multiple sequence alignment of the predicted *S. macrurus* myf5 protein with that of mouse (Fig. 1A), carp (*C. carpio*), zebrafish (*D. rerio*), striped seabass (*M. saxatilis*), rainbow trout (*O. mykiss*) and puffer fish (*T. rubripes*; Fig. 1B) revealed a high degree of conservation among these myf5 amino acid sequences. The similarity between myf5 of *S. macrurus*, mouse, and other piscine species ranged from 59% (mouse) to 79% identity (carp; Fig.1A,B). Mammalian myf5 contains distinct protein regions that are required for its transcriptional activation of genes expressed in skeletal muscle, i.e. the N-terminal (amino acids 1-46), basic helix-loop-helix (bHLH, amino acids 78-137) and helix III region within the C-terminal (amino acids 135-255) domain (Braun et al., 1990;Winter et al., 1992). Within the N-terminus, *S. macrurus* myf5 showed truncations, i.e. amino acids 4-8 and 26-35, and a 59% homology compared with the mouse protein (Fig. 1A). In contrast, the N-terminus of *S. macrurus* myf5 and that of other teleosts were of equal length (Fig. 1B) and showed a highly conserved region between amino acids 29 to 38 that is not shared with mouse. The bHLH and the helix III regions of *S. macrurus* myf5 were 82% and 79% similar to the respective mouse myf5 amino acid sequences.

Similarly, we isolated an 845 bp cDNA fragment of *S. macrurus MRF4* that contained a 242 amino acid open reading frame flanked by a 47 bp 5′ UTR and a 49 bp 3′ UTR (accession number DQ059552). Multiple sequence alignment of MRF4 proteins from mouse (Fig. 2A), and other piscine species (Fig. 2B) showed an amino acid sequence identity of 61%, 66% and 78%, with mouse, puffer fish and zebrafish, respectively. Compared with the N-terminal region of the mouse (amino acids 1-85), this region of the *S. macrurus* MRF4 contained four additional amino acids and showed 69% identity (Fig. 2A). Interestingly, the four additional amino acids are conserved in the zebrafish and pufferfish, and the N-termini of the three teleost proteins share 70% homology in this region (Fig. 2B). The bHLH and helix III regions of *S. macrurus* MRF4 were 90% and 94% similar to the mouse MRF4 amino acid sequences, respectively. In sum, *S. macrurus* myf5 and MRF4 proteins exhibit a high degree of sequence conservation in the specific amino acids that are important for their transcriptional activation (Bergstrom and Tapscott, 2001;Braun et al., 1990;Mak et al., 1992;Sirri et al., 2003;Winter et al., 1992). These results suggest that the *S. macrurus* myf5 and MRF4 proteins probably have similar myogenic regulatory functions to those observed for their mammalian counterparts.

MRF expression is restricted to skeletal muscle and EO in *S. macrurus*

We used RT-PCR to compare the tissue-specific patterns of MRF expression in *S. macrurus*. *MyoD* and *myogenin* transcripts were detected in adult skeletal muscle and EO (Fig. 3), an expression pattern consistent with previous findings (Kim et al., 2004). Moreover, *myf5* and *MRF4* transcripts were also detected in the EO, despite its incomplete ‘muscle-like’ phenotype (Fig. 3). The detectable levels of *myf5* and *MRF4* in the EO were of similar intensities to those found in muscle. None of the four MRF transcripts were detected in non-skeletal muscle tissues

such as liver or brain (Fig. 3), indicating that, as in other vertebrates, transcription of all four MRFS in *S. macrurus* is restricted to tissues in the myogenic lineage.

MRFs are differentially expressed in skeletal muscle and EO in *S. macrurus*

To determine whether differential levels of MRF transcripts correlated with differences in muscle protein expression profiles, we performed quantitative RT-PCR to establish levels of endogenous transcripts in skeletal muscle and EO. In skeletal muscle and EO, the relative transcript levels of each MRF followed a similar pattern: *MyoD>MRF4>myf5>myogenin*. However, the mean *MyoD* mRNA content in skeletal muscle was 3-fold lower than that found in EO. This difference was not statistically significant (P=0.07) due to a substantial inter-animal variability in *MyoD* content in the two tissues (Fig. 4). In contrast to the MyoD expression patterns, differences in *myogenin*, *myf5* and *MRF4* mRNA content between skeletal muscle and EO were significant, with mRNA levels for each MRF being higher in EO than in muscle (P=0.02, 0.02 and 0.005, respectively). Specifically, *myogenin* and *myf5* mRNA levels in EO were over 2-fold higher [5.58($\times 10^5$)±1.98 and 22.6($\times 10^5$)±6.78 molecules μl^{-1} mRNA] than the *myogenin* and *myf5* mRNA levels in muscle [2.39($\times 10^5$)±0.87 and 9.09($\times 10^5$)±4.10 molecules μl^{-1} mRNA; Fig. 4]. The largest difference in MRF expression patterns was observed for *MRF4* where mRNA levels in EO [65.68($\times 10^5$)±18.65 molecules μl^{-1} mRNA] were 3-fold higher (P=0.005) than those detected in muscle [18.17($\times 10^5$)±6.58 molecules μl^{-1} mRNA; Fig. 4]. These data demonstrate that full expression of the skeletal muscle program in *S. macrurus* occurs with the transcription of all four MRFs. Surprisingly, in the EO, which expresses only a subset of muscle genes, there was an even greater MRF transcript content.

Effects of electrical inactivity on MRF expression patterns

We previously demonstrated that removal of neural influences by ST leads to an up-regulation of MHC and tropomyosin in some mature electrocytes 2 weeks after ST (Unguez and Zakon, 1998b), whereas by 5 weeks post-ST surgery, all electrocytes contained different amounts of these proteins. Whether the re-expression of select sarcomeric genes was accompanied by specific changes in MRF profiles was not determined. In this study we found that the MRF expression pattern in the EO 5 weeks after ST was *MRF4>MyoD>myf5>myogenin* (Fig. 4). In contrast to control tissues, the mean transcript level of all four MRFs in skeletal muscle and EO was reduced after ST (Fig. 4). However, only the reduction in *MyoD* mRNA level in muscle and EO after ST was significant. In fact, *MyoD* mRNA level in muscle and EO was about 10- and 4-fold lower, respectively, than the control values (Fig. 4).

Expression of MRF co-regulators in *S. macrurus* tissues

The expression of all four MRF transcripts in electrocytes at levels higher than in skeletal muscle was unexpected given that electrocytes do not retain the full muscle program. Since the activities of MRFs are known to be under the control of both positive and negative regulatory factors including MEF2 and Id transcription factors, respectively (Benezra et al., 1990; Molkentin et al., 1995), we also determined the expression patterns of some of these co-regulators. Specifically, we isolated partial cDNA sequences of *Id1* (304 bp), *Id2* (101 bp) and *MEF2C* (265 bp) from *S. macrurus* muscle, EO, liver, brain and heart (Fig. 5). Qualitative RT-PCR showed that *Id1* is transcribed in all tissues analyzed although the band detected in muscle had a lower intensity than that detected in EO, liver or brain (Fig. 5). *Id2* transcript was also detected in all tissues analyzed with band intensities more or less similar to those observed for *Id1* (Fig. 5). A partial 141 bp cDNA sequence of *Id4* was isolated from *S. macrurus* brain, but was not detected in muscle, EO or liver (data not shown). The absence of *Id4* in muscle and EO together with the difficulty in cloning *Id3* may suggest that *Id1* and *Id2* are the predominant members of the Id family expressed in tissues of the myogenic lineage in *S. macrurus*. A *MEF2C* cDNA fragment corresponding to the C-terminal transactivating domain II in mouse

was isolated from *S. macrurus* muscle. Muscle, EO, liver, brain and heart showed detectable levels of *MEF2C* transcript. Interestingly, *MEF2C* in EO appeared to be present at higher levels than in muscle or non-skeletal muscle tissues based on band intensity (Fig. 5). The expression of both *Id1* and *Id2* transcripts after the 5 week ST period showed a pattern similar to that in control muscle and EO, i.e. skeletal muscle in ST fish showed detectable levels of *Id1* at a somewhat higher intensity than those found in EO, and *Id2* mRNA content in muscle and EO appeared similar before and after ST (Fig. 6). The differential expression of *MEF2C* mRNA in muscle and EO in control fish was also similar to that found in ST fish (Fig. 6).

Single copy genes encoding myogenic MRF factors in *S. macrurus*

Southern blots were used to estimate the number of copies of *MyoD, myogenin, myf5* and *MRF4* genes in *S. macrurus*. The distribution of restriction fragments of each MRF was detected following hybridization of genomic DNA digested with *Bam*HI, *Eco*RI, and *Hin*dIII, and double digests with *Bam*HI/*Eco*RI, *Bam*HI/*Hin*dIII, and *Eco*RI/*Hin*dIII (Fig. 7). The Southern blots were probed with ^{32}P-labeled *MyoD* (640 bp), *myogenin* (805 bp), *myf5* (718 bp) and *MRF4* (636 bp) fragments. In two of the three single digests, the *MyoD* probe hybridized to a single band, 6 kb *Bam*H1 or 11 kb *Eco*R1 fragment. In the *Hin*dII digest, two small fragments were detected, 740 bp and 2.5 kb. The double digests also produced very simple hybridization patterns, consistent with one gene for *MyoD*. A similar pattern was observed for *myogenin*; one fragment hybridized strongly in each of the digestions. *MRF4* hybridized to 4.6 and 2.1 kb fragments in the *Bam*H1 digest, a 6 kb fragment in the *Eco*R1, and a 4.6 kb fragment in the *Hin*dIII digest. *myf5* hybridized to single bands in the *Eco*R1 (6.8 kb) digest, and in the *Bam*H1/*Eco*R1 (4.9 kb) and *Eco*R1/*Hin*dIII (3.0 kb) double digests. Overall, these hybridization patterns are consistent with a simple organization of each of the four genes, suggesting the presence of one copy of each gene. Given the wide phylogenetic distribution and structure conservation (three exons, two introns) of the MRF genes across animal taxa, *S. macrurus* MRFs are expected to have two introns. The cDNA probes may hybridize to two restriction fragments generated by a restriction site in an intron. Previous northern blot analyses suggest a single *MyoD* and *myogenin* gene (Kim et al., 2004). Further, the full-length coding sequences of all four MRFs were obtained from 5′- and 3′-RACE fragments whose corresponding cDNA sequences after amplification showed no evidence suggestive of multiple MRF gene copy number. Together, the band patterning obtained from the Southern blot analysis is most easily explained by the presence of single *MyoD, myogenin, myf5* and *MRF4* genes in *S. macrurus.*

Sarcomeric proteins are re-expressed in electrically inactive electrocytes

In *S. macrurus,* all muscle fibers were intensely immunolabeled with antibodies against MHC and tropomyosin, whereas electrocytes were not - a labeling pattern similar to that reported previously (Unguez and Zakon, 1998b). F-actin was studied by positive phalloidin labeling (data not shown). Phalloidin label was readily detected in the cytoplasm of muscle fibers whereas in electrocytes, if detected, it was weak and found along the perimeter of only some electrocytes. Following ST, skeletal muscle properties were not different from those in un-operated control animals based on immunolabeling patterns of MHC and tropomyosin, consistent with what has previously been reported (Unguez and Zakon, 1998b). The fiber-type distribution characterized by smaller, slow MHC fibers located peripherally next to the epithelium and the larger, fast-MHC fibers being central to slow MHC fibers was also not affected (data not shown). However, ST did affect the biochemical composition of electrocytes. Consistent with earlier observations (Unguez and Zakon, 1998b), some electrocytes were immunolabeled by antibodies against MHC and tropomyosin. F-actin labeling in skeletal muscle 5 weeks after ST was not different from control, whereas F-actin was evident in more electrocytes after ST than in control fish (data not shown). In control and 5 week ST animals, the cholinergic endplates of muscle fibers and electrocytes were detected by immunolabeling

of acetylcholine receptors (data not shown). The presence of neurofilaments near the endplates after 5 weeks of ST suggests that synaptic contacts between spinal motoneurons and their target cells, muscle fibers and electrocytes, were not affected by ST as previously reported (Unguez and Zakon, 1998b).

DISCUSSION

Given the crucial role of MRFs in the activation of the vertebrate myogenic program, we presumed that the down-regulation of select, but not all, components of the muscle program in the EO was coupled to a MRF expression pattern that predicts the level and type of muscle proteins retained in mature electrocytes. In this study we have shown that the MRFs MyoD, myogenin, myf5 and MRF4 are expressed in mature electrocytes despite the fact that these cells do not maintain a normal muscle phenotype. Thus, these data do not support our original hypothesis. Further, 5 weeks of electrical inactivation did not affect the distribution of skeletal muscle fiber types. In contrast, although there was an up-regulation of some muscle proteins in the EO of ST fish, only *MyoD* mRNA levels were significantly different from control in EO after ST. Together, these data demonstrate that the expression of MRFs is not sufficient to induce the full skeletal muscle phenotype in some cells of the myogenic lineage, and the neural regulation of the myogenic program in *S. macrurus* may not require a MRF-dependent gene activation as observed in other vertebrates.

MRF transcript levels differ in adult skeletal muscle and EO

Our expression analysis showed that the relative MRF mRNA levels in both skeletal muscle and EO of *S. macrurus* follow this pattern: *MyoD>MRF4>myf5>myogenin*. This expression pattern is interesting given that fast muscle fibers give rise to electrocytes, and MyoD and myogenin are the predominant MRFs in fast glycolytic and slow oxidative muscle fibers, respectively, in other vertebrates (Charbonnier et al., 2002; Ekmark et al., 2003; Hughes et al., 1993; Voytik et al., 1993; Walters et al., 2000; Yutzey et al., 1990). Future studies using antibodies specific to *S. macrurus* MRF antigens will allow us to compare their expression patterns across different muscle fiber-type populations in electric fish.

As the relative expression patterns of MRFs were similar in muscle and EO, it is feasible that different levels of MRF transcript expression affect distinct aspects of the skeletal muscle program. Quantitative RT-PCR revealed that with the exception of *MyoD*, MRF transcript levels in the EO exceeded those detected in skeletal muscle. These data indicate that the differentiation of muscle fibers into electrocytes is accompanied by an up-regulation of myogenin, myf5 and MRF4 that is concurrent with a down-regulation of subsets of muscle protein systems including sarcomeric and sarcolemmal genes (Unguez and Zakon, 1998a). This MRF composition clearly negates the idea that the manifestation of a partial muscle program in electrocytes is correlated with MRF transcript expression levels that are lower than those in muscle.

Our analysis of transcript levels does not allow us to draw firm conclusions about MRF protein activity in electrocytes. Nevertheless, a recent analysis of randomly selected clones from an EO cDNA library revealed the presence of desmin, titin, muscle creatine kinase (MCK), α-actin, α-AChR and fast MHC mRNAs (Cuellar et al., 2006), genes that are known transcriptional targets of MRFs (Gilmour et al., 1991; Jaynes et al., 1988; Li and Capetanaki, 1994; Maleki et al., 2002; Simon and Burden, 1993; Wheeler et al., 1999; Yutzey et al., 1990). Further, all four *S. macrurus* MRFs can induce myotube formation and expression of sarcomeric proteins in mammalian 10T1/2 embryonic cells with similar conversion efficiency to their mammalian heterologs (Kim et al., 2007). Together with our present data in support of *S. macrurus* having single copies of *MyoD*, *myogenin*, *myf5* and *MRF4* genes with highly conserved functional protein domains, we interpret these findings to indicate that both muscle

fibers and electrocytes contain MRF proteins with similar myogenic functions to those observed in their mammalian counterparts. Nevertheless, development of antibodies that can be used to detect *S. macrurus* MRF proteins will be critical to fully evaluate their role in the EO and to characterize the mechanism of muscle gene regulation in mature electrocytes.

We should note that the present MRF expression patterns differ from those reported previously for muscle and EO in *S. macrurus* where myogenin, not MyoD, was the predominant MRF in both tissues (Kim et al., 2004). We consider the present analysis to be a more rigorous assessment of the endogenous MRF transcript levels because the quantitative assessment of MRFs was based on muscle and EO samples from five individual animals (*vs* quantification of MRFs from pooled muscles and EO samples taken from different adult fish). Further, real-time RT-PCR (*vs* competitive RT-PCR) yields a higher degree of reproducibility without the need for post-PCR processing and image analysis, which may introduce systemic errors (Wall and Edwards, 2002).

The expression of MRF co-regulators MEF2C, Id1 and Id2 is not unique to skeletal muscle or EO in *S. macrurus*

MRFs are regulated by co-activators and repressors. MEF2 proteins are co-activators of myogenic differentiation and their interaction with MRFs is required for the transcription of muscle-specific genes, i.e. MHC (Molkentin and Olson, 1996; Molkentin et al., 1996). In vertebrates, MEF2C is the only MEF2 protein that is restricted to the skeletal muscle, brain and spleen following differentiation (Martin et al., 1993; McDermott et al., 1993), whereas MEF2A, B and D proteins are ubiquitously expressed (Yu et al., 1992; Martin et al., 1993; McDermott et al., 1993). A recent cDNA library screen from the EO of *S. macrurus* revealed the presence of *MEF2C* transcript (Cuellar et al., 2006). To confirm the expression of MEF2C in the EO, and test the hypothesis that the differential expression of MEF2C is correlated with the muscle and EO phenotype, a partial fragment of *MEF2C* was isolated and cloned from both tissues. Our expression experiments confirmed the presence of *MEF2C* transcripts in EO. Hence, our data clearly demonstrate that expression of positive co-regulators of MRFs does not ensure that an electrocyte will fully express the skeletal muscle program.

Whether mature electrocytes up-regulate the expression of MRF inhibitors such as Id proteins was also addressed to further characterize the transcriptional program in these muscle-derived cells. Id proteins lack the basic domains necessary for DNA binding (Murre and Baltimore, 1992), and heterodimerize with MRFs to inhibit their transcriptional activity (Benezra et al., 1990). Data from the present study suggest that *Id1* and *Id2* are the predominant inhibitory transcripts in *S. macrurus*, and the two are transcribed in adult skeletal muscle and EO at relatively similar levels. Taken together, these data reveal that expression of central players in the regulation of the skeletal muscle program does not correlate with the level to which a muscle cell or an electrocyte in *S. macrurus* manifests the myogenic program.

Muscle properties in myogenically derived tissues in *S. macrurus* following changes in electrical activity patterns

Five weeks of electrical inactivation by ST had a differential effect on the phenotypic properties of muscle and EO. In muscle, there were no observable differences in the fiber-type composition. Consistent with a previous study (Unguez and Zakon, 1998b), these data suggest that skeletal muscle fibers of *S. macrurus* are more resistant to changes in MHC isoform composition compared with mammalian muscle fibers following neural inactivation (Talmadge et al., 1995; Talmadge et al., 1999). ST also resulted in the decreased expression of all four MRF transcripts in muscle, with the greatest effect observed in *MyoD* mRNA levels. However, even a 10-fold decrease in *MyoD* mRNA content in muscle did not seem to affect its biochemical properties, as the muscle protein profile appeared unchanged based on the

immunolabeling profile before and after ST (data not shown). This is consistent with previous studies on hindlimb muscles of rodents showing that changes in MRF expression are not sufficient to induce changes in muscle fiber-type composition (Mozdziak et al., 1999; Walters et al., 2000).

We are aware of only one study of vertebrate muscle that also shows a decrease in MRF expression levels following electrical inactivation. Nicolas and colleagues reported a marked down-regulation of *myf5* and *MRF4* mRNA expression at 11 days after denervation of forelimb muscles in adult *Xenopus* (Nicolas et al., 2000). Expression of *myf5* was only transiently decreased, whereas *MRF4* mRNA levels remained lower than control levels up to 30 days post-denervation. By comparison, *MyoD* levels were not affected and myogenin expression increased, but this was not observed before 20 days post-denervation. In their study (Nicolas et al., 2000), little to no change in muscle fiber size was also observed following the 30 day denervation period. The muscle response to denervation in *Xenopus* is somewhat analogous to what we found in *S. macrurus* muscle after 5 weeks of ST. Although MRFs have been isolated from other fish including zebrafish (Chen et al., 2001; Hinits et al., 2007), carp (Kobiyama et al., 1998), pufferfish (Fernandes et al., 2007) and striped bass (Tan et al., 2002), the present data represent the first quantitative analysis of mRNA content of all four MRFs in myogenic tissues of a teleost vertebrate. Determining whether skeletal muscle in these fish responds to electrical inactivation in a similar way to *S. macrurus* and *Xenopus* muscle would prove invaluable for elucidating the incidence of inactivation-induced cellular and molecular correlates in non-mammalian species.

The data from *S. macrurus* and *Xenopus* (Nicolas et al., 2000) show a muscle response that is contrary to that commonly found in mammals. For example, in rodents, removal of neural input to the hindlimb muscles is accompanied by rapid and sustained increases in all four MRF transcripts beginning as little as 24-48 h after denervation (Voytik et al., 1993; Walters et al., 2000). More recently, Hyatt and colleagues demonstrated that MyoD and myogenin mRNA levels increased after either electrical inactivation or denervation of fast- and slow-twitch skeletal hindlimb muscles, and reached maximum levels between 3 and 28 days after removal of neural input, depending on the muscle (Hyatt et al., 2003; Hyatt et al., 2006). The latter studies also showed that once maximum increases in MRF levels are reached, they decrease over time, but can remain elevated (even up to 1 month of disruption of nerve-muscle connections) to levels that are significantly greater than those found in control tissues (Hyatt et al., 2006). These data suggest the possibility that differences in the physiological response of muscle to removal of neural input may involve a MRF-dependent transcriptional signaling mechanism that is distinct among vertebrate species. Our quantitative assessment of mRNA levels was not normalized to cell size - a parameter that might be relevant. However, we are not aware of any study that has taken changes in cell size into account when assessing MRF mRNA levels following changes in neural input. It may be that the physical connection between the motoneuron and its target cell enables some regulatory role of the muscle and electrocyte plasticity through activity-independent mechanisms as some have suggested (Hyatt et al., 2003; Roy et al., 1991).

Unlike muscle fibers, there were pronounced phenotypic changes in electrocytes. We observed an up-regulation of MHC, tropomyosin and troponin-T proteins in many electrocytes, resembling the phenotype of their precursor skeletal muscle fibers, consistent with previous findings (Unguez and Zakon, 1998b). This reversal of differentiation in electrocytes after 5 weeks of ST is analogous to the re-expression of embryonic isoforms of contractile proteins and acetylcholine receptor (nAChR) subunits observed in adult mammalian muscles when activation patterns are removed (Buonanno et al., 1998; Buonanno and Fields, 1999; Salpeter and Loring, 1985; Schuetze and Role, 1987). Thus, removal of electrical activity from EMNs can trigger transcriptional and/or translational mechanisms within mature electrocytes. The

change in protein composition of electrocytes is consistent with a suppression of select features of the muscle program by the high frequency activation pattern exerted by the EMNs (Unguez and Zakon, 1998b). The 5 week period of ST also reduced *MyoD*, but not *myogenin*, *myf5* or *MRF4* transcript levels in the EO. We interpret our current data to suggest that the 5 week electrical inactivity period had a greater influence on the expression levels of MyoD, the most prevalent MRF in myogenically derived tissues in *S. macrurus*.

Absence of the full myogenic program in the presence of MRF expression

The present study provides the first quantitative assessment of all four MRFs in the EO of an electric fish and, to our knowledge, the first demonstration of a non-contractile cell that expresses all four MRFs. Other fully differentiated cell types such as myofibroblasts (Mayer and Leinwand, 1997) and Purkinje fibers of the heart (Takebayashi-Suzuki et al., 2001) also express some MRFs such as MyoD and myogenin despite the lack of much of the contractile apparatus. In these cell types, the absence of a full complement of all four MRFs could explain the observed partial myogenic program. Nevertheless, the distinct sets of muscle-like properties manifested by electrocytes, Purkinje fibers or myofibroblasts argue against a single ‘master’ MRF that controls different subsets of the myogenic program. Instead, these data implicate a mechanism of transcriptional control of muscle genes in electrocytes, myofibroblasts and Purkinje fibers that might differ from the one present in skeletal muscle cells. A deeper understanding of the possible roles of MRFs in the homeostasis of muscle-specific properties after differentiation will be gained through further studies of cells with a partial muscle program, as well as identification of mechanisms that affect different muscle protein systems for the evolution of highly specialized, non-contractile tissues.

Acknowledgements

This work was supported by grants from the NIH (S06-GM008136, RR16480 and U56-CA96286) to G.A.U. The authors wish to thank Leigh Murray for assistance with the statistical analyses, and two anonymous readers for critical suggestions on earlier drafts of the manuscript.

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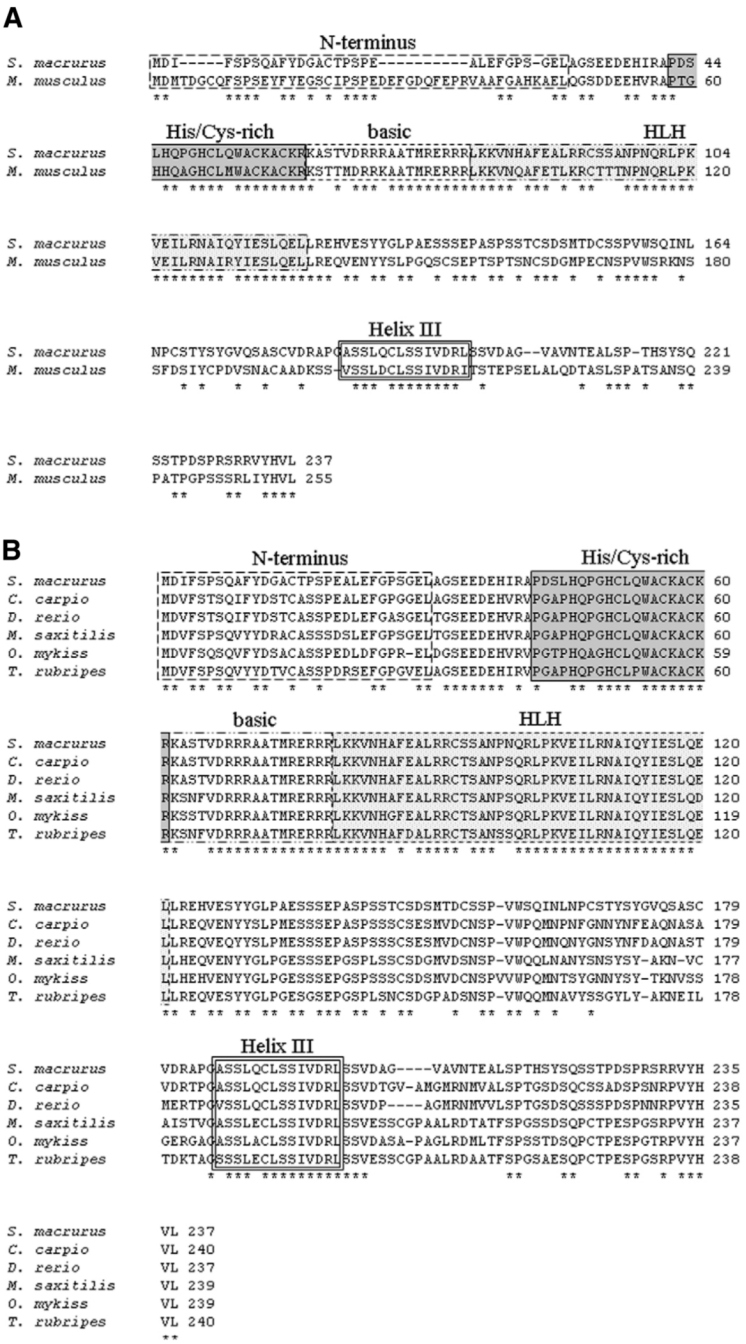

Fig. 1.

Sequence comparison of the deduced proteins encoded by *S. macrurus myf5* with comparable sequences from mouse (A) and other piscine species (B). Protein sequences from mouse (*M. musculus*, GenBank accession number NP_032682.1), carp (*C. carpio*, GenBank accession number BAA33566), zebrafish (*D. rerio*, GenBank accession number NP_571651.1), striped seabass (*M. saxitilis*, GenBank accession number AF463525_1), rainbow trout (*O. mykiss*, GenBank accession number AAV30214) and pufferfish (**T. rubripes**, GenBank accession number CAC39208.1) were used. The boxed areas are labeled to indicate the N-terminal, His/Cys-rich, basic helix-loop-helix (bHLH) and helix III domains. Asterisks represent amino acid similarities across sequences.

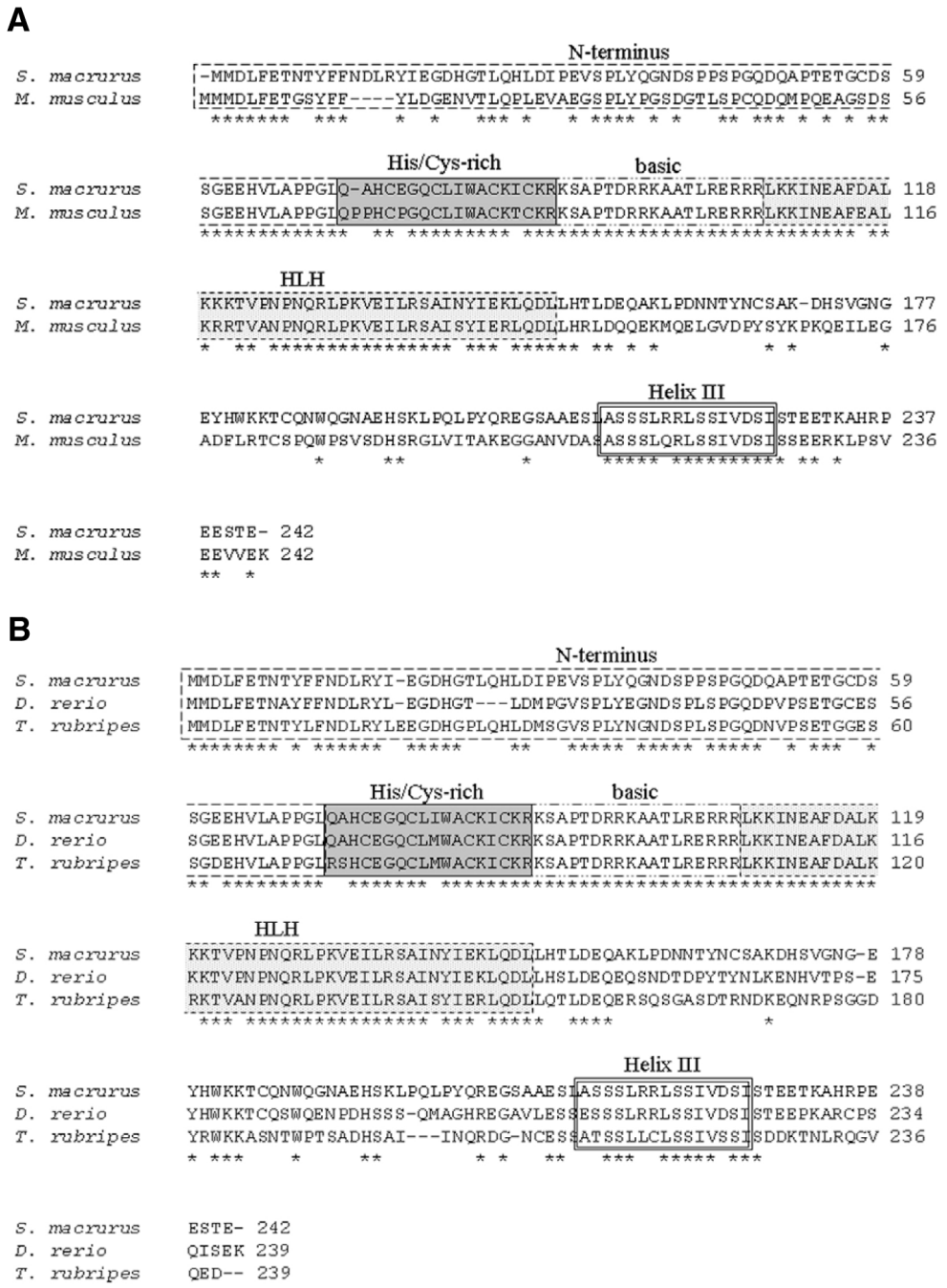

Fig. 2.

Sequence comparison of the deduced protein encoded by *S. macrurus MRF4* with comparable sequences from mouse (A) and other piscine species (B). Sequences from mouse (*M. musculus*, GenBank accession number 1604247A), zebrafish (*D. rerio*, GenBank accession number AAQ67704) and puffer fish (*T. rubripes*, GenBank accession number AAR20812) were used. The boxed areas are labeled to indicate the N-terminal, the His/Cys-rich, bHLH and Helix III domains. Asterisks represent amino acid similarities across sequences.

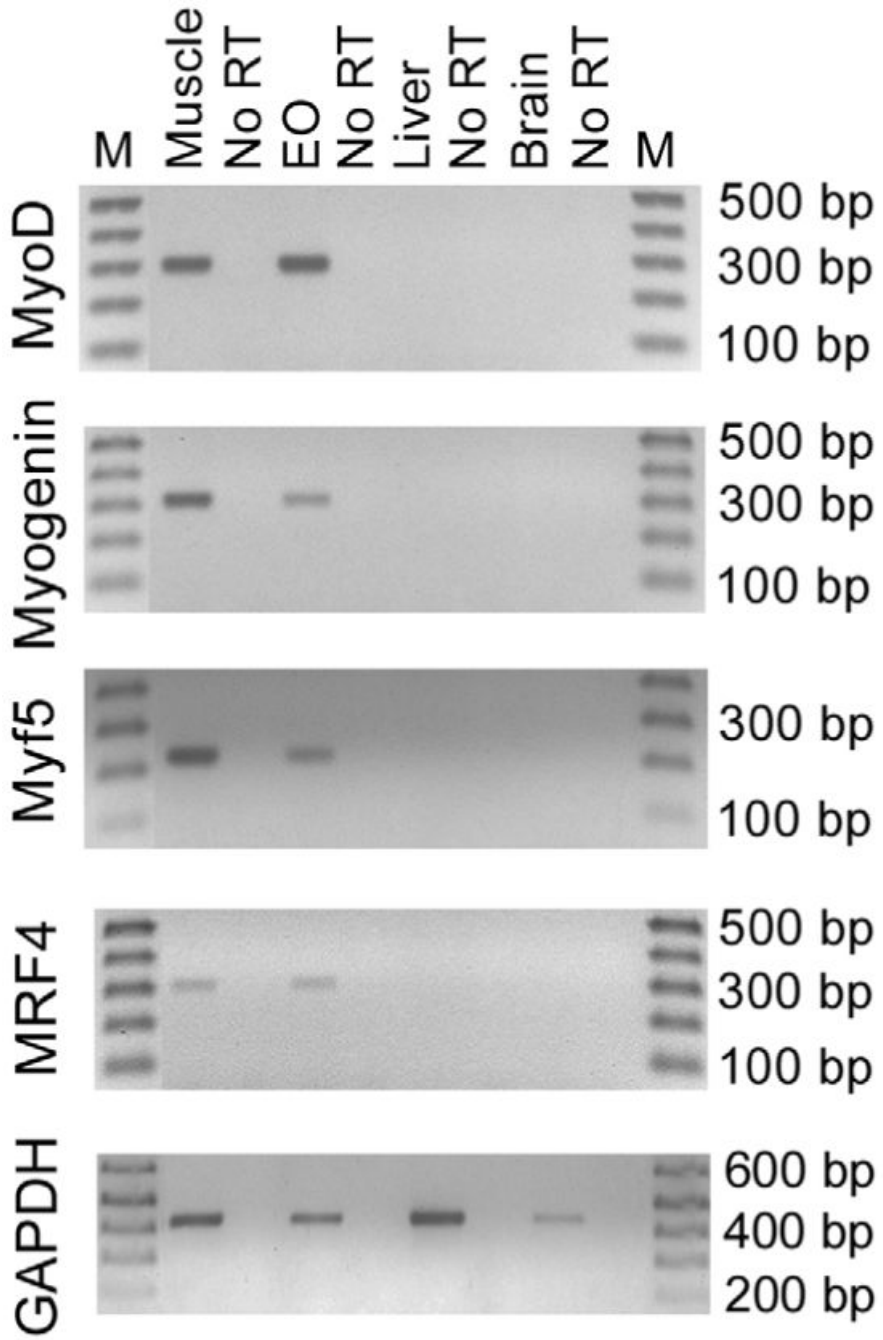

Fig. 3.

Expression of *MyoD*, *myogenin*, *myf5* and *MRF4* transcripts in adult tissues of *S. macrurus* by RT-PCR analysis. Total RNAs from skeletal muscle, EO, liver and brain were reverse transcribed and used for PCR. PCR products (1.0 μg per lane) were resolved on agarose gels containing ethidium bromide, and the resultant bands are presented as negative images of the original gels. Partial cDNA fragments of *MyoD* (290 bp), *myogenin* (312 bp), *myf5* (209 bp) and MRF4 (301 bp) were detected in skeletal muscle and EO, but not in liver or brain. Control reactions without reverse transcriptase (No RT lanes) were carried out for muscle (lane 2), EO (lane 4), liver (lane 6) and brain (lane 8) to ensure that PCR products were RNA dependent and not the result of genomic DNA amplification. RT-PCR analysis of glyceraldehyde

dehydrogenase (GAPDH) was used as a loading control. Lanes labelled M represent the 1 kb^+ DNA ladder.

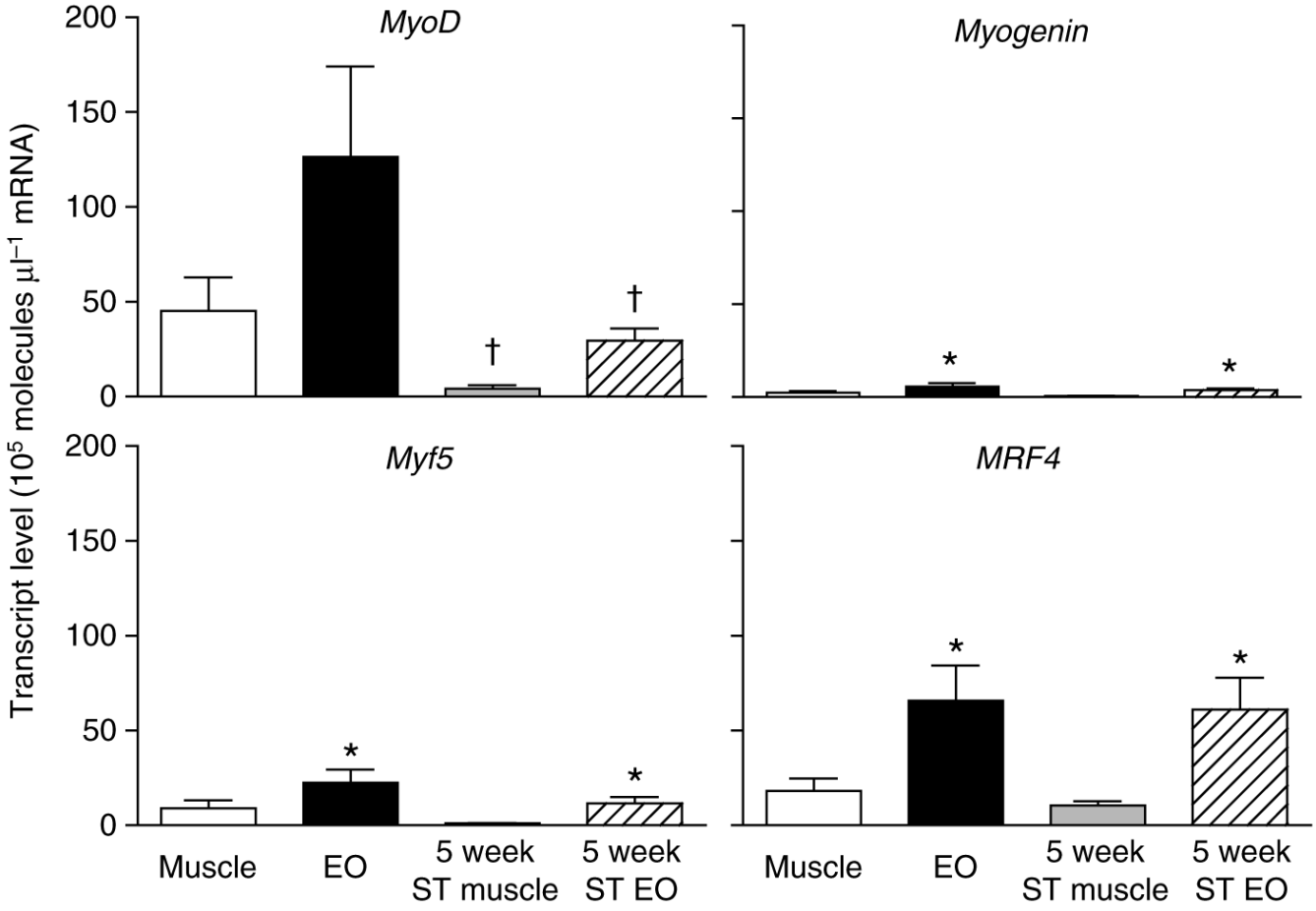

Fig. 4.

Expression of *MyoD*, *myogenin*, *myf5* and *MRF4* transcripts in muscle and EO from control fish (*N*=5) and fish after 5 weeks spinal transection (ST, *N*=5) by quantitative RT-PCR. The amount of *MyoD*, *myogenin*, *myf5* and *MRF4* transcript per tissue type was obtained from real-time RT-PCR experiments, and data are represented as molecules of transcript per µl of mRNA of each tissue type. Each column represents the mean ± s.e.m. †Significantly different from control; *significantly different from control or 5 week ST muscle groups at $P \leq 0.05$.

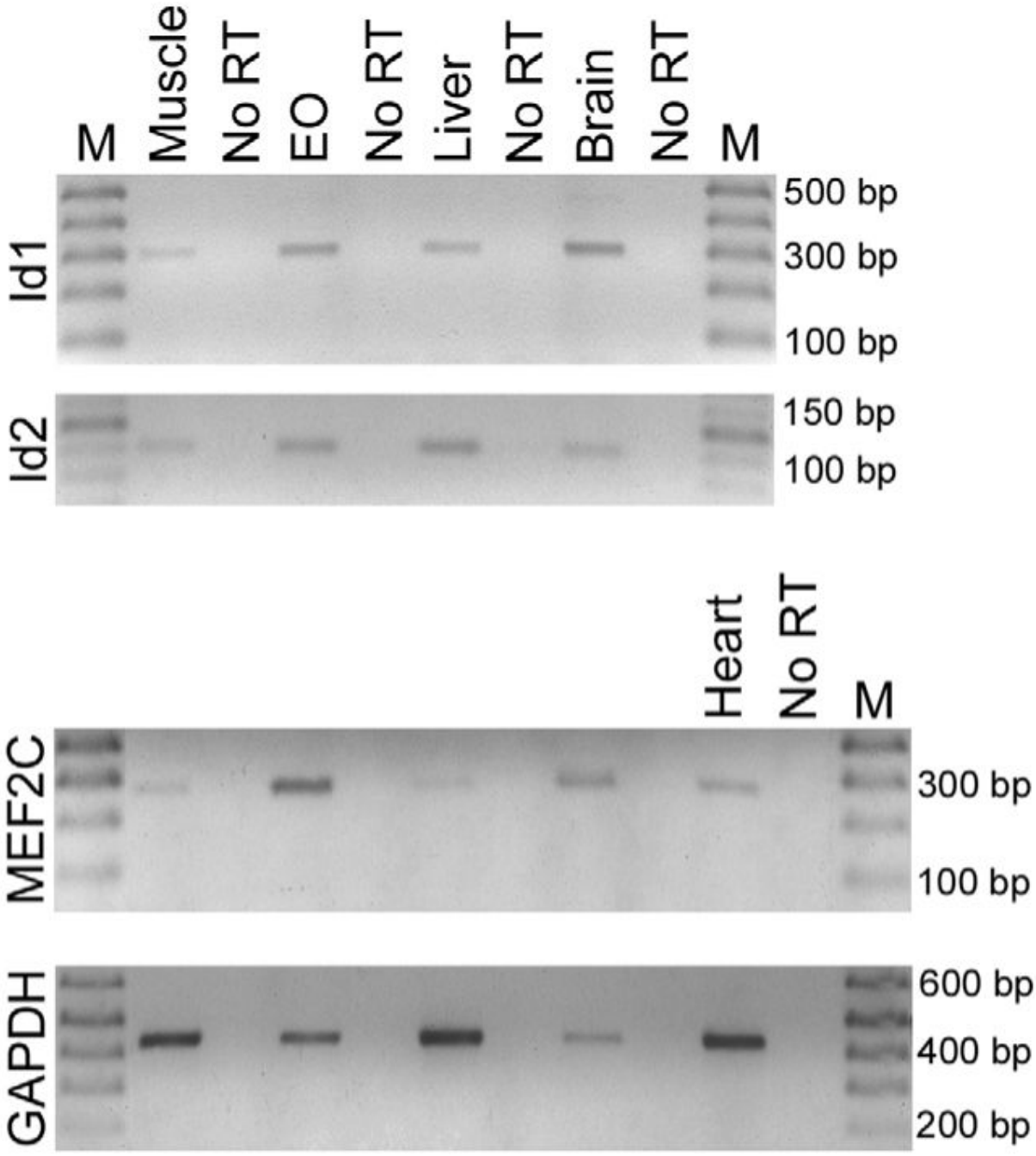

Fig. 5.

Expression of *Id1*, *Id2* and *MEF2C* in adult tissues of *S. macrurus* by RT-PCR. Agarose gel showing the expression of partial *Id1* (304 bp), Id2 (101 bp) and *MEF2C* (265 bp) cDNA fragments in skeletal muscle, EO, liver, brain and heart of *S. macrurus* using RT-PCR. For each transcript, 1 μg of cDNA was used for the PCR amplification. RT-PCR analysis of GAPDH was used as a loading control. Lanes labeled M represent the $1kb^{+}$ or 25 bp (*Id2*) DNA ladder. Other lanes as in Fig. 3.

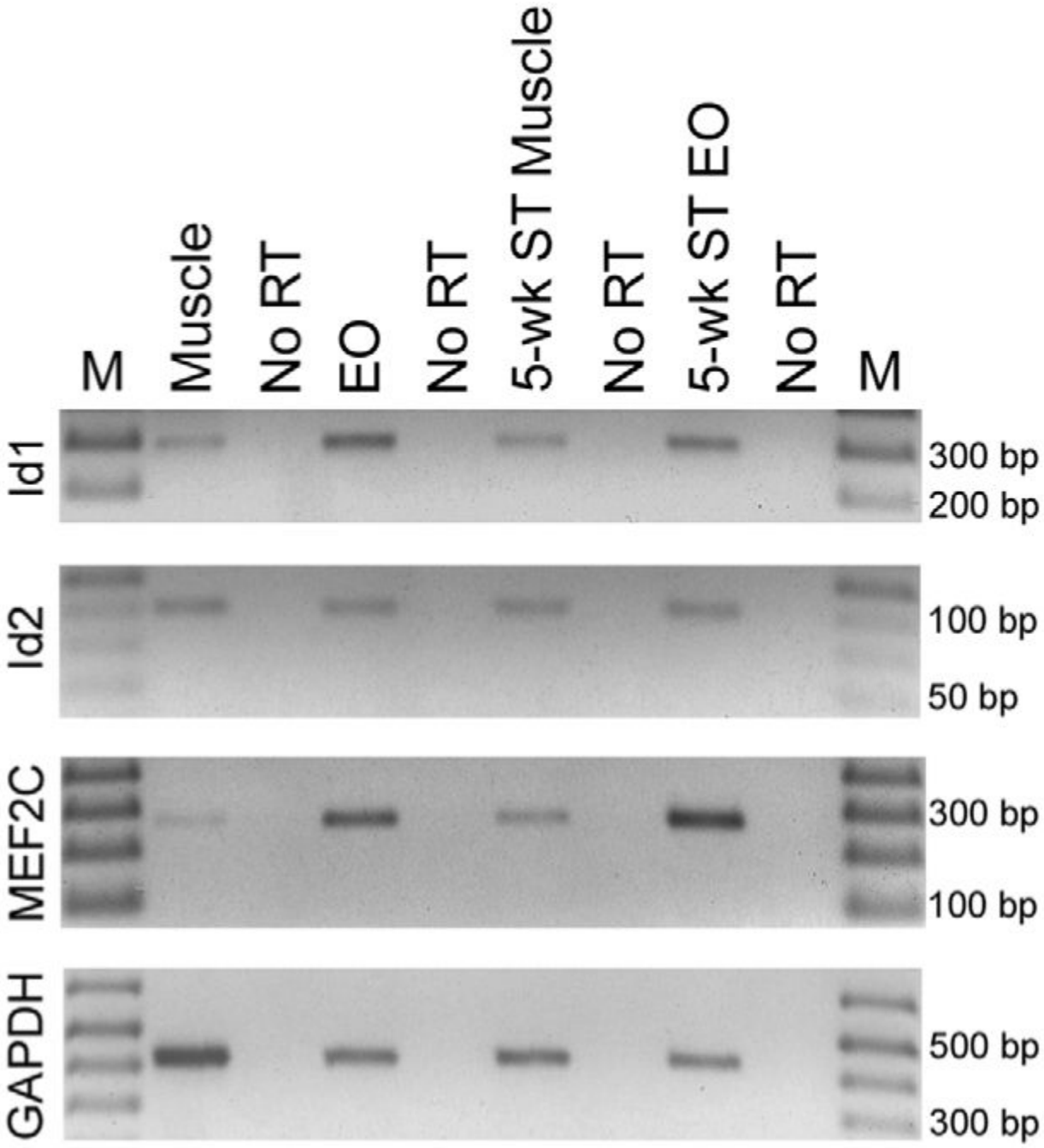

Fig. 6.

Expression of *Id1*, *Id2* and *MEF2C* in adult and 5 week ST muscle and EO by RT-PCR. Agarose gel showing the expression of *Id1*, *Id2* and *MEF2C* in skeletal muscle and EO from control un-operated and 5 week ST fish using RT-PCR. *Id1*, *Id2* and *MEF2C* transcripts were detected in all tissues analyzed. The size of the cDNA fragments for each transcript was the same as that in Fig. 5; 1 μg of cDNA was used for PCR amplification. RT-PCR analysis of GAPDH was used as a loading control. Lanes labeled M represent the 1 kb^{+} or 25 bp (*Id2*) DNA ladder. Other lanes as in Fig. 3.

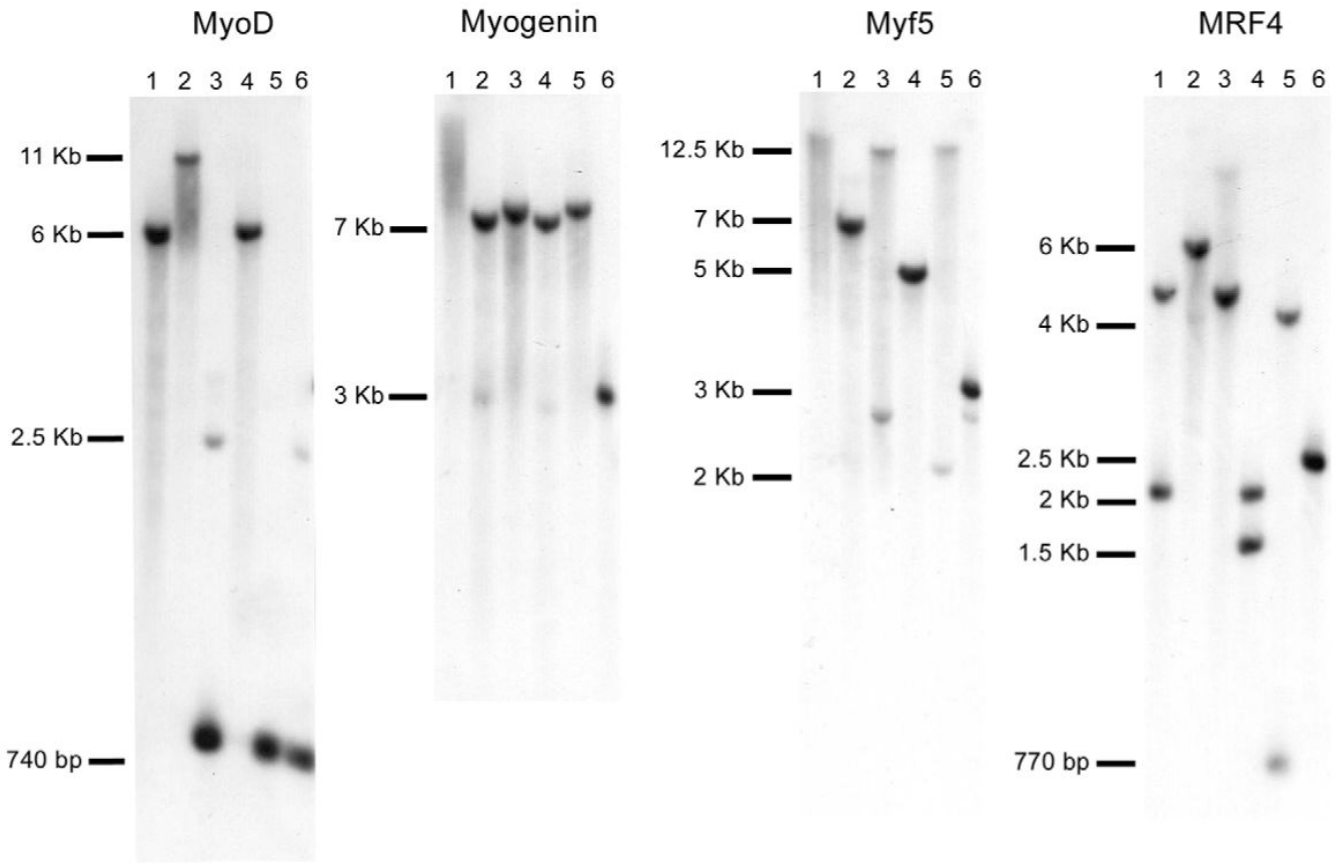

Fig. 7.

Genomic Southern blot analysis of the *MyoD*, *myogenin*, *myf5* and *MRF4* genes. Genomic DNA prepared from *S. macrurus* adults was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), *Bam*HI/*Eco*RI (lane 4), *Bam*HI/*Hin*dIII (lane 5) and *Eco*RI/*Hin*dIII (lane 6), resolved by 1% agarose gel electrophoresis, and transferred to a nylon membrane. Genomic DNA was loaded at 5 μg per lane. Blots were hybridized with ^{32}P-labeled probes specific to *S. macrurus MyoD*, *myogenin*, *myf5* and *MRF4*, and washed under high stringency conditions.

Table 1

Oligonucleotides and TaqMan fluorogenic probes

Gene	GenBank accession no.	Primer	Sequence 5′ → 3′	bp
MyoD	AY396566.1	s	TGCTCAGATGGCATGATGGA	77
		a	AAGTAGGAGTTGTCATAGCTGTTTCG	
		p	TAATGGCCCTACATGCCCGTCTGG	
myogenin	AY396565.1	s	GCAGCCCAGAGTGGAGCA	114
		a	GATGTCAGAGACCTCATGTTGGC	
		p	CCGCCTACAGCTCCACCCACGA	
myf5	DQ016032.1	s	GCTCCGACAGCATGACAG	72
		a	TGTAAGTGCTGCATGGGTTTA	
		p	TTGCAGCAGTCCTGTCTGGTCTCA	
MRF4	DQ059552.1	s	GCAAGCTAAACTCCCTGATAACAATA	82
		a	TTTCCAGTGATACTCACCGTTACC	
		p	AACTGTAGCGCCAAAGACCACAGCG	

Sequences are shown for sense (s) and antisense (a) primers and TaqMan probe (p), as well as the size of amplicon (in base pairs, bp). Primers for MyoD and MRF4 were between exons 1 and 2, and primers for myogenin and myf5 were between exons 2 and 3.