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Transcription of MyoD and Myogenin in the Non-contractile Electrogenic Cells of the Weakly Electric Fish, *S. macrurus*

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Running Title: Muscle regulatory factors in myogenically derived electrocytes

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ABSTRACT

The MyoD family of basic helix-loop-helix (bHLH) myogenic regulatory factors (MRFs) are transcriptional activators of skeletal muscle gene expression and are pivotal in inducing the full myogenic program. The expression of these factors after muscle differentiation is complete and the mechanism by which they modulate (or maintain) the muscle phenotype, is less well understood. The myogenically derived electric organ (EO) of the electric fish *Sternopygus macrurus* is an excellent model to address this question. The electrocytes, i.e., the electrogenic cells of the EO, are not contractile but they do retain some muscle proteins. In order to examine the molecular regulatory pathways that control the muscle-to-electrocyte cell conversion, we have cloned the MyoD and myogenin cDNAs from *S. macrurus*. Clustal-based alignments showed that the functional domains observed in mammalian MyoD and myogenin are highly conserved in these MRF homologs. Expression analyses revealed that mature electrocytes, which retain the muscle proteins dystrophin, desmin, acetylcholine receptors (AChRs), α-actin, and α-actinin, also transcribe the MyoD and myogenin genes. RT-PCR studies confirmed that expression of these MRFs is confined to the myogenic lineage. Surprisingly, the levels of MyoD and myogenin transcripts in skeletal muscle and EO could not be used to predict the level to which a cell manifests the muscle program. We conclude that expression of multiple MRFs is not sufficient to induce non-contractile cells to fully express the skeletal muscle program. These data also suggest that the MRF transcriptional program in *S. macrurus* may be distinct from MRF-dependent myogenesis in other vertebrate systems.

Key words: MyoD, myogenin, electrocytes, electric fish
**INTRODUCTION**

Electrocytes, the current-producing cells of electric organs (EOs), are unique in that they possess biochemical characteristics of both muscle and non-muscle cells. Electrocytes produce an electric field outside the body, which is essential to the organism as part of an electrosensory system (weakly electric fish) or as a defensive or offensive weapon (strong electric fish) (reviewed in Bennett, 1971). Electrocytes are found in species that have evolved independently at least six times (Darwin, 1859; Bennett, 1971), and derive from different skeletal muscle groups in different lineages including tail, extraocular, brachial, pectoral and axial muscles (Bass, 1986; Bennett, 1971). In each case, light and electron microscopy studies have shown that mesodermal cells initially differentiate into myoblast-like cells that subsequently form multinucleated myotubes. Conversion of these myotubes to mature electrocytes is characterized by the disassembly of sarcomeric structures, down regulation of sarcomeric proteins, the replacement of myofibrils with amorphous material, relocation of many organelles to the periphery, and changes in cell size and shape (Bennett, 1971).

In the weakly electric fish *Sternopygus macrurus*, electrocytes are similar to their muscle precursors in that they are multinucleated, express proteins common to muscle cells such as desmin, talin, acetylcholine receptors (AChRs), α-actin, sarcomeric α-actinin (Patterson and Zakon, 1993), a paralog of the muscle sodium channel gene Nav1.4 (Harold Zakon, personal communication), and are innervated at a single endplate (Bennett, 1971; Unguez and Zakon, 1998b). In contrast to muscle fibers, mature electrocytes do not contain sarcomeres, sarcoplasmic reticulum, or T-tubules, nor do they produce tropomyosin or myosin heavy chains (MHCs) (Patterson and Zakon, 1996). However, electrocytes do express keratin, a protein not found in muscle fibers (Patterson and Zakon, 1996). In addition, electrocytes have a cross-sectional area (range, 7181-43,292 µm²) that is remarkably larger
than adjacent muscle fibers (range, 175-1990 µm²; Unguez and Zakon, 1998a), and these cells are electrically driven by electromotoneurons at a continuous rate of 50-200Hz (Mills et al., 1992). This frequency of activation is significantly higher than found for muscle fibers, which are activated intermittently at frequencies lower than 10 Hz (Rome et al., 1992, 1996). The retention of both muscle and electrocyte characteristics reflects their common differentiation pathway. Yet, the presence of keratin in electrocytes demonstrates that unique transcriptional regulatory pathways control the electrocyte phenotype.

The transdifferentiation of skeletal muscle fibers to electrocytes is a developmental phenomenon that is recapitulated in the adult during regeneration events. Following amputation of the tail, regeneration proceeds by local formation of a blastema that consists of undifferentiated ependymal and mesenchymal cells that subsequently differentiate into spinal cord, skin, skeleton, muscle and electrocytes (Patterson and Zakon, 1996). Based on ultrastructural analysis, we found that mature muscle fibers fuse with one another to give rise to electrocytes (Unguez and Zakon, 1998a) – a process that accounts for the much larger cross-sectional area of electrocytes. Moreover, the skeletal muscle fibers that undergo fusion express the fast isoform of skeletal muscle myosin (Unguez and Zakon, 1998a), suggesting that only a subset of muscle fibers can transdifferentiate into electrocytes. The cellular and molecular mechanisms responsible for the fusion of fast-specific muscle fibers are not known. Similarly, the regulatory pathways that allow a differentiated electrocyte to downregulate some, but not all components of the muscle program is an intriguing problem in our current understanding of the transcriptional regulation of skeletal myogenesis.
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. Their pivotal role in the differentiation and commitment of cells to the skeletal muscle lineage is well established based on genetic studies inactivating individual MRF genes and their ability to activate the myogenic program following forced expression into nonmuscle cells (Choi et al., 1990; Delfini and Duprez, 2003; Weintraub et al., 1989). Transgenic and gene knock-out mice further demonstrate that MRFs are essential for skeletal muscle development (Nabeshima et al., 1993; Rudnicki et al., 1993).

The wide phylogenetic distribution of the MyoD family across animal taxa (Atchley et al., 1994; Zhang et al., 1999), and in the myogenically derived EO of Torpedo (Neville and Schmidt, 1992), suggested to us that the MRFs may also be pivotal to the proper development of *S. macrurus* tissues including skeletal muscle and electrocytes. In this study, we examined if the partial muscle phenotype of electrocytes in *S. macrurus* is due to an MRF expression pattern that correlates to the level and number of the muscle proteins maintained after their differentiation. We show for the first time that the *S. macrurus* genome contains functional MyoD and myogenin genes that are expressed in regenerating blastema, skeletal muscle, and EO cells, but not in liver or brain cells. Our studies reveal that MRF expression in *S. macrurus* is confined to tissues containing cells in the myogenic lineage and that the quantitative expression patterns of MyoD and myogenin in muscle fibers and electrocytes do not correlate with the level to which each cell manifests the myogenic program.
MATERIALS AND METHODS

Animal Tissue Preparation

*S. macrurus* is a fresh-water species of knife fish native to South America and was obtained commercially from Segrest Farms (Gibson, FL). Adult fish, 20-35 cm in length, were housed individually in 15 to 20-gallon aerated aquaria maintained at 25-28°C and fed three times weekly. In one group, fish were overdosed using 2-phenoxyethanol (1:500 in tank water) and the liver, brain, ventral skeletal muscle and EO were excised under a dissecting microscope. Tissues were immediately immersed in RNA later™ (Ambion, Austin, TX) and stored at -80°C until RNA extraction. In a second group of fish, the tail segment distal to the ventral fin was amputated under anesthesia (2-phenoxyethanol, 1:1500, 3 minutes) and frozen in isopentane cooled in liquid nitrogen for histochemical processing. Two weeks after tail amputation, fish were re-anesthetized and their regeneration blastemas were removed for RNA isolation. After each surgery, fish were returned to their tanks and monitored until they recovered fully from anesthesia. 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.

Immunohistochemistry

Serial transverse and longitudinal sections of control adult tails were cut at 12-µm thickness in a cryostat at –20°C, mounted on glass slides, and air-dried at room temperature. Tissue sections were rehydrated in phosphate-buffered saline (PBS) for 5 min, incubated in blocking solution (PBS, 2% bovine serum albumin, and 5% horse serum) for 30 min, and subsequently incubated overnight at room temperature in monoclonal antibodies specific to MHC (MF20), desmin (D76), tropomyosin (CH1), α-actinin (EA53), actin (JLA20), neurofilament-associated protein (3A10), and α-
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acetylcholine receptor (88b) at a dilution of 1:10. Primary antibodies were obtained from the Developmental Hybridoma Bank (Iowa City, IA) and were visualized with a fluorescein-conjugated secondary antibody (anti-mouse, 1:200; Cappel, ICN Pharmaceuticals, Aurora, OH).

The cellular distribution of dystrophin in skeletal muscle fibers and electrocytes was analyzed using monoclonal antibodies 5A3 and 3E7, kindly supplied by Dr. D. Mornet (Institut Bousson-Bertrand, Montpellier, France). Anti-dystrophin antibody labeling was visualized by using a biotinylated secondary antibody (Vectastain ABC kit, Vector Laboratories, Burlingame, CA), and a horseradish peroxidase reaction was run to amplify the signal by use of diaminobenzidine and hydrogen peroxide. Images of immunolabeled tissue sections were captured using a Zeiss Axioskop epifluorescence microscope connected to an Orca-100 digital camera (Hamamatsu, Bridgewater, NJ) interfaced to a Macintosh G4 running Openlab 2 (Improvision, UK).

**RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total cellular RNA was isolated from 2-week regeneration blastemas, ventral skeletal muscle, EO, liver, and brain of adult *S. macrurus* using a protocol adapted from the guanidinium thiocyanate method of Sambrook et al. (1989). To remove residual DNA, total RNA isolated from each tissue was treated with DNase I, Amplification Grade (Invitrogen, Carlsbad, CA) and then analyzed by electrophoresis and spectrophotometry (OD$_{260}$/OD$_{280}$). On average, total RNA isolations yielded 1-3% of starting material from each of the different tissues. mRNA was isolated from total RNA preparations using the *MicroPoly(A) Purist™* kit (Ambion). All RNA samples were stored at -80°C.
Cloning of partial MyoD and myogenin transcripts from *S. macrurus* was performed with RT-PCR using the One-Step RT-PCR Superscript™ with Platinum® Taq System (Gibco, BRL, Gaithersburg, MD) according to the manufacturer’s specifications. Total RNA from 2-week regeneration blastema was used because at this regeneration stage, the blastema is composed of a mass of cells at different stages of proliferation and differentiation including myogenic cells (Patterson and Zakon, 1997). Reverse transcription from 1 µg of total RNA was performed for 30 min at 50°C, followed by a 2-min incubation at 94°C. PCR amplification of the cDNAs corresponding to MyoD and myogenin mRNAs was performed in the same reaction tube immediately following the RT step. The standard PCR profile included 30-40 cycles of 15-sec denaturation at 94°C, 30-sec primer annealing at 52-55°C (depending on the primers, Table 1), and 30-sec elongation at 72°C, and finishing with one elongation step at 72°C for 7 min. RT-PCR conditions (primer concentrations, input RNA, cycling conditions) were initially optimized and these were identical for all samples. Heterologous degenerate oligonucleotide primers were synthesized for MyoD and myogenin based on respective GenBank sequences and published data on other teleosts (Table 1). The MyoD primers correspond to the cysteine-rich (WACKACK; sense primer) and serine-rich (SSPRSNC; antisense primer) domains of carp MyoD (Kobiyama et al., 1998; accession number AB012882). The myogenin primers correspond to MNPNQRL, the region between the first and second helix domains (sense primer) and the NLRSLTSI (antisense primer) domain of zebrafish myogenin (Chen et al., 2000; accession number AF202639).

To examine the integrity of the templates, a ubiquitously expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous marker in parallel amplifications. GAPDH primers were synthesized based on the rainbow trout cDNA (Saito et al., 2001; accession
number AB066373) with a 487-bp PCR product. PCR products were run out on a 1% agarose gel stained with ethidium bromide (10 µl per 100 ml). To monitor DNA contamination in all RT-PCR experiments, control reactions were performed without the cDNA template or reverse transcriptase enzyme. Primer-specific amplification was also tested in RT-PCR reactions in the absence of primers.

**Cloning and sequencing of RT-PCR products**

RT-PCR products for MyoD and myogenin were gel isolated using a gel extraction kit, (Qiagen, Valencia, CA), subcloned into the pCR®2.1-TOPO vector (Invitrogen) and transformed into TOPO cells (Invitrogen). GAPDH was subcloned into the pGEMT-Easy Vector System II (Promega, Madison, WI). Plasmids from ten to twenty cDNA clones of each transcript were isolated using the QIAprep Spin Miniprep Kit (Qiagen), sequenced in both directions, and analyzed by either a Li-Cor 4200L Global IR2 DNA Sequencer or an Applied Biosystems automated DNA sequencer (Model 377).

Following verification of the cloned sequences obtained from 2-week regeneration blastemas, homologous primers to MyoD and myogenin specific to *S. macrurus* were designed for qualitative RT-PCR experiments using total RNA (1 µg) or mRNA (100 ng) from 2-week regeneration blastema, skeletal muscle, EO, brain, and liver, using similar amplification parameters as described above. RT-PCR experiments using homologous primers resulted in partial sequences of 290 nt and 312 nt for MyoD and myogenin, respectively. The cloned partial sequences were subsequently used for quantitative RT-PCR, and 3’- and 5’-RACE experiments (see below).
Quantitative RT-PCR

We used the RT-PCR Competitor Construction Kit (Ambion) to design internal (“competitor”) DNA templates containing ends complimentary to, and amplifiable with *S. macrurus* MyoD, myogenin, and GAPDH-specific primers. The competitor DNA templates were synthesized to contain nucleotide deletions in the 5’ and 3’ ends resulting in templates that were 79 nucleotides shorter than the endogenous partial sequences for each transcript, transcribed in the presence of [α-32P] dATP, and purified by gel excision and ethanol extraction. The concentration of each of the competitor templates was calculated (µM or copies/µl) and known amounts of these competitor templates were added in known dilutions and co-amplified in a competitive one-step RT-PCR reaction (Invitrogen) with 100 ng of mRNA from EO, muscle, or 2-week regeneration blastema. RT-PCR conditions were similar to those used for qualitative RT-PCR (see above). The PCR products were run out on a 2% agarose gel and the lane that showed matching intensities between the competitor and the endogenous transcripts was used to determine the amount of MyoD, myogenin, or GAPDH in each tissue sample. Quantitative RT-PCR was performed in duplicate using muscle, EO, and 2-week blastemas from 4, 80, and 20 different fish, respectively. Transcript levels were normalized to 1 µg of mRNA per tissue. The data were expressed as the mean ± standard error of the mean (SEM) per transcript per tissue type.

Molecular cloning of full-length MyoD and myogenin sequences from *S. macrurus*

The full-length coding sequences of both MyoD and myogenin were obtained by rapid amplification of cDNA ends (RACE) prepared from mRNA (150 ng) of EO from *S. macrurus* with the 3’-RACE Adapter Primer (Gibco BRL) and 5’ GeneRacer Kit (Invitrogen). RACE first strand synthesis was performed using Oligo-ligated mRNA for 5’ and 3’ amplifications. MyoD specific primers for 5’-
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RACE were 5’-GAG CAG TTG GAC CTG GGA CTG TTG GGA TTA G-3’, and for 3’-RACE were 5’-GCA GGC AGG TGT CTT CTG TGG G-3’ and 5’-CTA ATC CCA ACC AGA GGC TTC C-3’. Myogenin specific primers for 5’-RACE included 5’-CAC TAT AGA CGT CAG AGA CCT C-3’ and 5’-GCT AC A GCA GGT GCT GCC AGA G-3’, and for 3’-RACE were 5’-GTG AGC ATG GAT AGG CGA CGG G-3’ and 5’-CGA GAG GCT CCA GGC GTT GGT C-3’.

Nucleotide and deduced amino acid sequences were analyzed using the Vector NTI Suite 8.0 software program (InforMax Inc., Bethesda, MD). BLAST searches were performed using the BLAST network service (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were generated with ClustalW from the European Bioinformatics Institute at http://www.ebi.ac.uk/clustalw/. The two novel sequences described here have been submitted to the DNA databases with the Accession numbers AY396566 and AY396565 for *S. macrurus* MyoD and myogenin, respectively.

**Northern Blot Analysis**

Partial RNA transcripts of MyoD (290 nt) and myogenin (312 nt) were produced from linearized plasmids using the MaxiScript SP6/T7 RNA Transcription Kit (Ambion), purified using the RNeasy Kit (Qiagen), and stored at –20°C in 25 µl aliquots for up to two weeks. These samples acted as standards to adjust for probe specific activities and thus, allow comparisons among different experiments. A random primer kit (Gibco BRL) was used to generate radiolabeled probes. Unincorporated radioactivity was removed with an Amersham G25 spin column. RNAs isolated from skeletal muscle, EO, and 2-week regeneration blastemas (10 µg of total RNA or 1 µg of
mRNA) were fractionated by electrophoresis on 1.2% agarose gels containing formaldehyde, transferred to a nitrocellulose membrane, and UV cross-linked (254 nm for 1200 joules) in a UV Stratalinker. Membranes were pre-hybridized for 1-4 h at 42°C with 10 ml prehybridization-hybridization-buffer (5X SSC; 40 % formamide; 5X Denhardt’s-solution; 1% SDS; 50 µg/ml heat-denatured sheared non-homologous DNA (Salmon sperm DNA). Radioactive labeled probes were denatured for 5 min at 95°C, cooled on ice, and added to the hybridization buffer at 42°C for 12-36 hours. Membranes were washed 2 times for 20 min with 2X SSC, 0.1% SDS at RT and washed 1 time with 0.1 SSC, 0.1% SDS at 50°C for 15 min. Semi-dry membranes were exposed to X-ray film under saran wrap (-80°C) for 2-7 days.

RESULTS
Given the importance of MRFs to muscle development, we suspected that expression of these key regulatory genes might be coupled to the partial level of muscle program expression in electrocytes. Initial tests using various antibodies against the mammalian MRF proteins failed to identify any cross-reactivity with S. macrurus tissues. Therefore, we sought to isolate cDNA clones of the S. macrurus MyoD gene family to determine the correlation between MRF transcript expression and the presence of muscle-specific proteins in electrocytes. Based on DNA sequence alignments of heterologous MRF sequences from teleosts and murine mRNAs, DNA primers were designed to clone partial MyoD and myogenin cDNAs from S. macrurus tissues (Table 1). 5’- and 3’-RACE strategies were then implemented to clone full-length MyoD and myogenin cDNAs.
Cloning and sequence analysis of *S. macrurus* MyoD

PCR-coupled RACE generated a 1,115 bp *S. macrurus* MyoD cDNA (accession number AY396566). This cDNA contains a 274 amino acid open reading frame that is flanked by a 213 bp 5’ untranslated region (UTR) and a 77 bp 3’ UTR. The open reading frame includes a predicted N-terminal activation domain followed by a His/Cys-rich domain that is required for chromatin remodeling, the bHLH dimerization and DNA binding domain, and the C-terminal helix III domain, an amphipathic alpha-helix that is necessary for the efficient initiation of the endogenous skeletal muscle gene expression program (Bergstrom and Tapscott, 2001; Gerber et al., 1997; Ma et al., 1994; Rhodes and Konieczny, 1989; Schwarz et al., 1992; Weintraub et al., 1991).

Multiple sequence alignments of the *S. macrurus* MyoD protein with mouse (Figure 1A), carp (*C. carpio*), zebrafish (*D. rerio*), tilapia (*O. aureus*), rainbow trout (*O. mykiss*), and gilthead seabream (*S. aurata*) (Figure 1B) revealed that the *S. macrurus* MyoD protein exhibits a high degree of sequence conservation with these other species. The degree of nucleotide similarity between MyoD of *S. macrurus*, mouse and other piscine species ranged between 53-64% while the protein similarity was considerably higher, ranging from 64% identity (mouse) to 88% identity (carp) (Figure 1A, B). However, the His/Cys-rich, bHLH, and helix III domains were highly conserved, with greater than 85% similarity across all species.

Cloning and sequence analysis of *S. macrurus* myogenin

Isolation of the *S. macrurus* myogenin cDNA generated a 1,401 bp fragment which contained a 252 amino acid open reading frame flanked by a 138 bp 5’ UTR and a 504 bp 3’ UTR (accession number AY396565). The degree of similarity between myogenin of *S. macrurus*, mouse and other piscine
species at the nucleotide sequence level ranged from 23-75%, with mouse having the lowest similarity (23%).

Multiple sequence alignment of myogenin proteins from mouse (Figure 2A) and other piscine species (Figure 2B) showed a sequence identity of 58%, 80%, 79%, 66%, and 70%, with mouse, carp, zebrafish, halibut (*H. hippoglossus*), and trout, respectively (Figure 2B). As expected, the His/Cys-rich, bHLH and C-terminal helix III domains were highly conserved among all species examined, including mouse. These results suggest that the *S. macrurus* MyoD and myogenin proteins likely have similar myogenic regulatory functions as observed with their mammalian counterparts.

**Differential expression of structural proteins in muscle and EO cells**

Electrocytes are unique cell types that are derived from mature muscle cells yet acquire the ability to produce strong electric fields. Interestingly, the mature EO retains a partial “muscle-like” phenotype suggesting that the muscle transcriptional machinery remains operative in these cells (Patterson and Zakon, 1993; 1997). To understand the regulatory mechanisms that operate during this transdifferentiation event, we examined electrocytes for the expression of various known skeletal muscle proteins. Control immunostaining of mature skeletal muscle cells with antibodies against skeletal muscle MHC, tropomyosin, sarcomeric actin, desmin, and α-actinin confirmed the normal expression pattern in all muscle fibers (Figure 3). Interestingly, mature electrocytes also express skeletal muscle-specific actin, desmin, and α-actinin, with a more intense immunolabeling obtained with antibodies against desmin and actin than with α-actinin (Figure 3). Although mature electrocytes continue to express a subset of skeletal muscle-specific proteins, they do not express a
complete complement of muscle gene products since sarcomeric MHC and tropomyosin are not detected in these samples (Figure 3).

We next examined the expression of dystrophin, a muscle-specific membrane associated protein, in EO and skeletal muscle cells. Dystrophin immunoreactivity was found in mature electrocytes using two different dystrophin antibodies (5A3, 3E7) (Figure 4C, F, and data not shown). The dystrophin protein was confined to the endplate region of electrocytes (Figure 4C, F) where co-localization of neurofilaments (Figure 4A, D) and α-acetylcholine receptors (Figure 4B, E) was found. As predicted, dystrophin was also localized to the muscle cell membranes (data not shown).

Muscle, electric organ, and regenerating blastema express MyoD and myogenin transcripts
Our data showed that electrocytes maintain the expression of several different sarcomeric and non-sarcomeric skeletal muscle proteins, despite their transdifferentiation from muscle to EO. The maintenance of muscle-specific proteins suggests that MyoD and/or myogenin may account for this partial “muscle-like” phenotype. To determine if MyoD and myogenin are expressed in EO, we used RT-PCR to compare their patterns of expression in skeletal muscle, EO, 2-week regeneration blastema, brain and liver tissues. As predicted, MyoD and myogenin transcripts were readily detected in adult skeletal muscle (Figure 5). Interestingly, MyoD and myogenin transcripts were also detected in the EO despite its incomplete “muscle-like” phenotype (Figure 5). Two-week blastemas undergoing regeneration also showed detectable levels of MyoD and myogenin transcripts, although at somewhat lower intensities than those found in either skeletal muscle or EO (Figure 5). MyoD and myogenin transcripts were not detected in nonmuscle cell types such as liver
or brain (Figure 5). The presence of MyoD and myogenin transcripts in skeletal muscle, EO, and 2-week regeneration blastema was confirmed by Northern blot analysis (data not shown).

The presence of MyoD and myogenin in EO is particularly noteworthy because this organ lacks many sarcomeric proteins that are characteristic of mature skeletal muscle cells including MHC, tropomyosin, sarcomeric actin and dystrophin (see Figures 3 and 4). To determine whether differential levels of MyoD and myogenin transcripts correlated with differences in muscle protein expression profiles, we performed quantitative RT-PCR to establish levels of endogenous transcripts in EO and skeletal muscle.

As shown in Figure 6, the transcript levels of MyoD and myogenin differed significantly between skeletal muscle and EO. Overall, myogenin mRNA content was approximately 5-fold higher in EO than in muscle, whereas MyoD mRNA levels were about 2-fold lower in EO than in muscle. Furthermore, the expression patterns of myogenin and MyoD in skeletal muscle were comparable to those exhibited in 2-week blastema. For example, the expression of MyoD was relatively uniform in muscle (3.11 x 10^8 ± 0.35 molecules/µg mRNA) and 2-week regeneration blastema (2.67 x 10^8 ± 0.29 molecules/µg mRNA). The MyoD mRNA levels of EO were slightly lower (1.64 x 10^8 ± 1.48 molecules/µg mRNA) than that of either muscle or 2-week regeneration blastema.

In contrast to the MyoD expression patterns, pronounced differences in myogenin mRNA content were found across muscle, 2-week regeneration blastema, and EO. The EO (39.10 x 10^8 ± 0.00 molecules/µg mRNA) exhibited almost a 5-fold higher transcript level than either muscle (7.40 x 10^8 ± 1.16 molecules/µg mRNA) or 2-week regeneration blastema (8.10 x 10^8 ± 0.00 molecules/µg mRNA).
mRNA) samples. The myogenin mRNA content of muscle resembled that of the 2-week regeneration blastema – an expression pattern similar to that found with MyoD levels. The expression levels of myogenin were higher than MyoD in all three tissues studied. The myogenin mRNA content in muscle and 2-week regeneration blastema was approximately 2.4- and 3-fold higher than the MyoD mRNA content, respectively. EO showed the greatest difference of all three tissues. The EO myogenin transcript levels were 24-fold higher than the corresponding MyoD transcript levels.

The predominance of myogenin over MyoD in all three tissues investigated was further illustrated by their specific ratios. The ratio of myogenin to MyoD across tissues was 2.38 for muscle, 23.84 for EO, and 3.03 for 2-week regeneration blastema. Further, myogenin and MyoD transcript copy number in each tissue was normalized to its GAPDH mRNA content and this is shown in Figure 6C. Although the choice of internal reference RNA can be tissue- or cell-dependent (Tricarico et al., 2002), comparisons of each MRF to GAPDH (Figure 6C) corroborate the relative differences in MRF expression patterns across tissue types when evaluating each MRF alone (Figure 6B). Data from the present study show that down regulation of the sarcomeric proteins MHC and tropomyosin in differentiated electrocytes occurs without transcriptional suppression of MyoD or myogenin. Instead, myogenin is expressed at high levels suggesting a transcriptional up regulation of the gene in electrocytes, despite their non-contractile muscle-like phenotype.

**DISCUSSION**

Our previous studies on the origin of electrocytes of the weakly electric fish *S. macrurus* described the fusion of fast muscle fibers, the subsequent disassembly of sarcomeres, and the
down regulation of a subset of sarcomeric genes to give rise to the non-contractile electrogenic cells with a partial muscle phenotype (Unguez and Zakon, 1998a). We also demonstrated that the formation of electrocytes via transdifferentiation of skeletal muscle fibers, and the maintenance of a partial muscle-like phenotype after differentiation, are dependent on input from the nervous system (Unguez and Zakon, 1998b; 2002). However, the transcriptional mechanisms that maintain the partial myogenic program of electrocytes are not known. In this study we show that the myogenic regulatory factors MyoD and myogenin are expressed in mature electrocytes despite the fact that these cells do not maintain a normal muscle phenotype.

MyoD and myogenin are differentially expressed in myogenically-derived tissues in S. macrurus

Quantitative RT-PCR revealed that both MyoD and myogenin are transcribed in mature skeletal muscle and EO, but myogenin transcript levels in EO cells far exceed the levels detected in muscle cells. This preferential accumulation of myogenin over MyoD in EO is intriguing. For one thing, electrocytes derive from fast muscle fiber types (Unguez and Zakon, 1998a), and MyoD, not myogenin, is known to be the predominant MRF in fast muscle cells in other vertebrates (Charbonnier et al., 2002; Hughes et al., 1993; Miller, 1991). Further, functional studies in mice have shown that the helix III domain of MyoD confers a higher efficiency of muscle gene activation than that of myogenin (Bergstrom and Tapscott, 2001), and this region is fully conserved in the S. macrurus MyoD (compare Figures 1A and 2A).

Transcript levels of MyoD and myogenin are known to differ in a fiber type-specific manner in other vertebrates (Charbonnier et al., 2002; Hughes et al., 1993), and this variation is thought to
correspond to a differential activation of muscle genes by these MRFs (Charbonnier et al., 2002; Chin et al., 1998; Hughes et al., 1993; Wheeler et al., 1999; Yutzey et al., 1990). To our knowledge, differential regulation of muscle genes like dystrophin, desmin, actin, and α-actinin by MyoD and myogenin is not known. This model system will allow the study of how distinct MRFs control the expression of clearly defined subsets of muscle proteins.

Detection of MRFs in cells with a partial muscle phenotype

The presence of MRFs in the partial muscle-like electrocytes in *S. macrurus* is reminiscent of the MRF profile found in the non-contractile Purkinje fibers, i.e., the electrical conductive cells of the heart (Takebayashi-Suzuki et al., 2001), myoid cells of the thymus (Grounds et al., 1992), and myofibroblasts (Mayer and Leinwand, 1997). In each of these cell types, the presence of MyoD and myogenin transcripts does not ensure the full expression of the skeletal muscle program. Since only a select number of muscle-specific genes are activated in Purkinje fibers, myoid cells, myofibroblasts, and electrocytes, it is unlikely that a single “master” transcription factor controls all programs underlying the unique gene expression of these partial muscle-like cell types. Further studies of cells with a partial muscle program are likely to provide insight into the possible roles of MRFs in the homeostasis of muscle-specific properties after differentiation.

Our analysis of transcript levels does not allow us to draw firm conclusions about MRF protein activity in electrocytes. There are several cases in which the presence of MRF mRNAs does not necessarily correlate with the presence of functional proteins. Myf5 mRNA, for example, has been reported in distinct regions of the mammalian brain, but the protein has not been detected
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(Daubas et al., 2000). Unfortunately, at this time we lack specific antibodies that can be used to detect *S. macrurus* MyoD and myogenin proteins. The development of these reagents will be critical to fully evaluate their role in the EO and to characterize the unique mechanism of muscle gene regulation that exists in electrocytes.

**Phylogenetic analysis of *S. macrurus* MyoD and myogenin**

Multiple MyoD genes have been reported for some vertebrates including *Xenopus* (Scales et al., 1991) and the teleosts gilthead seabream (Tan and Du, 2002) and rainbow trout (Delalande et al., 1999; Rescan and Gauvry, 1996). However, only one MyoD gene has been reported for other teleosts such as carp, zebrafish and puffer fish (*Fugu rubripes*). Hence, although increased gene copy number is a common feature in fish (Meyer and Schartl, 1999), differences in the number of MyoD genes across species may result from independent gene duplication events or from a genome duplication event followed by gene loss in some species (Robinson-Rechavi et al., 2001; Tan and Du, 2002). Although Northern blot analyses suggest that only a single MyoD and myogenin gene exists in *S. macrurus*, further studies are needed to evaluate the specific MRF gene number in *S. macrurus*. At present, the *S. macrurus* MyoD and myogenin mRNAs and protein sequences share the highest identity with the single MyoD and myogenin genes reported for carp and zebrafish.

**Expression of muscle properties in electric organs of other electric fish species**

The expression of MRF transcripts in electrogenic cells with a partial muscle phenotype is not unique to the EO of *S. macrurus*. As in *S. macrurus*, electrocytes in the strongly electric fish *Torpedo* derive from skeletal muscle fibers, and these continue to express desmin, dystrophin,
AChRs, and actin (Gullick and Lindstrom, 1982; Jasmin et al., 1990; Walker et al., 1985).

However, the non-contractile electrocytes in *Torpedo* contain loose clusters of myofibrils that do not assemble into sarcomeres (Fox and Richardson, 1979). Further, Neville and Schmidt (1992) showed that MyoD and Myf5 are transcribed in mature electrocytes with higher amounts of MyoD than Myf5 mRNAs (Neville and Schmidt, 1992). In this study, no myogenin transcripts were detected in either muscle or EO of *Torpedo*. To date, the MRF expression patterns in EO of electric fish other than *S. macrurus* (present study) and *Torpedo* (Neville and Schmidt, 1992) have not been examined. Nevertheless, these studies clearly demonstrate that expression of multiple MRFs does not ensure an electrocyte to fully express the skeletal muscle program.

The expression of MRFs in the electrocytes with a partial muscle phenotype in different electric fish species is interesting given that electrocytes in *S. macrurus* and *Torpedo* form from distinct muscle groups, and that both species evolved independently of each other (Bass, 1986; Bennett, 1971). Despite the large phylogenetic distance between *S. macrurus* and *Torpedo*, it is feasible that similar regulators and mechanisms underlie the phenotypic switch from muscle to EO, and the continued activation of a partial muscle program in electrocytes after differentiation. In conclusion, the electrocyte phenotype demonstrates that the expression of multiple MRFs is not sufficient to induce non-contractile cells of the myogenic lineage to fully express the skeletal muscle program. These data suggest that the MRF transcriptional program in electrocytes may be distinct from MRF-dependent myogenesis in other vertebrate systems.

*Future directions*
MyoD and myogenin may be regulating the expression of subsets of muscle proteins in mature electrocytes. However, it is worth noting that Myf5, also implicated in the establishment of muscle progenitor lineages in vertebrates (Pownall et al. 2002), has been detected in skeletal muscle and in electrocytes of *S. macrurus* (Clinton et al., 2003). Our preliminary data is based on cloning of a partial Myf5 cDNA from *S. macrurus* that includes a 471-bp cDNA fragment generated by PCR-coupled 3’-RACE. Alignment of its predicted protein sequence with mouse revealed high sequence conservation in the HLH and C-terminal helix III domains. We have not yet studied MRF4 expression in *S. macrurus*. The few studies that have examined the expression of MRFs in mammals postnatally have demonstrated that MRF4 is expressed at a higher level than any other MRF in mature skeletal muscle, and at the single cell level, MRF4 is preferentially expressed in slow oxidative muscle cells (Rhodes and Konieczny 1989; Voytik et al., 1993; Walters et al., 2000). Whether mature electrocytes also transcribe MRF4 will be interesting with respect to their fast muscle fiber type lineage (Unguez and Zakon, 1998a).

Further, our very preliminary data from *in situ* hybridization studies show that muscle fibers and electrocytes in regenerating blastema contain MyoD (data not shown). Cellular characterization of MRF expression at the mRNA and protein levels using *in situ* hybridization and antibodies generated specifically to *S. macrurus* will provide important information in understanding the role that MRFs play in the transdifferentiation of muscle fibers to electrocytes and the maintenance of the partial muscle phenotype of mature electrocytes. These additional experiments will complete the characterization of MRF expression patterns in the myogenic lineage in *S. macrurus* and further test the idea that the partial muscle phenotype of electrocytes is due to a MRF composition that differs from that of its skeletal muscle precursor.
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LEGENDS

Figure 1. Sequence comparison of the deduced proteins encoded by *S. macrurus* MyoD with comparable sequences from mouse (A) and other piscine species (B). Sequences from mouse (*M. musculus*, Davis et al., 1987), carp (*C. carpio*, Kobiyama et al., 1998), zebrafish (*D. rerio*, GenBank Accession number AF318503), tilapia (*O. aureus*, Chen et al., 2002), rainbow trout (*O. mykiss*, Rescan and Gauvry, 1996), and gilthead seabream (*S. aurata*, Tan and Du, 2002) were used. The boxed areas are labeled to indicate the N-terminal, His/Cys-rich, basic HLH, and Helix III domains. Asterisks represent amino acid similarities across sequences.

Figure 2. Sequence comparison of the deduced proteins encoded by *S. macrurus* myogenin with comparable sequences from mouse (A) and other piscine species (B). Sequences from mouse (Fujisawa-Sehara et al., 1990), carp (Kobiyama et al., 1998), zebrafish (Chen et al., 2000), atlantic halibut (GenBank accession number AJ487982), and rainbow trout (Rescan et al., 1995) were used. The boxed areas are labeled to indicate the His/Cys-rich, basic HLH, and Helix III domains. Asterisks represent amino acid similarities across sequences.

Figure 3. Phenotypic properties of muscle fibers (top panels) and electrocytes (bottom panels) in a normal adult tail of *S. macrurus*. Serial cross sections (12 μm-thick) of a fish tail were immunolabeled with MF20 (anti-MHC), CH1 (anti-tropomyosin), JLA20 (anti-actin), D76 (anti-desmin), and EA53 (anti-α-actinin). Muscle fibers were strongly labeled by all antibodies, whereas electrocytes were labeled by JLA20 (anti-actin) and...
D76 (anti-desmin), and more lightly labeled by EA53 (anti-α-actinin), but were not immunoreactive to MF20 (anti-MHC) or CH1 (anti-tropomyosin). In the bottom panel, arrowheads point to muscle fibers and arrows point to electrocytes. It is noted that all electrocytes are labeled by desmin and the regions between electrocytes represent regions taken by connective tissue, bone, and/or blood vessels. EO, electric organ; MHC, myosin heavy chain.

Figure 4. Detection of dystrophin at the endplate region of electrocytes (EC) by immunohistochemistry. Shown are cross- (A-C) and longitudinal (D-F) sections (12 µm-thick) of a control adult tail that were immunoreacted with anti-neurofilament antibody 3A10 (A, D), anti-α-acetylcholine receptor antibody 88b (B, E), and anti-dystrophin antibody 5A3 (C, F). As evident in cross-sections, neurofilaments are not uniformly distributed around electrocytes (A, arrow) – a distribution pattern also found for α-acetylcholine receptors (B, arrow) and dystrophin (C, arrow). Immunostaining of longitudinal sections showed that anti-dystrophin label was more intense at the posterior surface of electrocytes (F, arrow), the region innervated by axons (D, arrow) and location of darkest AChR immunolabel (E, arrow). Scale bar = 10 µm.

Figure 5. Expression of MyoD and myogenin transcripts in adult tissues of *S. macrurus* by RT-PCR analysis. Total RNAs from skeletal muscle, EO, 2-week regeneration blastema, liver, and brain were reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and used for PCR. PCR products (1.8 µg per lane) were resolved on agarose gels containing ethidium bromide. The resultant bands are
presented as negative images of the original gels. A 290-bp cDNA fragment of MyoD and a 312-bp cDNA fragment of myogenin were detected in skeletal muscle, EO, and 2-week blastema, but not in liver or brain. Control reactions without reverse transcriptase were done for muscle (lane 3), EO (lane 5), 2-week regeneration blastema (lane 7), liver (lane 9), and brain (lane 11) to ensure that PCR products were RNA dependent and not the result of genomic DNA amplification. The RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a ubiquitously expressed gene, was included to ensure consistency in reaction loading and execution. Lanes $M$ represent the 1 Kb$^+$ DNA ladder.

Figure 6. Expression of MyoD and myogenin transcripts in adult tissues of $S. macrurus$ by quantitative RT-PCR analysis. (A) Representative quantitative RT-PCR reactions of MyoD and myogenin transcript levels in tissues of 2-week regeneration blastema. For each transcript, 1 µl of different dilutions of known amounts of competitor was added to 1 µl (100 ng) of endogenous mRNA per each RT-PCR reaction. Eight reactions (lanes 1-8) were run per transcript and the lane that showed matching intensities between the competitor and endogenous mRNAs indicates the amounts of MyoD or myogenin in that tissue ($asterisk$). $M$ lanes represent the 1 Kb$^+$ DNA ladder. (B) The amounts of MyoD (white bars) and myogenin (black bars) transcripts per tissue type were obtained from quantitative RT-PCR reactions, and are represented as transcript number per µg of mRNA of tissue type. Each column represents the mean ± SEM. (C) Levels of MyoD (white bars) and myogenin (black bars) mRNA relative to GAPDH.
mRNA amounts in each tissue type are presented. End, endogenous; Comp, competitor.
Table 1. **Primers used for polymerase chain reaction (PCR) amplification of S. macrurus MyoD, myogenin, and GAPDH cDNA**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank No.</th>
<th>Orientation</th>
<th>Sequence</th>
<th>Nucleotide Position</th>
<th>PCR Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AB066373</td>
<td>Forward</td>
<td>5’ – CATGTTCAAAGTATGACTCCACC – 3’</td>
<td>188-209</td>
<td>486</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’ – GTGCCAGCCAGAACATCAT – 3’</td>
<td>655-673</td>
<td></td>
</tr>
<tr>
<td>MyoD</td>
<td>AB012882</td>
<td>Forward</td>
<td>5’ – TGGGCATGCAAAGCCTGCAAG – 3’</td>
<td>400-421</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’ – CAGTCCGAGATCCAACTGCTC – 3’</td>
<td>670-690</td>
<td></td>
</tr>
<tr>
<td>Myogenin</td>
<td>AF202639</td>
<td>Forward</td>
<td>5’ – ATGAACCCCAACCAGGCTGC – 3’</td>
<td>504-525</td>
<td>320</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’ – GAGGTCTCTGACGTCTATAGTG – 3’</td>
<td>803-824</td>
<td></td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde-3-phosphate dehydrogenase
A

N-terminal

S. macrurus  MELSDIPFN--------------ITSDADDYDDFCSENTNDHFEELDPRLVHV-SLLKPD  46
M. musculus  MELSSPLRDIDLTGSDLCSFETADDYDPCFDSPDLRFFEDLDPRLVHV-GALLKPE  60

His/Cys-rich

S. macrurus  EHSI------------EDEHRAAGRCAGRLWACKACKRKTNNARDKKAAATMRERR  95
M. musculus  EHAHFSTAVHPGAREDEHRAAGRCAGRLWACKACKRKTNNARDKKAAATMRERR 120

HLH

S. macrurus  PLSKYNDAFELKCRTSTNPQRLPKVEILNAISYIESLSQALLRSQEDN------YYP 148
M. musculus  PLSKVNEAFELKCRTSNPQRLPKVEILNAISYIESLSQALLRDQDAAPPAAAAFYAP 180

Helix III

S. macrurus  TNCPQP----VQDPIYQVL 274
M. musculus  DAAPCPAGSNPAIYQVL 318
**B**

### N-terminal

- **S. macrurus**: MELSDIPFNITSADDYDDPCFNTNDMFFEDLDPLVLHVSLLKPDE-
- **C. carpio**: MELSDIPFIPSADDYDDPCFNTNDMFFEDLDPLVLHVSLLKPDE-
- **D. rerio**: MELSDIPFIPSADDYDDPCFNTNDMFFEDLDPLVLHVSLLKPDE-
- **O. aurea**: MELPDISFPIPTADDFYDDPCFNTSDMHFFEDLDPRLVHVGLLKPDDSSSSSSSSPSSSS
- **O. mykiss**: MELSDISFPVTSADDYDDPCFNTSDMHFFEDLDPRLVHVGLLKPDD-
- **S. aurata**: MELSDIPFPAADDYDDPCFNTSDMHFFEDLDPVLHVSLLKPDDSSSSVSPSSSA

### His/Cys-rich

- **S. macrurus**: ----------HSHIE---DEHIRAPSGHQAQCLWACKACKRKTTNADRRKAATMRER
- **C. carpio**: ----------HHHLE---DEHRAPSGHQAQCLWACKACKRKTTNADRRKAATMRER
- **D. rerio**: ----------HHHIE---DEHRAPSGHQAQCLWACKACKRKTTNADRRKAATMRER
- **O. aurea**: SS-PSSLLHLHHAEVEDDEHRAPSGHQAQCLWACKACKRKTTNADRRKAATLRER
- **O. mykiss**: ----------HHYNE---DEHIRAPSGHQAQCLWACKACKRKTTNADRRKAATMRER
- **S. aurata**: SSSPSSLLHLHHAEGDEHRAPSGHQAQCLWACKACKRKTTNADRRKAATLRER

### HLH

- **S. macrurus**: RRLSKVNDAFETLKRCTSTNPQRLPKVNERALRNAISYIESLQALLRSQE-DNYYPVLEQY
- **C. carpio**: RRLSKVNDAFETLKRCTSNPQRLPKVNERALRNAISYIESLQALLRGQE-ENYYPVLEHY
- **D. rerio**: RRLSKVNDAFETLKRCTSTNPQRLPKVNERALRNAISYIESLQALLRGQE-DNYYPVLEHY
- **O. aurea**: RRLGKVNDAFENLKRCTSNNPNQRLPKVNERALRNAISLQLRGQDGENYYPVLEHY
- **O. mykiss**: RRLGKVNDAFENLKRCTSNNPQRLPKVNERALRNAISLQLRGQDGENYYPVLEHY
- **S. aurata**: RRLSKVNDAFETLKRCTSTNPQRLPKVNERALRNAISYIESLQALLRGQDGENYYPVLEHY

### Helix

- **S. macrurus**: SGDSASSPRSNCSGMDFNGPTCPSGRNYSYDN-SYFNETQNGDARNKNSTVWSSLD
- **C. carpio**: SGDSASSPRSNCSGMDFNGPTCPSGRNYSYDN-SYFNETQNGDARNKNSTVWSSLD
- **D. rerio**: SGDSASSPRSNCSGMDFNGPTCPSGRNYSYDN-SYFNETQNGDARNKNSTVWSSLD
- **O. aurea**: SGDSASSPRSNCSGMDFNGPTCPSGRNYSYDN-SYFNETQNGDARNKNSTVWSSLD
- **O. mykiss**: SGDSASSPRSNCSGMDFNGPTCPSGRNYSYDN-SYFNETQNGDARNKNSTVWSSLD
- **S. aurata**: SGDSASSPRSNCSGMDFNGPTCPSGRNYSYDN-SYFNETQNGDARNKNSTVWSSLD

### III

- **S. macrurus**: LSSIVERISTETPACPALAHEGSDGSCPSQEQGSTLSTETVAPTSPNCP-QPVDPIY
- **C. carpio**: LSSIVERISTETPACPVLSVPEGHEGSCPSQEQGSTLSTETVAPTSPNCP-QPVDPIY
- **D. rerio**: LSSIVERISTETPACPVLSVPEGHEGSCPSQEQGSTLSTETVAPTSPNCP-QPVDPIY
- **O. aurea**: LSSIVERISTDNP--SSLPPADGP-GSPPTTTT-----VPMQFADPTRRR--------
- **O. mykiss**: LSSIVERISTDTPSACTVLSQEGSEGSCEQGPTQCPSQEQGSLSNRGTVYTVPNCQ-QPSHPDIY
- **S. aurata**: LSSIVERISTDND--SSLPPADGP-ASPTTPTGEEAAGFQVIPSPASQ--DPNLIIY

### QVL

- **S. macrurus**: QVL 274
- **C. carpio**: QVL 275
- **D. rerio**: QVL 275
- **O. aurea**: ---
- **O. mykiss**: QVL 275
- **S. aurata**: QVL 297
**

A

**

*S. macrurus*  
DGE under Review

*S. macrurus*  
M. *macrurus*  
MELYETSPYFYPHYFDG-ENYLPHVHQF-EPFGYER-------TELSPSPEARGPL

*** ** ***     ** *  *  *  * * **  **              * * * **

**

*S. macrurus*  
His/Cys-rich         basic

*S. macrurus*  
DKLSPALGLRFLSPQEOGLHCGQCLPWACKVCKRKEVSMDFRRAATLREKRRLKVKNE

M. *musculus*  
E----EKGLGTP-------EHCQGCLPWACKVCKRKEVSSVDRRAATLREKRRLKVKNE

*** ** ***    ******************** *******************

**

*S. macrurus*  
HLH

*S. macrurus*  
AFEALKRSTLMNPQRLPKVEILRSAIQYIERLQALYSSFNOQEEOQAGLPRGSAPQRV

M. *musculus*  
AFEALKRSTLLNPQRLPKVEILRSAIQYIERLQALLSSLNQEERD---LRYRGGGPQP

********** ************************* ** ** *     * ***

**

*S. macrurus*  
Helix III

*S. macrurus*  
SSSSEQGSGSTCCSPWESSSAEHCQPAYSSSTHEDLLNEDSSSEHANNRSLSITSDS

M. *musculus*  
MVPSECNSHASCPS-EWGNALEFG-----PNFGHLLAADTPAHNLHSLTSIVDSI-V

********** ********** ** ** *     * ***

**

*S. macrurus*  
EATPVTYSVDIK--

M. *musculus*  
EDMSVAFPPDEMPN

* *
S. macrurus       MELFETNPYFFDQFYEGADNFPSRSLGGFEQGYGQERG-GVVGLCDSRLLPGAAQP 59
C. carpio       MELFETNPYFLQDFRYEQQDFFQSRLTGGFDQGYQDRS-SMMGCLGDRILSSNVGL 59
D. rerio       MELFETNPYFFNDQFYEGADNFQRSRSLGGYGQADQGGYQERG-SMMGLCGDSRLLPGAAQP 59
H. hippoglossus MELFETNPYFFDPQFYEGSDYFPSSLGGYDQAGYQDRN-SMMGLCAGGS---LSGNVGV 56
O. mykiss       MELFETNPYFFDQFYEGDNYQSLPGGDDQYQERGSSMMGLGAGGS---LSGNYGV 57

His/Cys-rich basic

His/Cys-rich basic

HLH

S. macrurus       RLKKVNEAFALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQEOHVT-DSSEQK 113
C. carpio       RLKKVNEAFALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQEOHVT-DSSEQK 113
D. rerio       RLKKVNEAFALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQEOHVT-DSSEQK 113
H. hippoglossus RLKKVNEAFALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQEOHVT-DSSEQK 113
O. mykiss       RLKKVNEAFALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQEOHVT-DSSEQK 113

Helix III

S. macrurus       RSLTSIVSDSTGSEATPVTY--SVDIK- 252
C. carpio       RSLTSIVSDYGTVEATPVTY--SVDISK- 253
D. rerio       RSLTSIVSDSTGTEATPVTY--SVDISK- 256
H. hippoglossus RALTSIVSDSEAD-AAAAP--PVDIPK 250
O. mykiss       RSLTSIVSDSTAAEAGPLAYPVDIPK 254