Molecular characterization of the \textit{MuRF} genes in rainbow trout: Potential role in muscle degradation

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\textbf{A B S T R A C T}

Muscle growth is determined primarily by the balance between protein synthesis and degradation. When rates of protein synthesis are similar between individuals, protein degradation is critical in explaining differences in growth efficiency. Studies in mammals showed that muscle atrophy results from increased protein breakdown, and is associated with activation of the ubiquitin proteasome pathway, including induction of the muscle-specific ubiquitin protein ligase, MuRF1. Animals lacking MuRF1 are resistant to muscle atrophy. In fish, little is known about the role of the proteasome/MuRF pathway in muscle degradation. The objectives of this study were to: 1) clone and characterize \textit{MuRF} genes in rainbow trout; and 2) determine expression of \textit{MuRF} genes in association with starvation- and vitellogenesis-induced muscle atrophy in rainbow trout. We have identified full-length cDNA sequences for three \textit{MuRF} genes (\textit{MuRF1}, \textit{MuRF2}, and \textit{MuRF3}). These genes encode proteins with typical MuRF structural domains, including a RING-finger, a B-box and a Leucine-rich coiled-coil domain. RT-PCR analysis showed that \textit{MuRF} genes are predominantly expressed in muscle and heart tissues. Real time PCR analysis revealed that expression of all \textit{MuRF} genes is up-regulated during starvation and \textit{MuRF3} is up-regulated in vitellogenesis-associated muscle degradation. These results suggest that \textit{MuRF} genes have an important role in fish muscle protein degradation. Further studies are warranted to assess the potential use of \textit{MuRF} genes as tools to monitor fish muscle growth and degradation.

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1. Introduction

Muscle is the largest protein reservoir in fish, serving as a primary reserve of amino acids that can be mobilized during fasting and disease to provide a source of amino acids for hepatic gluconeogenesis and energy production (Ketteler et al., 1988) as well as protein synthesis. In aquaculture, muscle growth and fillet quality are important traits that impact profitability. Expression of molecular/genetic markers can be used to evaluate muscle proteolysis and select for 1) increased muscle protein accretion during fish growth and 2) decreased postmortem protein degradation (less fillet softening).

Protein turnover is determined by rate of protein synthesis and protein degradation. The rate of protein turnover is important in determining efficiency of animal growth and muscle catabolism (Young et al., 1975; Hawkins et al., 1989; Mommsen, 2004; Salem et al., 2006b). When rates of protein synthesis are similar between individuals, protein degradation is critical in determining differences in growth efficiency. Therefore, detailed knowledge of protein degradation mechanisms in fish will benefit the aquaculture industry.

Protein degradation in animal cells is a highly selective, regulated and energy-dependent process controlled by activities of proteolytic enzymes (Hershko et al., 2000). There are four known proteolytic systems involved in muscle proteolysis; the calcium-dependent, calpain system; the lysosomal protease system (cathepsins); the ubiquitin (Ub)–proteasome system; and the apoptosis protease system (caspase) (Salem et al., 2006b; Argilés et al., 2008). The performance of these various systems has been widely discussed (Millward, 1985; Kumamoto et al., 2000; Lecker et al., 2004) with strong evidence that the ATP-dependent Ub–proteasome pathway is essential during the majority of protein degradation associated with muscle atrophy in mammals (Lecker et al., 1999; Jagoe and Goldberg, 2001). However, in fish, little is known about the proteolytic strategies of muscle protein degradation. Some studies suggested that the Ub–proteasome pathway is not involved in vitellogenesis-induced muscle atrophy (Salem et al., 2006a); other studies found down-regulated expression of the proteasome pathway in fasting-induced fish muscle degradation (Martin et al., 2002). A recent report showed that the polyubiquitination step of the Ub–proteasome route is regulated by starvation/refeeding status with little effect on 20 S proteasome activity (Seiliez et al., 2008).

The ubiquitin–proteasome pathway of protein degradation involves tagging the substrate protein by covalent attachment of
multiple Ub molecules, a 76-amino acid polypeptide (conjugation); and subsequent degradation of the tagged protein by the 26 S proteasome, resulting in short peptides of 7–9 amino acid residues. Ub–protein conjugation requires ATP, the Ub-activating enzyme (E1), Ub carrier proteins (E2 enzymes), and frequently, Ub–protein isopeptide ligases (E3 enzymes). The key enzyme responsible for targeting ubiquitination of specific substrate proteins is a Ub–protein ligase (E3) that catalyzes transfer of an activated form of Ub from a specific Ub–carrier protein (E2) to a lysine residue on the substrate (O’Brien et al., 1996). Recent results suggest that Atrog1–1 (muscle atrophy F-box) and MuRF1 (muscle RING finger 1) are two Ub–protein ligases (E3) that are key players in the regulation of ubiquitin–proteasome–mediated muscle atrophy in mammals (Lecker, 2003; Cao et al., 2005). As evidence of the role of MuRF1 in this process, MuRF1−/− mice displayed a partial protection against denervation-induced muscle wasting (Bodine et al., 2001). In addition under amino acid depletion, MuRF1−/− mice had less muscle wasting maintaining normal de novo protein synthesis compared to wild-type mice (Koyama et al., 2008).

MuRF2 and MuRF3 were identified in yeast two-hybrid studies, as binding proteins of MuRF1 (Spencer et al., 2000b). MuRF2 and MuRF3 were speculated to work together in muscle differentiation, via microtubule stabilizing and titin-based signaling mechanisms (Gregorio et al., 2005). MuRF2 was proposed to act as an adaptor between microtubules, myosin, and titin during myofibrillogenesis. Studies examining the function of MuRF2 showed importance for microtubule, intermediate filament and sarcomeric M-line maintenance in striated muscle development (McElhinny et al., 2004). Mice lacking MuRF3 had normal cardiac function but were susceptible to cardiac rupture after acute myocardial infarction (Fieltz et al., 2007). A recent study showed that MuRF1, but not MuRF2, plays important role in regulating cardiac hypertrophy (Willis et al., 2007). In contrast, another study showed that mice lacking both MuRF1 and MuRF2 proteins develop cardiac and skeletal muscle hypertrophy, whereas animals lacking either MuRF1 or MuRF2 are normal (Witt et al., 2008).

Muscle atrophy occurs in aging, denervation, disuse, muscular dystrophies cystic fibrosis, AIDS, and after high dose treatment with glucocorticoids (Lecker et al., 2004; Mittal et al., 2010). In fish, muscle deterioration occurs as a physiological response to the increased energetic demands associated with egg growth and development and in response to starvation (Mommesen, 2004; Salem et al., 2006b; Seiliez et al., 2008). A microarray gene expression analysis of atrophying rainbow trout muscle showed up-regulated expression of a muscle-specific RING finger protein 3-like transcript (Salem et al., 2006a). The primary objective of this study was to clone and characterize the MuRF gene family in rainbow trout and investigate its expression during starvation, and egg growth and development leading up to spawning.

2. Materials and methods

2.1. Identification of MuRF1, MuRF2 and MuRF3 cDNAs

Nucleotide and amino acid sequence homology searches, against the GenBank database (http://www.ncbi.nlm.nih.gov/), were used to examine rainbow trout (Oncorhynchus mykiss) expressed sequence tags (ESTs) to identify MuRF-like clones. We identified three rainbow trout cDNA clones (GenBank Accession No.: CA369980, CA342294, BX321103) that had high homology with mammalian MuRFs. Clones were obtained from the United States Department of Agriculture, National Center for Cool and Cold Water Aquaculture (USDA-NCCWCA) (Kearneysville, WV, USA). All clones were completely sequenced and aligned to generate full length sequences for rainbow trout MuRF1, MuRF2 and MuRF3 cDNAs. CLUSTALW version 1.81 (http://www.cmbi.kun.nl/cbi-bin) algorithm was used for protein multiple alignments. The characteristic domains of three MuRF proteins were assigned by blasting their sequences against the Protein Families Database of Alignment (Pfam, http://www.sanger.ac.uk/Software/ Pfam/search.shtml) and according to the work of Spencer and coworkers (Spencer et al., 2000a). Sequence analysis was done using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html).

2.2. Fish and tissue collection

Samples used in quantitative measurement of MuRF gene expression in spawning-induced muscle atrophy were collected as described previously (Salem et al., 2006a). Mature fertile (diploid) and sterile (triploid) female rainbow trout (500 g) were collected from Flowing Springs Trout Farm (Delray, WV, USA) during the spawning season in early October. Fish were cultured in identical raceways receiving water from a common spring at 13 ± 3 °C. Fish were fed ad libitum (Zeigler Gold; Zeigler Bros., Gardeners, PA, USA) via demand feeders. No difference of food consumption was noticed between groups. Fish had access to feed when sampled. As confirmed by dissection, fertile fish were gravid with a gonado-somatic index (GSI = ovary weight/fish weight X100) of 15.8 ± 0.3 (n = 5). The GSI of sterile fish was 0.3 ± 0.2 (n = 5). White muscle samples (20 g) from five fish of each group were collected from the dorsal musculature and flash frozen in liquid nitrogen and stored at − 80 °C until RNA extraction.

Samples used in quantitative analysis of MuRF gene expression in starvation-induced muscle atrophy were collected as described in a previous study (Salem et al., 2005). Rainbow trout fingerlings (15–20 g) were brought from Bowden State Fish Hatchery, WV Division of Natural Resources (Elkins, WV, USA) to the laboratory and acclimatized for 30 days. Temperature (14.0 °C ± 1), dissolved oxygen, pH, and other water quality parameters were held constant throughout the study. Fish were randomly divided into six experimental groups (60 fish each). Each group was assigned to a net pen (60 ×60 ×60 cm). To remove variation associated with potential differences in water quality, all pens were kept in the same aquarium (1100 L). Three control groups were manually fed a commercial fish diet (Zeigler Bros.) at 1% of fish body weight, twice per day. Three experimental groups were subjected to a starvation regimen for 35 days. This starvation regimen was based on Tripathi et al. (Tripathi and Verma, 2003) who showed that the maximum effect of fasting on rainbow trout muscle protein content occurs after 35 days of starvation. At the end of the experimental period, nine fish from each experimental group were randomly sampled. Fish were euthanized and weighed, and white muscle samples were collected.

2.3. Northern blot analysis

Total RNA was isolated from muscle or heart samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. Frozen tissue (0.2 g) was placed in 2 ml of Trizol reagent and homogenized using a Polytron homogenizer. Messenger RNA was isolated from total RNA using the polyAtract mRNA isolation system (Promega, Madison, WI, USA). Biotinylated oligo d(T) probe and streptavidin attached magnetic beads were used for this purpose. A 5-μg sample of mRNA was separated on a 1% denaturing agarose gel by electrophoresis along with an RNA marker (Promega). Messenger RNA was subsequently transferred to a Hybond N + nylon membrane (Amersham Biosciences, Piscataway, NJ, USA). The mRNA was cross-linked onto the membrane by UV rays, and then the membrane was prehybridized in DIG Easy Hyb solution (Roche Diagnostics, Indianapolis, IN, USA) for 1 h and then hybridized in the same solution containing DIG-labeled PCR probe (2 μL/ml in hybridization solution) overnight at 68 °C. DIG-labeled PCR probes, used for Northern blot analysis, were synthesized using a DIG PCR labeling kit (Roche Diagnostics). DIG labeled DNA fragments of rainbow trout MuRF1 (429 bp), MuRF2 (570 bp) and MuRF3 (565 bp) were synthesized by PCR using the corresponding cDNA clones as templates and gene-specific primers (Table 1). Following stringent washes (4 × 10 min with 2X SSC, 0.1% SDS
treated total RNA from each sample were converted to cDNA using reverse transcriptase. Prior to reverse transcription, the blocking solution for 30 min followed by additional incubation with a blocking solution that contained a 1:10,000 dilution of alkaline phosphatase conjugated, anti-DIG antibody (Roche Diagnostics). The second incubation was for 1 h at room temperature. After 2 washes in the washing buffer, the hyridized probe was detected with the chemiluminescent substrate, CSPD (Roche Diagnostics). Blocking and washing solutions were from a DIG wash and block buffer set (Roche Diagnostics). The mRNA expression of three MuRF genes in muscle samples were chosen as a control for normalization of the real-time PCR data based on our previous observation that expression of β-actin was not changed in spawning-induced muscle atrophy (Salem et al., 2006a).

2.4. RT-PCR

Total RNA from fish tissue samples was first treated with DNase I and then reverse transcribed to first-strand cDNA using oligo (dT)\textsubscript{18} primer and Superscript III reverse transcriptase (Invitrogen). First-strand cDNA from various tissues was used as a template for PCR amplification of the three MuRF genes using gene-specific primers (Table 1). The PCR reaction conditions (25-μL reaction mixture) included 5 min denaturation at 94 °C followed by 35 cycles of 94 °C for 30 s, 59 °C for 45 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min. Trout β-actin gene was used as internal control to verify RNA quality.

2.5. Quantitative real-time PCR assay

The mRNA expression of three MuRF genes in muscle samples were measured using quantitative real-time PCR. Prior to reverse transcription, total RNA was treated with DNase I (Ambion, Austin, TX, USA) to eliminate genomic DNA contamination. Two μg of DNase-treated total RNA from each sample were converted to cDNA using Superscript III reverse transcriptase (Invitrogen). Negative control cDNAs were prepared by reverse transcription reactions without adding the reverse transcriptase.

Real-time PCR primers (Table 1) for MuRF1, MuRF2 and MuRF3 genes and the endogenous control, β-actin gene, were designed based on the corresponding cDNA sequences (rainbow trout β-actin gene: GenBank accession No.: AJ438158) using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Quantitative PCR was performed in duplicate for each cDNA sample on a Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). IQ SYBR Green Supermix (Bio-Rad) was used in a 25-μL reaction with cDNA derived from 0.2 μg of total RNA. Trout β-actin gene was used as an internal control for normalization. Standard curves for the target and reference genes were constructed using 10-fold serial dilutions of the corresponding plasmids. Melting curve analyses were done to confirm amplification of a single product. Standard curves were run on the same plate with the samples. Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curve and cycles to threshold (Ct) were recorded. For each sample, the amount of the target and internal reference genes was determined from the appropriate standard curve. The amount of target gene was then divided by the amount of reference gene to obtain a normalized target value. The mean differences in expression levels were determined by a t-test (SigmaStat version 3.11, Aspire Software International, Leesburg, VA, USA). The β-actin gene was chosen as a control for normalization of the real-time PCR data based on our previous observation that expression of β-actin was not changed in spawning-induced muscle atrophy (Salem et al., 2006a).

2.6. Phylogenetic analysis

A phylogenetic analysis of the rainbow trout MuRF genes was performed using full MuRF protein sequences including Homo sapiens TRIM63 (19924163), H. sapiens ring finger protein 28 (30583585), H. sapiens TRIM-55 isoform 2 (34878821), Danio rerio hypothetical protein-LOC394070 (41055281), Tetraodon nigroviridis unnamed protein product (47207992), D. rerio hypothetical protein LOC415223 (50344922), D. rerio tripartite motif-containing 55 (50539776), D. rerio tripartite motif-containing 55 (50652813), Gallus gallus similar to RING finger protein 29 isoform 1 (118087028), G. gallus similar to ring finger protein 28 (118101656), Xenopus tropicalis TRIM 63 (118404880), X. laevis TRIM 54 (147905260), Salmo salar MuRF1-like (213511072), S. salar LOC394070 protein-like (213512264), D. rerio novel protein similar to TRIM54 (220941743), rainbow trout MuRF1- (HM357611), MuRF2 (HM357612) and MuRF3 (HM357613). Reconstruction and analysis of phylogenetic relationships between rainbow trout MuRF orthologues and paralogues sequences were performed using PhyML maximum likelihood based program (http://www.phylogeny.fr/version2/cgi/index.cgi) (Dereeper et al., 2008). Approximate likelihood ratio test was used to test the PhyML trees with program default settings. Values obtained from approximate likelihood ratio test are similar to standard 100 runs of bootstrapping.

3. Results

3.1. Identification and characterization of MuRF cDNA sequences

By searching the rainbow trout EST database at the GenBank, (http://www.ncbi.nlm.nih.gov/), we identified 3 rainbow trout MuRF-like cDNA clones. The complete sequences of the cDNA clones were determined by primer walking (Core Sequencing Facility, University of Illinois at Urbana-Champaign, Urbana, IL, USA). The longest open reading frame of each sequence was predicted, and the amino acid sequences were aligned against known mammalian MuRF proteins for annotation and characterization. Based on the amino acid homology, clones CA369980, CA342294 and BX321103 were designated as rainbow trout MuRF1, MuRF2 and MuRF3, respectively.

### Table 1

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
<th>Method</th>
<th>Amplicon size</th>
<th>qPCR efficiency</th>
</tr>
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<tbody>
<tr>
<td>MuRF1</td>
<td>Sense: CAAGCAGATTCAGGAGCAAGC</td>
<td>Standard RT-PCR</td>
<td>429 bp</td>
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</tr>
<tr>
<td></td>
<td>Antisense: TCTCTGTACCACTACATCA</td>
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<tr>
<td>MuRF1</td>
<td>Sense: CTTATTAGTGGCAAGACGTCG</td>
<td>Real time PCR</td>
<td>101 bp</td>
<td>92.1%</td>
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<tr>
<td></td>
<td>Antisense: GAAAGGCTTCACTGAGCTCG</td>
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<tr>
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<td>Sense: AGGCACACTAATCTCCACCT</td>
<td>Standard RT-PCR</td>
<td>570 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: ACAGAAGAAAAAGAGCAAGACG</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MuRF2</td>
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<td>Real time PCR</td>
<td>88 bp</td>
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<tr>
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<td>Real time PCR</td>
<td>143 bp</td>
<td>97.3%</td>
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<td></td>
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<tr>
<td>MuRF3</td>
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<td>Standard RT-PCR</td>
<td>565 bp</td>
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<td></td>
<td>Antisense: GCGCAGGCGAATGGCCTGTA</td>
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<td>β-actin</td>
<td>Sense: GCCGCGCCCACCTCAAGCCTAC</td>
<td>Real time PCR</td>
<td>73 bp</td>
<td>100%</td>
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<tr>
<td></td>
<td>Antisense: GCCCCCCGGTGCTGAACTGTGAAC</td>
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</table>
The complete nucleotide sequence of MuRF1 cDNA is 1791 bp in length and contains an open reading frame encoding a protein of 359 amino acids (GenBank accession number: HM357611). The amino acid sequence shows 67% similarity with salmon MuRF1 (NP_001133124.1) and 54% with mouse MuRF1 (NP_001034317.2). The cDNA sequence for MuRF2 is 2193 bp long, and it codes for a protein of 490 amino acids (HM357612). The deduced amino acid sequence is also closely related to the known MuRF2 from other species as it shares 59% sequence identity with zebrafish (NP_001002358.1) and 66% with mouse MuRF2 (NP_001074750.1). The nucleotide sequence of MuRF3 consists of 1785 bp, and it has an open reading frame coding for 350 amino acids (HM357613). The deduced amino acid sequence displayed high sequence homology with previously reported MuRF3 in other species, with 85% amino acid identity to zebrafish (CAX15444.1) and 53% to mouse MuRF3 (NP_067422.1).

Amino acid sequence analysis of MuRF1, MuRF2 and MuRF3 revealed that all three MuRF proteins contain the characteristic RING-finger domain, B-box zinc finger domain and Leucine-rich coiled-coil domain (Fig. 1). Each domain contains the highly conserved sequence motif that is essential for MuRF activity. The RING-finger domain is located at the N-terminus, which is functional in protein–protein interaction. Trout MuRF1 and MuRF2 share 49% protein sequence identity (45% nucleotide identity), MuRF1 and MuRF3 share 67% protein sequence identity (64% nucleotide identity), and MuRF2 and MuRF3 share 57% protein sequence identity (47% nucleotide identity).

3.2. Tissue distribution of rainbow trout MuRF genes

The tissue distribution patterns of MuRF1, MuRF2 and MuRF3 transcripts were determined by RT-PCR analysis (Fig. 2). Rainbow trout spleen, kidney, white muscle, liver, gill, heart, brain, skin, testis and egg were selected for analysis of MuRF gene expression patterns. MuRF1 exhibited muscle specific expression because it was only seen in muscle and heart. MuRF2 is expressed primarily in muscle and heart, with lesser amounts in brain. MuRF3 mRNA is also predominantly expressed in muscle and heart, with greater amounts in heart (Fig. 2).

3.3. Northern blot analysis

Northern blot analysis, using mRNA isolated from muscle or heart, revealed a single transcript of ~1.9 kb for MuRF1, two transcripts of ~2.0 and ~2.3 kb for MuRF2 and a single transcript of ~1.9 kb for MuRF3 (Fig. 3). The size of each transcript is consistent with the expected size of the corresponding cDNA (MuRF1, 1791 bp; MuRF2, 2193 bp; MuRF3, 1785 bp). The additional band of 2.0 kb detected by the MuRF2 probe may represent an alternative splice variant of MuRF2 or a transcript from a paralogous gene due to partially tetraploid trout genome (Allendorf and Thorgaard, 1984) or alternative use of polyadenylation signals.
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with lesser amounts in brain. Only seen in muscle and heart.

A single transcript of 1.9 kb for TRIM54; Danio rerio TRIM 54; Tetraodon nigroviridis MuRF proteins similar to RING–finger domain, MuRF1 is a protein–protein interaction domain and any splice variants of MuRF2 and MuRF3 in spawning-induced and starvation-induced muscle atrophies. To investigate mRNA expression of MuRF genes in spawning-induced muscle atrophy in rainbow trout females, fertile (2 N) and sterile (3 N) fish muscle samples were collected at the spawning season and subjected to quantitative real time PCR analysis. As shown in Fig. 5, numerical increase of expression for all three MuRF genes was observed in 2 N females, however, only MuRF3 expression was increased significantly in 2 N females (9.6-fold, P<0.05). Expression of all three MuRF transcripts was up-regulated in starved fish muscle compared to normal-fed fish muscle. Abundance of mRNA was increased by 6.5-fold for MuRF1, 2.1-fold for MuRF2 and 3.5-fold for MuRF3 (P<0.01) in fish that had food withdrawn for 35 days compared to fed controls (Fig. 6).

4. Discussion

In this study, we identified and characterized three full-length cDNA sequences for MuRF genes in rainbow trout. The cDNA sequences of these three genes contain complete open reading frames encoding MuRF proteins with characteristic N-terminal RING-finger, B-box zinc finger and Leucine-rich coiled-coil domains (RBCC). The RING-finger domain is an unusual type of Cys-His zinc-binding motif, with a specialized type of Zn-finger domain containing 40 to 60 residues that bind two atoms of zinc (Freemont, 1993; Borden and Freemont, 1996; Saurin et al., 1996). The RING-finger domain is probably involved in mediating protein–protein interactions, and is identified in proteins with a wide range of functions such as signal transduction, gene transcription, differentiation, ubiquitination, morphogenesis and microtubule stabilization (Spencer et al., 2000b). The B-box zinc finger domain is also a protein–protein interaction domain (Borden, 1998). The leucine-rich coiled-coil domain mediates association with microtubules (Spencer et al., 2000b). Depending on the protein–protein interactions of the RING-finger domain, MuRF1 is a bona fide RING finger-dependent, ubiquitin ligase that catalyzes a rate-limiting step in troponin I degradation. Troponin I is a MuRF1 interaction partner, and MuRF1-troponin I interactions can be detected in cardiomyocytes (Kedar et al., 2004).

The MuRF genes are predominantly expressed in muscle and heart tissues (Fig. 2). Northern blot analyses showed a single transcript corresponding to the cDNA size for MuRF1 and MuRF3. In addition to the transcript corresponding to the cDNA size for MuRF2, another transcript of 2.0 kb was detected by the MuRF2 probe. This transcript may represent an alternative splice variant of MuRF2 or a paralogous sequence. However, we were not able to find any splice variants of MuRF2 by BLAST search of the well characterized rainbow trout EST database (258,973 Sanger-based and ~1.3 million pyrosequencing ESTs, available through NCBI). As a member of the family Salmonidae, rainbow trout has descendend from a tetraploid ancestor (Allendorf and Thorgaard, 1984). About 50% of protein-coding loci in salmonid species show duplicate gene expression (Bailey et al., 1978). Therefore, it is more likely that the two MuRF2 transcripts are produced from paralogous sequences. It would be hard to determine the origin of the additional transcript without a characterized rainbow trout genome sequence.

The phylogenetic tree topology (Fig. 4) suggests the existence of a MuRF-like ancestral gene in all vertebrate superfamily members. Rainbow trout MuRF2 was found clustered with homologous protein from fish, amphibian, avian and mammalian species (Fig. 4). However, MuRF1 and MuRF3 were found on a separate branch of the tree with only fish members suggesting a significant paralogous divergence from other vertebrate MuRF-like proteins. The phylogenetic tree identifies MuRF homologues from fish species (Danio rerio, Salmo salar and Tetraodon nigroviridis) that warrant further studies.

### 3.4. Phylogenetic analysis

Fig. 4 presents the proposed phylogenetic tree of the MuRF genes. This tree includes the deduced rainbow trout MuRF proteins; Homo sapiens TRIM63, ring finger protein 28, TRIM-55 isoform 2; Gallus gallus protein similar to RING finger protein 29 isoform 1 and protein similar to ring finger protein 28; Xenopus tropicalis TRIM 63; X. laevis TRIM 54; Danio rerio hypothetical protein LOC394070, hypothetical protein LOC415223, TRIM55, TRIM55b and a novel protein similar to TRIM54; Salmo salar MuRF1-like and LOC394070 protein-like; and pufferfish (Tetraodon nigroviridis) unnamed protein. All proteins used to construct the phylogenetic tree were found to show similarity to trout MuRF proteins by NCBI blast search. The topology of the phylogenetic tree shows the evolutionary relationships between rainbow trout MuRF orthologues and paralogues. The tree shows two main branches: 1) fish MuRF1/3 (Fig. 4 cluster A) and 2) fish MuRF2 in addition to MuRF1-3 of other species (Fig. 4 clusters B1 & B2). The later branch is subdivided into the MuRF2 like cluster (B1) and MuRF1 like cluster (B2). Homologous sequences that differ because they are found in different species appear to be orthologues. The tree suggests a paralogous mode of evolution of trout MuRF1-3. Fish MuRF1 and MuRF3 are closer to each other than to MuRF2.

### 3.5. Real time PCR quantification of MuRF gene expression in spawning-induced and starvation-induced muscle atrophies

To investigate mRNA expression of MuRF genes in spawning-induced muscle atrophy in rainbow trout females, fertile (2 N) and sterile (3 N) fish muscle samples were collected at the spawning season and subjected to quantitative real time PCR analysis. As shown in Fig. 5, numerical increase of expression for all three MuRF genes was observed in 2 N females, however, only MuRF3 expression was increased significantly in 2 N females (9.6-fold, P<0.05). Expression of all three MuRF transcripts was up-regulated in starved fish muscle compared to normal-fed fish muscle. Abundance of mRNA was increased by 6.5-fold for MuRF1, 2.1-fold for MuRF2 and 3.5-fold for MuRF3 (P<0.01) in fish that had food withdrawn for 35 days compared to fed controls (Fig. 6).
MuRF genes are described as ubiquitin E3 ligases, as such they are highly likely to be involved in proteasome protein degradation (Lecker, 2003). However, Salem and coworkers showed that expression of the proteasome pathway genes is not associated with vitellogenesis-induced atrophy in rainbow trout (Salem et al., 2006a). Additionally, Martin and coworkers reported down-regulated expression of proteasome genes in fasting-induced rainbow trout muscle degradation (Martin et al., 2001; Martin et al., 2002). Conversely, Insulin and IGF-I independently reduced the abundance of the ubiquitin ligase mRNA in primary cultures of rainbow trout myocytes (Cleveland and Weber, 2009, 2009). Therefore, MuRF genes may be involved in different mechanisms regulating muscle growth and degradation in fish that may not include the proteasome pathway. Further studies are needed to explain the role of MuRF genes in fish muscle degradation.

Muscle atrophy refers to wasting of muscle tissue resulting from conditions such as starvation or systemic diseases (cancer, diabetes, sepsis). Muscle atrophy also occurs as a physiological response to the increased energetic demands placed on muscle during fish spawning and starvation (Mommsen, 2004; Salem et al., 2005; Rescan et al., 2007; Salem et al., 2007). These responses represent suitable models to study mechanisms of muscle degradation/regeneration in fish. During the spawning phase, white muscle acts as the major energy source once lipids are depleted (Love, 1979; Mommsen et al., 1981; Ando et al., 1998; Mommsen, 2004). Significant decreases in body mass and separable muscle were reported in sexually maturing rainbow trout and salmon. This process also involves a decline in net protein concentration (Martin et al., 1993; Salem et al., 2006b). Muscle acts as a major energy source to support vitellogenesis-induced increases in energy demand. Our data showed significantly increased expression of MuRF3 and numerically increased expression of MuRF1 and MuRF2 in spawning 2 N females. These results are consistent with our previous microarray gene expression analysis of atrophying rainbow trout muscle which showed up-regulated expression of a muscle-specific RING finger protein 3-like transcript (Salem et al., 2006a). The increased MuRF gene expression is consistent with elevated

![Image](https://example.com/image.png)

**Fig. 4.** A phylogram of the evolutionary relationships between rainbow trout MuRF orthologues and paralogues generated by using the PhyML maximum likelihood analysis. The tree shows two main branches: 1) fish MuRF1/3 (A) and 2) fish MuRF2 in addition to MuRF1-3 of other species (B1&2). The later is subdivided into the MuRF2 like cluster (B1) and MuRF1 like cluster (B2). Homologous sequences that differ because they are found in different species appear to be orthologues. The tree suggests a paralogous mode of evolution of trout MuRF1-3. Fish MuRF1 and MuRF3 are closer to each other than to MuRF2.

![Image](https://example.com/image.png)

**Fig. 5.** Effect of spawning-induced muscle atrophy on the expression of MuRF genes. Expression of the MuRF genes was analyzed by real-time PCR. Quantity of each MuRF mRNA was normalized to β-actin. The means of the normalized gene expression values were calculated and expressed as relative fold changes (n = 5, mean ± SEM). * Indicates significant difference (P < 0.05).

![Image](https://example.com/image.png)

**Fig. 6.** Effect of starvation-induced muscle atrophy on the expression of MuRF genes. Expression of the MuRF genes was analyzed by real-time PCR. Quantity of each MuRF mRNA was normalized to the trout β-actin gene. The means of the normalized gene expression values were calculated and expressed as relative fold changes (n = 15, mean ± SEM). * Indicates significant difference (P < 0.01).
protein degradation needed to supply carbon skeletons for intermediary metabolism and thus ATP synthesis, suggesting a role of MuRF genes in spawing-induced muscle atrophy and protein mobilization. The discrepancy of MuRF gene expression (MuRF1/MuRF2 vs. MuRF3) in spawning fish may indicate difference in relative importance and functionality of the three MuRF isoforms.

Starvation triggered up-regulated expression of all three MuRF genes (Fig. 6). These data are consistent with recent studies that showed dramatic increase in abundance of the muscle-specific ubiquitin ligase atrogin-1 (MAFbx) mRNA in starved rainbow trout and salmon (Cleveland and Weber, 2009; Bower et al., 2010) and inhibition of MuRF and MAFbx in responses to re-feeding (Bower et al., 2009). White muscle responds to starvation with rapidly declining protein synthesis rates (Pocrnjic et al., 1983; Fauconneau and Arnal, 1985; Houlihan et al., 1988). Sensitivity of white muscle protein synthesis during starvation is important to overall growth; any decrease in white muscle protein synthesis will have a substantial effect on growth and metabolism (Houlihan et al., 1988). The observed decrease in white muscle protein synthesis during starvation appears to be highly protein specific, with a 90% decrease in myofibrillar protein synthesis observed during starvation (Lowery and Somero, 1990). Considerably less is understood about the effects of starvation on protein degradation. Martin et al. demonstrated that activity of the proteasome pathway, a major vehicle of protein degradation, is reduced after 2 weeks starvation in rainbow trout. In contrast, lysosomal and the calpain enzyme activities appears to increase during starvation (Martin et al., 2000; Salem et al., 2005). Additionally, Dobly and coworkers reported a negative correlation between trout liver, but not white muscle, 20 S proteasome activity and specific growth rate (Dobly et al., 2004). Feed deprivation caused remarkable increase in abundance of MAFbx mRNA, without a corresponding change in the proteasome transcripts (Cleveland and Weber, 2009, 2009). Together, these data suggest a significant role of the MuRF ubiquitin ligases in fish muscle degradation. However, how the MuRF genes work in the proteolytic pathways is still not understood, and a larger scale, follow-up investigation is warranted.

In summary, we identified cDNA sequences for rainbow trout MuRF1, MuRF2 and MuRF3 genes. These genes are predominately expressed in skeletal (white) and cardiac muscle, and encode proteins with characteristic MuRF structural domains. Expression of these genes in rainbow trout was up-regulated during starvation- and spawning-induced muscle atrophy, suggesting important roles in muscle protein degradation. Future studies are necessary to 1) determine the roles of MuRF genes in regulation of muscle degradation in fish and 2) evaluate the potential use of MuRF genes as genetic markers for muscle growth and fillet firmness in trout, a significant teleost, aquatic food species. In addition, these markers may be used for conservation management of wild populations (Vignal et al., 2002).

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