FEBS Letters 491 (2001) 212–216 FEBS 24639

Differential skeletal muscle expression of myostatin across teleost species, and the isolation of multiple myostatin isoforms

Steven B. Roberts, Frederick W. Goetz*

University of Notre Dame, Department of Biological Sciences, P.O. Box 369, Notre Dame, IN 46556, USA

Accepted 30 January 2001

First published online 13 February 2001

Edited by Amy M. McGough

Abstract Two myostatin (MSTN) isoforms were isolated from brook trout with 92% identity in corresponding regions at the nucleotide level. One isoform was isolated from muscle and brain and the second from ovarian tissue. To our knowledge this is the first time two MSTN isoforms have been isolated from a given vertebrate species. Within the brain, MSTN transcripts were localized to the optic lobes, hindbrain, and hypothalamus. In the trout ovary, MSTN transcripts were upregulated at ovulation in several females. MSTN cDNA fragments were also isolated from several other fish species and differential expression of MSTN among muscle fiber types was observed.

Key words: Myostatin; Trout; Muscle; Ovulation; Ovary

1. Introduction

Myostatin (MSTN), a member of the Transforming Growth Factor-β (TGF-β) superfamily, has been established as a regulator of development and growth in several vertebrates. MSTN, originally termed growth and differentiation factor-8, was first characterized in the mouse where it is expressed during embryogenesis in developing somites [1]. Adult mice that did not express the MSTN gene were 25–30% heavier than wild-type littermates and the individual muscles of the MSTN null mice were 2–3 times heavier. This increase in muscle mass was attributed to an increase in fiber number (hyperplasia) and size (hypertrophy). Subsequently, the double muscling phenotype found in Belgian Blue and Piedmontese cattle was attributed to mutations in the MSTN gene [2–4]. Research on cattle [2], pigs [5], and chickens [6] has indicated a similar developmental expression pattern of MSTN that begins early and continues through gestation. These studies also show that MSTN expression roughly coincides with primary and secondary muscle formation followed by reduced levels at birth. MSTN expression has been reported in adults of several vertebrate species [2–5]. During development and adulthood, MSTN is predominately expressed in skeletal muscle, though there have been reports of myostatin protein in cardiomyocytes and Purkinje fibers of the heart [7], as well as MSTN mRNA expression in the mammary gland [5]. Recently, Rodgers et al. [8] have also reported the presence of MSTN in a variety of tissues in tilapia.

Originally, the MSTN gene was cloned in representatives of several vertebrates including zebrafish [3]. The gene is highly conserved among species and the predicted amino acid sequences of human, rat, mouse, porcine, chicken, and turkey are identical within the active carboxy-terminal region [3]. Though MSTN has been cloned in representative lower vertebrates, MSTN gene expression has not been characterized in these animals. To understand the role that MSTN could have in the growth and development of fish, the present study examined MSTN gene expression in several fish species.

2. Materials and methods

2.1. Animals and tissue collection

Experiments and animal care were conducted according to the guidelines specified by the University of Notre Dame Institutional Animal Care and Use Committee. Mature brook trout (*Salvelinus fontinalis*) were purchased from a commercial hatchery in Grand Haven, MI and held in 300 gallon tanks in 12°C well water. Yellow perch (*Perca flavescens*) were obtained from the Lake Mills State Fish Hatchery in Lake Mills, WI. Mahi-mahi (*Coryphaena hippurus*), little tunny (*Euthynnus alletteratus*), and king mackerel (*Scomberomorus cavalla*) were obtained by hook and line in the Atlantic Ocean offshore of South Carolina. For obtaining ovaries, the reproductive stage of individual brook trout was determined by sampling follicles in vivo as previously described [9]. Ovarian tissue was collected from females before germinal vesicle breakdown, during ovulation (20–100% of the ovary ovulated at time of sampling), and 48 h after ovulation. In all cases, fish were overanesthetized and tissue samples were dissected and placed into ice-cold Tri-Reagent (Molecular Research Center Inc.). Tissue was homogenized with a TissueTearor (Biospec) and RNA isolation was completed as previously described [10,11]. When used, mRNA isolation was performed using the Poly-A-Tract mRNA Isolation System (Promega).

2.2. Cloning fish MSTNs

Two sets of degenerative primers were designed based on known MSTN sequences. These consisted of two forward primers: AA-(A,G)CCI(inosine)AA(A,G)TG(C,T) TG(C,T)TT(C,T)TT(C,T) [Fw1] and CAAAT(T,C)CT(T,C)AG(C,T)AAACT(C,G,T) [Fw2] and two reverse primers: (A,G)TGIGT(A,G)TGIGG(A,G)TA(C,T)TT(C,T)-TG [Rv1] and ATAATCCA(G,A)TCCCA(G,T)CCAAA [Rv2].

Total RNA from brook trout ovaries, brain, and red muscle, as well as red muscle from all other fish species, was reverse transcribed using AMV reverse transcriptase (Promega) and the resulting cDNA was used for PCR. Polymerase chain reactions were separated on agarose gels, visualized under UV light and the appropriate size band was cut, gel purified, and cloned in pCR 2.1 (Invitrogen). Positive clones were grown for plasmid preparation and the cDNAs were sequenced using a modified dideoxy chain termination method (SequiTherm EXCEL II Long-Read, Epicentre). The sequencing reactions were separated and analyzed using an ALFexpress Sequencer (Amersham Pharmacia Biotech).

*Corresponding author. Fax: (1)-219-631 7413.
E-mail: goetz.1@nd.edu

PII: S0014-5793(01)02196-2

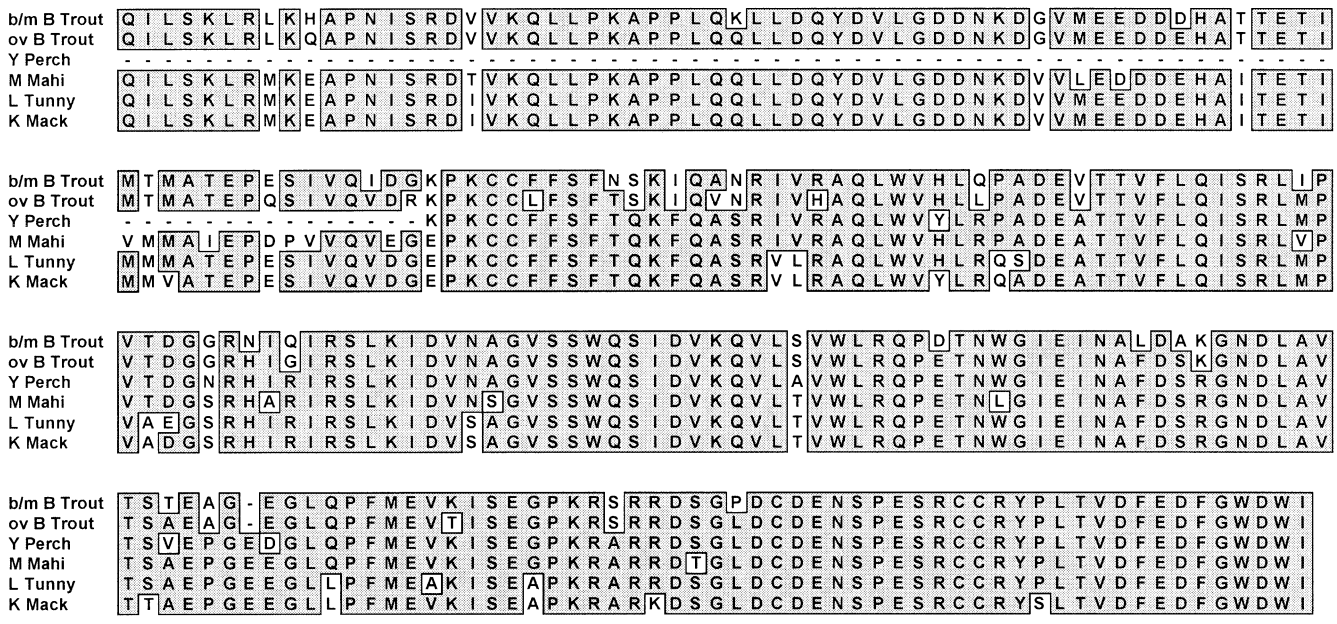

Fig. 1. Amino acid alignment of MSTN fragments from brook trout ovarian tissue (ov B Trout) (accession number AF313912), yellow perch red muscle (Y Perch) (accession number AF319959), mahi-mahi red muscle (M Mahi) (accession number AF317665), little tunny red muscle (L Tunny) (accession number AF317666), and king mackerel red muscle (K Mack) (accession number AF317667), compared with the corresponding section of the full-length brook trout brain/muscle MSTN (b/m B Trout) (accession number AF247650). Shading indicates amino acid identity.

A cDNA fragment obtained from RT-PCR of brain and muscle tissue (b/m BT MSTN) was used to screen a brook trout multiple tissue (liver, brain, testes, skin, and spleen) cDNA library constructed in Zap Express (Stratagene). Library screening was conducted under high stringency as previously described [12]. Positive clones were re-screened once to homogeneity, in vivo excised, and the resulting plasmids were completely sequenced on both strands as described above.

2.3. Northern analysis

Northern blot analysis was performed as previously described [12]. The full-length b/m BT MSTN clone obtained from library screening and the brook trout cDNA fragment from ovarian tissue (ov BT MSTN) were used to probe brook trout tissue blots. Northern blots of tissue from other fish species were probed using MSTN cDNA fragments cloned from each corresponding fish species.

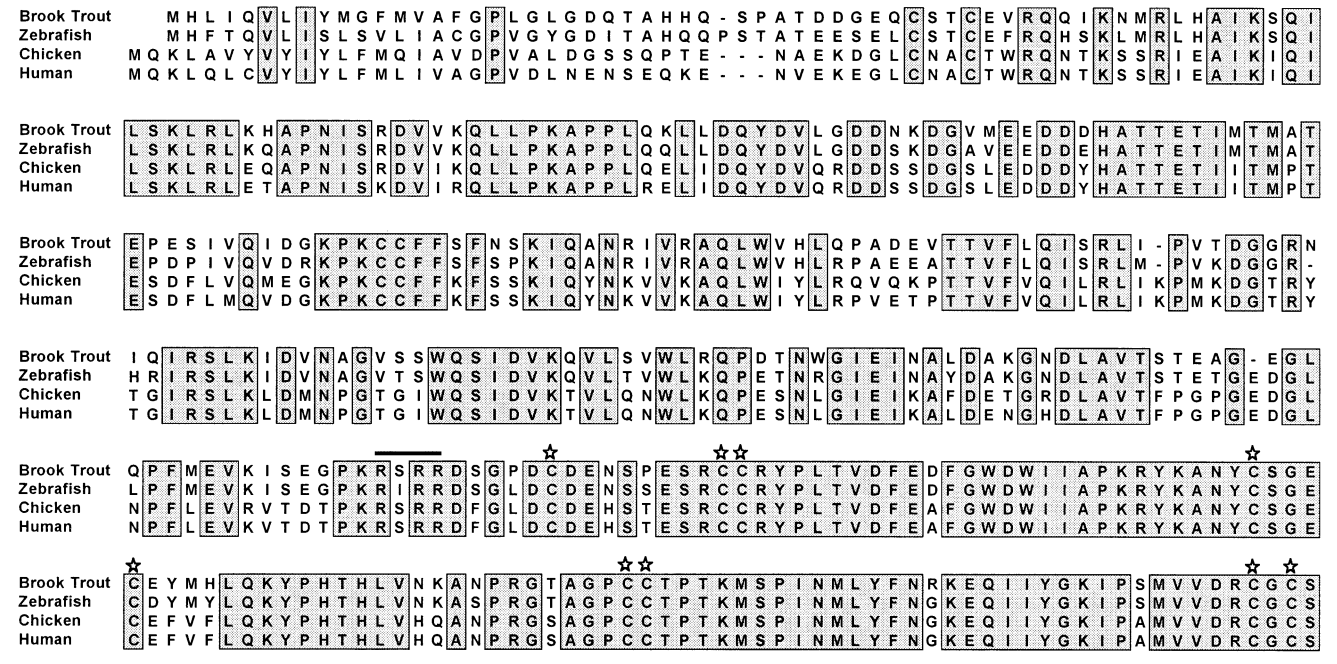

Fig. 2. Amino acid alignment of brook trout brain/muscle (accession number AF247650), zebrafish (accession number AF019626), chicken (accession number AF019621), and human MSTNs (accession number AF019627). Consensus sequence is shaded. Solid bar indicates RXXR proteolytic cleavage domain and conserved cysteines are indicated with stars.

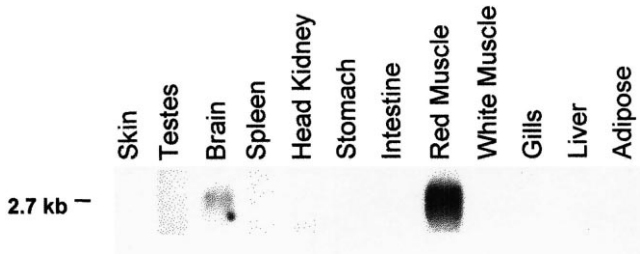

Fig. 3. Northern blot of mRNA (0.5 μg/lane) taken from various brook trout tissues. Blot probed with full-length b/m BT MSTN cDNA.

3. Results

The degenerative primer pair Fw2:Rv2 produced cDNA fragments of 716 bp using brook trout ovarian, muscle, and brain RNA. However, the fragment obtained from the ovary was different from that obtained from muscle and brain. The ov BT MSTN cDNA fragment was 91% identical to the corresponding sequence of b/m BT MSTN at the amino acid level (Fig. 1) and 92% identical at the nucleotide level. The identical ov BT MSTN form was independently isolated by RT-PCR performed on mRNA from ovaries taken from four different female brook trout. When the b/m BT MSTN fragment was used to screen the multiple tissue cDNA library, a 2278 bp clone was obtained. Compared to zebrafish, the full-length b/m BT MSTN clone is 84% identical throughout and 93% identical downstream of the proteolytic cleavage site (Fig. 2). Both human and chicken MSTNs are 65% identical to the brook trout MSTN and 88% identical downstream of the proteolytic cleavage site at the amino acid level (Fig. 2).

Based on known MSTNs, the degenerative primer pair Fw1:Rv1 produced an appropriate size (573 bp) cDNA band when used in PCR with RNA from yellow perch brain tissue. The Fw2:Rv2 primer pair produced cDNA fragments of 719 bp when used in PCR with mahi-mahi, little tunny, and king mackerel muscle RNA. As would be expected, the cloned fragments from king mackerel, little tunny, mahi-mahi, and yellow perch, all had high homology with one another and with b/m and ov BT MSTNs (Fig. 1). Just as other members of the TGF-β superfamily, all of the cDNAs have conserved cysteine residues and a RXXR proteolytic cleavage site. The one exception is that king mackerel MSTN contains the amino acids RARK in the corresponding site.

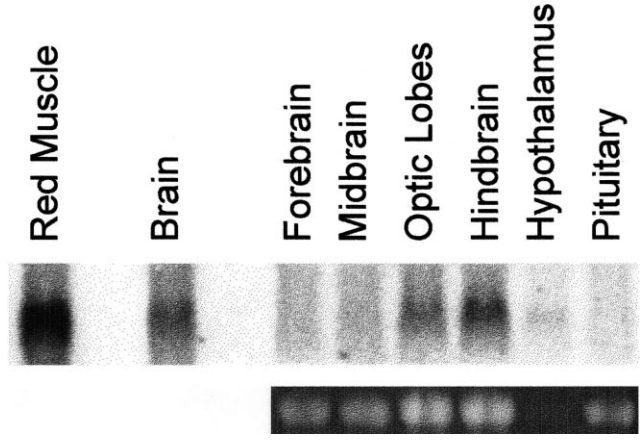

Fig. 4. Northern blot of brook trout red muscle and brain mRNA (0.5 μg/lane) (identical tissue samples as in Fig. 1) adjacent to samples of total RNA from specific regions of the brook trout brain (note: 'Midbrain' = midbrain–optic lobes). For all brain sections, 15 μg of total RNA was loaded per lane except for the hypothalamus for which as much RNA was loaded as was available. 18S rRNA bands of the brain tissue samples are shown below the blot. Blot probed with full-length b/m BT MSTN cDNA.

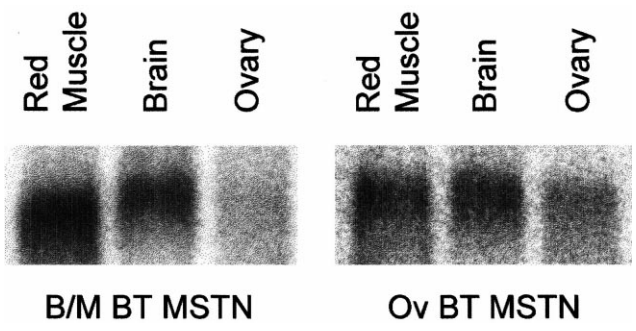

Fig. 5. Northern blots of brook trout red muscle, brain and ovarian mRNA (0.5 μg/lane). The ovarian sample is the same sample as in Fig. 6 (lane 5). A single gel was loaded in replicate with identical samples and after transfer, the blot was cut and replicates were separately hybridized with either the full-length b/m BT MSTN (2278 bp) or the ov BT MSTN cDNA fragment (716 bp).

On Northern blots, a 2.7 kb transcript was observed in red muscle when probed with the b/m BT MSTN cDNA (Fig. 3). A less abundant transcript was present in brain (Fig. 3). On Northern blots of brain tissue dissected into six distinct regions, the transcript was prominent in the optic lobes and hindbrain, and faint in the hypothalamus (Fig. 4). When the full-length b/m BT MSTN cDNA was used as a probe with ovarian tissue taken at ovulation, no hybridization was observed (Fig. 5). This was also true for Northern blots of ovarian tissue taken at stages prior to and following ovulation (results not shown). Transcripts were observed (Fig. 5), however, in red muscle, brain, and ovary when the ov BT MSTN cDNA fragment was used to probe a duplicate blot of the same tissues as probed with the b/m BT MSTN cDNA. On Northern blots of ovarian tissue taken at different reproductive stages and probed with the ov BT MSTN cDNA fragment, increased transcript levels were observed during ovulation in several individuals (Fig. 6).

To evaluate the distribution of MSTN in red and white muscle tissue across different species, the muscle of four teleosts was examined by Northern analysis (Fig. 7). In brook trout, king mackerel, and yellow perch, MSTN was predominately expressed in red muscle. In little tunny, higher levels of MSTN were observed in white muscle while in mahi-mahi MSTN expression was similar in both red and white muscle (Fig. 7).

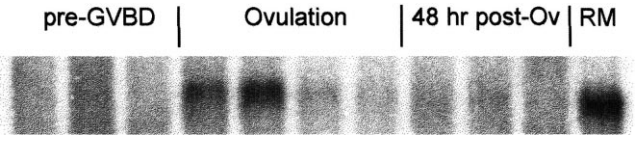

Fig. 6. Northern blot of brook trout ovarian mRNA (0.5 μg/lane) taken prior to germinal vesicle breakdown (pre-GVBD), during ovulation (Ovulation), and 48 h after ovulation (48 h post-Ov). Adjacent to the ovarian samples is a brook trout brain mRNA sample (0.5 μg/lane). Blot probed with the ov BT MSTN cDNA fragment.

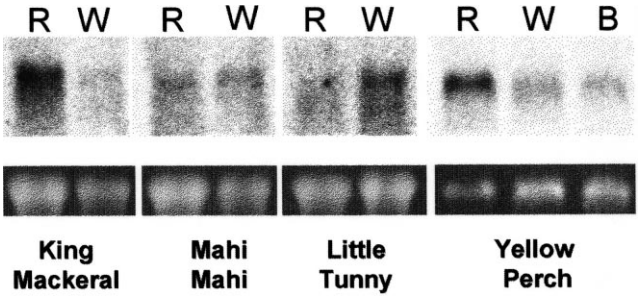

Fig. 7. Northern blots of total RNA (10 μg/lane) from red (fast) 'R' and white (slow) 'W' muscle fibers from four different fish species. Yellow perch blot also contains brain tissue 'B'. Each blot is probed with the corresponding species-specific MSTN cDNA fragment isolated from red muscle total RNA by RT-PCR. 18S rRNA bands for each lane are shown below the blot.

4. Discussion

Since the recent identification of MSTN there has been considerable knowledge gained concerning the role that MSTN plays in mammalian muscle development. Though MSTN has been cloned in representative lower vertebrates it is not known if MSTN has the same function in these animals as it does in higher vertebrates. The results of the present study clearly demonstrate several major differences between MSTN expression in fish and other vertebrates. To date, there has been only one MSTN cDNA cloned within a given vertebrate species. Surprisingly, in the present study two distinct cDNA clones were isolated from brook trout tissue using RT-PCR. These two clones were only 92% identical at the nucleotide level when comparing corresponding regions. When the specific cDNA fragment isolated from ovarian tissue (ov BT MSTN) was used as a probe, hybridization was observed in ovaries of several individuals undergoing ovulation. When the full-length b/m BT MSTN was used to probe the ovary of one of the same individuals undergoing ovulation no hybridization was observed and in other ovarian Northern blots we have not detected a signal using the b/m BT MSTN as a probe. While the MSTN isoform isolated from the brain and red muscle appears to hybridize only with those tissues, it should be noted that in RT-PCR reactions of ovarian mRNA one clone of the b/m BT MSTN form was isolated indicating that it may be in the ovary but expressed at low levels or at some reproductive stage not yet studied. The ov BT MSTN cDNA fragment did hybridize with red muscle and brain in addition to the ovary. Thus it is not known if the isoform isolated from the ovary is also found in other tissues or if hybridization was a result of using a highly homologous cDNA fragment (within the open reading frame) as a probe. Hybridization with brain and muscle might not occur if the full-length ov BT MSTN is used as a probe. However, so far we have been unable to clone this full-length cDNA. To our knowledge this is the first time two MSTN isoforms have been isolated from a given vertebrate species. At this time the role of MSTN in the ovary is unknown but the expression pattern indicates a possible role in ovulation.

A second difference between fish and other vertebrates is the presence of MSTN in multiple tissues. Originally it was believed that MSTN was limited to skeletal muscle. However, recently there have been reports of myostatin protein in cardiomyocytes and Purkinje fibers of the heart [7], as well as MSTN mRNA expression in the mammary gland [5]. In addition, MSTN has recently been isolated in multiple tissues from tilapia including the ovary and the brain [8]. In the current study, MSTN mRNA expression was observed in brook trout and yellow perch brains. Further, by dissecting the trout brain into distinct regions, Northern blots localized brook trout MSTN expression specifically to the optic lobe, hindbrain, and hypothalamus. The function of MSTN in the brain is unknown though other growth and differentiation factors in the TGF-β superfamily have been isolated in the brain of rodents [13–15] and *Xenopus* [16]. Expression of MSTN in the hypothalamus could indicate a possible endocrine function for MSTN. A MSTN immunoreactive substance has been found in human serum [17], suggesting a circulatory role for MSTN.

As expected, MSTN was expressed in the muscle tissue of all fish examined. However, what was unexpected was the differential expression of MSTN among fiber types within various fish species. In adult brook trout, king mackerel, and yellow perch, Northern analysis indicated that MSTN mRNA was specific to red muscle tissue (slow fibers). However, in other species MSTN was either expressed predominately in white muscle (little tunny) or equally in both fibers (mahi-mahi). During muscle unloading in mice, expression of MSTN mRNA is higher in fast muscle [18], and higher mRNA and protein concentrations were also observed in fast muscle in rats [19]. Recent research on rats has indicated that during muscle fiber damage MSTN protein is additionally expressed in slow fibers [20]. Most fish have distinct regions of muscle primarily containing fast twitch or slow twitch myofibers. In contrast, in most mammals there is a more heterologous arrangement of myofibers in muscle. The differences in MSTN expression in red and white muscle observed between fish species could be related to several aspects involving locomotion. Recently, evidence has been presented that MSTN plays a role in muscle regeneration [20–22] and loading/unloading processes [18,19,22] in mammals. It is likely that MSTN could also be involved in the same processes in fish. The species examined in the present study have slightly different red:white muscle ratios and use different strategies for locomotion. This, in conjunction with the fact that fish were taken from environments where varying degrees of muscle loading/unloading occur (e.g. tanks versus open ocean), might also explain the variation in MSTN expression in red and white muscle.

In conclusion, this study demonstrates the presence of MSTN in adult piscine skeletal muscle tissue and the differential expression in red and white muscle types. This study also provides evidence that MSTN is not limited to skeletal muscle, but is present in other tissues such as the brain and ovary. Finally, the data suggest the possibility of multiple MSTN isoforms that could be expressed in a tissue-specific manner.

Acknowledgements: The authors wish to express their thanks to Jeff Malison for providing yellow perch, Steve Roberts (senior) for providing pelagic fish species, Buel Rodgers for access to data in press, and Priscilla Duman for technical assistance. This study was supported by the Illinois-Indiana Sea Grant under the U.S. Department of Commerce's National Oceanic and Atmospheric Administration (Project R/A-05-99, Grant NA86RG0048) and USDA Grant 99-35203-7718. The views expressed herein do not reflect the views of any of those organizations.

References

[1] McPherron, A.C., Lawler, A.M. and Lee, S.-J. (1997) Nature 387, 83–90.
[2] Kambadur, R., Sharma, M., Smith, T.P. and Bass, J.J. (1997) Genome Res. 7, 910–916.
[3] McPherron, A.C. and Lee, S.J. (1997) Proc. Natl. Acad. Sci. USA 94, 12457–12461.
[4] Grobet, L., Martin, L.J., Poncelet, D., Pirottin, D., Brouwers, B., Riquet, J., Schoeberlein, A., Dunner, S., Menissier, F., Massabanda, J., Fries, R., Hanset, R. and Georges, M. (1997) Nature Genet. 17, 71–74.
[5] Ji, S., Losinski, R.L., Cornelius, S.G., Frank, G.R., Willis, G.M., Gerrard, D.E., Depreux, F.F. and Spurlock, M.E. (1998) Am. J. Physiol. 275, R1265–1273.
[6] Kocamis, H., Kirkpatrick-Keller, D.C., Richter, J. and Killefer, J. (1999) Growth Dev. Aging 63, 143–150.
[7] Sharma, M., Kambadur, R., Matthews, K.G., Somers, W.G., Devlin, G.P., Conaglen, J.V., Fowke, P.J. and Bass, J.J. (1999) J. Cell Physiol. 180, 1–9.
[8] Rodgers, B.D., Weber, G.M., Sullivan, C.V. and Levine, M.A. (2001) Endocrinology (in press).
[9] Goetz, F.W., Smith, D.C. and Krickl, S.P. (1982) Gen. Comp. Endocrinol. 48, 154–160.
[10] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[11] Chomczynski, P. (1993) BioTechniques 15, 536–537.
[12] Roberts, S.B., Langenau, D.M. and Goetz, F.W. (2000) Mol. Cell. Endocrinol. 160, 89–97.
[13] Bottner, M., Suter-Crazzolara, C., Schober, A. and Unsicker, K. (1999) Cell Tissue Res. 297, 103–110.
[14] Zhao, R., Lawler, A.M. and Lee, S.J. (1999) Dev. Biol. 212, 68–79.
[15] Soderstrom, S. and Ebendal, T. (1999) J. Neurosci. Res. 56, 482–492.
[16] Vokes, S.A. and Krieg, P.A. (2000) Mech. Dev. 95, 279–282.
[17] Gonzalez-Cadavid, N.F. et al. (1998) Proc. Natl. Acad. Sci. USA 95, 14938–14943.
[18] Carlson, C.J., Booth, F.W. and Gordon, S.E. (1999) Am. J. Physiol. 277, R601–R606.
[19] Wehling, M., Cai, B. and Tidball, J.G. (2000) FASEB J. 14, 103–110.
[20] Kirk, S., Oldham, J., Kambadur, R., Sharma, M., Dobbie, P. and Bass, J. (2000) J. Cell Physiol. 184, 356–363.
[21] Yamanouchi, K., Soeta, C., Naito, K. and Tojo, H. (2000) Biochem. Biophys. Res. Commun. 270, 510–516.
[22] Sakuma, K., Watanabe, K., Sano, M., Uramoto, I. and Totsuka, T. (2000) Biochim. Biophys. Acta 1497, 77–88.