FEBS Letters 509 (2001) 36–40 FEBS 25513

A novel second myostatin gene is present in teleost fish

Lisa Maccatrozzo[a], Luca Bargelloni[a], Barbara Cardazzo[a], Giorgia Rizzo[a], Tomaso Patarnello[a,b,*]

[a] *Dipartimento di Biologia, Università di Padova, Via Ugo Bassi 58/B, I-35121 Padova, Italy*
[b] *Facoltà di Medicina Veterinaria-Agripolis, Università di Padova, Via Romea 16, I-35020 Legnaro, Italy*

Received 18 October 2001; revised 29 October 2001; accepted 30 October 2001

First published online 8 November 2001

Edited by Ned Mantei

Abstract We report on the isolation and characterisation of the complete cDNA sequence encoding a novel bone morphogenetic protein-like protein (sbMSTN-b) in the teleost fish *Sparus aurata*. The encoded protein is 68% identical to *S. aurata* MSTN at the amino acid level, and homologues were also found in *Umbrina cirrosa* and *Tetraodon nigroviridis*. Phylogenetic analysis suggests that the *MSTN-b* gene may be present in most, perhaps all, teleost fish species. RT-PCR on different tissues/stages indicates that MSTN-b is expressed almost exclusively in the central nervous system, starting from late larval stages. Quantitative analyses indicate an increase of *sbMSTN-b* expression in the brain associated with metamorphosis, at the same time as completion of nervous system differentiation.

Key words: Teleost fish; Brain; Evolution; *MSTN*; *BMP-11*

1. Introduction

The transforming growth factor-β/bone morphogenetic protein (BMP) superfamily includes a large number of proteins implicated in early embryonic patterning and in development of several organs and tissues. BMP-like molecules regulate biological processes as diverse as cell proliferation and differentiation, cell-fate determination and morphogenesis [1]. Within the BMP superfamily, myostatin (*MSTN*) has been recently characterised as a negative regulator of skeletal muscle growth in mice [2] and cattle [3–5]. In consideration of its pivotal role in muscle growth control, *MSTN* represents an extremely interesting locus for genetic improvement of farmed animals. Such potential applications in animal husbandry have prompted the sequencing of *MSTN* cDNA from several mammalian and avian species, as well as from zebrafish [4]. More recently, *MSTN* cDNA sequence and expression pattern have been characterised in several fish species of commercial importance [6–10]. In mammals, *MSTN* mRNA is expressed mainly in the skeletal muscle, and at lower levels in the adipose tissue [2,11], mammary gland [12] and cardiac muscle [13]. In fish, although differences exist among species, *MSTN* mRNA was detected also in other tissues/organs (brain, eye, intestine, gill filaments, gonad, kidney) [6,7,9,10]. Particularly, two isoforms of *MSTN* have been recently reported in the brook trout *Salvelinus fontinalis* [7]. In that case, two different mRNAs were found, one specifically expressed in the ovary (BT ovMSTN), the other in the red muscle and brain. In mammals, a second gene, *BMP-11*, with high similarity to *MSTN*, is expressed in the brain and the eye [14–16]. In a previous paper [9], we demonstrated the presence of a *MSTN* ortholog (*sbMSTN*) in the gilthead seabream *Sparus aurata*, a marine teleost fish of great interest for fisheries and aquaculture. Here we report on the isolation and characterisation of a cDNA encoding a second BMP-like protein from *S. aurata*, and the finding of a second isoform also in other fish species (*Umbrina cirrosa* and *Tetraodon nigroviridis*). This novel gene appears to be closely related to *MSTN* and *BMP-11* and is expressed almost exclusively in the brain.

2. Materials and methods

2.1. Animals and tissues collection

Seabreams were collected at the fish farm 'Impianto di Acquacoltura Ca' Zuliani' (Monfalcone, Italy). Samples consisted of different development stages and different tissues/organs dissected from adults: adipose tissue, brain (both whole and the optic and olfactory lobe separately), eye, hermaphrodite gonad (consisting of both ovarian and testicular portions), heart, intestine, kidney, liver, muscle (a section of lateral muscle, including both red and white type fibres, although in different proportions) and spleen. Brain samples were collected from different ontogenetic stages: larvae immediately before metamorphosis, fry immediately after metamorphosis, older fry, young and mature adults.

All samples were dissected from over-anaesthetised animals, rapidly frozen in liquid nitrogen and stored at −80°C; subsequently, samples were homogenised in Trizol Reagent (Gibco-BRL, Gaithersburg, MD, USA), and total RNA isolation was completed as previously described [9].

2.2. Full-length cDNA isolation

One microgram of total RNA from adult brain tissue was reverse transcribed using a reverse transcriptase (Superscript II, Gibco-BRL) and random hexamers to obtain first-strand cDNA. The cDNA was then used as template for the subsequent PCR reactions. All PCR reactions were performed using a GeneAmp PCR System 2400 (Perkin Elmer, Branchburg, NJ, USA), under the following conditions: 3 min at 94°C (initial denaturation), 40 cycles with 50 s at 94°C, 50 s at annealing temperature (see below), 50 s at 72°C, and 2 min at 72°C (final extension). The annealing temperature was progressively reduced from 53°C to 45°C in the first 10 cycles (−0.8°C per cycle), then set at 49°C for the remaining 30 cycles. Five microlitres of each PCR product was electrophoresed on a 1.8% agarose gel, stained with ethidium bromide, and visualised under UV light.

Initially, a partial fragment (182 bp) was amplified using a pair of degenerate primers, M2F and M2R, designed on the basis of MSTN amino acid sequences in other species. The primer sequences were GTAA(G,A)(G,A)GC(I)CAGCT(I)TGG(G,A)TT(C,T)AT(C,T)-TGAG and A(G,A)CAC(I)TGCTT(C,T)AC(G,A)TC(A,T)AT(I)CT-CTGCCA, respectively. The PCR product was cloned into a plasmid

*Corresponding author. Fax: (39)-49-8276209.
E-mail address: patarnel@civ.bio.unipd.it (T. Patarnello).

PII: S0014-5793(01)03124-6

vector (pCR-II, Invitrogen, Carlsbad, CA, USA) using a TOPO-TA cloning kit (Invitrogen) and sequenced using the thermosequenase pre-mixed cycle sequencing kit (Amersham-Pharmacia Biotech, Uppsala, Sweden). At least three independent clones were sequenced. Based on the sequenced fragment, a specific primer was designed to amplify, with a third degenerate primer (M3R, primer sequence as in [9]), a larger cDNA region (534 bp), that was cloned and sequenced as described above. Based on obtained sequence information, specific oligos were designed to isolate the complete cDNA by means of a *R*apid *A*mplification of *C*DNA *E*nds (RACE) technique [17]. The SMART RACE cDNA Amplification kit (Clontech, Palo Alto, CA, USA) was used to obtain the 5′ and 3′ unknown regions, according to the manufacturer's instructions. PCR products of 5′ and 3′ RACE reactions were cloned and sequenced as described above. To confirm the full-length cDNA sequence, two specific primers were designed on 5′- and 3′-end regions, and the PCR product was directly sequenced.

Degenerate PCR primers (b1F GAGCACAGCAAGCAGATG-(AC)G(GA)(CT)T(i)(CA)AC(AG)(GC)CA-3′ and b2R CTCTTGGG-GTTGTCGAG(i)AT(CT)TTCAC(TC)TC(GC)AT) were designed, on the basis of the seabream cDNA sequence, to amplify a fragment of the novel gene from another fish species (*U. cirrosa*), using the same approach described above.

2.3. Sequence analysis

The putative amino acid sequence was used to perform a BLASTP search (http://www.ncbi.nlm.nih.gov/blast/) with default settings on the complete, non-redundant GenBank database of translated coding sequences (release 2.2.1, April 13, 2001), as well as a BLASTN search on the genome survey sequence section of GenBank nucleotide database. The BLASTN search was limited to fugu (*T. nigroviridis*) genome sequences. Sequences closely related to the novel isolated protein (MSTN and BMP-11 protein from different species) were aligned using the Dialign software (http://genomatix.gsf.de/products/index_dialign.htlm). Two sequences were included as outgroups. Based on this multiple protein alignment, Poisson-corrected distances were estimated for every possible sequence pair. Regions where alignment was unreliable (e.g. signal sequences) and gap-containing positions were excluded from the analysis. The neighbour-joining method [18] was used to reconstruct the phylogenetic tree based on the obtained distance matrix; node robustness was assessed using a bootstrap approach [19]. All phylogenetic analyses were implemented in the programme MEGA2 [20].

2.4. Qualitative and quantitative RT-PCR

A qualitative RT-PCR analysis was carried out on total RNA extracted from different tissues/organs of adult seabreams as well as from whole larval stages. For each sample the same amount of RNA (1.5 μg) was reverse transcribed using as primer a mixture of random hexamers. Specific primers were used to amplify a short cDNA fragment 296 bp in size. To exclude false positive results due to genomic DNA contamination, primers were designed to encompass an intronic sequence (data not shown). PCR conditions were: 94°C 45 s, 58°C 45 s, 72°C 1 min for 35 cycles. Ten microlitres of PCR product from each sample was electrophoresed on a 1.8% agarose gel. A fragment of the seabream *β-actin* gene was amplified to test the quality of extracted RNA and the efficiency of RT reaction as previously described [9].

A quantitative RT-PCR was performed to estimate relative expression levels in brain samples at different stages of development. Quantitative RT-PCR assay was carried out through the use of an internal competitor DNA. The competitor template was constructed as follows: a completely unrelated sequence (a fragment of seabream *Sox9* gene) was amplified using specifically designed primers (GGGAAGGAGGGAAACAACAATGAAGATGACAGAAGAAC, GCAGACTCTTCGTCACAGTTAGACCATGAAGGCGTT). The oligos bear a tail (underlined) that is complementary respectively to E2F and MAO2R primers. The amplified competitor was then cloned into a plasmid vector as described above. Therefore, using primers E2F–MAO2R, it was possible to amplify with similar efficiency two products: the target cDNA (296 bp) and the internal standard (355 bp). Moreover, competitor DNA shared with target DNA only the primer regions, avoiding the possibility of heteroduplex formation [21]. The number of PCR cycles was optimised for each template by performing 20, 24, 28, 32, 36, or 40 cycles of the same reaction, and selecting the appropriate conditions to ensure that PCR reactions ended during the exponential phase.

To quantify the target cDNA, a dilution series of competitor DNA (1, 0.5, 0.2, 0.1, 0.05 pg/μl) was amplified with a constant amount of target cDNA (2 μl of RT reaction), under the following conditions: 94°C 50 s, 58°C 50 s, 72°C 50 s for 32 cycles. Ten microlitres of product was run on a 3% agarose gel. Concentration of target cDNA was determined by image analysis comparing band intensities respectively of competitor DNA dilutions and cDNA amplification products.

To ensure repeatability, RNA extractions were performed in duplicate either on tissues from larval pools (6–10 individuals), or two to three adult individuals. Two separate RT reactions for each duplicated extraction were carried out, and competitive PCR amplifications were always performed in duplicate on each RT product. This yielded 8 and 16 competitive-PCR replicates respectively for larval and adult stages. Expression levels were averaged across replicates for each examined stage.

Detailed information on primer sequences is available from T.P. upon request.

3. Results and discussion

The adopted RT-PCR/RACE approach yielded a putative full-length cDNA of 1464 bp (GenBank AY046314), which we named *MSTN-b*. The first possible start codon was located at position 25, with an open reading frame of 1077 bp, encoding a peptide of 359 amino acids. Compared to other members of the BMP superfamily, the putative amino acid sequence showed a highly conserved carboxy-terminal portion, corresponding to the mature processed protein. In particular, a potential proteolytic processing site (RSRR, matching the RXXR consensus site) and nine cysteine residues were present, as in all vertebrate MSTNs and BMP-11s.

A BLASTP search on the complete non-redundant database of translated coding sequences confirmed that the obtained sequence is most similar to fish MSTNs, with 248–258 identical residues over 373 compared amino acid positions, and other vertebrate MSTNs (230–234/373). High scores were also observed in comparisons with human and murine BMP-11 (respectively 214/353 and 206/330). Pairwise alignment between the two seabream isoforms, sbMSTN (GenBank AF258448) and the new isolated sequence, revealed 244 identical amino acid residues over 357 (68% similarity), while at the nucleotide level, overall similarity was 72%. On the other hand, the highest identity values for sbMSTN were observed in comparison with the striped bass MSTN, (89.5% and 93% respectively for amino acid and nucleotide identity). When the newly isolated seabream MSTN isoform was compared with the striped bass MSTN, much lower values were obtained (69% and 70% respectively). Sequence similarity therefore indicates that the isolated cDNA encodes a novel protein, closely related though not orthologous to fish MSTNs. However, evolutionary relationships among sequences are best investigated using gene genealogies. To this end, a phylogenetic tree was reconstructed including all complete MSTN and BMP-11 vertebrate sequences together with two related BMP-like proteins as outgroups (Fig. 1A). The obtained tree topology showed the presence of three well-supported clusters (93–100% bootstrap values). A first cluster containing all teleost MSTNs together with the newly isolated sequence, a second one grouping mammalian and avian MSTN sequences, and a third one comprising murine and human BMP-11 proteins. All vertebrate MSTNs (fish, avian and mammalian) were clustered together (68% bootstrap value), whereas BMP-11s diverged earlier in the tree. While this result suggests that teleost and higher vertebrate *MSTNs* are

orthologous genes, the position of the novel gene is indicative of further complexity. The new MSTN-like sequence branches out after the divergence of avian and mammalian MSTNs, yet before the diversification of all fish MSTNs, with strong statistical support (100% bootstrap value). This evidence might be the result of an ancestral event of gene duplication before the evolution of teleost lineages. Therefore, to further elucidate evolutionary relationships within teleost MSTNs, an additional analysis was performed, using all available fish sequences, even partial ones. A putative fugu MSTN coding sequence, assembled from genomic sequences of two clones available on NCBI database (GenBank AL174773, AL274449), and MSTN isoforms isolated in three salmonid species (BT ovMSTN [7]; rainbow trout MSTN 1, GenBank AF273035; Atlantic salmon MSTN II, GenBank AJ344158) were also analysed. In addition, two putative orthologs of sbMSTN-b from shi drum (*U. cirrosa*) and fugu (*T. nigroviridis*) were included in the analysis. With respect to *S. aurata*, these two species belong respectively to a different taxonomic family (shi drum) and a different order (fugu). In the case of shi drum, we experimentally obtained a partial cDNA sequence of *MSTN-b* (791 bp, GenBank AY059386) from total RNA extracted from adult brain; for fugu the putative coding sequence was assembled from different genomic sequences obtained from public sequence database (GenBank AL200779, AL216859, AL258020, AL270849). The phylogenetic tree obtained from the analysis of all these fish sequences (Fig. 1B) provides compelling evidence for two independent duplications within fish *MSTN* gene cluster. In consideration of the basal position of the tree branch leading to the *MSTN-b* gene cluster, it is likely that a gene duplication (D1 in Fig. 1A,B) in an early fish ancestor generated two *MSTN*-like genes, one giving rise to all fish *MSTNs*, the other to the novel *MSTN*-like genes found in this study. While loss of duplicated genes might have occurred during teleost evolution, retention of two copies in three independent fish lineages suggests that *MSTN-b* should still be present in several fish genomes. A more recent duplication (D2 in Fig. 1B) appears to have occurred only in the salmonid lineage, generating the second isoform found in the BT, in the rainbow trout and in the Atlantic salmon. The hypothesis of a second, salmonid-specific duplication is in agreement with the well-known tetraploidisation of the salmonid genome [22].

Reconstruction of homology relations among vertebrate MSTN sequences reveals a complex evolutionary history. A similarly intricate pattern is observed when examining expression profiles of *MSTN* genes in different lineages. In mammals, *MSTN* is mainly expressed in the skeletal muscle, while

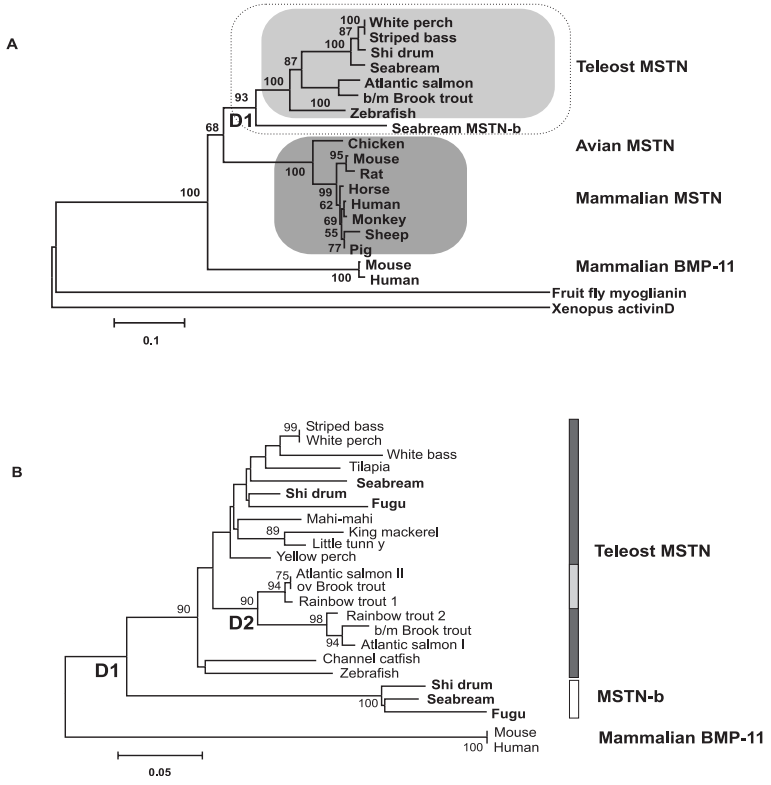

Fig. 1. A: Neighbour-joining tree of sbMSTN-b, vertebrate MSTNs and related sequences (fruit fly myoglianin, AF132814; *Xenopus* activinD, D49543). Numbers at tree nodes refer to percent bootstrap values after 1000 replicates; the scale bar refers to a phylogenetic distance of 0.1 amino acid substitutions per site. B: Phylogenetic tree of sbMSTN-b, teleost MSTNs and vertebrate BMP-11s. D1 and D2 indicate tree nodes where gene duplications might have occurred during evolution of teleost lineage. Several of these sequences are only partial (see text). The grey (dark and light) bar groups together all teleost MSTNs (light grey indicates the salmonid MSTN isoforms), and the white bar indicates the novel fish MSTN-b. The scale bar refers to a phylogenetic distance of 0.05 amino acid substitutions per site. Vertebrate MSTN GenBank accession numbers: Atlantic salmon I AJ297267, Atlantic salmon II AJ344158, BT red muscle and brain AF247650, BT ov AF313912, channel catfish AF396747, king mackerel AF317667, little tunny AF317666, mahi-mahi AF317665, rainbow trout 1 AF273035, rainbow trout 2 AF273036, seabream AF258448, shi drum AF316881, striped bass AF290910, tilapia AF197193, white bass AF197194, white perch AF290911, yellow perch AF319959, zebrafish AF019626, chicken AF346599, horse AB033541, human AF019627, monkey AF019619, mouse NM010834, pig AF019623, rat NM019151, sheep AF019622. Fish MSTN-b GenBank accession numbers: seabream AY046314, shi drum AY059386. Mammalian BMP-11 GenBank accession numbers: human AF100907, mouse AF100906.

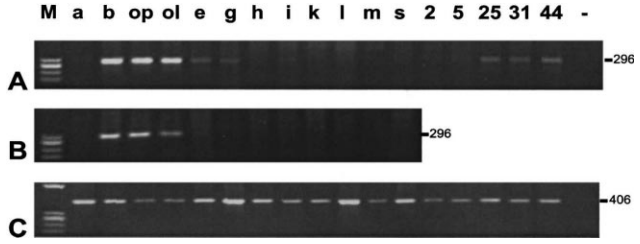

Fig. 2. RT-PCR assay on different tissues/stages. Samples were loaded onto agarose gels in the same order. Lanes correspond to: adipose tissue (a), whole brain (b), optic lobe (op), olfactory lobe (ol), eye (e), gonad (g), heart (h), intestine (i), kidney (k), liver (l), muscle (m), spleen (s) (adult tissues); 2-day-old embryos (2), 5-day-old larvae (5), 25-day-old larvae (25), 31-day-old larvae (31), 44-day-old larvae (44), negative control (−). Size standard (M) used was DNA molecular weight marker IX (Roche Molecular Biochemicals). Ten microlitres of product was loaded in each lane. Primers used were E2F-MAO2R. A: RT-PCR analysis of *MSTN-b* expression in 2-year-old seabream tissues and in different developmental stages. B: RT-PCR expression profile in 1-year-old seabream tissues. C: Results of amplification of a 406 bp fragment of the *β-actin* mRNA (see text).

in fish *MSTN* mRNA was detected also in other tissues/organs (brain, eye, intestine, gill filaments, gonad, kidney). In contrast to the wide expression pattern of MSTN in fish, RT-PCR analysis (Fig. 2) revealed that expression of the *sbMSTN-b* gene in the adult seabream is observed almost exclusively in the brain. When examined separately, both the optic and olfactory lobe yielded a positive signal. A very weak signal was detected also in the eye and in the gonad (Fig. 2A). Intriguingly, the latter result was observed only starting from mature (2 years old) adults, as in immature (1 year old) individuals, *sbMSTN-b* was expressed only in the brain (Fig. 2B). Seabreams are sequential hermaphrodites, and their gonads comprise both testicular and ovarian regions. For this reason, it is difficult to assess a possible relationship between levels of *MSTN-b* mRNA and reproductive state (immature–mature, male–female), and further studies providing evidence for *sbMSTN-b* expression in situ on gonad sections would be needed.

In general, expression of MSTN-b seems to be limited to later phases of ontogenesis, as at early developmental stages (2-day-old embryos and larvae immediately after hatching), no signal was observed in RT-PCR on RNA from whole individuals (Fig. 2A), nor in whole-mount in situ hybridisation experiments (data not shown). Absence of amplified product for *sbMSTN-b* cannot be ascribed to poor quality of extracted RNA or inefficient RT reaction, as shown by control amplification of a seabream *β-actin* fragment (Fig. 2C). A weak product was present at later larval stages (25–31 days), while the RT-PCR band intensity increased for 44-day-old whole larvae. The latter stage was also the first one for which it was feasible to microdissect the brain and quantitatively analyse tissue-specific expression. Thus, starting from larvae immediately before metamorphosis (40–45 days old), relative expression levels in the brain were quantitatively determined for different ontogenetic stages (fry immediately after metamorphosis, later fry stages and adults). Results of competitive RT-PCR revealed that expression of *sbMSTN-b* is approximately two-fold–four-fold lower in pre-metamorphosis larvae compared to post-metamorphosis fry (Table 1, Fig. 3). On the other hand, a comparable degree of expression was detected for all post-metamorphosis stages (Table 1), from young fry (50–150 days old) to adults (1.5 years old), although *sbMSTN-b* seems to be expressed at slightly higher levels in the adults.

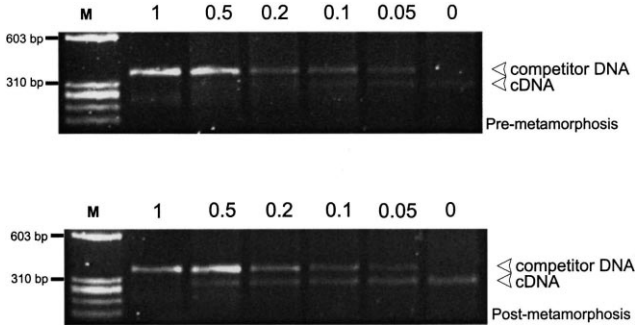

Fig. 3. Competitive RT-PCR of larval (pre-metamorphosis; upper panel) and fry (post-metamorphosis; lower panel) seabream stages. *SbMSTN-b* cDNA was amplified, using E2F–MAO2R primers, with decreasing amounts of competitor DNA (1, 0.5, 0.2, 0.1, 0.05, 0 pg/μl).

Table 1
SbMSTN-b cDNA expression level in the brain of different ontogenetic stages

Stage	Relative amount of *sbMSTN-b* cDNA (pg/μl)[a]
Pre-metamorphosis larva	<0.05
Post-metamorphosis fry	0.1–0.2
Fry	0.2
Mature adult	0.2

[a]Calculated with reference to dilutions of competitor DNA.

In the seabream, as in other teleosts, metamorphosis represents a crucial transition during ontogenesis, with a change of external morphology from larval to adult form. The appearance of a swimming bladder, and all related structures, is associated with this transition, and the anatomy of several organs acquires adult features. In particular, at the end of the larval stage, the aspect of the nervous system is similar to the definitive one [23].

Results presented here reveal that *sbMSTN-b* is expressed almost exclusively in the central nervous system, and only from late larval stages. Moreover, a marked increase is observed in relation to metamorphosis, thus, at the same time as the completion of nervous system differentiation. In consideration of the function of MSTN in mammals as an inhibitor of skeletal muscle growth, one could speculate that a similar (negative) role is played in brain development by sbMSTN-b, once the nervous system has concluded its pathway of differentiation. Moderately higher levels of expression in the adult are likewise suggestive of a growth-limiting function for sbMSTN-b in the nervous system.

Acknowledgements: We acknowledge Dr. P. Patarnello and the 'Impianto Ca' Zuliani' (Monfalcone, Italy) for providing samples, and Profs. F. Mascarello and G. Radaelli for useful discussions. This work was supported by 'ex-40% MURST' to T.P.

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