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β-Thymosin is required for axonal tract formation in developing zebrafish brain

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SUMMARY

β-Thymosins are polypeptides that bind monomeric actin and thereby function as actin buffers in many cells. We show that during zebrafish development, β-thymosin expression is tightly correlated with neuronal growth and differentiation. It is transiently expressed in a subset of axon-extending neurons, essentially primary neurons that extend long axons, glia and muscle. Non-neuronal expression in the brain is restricted to a subset of glia surrounding newly forming axonal tracts. Skeletal muscle cells in somites, jaw and fin express β-thymosin during differentiation, coinciding with the time of innervation. Injection of β-thymosin antisense RNA into zebrafish embryos results in brain defects and impairment of the development of β-thymosin-associated axon tracts. Furthermore, irregularities in somite formation can be seen in a subset of embryos. Compared to wild-type, antisense-injected embryos show slightly weaker and more diffuse engrailed staining at the midbrain-hindbrain boundary and a strong reduction of Isl-1 labeling in Rohon Beard and trigeminal neurons. The decreased expression is not based on a loss of neurons indicating that β-thymosin may be involved in the maintenance of the expression of molecules necessary for neuronal differentiation. Taken together, our results strongly indicate that β-thymosin is an important regulator of development.

Key words: β-Thymosin, Zebrafish, Neuronal growth, Muscle differentiation, Actin, Antisense injections

INTRODUCTION

To reach the appropriate target, axon-extending neurons depend on the ability to rapidly polymerize and depolymerize their cytoskeleton. Treatment with cytochalasin, a drug that causes disruption of the actin network, results in the inability of axons to respond to environmental cues that normally guide their growth, leading to uncontrolled extension (Bentley and Toroian-Raymond, 1986; Chien et al., 1993). Furthermore, rapid changes in the arrangement of filamentous actin within a particular region of the cell underlie such diverse processes as vesicular transport, endocytosis and exocytosis (Theriot, 1994). The rate of actin polymerization and depolymerization is influenced by the concentration of free filamentous actin ends and free actin monomers. Actin-binding proteins sequester actin monomers in the cytoplasm and thereby influence the rate of actin assembly.

Among the different actin monomer-binding proteins is the highly conserved family of the β-thymosins. It includes several isoforms with β_{10} and β_4 being the major isoforms in mammals (Hannappel et al., 1982; Erickson-Viitanen et al., 1983). It is thought that they constitute the major actin monomer buffering proteins in many cells (Safer and Nachmias, 1994; Sun et al., 1995). In vivo, both isoforms are developmentally regulated and are preferentially found in the nervous system. Thymosin β_{10} peaks at embryonic day 15 and then declines 20 fold by postnatal day 14 in the rat central nervous system (CNS; Lugo et al., 1991). A similar regulation was observed for thymosin β_4 during development of the human brain (Condon and Hall, 1992) and the *Xenopus laevis* nervous system (Yamamoto et al., 1994). These studies indicate a role for β-thymosin in nervous system differentiation but its function in this process is not known. Outside the nervous system, thymosin β_4 has been reported to be necessary for endothelial cells to form capillary-like tubes in vitro (Grant et al., 1995), suggesting a role in controlling cell shapes. Other reports, focusing on β-thymosin in cancer cells (Yamamoto et al., 1993) and in PC 12 cells after stimulation with NGF (Leonard et al., 1987) found no general relationship between increased β-thymosin levels and growth or differentiation.

Several other actin regulating proteins that have been associated with neuronal development are, in contrast to β-thymosin, directly controlled by second messengers. A well-studied example is profilin that binds, as does β-thymosin, actin monomers (Sohn and Goldschmidt-Clermont, 1994). It is phosphorylated by protein kinase C, a process regulated by phosphatidylinositol bisphosphate (Hansson et al., 1988). Other examples are gelsolin and cofilin. Under the control of Ca^{2+} and membrane polyphosphoinositides, gelsolin severs actin filaments, caps the barbed ends and nucleates actin polymerization (Matsudaira and Janmey, 1988). Cofilin, a ubiquitous actin-binding protein is regulated by Rac and LIM-kinase 1 and determines the number of filopodia in growth cones in vitro (Arber et al., 1998).

Here, we report that β-thymosin is tightly regulated on the transcriptional level in developing zebrafish. It is primarily expressed in regions where neuronal growth takes place and in differentiating skeletal muscle. Injection of antisense RNA obstructs the formation of normally β-thymosin-positive axonal tracts in the developing brain and shows effects on segmentation. This is a first indication that β-thymosin is not a passive actin buffering protein, but is essentially involved in specific developmental processes.

MATERIALS AND METHODS

Cloning of β-thymosin

Zebrafish β-thymosin cDNA has been isolated in a screen for genes that are upregulated in regenerating retina by differential display. An amplification product from the 3′ end of the β-thymosin cDNA was obtained using the primers 5′ -T_{14}CG-3′ and 5′ -CCAAGGAGAG-3′. The subcloned fragment was used to screen a λZAP II cDNA library, generated from regenerating and normal adult zebrafish retinas.

All sequencing reactions were performed using an ABI Prism dye terminator sequencing kit (No. 402080) and a GeneAmp 9600 thermal cycler. The products were run on a ABI 373A automated sequencer (all Perkin Elmer Corporation, California, USA). Sequence analysis was performed using the Wisconsin Sequence Analysis Package VMS version 8.0 (Genetics Computer Group, Madison, USA) and the BLAST network service of the NCBI (National Center for Biotechnology Information, USA). The zebrafish β-thymosin sequence data is available in GenEMBL, accession number AF006831.

Northern blot hybridization

Northern blot hybridization was performed following the protocols of Sambrook et al. (1989) and the DIG System User Guide for Filter Hybridization (Boehringer Mannheim, Mannheim, Germany). Total RNA was isolated from embryos of different developmental stages and adult brain, according to the instructions given in the RNeasy Total RNA kit (Qiagen AG, Basel, Switzerland). DNA was removed by treatment with DNAse (Promega, Catalys AG, Wallisellen, Switzerland). The transcripts were separated in a formaldehyde gel. 4 μg of total RNA was loaded per lane. To analyze the effect of antisense RNA injection on endogenous β-thymosin mRNA levels, RNA isolated from an equal number of injected and control embryos was loaded per lane (24 h, 120 embryos; 36 h, 30 embryos). The RNA was transferred to a nylon membrane (Schleicher & Schuell NY 13 N, Keene, NH) using a pressure blotter (Posiblot 30-30, Stratagene, La Jolla, CA) and UV crosslinked (Stratalinker 2400, Stratagene, La Jolla, CA). To control for equal loading, RNA was visualized with 0.5 M sodium acetate, containing 0.04% methylene blue. Hybridization with DIG-labeled probes (see below) was performed at 68°C overnight in a Hybritube (Gibco BRL, Life Technologies AG, Basel, Switzerland), followed by several washes at a maximal stringency of 0.1× SSC, 0.5% SDS at 68°C. Hybridized probes were visualized using anti-digoxigenin-AP Fab fragments (diluted 1:10’000 in Boehringer buffer; Boehringer Mannheim, Mannheim, Germany, No 1093 274) and the chemoluminescence reagent CDP-Star (TROPIX, Massachusetts, USA, No MS100R).

In situ hybridization

Digoxigenin-labeled riboprobes were generated from the β-thymosin and zebrafish actin (provided by Dr P. Bormann, Biozentrum, Basel) cDNAs, according to the instructions from the DIG labeling kit (Boehringer Mannheim, Mannheim, Germany). Unincorporated nucleotides were removed using purification columns from the RNeasy Total RNA kit (Qiagen AG, Basel, Switzerland). The labeled probe was eluted in 30 μl H_2O, hybridization solution was added to 100 μl (Westerfield, 1995) and stored at −20°C until use.

In situ hybridization analysis followed the protocol of Westerfield (1995). Proteinase K treatment (10 μg/ml; Boehringer Mannheim, Mannheim, Germany) was performed for 10 minutes or longer (up to 50 minutes for older embryos) at room temperature. Washes after hybridization were at a maximal stringency of 0.2× SSC, 0.1% Tween-20 at 55°C. The hybridized probes were visualized using anti-digoxigenin-AP Fab fragments (diluted 1:2000 in PBST; Boehringer Mannheim No 1093274, Mannheim, Germany). Times for color development (chosen according to the probes used and the developmental stage of the embryos) ranged from 4 to 8 hours. Embryos were refixed in 4% PFA and either cleared in a graded series of glycerol/PBS for whole-mount microscopy or cryoprotected in 20% sucrose/PBS and embedded in OCT Tissue-tek (Miles Inc., Elkhart, IN) for cryosectioning. 12 μm sections were cut, mounted on slides and examined by microscopy.

In vitro transcription

A PCR product of the coding region of zebrafish β-thymosin was cloned into the vector pCS2+MT (Roth et al., 1991; Rupp et al., 1994; Turner and Weintraub, 1994). Full-length sense and antisense RNAs were synthesized using SP6 and T7 RNA polymerase, respectively (Boehringer Mannheim, Mannheim, Germany). In short, 2.5 μg DNA was linearized and purified for in vitro transcription (Qiaquick, Qiagen AG, Basel, Switzerland). After transcription and capping, the DNA template was digested and the RNA purified (RNeasy, Qiagen AG, Basel, Switzerland). An estimated 0.5 ng RNA was pressure-injected into one-cell zebrafish embryos. Embryos were then analyzed at 24 and 33 hours after fertilization (h) by immunohistochemistry, in situ hybridization and northern blot.

Whole-mount immunohistochemistry

Embryos were pretreated for immunohistochemistry as described by Westerfield (1995). The primary antibodies (supernatants from the Developmental Studies Hybridoma Bank, Iowa City, IA) against engrailed (4D9; Patel et al., 1989) and Islet-1 (39.4D5; Ericson et al., 1992) were diluted 1:50 in BDP+N (1% BSA, 1% DMSO in PBSTx + 10% normal sheep serum) and incubated for 3 hours at room temperature. After several washes, the secondary antibody (sheep anti-mouse IgAP; Boehringer Mannheim, Mannheim, Germany) was applied overnight at 4°C and the alkaline phosphatase visualized as described by Westerfield (1995).

In double labeling experiments, embryos were first analyzed by in situ hybridization, followed by immunohistochemistry. Embryos were washed several times in PBST after mRNA visualization and then incubated for 10 minutes in glycine (0.1 M, pH 2.2) and washed again in PBS, 0.1% Triton X-100 (PBSTx). The primary antibody (mouse anti-acetylated tubulin, T-6793; Sigma, Buchs, Switzerland) was diluted 1:1000 in BDP+N and incubated for 3 hours at room temperature. After several washes, the secondary antibody (sheep anti-mouse IgAP; Boehringer Mannheim, Mannheim, Germany) was applied overnight at 4°C. For detection of the alkaline phosphatase, Fast Red tablets (Boehringer Mannheim, Mannheim, Germany) were used (Hauptmann and Gerster, 1994).

RESULTS

β-Thymosin is primarily expressed during zebrafish development

In a search for genes that are transiently expressed in zebrafish regenerating retinal ganglion cells, we isolated a partial sequence of the actin monomer-binding protein β-thymosin. By screening an adult zebrafish retina cDNA library, six independent full-length clones were obtained that all coded for an identical β-thymosin (Fig. 1A). In other species, β-thymosin isoforms have been identified that range in size between 39 and

A

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  1  AGCACACAGCCTGACACTTTCTCTTTCAGCACTTCAGCCCATTCACTGCTTTACGCTCAA   60

 61  AAAAACATGGCCGACAAACCCAACATGACTGAAATTACATCCTTTGATAAAACTAAACTC   120
           M  A  D  K  P  N  M  T  E  I  T  S  F  D  K  T  K  L

121  AGAAAGACAGAGACCCAAGAAAAGAACCCTTTGCCAACCAAAGAAACTATTGAACAGGAG   180
     R  K  T  E  T  Q  E  K  N  P  L  P  T  K  E  T  I  E  Q  E

181  AGACAAGGAGAGTCCACACCGTGAATGACGTCTGTAACACAGCAGAAAGTGAAGACCAGA   240
     R  Q  G  E  S  T  P  *

241  GACTTCAGACGAACTCAAGCACTCACTAATGTGTCTTTTTTTATAACTTAGATTGTCCGT   300

301  ATAAGAACTAAATCAAATGTGAGGAATGTATTTCACCCTGTGAGTTCAGAAAACCAAATA   360

361  AAATGCACCTCATTTTTGACACG  383
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B

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zebrafish β-thymosin:              MADKPNMTEITSFDKTKLRKTETQEKNPLPTKETIEQERQGESTP
rainbow trout thymosin β-11:    ACSDKPNLEEVASFDKTKLKKTETQEKNPLPTKETIEQEKQAS
Homo sapiens thymosin β-10:        MADKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS
Rat thymosin β-10:                 MADKPDMGEIASFDKAKLKKTETQEKNTLPTKETIEQEKRSEIS
Rat thymosin β-4:                  MSDKPDMVEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES
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C

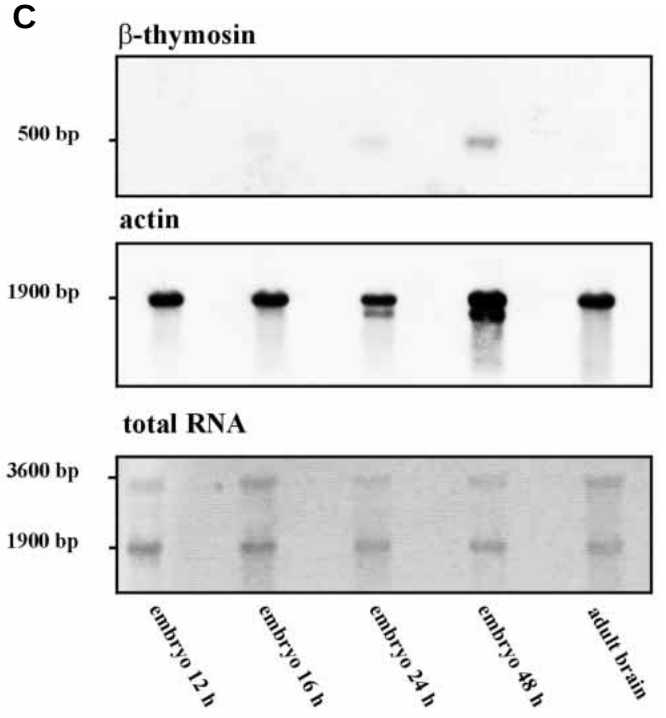

Fig. 1. Molecular characterization and expression analysis of zebrafish β-thymosin. (A) Nucleotide sequence of the full-length zebrafish cDNA. (B) Comparison of the zebrafish β-thymosin with different vertebrate isoforms. The boxed amino acids are identical in all isoforms. (C) Northern blot analysis of β-thymosin and actin mRNA expression during development and in the adult. The RNA analyzed was isolated from zebrafish embryos at different developmental stages (12, 16, 24, 48 h) and adult brain. Per lane, 4 μg total RNA was loaded and visualized by methylene blue staining after the transfer to control for equal loading (bottom panel). A single band of approximately 500 bp is seen after hybridization with a probe to β-thymosin (top panel). The same blot was hybridized with a probe to actin (middle panel). Cytoplasmic actin mRNA reveals a band at 1,900 bp and is expressed constitutively. A smaller, muscle-specific isoform arises at 24 h, when myoblasts start to differentiate.

45 amino acids (Nachmias, 1993). The zebrafish β-thymosin we cloned shows identities of 70-74% at the nucleic acid level to the rat and human β_4 and β_{10} isoforms (not shown) and at the amino acid level 82% and 78% identities to the trout thymosin β_{11} and human thymosin $\beta_{10,}$ respectively (Fig. 1B). Genomic Southern blot analysis reveals a single band (not shown). This indicates that there is only one β-thymosin isoform in zebrafish.

High levels of β-thymosin mRNA are present during zebrafish development at times of extensive neurite outgrowth, as shown by northern blot analysis (Fig. 1C). A single β-thymosin mRNA-positive band was obtained, confirming the genomic Southern blot analysis. In the adult brain, its mRNA concentration declines to undetectable levels. The same northern blot was incubated with a probe to actin. (Fig. 1C; actin). Densitometric analysis revealed that during development, increased β-thymosin mRNA levels are paralleled by increasing mRNA levels for actin (not shown) whereas in the adult, only actin is expressed at detectable levels. The actin probe used consists of 420 bp, located within the coding region and therefore recognizes different actin mRNA species. The band at approximately 1,900 nucleotides derives from cytoskeletal actin mRNA that is present in all tissues tested. An additional lower band that reflects the onset

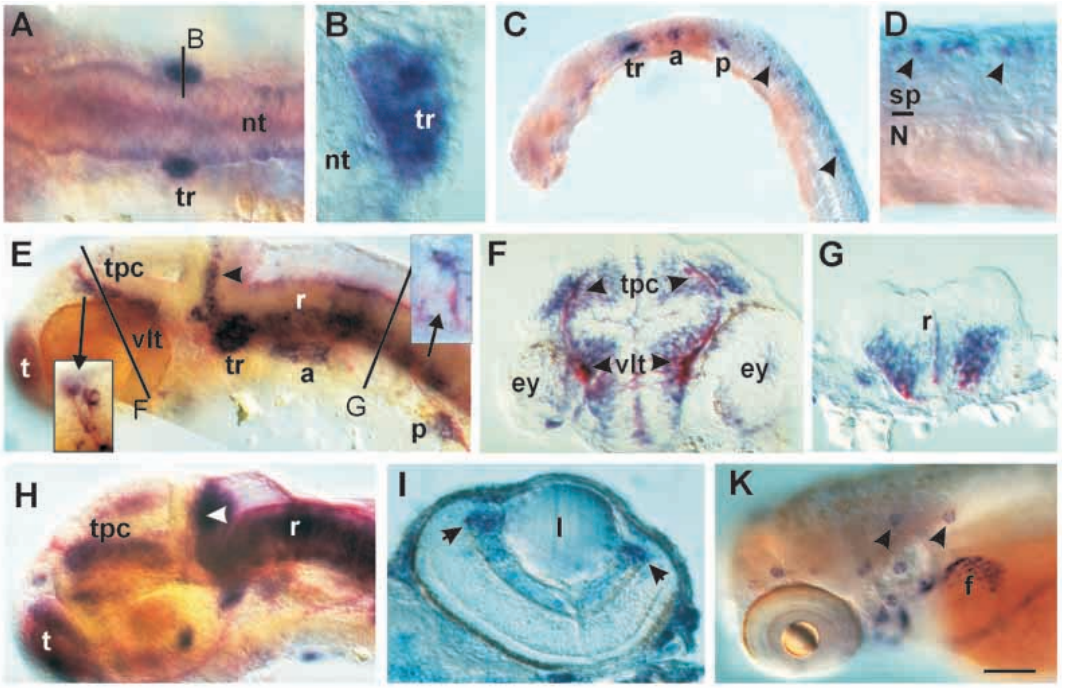

Fig. 2. In situ hybridization analysis of β-thymosin expression in 12 h to 5 day zebrafish embryos. Axonal tracts are visualized in E, F, G, and H (in red), using an antibody to acetylated tubulin. (A) The first distinct signal appears in the presumptive trigeminal ganglion (tr), laterally to the neural tube (nt) at about 12 h. The vertical line indicates the plane of section shown in B. (B) A cross section reveals that the positive structures are located outside the neural tube. (C) At around 17 h, the trigeminal, anterior (a) and posterior (p) lateral line ganglia and individual cells in the hindbrain and spinal cord (arrowheads) express β-thymosin. (D) Positive cells in the spinal cord (sp), above the notochord (N), represent Rohon Beard neurons. (E) In 24 h embryos, prominent staining is seen in the telencephalon (t), along the tract of the posterior commissure (tpc), in the hindbrain at the midbrain-hindbrain boundary (arrowhead), in rhombomeres (r) and the three sensory ganglia. Individual neurons with acetylated-tubulin-positive axons express β-thymosin (insets). The black lines indicate the planes of sections shown in F and G. (F) Coronal section through the midbrain, at the level of the eyes (ey) of a 24 h embryo. β-Thymosin mRNA is found in ventral midbrain cells surrounding the ventral longitudinal tract (vlt) and along the tract of the posterior commissure. (G) Coronal section through a rhombomere of a 24 h embryo. Ventromedial and ventrolateral cells, presumably reticulospinal neurons, express β-thymosin. (H) At 48 h, the tract of the posterior commissure and the telencephalon are still positive. Expression is stronger in the hindbrain, including the midbrain-hindbrain boundary (arrowhead) and the rhombomeres. (I) Transverse section through the eye at 60 h. Axon-extending retinal ganglion cells and possibly mitotically active neuroepithelial progenitor cells at the retinal margin (arrows, encompassing the lens (l), express β-thymosin. (K) In 5 day embryos, the neuromasts (arrowheads) and cells of mesodermal origin in the fin (f) are the only structures left that express β-thymosin. The size bar corresponds to, 15 μm (B); 35 μm (I,F,G); 80 μm (A,E,H); 120 μm (K,C) and 50 μm (D).

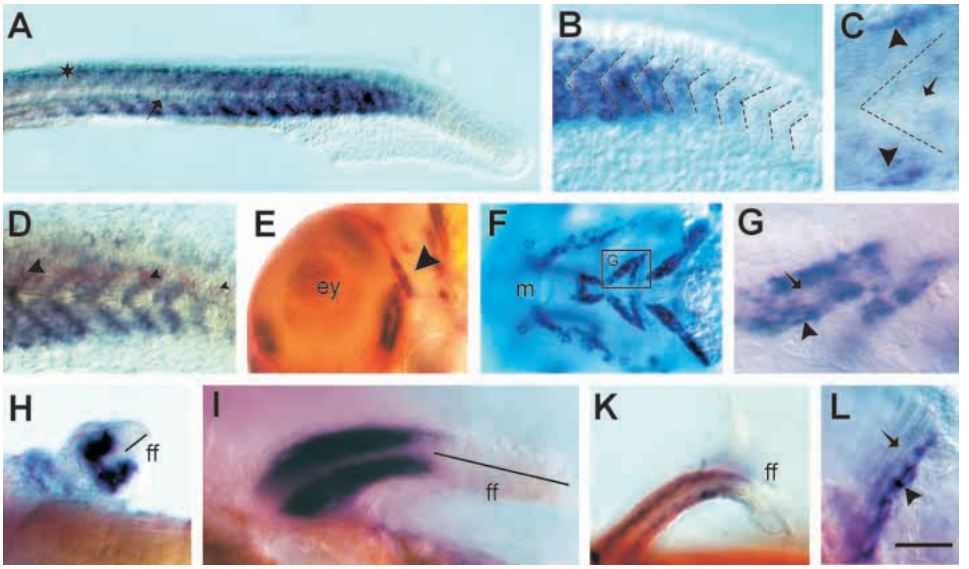

Fig. 3. β-Thymosin is transiently expressed during skeletal muscle development. (A,B) β-Thymosin mRNA is first detected in the medial posterior compartment of individual, pinched off somites. Expression then spreads dorsally, ventrally and anteriorly, to occupy the entire somite. Transcripts disappear in a wave from anterior to posterior, being absent in the horizontal myoseptum (arrow). The star indicates expression in Rohon Beard neurons. (C) The horizontal myoseptum (arrow) and birefringent muscle fibers are β-thymosin negative. Arrowheads point to β-thymosin positive cells that are not fully extended and have large nuclei. (D) Muscle cells express β-thymosin during innervation. Axon-extending motoneurons (arrowheads), labeled with an antibody to acetylated tubulin are shown in red. Embryos in A-D are approximately 22 h. (E) Oculomotor muscles express β-thymosin during the time of differentiation and innervation (arrowhead), at 60 h. (F) At 3 days, muscles in the region of the jaw (ventral view; mouth, m) express β-thymosin. (G) As in somites, only muscle cells with large nuclei (arrowhead) are positive. Birefringent muscle (arrow) is negative. (H-I) Two mesodermal sheets in pectoral fin express β-thymosin between 36 h and 60 h. The fin fold (ff) is negative. (K-L) Expression of β-thymosin in differentiating myoblasts (arrowhead) starts to decrease in 2- to 3-day embryos and is almost absent in 5-day larvae (comp. Fig. 2K), when most of the muscle fibers display birefringence (arrow). The size bar represents, 10 μm (G,L); 30 μm (L,H,I); 50 μm (K); 60 μm (B); 80 μm (E); 90 μm (D) and 120 μm (A,F).

of muscle-specific actin expression appears at 24 h (Mohun et al., 1984).

β-Thymosin is expressed by growing neurons and glial cells that delineate neuronal pathways

We further analyzed, by in situ hybridization, which cells express β-thymosin during development. The first prominent sites of expression are seen in 12 h embryos (Fig. 2A,B). The location of the positive, bilateral structures indicates that the trigeminal placodes express β-thymosin. At approximately 17 h, the trigeminal ganglion neurons and the Rohon Beard neurons in the caudal hindbrain and spinal cord have growing axons and are β-thymosin mRNA positive (Fig. 2C,D). Furthermore, the anterior and posterior lateral ganglion primordia start to express β-thymosin at this time (Fig. 2C). Continuous expression is also seen in the primordium during migration along the midbody line (not shown). Towards the end of the first day, many more cells of neuroepithelial origin start to express β-thymosin. At 24 h, accumulation of β-thymosin mRNA is seen in selected areas of the brain, such as the rhombomeres, the region of the midbrain-hindbrain boundary (MHB) in the hindbrain, the ventral longitudinal tract and the tracts to the posterior commissure and the postoptic commissure, including the postoptic commissure itself (Fig. 2E). To visualize the association of β-thymosin expression with axon-extending neurons, double labeling was performed using antibodies to acetylated tubulin (Fig. 2E-H). This shows that β-thymosin mRNA (purple reaction product) is found in

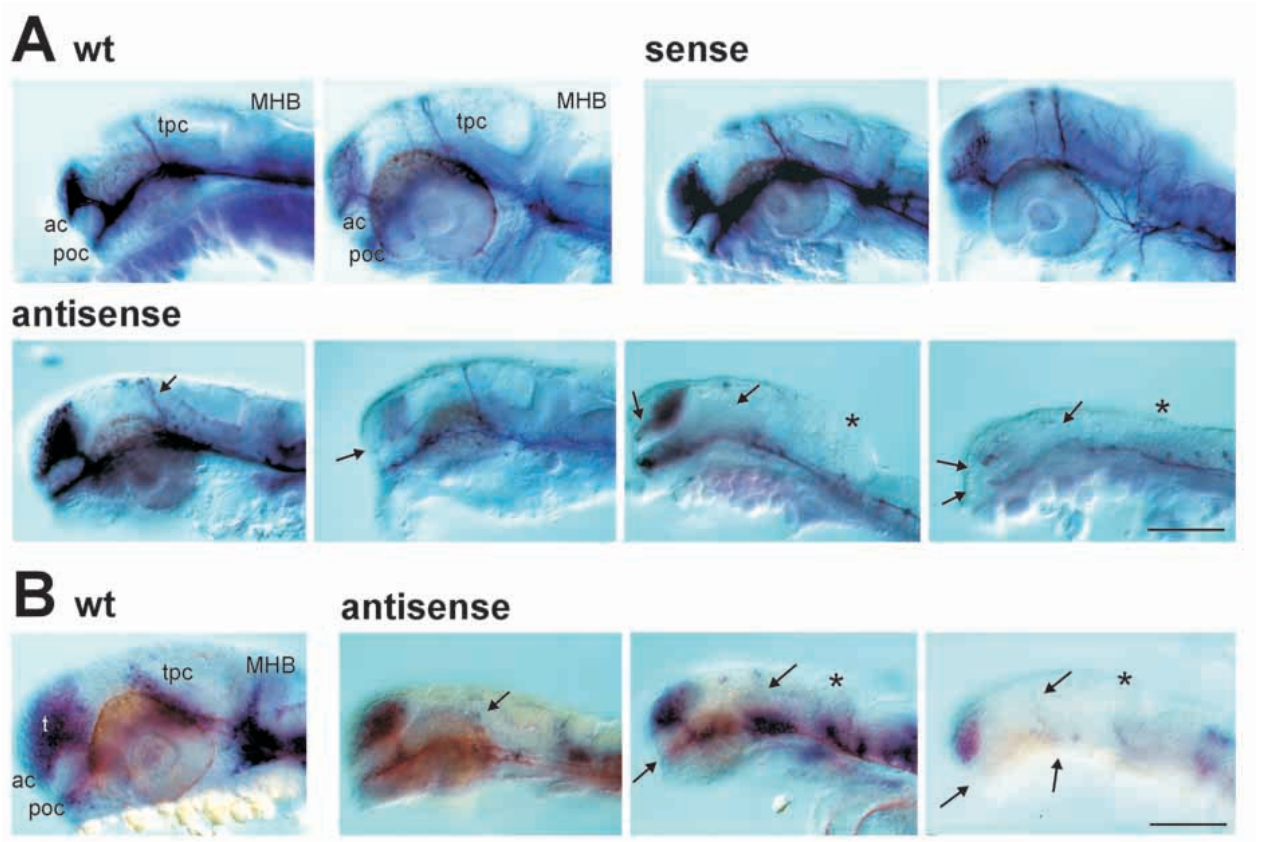

Fig. 4. Effects of β-thymosin sense and antisense injections on zebrafish brain development. Injected embryos were analyzed at 24 h. Axonal tracts were visualized with an antibody to acetylated tubulin (A, in blue; B, in red). (A) No obvious difference in acetylated tubulin-positive tracts and commissures is seen between wild-type (wt) and β-thymosin sense RNA injected embryos. In contrast, injection of antisense RNA leads to a slight delay of development and specific brain defects at various degrees. Strongly reduced or absent acetylated tubulin-positive tracts and commissures are indicated by arrows. Deformed or missing midbrain-hindbrain boundaries are marked by asterisks. (B) β-Thymosin antisense RNA injected embryos were further analyzed by in situ hybridization using a probe to β-thymosin (purple reaction product). Many embryos show reduced hybridization. Places of decreased β-thymosin mRNA concentrations correlate with places of reduced or absent acetylated tubulin-positive tracts (arrows). Embryos that have no morphologically distinct midbrain-hindbrain boundary (asterisk) are β-thymosin mRNA negative in this region. Abbreviations: ac, anterior commissure; MHB, midbrain-hindbrain boundary; poc, postoptic commissure; t, telencephalon; tpc, tract of the posterior commissure. The size bars 130 μm (A,B). (C) Northern blot analysis of injected and control embryos. Total RNA isolated from an identical number of embryos was loaded in antisense-injected and control lanes. Antisense injection leads to a reduction of endogenous β-thymosin mRNA levels at 24 h, but not at 36 h. The upper band in lanes of RNA isolated from antisense-injected embryos probably represents injected antisense RNA.

neurons that extend acetylated tubulin-positive processes (red reaction product; insets in Fig. 2E). β-Thymosin expression is not restricted to neurons but is also found in glial cells, e.g. along the tract to the posterior commissure and the ventral longitudinal tract (Fig. 2F). At 48 h, expression in the hindbrain becomes very prominent (Fig. 2G,H). At around 60 h, growing retinal ganglion cells in the retina express β-thymosin (Fig. 2I). The strongest expression is seen at the retinal margin, where neurons start to differentiate and extend axons. Possibly, the mitotically active neuroepithelial progenitor cells in the germinal zone also express β-thymosin. At 5 days, the neuromasts and muscle cells in the fin are the only sites of β-thymosin expression (Fig. 2K). Neuromasts constitute the volcano-shaped lateral line sensory organ. They are located in characteristic positions within the skin epithelium and contain hair cells and their support elements.

Developing muscle expresses β-thymosin

As in other vertebrates, formation of skeletal muscle in zebrafish involves commitment of mesodermal cells into the myogenic pathway and subsequently differentiation of the skeletal myoblasts into fully differentiated myotubes, resulting in multinucleated muscle fibers. Somites appear sequentially from anterior to posterior in the trunk and tail. β-Thymosin expression is seen in trunk and tail mesoderm between 19 h and 36 h, after the time when the majority of segments have been formed (Fig. 3A-D). β-Thymosin mRNA is first detected in the medial posterior compartment of pinched off somites that exhibit the characteristic chevron shape (Fig. 3B). As a somite matures, the territory of β-thymosin expression spreads first dorsally and ventrally and eventually anteriorly, to occupy the entire somite (Fig. 3B,D). Similar to its appearance, β-thymosin mRNA disappears from paraxial muscle in a wave, from anterior to posterior (Fig. 3A). Within a single segment, labeling decreases from medial to peripheral (Fig. 3A,C). The horizontal myoseptum, elongated muscle pioneers in that region (Felsenfeld et al., 1991) and fully differentiated muscle cells showing birefringence due to cross-striations, are never β-thymosin mRNA positive (Fig. 3A,B). Expression is restricted to muscle cells that are not fully extended, have large nuclei within their cytoplasm and lack evident sarcomeres (Fig. 3C). Fig. 3D shows axon-extending motoneurons labeled with an antibody to acetylated tubulin in red. Segments showing overall β-thymosin expression in the myotome contain motoneurons that have started to send processes into the periphery. In contrast, motoneurons located in somites that are β-thymosin negative have not yet extended long axons (Fig. 3D). Thus, the time of β-thymosin expression in the trunk and tail correlates with maturation of somitic muscle and with innervation by primary and secondary motoneurons (Felsenfeld et al., 1991; Myers et al., 1986). The same paradigm is found in other skeletal muscles, such as the oculomotor muscles (Fig. 3E) and the musculature of the jaw, formed from the first and second pharyngeal arches (Fig. 3F,G). No hybridization is seen in the mesoderm around the branchial arches that will form the gills (not shown). The zebrafish pectoral fin buds appear at around 28 h and are formed from protruding mesodermal cells surrounded by an ectodermal layer (Kimmel et al., 1995). β-Thymosin expression can first be seen in the two mesodermal sheets at around 36 h (Fig. 3H) and is maintained at a high level until around 60 h (Fig. 3I). For comparison, muscle differentiation markers, such as the myoD family (de la Brousse and Emerson, 1990), Pax-3 (Williams and Ordahl, 1994) and snail1 (Thisse et al., 1993) are found in the same tissue. Expression of β-thymosin is almost absent in pectoral fins of larvae at 5 days, when most of the muscle fibers display birefringence (Fig. 3K,L). As in axial muscle, β-thymosin mRNA is not found in birefringent fibers in the fin (Fig. 3L). Immunohistochemical staining with anti-acetylated tubulin antibody reveals innervation of the fin at this stage (not shown). No expression is seen in the caudal fin at any stage of development (not shown).

Perturbation of endogenous β-thymosin expression

To study the function of β-thymosin during development of the zebrafish nervous system, we injected β-thymosin sense and antisense RNA into one-cell zebrafish embryos. Injected embryos were allowed to develop and were compared to their uninjected siblings. Embryos that died prior to the onset of neurulation were excluded from further analysis. In total, we analyzed 180 embryos injected with sense transcripts and 222 embryos injected with antisense transcripts for brain defects. Axonal tracts were visualized by anti-acetylated tubulin staining. Subsequently, embryos were examined for morphological abnormalities. Ectopic expression of β-thymosin, by injection of sense transcripts, does not disturb normal development. No severe, quantifiable changes are observed (Fig. 4A, sense; Table 1). A few embryos show more pronounced acetylated tubulin staining than control embryos and in some cases, increased branching of tracts, e.g. the tract to the posterior commissure, which is never seen in control embryos or embryos injected with antisense RNA (not shown). In contrast, injection of β-thymosin antisense RNA results in clear embryonic defects. Only 50% of the antisense injected embryos show a normal phenotype, compared to 85% in sense experiments (Table 1). The percentage of cripples is similar in both groups and may be attributed to injection artefacts. Up to 55% of antisense-injected embryos show morphological defects in the brain at 24 h (Table 1). The predominant phenotype is a reduction or loss of individual tracts and commissures, such as the anterior commissure, the post optic

Table 1. Effect of β-thymosin sense and antisense mRNA injections on zebrafish brain development

	RNA injected								
	Sense				Antisense				
approx. stage	24h	24h	33h	33h	24h	24h	24h	24h	33h
number	62	44	54	20	31	45	38	65	43
normal (%)	87.1	86.4	79.6	70	48.4	44.4	50	41.5	67.4
cripples (%)	9.7	13.6	14.8	30	22.6	28.9	13.2	3.1	18.6
brain defects (%)	3.2	0	5.6	0	29	26.7	36.8	55.4	14

commissure or tract to the posterior commissure (Fig. 4A,B, antisense). In addition, the MHB is often distorted or absent. Fig. 4A shows varying degrees of defects in response to antisense injections. In the most severe cases, axon tracts in the midbrain and forebrain, but not in the rhombomeres and spinal cord are completely lost and no distinct MHB is visible. In general, injection of β-thymosin antisense RNA seems to slightly retard development, without affecting the survival rate. However, since the first axon tracts appear at 17 h and all major tracts are present at 22 h (Chitnis and Kuwada, 1990), the observed phenotypes cannot be attributed to the general retardation of development. In order to further test the specificity of the antisense-associated brain defects, we performed in situ hybridization analysis using the β-thymosin antisense probe on injected embryos. A high percentage of embryos show reduced hybridization signals for endogenous β-thymosin (Fig. 4B, in blue). The place and degree of reduced labeling vary but correlate with the observed defects. Virtually all embryos exhibiting strongly reduced or absent labeling in places like the MHB, the tract to the posterior commissure or the postoptic commissure, show defects in axonal tract formation and development of the MHB. Northern blot analysis confirms these results. Antisense-injected embryos show decreased endogenous β-thymosin mRNA levels at 24 h (Fig. 4C). Densitometric analysis reveals a reduction of at least 30% (not shown). In addition to the band representing endogenous β-thymosin mRNA, a second higher band that corresponds in size to the injected antisense RNA appears in lanes of RNA isolated from injected embryos (Fig. 4C). Antisense RNA contains flanking sequences of the pCS2 vector that are similar to the flanking regions present in the mRNA probe. This may lead to hybridization of the probe to the injected RNA. Interestingly, no widespread labeling, resulting from hybridization to injected RNA is observed by in situ hybridization analysis. Possibly, the conditions used for in situ hybridization are more stringent than for northern blot analysis. Alternatively, the still intact antisense RNA is concentrated in the yolk. Accordingly, Barth and Wilson (1995) reported that most of the injected transcripts are degraded in the embryo proper during the first day of development. At 36 h, no difference in the amount of endogenous β-thymosin transcript is detected between injected and control embryos. In addition, the ratio of the amount of RNA in the upper band to the lower band is reduced at 36 h, compared to 24 h. Two thirds of the antisense-injected embryos appear normal at 33 h (Table 1). At this time, endogenous β-thymosin expression is high (Fig. 1C) and may allow recovery from the antisense-specific defects seen at 24 h.

A few antisense-injected embryos show defects in segmentation that are not seen in sense-injected embryos of the same batch (Fig. 5). Normal embryos show regular segmentation at 24 h with β-thymosin expression being restricted to the posterior part of the younger somites (Fig. 5A). The antisense effects observed are irregularities in the shapes of somites to varying degrees (Fig. 5B-D). β-Thymosin mRNA is present in these somites. It is much less reduced than in brain regions showing morphological defects (compare Fig. 4B). Possibly, mRNA levels are reduced in individual muscle cells that are located more interiorly in deformed segments. Alternatively, high endogenous β-thymosin levels seen at 24 h can not rescue the phenotype caused by earlier antisense action.

Influence of β-thymosin antisense injection on Engrailed and Isl-1 expression

To understand the β-thymosin antisense effects in more detail, we examined expression of the marker genes *engrailed* and *Isl-1* in injected embryos. Engrailed is found at the MHB, starting from the 1-2 somite stage throughout adulthood (Hatta et al., 1991), while β-thymosin is transcribed there during a short period only (Fig. 2E,H). Engrailed expression is not drastically influenced by β-thymosin antisense RNA injections (Fig. 6A). Embryos that have a distorted or missing MHB as a result of antisense injection exhibit engrailed expression at the appropriate location. The stripe appears somewhat weaker and less defined in some embryos (Fig. 6A), an effect that may be due to the morphological distortion.

Isl-1, a member of the LIM homeodomain family, is an early marker of pioneering neurons, such as Rohon-Beard cells and cranial ganglia (Korzh et al., 1993) that are the first β-thymosin positive cells in the embryo (Fig. 2A-D). Isl-1 is present in these neurons prior to axogenesis and prior to β-thymosin expression. β-Thymosin antisense injections result in a decrease of Isl-1 labeling in a subset of cells in the trigeminal ganglion and in Rohon Beard neurons in the hindbrain and in the spinal cord (Fig. 6B-D). Analysis by Nomarski interference contrast optics shows that normally Isl-1 positive cells are still present (Fig. 6B,C). Therefore, the reduction of labeling is not based on a loss of trigeminal ganglion or Rohon Beard neurons, but rather on a decreased Isl-1 expression level in these cells. This indicates that β-thymosin may indirectly be involved in the maintenance of Isl-1 expression and supports the observation that embryos recover from antisense injections at later stages.

DISCUSSION

Neuronal and non-neuronal expression of β-thymosin correlates with axonal growth

Expression of the actin-monomer binding protein β-thymosin in zebrafish embryos is tightly correlated with nervous system development. In all places examined except the MHB, β-thymosin mRNA is associated with axon-extending neurons, visualized by acetylated tubulin staining (Figs 2, 3). β-Thymosin is expressed by a subset of neurons and in glia delineating axonal pathways, such as the ventral longitudinal tract and the tracts to the postoptic and posterior commissures. β-Thymosin is not expressed along all tracts in the embryo. Neither the supraoptic tract nor the dorsoventral diencephalic tract to the epiphysis, including the epiphysis itself are β-thymosin positive. Neuronal expression is conclusive in the retina, the posterior commissure, the telencephalon and in Rohon Beard neurons in the trunk and tail (Fig. 3). Similar to the transient glial expression, β-thymosin is expressed in muscle during the time of innervation (Fig. 3).

The presence of a protein in growing neurons and in non-neuronal cells along their trajectory has been reported for the membrane protein commissureless in *Drosophila* (Tear et al., 1996) and for recognition molecules in vertebrates (Martini, 1994; Bernhardt et al., 1996). This dual expression is thought

to contribute to appropriate glia-axon interactions that are essential for the establishment of the nervous system and have been shown to influence gene expression in both cell types (Wu et al., 1994). In what way the presence of β-thymosin in growing neurons and their surrounding glia may contribute to cell-cell communication remains to be elucidated.

β-Thymosin is required for normal development of axonal tracts in the brain

To investigate the role of β-thymosin during development of the zebrafish brain, we used an antisense 'knock-out' approach. Injection of β-thymosin antisense transcripts into one-cell embryos led to severe defects or complete lack of normally β-thymosin-positive tracts and distorted MHBs (Fig. 4). These defects are associated with a reduction of endogenous β-thymosin mRNA levels, as shown by in situ hybridization and northern blot analysis. The most common and severe defects are seen in the fore- and midbrain, where the endogenous β-thymosin expression is high during development of the nervous system. The variability in the severity of the phenotypes is probably due to varying amounts of injected RNA and its mosaic distribution in the embryo (Alexandre et al., 1996). The fact that antisense-injected embryos analyzed at 33 h show normal β-thymosin mRNA levels and a higher percentage of normal embryos, compared to analysis at 24 h (Fig. 4, Table 1), supports the specificity of the effect. Likely, strong endogenous β-thymosin expression allows recovery from defects to a certain degree. Furthermore, neither sense nor unrelated control RNA injections ever lead to comparable phenotypes.

Analysis of the antisense-injected embryos by early marker proteins revealed that the brain defects are not due to a loss of cells. Engrailed- and normally Isl-1-positive cells are still present, but do not express the marker proteins (in particular Isl-1) at normal levels (Fig. 6). Therefore, β-thymosin may indirectly influence gene expression. Golla et al. (1997) showed that β-thymosin overexpressing fibroblasts coordinately increase several cytoskeletal and adhesion plaque proteins and thereby restore the balance between monomeric and filamentous actin. This effect may be particularly important in vivo and may explain why no significant effects were detected in zebrafish embryos overexpressing β-thymosin (Fig. 4). The actin cytoskeleton has been shown to influence gene transcription. Schwann cells in culture did not express myelin-specific genes after depolymerization of filamentous actin by cytochalasin

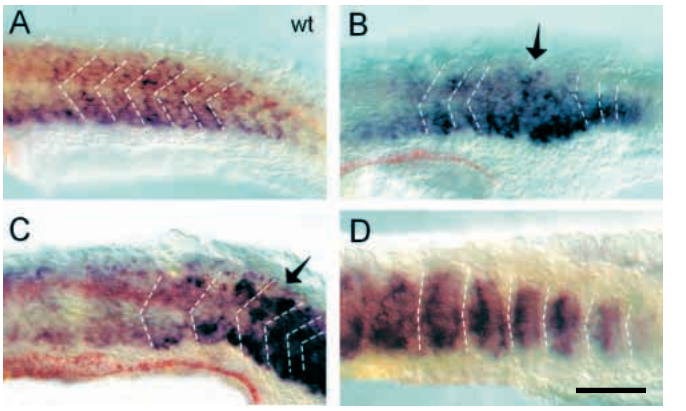

Fig. 5. Effect of β-thymosin antisense injections on myotome development. Embryos were analyzed at 24 h. (A) Control embryos show the typical chevron shaped segments, transiently expressing β-thymosin. (B-D) Antisense injection can lead to segmentation defects at various degrees. The shape of individual (arrows) or adjacent segments is distorted. Irregularities are accompanied by abnormal β-thymosin expression. The size bar corresponds to 80 μm.

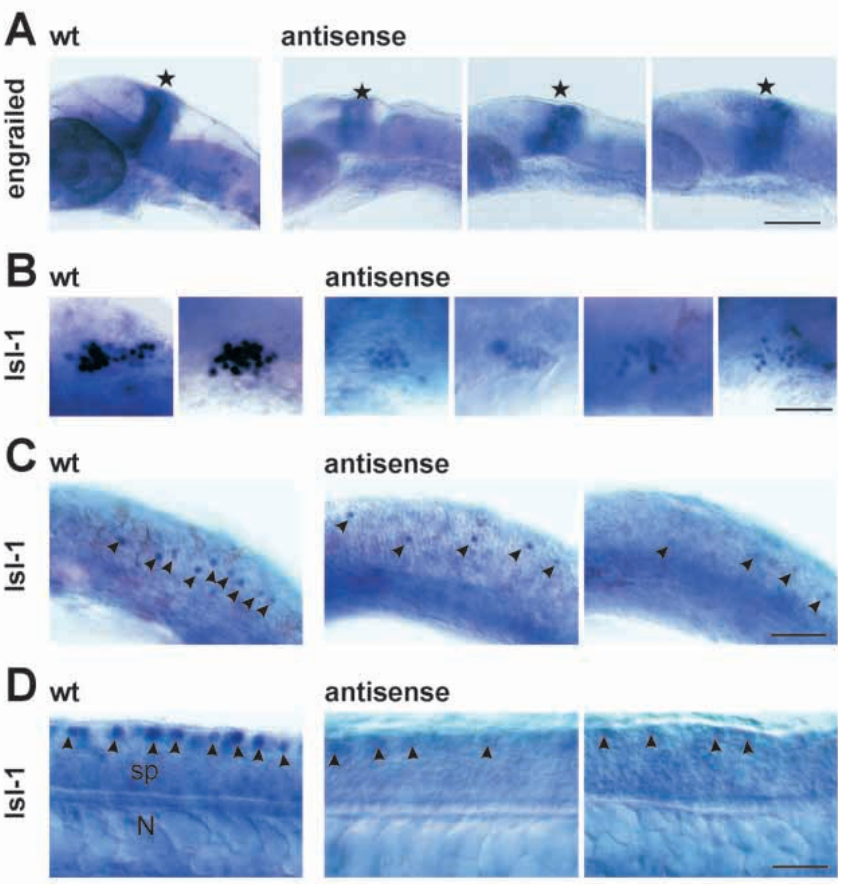

Fig. 6. Effect of β-thymosin antisense injections on engrailed and Isl-1 expression in 24 h embryos. (A) Engrailed is found at the MHB (asterisk) in wild-type embryos. Embryos that exhibit a distorted or missing MHB as a result of antisense injection show engrailed expression at the appropriate location (asterisk) though the stripe appears somewhat weaker and less defined. (B-D) Isl-1 is strongly expressed in trigeminal ganglion and Rohon Beard neurons (arrowheads) in wild-type embryos. Injected embryos show reduced Isl-1 expression in individual cells in the trigeminal ganglion (B), Rohon Beard neurons in the hindbrain (C) and in the spinal cord (D). Analysis by Nomarski interference contrast optics shows that normally Isl-1-positive cells are still present (antisense; arrowheads). The size bar corresponds to, 115 μm (A), 50 μm (B), 65 μm (C) and 25 μm (D).

(Fernandez-Valle et al., 1997). This may be explained by actin being part of a signal transduction pathway, connecting basal lamina via integrins to gene expression.

β-Thymosin is expressed in developing muscle

In zebrafish, somites form as epithelial spheres from the mesoderm in an anterior to posterior direction. Myoblasts then cease dividing, elongate and fuse into myotubes. β-Thymosin is transiently expressed in myoblasts during this differentiation process (Fig. 3). Expression starts in myoblasts located in the medial posterior part of pinched off somites and then spreads dorsally, ventrally and anteriorly to occupy the entire somite. Striated, birefringent muscle is always β-thymosin mRNA negative.

According to its expression in differentiating myotome, a subset of β-thymosin antisense-injected embryos show segmentation defects. Most obvious is a loss of the typical chevron shape of individual segments (Fig. 5). A likely cause is improper differentiation or organization of single, antisense containing myofibrils that results in a loss of regularity in segmentation. Several zebrafish mutants have been described that show defects in somite patterning (Felsenfeld et al., 1991; Granato et al., 1996; van Eeden et al., 1996). Interestingly, several of these mutants show a general defect in skeletal muscle, including the extrinsic ocular muscles, jaw and pectoral fin muscles that transiently express β-thymosin during development.

β-Thymosin and the modulation of the actin cytoskeleton

Actin rearrangements at specific locations within cells is necessary for a multitude of developmental processes. The de- and repolymerization of actin filaments is controlled by specific actin-binding proteins. Several studies showed that β-thymosin can influence the polymerization state of actin in vitro (Safer and Nachmias, 1994; Sun et al., 1995). It evokes a dose-dependent decrease in intensity of rhodamine staining of stress fibers and of actin filaments in non-neuronal cells (Hall, 1995; Muallem et al., 1995). The ability of β-thymosin to hinder actin assembly appears to depend on its concentration and the concentration of other monomeric actin-binding proteins in the cytoplasm.

Complex and rapid actin rearrangements in filopodia underlie the directionality of growth cone advance (Chien et al., 1993; Bentley and O'Connor, 1994). Possibly, local changes of β-thymosin concentrations in extending neurites result in confined actin rearrangements that are necessary for appropriate pathfinding. The actin cytoskeleton has been shown to regulate additional processes, including the distribution of integral membrane proteins (Mays et al., 1994), membrane and nuclear ion channel activation (Cantiello et al., 1991; Prat and Cantiello, 1996), exocytosis and endocytosis (Gottlieb et al., 1993; Lamaze et al., 1997). β-Thymosin has been shown to directly regulate receptor-mediated endocytosis (Lamaze et al., 1997). Interestingly, suppression of molecules involved in endocytosis has a potent inhibitory effect on neurite outgrowth and induces growth cone collapse (Mundigl et al., 1998). The L1 subfamily of cell adhesion molecules whose expression is correlated with neuronal growth has recently been reported to be endocytosed, preferentially at the rear of axonal growth cones (Kamiguchi et al., 1998).

The tightly regulated β-thymosin expression in myoblasts suggests a role in the rearrangement of the actin cytoskeleton prior to myofibril formation. Myofibril assembly occurs in conjunction with preexisting actin filament bundles (Dlugosz et al., 1984). For example, the zebrafish mutant *fub-1* displays disorganized myofibrils in all skeletal muscle cells, a phenotype related to the absence of ordered actin filament bundles (Felsenfeld et al., 1991).

A β-thymosin null mutant would permit the examination, in more detail, of the possible functions of β-thymosin during embryonic development. However, our data clearly indicate that β-thymosin is crucially involved in neural development and myogenesis.

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