Induction of α1-Tubulin Gene Expression during Development and Regeneration of the Fish Central Nervous System

Virginia Hieber, Xinhua Dai, Mark Foreman, Daniel Goldman

Mental Health Research Institute and Department of Biological Chemistry, 205 Zina Pitcher Place, University of Michigan, Ann Arbor, Michigan 48109

Received 28 April 1998; accepted 18 June 1998

ABSTRACT: The α1- and α2-tubulin encoding genes were cloned from a goldfish genomic DNA library. α1- and α2-tubulin RNA expression was examined in developing and adult retinas. These studies demonstrated increased α1-tubulin RNA in presumptive ganglion cells that grow axons early in retinal development and in adult retinal ganglion cells whose optic axons had been damaged. The α2-tubulin RNA was undetectable in developing retina and constitutively expressed in adult retinal ganglion cells regardless of optic nerve crush. To determine if these changes in α1-tubulin RNA reflected changes in α1-tubulin promoter activity, we introduced into zebrafish embryos and adult goldfish retinal explants expression vectors harboring the α1-tubulin gene’s promoter. These studies showed that the α1-tubulin promoter confers a developmentally regulated, neuron-restricted pattern of reporter gene expression in vivo and its activity is increased in adult retinal neurons induced to regenerate their axons. Promoter deletions defined regions of α1-tubulin DNA necessary for this pattern of expression. These results suggest that DNA sequences necessary for α1-tubulin gene induction during central nervous system development and regeneration are contained within the α1-tubulin gene’s 5’-flanking DNA and that this promoter will be useful for identifying these elements and their DNA binding proteins.

Keywords: CNS development; tubulin; gene expression; regeneration; zebrafish

The elaboration of an axon during nervous system development allows cells that are separated by relatively long distances to communicate with each other. In the adult, axon regrowth is a prerequisite to the reestablishment of appropriate connections and return of function following nerve damage. Although axon growth is necessary for establishing connections between cells during development and following nerve damage, very little is known about the molecular mechanisms mediating this process.

One approach to identifying these mechanisms is to identify proteins specifically necessary for axonogenesis. Some of these proteins would presumably be induced at times when the cell needs to generate an axon. Such proteins could then serve as probes for the mechanisms mediating their regulated expression, which would also contribute to successful growth of an axon.

One such protein is that encoded by the α1-tubulin gene. Tubulins are required for the formation of the cytoskeleton that accompanies axonal growth. Of all the α-tubulins, the α1-isof orm is specifically induced during development and regeneration of axons (Lewis et al., 1985; Miller et al., 1987, 1989; Skene, 1989; Gloster et al., 1994). Therefore, α1-tubulin expression may be a good probe for characterizing some of the mechanisms mediating axonal growth. Indeed, the creation of transgenic mice harboring the rat α1-tubulin (Tα1) promoter driving lacZ expression iden-
tified a 1.1-kb piece of Tα1 5′-flanking DNA that directed transgene expression to developing and regenerating neurons (Gloster et al., 1994).

We chose to examine α1-tubulin gene expression in zebrafish and goldfish because they offer several advantages over mammalian systems for studying axon growth during development and regeneration. First, unlike mammals, fish are able to mount a robust regenerative response following damage to their central nervous system (CNS). This is clearly demonstrated by the use of the goldfish visual pathway to successfully identify proteins that are induced during optic nerve regeneration and may participate in the successful reestablishment of retinotectal connections (Grafstein, 1991; Murray and Grafstein, 1969; Heacock and Agranoff, 1982; Stuermer et al., 1992). Second, goldfish retina can be readily explanted into culture and extend neurites in response to an in vivo conditioning optic nerve crush (Landreth and Agranoff, 1976, 1979), facilitating studies of retinal ganglion cell axon regeneration. Third, zebrafish develop rapidly and are relatively transparent at early times of development when the nervous system is forming, facilitating visualization of neural structures (Ross et al., 1992). In addition, zebrafish embryos can be injected with DNA/RNA at the single-cell stage to investigate gene expression and protein function in vivo (Meng et al., 1997). With these advantages in mind, we chose to clone the α1-tubulin gene from goldfish and characterize its expression during nervous system development and regeneration. Our immediate aim is to determine if α1-tubulin gene expression in fish would serve as a suitable probe for unraveling mechanisms mediating neurite outgrowth in the developing and regenerating CNS. Our long-term goal is to use α1-tubulin gene expression as a probe for these mechanisms.

We report here that the α1-tubulin gene is linked to the α2-tubulin gene in the goldfish genome. RNase and in situ hybridization assays showed that α1-tubulin RNA is increased in retinal ganglion cells following optic nerve damage, while the α2-tubulin RNA is constitutively expressed. To determine if these changes in RNA expression are mediated by changes in α1-tubulin promoter activity, we cloned the α1-tubulin promoter and showed it is most active during CNS development and regeneration and that promoter deletions abrogate this activity. These results suggest the α1-tubulin promoter will be a useful probe for characterizing the mechanisms mediating CNS differentiation and regeneration in fish.

**MATERIALS AND METHODS**

**Goldfish Maintenance and Surgery**

Maintenance of goldfish and optic nerve crush were carried out as previously described (Hieber et al., 1992). Briefly, common goldfish (Carassius auratus) of 5–7 cm length were maintained at 25°C in well-aerated tanks. Right optic nerve crush was carried out just behind the orbit in tricaine methane sulfonate (0.1%) anesthetized fish. The left optic nerve of each fish was left intact, with the left retina serving as an unoperated control.

**Isolation of Genomic α1- and α2-Tubulin Clones**

A genomic goldfish library, cloned into the lambda dash vector (Stratagene), was kindly provided by Dr. Nisson Schecter (State University of New York at Stony Brook, Stony Brook, New York). Approximately $0.5 \times 10^6$ clones were screened with a radiolabeled goldfish α-tubulin cDNA (clone 6 in Hieber et al., 1992). Three unique clones were purified. One of these clones, GF6, contained a 14.5-kb insert and was further characterized as reported in this article. DNA sequencing was performed using an Applied Biosystems automated DNA sequencer using thermal cycling procedures recommended by the manufacturer.

**RNase Protection Assays**

Total cellular RNA was isolated from goldfish retinas as previously described (Hieber and Goldman, 1993). RNase protection assays were performed as previously described (Chahine et al., 1993). The α1-tubulin probe is an antisense RNA corresponding to 49 bases of the 3′ end of the α1-tubulin coding sequence and extending into the 3′-untranslated sequence for an additional 251 nucleotides. The α2-tubulin probe is an antisense RNA corresponding to 98 bases of the 3′ end of the α2-tubulin coding sequence and extending into the 3′-untranslated sequence for an additional 245 nucleotides. $^{32}$P-radiolabeled RNA probes were prepared by run-off transcription of linearized vectors. The RNA probe was hybridized with 20 μg of RNA overnight at 55°C and digested with RNase A for 1 h, and the products were analyzed on a denaturing polyacrylamide gel. Probe integrity was confirmed by omitting RNase from the procedure. Controls consisted of using a sense strand probe, which consistently resulted in complete digestion of the probe.

**In Situ Hybridization**

Digoxigenin-labeled probes were prepared according to manufacturer’s protocol (Boehringer Mannheim). The α1- and α2-tubulin probes both contain the last nine nucleotides of coding sequence from exon 4 and extend 3′ into the untranslated region for an additional 341 and 231 nucleo-
tides, respectively. Tissue sections were prepared and hybridized with digoxigenin-labeled probes (approximately 2 μg/ml) as previously described for radioactive probes (Hieber et al., 1992), except diethiothreitol (DTT) was omitted and trNA (10 μg/ml) was included in the hybridization solution. Posthybridization treatments are identical to that previously reported through the RNase step (Hieber et al., 1992). Following RNase digestion slides were immersed in 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8) for 30 min at 55°C and then transferred to a solution of 2× SSC, 1% nonfat dry milk, and 0.05% Triton X-100 and incubated for 2–3 h at 55°C. Slides were then washed in buffer 1 containing 0.3% Triton X-100 and 1% nonfat dry milk and 0.05% Triton X-100 for 30 min at room temperature. Following the antibody incubation, slides were washed in buffer 2 for 2–3 h at 55°C. Slides were then washed in buffer 1 (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl) containing 1% nonfat dry milk and 0.05% Triton X-100 for 30 min at room temperature. A 1/3000 dilution of alkaline phosphatase coupled anti-digoxigenin antibody (Boehringer Mannheim) was prepared in buffer 1 containing 0.3% Triton X-100 and 1% nonfat dry milk. A few drops of this solution were added over sections and coverslipped. Slides were then placed in a humid chamber and incubated for 2–3 h at room temperature. Following the antibody incubation, slides were washed in buffer 1 for 30 min at room temperature (three changes) and then buffer 2 (0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM MgCl₂) for 30 min at room temperature (three changes). Chromogen solution (buffer 2 containing 0.175 mg/mL 5-bromo-4-chloro-3-indolylphosphate and 0.33 mg/mL nitroblue tetrazolium salt) was then applied to the slides and incubated for 2 h to overnight in the dark.

Expression Vectors

The α1-transcriptional start site was identified by an RACE polymerase chain reaction (PCR) protocol using goldfish RNA (Frohman, 1990) followed by cloning and sequencing of the amplified product. α1-Tubulin expression vectors were created by subcloning α1-tubulin 5′-flanking DNA in front of the green fluorescent protein (GFP) reporter of the pEGFP-1 vector (Clontech). The α1TIpEGFP vectors contain various lengths of 5′-flanking DNA (designated by the number at the beginning of the name), exon 1, intron 1, and the first codon of exon 2 (amino acid 2 begins exon 2) fused in frame with the GFP sequence (see Fig. 5). 5′ deletions were created by taking advantage of convenient restriction enzyme sites. pCS2 contains the cytomegalovirus promoter driving GFP expression.

Retinal Explants and Electroporation

The optic nerve emanating from the right retina was crushed in vivo. Seven days later, retinas were removed from dark-adapted goldfish (we generally use 12 fish for each experiment) and placed in sterile phosphate-buffered saline (PBS). At this point, all subsequent procedures were carried out in a laminar flow hood. Retinas were washed with PBS and the peripheral outer third of the retina, containing undifferentiated precursor cells, was cut away and discarded. The remaining retina was cut into small pieces (1–3 mm) with scissors. Retinal pieces were washed four to five times with PBS and resuspended in electroporation buffer (100 mM Hepes [pH 7.4], 137 mM NaCl, 6 mM dextrose, 4 mM Na₂HPO₄). We use 0.4 ml of electroporation buffer for two retinas. Then, 10 μg of α1TIpEGFP DNA and 40 μg of BSSK (Stratagene) DNA was added to the 0.4 ml of retinal pieces in electroporation buffer. A BTX Electro Cell Manipulator 600 was used for electroporation. Settings were: resistance = 86; capacitance = 950 μF; and voltage = 326 V. Immediately following electroporation, the DNA/retinal suspension was transferred to a tissue culture dish containing L-15 media supplemented with 1% FCS. Explants were maintained at room temperature in humidified chambers. Approximately 7 days later, retinal explants were examined using fluorescent microscopy to identify cells expressing the electroporated DNA. Quantitation of expression was performed by counting expressing cells in the explants. We found that electroporation resulted in numerous clumps of cells expressing the reporter gene (see Fig. 7 for an example) and the total number of cells comprising a clump was quite variable (1–100 cells). Regardless of whether we counted the total number of expressing cells or the total number of expressing clumps, we obtained similar results. Therefore, we reported our data as number of expressing cell clumps in right versus left retina.

Zebrafish Injections

Zebrafish embryos were obtained by natural crosses. Solution for DNA injection contained 50 ng/μl α1TIpEGFP in 250 mM KCl and 0.1% Phenol red. Approximately 300 pL of DNA solution was injected into one or two cell embryos with a glass micropipette. At 24–72 h postinjection, animals were anesthetized and examined using fluorescent microscopy. Pictures were taken of either developing fish or fish smashed under a coverslip. Quantitation of GFP expression was performed by counting the number of neural cells expressing GFP divided by the total number of cells expressing GFP in a microscopic field. Neural cells include developing neurons and mature neurons and were defined as cells extending axons >2 cell diameters. Three different fields were counted and averaged. Cell counts were made using both whole-mount embryos and embryos smashed under a coverslip. Both methods of analysis gave similar results.

RESULTS

α1- and α2-Tubulin Genes Are Linked in Goldfish Genome

Although α1-tubulin gene expression has been shown to increase during development of the mammalian nervous system and upon regeneration of damaged peripheral neurons (Lewis et al., 1985; Miller et al., 1987, 1989; Gloster et al., 1994), it is not known if
this gene is regulated in a similar fashion in fish. Therefore, it was necessary to clone the fish α1-tubulin gene to provide a specific probe for α1-tubulin gene expression.

An α-tubulin cDNA (Heiber et al., 1992) was used to isolate λGF6 from a goldfish genomic library. λGF6 contains approximately 14.5 kb of insert DNA. Southern blotting and DNA sequencing identified tubulin coding sequences residing within this genomic clone. This analysis revealed that clone λGF6 contains two α-tubulin genes in tandem with their coding sequences residing in EcoR1 restriction fragments 6B/6D and 6A/6C, respectively (Fig. 1). Introns/exon boundaries were identified by comparison of genomic DNA with that of published tubulin cDNAs. Because 3′-untranslated sequences are not conserved between goldfish and other α-tubulins, we did not define the exact 3′ boundary of exon 4. However, both 6B/6D and 6A/6C tubulin genes contained a consensus polyadenylation signal sequence in exon 4, which generally precedes the site of polyadenylation by about 10–30 nucleotides and provides an approximation of where exon 4 ends.

The deduced amino acid sequence for goldfish tubulins 6B/6D and 6A/6C revealed that they encode tubulin proteins that are identical to the mammalian α1 and α2 genes, respectively (Lemischka and Sharp, 1982; Cowan et al., 1983; Lewis et al., 1985; Ginzburg et al., 1986; Villasante et al., 1986). These two tubulins differ by a single amino acid at position 232 (Villasante et al., 1986).

α1-Tubulin RNA Increases during Development of the Retina

We compared the expression of α1- and α2-tubulin RNA in developing goldfish retina. α1- and α2-specific probes were prepared from 3′-untranslated sequences and incubated with stage 22 goldfish embryo retinal sections (Sharma and Ungar, 1980) for in situ hybridization assays. These assays showed increased α1-tubulin RNA in retinal cells dispersed throughout the central portion of the retina, with highest expression in those cells that are either approaching or
residing closest to the inner limiting membrane (Fig. 2). In contrast, α₂-tubulin RNA expression was undetectable at this stage of retinal development (Fig. 2).

a₁-Tubulin RNA Is Specifically Induced in Retinal Ganglion Cells upon Optic Nerve Regeneration

RNase protection assays were performed to determine if the identified goldfish a₁- and α₂-tubulin genes were expressed in adult retina and if their expression was regulated during optic nerve regeneration. For these experiments, we crushed the right optic nerve 10 days prior to isolating left and right retinas. Total retinal RNA was prepared and a₁- and α₂-tubulin specific probes were used to quantitate their corresponding RNA levels. These experiments revealed a large increase in a₁ RNA following optic nerve crush (Fig. 3). Quantitation by scanning densitometry identified a 10-fold increase in a₁-tubulin RNA, while the α₂ RNA only increased twofold.

The above RNase protection assays average RNA levels across the entire retina and therefore may underestimate cell specific changes in RNA levels. To circumvent this problem, we used in situ hybridization assays. a₁-Tubulin RNA was undetectable in normal goldfish retina (Fig. 4). However, by 10 days postoptic nerve crush, we observed a large increase in a₁-tubulin RNA residing specifically in retinal ganglion cells. In contrast, α₂-tubulin RNA appears to be constitutively expressed at a relatively low but detectable level, and, like the a₁ RNA, α₂ transcripts were selectively expressed in retinal ganglion cells.

DNA Sequence of the Goldfish a₁-Tubulin Gene’s 5’-Flanking Region

The above experiments showed a specific increase in a₁-tubulin RNA during retinal development and upon regeneration of damaged adult optic axons. This change in tubulin RNA level may represent transcriptional and/or posttranscriptional mechanisms. To explore the possibility that transcriptional mechanisms mediate changes in a₁-tubulin RNA levels, we cloned and sequenced the a₁ gene’s 5’-flanking DNA.

Approximately 1.7 kb of DNA upstream from the a₁ gene’s transcription start site was sequenced (Fig. 5). Inspection of this sequence identified a number of putative regulatory elements (Fig. 5). Consensus TATAA and CCAAT boxes are located at position −28 and −71, respectively. Upstream of these sequences are a variety of DNA elements that may participate in directing appropriate developmental and tissue-specific expression. These elements include a retinoic acid response element, E-box basic helix–loop–helix consensus sequence, AP2 consensus sequence, and a homeodomain consensus motif for Drosophila antennapedia, fushi tarazu, and ultrabithorax homeodomain proteins (Fig. 5). In addition to these putative regulatory elements, we identified an AT repeat beginning at position −1461 which is immediately followed by a GT repeat and a TAGA repeat beginning at position −1138.

We also sequenced approximately 3 kb of DNA extending 5’ of the α₂-tubulin gene’s transcription start site and found little similarity to the a₁ gene’s 5’-flanking DNA (data not shown).

The a₁-Tubulin Promoter Restricts Reporter Gene Expression to the Developing Nervous System In Vivo

To determine if the cloned a₁-tubulin promoter contained sequences that directed expression to developing neurons in vivo, we injected zebrafish embryos with the −1696a₁TIpEGFP expression vector. We chose to use an expression construct containing the a₁-tubulin gene’s first intron since it showed significantly higher expression in vivo than those constructs lacking the first intron. Injected fish were analyzed at 24, 48, and 72 h postinjection (data only shown for
48 h). This analysis revealed maximal transgene expression around 48 h postinjection. Expression patterns were mosaic with fish exhibiting as few as two expressing cells to well over 1000 expressing cells. At 48 h of development, α1TIpEGFP-injected embryos showed preferential and highest expression in cells extending axons [Fig. 6(A,B)]. GFP expressing cells could be found in retina, forebrain, mesencephalon, hindbrain, and spinal cord. Quantitation of this expression pattern (Table 1) showed that of those fish expressing GFP, 98% had at least some neural expression. Quantitation of the number of GFP-positive cells in these fish revealed that 82% of the expressing cells are neurons. This is in contrast to an expression vector harboring the CMV promoter driving GFP expression which showed widespread expression in most cell types of the developing zebrafish embryo, with only about 10% of the GFP expressing cells representing neurons [Fig. 6(C,D) and Table 1].

Unidirectional deletions of the α1-tubulin promoter identified regions of DNA that appear to contribute to a robust developmentally induced neuron-specific expression pattern (Table 1). First, deletion of approximately 900 bp from the 5' end of the α1 promoter (−808α1TIpEGFP) resulted in a small (15%) reduction in the number of embryos exhibiting neural-specific expression and a similar decrease in the percentage of GFP cells that are neural. However, deletion of an additional 500 bp (−302α1TIpEGFP) caused a 19% decrease in the number of embryos showing neural expression and also reduced the percentage of GFP-expressing cells that are neurons to 42%. However, this expression pattern is still neurally restricted compared to the promiscuous expression driven by the CMV promoter which only resulted in about 10% of the total GFP-expressing cells representing neurons. Finally, deletion of an additional 120 bp (−182α1TIpEGFP) dramatically reduced the number of embryos exhibiting expression in the nervous system (10%) and the percentage of total GFP-expressing cells representing neurons (8%). The pEGFP vector without a promoter showed no significant expression.

Figure 4  A specific induction of α1-tubulin RNA in retinal ganglion cells following optic nerve crush. Adult goldfish were induced to regenerate their right optic nerve by crushing the nerve emanating from the right retina. The left retina served as a normal control. Ten days following optic nerve crush, retinas were isolated and sectioned for in situ hybridization. α1- and α2-tubulin specific probes were hybridized with left (normal) and right (crush) retinal sections. The α1 probe identified a large increase in α1-tubulin RNA following optic nerve crush that is specific to the retinal ganglion cells, while the α2-tubulin probe identified a low basal level of α2-tubulin RNA expression in both normal and optic nerve crush retina. ONL = outer nuclear layer; INL = inner nuclear layer; GCL = ganglion cell layer. Scale bar = 100 μm.
α1-Tubulin Promoter Activity in Regenerating CNS Neurons

Explants of adult goldfish retina have been shown to regenerate their ganglion cell axons in tissue culture (Landreth and Agranoff, 1979). To determine if the α1-tubulin promoter was activated during CNS regeneration, we took advantage of the observation that explanted adult goldfish retinas do not extend neurites efficiently unless their optic nerve was crushed in vivo a few days prior to explantation (Landreth and Agranoff, 1976). Quantitation of the number of explants exhibiting outgrowth indicates that an in vivo optic nerve crush can increase the number of explants exhibiting neurite outgrowth by 3- to 10-fold. Therefore, we crushed the optic nerve emanating from the right retina of adult goldfish 7 days prior to isolating right and left retinas. Retinas were electroporated with the α1TIpEGFP expression vector as described in Materials and Methods. Following electroporation, retinas were placed in tissue culture dishes and assayed for reporter gene expression 7 days later. This time point was chosen because it allowed sufficient time for cells to regrow axons which facilitated the identification of regenerating neurons. The number of expressing cells and the level of expression did not appear to decrease from 2 to 7 days following electroporation.

These assays revealed robust expression of α1-Tubulin Promoter Activity in Regenerating CNS Neurons. Figure 5 DNA sequence of the α1-tubulin gene’s 5′-flanking DNA, exon 1 and intron 1. Sequences in bold represent exons. Sequences in italics are repeats. Putative regulatory sequences are identified as follows: dashed box = retinoic acid response element; underline = E-box, basic helix–loop–helix consensus sequence; solid box = homeodomain, tandem consensus motifs for Drosophila antennapedia, fushi tarazu, and ultrabithorax homeodomain proteins; even dashed line = consensus TATA and CAAT box promoter sequences; uneven dashed line = AP-2 consensus sequence; dotted line = sequences that show no similarity to known cis-acting regulatory elements, but are similar to sequences in the rat α1 promoter (Gloster et al., 1994).
**Figure 7** $-1696\alpha1TIpEGFP$ expression in retinal explants. The right optic nerve of adult goldfish was crushed *in vivo* and retinas isolated 7 days later. Retinas were electroporated with expression vector DNA ($-1696\alpha1TIpEGFP$) and placed culture for 7 days. Photos show two examples of expression patterns observed in these retinas. Photos were taken with a $\times20$ objective and fluorescent optics. Scale bar = 100 $\mu$m.

**Figure 6** $\alpha1$-Tubulin and CMV promoter-directed expression patterns in developing zebrafish. (A–D) Single-cell zebrafish embryos were injected with either the $-1696\alpha1TIpEGFP$ (A,B) or the pCS2 vector (CMV promoter driving GFP expression) (C,D) and examined using fluorescent optics at 48 h of development. Yellow/green cells and axons represent GFP-expressing cells. (A) Whole-mount embryo. Note that many cells are out of the plane of focus, and thus their axons are obscured. The asterisk marks a cluster of three nonneuronal cells expressing the reporter gene. (B) A 48-h zebrafish embryo smashed under a coverslip to get most of the expressing cells in the plane of focus. The asterisk marks a nonneuronal cell expressing the reporter gene. (C) The CMV promoter directs expression to mostly nonneuronal cells in the 48-h zebrafish embryo. (D) The CMV promoter directs expression to both neural and nonneuronal cells. The asterisk in (D) marks a region just behind the eye, where neurons are expressing the reporter gene. Note the $\alpha1T$ promoter preferentially directs expression to the nervous system, while the CMV promoter does not show neural specificity and is expressed throughout the embryo (see Table 1 for quantitation of these data). Photos were taken with either a $\times10$ objective [(C), scale bar = 50 $\mu$m] or a $\times20$ objective [(A,B,D), scale bar = 100 $\mu$m].
The data were generated by counting GFP-expressing cells in injected embryos. A minimum of 200 cells were analyzed for each construct. The percentage of embryos expressing GFP, a neuron expressing the GFP reporter gene. The column reporting percentage of expressing cells that are neural indicates the relative number of GFP-expressing cells with a clearly defined axon (at least two cell diameters long) divided by the total number of cells expressing GFP.

Table 1  $\alpha_1$TIpEGFP Expression in 48-h Zebrafish Embryos

<table>
<thead>
<tr>
<th>Expression Vector</th>
<th>Embryos Expressing Injected DNA</th>
<th>Embryos with CNS Expression (%)</th>
<th>Expressing Cells That Are Neural (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$-1696$ $\alpha_1$ TI pEGFP</td>
<td>305</td>
<td>97</td>
<td>82</td>
</tr>
<tr>
<td>$-808$ $\alpha_1$ TI pEGFP</td>
<td>192</td>
<td>82</td>
<td>70</td>
</tr>
<tr>
<td>$-302$ $\alpha_1$ TI pEGFP</td>
<td>234</td>
<td>79</td>
<td>42</td>
</tr>
<tr>
<td>$-182$ $\alpha_1$ TI pEGFP</td>
<td>276</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>CMV pEGFP</td>
<td>64</td>
<td>75</td>
<td>10</td>
</tr>
</tbody>
</table>

Expression vectors harboring different lengths of the $\alpha_1$-tubulin promoter or the CMV promoter driving GFP expression were injected into one- to two-cell stage embryos and analyzed 48 h later. Percentage of embryos with CNS expression indicates embryos having at least one neuron expressing the GFP reporter gene. The column reporting percentage of expressing cells that are neural indicates the relative number of GFP-expressing cells with a clearly defined axon (at least two cell diameters long) divided by the total number of cells expressing GFP. The data were generated by counting GFP-expressing cells in injected embryos. A minimum of 200 cells were analyzed for each construct.

500-bp deletion ($-302\alpha_1$TIpEGFP) had no further effect on expression (Table 2). Nonetheless these constructs were expressed at a higher level (approximately fivefold) in retinas that received a prior in vivo crush (Table 2). Interestingly, an additional deletion of 120 bp ($-182\alpha_1$TIpEGFP) abrogated regeneration-dependent induction of this promoter (Table 2). In contrast to the regulated expression of the $\alpha_1$-tubulin promoter, we observed no significant difference between right and left retinas electroporated with an expression vector harboring the CMV promoter driving GFP expression (Table 2). However, the expression of the CMV promoter was much more robust than the $\alpha_1$-tubulin promoter in these retinal explants, consistent with its nonspecific high-level expression pattern observed in vivo [Fig. 6(C,D)].

**DISCUSSION**

We report here the cloning and expression of the goldfish $\alpha_1$- and $\alpha_2$-tubulin genes. These genes are linked in the goldfish genome and were designated $\alpha_1$- and $\alpha_2$-tubulins based on the similarity of their deduced amino acid sequence with various vertebrate $\alpha$-tubulins (Sullivan, 1988; Ginzburg et al., 1986; Lemischka and Sharp, 1982; Cowan et al., 1983; Lewis et al., 1985; Villasante et al., 1986). The reason for maintaining two very similar tubulin genes whose products differ by a single amino acid is not clear. However, it has been suggested that multiple genes encoding functionally similar proteins may provide an opportunity for multiple independent transcriptional controls (Sullivan, 1988). This is consistent with the observed regulation of the $\alpha_1$-tubulin gene and the constitutive expression of the $\alpha_2$ gene.

Although the $\alpha_2$ gene is expressed in many tissues, it is also expressed in the developing brain where the $\alpha_1$ gene is preferentially expressed (Lewis et al., 1985). One way to ensure coexpression of two genes is for them to share common cis-acting regulatory elements. For example, regulatory elements located between particular Hox genes are thought to influence the expression of those Hox genes flanking this region of DNA (Sham et al., 1990; Whiting et al., 1991). Therefore, the tight linkage of the $\alpha_1$- and $\alpha_2$-tubulin genes may also reflect a requirement for shared regulatory elements, perhaps allowing expression of these genes in the nervous system (Lewis et al., 1985). Furthermore, it is likely the $\alpha_2$ gene contains additional regulatory elements mediating its expression in other tissues, while the $\alpha_1$ gene may contain elements that repress its expression in nonneural cells.

Although the goldfish $\alpha_1$- and $\alpha_2$-tubulin genes encode almost identical proteins that are expressed in neural tissue, they are regulated very differently during CNS development and regeneration (Figs. 2–4). The fish $\alpha_1$-tubulin RNA, like its mammalian counterpart (Miller et al., 1987, 1989), increases at a time when axons are growing (Figs. 2–4). Developmentally, this is illustrated in the retina where $\alpha_1$ gene expression is localized to the middle third, a region enriched in neuroblasts that are differentiating into retinal ganglion cells (Sharma and Ungar, 1980). Within this area of the retina, $\alpha_1$ expression is highest in those cells approaching the inner limiting membrane. Based on this location and their high level of $\alpha_1$-tubulin gene expression, these cells likely represent ganglion cells that are extending axons. The lower level of expression observed throughout the middle third of the developing retina may represent dividing neuroblasts committed to a neuronal fate and/or postmitotic cells migrating to their final position. This expression profile is similar to that of the rat Tα1 promoter driving reporter gene expression in developing mouse retina (Gloster et al., 1994).

In contrast to the regulated expression of the $\alpha_1$ gene, $\alpha_2$-tubulin gene expression does not correlate...
Table 2 α1TIP Expression in Retinal Explants

<table>
<thead>
<tr>
<th>Expression Vector</th>
<th>Retinas Examined (Right or Left) (n)</th>
<th>Total No. of GFP-Expressing Cell Clumps</th>
<th>GFP-Expressing Cell Clumps (Right/Left)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1696 α1 TI pEGFP</td>
<td>48</td>
<td>36 Left Retina, 176 Right Retina</td>
<td>4.9 Left Retina/Right Retina</td>
</tr>
<tr>
<td>−808 α1 TI pEGFP</td>
<td>24</td>
<td>8 Left Retina, 40 Right Retina</td>
<td>5 Left Retina/Right Retina</td>
</tr>
<tr>
<td>−302 α1 TI pEGFP</td>
<td>24</td>
<td>8 Left Retina, 48 Right Retina</td>
<td>6 Left Retina/Right Retina</td>
</tr>
<tr>
<td>−182 α1 TI pEGFP</td>
<td>24</td>
<td>6 Left Retina, 8 Right Retina</td>
<td>1.3 Left Retina/Right Retina</td>
</tr>
<tr>
<td>CMV pEGFP</td>
<td>12</td>
<td>77 Left Retina, 69 Right Retina</td>
<td>0.9 Left Retina/Right Retina</td>
</tr>
</tbody>
</table>

Expression vectors harboring different lengths of the α1-tubulin promoter or the CMV promoter driving GFP expression were electroporated into adult retinas as described in Methods. The optic nerve emanating from the right retina was crushed in vivo 7 days prior to isolating left and right retinas. Positive expressing cells were identified using a Zeiss Axiophot microscope equipped with fluorescent optics. GFP-expressing cells were found in clumps containing anywhere from 1 to 100 cells. We obtained similar results regardless of whether we counted individual cells or clumps of expressing cells. Over 70% of the expressing cells had a clearly defined axon.

With axon outgrowth (Figs. 2–4). These results are reminiscent of the rat Tα1 and T26 expression patterns (Miller et al., 1987, 1989), where Tα1 is regulated by nerve growth and regeneration and the T26 RNA is constitutively expressed. Although the mouse α2-tubulin RNA has been reported to be expressed in the developing brain, it is expressed at a significantly lower level than the α1-tubulin RNA (Lewis et al., 1985). This lower level of expression may explain why we did not detect this RNA in the developing retina. Alternatively, the α2-tubulin RNA may be induced in the developing retina at a later time. Finally, these results are consistent with our previous reports showing a large increase in α-tubulin RNA following optic nerve crush in fish (Hieber et al., 1992; Hieber and Goldman, 1995). However, in those studies, we did not distinguish between α1- and α2-tubulin isoforms.

The above described increases in α1-tubulin RNA may reflect transcriptional and/or posttranscriptional mechanisms. To explore transcriptional mechanisms, we isolated the α1-tubulin gene’s 5′-flanking DNA and assayed its activity during CNS development and regeneration. These experiments showed that a 1696-bp α1-tubulin promoter directs reporter gene expression to the nervous system (Fig. 6 and Table 1). We found that reporter gene expression was highest around 24–48 h of development, corresponding to a time when the nervous system is experiencing extensive axonogenesis (Ross et al., 1992). This pattern of expression was not mimicked by our control CMVpEGFP vector, which was expressed in many cell types located throughout the developing zebrafish.

To confirm that α1-tubulin promoter sequences are necessary for conferring a developmentally regulated, neuron-specific pattern of reporter gene expression, we generated a number of α1TIP constructs that contained various promoter deletions (Table 1). In vivo expression assays using these constructs identified two main regulatory regions. First, deletion of sequences between −808 and −302 reduced neuron-specific expression from 70% (−808α1TIPEGFP) (Table 1) to about 42% (−302α1TIPEGFP) (Table 1), yet maintained it above that of the promiscuous CMV promoter (10%) (Table 1). Second, removal of an additional 120 bp from −302α1TIPEGFP to generate −182α1TIPEGFP resulted in a large decrease in the number of embryos exhibiting expression in the nervous system (10% neural) (Table 1) and reduced the percentage of expressing cells that are neurons to 8%. These results suggest that multiple α1-tubulin promoter elements contribute to the expression pattern of this gene.

Although both −182α1TIPEGFP- and CMVpEGFP-injected embryos resulted in approximately 8–10% of the total expressing cells representing neurons (Table 1, column 4), the number of embryos exhibiting neural expression was dramatically different (10% for −182α1TIPEGFP and 75% of CMVpEGFP) (Table 1, column 3). These results suggest that the α1-tubulin promoter, containing only 182 bp of 5′-flanking DNA, is a much weaker promoter than the CMV promoter and appears to have lost elements directing its expression to the developing nervous system (i.e., only 8% of total expressing cells represent neurons) (Table 1).

A number of neural-specific genes have been analyzed for elements mediating their specific expression pattern. These studies have identified a cell type-specific silencer element in the SCG10 and type II sodium channel gene that suppresses expression in nonneural cells (Mori et al., 1990; Kraner et al., 1992). This element was also found in the GAP-43 gene (Nedivi et al., 1992) and contains the core sequence CCAGG. We note that this core sequence
exists in the α1-tubulin promoter at position −535 and may contribute to the loss of neural specificity observed with −302α1TIpEGFP (Table 1). However, there are a number of putative regulatory elements located in the region −808 to −302 (Fig. 5) that may also participate in directing gene expression to the nervous system.

To determine if the cloned fish α1-tubulin promoter is capable of being activated in regenerating CNS tissue, we introduced our α1TIpEGFP expression vector into retinal explants. For these experiments, we took advantage of the observation that adult goldfish retinas explanted to tissue culture do not regenerate axons efficiently unless their optic nerve was crushed in vivo a few days prior to explantation (Landreth and Agranoff, 1976). These experiments showed that an α1-tubulin promoter containing as little as 302 bp of 5′-flanking DNA was able to increase reporter gene expression in retinas induced to regenerate their neurons (Table 2). However, deletion of an additional 120 bp (−182α1TIpEGFP) prevented increased GFP expression in retinas induced to regenerate (Table 2).

It is interesting that both developmentally induced, neuron-specific and regeneration-dependent induction of the α1-tubulin gene required sequences located within a 302-bp region upstream of the α1-tubulin gene’s transcription start site (Tables 1 and 2). This result may indicate that nerve regeneration recapitulates development and that common elements may mediate α1-tubulin gene induction during CNS development and regeneration.

Like the fish gene, the mammalian α1-tubulin gene is also induced in developing and regenerating neurons (Gloster et al., 1994). This similar regulation may indicate that conserved elements mediate their expression profiles. Comparison of the fish and rat α1-tubulin promoter sequences identified three regions of conserved DNA extending beyond eight nucleotides. Two of these conserved sequences are located within a 100-bp region just upstream of the transcription start site (dotted underline in Fig. 5). Neither of these sequences resembles previously identified transcription factor consensus binding sites. The third sequence is located 426 and 560 bp upstream of the fish and rat α1-tubulin gene’s transcription start sites, respectively. This sequence represents a tandem consensus sequence for homeodomain proteins antennapedia, fushi tarazu, and ultrabithorax (Biggin and Tjian, 1989). We also found an 8-bp consensus retinoic acid response element that is identical in both fish and rat promoters. Both homeobox binding sites and retinoic acid response elements have been implicated to play important roles in nervous system development (Chisaka et al., 1991; Guillemot et al., 1993; Kaplan et al., 1993) and may also participate in regulating α1-tubulin gene expression. Future experiments will focus on analyzing the functional significance of these putative regulatory elements in the fish system. In addition, we will focus our efforts on identifying those specific DNA sequences mediating developmental and regeneration-dependent induction of the fish α1-tubulin promoter.

The Genbank accession number for the sequence reported here is AF002654. The authors thank Dr. P. Raymond for providing the stage 22 goldfish embryos, Dr. P. Hitchcock for use of Picospritzer, Dr. J. Kuwada for help in setting up our zebrafish colony, and Dr. B. Agranoff for helping house goldfish. This work was made possible by the Lucille P. Markey Charitable Trust, and the Mary Hadly Hughes Memorial/DaPuzzos and the Cowen and Company Optic Nerve Research Fund of the Glaucoma Foundation.

REFERENCES


