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Transgenic zebrafish for studying nervous system development and regeneration

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Abstract

α1 tubulin gene expression is induced in the developing and regenerating CNS of vertebrates. Therefore, α1 tubulin gene expression may serve as a good probe for mechanisms underlying CNS development and regeneration. One approach to identify these mechanisms is to work backwards from the genome. This requires identification of α1 tubulin DNA sequences that mediate its developmental and regeneration-dependent expression pattern. Therefore, we generated transgenic zebrafish harboring a fragment of the α1 tubulin gene driving green fluorescent protein expression (GFP). In these fish, and similar to the endogenous gene, transgene expression was dramatically induced in the developing and regenerating nervous system. Although transgene expression generally declined during maturation of the nervous system, robust GFP expression was maintained in progenitor cells in the retinal periphery, lining brain ventricles and surrounding the central canal of the spinal cord. When these cells were cultured *in vitro* they divided and gave rise to new neurons. We also show that optic nerve crush in adult fish re-induced transgene expression in retinal ganglion cells. These studies identified a relatively small region of the α1 tubulin promoter that mediates its regulated expression pattern in developing and adult fish. This promoter will be extremely useful to investigators interested in targeting gene expression to the developing or regenerating nervous system. As adult transgenic fish maintain transgene expression in neural progenitors, these fish also provide a valuable resource of labeled adult neural progenitor cells that can be studied *in vivo* or *in vitro*. Finally, these fish should provide a unique *in vivo* system for investigating mechanisms mediating CNS development and regeneration.

Introduction

Tubulin is the protein that makes up microtubules. In neurons, tubulins are crucial for development of cellular polarity and formation of dendrites and axons (Laferriere et al., 1997). Microtubules may also play a role in neuronal plasticity, allowing the elaboration of new processes and modification of synaptic interactions (Heidemann, 1996; Laferriere et al., 1997).

Vertebrates express a number of α and β tubulin isoforms that are encoded by distinct genes (Sullivan, 1988). Of the six different α tubulin isoforms expressed in mouse, only α1 expression is enriched in the developing and regenerating nervous system (Miller et al., 1987; 1989; Hieber et al., 1998). Therefore, α1 tubulin gene expression may serve as a useful probe for studying mechanisms underlying nervous system development and regeneration. Indeed, transgenic mice harboring the rat α1 tubulin promoter (Tα1) driving lacZ expression exhibited a neuron-restricted pattern of transgene expression that

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was panneuronal during development (Gloster et al., 1994). Transgene expression declined postnatally coincident with neuronal maturation, but was reinduced following damage to peripheral neurons.

In contrast to mammals, zebrafish offer several advantages in studies of CNS development and regeneration. Zebrafish embryos are transparent, facilitating visualization of internal structures and cells in living animals. Zebrafish are amenable to large-scale genetic screens that have generated interesting mutations in the nervous system (Driever et al., 1996; Haffter et al., 1996). Zebrafish can regenerate injured CNS neurons, a process which coincides with increased α1 tubulin RNA expression (Hieber et al., 1998; Bormann et al., 1998). Direct DNA/RNA injection into zebrafish embryos provides a rapid and convenient *in vivo* transient expression assay for characterizing promoters and examing the effect specific molecules have on development (Meng et al., 1997; Hieber et al., 1998; Griffin et al., 1998; Higashijima et al., 2000).

These properties, along with the observation that α1 tubulin gene expression is correlated with CNS development and regeneration, prompted us to develop transgenic zebrafish harboring a fragment of the α1 tubulin gene driving GFP (green fluorescent protein) expression in their germ-line cells. Here we present a detailed characterization of transgene expression in developing and adult zebrafish. This analysis showed that a relatively small fragment of the α1 tubulin gene harbors all the necessary elements for specifically directing reporter gene expression to the developing CNS. Interestingly this promoter fragment also directed gene expression to adult neural progenitor cells and adult CNS neurons that are regenerating damaged axons. These fish should provide a useful system for studying mechanisms mediating α1 tubulin gene induction and therefore, CNS development and regeneration. In addition, adult transgenic fish provide a convenient source of GFP-labeled neural progenitor cells for both *in vivo* analysis and their isolation for *in vitro* studies.

Methods

Zebrafish maintenance and surgery

Adult zebrafish (*Danio rerio*) were obtained from the local pet store and maintained in our own fish facility with a controlled light cycle of 14 h light/10 h dark at 28°C. They spawned soon after the onset of light, and the fertilized eggs were collected at the one-cell stage.

Right optic nerve crush was carried out just behind the orbit in tricaine methane sulfonate (0.02%) anesthetized fish. Following the onset of deep anesthesia, the fish was removed from the tricaine solution and its right eye visualized under a dissecting microscope. The conjuctiva of the right eye was cut, and the eyeball was partly deflected from the orbit using a forceps and the right intraorbital nerve was crushed with a fine watchmaker's forceps. Care was taken to avoid damage to the opthalmic artery and any fish showing signs of damage were discarded. The left optic nerve of each fish was left intact, with the left retina serving as an unoperated control. Following surgery, fish were returned to tank water and usually recovered from anesthesia within 5 min. Six fish from two different transgenic lines were analyzed for transgene expression following optic nerve crush and all gave similar results.

Generation of α1TIpEGFP transgenic zebrafish

Our −1696α1TIpEGFP expression vector harbors 1696 bp of 5′ flanking α1 tubulin DNA, exon 1 and the first intron fused in frame to the GFP sequence in the pEGFP vector (Clontech) (Hieber et al., 1998). Zebrafish embryos were obtained by natural crosses. DNA solutions for injection contained 50 ng/μl supercoiled −1696α1TIpEGFP 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 48 h postinjection, fish were examined using fluorescent microscopy and GFP-expressing fish were saved. Germ-line integrated transgenic zebrafish were selected from these GFP-positive fish by raising them to sexual maturity and breeding them with wild-type fish. Progeny from these fish (at least 300 progeny) were screened for GFP expression and GFP-positive fish were saved for further analysis and breeding. We have maintained these lines for four generations.

Our studies suggest that approximately 2–5% of the fish expressing injected DNA will integrate the DNA into their germ-line. However, because this integration is a relatively random event only a fraction of the germ-line cells will have integrated the DNA (approximately 1–5%). Therefore, it is necessary to screen a large number of embryos (generally over 300) to identify transgenic founders.

Over 60 fish from each transgenic line have been evaluated for transgene expression during development. Although all lines of fish expressed the

transgene in a similar fashion, one of our lines (L5) clearly had reduced transgene expression compared to the others. We attribute this to the site of integration.

Immunohistochemistry

Dividing cells in adult fish were identified by placing fish in a small amount of fish water containing 2 mg/ml of BrdU (Sigma, St. Louis, MO). All fish were fixed by immersion overnight at 4°C in a solution of 3.7% formaldehyde in Zebrafish MOPS buffer (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$, pH 7.4). Zebrafish were prepared for cryosectioning according to Barthel and Raymond (1990). Sections were cut at 3 or 10 μm, placed on gelatin-subbed slides and stored at −80°C.

Standard immunohisotochemical procedures were used to stain zebrafish sections (Westerfield, 1993). Primary antibodies: anti-GFP (CloneTech, Palo Alto, CA) used at 1:500; anti-acetylated tubulin (Sigma, St. Louis, MO) used at 1:1000; anti-GFAP (Chemicon, Temecula, CA) used at 1:100; anti-BrdU (Sigma, St. Louis, MO, clone BU 33) used at 1:1000. Secondary antibodies: Cy2-conjugated rabbit anti-IgG used at 1:1000; Cy3-conjugated mouse anti-IgG used at 1:200; Alexafluor 488 anti-rabbit used at 1:1000; Alexfluor 350 anti-mouse used at 1:1000. Following antibody staining slides were stained with bis-Benzimide (Hoechst 33258; 0.25 μg/ml) for 5 min, rinsed again and coverslipped using MOWIOL medium (Calbiochem, San Diego, CA). Approximately 12 individual fish from each line were used to assay GFP and acetylated tubulin expression at 24 and 72 hpf (hours post firtilization), while 2–6 fish were used to analyze expression at 1 and 3 months of development.

In situ *hybridization*

Digoxygenin-labelled α1 tubulin antisense and sense RNA probes were synthesized by *in vitro* transcription of linearized template DNA (containing 870 bp of exon 4 and 3′ untranslated sequence) according to the manufacturer's directions (Boehringer Mannheim, Indianapolis, IN). *In situ* hybridizations were carried out on whole-mount embryos as previously described (Westerfield, 1993; Jowett, 1997). *In situ* hybridizations were performed on batches of embryos (approximately 12) and repeated at least three times.

Primary cultures

Primary cultures were prepared from adult brain. Brains were dissected, minced and then digested with trypsin (0.25%) at room temperature for 10 min with trituration. Dissociated cells were centrifuged and resuspended in L-15 media with 0.3 mg/ml glutamine, 50 U/ml penicillin, 0.05 mg/ml streptomycin, 10% FCS, 1X insulin/transferrin/selenium (ITS, Gibco), bFGF (20 ng/ml), and PDGF (20 ng/ml). GFP-positive cells were selected using fluorescent activated cell sorting and plated in the above media. Of the total cells sorted, approximately 20% were GFP-positive. We identified two populations of GFP-positive cells in adult brains: strong expressing cells were found lining brain ventricles; weak expressing cells were found throughout the brain. The strong expressing cells represent less than 1% of the total GFP expressing cells and these cells were selected for plating in primary culture. Proliferating cells were identified by including BrdU (0.15 mg/ml) in the culture media. Cells were plated on poly-L-lysine-coated glass coverslips in tissue culture wells and incubated at 28°C in a humidified chamber. Primary cultures were generally prepared from 2–4 fish and repeated two times using two different lines of transgenic fish.

Results

Generation of −1696α1TIpEGFP transgenic zebrafish

Five independent lines of transgenic zebrafish were created by injecting our −1696α1TIpEGFP expression vector into single-cell zebrafish embryos. To confirm germ-line integration of the transgene, transgenic F1 progeny were raised to sexual maturity and bred with either wild-type fish or siblings to obtain heterozygote and homozygote transgenic fish. F2's were generated at about a 50% frequency when an F1 transgenic fish was mated with a wild-type fish and about a 75% frequency when mated with another transgenic F1 fish. These breedings demonstrated that the transgene was inherited in a Mendelian fashion, consistent with germ-line integration.

Expression of −1696α1TIpEGFP in the developing zebrafish

All five lines of transgenic fish exhibited GFP expression that was restricted to the developing nervous

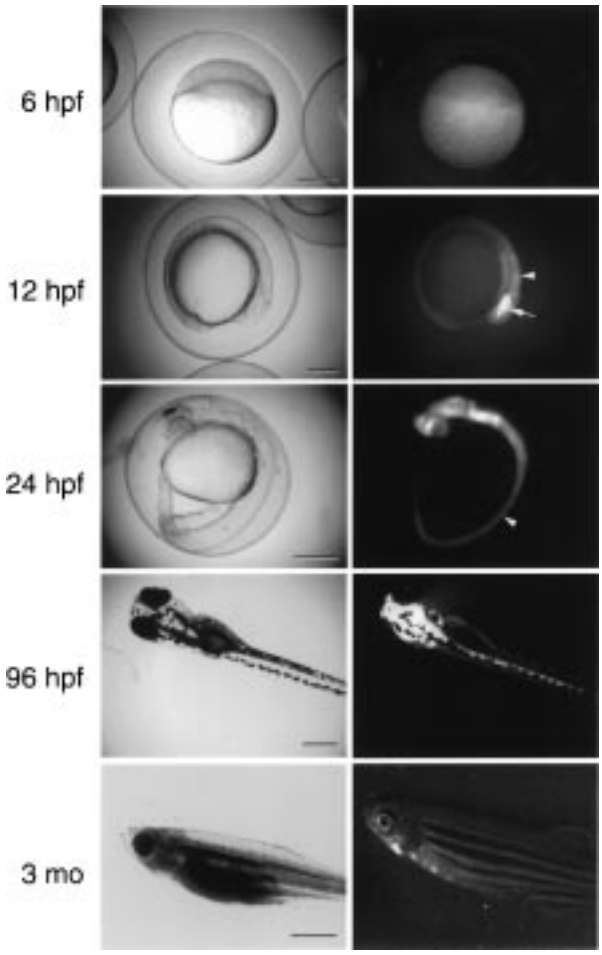


Figure 1. Transgene GFP expression in developing transgenic zebrafish. Brightfield and fluorescent photomicrographs were obtained at various stages of transgenic zebrafish development. Fish harbor the −1696α1TIpEGFP transgene in their germ-line cells. Note expression is undetectable at 6 hpf and first becomes detectable around 12 hpf. Expression is highest in the optic primordium (arrow) and brain rudiment (arrowhead). By 24hpf transgene expression is throughout the retina, brain and spinal cord (arrowhead). This expression pattern is maintained at 96 hpf. By 3 month of development, however, transgene expression becomes undetectable in whole mount fish. Note autofluorescence in the 3 months fish near the eye and the ventral surface. Scale bars: 250 μm for 6, 12, and 24 hpf; 500 μm for 96 hpf; 3 mm for 3 months.

system (Figure 1). Transgene expression was first detected at 12 hpf in the brain and rostral spinal cord rudiments (Figure 1). During the next 24 h there was a large increase in GFP expression that could be observed throughout the developing brain, retina and spinal cord (Figure 1). This pattern and level of GFP expression was maintained for the first week of development and then gradually declined as the fish matured.

As GFP is a relatively stable protein, we used whole mount *in situ* hybridization assays to compare transgene RNA expression with that of the endogenous α1 tubulin gene. This analysis showed a remarkably similar pattern of expression in the developing embryo, with endogenous and transgene expression reaching maximal levels in brain and spinal cord about 24 hpf (Figure 2) and declining thereafter. *In situ* hybridization to tissue sections confirmed this pattern of gene expression and showed endogenous and transgene expression in both recently differentiated neurons and proliferating neural progenitors (data not shown). This analysis suggests that elements necessary for directing α1 tubulin gene expression to the nervous system and regulating its expression during development are contained within our transgene construct.

Line 2 transgenic fish were chosen for more detailed analysis of transgene expression. Fish varying in age from 24 hpf to 3 months were sectioned, and retina, tectum, and spinal cord were examined for GFP expression and immunostained for acetylated tubulin to identify differentiated neurons (Schier et al., 1996). Bisbenzimide (Hoechst) staining was used to visualize cell nuclei.

At 24 hpf, the absence of acetylated tubulin immunoreactivity indicated that retinal cells were undifferentiated (Figure 3C). Nonetheless, we observed robust GFP expression in these cells (Figure 3B). At 72 hpf, bisbenzamide staining revealed morphologically identifiable cell layers corresponding to the ganglion cell, inner nuclear and outer nuclear layers (GCL, INL and ONL in Figure 3D). In addition, acetylated tubulin staining identified differentiated cells in the GCL and INL (Figure 3F). Transgene expression was very robust at this age and was found throughout all cell layers of the retina, with highest expression in the GCL and INL (Figure 3E). However, not all cells were GFP positive (Figure 3E and inset, arrowheads). In addition, GFP expression was undetectable in the retinal periphery where undifferentiated cells reside (Figures 3E and F, arrows). In contrast, at 1 month of development GFP expression was low throughout the retina except in the periphery, where transgene expression was very high (Figures 3H and I). Note that this latter region of the retina does not stain for acetylated tubulin, consistent with the location of undifferentiated precursor cells (Figures 3J, arrow). Transgene expression in the 3 month retina was very similar to that at 1 month of age, except the proliferative zone at the retinal margin was much smaller (Figure 3L). Transgene expression was detectable at a low level in

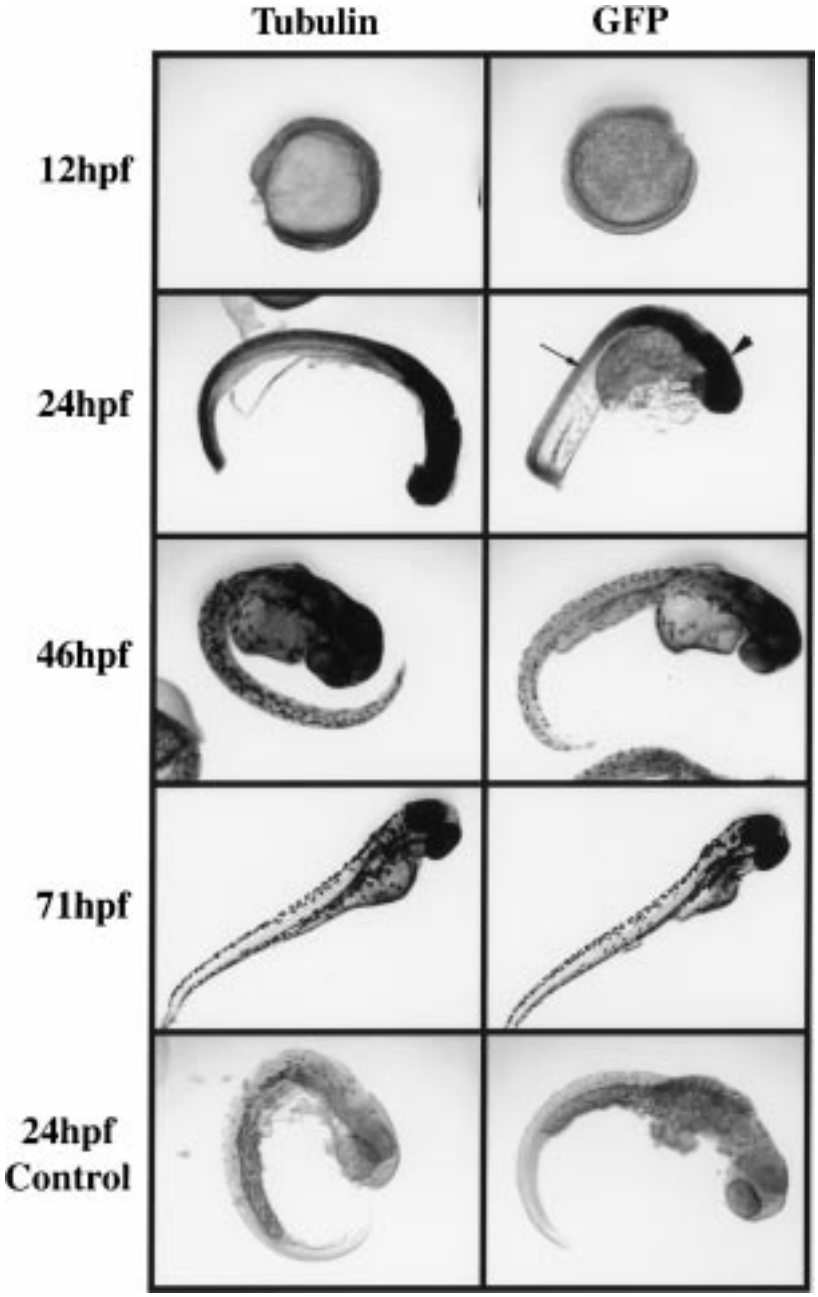


Figure 2. Endogenous α1 tubulin and transgene GFP RNA expression in developing transgenic zebrafish. Brightfield photomicrographs of transgenic zebrafish that have undergone whole-mount *in situ* hybridization to detect endogenous α1 tubulin and transgene GFP RNA. Transgenic zebrafish were harvested at various stages of development for whole-mount *in situ* hybridization assays. Fish were hybridized with either an α1 tubulin or a GFP antisense RNA probe. Control fish were hybridized with a sense-strand probe. Positive *in situ* hybridization signals appear as a dark product. Note that both endogenous and transgene RNA exhibit a similar pattern of expression that is restricted to the developing nervous system, peaks about 24 hpf and then begins to decline. The arrow and arrow head at 24 hpf point to positive GFP RNA expression in spinal cord and brain, respectively.

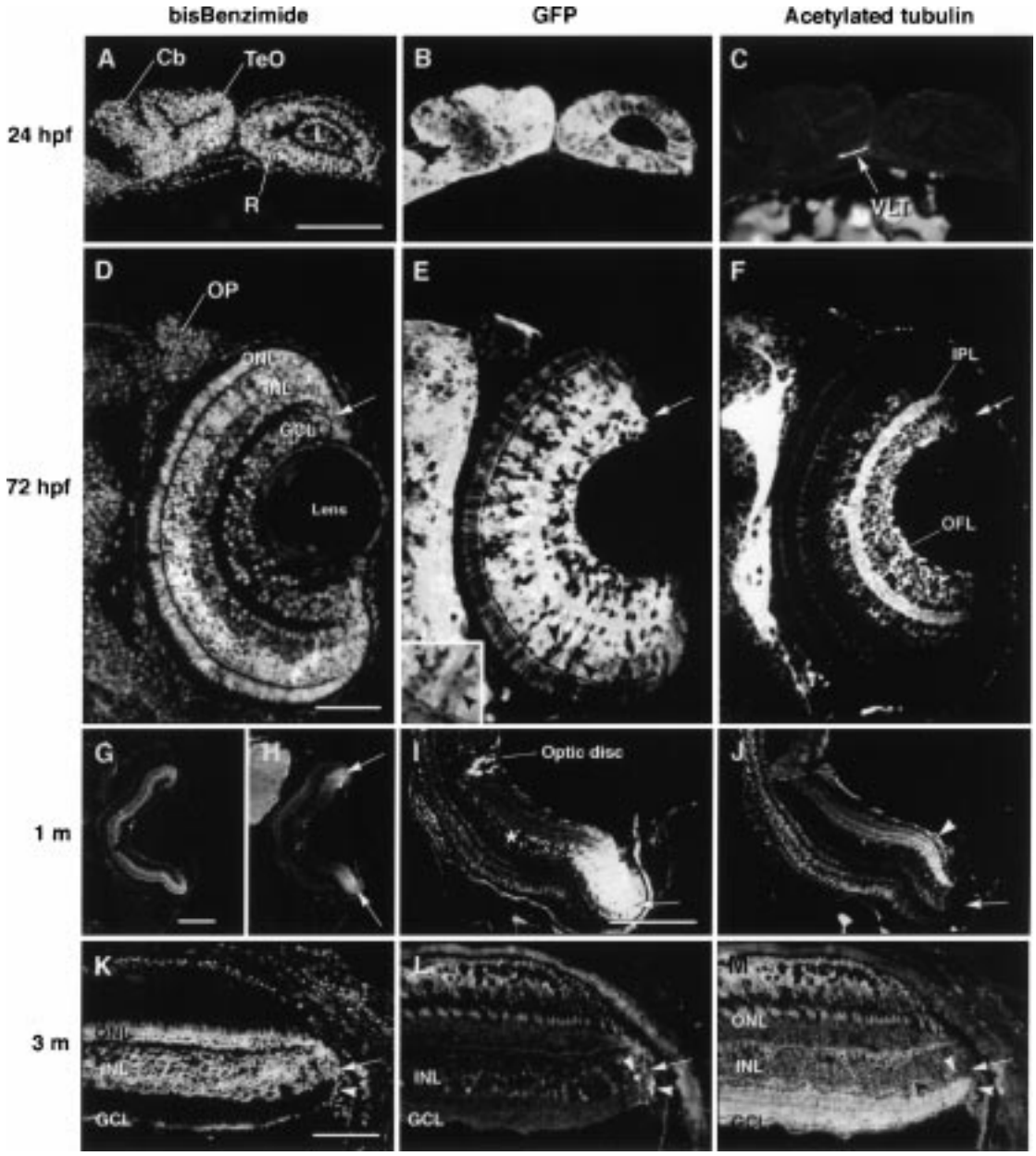


Figure 3. Transgene expression in the developing and mature retina. Triple stained sections showing nuclei with bisBenzimide (A, D, G and K), GFP expression (B, E, H, I and L), and acetylated tubulin (C, F, J and M). At 24 hpf (A–C), transgene expression was observed at relatively homogeneous levels throughout the neuraxis, including the retina (B). The lens did not show evidence of expression. Although axons were not detected in the retina at this stage, the first fiber tracts (e.g., VLT) appear at this time in the central nervous system. By 72 hpf (D–F), strong transgene expression was detected in the GCL and INL, as well as throughout the developing IPL and OFL of the inner retina. Transgene expression was, however, not expressed in all retinal cells: (1) the peripheral germinal zone (D, *arrow*) did not express GFP (E, *arrow*), (2) a population of cells in the inner retinal cell layers did not express GFP ('holes' in E, *arroweads*) – the 'holes' contain unlabeled cells (E-inset: *arrowheads*), and (3) expression in the outer nuclear layer (ONL) was weak (E). By 1 m (G–J), strong transgene expression was observed in the peripheral retina (H and I, *arrows*); in more central portions of the retina, GFP was only detected in ganglion cell axons (I, at the optic disc) and in a subpopulation of cells in the INL (I, *asterisk*). In the 3 month retina (K–M), transgene expression was largely restricted to a subpopulation of cells in the peripheral germinal zone (L, *arrow*). In addition, a small number of cells adjacent to the peripheral germinal zone (L, *arrowhead*) and a subpopulation of cells in the inner portion of the INL expressed GFP. Cb – cerebellum; GCL – ganglion cell layer; INL – inner nuclear layer; IPL – inner plexiform layer; L – lens; OFL – optic fiber layer; ONL – outer nuclear layer; OP – olfactory placode; R – retina; TeO – optic tectum; VLT – ventral longitudinal tract. Scale Bars: A–C = 100 μm; D–F = 50 μm; G–H = 250 μm; I–J = 200 μm; K–M = 100 μm.

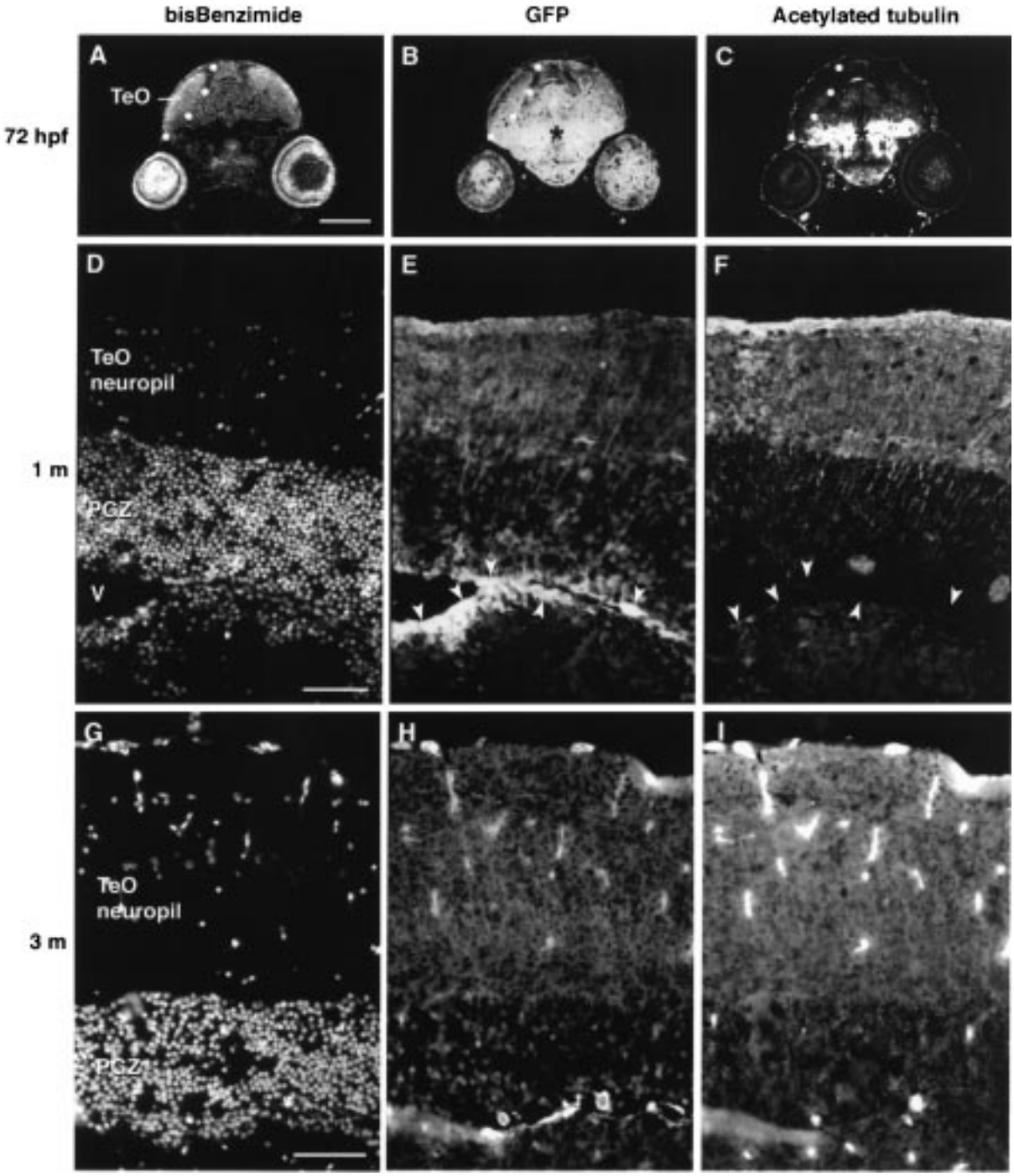
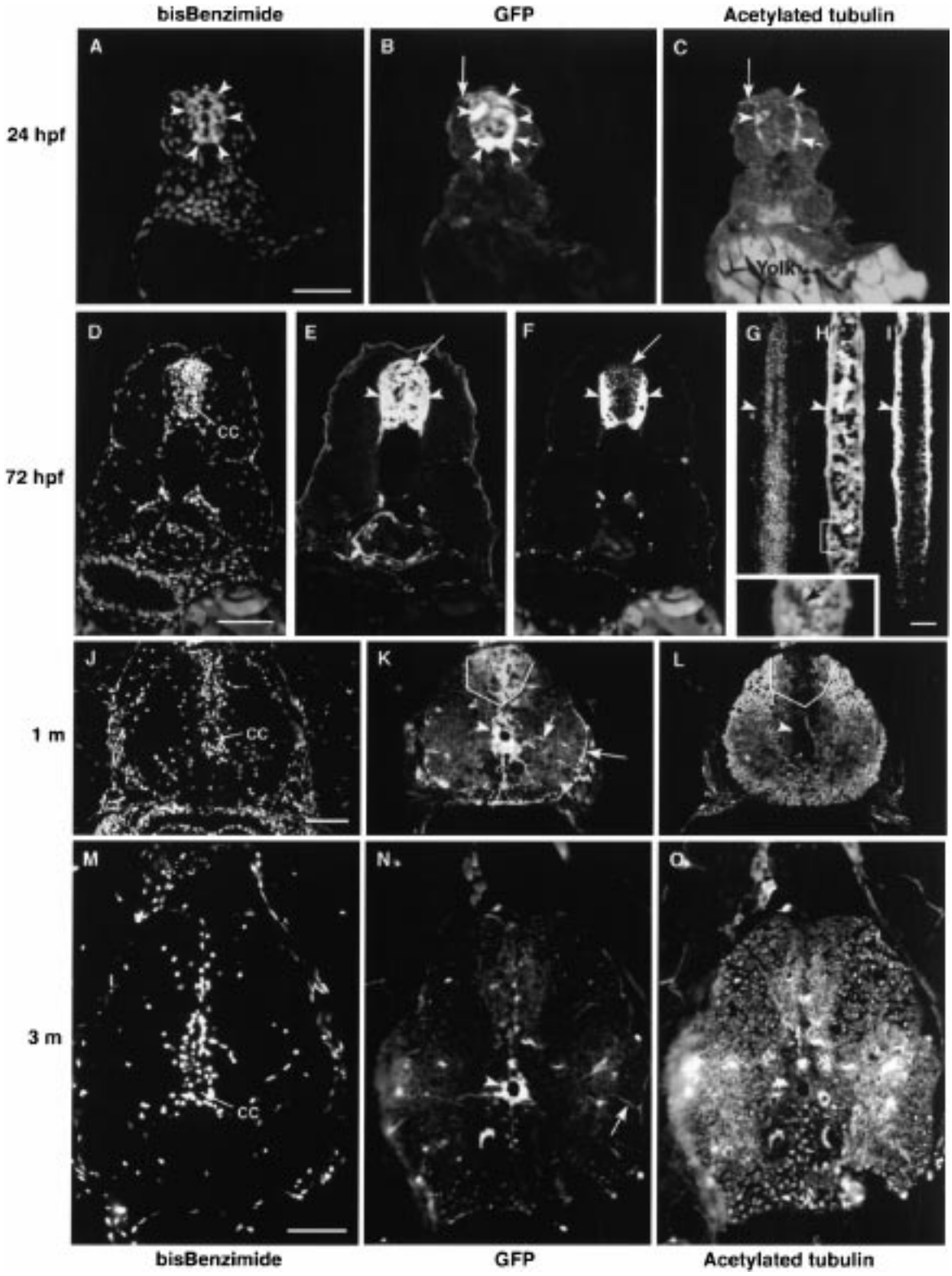


Figure 4. Transgene expression in the developing and mature optic tectum. Triple stained sections showing nuclei with bisBenzimide (A, D and G), GFP expression (B, E and H), and acetylated tubulin (C, F and I). At 72 hpf (A–C), transgene expression was observed in the mostly cellular optic tectum, as well as in fiber layers found deeper in the mesencephalon (B and C, *asterisks*). In the one-month-old zebrafish (E–F), GFP expression was observed in a subpopulation of cells in the periventricular gray zone (PGZ) and in the overlying tectal neuropil. In addition, a population of ependymal cells showed strong GFP expression (E, *arrowheads*); these cells expressed little or no tubulin (F, *arrowheads*). A similar patter of transgene expression was seen in the 3 month optic tectum (H–I). PGZ – periventricular gray zone; TeO – optic tectum. Scale bars: A–C = 100 μm; D–I = 50 μm.


bisBenzimide
GFP
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O
Yolk
CC
CC
CC
bisBenzimide
GFP
Acetylated tubulin

Figure 5.

the inner and outer nuclear layers and in the periphery where progenitor cells and their recently differentiated progeny reside. No expression was detected in ganglion cells.

In the 24–72 h tectum, transgene expression was widespread (Figure 3B and Figure 4B). At 24 hpf (see Figures 3A–C), very little differentiation has occured, although we do observe a small band of acetylated tubulin immunoreactivity (Figure 3C, arrow) that may represent differentiated cells contributing to the ventral longitudinal tract (Ross et al., 1992). At 72 hpf (Figures 4B and C), GFP expression was detected in both dorsally located tectal cell bodies and ventrally located axon tracts in the mesencephalon (identified by acetylated tubulin staining). At 1–3 months of development transgene expression was dramatically reduced in the tectal cells forming the dense paraventricular layer (Figures 4D, E, G, F). Note however the high expression in ependymal cells lining the tectal ventricles (Figure 4E).

In the 24 h spinal cord, strong GFP expression was observed in peripherally located cells (Figure 5B), some of which also stained prominantly for acetylated-tubulin (Figure 5C, arrowheads). At 72 hpf many cells (Figures 5E and F, arrows) and fiber tracts (Figures 5E, F and G–I, arrowheads) showed strong GFP expression. Interestingly, like the 72 h retina (Figure 3E), longitudinal sections of the spinal cord identified a population of bisbenzimide-stained cells that did not express GFP (Figure 5H, arrowhead). From 1–3 months of development GFP expression becomes restricted to acetylated tubulin-negative ependymal cells lining the central canal of the spinal cord and scattered cells located throughout the neuropil (Figures 5J–O).

Figure 5. Transgene expression in the developing and mature spinal cord. Triple stained sections showing nuclei with bisBenzimide (A, D, G, J and M), GFP expression (B, E, H, K and N), and acetylated tubulin (C, F, I, L and O). At 24 hpf (A–C), a population of cells located in the periphery of the developing spinal cord showed strong GFP expression (A and B, *arrowheads*). Some of the GFP expressing cells also showed acetylated tubulin immunoreactivity (C, *arrowheads*), though tubulin was also present in the developing longitudinal fiber tracts (C, *arrow*). The yolk (Y) showed auto-fluorescence with the red filter (C). By 72 hpf (D–I), many cells (E and F, *arrows*) and fiber tracts (E, F and G–I, *arrowheads*) showed strong transgene expression. As in the 72 hpf retina, the 72 hpf spinal cord also revealed 'holes' in GFP expression (H, *arrow*) that were filled with cells that did not express the transgene (inset, combined bisBenzimide/GFP, shows the area indicated by bracket in H). By 1 month (J–L), strong GFP expression was most notable in tubulin-negative ependymal cells (K and L, *arrowheads*) around the central canal (CC). In addition, a subpopulation cells and processes located dorsal to the central canal (K and L, *outlined area*), radial processes (K, *short arrow*) and pial endfeet (K, *long arrow*) expressed GFP. At 3 month (M–O), strong transgene expression was restricted to tubulin-negative ependymal cells (N and O, *arrowheads*), to scattered cells located throughout the neuropil, and to some radial processes (N, *arrowi*). CC – central canal. Scale bars: A–F and J–O = 50 μm; G–I = 25 μm.

Transgene expression in adult fish identifies neural progenitor cells

In adult fish, transgene expression was generally very low. However, in the CNS, expression was maintained in specific cells that reside in locations previously reported to harbor neural progenitor cells. These include the retinal periphery (Figures 6G, H and I) (Marcus et al., 1999) and the ependymal and subependymal zones surrounding the brain ventricles (Figures 6A–F) (Zupanc & Horschke, 1995). Consistent with their tentative assignment as neural progenitors, some of these GFP-positive cells were also labeled *in vivo* with the proliferative marker BrdU (Figures 6B, E and H).

To confirm these GFP-positive cells as neural progenitors, they were isolated using fluorescent activated cell sorting and cultured in the presence of BrdU. Cells were stained with antibodies 7–14 days later to identify proliferating cells (anti-BrdU) and neurons (anti-acetylated tubulin). Figures 6J–O shows two examples of GFP-positive cells that proliferated in culture (BrdU-positive) and gave rise to neurons (acetylated tubulin-positive). Approximately 5% of our GFP-selected cells divided and differentiated while in culture. This suggests that some of the selected cells may not be progenitors or may be progenitors that proliferate and differentiate very slowly in culture. More stringent selection criteria may be necessary to enrich further for neural progenitors.

The α1 tubulin promoter is reactivated in the regenerating CNS

The fish visual system has served as an ideal model for studying CNS regeneration following nerve damage. Optic nerve crush results in reinduction of α1 tubulin RNA expression in retinal ganglion cells (Hieber et al., 1995, 1998; Figure 7, top panels). To determine whether our transgene construct contained regulatory elements that would confer a CNS regeneration-dependent increase on reporter gene expression we crushed the right optic nerve of 3 month transgenic fish. The unoperated left optic nerve and retina served

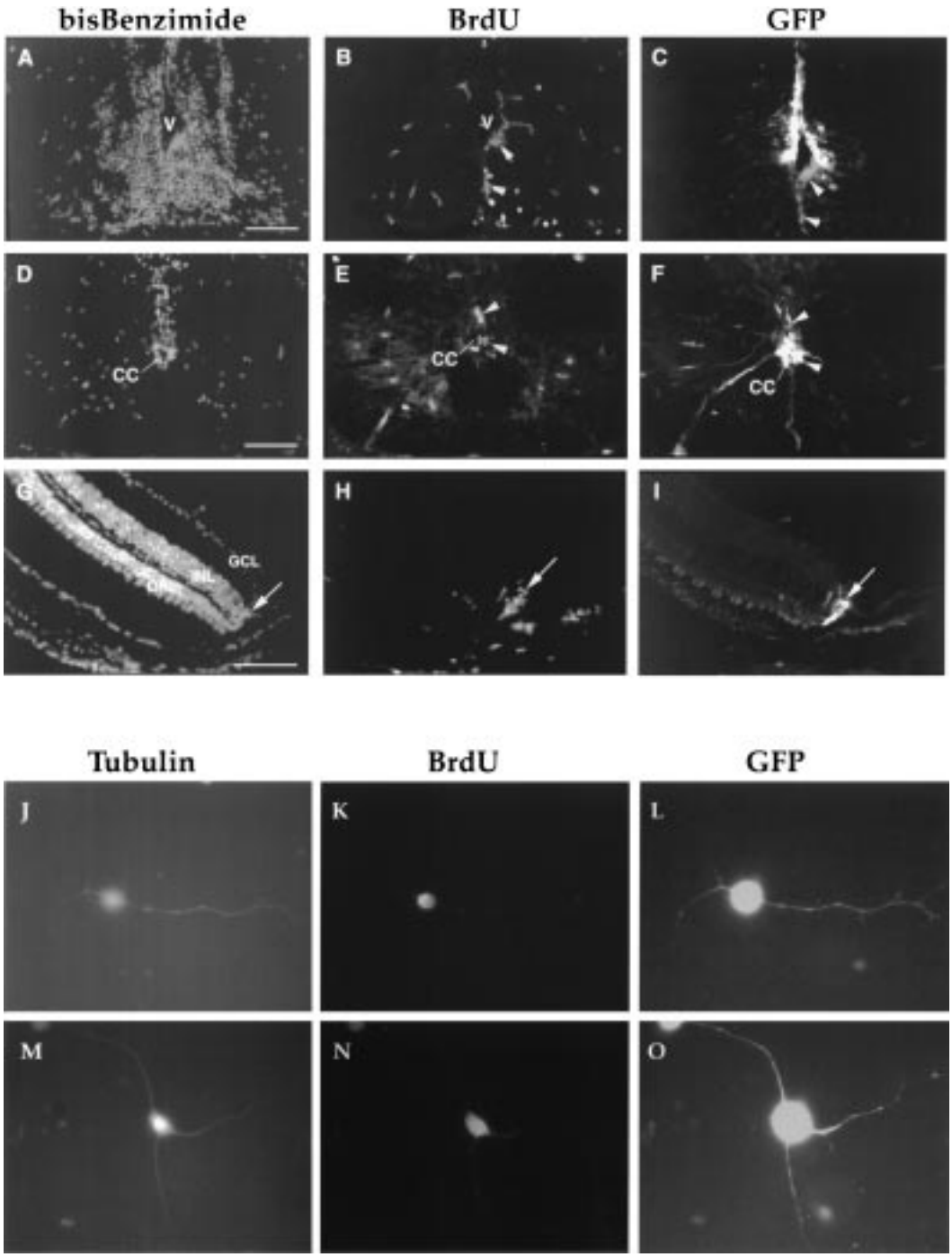

Figure 6. Neural progenitors express the transgene in adult zebrafish. Top panels (A–I): Transgenic fish at 3–5 months of age were labeled for 5 days with BrdU. Triple-stained sections through the rhombencephalic ventricle (A–C), spinal cord (D–F), and peripheral retina (G–I) are shown. BisBenzimide identifies cell nuclei. Arrowheads indicate BrdU-positive/GFP-positive cells lining the rhombencephalic ventricle (B and C) and central canal of the spinal cord (E and F). Arrows (G–I) point to the proliferative zone containing BrdU-positive/GFP-positive retinal progenitor cells. Bottom panels (J–O): Primary cultures of GFP-positive cells from adult brains isolated by fluorescent activated cells sorting. Cells were cultured in the presence of BrdU for 10 days prior to fixation and staining with anti-acetylated tubulin antibody to identify neurons, and anti-BrdU to identify dividing cells. CC – central canal; GCL – ganglion cell layer; INL – inner nuclear layer; ONL – outer nuclear layer; V – ventricle. Scale bars: 100 μm.

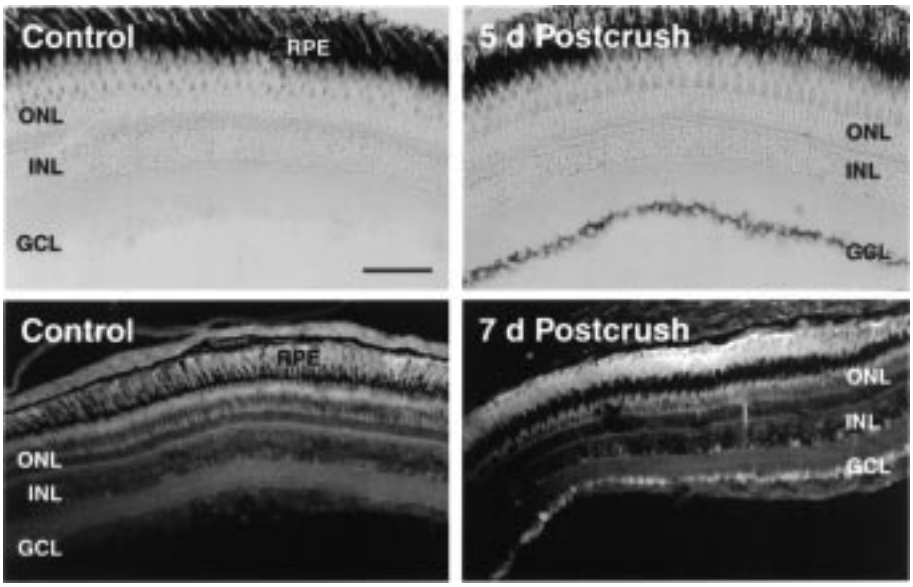

Figure 7. Optic nerve crush reinduces transgene expression in retinal ganglion cells that are regenerating their optic axons. Adult (3 months) zebrafish were used to study the effect of optic nerve crush on endogenous α1 tubulin RNA expression (top panels, *in situ* hybridization) and transgene GFP expression (bottom panels, fluorescent microscopy). The right optic nerve was crushed; the left optic nerve was left intact (control). After 5–7 days, digoxygenin-labeled probes were used to identify endogenous α1 tubulin RNA in retinal sections (dark deposit in 5-day postcrush retina GCL). The dark color at the top of the panels is the pigment epithelium. Specific induction of GFP expression was observed in retinal ganglion cells (GCL) and in a subpopulation of INL cells. GCL – ganglion cell layer; INL – inner nuclear layer; ONL – outer nuclear layer. Scale bar: 100 μm.

as a control. Fish were examined for transgene expression 5–7 days later. No GFP expression was observed in the ganglion cell layer of control retinas (Figure 7, lower left panel). In contrast, retinas that received an optic nerve crush exhibited robust GFP expression in their retinal ganglion cells and other cells scattered throughout the inner and outer nuclear layers (Figure 7, lower right panel). Therefore, our transgenic promoter fragment contains elements mediating increased transgene expression in the regenerating CNS.

Discussion

Zebrafish have recently emerged as a preferred model system for studying vertebrate development. In addition, to developing outside the mother, being optically clear and amenable to large-scale mutagenesis, zebrafish are also able to regenerate their CNS following damage. We had previously used transient expression assays to show that our −1696α1TIpEGFP expression vector directs reporter gene expression to the developing CNS of zebrafish embryos (Hieber et al., 1998). However, because transient expression assays result in mosaic expression (average of 30 cells express the transgene per embryo), we were unable to determine if these α1 tubulin sequences recapitulated the endogenous panneuronal pattern of α1 tubulin gene expression. In addition, if not integrated into the genome, injected DNA will be lost from the cell as the fish develops precluding analysis of transgene promoter activity in the adult CNS and during CNS regeneration. To resolve these issues we generated transgenic zebrafish harboring our −1696α1TIpEGFP expression vector in their germ-line cells.

These fish exhibited a transgene pattern of expression that was remarkably similar to that of the endogenous α1 tubulin gene (Figures 1 and 2). Expression was maximal around 24 hpf when the nervous system is maximally expanding and differentiating (Ross et al., 1992). At this time transgene expression could be detected in both neural progenitors and their differentiated progeny (Figures 3–5), similar to the endogenous gene (Hieber et al., 1998). This is different from the rat α1 tubulin promotor which is preferentially expressed in the postmitotic progeny of progenitor cells (Gloster et al., 1994, 1999).

In fish, retinal progenitor cells reside in the periphery (Marcus et al., 1999). These cells are responsible for growth of the retina as fish mature and their

numbers decline as the growth of the retina slows. We observed robust transgene expression in the retinal periphery at 1 month of age (Figures 3H and I). However, by 3 months there was a dramatic decrease in the number of GFP-expressing cells in the retinal periphery (Figure 3L), reflecting the decrease in progenitor cells (Figure 6G-I).

Interestingly, we did not detect GFP positive cells in the retinal periphery at 72 hpf (Figure 3E), although bisbenzimide staining showed that this region of the retina contained cells (Figure 3D). Perhaps this indicates a different developmental potential of these cells in the 72 hpf versus 1 month old retina. One possibility is that GFP-negative cells located in the periphery of the 72 hpf retina represent multipotent neural stem cells that, as the retina matures, become progenitors whose fate is restricted to retinal neurons. Coincident with the conversion from a neural stem cell to a retinal progenitor cell is the turning on of GFP.

In the adult zebrafish, transgene expression was enriched in neural progenitor cells located in the retinal periphery, lining brain ventricles and the central canal of the spinal cord. These cells were assigned as progenitors based on the observation that they can divide and differentiate into neurons (Figure 6). These cells are responsible for growth of the nervous system as the fish mature and recovery of function following CNS damage. The ability to readily identify these cells in the mature fish nervous system and purify them provides a powerful tool for obtaining homogeneous populations of progenitor cells for studying mechanisms underlying their proliferation and differentiation.

In addition to CNS regeneration via progenitor cells, fish can also regenerate damaged adult CNS axons. In the case of optic nerve regeneration we have clearly shown that our transgene construct contains elements mediating this increased gene expression following nerve damage (Figure 7). This is the first direct demonstration that increased α1 tubulin expression in the regenerating CNS is a transcriptional response and sequences upstream of the second codon mediate this expression *in vivo*.

As the −1696α1TIpEGFP transgene is expressed in specific populations of cells at specific times of development or following injury it provides a unique *in vivo* probe for signaling mechanisms that regulate α1 tubulin gene expression. These signals are likely to be crucial for successful CNS development and regeneration and our transgenic fish allow for mechanistic evaluations in the complex environment of an intact animal. These studies along with α1 tubulin promoter mutagenesis should facilitate the identification of important regulatory elements that respond to the identified signaling cascades and facilitate identification of their *trans*-acting binding proteins. Finally, these fish will be very useful in large-scale genetic screens for molecules regulating α1 tubulin promoter activity and nervous system development.

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