GAP-43 promoter elements in transgenic zebrafish reveal a difference in signals for axon growth during CNS development and regeneration

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SUMMARY

A pivotal event in neural development is the point at which differentiating neurons become competent to extend long axons. Initiation of axon growth is equally critical for regeneration. Yet we have a limited understanding of the signaling pathways that regulate the capacity for axon growth during either development or regeneration. Expression of a number of genes encoding growth associated proteins (GAPs) accompanies both developmental and regenerative axon growth and has led to the suggestion that the same signaling pathways regulate both modes of axon growth. We have tested this possibility by asking whether a promoter fragment from a well characterized GAP gene, GAP-43, is sufficient to activate expression in both developing and regenerating neurons. We generated stable lines of transgenic zebrafish that express green fluorescent protein (GFP) under regulation of a 1 kb fragment of the rat GAP-43 gene, a fragment that contains a number of evolutionarily conserved elements. Analysis of GFP expression in these lines confirms that the rat 1 kb region can direct growth-associated expression of the transgene in differentiating neurons that extend long axons. Furthermore, this region supports developmental down-regulation of transgene expression which, like the endogenous gene, coincides with neuronal maturation. Strikingly, these same sequences are insufficient for directing expression in regenerating neurons. This finding suggests that signaling pathways regulating axon growth during development and regeneration are not the same. While these results do not exclude the possibility that pathways involved in developmental axon growth are also active in regenerative growth, they do indicate that signaling pathway(s) controlling activation of the GAP-43 gene after CNS injury differ in at least one key component from the signals controlling essential features of developmental axon growth.

Key words: GAP-43, Neuron, Regeneration, Retina, Spinal cord, Zebrafish

INTRODUCTION

The ability of postmitotic neurons to extend long axons is essential both to the establishment of neural circuitry during development, and to the regrowth of injured axons in adults. Axon elongation is generally confined to a period beginning shortly after neurons undergo their final mitosis. Once mature synapses have been formed axon growth is sharply curtailed. Although most adult neurons lose the capacity to grow long axons, in some neurons this capacity can be regained following injury. It is not known whether the pathways that govern developmental axon growth and repression also control regenerative axon growth. One possibility is that injury reverses the diminished axon growth capacity of mature neurons by re-activating the developmental program. This hypothesis is supported by the fact that many growth-associated genes that are turned off in neurons during maturation are re-expressed during regeneration (reviewed by Skene, 1989). Alternatively, developmental growth and regenerative growth may have distinct requirements.

Distinguishing between these two possibilities is crucial to our understanding of the intrinsic differences between neurons that are capable of regeneration and those that are not.

Likely targets of the signaling pathways that regulate axon growth are genes encoding proteins that are abundant in axonal growth cones but absent from mature synapses. Expression of a number of such neuronal proteins, referred to as “growth associated proteins” (GAPs), is tightly correlated with axon growth during development and regeneration (reviewed by Skene, 1989). Expression of GAP genes is initiated in post-mitotic neurons immediately prior to axon elongation, and is arrested subsequent to synapse formation. GAP gene expression can be reinitiated in response to injury in neurons undergoing regenerative growth. Given this tight correlation, it appears that signaling pathways that regulate axon growth also regulate GAP gene expression.

To investigate signaling pathways that regulate axon growth during development and regeneration, we have attempted to separate the targets of these pathways within the regulatory sequences of GAP genes. This work has focused on the
regulatory region of the GAP-43 gene, which has been extensively studied by this laboratory and others (Eggen et al., 1994; Nedivi et al., 1992; Ortoft et al., 1993; Reinhard et al., 1994; Starr et al., 1994; Vanselow et al., 1994; Weber and Skene, 1997; Weber and Skene, 1998). Dissection of the rat GAP-43 regulatory region has led to the identification of a 1 kb fragment that directs the neuron-specific expression of GAP-43 in cultured cells (Nedivi et al., 1992; Weber and Skene, 1997; Weber and Skene, 1998). This region contains two promoter regions corresponding to multiple transcriptional start sites described previously (Eggen et al., 1994).

The importance of this region is suggested by the extensive sequence conservation between the human, rat and frog promoters (Weber and Skene, 1997 and unpublished). Further evidence of functional conservation comes from analysis of mosaically expressing transient transgenic embryos, which indicate that the 1 kb promoter from the rat GAP-43 gene is sufficient to direct appropriate expression in the developing nervous system of zebrafish embryos (Reinhard et al., 1994). These assays provide a powerful screen for gross analysis of promoter function. However, the mosaic expression of transgenes in the transient transgenic assays makes this approach unsuitable for detailed analysis of specific populations of neurons, particularly in adults. We have now created stable lines of transgenic zebrafish expressing the green fluorescent protein (GFP) under the regulation of the 1 kb rat GAP-43 promoter region. These lines have allowed us to monitor discrete populations of transgene-expressing neurons over time in live developing embryos. Furthermore, these lines have allowed us to examine whether the conserved sequences within the 1 kb GAP-43 promoter also contain targets for signaling pathways that activate regenerative axon growth in adults.

MATERIALS AND METHODS

Zebrafish maintenance and surgery

The laboratory zebrafish colony was started from fish originally obtained from Carolina Biological Supply Company (Burlington, NC) and have been maintained at 28.5°C as described previously (Westerman, 1995).

Optic nerve crush lesions were performed on adult zebrafish as previously described (Bormann et al., 1998). Briefly, the left optic nerve of anesthetized fish was exposed and crushed behind the orbit using watchmaker’s forceps. The intact right optic nerve served as the unoperated control. Fish were sacrificed 4-8 days post-crush and eyes were removed with RNase-free DNase treatment. In situ hybridizations from Dr Eva Reinhard) was used to generate labeled probes for in situ hybridization. Digoxigenin-incorporated riboprobes were synthesized according to the manufacturer’s specifications (Boehringer Mannheim; Mannheim, Germany). Template DNA was subsequently removed with RNase-free DNase treatment. In situ hybridizations were carried out on whole-mount adult zebrafish retina as previously described (Thissie et al., 1993). After 1-3 hours in developing solution, samples were fixed for 30 minutes in 4% PFA, and prepared for cryosectioning as described below.

In situ hybridization

A plasmid containing zebrafish GAP-43 cDNA sequence (kind gift from Dr Eva Reinhard) was used to generate labeled probes for in situ hybridization. Digoxigenin-incorporated riboprobes were synthesized according to the manufacturer’s specifications (Boehringer Mannheim; Mannheim, Germany). Template DNA was subsequently removed with RNase-free DNase treatment. In situ hybridizations were carried out on whole-mount adult zebrafish retina as previously described (Thissie et al., 1993). After 1-3 hours in developing solution, samples were fixed for 30 minutes in 4% PFA, and prepared for cryosectioning as described below.

Immunohistochemistry

Embryos, larvae and adult retinas were prepared for cryosectioning as previously described (Barthel and Raymond, 1990). Samples were fixed overnight in 0.1 M phosphate buffer (pH 7.3) containing 4% parafomaldehyde (PFA) and 5% sucrose, and cryoprotected in increasing concentrations of sucrose with an overnight incubation in 20% sucrose. Samples were then infiltrated for 30 minutes in 2:1, 20% sucrose: OCT embedding medium (Miles Inc.; Elkhart, IN). Samples were embedded in this same infiltration medium, frozen on dry ice, and then equilibrated to -20°C. Sections, 10-20 μm thick, were collected on SuperFrost/Plus slides (Menzel-Glaser; Germany) and dried overnight at room temperature in the presence of desiccant. Sections were stained using an antibody staining protocol adapted from that of Dr Anne Unger (University of Virginia – personal communication). Sections were rehydrated in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO₄, 1.4 mM KH₂PO₄) and blocked 1 hour in TNB buffer (0.1 M Tris, pH 8.0, 0.15 M NaCl, 0.1% Tween 20, 2% BSA, 10% goat serum). Sections were incubated overnight at 4°C with primary antibody diluted in TNB. Anti-GFP (# A6455 Molecular Probes; Eugene, OR) was used at 1:500. Following several washes in TNT (0.1 M Tris, pH 8.0, 0.15 M NaCl, 0.1% Tween 20), sections were incubated for 2 hours with 2 μg/ml cy-3-conjugated goat anti-rabbit IgG (Chemicon; Temecula, CA). Sections were washed several times in TNT buffer and mounted in Vectashield with DAPI (Vector Laboratories; Burlingame, CA).
**Table 1. Tissue distribution of GFP expression in stable lines of GAP-43/GFP transgenic zebrafish**

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<th></th>
<th>*Founder #1 line #1</th>
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<td>Spinal cord</td>
<td>+++</td>
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<td>Rohon Beard sensory neurons</td>
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<tr>
<td>Interneurons</td>
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<td>2° motor neurons</td>
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<td>Brain</td>
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<td>Trigeminal ganglion</td>
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Embryos at the one-cell stage were injected with either supercoiled (*) plasmid, or plasmid linearized at a unique XhoI site (**). Out of 119 injected embryos raised to maturity, 3 males gave rise to GFP-expressing progeny. One of these founders, founder #2, gave rise to progeny with 2 distinct patterns of expression (line #2 and line #3). Transgenic embryos were visually assessed for GFP expression using fluorescence microscopy over the first 3 days postfertilization. Expression levels peaked in different cell types at different times. The table denotes peak expression levels for each cell type: ++, strongly expressing; +, weakly expressing; --, no detectable expression; EVL, enveloping layer cells.

**RESULTS**

**GFP-expressing transgenic fish lines using the rat GAP-43 promoter**

In order to investigate signaling pathways regulating neuronal growth-associated gene expression of GAP-43 during development and regeneration, we generated stable lines of transgenic zebrafish expressing GFP under the regulation of a 1 kb fragment from the GAP-43 gene promoter. Analysis of four different lines has revealed that lines 1 and 2 display prominent neural expression of GFP while lines 3 and 4 express the transgene primarily in the notochord (Table 1). Based on prior characterization in transient transgenic assays of zebrafish, the neural expression can be attributed to the 1 kb GAP-43 promoter sequences. In contrast, we have two lines of evidence indicating that the notochord expression is an artifact induced by plasmid sequences outside the transgene. First, we used transient transgenic assays to compare embryos injected with DNA containing only the transgene sequences to embryos injected with DNA containing both the plasmid and transgene sequences (black bars) or with just the transgene fragment alone (grey). The percentage of fish expressing GFP in other tissues dramatically decreased in the absence of vector sequences. A five-fold increase in notochord expression is seen when plasmid backbone sequences are included. EVL, enveloping layer cells.
GAP-43/GFP transgene is temporally regulated within neurons

During development, onset of neuronal transgene expression in lines 1 and 2 closely follows that of endogenous GAP-43, with the earliest expression observed around 18 hpf. Transgene expression on the first day postfertilization (dpf) is most prominent in the spinal cord and cranial ganglia, although sparse expression in the hindbrain and cerebellum is also observed (Fig. 3 and not shown). By the second dpf, expression has extended throughout the nervous system including retina, forebrain, midbrain, hindbrain and olfactory epithelium (Figs 2 and 3). Although expression is most prominent in the nervous system, both lines also have some expression outside the nervous system in notochord and muscle (Fig. 1). This ectopic expression is most likely due to the fact that the promoter fragment we are using is relatively small and may be missing some elements required for complete repression of extraneuronal expression (Vanselow et al., 1994). Nonetheless, the appearance of transgene expression within the nervous system, as with the endogenous GAP-43, generally coincides with neuronal differentiation. For a more detailed analysis on developmental regulation of the transgene within neurons, we focused on two regions where individual types of neurons are easily identified, the spinal cord and the retina.

The developing spinal cord in zebrafish is a region where discrete classes of neurons are easily visualized and can be distinguished by morphology and location (Bernhardt et al., 1990). Expression of the zebrafish GAP-43 gene, detected by in situ hybridization, is first observed in the spinal cord around 17 hpf when spinal neurons are first starting to extend axons (Reinhard et al., 1994). By 18 hpf, five classes of neurons are undergoing axonogenesis, including Rohon Beard sensory neurons, three classes of interneurons and the primary motor neurons (Eisen et al., 1986; Kuwada et al., 1990). Secondary motor neurons, on average, begin axonogenesis approximately 6 hours later (Myers et al., 1986). The developmental pattern of transgene expression in the spinal cord of GAP-43/GFP lines roughly follows the time course of differentiation. As early as 18 hpf transgene expression is observed in Rohon Beard sensory neurons located in the dorsal spinal cord. At these early stages, transgene expression in the muscle obscures more ventrally located neurons, but by 25 hpf, both Rohon Beard neurons and interneurons are clearly expressing the transgene (Fig. 4A,B,D,E). Interestingly, we do not generally see expression in the primary motor neurons. It is possible that the early ectopic expression in the muscles makes it difficult to detect these cells. Alternatively, expression in primary motor neurons may require promoter elements outside the 1 kb region. However, by 40 hpf, secondary motor neurons are observed in addition to the Rohon Beard and interneurons (Fig. 4C,G). As previously shown with endogenous GAP-43, by the third day of development, transgene expression in the spinal neurons is very faint or falls below the limit of detection (Fig. 4D,H).

The retina provides another ideal context for studying axon growth during development and regeneration since it contains a single population of neurons that project long axons, the retinal ganglion cells. In mammals GAP-43 expression is primarily restricted to these cells during development, but is also constitutively observed in a limited number of cells in the inner nuclear layer (Kapfhammer et al., 1997; Reh et al., 1993). We observe the same pattern in zebrafish (data not shown). Retinal ganglion cells are the first neurons to differentiate within the retina and begin to extend axons around 32 hpf (Hu and Easter, 1999; Schmitt and Dowling, 1999). By 68 hpf, differentiation of subsequent layers of retinal neurons has progressed sufficiently for the fish to have sight (Easter and Nicola, 1996). In GAP-43/GFP fish, transgene expression is observed in the ganglion cell layer beginning around 37 hpf. Interestingly, in contrast to endogenous GAP-43, which is first detected in differentiated retinal ganglion cells, we first observe transgene expression in the retina several hours prior to ganglion cell differentiation (Fig. 5A,D). Whether these early expressing cells represent a population destined to become ganglion cells, or whether expression is simply turned off in non-ganglion cells, transgene expression eventually becomes...
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localized to the retinal ganglion cell layer as differentiation progresses (Fig. 5B,E). By 48 hpf, expression is primarily localized to the ganglion cell layer where it remains even after the other neuronal layers of the retina have formed (Fig. 5C,F).

Since heavy autofluorescence of pigment cells around the eye make it difficult to detect GFP expression in live animals past 4 dpf, in older larvae we visualized transgene expression by immunostaining horizontal tissue sections taken through localizing to the retina of the ganglion cell layer in retinal ganglion cells. Transgene expression localizes to ganglion cell layer (gcl) in the retina in both line 1 (A-C) and line 2 (D-F). (A,D) At 25 hpf, transgene expression occurs in undifferentiated neural retina (nr). L, lens. (B,E) At 48 hpf, transgene expression becomes localized to the ganglion cell layer (gcl) as it develops. (C,F) At 72 hpf, transgene expression remains localized mainly to the ganglion cell layer even after other layers of differentiated neurons have formed. Scale bars, 20 µm.

Fig. 3. Activation of the GAP-43/GFP transgene in differentiating neurons. Transgene expression is primarily in cranial ganglia and spinal neurons on the 1st day postfertilization, and becomes prominent in the brain by the 2nd dpf. Examples of neuronal expression typically observed in both lines using confocal images of GFP expression in live zebrafish embryos, 25-55 hpf, immobilized in agarose. (A-D,G,H) Lateral views (dorsal up and rostral left) with the fluorescent image superimposed on the bright-field image. (E,F) Ventral views with rostral up. (A) Expression in trigeminal ganglion (tg) but not in midbrain (mb) in 30 hpf embryo (line 2). (B) Posterior lateral line ganglion (plg) and hindbrain (hb) in 30 hpf embryo (line 2). (C) Interneurons located laterally and centrally in 25 hpf spinal cord (line 1); enlarged view of interneurons and axon bundles in inset. (D) Rohon Beard sensory neurons located medially and dorsally in 25 hpf spinal cord (line 1). (E) Ganglion cell layer in retina (gcl), optic chiasm (chi) and tectum (tec) in 55 hpf embryo (line 1). (F) Forebrain with olfactory epithelium (oe) and olfactory bulb (ob) in 55 hpf embryo (line 1). (G) Hindbrain (hb) and cerebellum (cb) in 52 hpf embryo (line 1). (H) Rohon Beard sensory neurons (rb) and 2nd motor neurons (mn) in medial spinal cord of 52 hpf embryo (line 1). mus, muscle; nt, notochord; 4th vent, 4th ventricle; ov, otic vesicle. Scale bars, 20 µm.

Fig. 4. The GAP-43/GFP transgene is temporally regulated in developing spinal neurons. In both line 1 (A-D) and line 2 (E-H) transgene expression is first observed in the spinal cord at 18 hpf and mostly disappears by 70 hpf. In detail, expression is seen at 25 hpf (A,B,E,F) in (A,E) Rohon Beard sensory neurons; (B,F) primary interneurons; (C,G) at 40 hpf, Rohon Beard sensory neurons located dorsally, secondary motor neurons located ventrally and interneurons located centrally; (D,H) at 72 hpf, faintly in a few interneurons and secondary motor neurons. Dorsal is up and rostral is left. Scale bar, 20 µm.

Fig. 5. GAP-43/GFP transgene expression precedes axon growth in retinal ganglion cells. Transgene expression localizes to ganglion cell layer (gcl) in the retina in both line 1 (A-C) and line 2 (D-F). (A,D) At 25 hpf, transgene expression occurs in undifferentiated neural retina (nr). L, lens. (B,E) At 48 hpf, transgene expression becomes localized to the ganglion cell layer (gcl) as it develops. (C,F) At 72 hpf, transgene expression remains localized mainly to the ganglion cell layer even after other layers of differentiated neurons have formed. Scale bars, 20 µm.
the eyes of fixed animals. Antibody staining against GFP clearly identifies cells in the ganglion cell layer through 6 dpf. However, by 8 dpf staining in the retina is undetectable (Fig. 6). Thus, as observed in spinal neurons, developmental down-regulation of the transgene is also observed in the retina. Taken together, these analyses demonstrate that like the endogenous promoter, the 1 kb fragment of the rat GAP-43 promoter used in these studies is sufficient to both promote transgene expression in distinct populations of developing neurons, and to arrest transgene expression as these neurons mature.

1 kb fragment is not sufficient to drive transgene expression in regenerating retina

It has previously been demonstrated that GAP-43 expression can be induced in retinal ganglion cells after crushing the optic nerve, maintaining maximal levels in zebrafish between 4 and 8 days post-crush (Bormann et al., 1998). Given that the relatively small promoter fragment in our transgenic lines contains elements that promote the appropriate localization and down-regulation of expression in retinal ganglion cells, these fish provided the necessary tool to investigate the sufficiency of these elements for regeneration-induced expression. We compared expression of transgene and endogenous GAP-43 following optic nerve crush. In both lines, we found that the regenerating ganglion cells clearly expressed the endogenous gene while the transgene remained undetectable in these cells (Fig. 7). We do detect occasional inner nuclear layer cells that constitutively express GFP in both control and regenerating retina demonstrating this assay is capable of identifying expressing cells.

Using RT-PCR we again see a strong induction of the endogenous GAP-43 gene, but fail to detect induction of transgene in regenerating retina (Fig. 8). We detect faint bands for the transgene in control and regenerating retina indicating that this assay is sensitive enough to detect trace amounts of expression (Fig. 8A). In embryos, we detect a strong band for the transgene in control retina confirming that our reaction conditions are not saturating (Fig. 8A). Thus even though zebrafish contain the signaling pathways necessary to clearly activate the rat 1 kb promoter in neurons undergoing developmental axon growth, in two separate lines, they fail to induce expression from this same promoter in neurons undergoing regenerative axon growth. These results strongly argue that the pathways used in development are not sufficient for regeneration.

DISCUSSION

The extent to which axon regeneration simply recapitulates the events of development has been the subject of much speculation. However, while the fundamental mechanisms

Fig. 6. The GAP-43/GFP transgene is developmentally down regulated in retina. (A,C) 65 hpf and (B,D) 8 dpf larvae were fixed overnight in 4% PFA. 10 μm horizontal sections through the head of fixed larvae were immunostained with α-GFP/cy3 (A,B). Sections were also stained with DAPI (C,D) to show nuclear layers of retina. This result is observed in both lines 1 and 2. Line 1 is shown. gcl, retinal ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer. Scale bar, 20 μm.

Fig. 7. A 1 kb fragment is not sufficient to drive transgene expression in regenerating retina. The left optic nerve of adult transgenic fish was crushed using watchmakers forceps (A,C,E). Right eye served as unoperated control (B,D,F). Animals were sacrificed 8 days post-crush and eyes were removed. The lens was removed and the remainder of the eye was fixed overnight in 4% PFA. A wedge from each eye was cut out for in situ hybridization analysis with a zebrafish mRNA GAP-43 probe. In situ-stained tissue was refixed and sectioned (A,B). The remainder of the eye was cryosectioned and immunostained with α-GFP/cy3 (E,F) and counterstained with DAPI (C,D). In situ hybridization shows: (A) endogenous GAP-43 expression in the gcl induced by optic nerve crush, but (B) no endogenous GAP-43 mRNA in unoperated control retina. Immunostaining shows no transgene expression in gcl of either regenerating (E), or control (F) retina. These results are observed in both lines 1 and 2. Line 1 is shown. Asterisks indicate inl cells positive for endogenous GAP-43 or the transgene. gcl, retinal ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer. Scale bar, 20 μm.
of axon growth and guidance have been highly conserved throughout metazoan evolution, there has been a profound divergence of mechanisms controlling axon regeneration over the last 400 million years of vertebrate evolution. This divergence is evidenced by the fact that interruption of CNS axons in fish and amphibians leads to robust regrowth and functional recovery, while axons in the brains or spinal cords of birds and mammals rarely regenerate.

The GAP-43 gene illustrates both the conservation of axon growth mechanisms, and the evolutionary divergence in the regulation of these mechanisms in the adult CNS. In all vertebrates, the GAP-43 gene is activated in differentiating neurons shortly before the onset of axon extension, providing one of the major protein constituents of axonal growth cones (Jacobson et al., 1986). Like many other growth cone components, GAP-43 is then downregulated as axon growth is completed and neurons mature. Subsequent regeneration of adult axons is linked to re-expression of GAP-43 and other growth-associated proteins (reviewed by Skene, 1989), indicating that regeneration does employ growth cone components involved in developmental axon outgrowth. In contrast to fish and amphibians, however, interruption of CNS axons in mammals activates the GAP-43 gene only under limited circumstances, and then in relatively few cells (Doster et al., 1991; Fernandes et al., 1999; Schaden et al., 1994). Thus what has diverged in vertebrate evolution are the conditions under which the GAP-43 gene and neuronal competence for axon regeneration are re-activated in adult neurons. If re-activation of growth-associated genes in the adult CNS is mediated by the same signaling pathways that control their developmental expression, then the differential responses of the GAP-43 gene to CNS injury in fish and mammals would have to reflect a fundamental evolutionary divergence in the developmental regulation of axon growth.

Our results show, however, that the molecular signals involved in the onset and later decline in GAP-43 expression during embryonic development are very highly conserved between fish and mammals. We have demonstrated that as little as 1 kb of the 5′ flanking sequences from a mammalian GAP-43 gene can direct preferential expression in developing neurons in zebrafish. Some extraneuronal expression is observed as previously noted in transgenic mice using such a small promoter fragment (Vanselow et al., 1994). The predominant expression, however, is neuronal and more importantly, is restricted to specific populations of neurons at developmentally appropriate times. These results imply that conserved elements with the 1 kb fragment are sufficient for these aspects of GAP-43 gene regulation and that the signaling pathways that converge on these elements are also strongly conserved between fish and mammals.

In contrast, both immunostaining and RT-PCR showed that optic nerve injury fails to activate transgene expression in regenerating retinal ganglion cells, even under conditions in which the endogenous GAP-43 gene is strongly induced. Confirmation of this finding in two independent lines of transgenic fish suggests that the failure of transgene induction does not arise from a specific positional effect at the transgene’s integration site. It is also unlikely that more widespread inactivation of chromatin can explain the lack of transgene induction in adult fish. Previous studies from Goldman and colleagues, using a 1.7 kb promoter fragment from the goldfish alpha-1 tubulin gene, showed that optic nerve injury could elicit strong re-expression of an EGFP reporter gene in retinal ganglion cells in 4 out of 4 independent lines of transgenic zebrafish (Goldman and Ding, 2000; Goldman et al., 2001). The absence of transgene induction in our fish under similar conditions therefore appears to reflect a specific inability of the GAP-43 1 kb promoter region to respond to signals responsible for induction of the endogenous GAP-43 gene after axon injury.

The fact that the rat 1 kb promoter is capable of gene activation and repression during zebrafish development suggests that the signaling pathways governing these aspects of regulation are conserved between fish and mammals. However given that this fragment is not capable of re-activating expression during regeneration we conclude that the signaling pathways leading to gene activation in developing neurons are not sufficient for activation in regenerating neurons. It is possible that sequences required for regenerative expression are contained within the 1 kb fragment, but that they are not
as well conserved as the sequences required for developmental activation. This difference may involve entirely novel transcription factor binding sites or could be the result of small variations in the sequences of known binding sites, such as AP-1 and E-box, that bind heterodimeric complexes. In this scenario, the sequences would be conserved enough to bind the developmentally active heterodimeric complex, but sufficiently divergent to prevent binding of the complex active in regenerating neurons. It is equally possible that sequences required for regenerative expression, conserved or otherwise, are in a completely different region of the gene. Nevertheless, our results indicate that signaling pathway(s) controlling activation of the GAP-43 gene after CNS injury differ in at least one key component from the signals controlling essential features of developmental axon growth.

Our results show that the developmental regulation of the GAP-43 gene is tightly conserved and can be maintained, even when signaling elements required for activation after CNS injury have been lost or altered in evolution. Thus we conclude that the control of growth-associated gene expression in the adult CNS is free to change over the course of vertebrate evolution without disrupting the more fundamental control of axon growth during development.

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