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3.6 kb genomic sequence from *Takifugu* capable of promoting axon growth-associated gene expression in developing and regenerating zebrafish neurons

Ava J. Udvardia

University of Wisconsin – Milwaukee, Department of Biological Sciences, Great Lakes WATER Institute UWM-GLWI, 600 E. Greenfield Ave., Milwaukee WI 53204, email: audvardia@uwm.edu, telephone: 414-382-1750, fax: 414-382-1705

Abstract

Unlike mammals, fish have the capacity for functional adult CNS regeneration, which is due, in part, to their ability to express axon growth-related genes in response to nerve injury. One such axon growth-associated gene is *gap43*, which is expressed during periods of developmental and regenerative axon growth, but is not expressed in CNS neurons that do not regenerate in adult mammals. We previously demonstrated that *cis*-regulatory elements of *gap43* that are sufficient for developmental expression are not sufficient for regenerative expression in the zebrafish. Here we have identified a 3.6 kb genomic sequence from *Fugu rubripes* that can promote reporter gene expression in the nervous system during both development and regeneration in zebrafish. This compact sequence is advantageous for functional dissection of regions important for axon growth-associated gene expression during development and/or regeneration. In addition, this sequence will also be useful for targeting gene expression to neurons during periods of growth and plasticity.

Keywords

GAP-43; axon growth; gene expression; neuronal development; regeneration; adult neurogenesis; transgenic zebrafish; *fugu*; CNS; PNS; GFP; reporter gene

1. Results and Discussion

Gap43 is a well-characterized gene associated with axon growth during development that is also expressed following injury in neurons capable of regeneration. Previously, it was shown in transgenic reporter mice that a 17 kb sequence, including 5' flanking and intronic sequences, from the rat *gap43* gene was sufficient to recapitulate the spatio-temporal pattern of gene expression observed for the endogenous *gap43* gene in developing neurons and in adult PNS neurons after peripheral nerve injury (Vanselow et al., 1994). Subsequently, we demonstrated that as little as 1 kb of the rat *gap43* 5' flanking sequences was sufficient to promote neuron-specific reporter gene expression in developing zebrafish as well as subsequent down-regulation of expression in the mature CNS of zebrafish, similar to the expression pattern of the endogenous *gap43* (Udvardia et al., 2001). However, we discovered that this 1 kb region was not sufficient for *gap43* re-expression in regenerating zebrafish neurons. These results

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demonstrated that sequences regulating developmental expression of *gap43* were evolutionarily conserved, but were distinct from sequences required for regenerative expression.

In contrast to mammals, adult teleosts have a remarkable capacity for functional recovery after sustaining CNS injuries (Becker and Becker, 2007; Becker et al., 1997). Several factors contribute to the limited regenerative potential in mammals including the inability of mammalian CNS neurons to re-express genes that are required for axon growth. This difference in ability to re-express axon growth genes could be a result of differences in a number of components of gene regulatory pathways including the regulatory sequences found in the non-coding regions of DNA surrounding genes, the transcription factors that recognize these sequences, or the signaling pathways that mediate transcription factor activity. Here we describe the discovery of a 3.6 kb *gap43* promoter/enhancer sequence from the pufferfish, *Takifugu rubripes* (fugu), which is capable of recapitulating all aspects of the endogenous pattern of *gap43* expression in developing, mature, and regenerating neurons in zebrafish.

1.1 Evolutionary comparisons of the *gap43* locus

In order to identify the smallest promoter/enhancer region capable of promoting *gap43* gene expression in developing and regenerating neurons, we compared *gap43* cDNA and genomic sequences from a variety of vertebrate species. A comparison of the predicted GAP-43 protein sequences shows that the amino-terminal 60 amino acids are highly conserved across fish, amphibians, birds, and mammals (Fig. 1; LaBate and Skene, 1989). This high degree of sequence conservation allowed us to easily identify the entire *gap43* gene within publicly available genomic sequences of a number of species. This analysis indicates that *gap43* is present as a single copy gene in all the species examined except in *Xenopus laevis* where it was previously shown to be duplicated (Shain et al., 1995).

In order to facilitate subsequent analysis, we compared genomic regions surrounding *gap43* in different vertebrates to identify the species with the most compact potential regulatory region (i.e. 5' flanking region and first intron). These criteria were met by a species of pufferfish, *Takifugu rubripes* (fugu). Pufferfish genomes are several-fold smaller than those of other vertebrates, yet contain similar numbers of genes (Elgar, 1996). This is due to shorter introns and intergenic regions of the pufferfish genome (McEwen et al., 2006). Comparison of the genomic structure within and surrounding the *gap43* gene from several vertebrate species revealed that in pufferfishes (fugu and *Tetraodon nigroviridis*) the distance from the 5' end of the *gap43* gene to the end of the next upstream coding sequence is approximately 1/70 the size of the equivalent region in the chicken genome and less than 1/100 the size of the region in mammalian genomes (Fig. 2). In addition, the total length of the *gap43* gene, including exons and introns, is 13 times shorter in pufferfishes than in mice or humans. Together these features indicated that fugu had the shortest potential *gap43* regulatory region and was thus chosen for the subsequent analyses.

1.2 Fugu *gap43/gfp* transgene expression mimics endogenous *gap43* expression in the developing zebrafish nervous system

In order to track the temporal and spatial regulation of gene expression by the fugu *gap43* promoter/enhancer region, we used regions 5' to the fugu *gap43* gene to drive the expression of a reporter gene encoding Green Fluorescent Protein (GFP). A search of fugu Bacterial Artificial Chromosome (BAC) library sequences yielded 3 BAC clones that contained fugu *gap43* exon 1 and surrounding sequences. Using PCR, we amplified and cloned a 3.6 kb sequence that encompasses most of the region flanking the 5' end of *gap43*, as well as exon 1, which encodes the first 10 amino acids of the protein. The N-terminal 10 amino acid region contains two cysteine residues that are palmitoylated, which promote the association of

GAP-43 with the cell membrane (van't Hof and Resh, 1997). The 3.6 kb DNA fragment was subcloned into a plasmid upstream and in frame with the GFP gene. The resulting plasmid (GfG43SA) encodes a GFP fusion protein that is targeted to cell membranes and allows detection of both cell bodies and neuronal processes.

Using the GfG43SA plasmid, we created two stable transgenic lines carrying the 3.6 kb fugu *gap43* promoter fragment (*tg(3.6fgap43:GFP)^{SA1}* and *tg(3.6fgap43:GFP)^{SA2}*). Both stable transgenic lines exhibited robust expression of the transgene throughout the developing nervous system (Fig. 3 and Fig. 4. Note: images throughout the results section are of *tg(3.6fgap43:GFP)^{SA1}* fish, however, similar expression was observed in both lines). We analyzed GFP expression using wide field fluorescence and confocal microscopy in live zebrafish embryos and larvae from 17 hours post fertilization (hpf) through 8 days post fertilization (dpf). In addition to neuronal GFP expression, there was some GFP in the enveloping layer (EVL) at earlier stages (17–27 hpf). However, after this time, GFP was restricted to the nervous system. Neuronal expression of GFP was observed as early as 17 hpf in spinal neurons (data not shown). By 48 hpf, expression extended throughout the nervous system (Fig. 3). Similar expression patterns were found in both lines, indicating the 3.6 kb fugu promoter/enhancer fragment is sufficient to direct expression in the developing nervous system.

We also confirmed that transgene expression followed the same spatial and temporal pattern of endogenous *gap43* during embryonic and larval development. We compared the spatial pattern of GFP expressed from the transgene with normal *gap43* mRNA expression at 24, 48, and 72 hpf using *in situ* hybridization. There was a clear correlation between transgene expression (GFP fluorescence) and endogenous *gap43* staining in many structures and at numerous stages of development (Fig. 4). At 24 hours, expression was most prominent in the peripheral nerve ganglia, spinal cord, and hindbrain, although there were also GFP- and *gap43*-expressing neurons in the midbrain (Fig. 4 A–H). By 48 hpf, there was robust expression in the hindbrain, midbrain, olfactory epithelium, and retinal ganglion cell layer (Fig. 4 I–L). By 72 hpf, expression remained prominent throughout the brain, but decreased in the ganglion cell layer (GCL) of the retina (Fig. 4 M–P).

In the spinal cord, high levels of GFP expression were observed in longitudinal axonal tracts throughout the eight-day period of observation (Fig. 5 and data not shown). Expression in spinal motor neuron axon bundles exiting the spinal cord was low at 24 hpf (5A), but increased over time (5B, C) corresponding to an increase in the number of motor neurons extending axons into the periphery at this time. Conversely, expression in the membranes surrounding the spinal neuron cell bodies was prominent at 24 and 48 hpf and diminished significantly by 96 hpf (Fig. 5). Expression of endogenous *gap43* mRNA also decreases over time in the developing spinal cord (Reinhard et al., 1994). Thus, as with the endogenous *gap43*, transgene expression peaks in neurons shortly after differentiation and diminishes as neurons mature.

1.3 Fugu *gap43/gfp* transgene expression in developing and regenerating retina

In retina, the transgene and *gap43* are transiently expressed and temporally regulated during development and continue to be expressed near regions of ongoing neurogenesis in the adult. Endogenous zebrafish *gap43* transcription is temporally regulated during development of retinal ganglion cells, and is down regulated in the adult retina (Udvadia et al., 2001). In *tg(3.6fgap43:GFP)* transgenic lines, strong GFP expression in the retina became apparent around 30 hpf when the first postmitotic retinal neurons appear (Hu and Easter, 1999). By 48 hpf, GFP was expressed in cell bodies throughout the retinal ganglion cell layer (GCL, Fig. 6B,C) and in the optic nerves (Fig. 4K). By 4 dpf expression was no longer observed in the GCL, but was prominent in the inner plexiform layer (IPL) and in some cells within the inner nuclear layer INL (Fig. 6E,F). In the adult retina we observed strong expression in the IPL and INL near the

ciliary marginal zone (CMZ) (Fig. 6H, I), a site of continuous neurogenesis in anamniotes (Johns, 1977). Weak expression in the IPL and in sporadic cells of the INL also occurs in the central retina (Fig. 6K,L). We previously showed *gap43* mRNA expression in the INL of the adult zebrafish retina (Udvardia et al., 2001). Others have described constitutive *gap43* expression within the mammalian IPL corresponding to a population of tyrosine hydroxylase (TH)-positive or dopaminergic amacrine cells (Kapfhammer et al., 1997). Thus, transgene expression closely parallels that of *gap43* in the developing and adult retina.

The *fugu* transgene also mirrors the temporal pattern of expression of endogenous *gap43* in the regenerating retina. *Gap43* is upregulated in damaged, adult CNS neurons that are capable of mounting a regenerative response. In zebrafish, *gap43* expression peaks 4 days after optic nerve crush and remains at elevated levels for 8 days after crush (Bormann et al., 1998). We previously reported that *gap43/gfp* transgenic lines with a truncated rat *gap43* promoter, which was capable of promoting developmental expression, did not express the transgene after optic nerve crush (Udvardia et al., 2001). In contrast, high levels of GFP were present 4–8 days after optic nerve crush in lines containing the *fugu* promoter/enhancer (Fig. 6N, O; 7 days post crush shown). Together, these results indicate that sequences in the 3.6 kb promoter/enhancer are sufficient to recapitulate the spatial and temporal pattern of endogenous *gap43* in regenerating as well as developing neurons.

The regulatory region of the *gap43* gene contains conserved sequences that are activated differentially during development and regeneration. Previously we demonstrated that *cis*-elements regulating *gap43* gene expression during development were distinct from those regulating regenerative expression, suggesting that the different elements became unlinked during vertebrate evolution. Here we have described a 3.6 kb *fugu gap43* promoter/enhancer sequence capable of regulating reporter gene expression during both development and regeneration, in a pattern that mimics endogenous *gap43* in zebrafish. A reporter gene regulated by the 3.6 kb fragment is expressed in newly differentiating neurons of the embryo as well as in regenerating retinal ganglion cells in the adult after optic nerve crush.

This relatively compact sequence will facilitate the dissection of promoter/enhancer elements that are necessary and/or sufficient for expression in the developing versus regenerating nervous system. Furthermore, this sequence will be useful for overexpression and misexpression of genes in fish specifically in neurons that are actively undergoing developmental or regenerative axon growth.

2. Methods

Zebrafish husbandry and all experimental procedures were approved by the *Institutional Animal Care and Use Committee* (IACUC). Zebrafish colonies were maintained as previously described (Westerfield, 2000). Adult fish were housed in recirculating rack systems (Aquatic Habitats and Marine Biotech) at 28.5°C on a 14 hour light, 10 hour dark cycle, and fed twice daily with brine shrimp (*Artemia*) and/or TetraMin flakes. Fry were raised in an incubator under the same temperature and light conditions as the adults, in static tanks with daily water changes for two-three weeks, and fed with dry larval food and *Artemia*.

2.1 Reporter Gene Constructs and Transgenesis

A Green Fluorescent Protein (GFP) reporter plasmid was constructed by subcloning the EGFP coding sequence (Clontech) into the pW1 cloning vector which contains the SV40 *small t* intron and poly adenylation site (Linney and Udvardia, 2004). A search of the *fugu* Bacterial Artificial Chromosome (BAC) library sequences ("Fugu Assembly Release 2"; <http://fugu.biology.qmul.ac.uk/>), yielded 3 BAC clones that contained *fugu* GAP-43 exon 1 and promoter sequences. Using the *fugu* BAC clones as templates, the polymerase chain

reaction (PCR) was used to amplify a 1.7 kilobase (kb) sequence flanked by Sal I and Bgl II restriction sites and the downstream 1.9 kb sequence flanked by Bgl II and Age I restriction sites. These two fragments were joined together at the Bgl II restriction site to form a 3.6 kb fragment that included promoter/enhancer sequences and exon 1 sequences. This sequence was subcloned in-frame into pW1EGFP with naturally occurring restriction sites, Sal I and Age I. The resulting plasmid (pW1GfG43SA) was verified by sequencing.

Initial analysis of transgene expression patterns was carried out using transient transgenic assays of 24–30 hour old embryos injected at the 1-cell stage with linearized transgene DNA. GFP expression was visualized and documented in live embryos using fluorescence microscopy (Nikon TE 2000 inverted microscope; see below). We generated stable transgenic lines by injecting purified, linear transgene DNA into the yolk-free cytoplasm of 1-cell stage embryos (Linney and Udvardia, 2004). Injected embryos were raised to maturity. Progeny from injected fish were screened for GFP expression using fluorescence microscopy to test for germline transmission of the transgene.

2.2 Visualization and Analysis

In situ hybridization—A plasmid containing zebrafish GAP-43 sequences (gift of Dr. Eva Reinhard) was used to generate labelled probes for non-radioactive *in situ* hybridization. Digoxigenin-incorporated riboprobes were synthesized according to the manufacturer's specifications (Roche; Basel, Switzerland). Template DNA was subsequently removed with RNase-free DNase treatment. *In situ* hybridizations were carried out on whole mount zebrafish embryos as previously described (Thisse et al., 1993). After 1–3 hours in developing solution, samples were fixed for 30 minutes in 4% paraformaldehyde, and prepared for digital imaging.

Optic nerve crush and histology—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 for 10 seconds using #5 forceps. The intact right optic nerve served as the unoperated control. Fish were sacrificed 4–8 days after optic nerve crush. Eyes were dissected and fixed overnight in 4% paraformaldehyde and prepared for frozen sectioning as previously described (Barthel and Raymond, 1990). 20 micron sections were collected on Superfrost plus slides, dried at room temperature and mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA).

Microscopy and Image analysis—Fixed embryos subjected to *in situ* hybridization were de-yolked and immersed in glycerol for photography. Stained embryos were analyzed using a dissecting scope (Olympus SZX12) equipped with a cooled CCD color camera (Olympus DP70). Live imaging of transgenic fish was carried out on specimens from the F2–F6 generation. Embryos were anesthetized in Tricaine and embedded in 0.5% agarose to immobilize them for image capture using an upright laser confocal scanning microscope (Leica TLS SP2), or an inverted fluorescence microscope (Nikon TE 2000) equipped with a cooled CCD camera (Photometrics Cool Snap ES, Roper Scientific) using Metamorph (Molecular Devices, Sunnyvale, CA) software for image capture and analysis. Images of retinal sections from adult fish were also captured and analyzed using the same procedure. Images of GFP fluorescence and DAPI staining in sections were merged in Metamorph. Images of the whole embryos (Fig. 3) were captured as series of images using standard settings for each specimen to maintain relative differences in fluorescence intensity within the specimen. Images were assembled in Photoshop to form a montage.

2.3 Bioinformatics

In order to find the known or predicted amino acid sequences of GAP-43 homologues from pufferfishes, medaka fish, goldfish, African clawed frog, chicken, mouse, rat and human, we

used the zebrafish *gap43* cDNA sequence (accession number [NM 131341](#)) to compare the publicly available sequences databases at NCBI and Ensembl (<http://www.ncbi.nlm.nih.gov/BLAST/>; <http://www.ensembl.org/index.html>) using BLAST (basic local alignment search tool). GAP-43 amino acid sequences were compared using MultAlin (<http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>; (Corpet, 1988). Genomic organization of the *gap43* gene was compared between seven species, *Takifugu rubripes* (Japanese pufferfish; FUGU assembly 4.0), *Tetraodon nigroviridis* (Green spotted pufferfish; TETRAODON assembly 7), *Oryzias latipes* (medaka; MEDAKA assembly 1), *Gallus gallus* (chicken; assembly WASHUC2), *Mus musculus* (mouse; NCBI assembly m36), *rattus norvegicus* (rat; RGSC assembly 3.4) and *Homo sapiens* (human; NCBI assembly m36), using the Ensembl genomic databases. In some cases, annotation of exons within the *gap43* genomic regions in Ensembl genomic sequence databases did not match the known cDNA sequence, therefore *gap43* orthologues for the different species were identified using the *gap43* cDNA sequences to query species specific genomic sequence databases at the Ensembl website using BLAST. A similar method was used to identify *eval* and *zbtb20* in medaka and chicken respectively.

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Fig. 1. Amino terminus sequence of the GAP-43 protein is highly conserved among vertebrates
 A. The N-terminal 60 amino acids are highly conserved among GAP-43 homologues from fish (1–5), frog (6), chicken (7), and mammals (8–10). Red = conserved across species. Blue = conserved in 5 or more species. Black = conserved in less than 5 species. B. Dendrogram generated using MultAlign based on GAP-43 amino acid sequence similarities.

A.

B.

	5' intergenic	intron 1	total gene	3' intergenic
fugu	-	-	-	-
tetraodon	1	1	1	1
medaka	7	1	1	4
chicken	74	6	7	3
mouse	129	9	12	14
rat	308	9	13	12
human	115	10	13	12

Fig. 2. Genomic organization of *gap43*

A. Orientation of *gap43* and flanking genes (*eva1*, *zbtb20*, and *lsamp*) are indicated by the arrows. The total length (in kilobases) of *gap43* as well as the length of the first intron of *gap43* is indicated below the yellow arrow representing *gap43*. Distances between *gap43* and flanking genes on both the 5' and 3' side are indicated below the dotted lines that separate *gap43* and *eva1* (blue), *zbtb20* (green), or *lsamp* (orange). Distances determined by comparison of genomic sequences in Ensembl databases. B. Size comparison of 5' and 3' *gap43* flanking regions, intron 1, and the total gene relative to fugu *gap43*.

Fig. 3. Fugu *gap43* promoter drives expression in newly differentiating neurons of the embryo 48 hpf zebrafish embryos: A. Yolk autofluorescence of non-transgenic zebrafish; B. *tg (3.6fgap43:GFP)^{SA1}* transgenic zebrafish displays transgene (GFP) expression throughout the developing nervous system: retina (ret), olfactory epithelia (olf), optic tectum (tec), hindbrain (hb), and spinal cord (sc). Scale bar = 200 μ m.

Fig. 4. Fugu *gap43* promoter mimics endogenous *gap43* expression pattern in developing embryos
 A–C, E, and G show neurons from 24 hpf *tg(3.6fgap43:GFP)^{SA1}* embryos expressing GFP in the midbrain (mb) and hind brain (hb), as well as neurons in the trigeminal ganglion (tg), and spinal cord (sc). Expression of endogenous *gap43* determined by in situ hybridization show similar patterns of expression (D, F, H). 48 hpf: expression in the retina(r), cerebellum (cb), hindbrain (hb), olfactory bulb (ob), optic chiasm (oc), optic tectum (ot) observed for transgene (I, K) and endogenous *gap43* (J, L). 72 hpf: expression in the retina (r), hindbrain (hb), optic tectum (ot) observed for transgene (M–O) and endogenous *gap43* (P). A–F, I, J; lateral views. G, H, M–P; dorsal views. K, L; ventral views. Scale bars = 100 μ m.

Fig. 5. Transgene expression in spinal neurons is developmentally regulated
 Lateral view of maximum projections of confocal stacks taken through *tg* (*3.6fgap43:GFP*)^{SA1} spinal cord at 24, 48, and 96 hpf. **A.** Motorneurons (**mn**), interneurons (**in**), and sensory neurons (**sn**), as well as longitudinal axonal tracts (**lat**) are observed at 24 hpf. Signal from axons of motorneurons exiting the spinal cord is weak, but increases over time as the number of axons increase (**arrowheads**). **B.** GFP-expressing cell bodies are observed throughout the dorso-ventral axis of the spinal cord, and axons of motorneurons (**arrowheads**) are observed exiting the spinal cord at 48 hpf. **C.** By 96 hpf, expression in the cell bodies of spinal neurons is diminished, but longitudinal axonal tracts and axons of

motoneurons exiting the spinal cord continue to express GFP. Arrowheads indicate motoneuron axons exiting the spinal cord. Scale bar = 100 μ m.

Fig. 6. Transgene expression is reinitiated in retinal ganglion cells following optic nerve injury
 Transgene expression in sectioned retinas from developing retinas (sagittal sections: A–C, 24 hpf; D–F, 4 dpf) and adult retina (cross sections: control, G–L; regenerating, M–O). DAPI staining (A, D, G, J, M), GFP fluorescence (B, E, H, K, N), and merged images (C, F, I, L, O) are shown. Transgene expression is localized to the differentiated ganglion cell layer (GCL, arrows) at 48 hpf (B, C). By 96 hpf all layers of the retina have formed and expression is localized to the inner plexiform layer (IPL), with a few cells in the inner nuclear layer (INL, asterisk in E) and absent from the GCL (arrows; E, F). In control adult retina (G–L) GFP expression remains localized to the IPL (K, L) and a few cells in the INL (asterisk) and absent from the GCL (arrows). There is also bright expression at the retinal margin (arrowheads H,

I). High levels of GFP expression are observed in the GCL 7 days after optic nerve crush (**arrows** N, O). **ONL**, outer plexiform layer. Scale bars = 100 μ m.