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Laser-induced gene expression in specific cells of transgenic zebrafish

Mary C. Halloran[1,*], Mika Sato-Maeda[2], James T. Warren, Jr[1,‡], Fengyun Su[1], Zsolt Lele[3,§], Patrick H. Krone[3], John Y. Kuwada[1,¶] and Wataru Shoji[2]

[1]Department of Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA
[2]Institute of Development, Aging, and Cancer, Tohoku University, Sendai 980-8575, Japan
[3]Department of Anatomy and Cell Biology, University of Saskatchewan, Saskatoon, Saskatchewan Canada S7N 5E5
*Present address: Department of Zoology, University of Wisconsin, Madison, WI 53706, USA
‡Present address: Division of Science, Behrend College, Penn State University Erie, Erie, PA 16563-0203, USA
§Present address: Max-Planck-Institute for Immunbiology, D-79104 Freiburg, Germany
¶Author for correspondence (e-mail: kuwada@umich.edu)

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SUMMARY

Over the past few years, a number of studies have described the generation of transgenic lines of zebrafish in which expression of reporters was driven by a variety of promoters. These lines opened up the real possibility that transgenics could be used to complement the genetic analysis of zebrafish development. Transgenic lines in which the expression of genes can be regulated both in space and time would be especially useful. Therefore, we have cloned the zebrafish promoter for the inducible *hsp70* gene and made stable transgenic lines of zebrafish that express the reporter green fluorescent protein gene under the control of a *hsp70* promoter. At normal temperatures, green fluorescent protein is not detectable in transgenic embryos with the exception of the lens, but is robustly expressed throughout the embryo following an increase in ambient temperature. Furthermore, we have taken advantage of the accessibility and optical clarity of the embryos to express green fluorescent protein in individual cells by focussing a sublethal laser microbeam onto them. The targeted cells appear to develop normally: cells migrate normally, neurons project axons that follow normal pathways, and progenitor cells divide and give rise to normal progeny cells. By generating other transgenic lines in which the *hsp70* promoter regulates genes of interest, it should be possible to examine the in vivo activity of the gene products by laser-inducing specific cells to express them in zebrafish embryos. As a first test, we laser-induced single muscle cells to make zebrafish Sema3A1, a semaphorin that is repulsive for specific growth cones, in a *hsp70-sema3A1* transgenic line of zebrafish and found that extension by the motor axons was retarded by the induced muscle.

Key words: Transgenic, Green fluorescent protein, Heat-shock gene promoter, Zebrafish, Semaphorin

INTRODUCTION

The zebrafish embryo has proven to be useful for examining a variety of different issues in developmental biology, both through genetic screens (Streisinger et al., 1981; Driever et al., 1996; Haffter et al., 1996) and manipulation through the generation of transgenic fish (Stuart et al., 1990; Culp et al., 1991; Bayer and Campos-Ortega, 1992; Lin et al., 1994; Amsterdam et al., 1995; Long et al., 1997; Higashijima et al., 1997).

In transgenic organisms, transgenes can be regulated by cell- or tissue-specific promoters or induced by promoters such as those of heat-shock protein (*hsp*) genes. The former is attractive because transgenes can be controlled in specific and intricate patterns, but require the cloning of appropriate promoters for each pattern of interest. The latter has the advantage that transgenes can be induced at a specified time, although spatial regulation is difficult to achieve by the usual heat-shock methods. Spatial control can be achieved in *hsp*-promoter-driven transgenic organisms using localized sources of heat. This was demonstrated by Monsma et al. (1988) by applying a heated needle to a region of *Drosophila* embryos transformed with a *hsp26-lacZ* transgene. More recently single-cell induction of transgenes was achieved by using a low-intensity laser microbeam focussed onto individual cells in both *Caenorhabditis elegans* and *Drosophila* (Stringham and Candido, 1993; Harris et al., 1996; Haflon et al., 1997). This strategy for targeting gene expression may be especially important for vertebrate embryos since methods for directed gene expression are more difficult in vertebrates than in *C. elegans* and *Drosophila*. The laser induction method is particularly useful in zebrafish, as it is accessible at all stages of development, optically clear and develops at a fast pace. Specific cells in living zebrafish embryos have been laser ablated under direct visual control (Eisen, 1991; Bernhardt et al., 1992; Hatta, 1992; Pike et al., 1992; Greenspoon et al., 1995).

To see if one could direct gene expression by focussing a

sublethal laser microbeam onto specific cells in zebrafish embryos, we cloned the promoter for a zebrafish *hsp70* gene that shows little to no expression at their normal temperature (28.5°C) but is robustly induced at 37°C (Lele et al., 1997). Furthermore, we generated transgenic lines of zebrafish in which the *hsp70* promoter controlled the expression of the green fluorescent protein (EGFP) gene (Chalfie et al., 1994; Cormack et al., 1996). Using these lines, we specifically induced EGFP in single cells with a sublethal laser microbeam. The targeted cells developed normally: cells migrated normally, neurons projected axons normally, and progenitor cells divided and gave rise to progeny cells normally. These results suggest that laser induction of gene expression should be a useful tool for examining the in vivo activity of genes in zebrafish embyros. Therefore, we examined the action of zebrafish Sema3A1 (formerly Sema Z1a), a secreted semaphorin that is repulsive to specific growth cones and hypothesized to be repulsive to spinal motor axons (Shoji et al., 1998; Yee et al., 1999). Motor axons were retarded by individual muscle fibers laser-induced to express Sema3A1 in a transgenic line in which the *hsp70* promoter regulated the expression of *sema3A1*.

MATERIALS AND METHODS

Zebrafish

Zebrafish embryos were collected from a laboratory breeding colony and kept at 28.5°C as previously described (Westerfield, 1995). Embryos were staged according to hours postfertilization (hpf).

Isolation of the zebrafish *hsp70* promoter and generation of the expression constructs

A human *hsp70* cDNA (Hunt and Morimoto, 1985) was used as a probe to screen 1 million plaques of a zebrafish λDASHII gDNA library (kindly provided by Dr M. Petkovich) under moderate stringencies, yielding 48 double-positive clones. These clones were screened further with a 642 bp PCR fragment corresponding to a heat-inducible *hsp70* gene (*hsp70-4*) (Lele et al, 1997). Three identical clones (HS1, HS4 and HS8) were generated by this second round of screening. The 642 bp probe hybridized to a 5 kb *Bam*HI fragment from clone HS1, and this fragment was subcloned into pBluescriptII SK– (to yield clone pHS1BamSC1). Sequence analysis revealed that this clone corresponded to the heat-inducible *hsp70-4* (Lele et al, 1997). The expression plasmid pHSP70/4-EGFP was made by cloning the 1.5 kb promoter fragment from pHS1BamSC1 into the *Pst*I site of the expression plasmid pEGFP-1 (Clontech). The expression plasmid pHSP70/4-EGFP was used to make two different promoter/GFP reporter fragments. HSP-EGFP(HA) is a *Hin*dIII-*Afl*II fragment from pHSP70/4-EGFP that includes all of the 1535 bp of zebrafish HSP70/4 promoter region, and the EGFP-coding region and SV40 polyadenylation site from pEGFP-1. HSP-EGFP(EA) is an *Eco*RI-*Afl*II fragment identical to HSP-EGFP(HA) except that it contains only the 3′ portion of the of zebrafish *hsp70-4* promoter sequence and is 638 bp. For microinjection, DNA was isolated and purified using the GeneClean II kit (Bio 101).

Generation of transgenic zebrafish

Embryo injection chambers were made by gluing a piece of a microscope slide onto another slide so that a wall was formed by the glued piece. Recently fertilized embryos (1- to 4-cell stage) still in their chorions were lined up against the wall so that the animal poles were against the wall. Water was pipetted out to leave enough water against the wall to just cover the embryos. The surface tension of the water held the embryos in place. Embryos were transferred to a Zeiss upright compound microscope for DNA injection. Micropipettes were pulled using thin-walled glass tubing (1 mm OD) with a Sutter microelectrode puller, and backfilled by capillary action with DNA (50 μg/ml) in distilled water containing 0.1% phenol red. Micropipettes were inserted in a pressure injection microelectrode holder (WPI, Inc.) and mounted on a Leitz micromanipulator. The tips of the micropipettes were broken to about 1 μm in diameter against the wall of the chamber just before injections. Embryos were viewed at ×50 magnification, and DNA was injected by inserting the micropipette into the embryos from their vegetal poles to the interface of the cytoplasm and yolk. DNA was injected into embryos with several pressure pulses (10-20 psi, 100 ms) delivered by a Picospritzer (General Valve Corp.). The phenol red was visible in successfully injected embryos. Following injection, the micropipette was removed and the embryos transferred to dishes. As others have reported, the survival rate depended upon the amount of DNA injected and varied from 46% to 80% at 24 hpf.

Injected embryos were raised to sexual maturity and pairwise crosses were done to identify fish that passed the transgene onto progeny. DNA was extracted from pools of 75-150 2-3 day embryos from pairwise crosses by incubating overnight at 55°C in equal volume of lysis buffer (100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS and 200 mM NaCl) with the addition of fresh proteinase K (100 μg/ml) and RNAse A (20 μg/ml). After digestion, samples were chloroform extracted and isopropanol precipitated. DNA was spooled out for use as template in PCR reactions. PCR reactions were carried out using an upstream primer from the zebrafish *hsp70-4* promoter sequence and a downstream primer in the EGFP sequence that yielded a 942 bp product. PCR was performed on approximately 100-200 ng of DNA in 1× PCR buffer (Promega) with 2 mM $MgCl_2$, and 8 ng/μl of each primer and 0.2 mM dNTPs. PCR reactions consisted of an initial denaturation step of 5 minutes and 30 cycles of 1 minute at 94°C, 45 seconds at 60°C, and 1 minute 30 seconds at 72°C. PCR products were analyzed on a 1% agarose gel for the presence of an amplification product.

Once a pair was identified, the male and female were crossed with wild-type fish to identify the founder fish again by assaying the DNA from offsprings with PCR. The F_1 embryos from the founder were assayed for EGFP expression by heat shocking them (see below) and examining them for fluorescence on a compound microscope.

GFP-expressing lines were analyzed by genomic Southern analysis using the 638 bp fragment of the *hsp70-4* cDNA to probe genomic DNA digested with *Pst*I from line 57 embryos. This identified a 3.2 kb band that presumably represents a fragment of the wild-type *hsp70-4* gene. A second genomic Southern analysis using the 1.5 kb fragment of the *hsp70-4* promoter was used to probe genomic DNA digested with *Pst*I from wild-type and line 57 embryos. This identified the 3.2 kb band in wild-type DNA representing a fragment of the wild-type *hsp70-4* gene, and a 3.2 kb and 1.5 kb bands in line 57 embryos representing fragments of the wild-type *hsp70-4* gene and the *hsp70-egfp* transgene, respectively. The 1.5 kb band was expected since the 1.5 kb fragment of the *hsp70-4* promoter was flanked by *Pst*I sites in the construct that was injected to make the transgenic line. The two bands were analyzed with the Scion Image program to estimate the copy number of the transgene in line 57.

In situ hybridization and immunocytochemistry

Sense and antisense riboprobes for *hsp70* labeled with digoxigenin-labeled UTP (DIG-UTP) were generated by in vitro transcription using a plasmid containing 642 bp fragment of the zebrafish *hsp70-4* cDNA (Lele et al., 1997). The probes were hydrolyzed to 300-1000 bases in 100 mM Na_2CO_3/$NaHCO_3$ pH10.2. In situ hybridization to whole-mount embryos was performed according to previously described protocols (Westerfield, 1995). Whole-mount embryos were labeled with a polyclonal antibody against GFP (Clonetech) at 1:1000 dilution or with a monoclonal antibody against acetylated α-tubulin

(Sigma) at 1:1000 dilution using previously described protocols (Westerfield, 1995). In some cases, anti-GFP-labeled embryos were embedded in gelatin and sectioned (approximately 100 μm) with a razor by hand.

Heat and laser induction

For heat-shock induction of transgenic embryos, a dish of embryos in approximately 120 ml of water was placed into a 37° to 40°C water bath for 1 hour. For laser induction of individual cells, a MicroPoint laser unit (Photonic Instruments, Arlington Heights, IL) was employed. The unit contained a nitrogen laser-pumped dye laser, which uses Coumarin 440 dye, directed into the epifluorescence port of a Zeiss Standard microscope. A graded neutral density filter slider in the laser beam path allows controlled attenuation of the beam intensity. The laser beam was focussed through a ×50 objective to give a spot of approximately 1×1 to 4×7 μm on a mirrored surface depending upon the intensity of the laser beam.

Embryos were dechorionated manually and mounted in 3% methyl cellulose on glass slides. Those embryos older than 16 hpf were anesthetized with 0.02% tricaine to stop movement of the embryo. Coverslips supported by small pieces of plasticine were placed over the embryos. Individual cells were visualized with DIC optics, and heat shocked with a 2 minute burst of 4 ns laser pulses delivered at a frequency of 3-4 Hz. After laser heat shock, embryos were removed from the methyl cellulose and allowed to recover for 4-24 hours at 28.5°C.

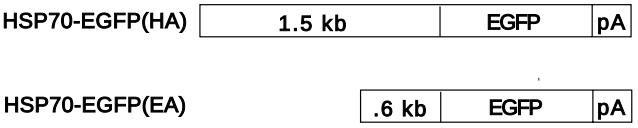

Fig. 2. Maps of the constructs injected into embryos to examine transient expression and to generate stable transgenic lines of zebrafish. The HSP70-EGFP (HA) construct included 1535 bp of the zebrafish *hsp70-4* promoter region (1.5 kb), the *egfp* gene (EGFP), and the SV40 polyadenylation signal (pA). The HSP70-EGFP (EA) construct was the same as the HSP70-EGFP (HA) except that only 638 bp of the zebrafish *hsp70-4* promoter region (0.6 kb) was used.

RESULTS

Cloning of the zebrafish *hsp70* promoter

A human *hsp70* cDNA (Hunt and Morimoto, 1985) was used to screen 10^6 plaques of a zebrafish λDASHII gDNA library (gift of M. Petkovich) under moderate stringency conditions. 48 clones were identified and further screened with a 642 bp PCR fragment corresponding to the inducible *hsp70-4* gene from zebrafish (Lele et al., 1997). Three identical clones were identified by the second screen. The 642 bp zebrafish probe hybridized to a 5 kb *Bam*HI fragment from one of the clones, and this fragment was subcloned into pBluescriptII SK− and sequenced. The sequence (Genbank accession number AF158020) revealed that this clone corresponded with the heat-inducible *hsp70-4* gene. An alignment of this clone with *hsp70* genes from other species suggested that it contained 1.5 kb of DNA that is upstream of the putative ATG translation start site (not shown). An analysis

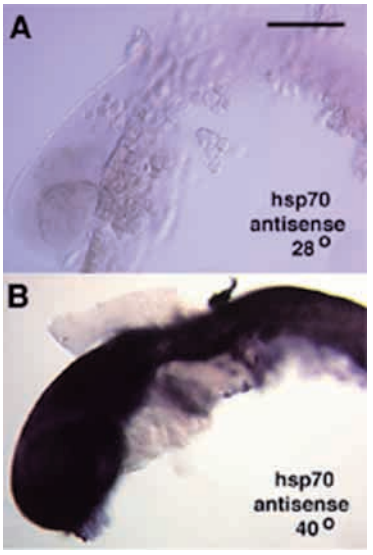

Fig. 1. A zebrafish *hsp70* gene is induced throughout the embryo by elevated temperatures. (A) In situ hybridization to whole-mount 24 hpf embryos with a *hsp70-4* antisense riboprobe showing that *hsp70-4* mRNA is not expressed at 28°C. (B) In situ hybridization to whole-mount 24 hpf embryos with a *hsp70-4* antisense riboprobe showing that *hsp70-4* mRNA is robustly expressed following elevation of the temperature to 40°C. Scale bar, A,B, 200 μm.

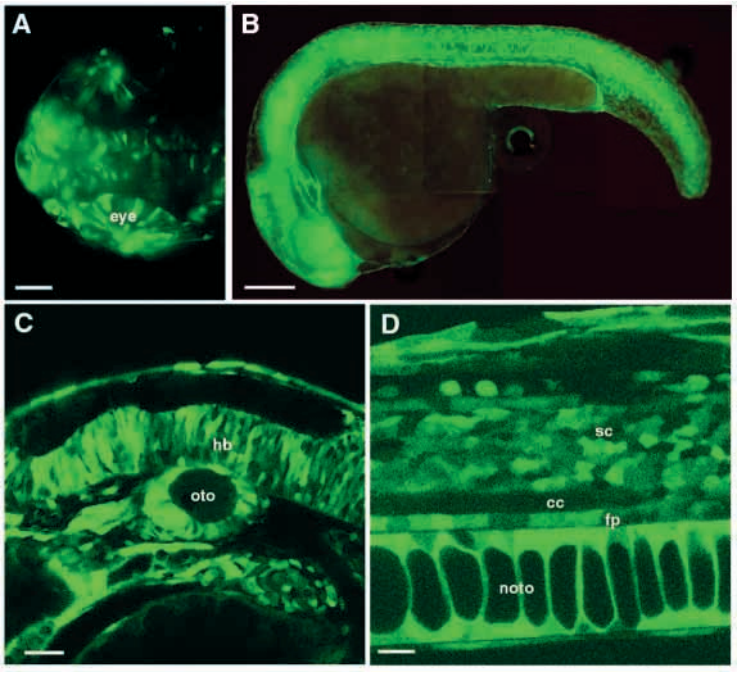

Fig. 3. The *hsp70-egfp* transgene is induced in embryos by elevating the ambient temperature. (A) Dorsal view of the head of a 24 hpf embryo injected with HSP70-EGFP (HA) at the 1-cell stage and heat shocked at 40°C for 1 hour at 15 hpf. The embryo shows a mosaic pattern of transient transgene expression. (B) Sideview of a 24 hpf *hsp70-egfp* transgenic embryo (line 57) showing ubiquitous EGFP expression 2 hours after bath heat shock at 37°C for 1 hour. (C,D) Confocal images of 24 hpf *hsp70-egfp* transgenic embryos showing EGFP expression in all cell types, even within deep tissues of the embryos. Images are single optical sections through the hindbrain (C) and spinal cord (D). Note that expression levels appear to vary from cell to cell, but nearly all cells express EGFP. cc, central canal; fp, floor plate; hb, hindbrain; noto, notochord; oto, otocyst; sc, spinal cord. Scale bars, A, 100 μm; B, 200 μm; C, 50 μm; D, 10 μm.

of the zebrafish promoter with the Transcription Element Search Software suggested that the TATAAA, CCAAT, GC and heat-shock elements were all within the first 638 bp upstream of the putative initiation methionine (not shown). The 1.5 kb upstream of the putative initiation methionine was then cloned into pBluescriptII SK–.

Generation of and expression in transgenic zebrafish

We confirmed that the zebrafish *hsp70-4* gene is inducible (Lele et al., 1997) by placing 24 hpf embryos into 37°C to 40°C water for 1 hour and examining expression with in situ hybridization to whole-mount embryos. Robust expression was induced and appeared to be present throughout the embryo at this stage (Fig. 1). We then generated an expression plasmid (pHSP70-4-EGFP) by cloning the 1.5 kb *hsp70-4* promoter into pEGFP-1.

Two DNA constructs derived from the expression plasmid were tested for expression by injecting them (50 μg/ml) into recently fertilized embryos. One construct (HSP70-EGFP(HA)) consisted of a *Hin*dIII-*Afl*II fragment of pHSP70-4-EGFP that contained the entire 1.5 kb *hsp70-4* promoter upstream of EGFP and the other (HSP70-EGFP(EA)) an *Eco*RI-*Afl*II fragment that contained 0.6 kb of the *hsp70-4* promoter upstream of EGFP (Fig. 2). Both DNA constructs were effective at inducing EGFP in the expected mosaic pattern when the injected embryos (15-24 hpf) were placed into water at 37°C to 40°C for 1 hour (Fig. 3A).

To make transgenic lines, linearized HSP70-EGFP(HA) was injected (50 μg/ml) into recently fertilized embryos and the embryos raised to sexual maturity (approximately 3 months). The mature fish were then pairwise mated and their F_1 progeny screened for the presence of the transgene by PCR. Of 71 pairs checked 3 pairs were positive for the transgene. Each of the 6 fish were then crossed with wild-type fish and assayed for the transgene with PCR to identify 3 transgenic founders. Two founders (lines 57 and 45) produced progeny that exhibited GFP fluorescence following heat induction (Fig. 3B) while the third (line 69) did not. The germ line of founder 57 was severely mosaic with GFP expression observed in 9/905 embryos from founder 57. Founder 45 was lost but 1/25 sexually mature F_1 fish was found to be positive for the transgene by PCR analysis of DNA extracted from clipped fins. 50% of the embryos from this F_1 expressed GFP fluorescence. None of 565 embryos expressed GFP from founder 69. The expressing embryos from both founders 57 and 45 were raised to sexual maturity. When these fish were outcrossed, 50% of the F_2 embryos expressed GFP. Examination of the intensities of the wild-type *hsp70* gene and the transgene fragments from genomic Southerns of DNA from transgenic embryos suggested that line 57 had integrated approximately 3 copies of the transgene (see Materials and Methods for details; data not shown).

Induced expression of GFP was seen throughout the embryos (Fig. 3B), and was more robust in line 57 compared with line 45. When embryos from an outcross were not heat shocked no GFP fluorescence was detectable during the first 72 hpf with the exception of the lens which began to fluoresce by 48 hpf in 50% of the cases for line 57. These presumably were the transgenic offsprings. For line 45, no embryos expressed GFP at least up to 72 hpf without induction. Furthermore, no GFP was detected by GFP fluorescence or anti-GFP in any of the 24 hpf embryos from an incross that were not heat shocked, while 75% of embryos fluoresced and were labeled with anti-GFP following heat shock. Examination of optical sections of transgenic embryos with a confocal microscope showed that most of the cells of the embryos express GFP following heat shock (Fig. 3C,D). Embryos showed induced GFP expression at all stages tested (15 to 48 hpf). These results demonstrate that GFP expression is undetectable at normal temperatures with the exception of the lens in line 57, but robustly expressed in nearly all cells of the embryo following heat induction in the transgenic lines.

Transgenic embryos develop normally following heat induction

In order for heat induction of transgenes to be a useful method for analyzing gene function, ideally the procedures for heat shock and the induction of transgenes, per se, should not interfere with the developmental processes of interest. We examined this issue by analyzing several different aspects of development in transgenic embryos following heat induction of EGFP. First, there were no obvious morphological defects evident following heat induction for 1 hour at 20 hpf of transgenic embryos (n=40) or their wild-type siblings (n=40) for at least up to 48 hpf. Second, the pattern of axonal tracts and nerves in transgenic embryos (n=20) and in their wild-type siblings (n=12) and in wild-type embryos (n=100) from wild-type parents was normal following heat induction (Fig. 4). Examination of axons in heat-shocked embryos labeled with anti-acetylated α-tubulin showed that all the major tracts in the CNS and the peripheral nerves were normal (Chitnis and Kuwada, 1990; Wilson et al., 1990). These included the RB axons in the dorsal longitudinal fasciculus (DLF) and the commissural axons in the spinal cord (Bernhardt et al., 1990), the spinal motor nerves (Myers et al., 1986) and the nerve of the posterior ganglion of lateral line (Metcalfe et al., 1985). Third, transgenic F_1s that were identified by GFP fluorescence following heat induction developed into normal adults that reproduced successfully. These results suggest that heat induction of EGFP does not by itself lead to developmental defects.

Targeted expression of GFP

To determine whether transgene expression could be targeted to individual cells in living embryos, specific cells in transgenic embryos were heated by exposure to a laser microbeam. Following further development, embryos were assayed for EGFP expression by fluorescence and/or anti-GFP antibody labeling. Single cells in a variety of tissues were targeted and exposed to a series of laser pulses at frequencies from 3 to 4 Hz for 2 minutes. We found that this protocol yielded induction of EGFP in 24-94% of cells, depending on the cell type targeted (n=70; Fig. 5). Cells adjacent to the targeted cells in the plane of focus did not express EGFP. Control of targeting was less accurate in the z-axis. Occasionally cells above or below the targeted cell would be induced instead of the cell in focus (7% of targeted cells). Importantly, the targeted cells appeared not to be damaged. They were indistinguishable from nontargeted cells in appearance when viewed with DIC optics several minutes to 24 hours after laser induction (Fig. 5), and showed no signs of damage such as the bloating and granular

cytoplasm normally seen following laser ablations (Kuwada, 1986). We consistently found strong EGFP expression for more than 24 hours after induction.

Not only were the targeted cells undamaged, but they appeared to develop normally. We tested tailbud progenitor cells for their ability to divide and differentiate into appropriate progeny and migrate to correct final positions, and spinal neurons for their ability to extend axons normally along their correct pathways. First, we targeted cells in the region of the developing tailbud posterior to Kupffer's vesicle during initial tailbud extension (15-17 hpf; Fig. 5). Previous fate-mapping experiments demonstrated that individual cells in this region migrate ventrolaterally and give rise to somite cells located bilaterally in the posterior tail region (Kanki and Ho, 1997). We found that 94% (n=16) of posterior tailbud cells targeted at 15-17 hpf appropriately gave rise to clusters of 2-15 somite cells positioned bilaterally in the posterior tail at 36 hpf (Fig. 5). Spinal neurons were less readily laser-induced. We targeted 54 spinal cells at 15 hpf, before they normally project their axons, and later (24-36 hpf) found EGFP-expressing neurons in 13 (24%) cases. In 5 other cases, somite cells out of the plane of focus were induced instead. When we did successfully induce neurons, they had extended axons along their normal pathways with normal timing (Fig. 5). These included CoPA, RB and CiD neurons (Bernhardt et al., 1990). Furthermore, the level of resolution afforded by the labeling was good, with many of the fine features of growth cones visible in living embryos. The targeted cells or their progeny were assayed both by EGFP fluorescence and anti-GFP. These results demonstrate that targeted gene expression by laser induction is efficient and specific, and that the targeted cells appear to develop normally.

Motor axons are repulsed by muscles laser-induced to make Sema3A1

To examine the usefulness of laser targeted expression of transgenes, we investigated the action of a laser-induced zebrafish semaphorin on motor axons. There are two secreted zebrafish semaphorins formerly called Sema Z1a (Shoji et al., 1998; Yee et al., 1999) and Sema Z1b (Roos et al., 1999) but now known as Sema3A1 and Sema3A2, respectively, that are highly homologous to chick Collapsin 1 (Luo et al., 1993) and mammalian Sema III/D (Messersmith et al., 1995; Puschel et al., 1995; Taniguchi et al., 1997). Sema3A1 is expressed by dorsal and ventral axial muscles in the trunk and tail but not by the muscle in the region of the horizontal myoseptum in zebrafish embryos. Since motor axons are initially restricted to extending on the Sema3A1-free myotome cells in the horizontal myoseptal region including the muscle pioneers (Westerfield et al., 1986), we hypothesized that Sema3A1 was repulsive to motor axons during this early portion of their pathway (Shoji et al., 1998; Yee et al., 1999). To test this, we generated transgenic lines of zebrafish in which the *hsp70* promoter regulated a ^{egfp}*sema3A1*^{myc} transgene (unpublished data). Individual myotome cells were laser-induced at stages in which the early motor axons are initially extending out of the spinal cord (15-18 hpf) (Eisen et al., 1986). Later (22-24 hpf) these embryos were assayed for expression of ^{egfp}Sema3A1^{myc} with an antibody against the myc epitope and for motor axons with the znp1 monoclonal antibody, which labels the early motor axons (Trevarrow et al., 1990). In 5 of the 9 embryos in which myotome cells were laser-induced to express ^{egfp}Sema3A1^{myc}, the induced cells were muscle pioneers as judged by their position within the horizontal myoseptal region of the axial muscles (Devoto et al., 1996). Muscle pioneers normally do not express *sema3A1* (Yee et al., 1999). In these embryos, the motor axons stalled just outside of the spinal cord in the experimental segments while the motor axons from the adjacent segments in which no cells were laser-induced were normal (Fig. 6). In the other 4 cases, the induced cells were muscle cells located either ventral or dorsal to the horizontal myoseptal region. These muscle cells normally do express *sema3A1*. In these cases, the motor axons in the experimental segments were normal (not shown). Following similar laser-induction of EGFP in horizontal myoseptal muscle fibers in *hsp70-egfp* embryos, all the motor axons were normal (n=11). The finding that motor axons are retarded only when cells that normally serve as their substratum are induced to make Sema3A1 suggests that motor axons are repulsed by Sema3A1 and that they are restricted initially to extend on the Sema3A1-free muscles due to the expression of Sema3A1 by the dorsal and ventral axial muscles.

DISCUSSION

We have generated *hsp70*-promoter-controlled transgenic lines of zebrafish and methods for targeting transgenes in specific cells with a low-level laser microbeam. This strategy takes advantage of the accessibility, optical clarity and fast pace of development of the zebrafish embryo. Using this flexible method, it should be possible to answer a number of vertebrate development questions.

Laser-induced GFP as a marker for developmental studies

Our initial demonstration of transgene targeting in the *hsp70-egfp* lines showed that neurons labeled with laser-induced EGFP project axons that follow normal pathways and migrating cells reach their normal destinations. This suggests that it should be possible to examine axonal outgrowth of a wide variety of neurons and cell migration by laser induction of EGFP. In principle, it should be possible to record the dynamic activity of living growth cones or migrating cells following EGFP induction.

Our experiments also showed that targeted progenitor cells divide and give rise to appropriate progeny. This suggests that laser targeting of EGFP can be a powerful method for tracing out the lineages of specific cells and generating highly specific fate maps. Lineage and fate-mapping analysis with zebrafish has primarily involved the injection of heritable dyes into individual cells up to mid to late blastula stages and recording the progeny some time later (Kimmel and Warga, 1986; Kimmel et al., 1990; Strehlow et al., 1994). Although these experiments have provided useful lineages and fate maps, it has been more difficult to fate map later stages, e.g. gastrulation and neurulation, because of the small size of the cells (but see Woo and Fraser, 1995). The laser microbeam can be focussed to a diameter of about 1 μm, and our experiments show that single cells can be targeted with high fidelity without inducing adjacent cells. The use of this technique may be especially useful for fate mapping and lineage analysis in the central nervous system (CNS), since many neurons and glia are

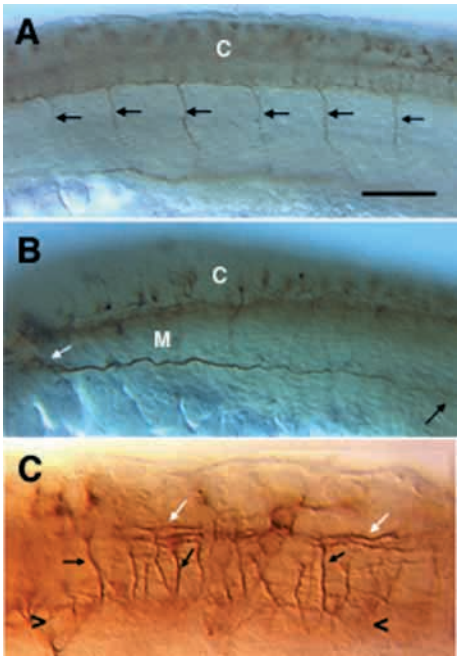

Fig. 4. Axon outgrowth in 24 hpf embryos appears normal following an increase in temperature by 10°C for 1 hour at 15 hpf. All panels show portions of whole-mount embryos in which axons were labeled with anti-acetylated α-tubulin. (A) Side view of the trunk showing that the spinal motor nerves (arrows) are normal following heat shock. Anterior is to the left and dorsal up in all panels. C denotes the spinal cord. (B) Side view of the trunk showing that the nerve extending from the posterior ganglion of the lateral line (white arrow) is normal following heat shock. Black arrow denotes the distal end of the nerve and M denotes the axial muscles. (C) Side view of the spinal cord showing that the RB axons in the DLF (white arrows) and commissural axons (black arrows) are normal following heat shock. Open arrowheads denote the ventral border of the spinal cord. Scale bar, A,B, 40 μm; C, 20 μm.

generated at later stages in development. Using this method, it may be possible to construct highly detailed fate maps of the CNS of a vertebrate embryo.

Targeted expression of genes

Laser targeting of gene expression offers a flexible method for gain-of-function analysis (Harris et al., 1996; Haflon et al., 1997). It may prove very useful for some genes that do not show an obvious phenotype when expressed ubiquitously but may do so when expressed in patterns. For instance, axonal guidance signals are effective because they are distributed in specific patterns such as at borders or in a gradient (Tessier-Lavigne and Goodman, 1996). In fact, growth cones will avoid areas containing a higher level of a repulsive molecule, but can extend over a uniform field of the same molecule (Polleux et al., 1999). Furthermore, some growth cones seem to respond to the rate in change of inhibitory molecules rather than to the absolute concentration of the molecules (Baier and Bonhoeffer, 1992). Ubiquitous misexpression of such a molecule may mask the action of the endogenous molecule, but other redundant signals may, nevertheless, be sufficient to guide the axon. Expressing the molecule in discrete cells, however, would present growth cones with a choice of extending upon these cells, towards them or away from them, and should, therefore, be much more effective at altering their pathways.

One especially useful way to analyze the effects of laser induction would be to cross an *hsp70-egfp* line with another transgenic line in which a gene of interest is regulated by the *hsp70* promoter as well. In such double transgenic embryos, GFP fluorescence could be used to visualize and confirm successful transgene induction. This would allow assays for the effect of transgene induction to be performed in living embryos, for example, time lapse analysis of extending growth cones or migrating cells or the lineage pattern of progenitor cells in the CNS. For instance, one could generate double transgenics for GFP and a receptor for a growth cone guidance

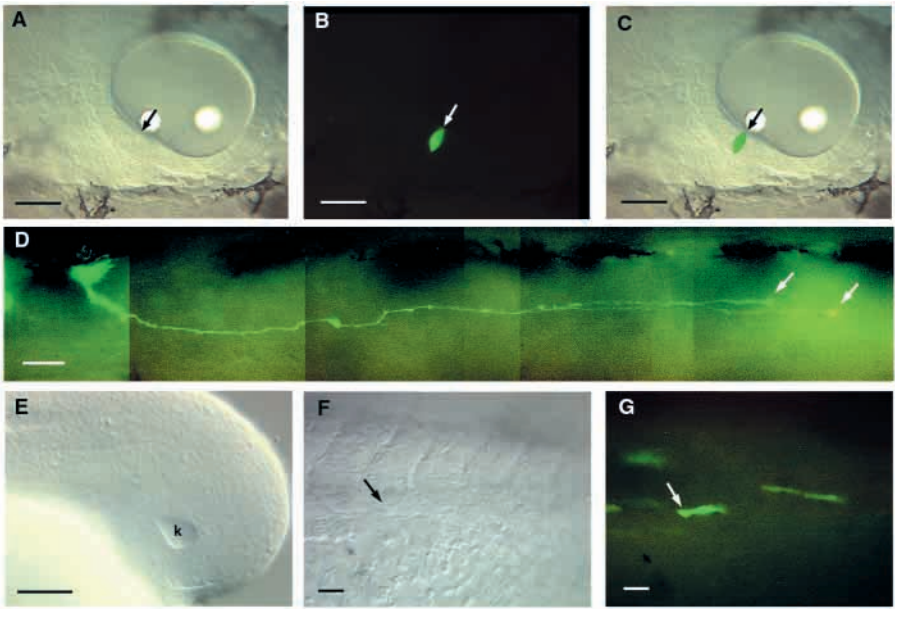

Fig. 5. Individual cells in *hsp70-egfp* transgenic embryos can be induced to express EGFP by targeting them with a laser microbeam. All panels are side views with anterior to the left and dorsal up. (A-C) Images of the developing ear in a living transgenic embryo 1 day after laser induction of an individual cell in the otocyst at 16 hpf, demonstrating that a single cell can be specifically induced and that the targeted cell appears undamaged. (A) DIC image; (B) fluorescence image of the same field as A; (C) superposition of A and B. Arrows indicate targeted cell. (D) Fluorescence image of a laser targeted CiD neuron in the spinal cord of a 33 hpf living transgenic embryo. The neuron was targeted at 15 hpf, before axon outgrowth, and has subsequently extended an axon several hundred microns down the spinal cord. The axon has branched and each branch is tipped with growth cones (arrows). The cell body is at level of somite 4 and growth cones at somite 9. (E) A DIC image of the tailbud at the stage tailbud cells were laser targeted (16 hpf). Individual progenitor cells in the region posterior to Kupffer's vesicle (k) were induced. (F,G) DIC (F) and fluorescence (G) images showing the tail somites of the same 36 hpf embryo in which one tailbud progenitor cell had been induced at 16 hpf. Arrows indicate the same region of both images. In this embryo, 6 EGFP-expressing somite cells were present in the posterior tail somites at 36 hpf. Scale bars, A-D,F,G, 20 μm; E, 50 μm

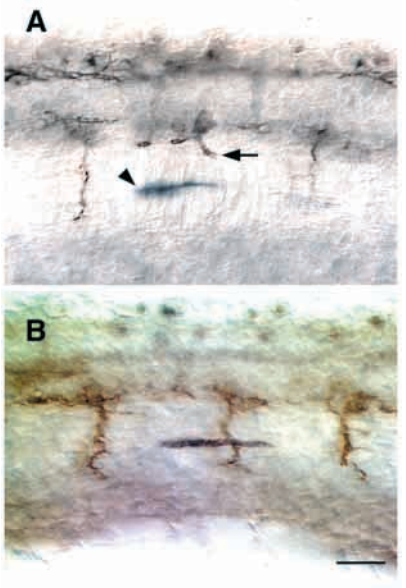

Fig. 6. Early motor axons are retarded by laser-induction of Sema3A1 in muscle cells that they normally extend upon. (A) Side view of the trunk of a 22 hpf *hsp70-*^{egfp}*Sema3A1*^{myc} embryo in which a muscle pioneer cell had been laser-induced to express ^{egfp}Sema3A1^{myc} (arrowhead) at 17 hpf showing that the znp-1 labeled motor axon (arrow) had stopped upon exiting the spinal cord in the segment in which the anti-myc labeled, Sema3A1-expressing muscle pioneer is located. The motor axons in the adjacent segments are normal. (B) Side view of the trunk of a 23 hpf *hsp70-egfp* embryo showing that the motor axon is normal following laser-induction of EGFP in a muscle pioneer at 17 hpf. The induced muscle pioneer is labeled with anti-GFP. Scale bar, 20 μm.

signal. Since laser targeting of a neuron in such a double transgenic would induce both the receptor and EGFP in the neuron, it would be possible to examine how the expression of such a receptor might affect the dynamic behavior of the targeted growth cone. Similarly, lines could be generated so that multiple transgenes could be simultaneously laser induced in cells. Laser induction of transgenes could also be potentially used for loss-of-function analysis by expression of dominant negative forms of a molecule or antisense RNA in specific cells. Thus, the ability to make transgenic lines of zebrafish combined with the accessibility and optical clarity of the embryos make laser targeting of gene induction an especially attractive strategy for the analysis of zebrafish development.

Motor axons are repulsed by laser-induced Sema3A1

As an initial test of the usefulness of laser targeted expression of genes, we showed that motor axons were repulsed by individual muscle cells following laser-induction of Sema3A1. Sema3A1 was previously demonstrated to be repulsive for the growth cones of the posterior lateral line neurons (Shoji et al., 1998). These experiments involved the analysis of embryos that mosaically expressed Sema3A1 following heat induction in embryos previously injected with the *hsp70-sema3A1*^{myc} construct. Since expression was usually limited to a random small fraction of cells, these experiments required the analysis of many embryos to find ones in which the appropriate cells were expressing *hsp70-sema3A1*^{myc}. This hurdle was overcome by directly targetting expression of *sema3A1* with a laser microbeam. When *sema3A1* was induced in muscle pioneers, motor axons stalled just outside of the spinal cord. These results suggest that, when motor growth cones are extending out the spinal cord upon the cells from the horizontal myoseptal region (Zeller and Granato, 1999), the growth cones are repulsed by Sema3A1 and are restricted to the horizontal myoseptal portion of the pathway by the Sema3A1 made by the dorsal and ventral muscles. However, since some of the motor axons then proceed to extend into the dorsal or ventral muscles, responsiveness to Sema3A1 is presumed to be dynamic. For these axons, we hypothesize that they are repulsed by Sema3A1 while in the horizontal myoseptal region, but are not repulsed beyond the initial portion of the pathway. One attractive possibility is that this change in response to Sema3A1 may be regulated by interactions with cells in the horizontal myoseptal region.

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