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Review

Application of heat shock promoter in transgenic zebrafish

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The heat shock promoter is useful for regulating transgene expression in small water-living organisms. In zebrafish embryos, downstream gene expression can be greatly induced throughout the body by raising the temperature from 28.5°C to 38.0°C. By manipulating the local temperature within an embryo, spatial control of transgene expression is also possible. One such way for inducing heat shock response in targeted cells is by using a laser microbeam under the microscope. In addition, random mosaic expression by transient gene expression and transplantation of the transgenic embryo into a wild type host can be considered a powerful tool for studying gene functions using this promoter. In this paper, we review the applications of the zebrafish heat shock protein promoter as a gene expression tool and for lineage labeling and transcription enhancer screening.

Key words: hsp70, transgenic, zebrafish.

Introduction

Heat shock protein (hsp) promoters have been used to regulate exogenous gene expression in a broad range of studies, including developmental studies. These promoters can control downstream gene expression induction at higher ambient temperature, and yet they can be silenced at normal temperature. This inducible nature provides benefits in studying gene functions *in vivo*, by circumventing undesirable effects that may occur during earlier stages of development. Over the past decade, hsp 70 promoter (Shoji *et al*. 1998; Halloran *et al*. 2000) has been extensively used for studying gene functions in zebrafish embryos. Although many tissue-specific gene promoters are being isolated, the hsp 70 promoter is still a preferred choice as a tool for raising ectopic gene expression. In addition, controlling localized activation, which is often crucial for examining gene functions, can be achieved by using a dye-laser equipped microscope (Sato-Maeda *et al*. 2006) or temperature controlled iron (Hardy *et al*. 2007). In this article, we present various applications of the hsp 70 promoter in studying gene functions, searching transcription enhancers, and lineage labeling.

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Zebrafish heat shock promoter and its *in vivo* response

The zebrafish heat shock promoter is available as a 1.5 kb genomic fragment located 5′-upstream of the hsp 70 gene (Halloran *et al*. 2000). In this fragment, contiguous arrays of 5 bp DNA consensus sequences, known as the heat shock elements (HSEs), are found in the 3′-region of the fragment, which corresponds to 80–560 bases upstream from the Start codon of the hsp 70 gene (Fig. 1). These elements are controlled by a transcription factor, the heat shock factor (HSF), which is repressed by an hsp 90 containing protein complex under the unstressed condition (Zou *et al*. 1998). As protein unfolding increases with temperature, the chaperon protein hsp 90 is recruited from the HSF repressing protein complex. HSF is then released from repression, where it binds to the heat shock elements and activates transcription. The heat shock treatment of live zebrafish is typically carried out by soaking embryos in water at 38°C for 30 min. As shown in Figure 2, expression of the downstream gene product is observed in 30 min after the heat exposure. The level of protein expression depends on the innate stability of the protein. For example, in

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-700  CAGCTGAAGAAGTGACGGCGGCTGCCTGCTGTGTTTTGATTGGTCGAATT
      CACTGGAGGCTTCCAGAACAGTGTAGAGTCTGAACGGGTGCGCGCTCTGC
-600  TGTATTTAAAGGGCGAAAGAGAGACCGCAGAGAAACTCAACCGAAGAGAA
      GCGACTTGACAAAGAAGAAAAGAGCAGCCTGACAGGACTTTTCCCCGACG
-500  AGGTGTTTATTCGCTCTATTTAAGAATCTACTGTAAGGTAAGTCTCAATA
      TATTGTACTCTATTGGCTAATCAGAATTATATAGAGATTATATGTACTTA
-400  ATGTCAAAAAATCAACTTTGTATATGTAATCTTTTTACATGTGGACTGCC
      TATGTTCATCTTATTTTAGGTCTACTAGAAAATTATATTTCCCGTTTTCA
-300  CAATAAGGATTTTAAAAAAAGCAATGAACAGACGGGCATTTACTTTATGT
      TGCTGACATTATTTTATATGAGCATAATAACCATAAATACTAGCAAATGT
-200  CCTAAATGAATTTGTGTTAATGTTGTCTACAAAAGAAAATTAGCGTTTTA
      CTTGTACAACTAATAATAACTTGGTTATTAAGAGAATTTCACTTGTTGAC
-100  TAGAAAAATCCTTTCATAATGAAACAATTGCACCGATAAATTGTATAAAT
      ATAAAATTAATTCTAATTGTTTTTTTTATTGCAGTTTCTTGAAATCAATC
      ATG
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Fig. 1. The DNA sequence of zebrafish heat shock protein (hsp) 70 promoter. The proximal region of the promoter, 700 bases from the first ATG, is presented. Contiguous arrays of heat shock elements (nGAAn/nTTCn, a single nucleotide exception is permitted) are boxed. TATA box and first ATG are underlined.

case of a secreted type of axon guidance molecule Sema3aa, even after several hours of heat induction, a certain level of the product could be observed.

Transient ectopic gene expression

When studying gene functions in zebrafish embryo, transient gene expression without establishing a transgenic strain is the fastest way to achieve ectopic gene expression. This can be carried out by injecting a DNA construct into the cytoplasm of fertilized eggs, where the construct can randomly express the exogenous gene in about 5–10% of the embryos after heat treatment. Although many embryos are required to be screened to obtain the desired expression, hsp promoter can substitute for tissue-specific promoters, especially when appropriate promoters are not available. Examples of hsp promoter usage in transient expression are shown in Figure 3. In one case, fasciculated lateral line nerves split abnormally in the presence of ectopic Sema3aa as if they avoided the cell (Fig. 3A). It has been shown that Sema3aa collapses the neural growth cones of the lateral line (Shoji *et al.* 1998). The data suggest that the repulsing activity of Sema3aa alters the route of the lateral line axons. In another case, a cell body of the caudal primary (CaP) spinal motor neuron was abnormally shifted away from the cell expressing ectopic Sema3ab (Fig. 3B), which again suggests that Sema3ab changes the position of the CaP cell body in a repulsing manner. The potential disadvantage of this method is that some cells occasionally misexpress exogenous gene before heat treatment. This may impose a problem, especially when timing of gene expression is important and needs to be tightly regulated to determine the functions. In this regard, the transient gene expression by the hsp promoter proves to be a very useful tool with its high transcription level and relatively small DNA size.

Transgenic gene induction by local heating and sublethal laser irradiation

If the temperature is raised locally within transgenic animals, ectopic gene expression is spatially controlled by the hsp promoter. The first trial of local heating was reported 20 years ago by Monsma *et al.* (1988).

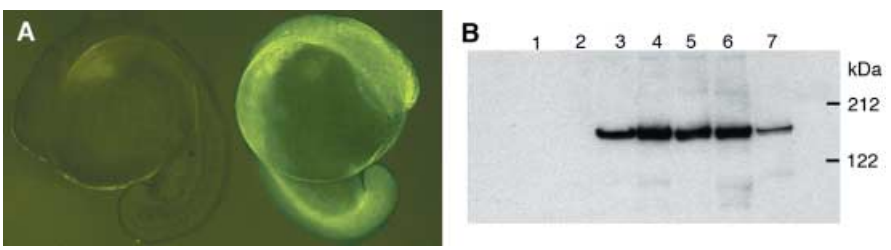

Fig. 2. Heat induction of transgene by heat shock protein (hsp) promoter in zebrafish embryos. (A) On the right, hsp:gfp-Sema3aa-mt transgenic embryos, after heat induction, exhibit ubiquitous green fluorescent protein (GFP) fluorescence, indicating that the transgene was induced. On the left, transgenic embryos, which were not heat induced, exhibit no GFP fluorescence. 'mt' means myc tag. (B) After heat induction of transgenic embryos, the GFP-Sema3aa-Mt fusion protein was detected by Western blotting using an anti-Myc antibody. No band corresponding to the fusion protein was detectable in wild-type embryos (lane 1), or the non-heat induced transgenic embryos (lane 2). An approximately 150-kDa band corresponding to the fusion protein is labeled immediately after the heat treatment (lane 3), 1 h (lane 4), 3 h (lane 5), 7 h (lane 6), and 15 h (lane 7) after heat treatment of transgenic embryos. In lanes 3–7, heat treatment consisted of raising the water temperature from 28.5°C to 38°C over 15 min, then maintaining at 38°C for 30 min, and finally decreasing it to 28.5°C over the next 15 min in a programmable water bath (Shoji *et al.* 2003).

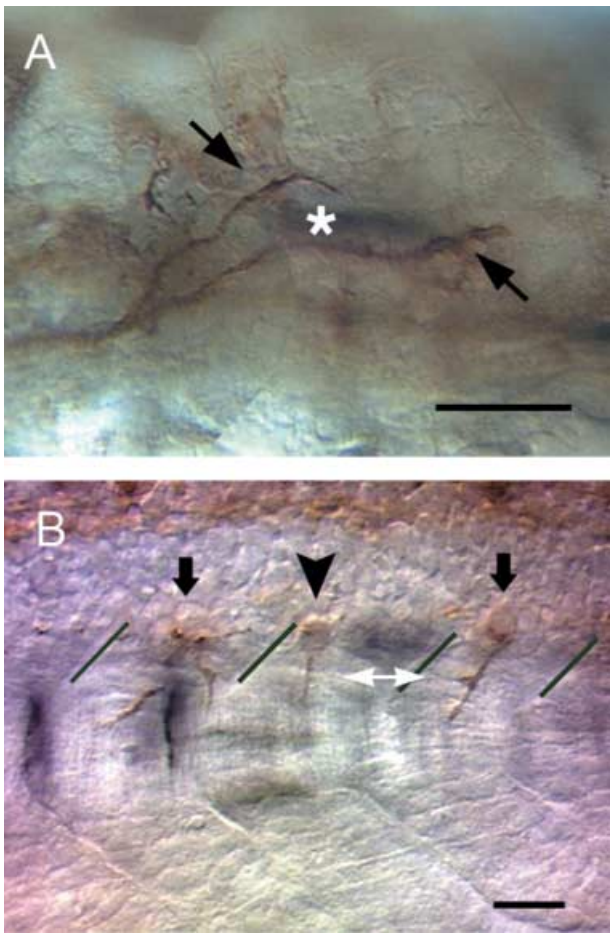

Fig. 3. Transient ectopic expression by injecting DNA constructs. (A) Lateral line axons appear to avoid cells, which were induced to misexpress Sema3aa along their normal pathway. The nerve branch with one arm extending dorsally and the other branch ventrally (arrows) to a horizontal myoseptal cell expressing Sema3aa in the first somite (star). (B) Ectopic Sema3ab has been expressed in floor plate cells (white double arrows), the CaP cell body was shifted anteriorly (arrowhead) away from the cells expressing Sema3ab. Lines denote somitic borders. Arrows indicate normal positions of CaP cell bodies in the middle between the borders. Cells expressing ectopic protein were labeled black with anti-myc tag and neuronal cells and axons stained brown with anti-HNK1 (A) or mAb Sv2 (B). Bar, 20 μm (Shoji *et al*. 1998; Sato-Maeda *et al*. 2008).

A heated needle was placed on *Drosophila* larvae, which successfully induced exogenous lacZ protein at superficial cuticles touched by the needle. A more sophisticated technique of direct heating has been recently reported by Hardy *et al*. (2007) for transgenic zebrafish. They used a thin copper soldering iron tip, which was reduced to a diameter of 15 μm, and heated the embryos locally in a chamber with water perfusion apparatus. The perfusion enabled cooling of the medium surrounding the embryo, thereby activating the hsp promoter at a depth of 20 μm without causing heat injures at the tip-contacted regions.

Irradiation by laser microbeam can also activate the heat shock promoter probably by increasing protein unfolding. This method was introduced by Stringham & Candido (1993), who used cell ablation laser to induce lacZ protein at sublethal levels. Currently, the induction of heat shock promoters by laser has been successfully used in various transparent model organisms such as *C. elegans*, *Drosophila*, and Zebrafish embryos (Harris *et al*. 1996; Halfon *et al*. 1997; Sato-Maeda *et al*. 2007). To induce transgene expression in zebrafish embryos, a single cell nucleus was targeted by microbeam of 440 nm wavelength from a nitrogen pumped laser for 1–2 min at a frequency of 2–5 Hz. Using this technique, any cell can be activated conceptually as long as their nuclei are visible under the microscope. In practice, we were able to activate cells at a depth of more than 40 μm from the epidermis (W. Shoji, unpubl. data, 2003), which includes the depth of either side of the hemi-body of the zebrafish trunk region at 24 h postfertilization (hpf). An example of laser induction can be seen in Figure 4(B), in which Sema3aa was expressed in three myotomes (asterisks) located on the common route of spinal motor axons. A CaP spinal motor axon (whose cell body is indicated as C) that should have extended longer was inhibited from reaching the Sema3aa expressing cells. On the other hand, another spinal motor neuron, mid primary (MiP, indicated as M) axon was not affected by Sema3aa and it followed the normal pathway. Thus, the laser activation of transgene expression is useful to investigate cell behavior *in vivo* in a single cell resolution.

Transplantation of transgenic cells into wild type hosts

Although the laser microbeam can activate cells at a certain depth, cells at the ventral portion of the zebrafish embryo are hidden by the yolk ball, and the head region behind the eyecup is not clearly visible under the microscope. These cells cannot be considered for laser irradiation. An alternative way to generate ectopic gene expression in these regions is by transplanting transgenic cells into a wild type host embryo. The transplant can produce a higher probability and larger cell mass of transgene expression than the abovementioned method of injecting DNA. The zebrafish cell transplant is typically carried out at the blastula and shield stages. The former stage is technically easier, and thus should be the

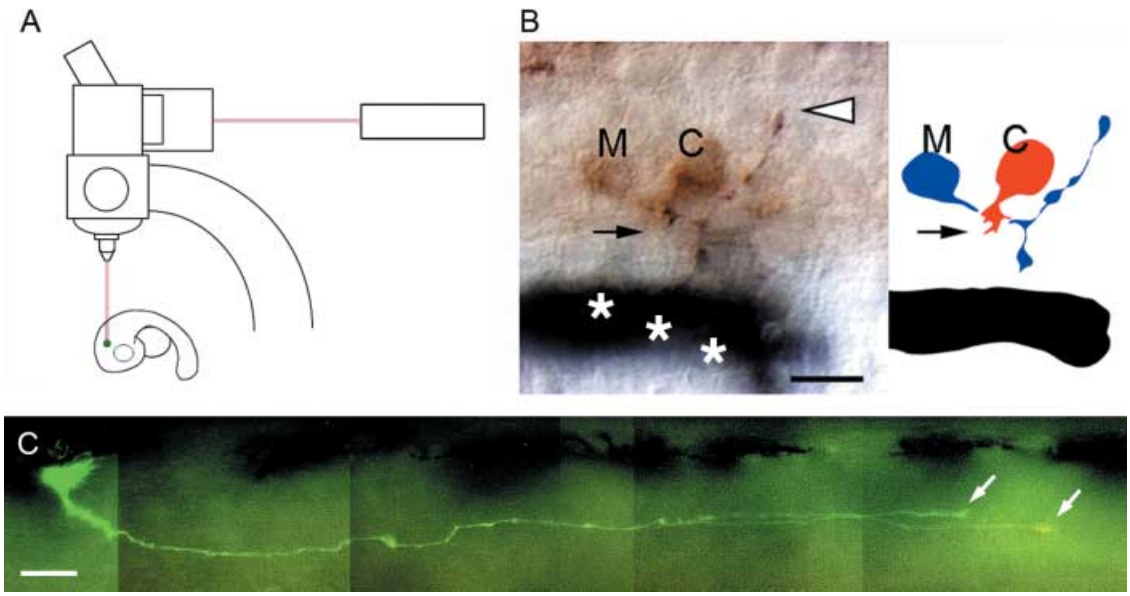

Fig. 4. Laser activation of heat shock protein (hsp) promoter for transgene expression. (A) Schematic representation of laser irradiation on living zebrafish embryos as observed under the microscope. (B) A CaP axon (arrow) should have extended longer toward the ventral region of the body (to the bottom of the figure), but is stalled in the vicinity of three myotome cells that express ectopic Sema3aa (asterisks). On the other hand, a presumptive MiP axon (white arrowhead) is normal. Right panel shows a camera lucida drawing of the CaP and MiP motoneurons. (C) The CaP cell body; M, the MiP cell body. (C) Fluorescence image of a laser targeted CiD neuron in the spinal cord of a 33 h postfertilization (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. Bar, 20 μm (Halloran *et al.* 2000; Sato-Maeda *et al.* 2007).

first choice. The latter can generate targeted chimera by referencing the fine fate map. Figure 5 is an example of the transgenic transplant at the blastula stage. Cells derived from hsp:sema4e donor were localized in the second or forth gill arches of wild type host embryo (asterisks). The branchio-motor axons that innervate gill arches were unable to penetrate these donor cells, which suggests Sema4E inhibits the branchio-motor axons from entering the gill arches.

Applications of the heat shock promoter

In addition to examining gene functions, the hsp promoter can be used for searching *cis*-acting transcriptional elements during zebrafish embryogenesis. Because of its silent nature at normal temperature, the hsp promoter is suitable as a proximal minimal promoter. It can be linked with the enhanced green fluorescence protein (EGFP) reporter gene (hsp:egfp) and injected into fertilized eggs with distal DNA fragments for tissue- or cell type specific activation (Uemura *et al.* 2005; Islam *et al.* 2006). In fish, injected foreign DNA forms extra-chromosomal concatamers independently of the DNA ends (Stuart *et al.* 1988), thus hsp:egfp and presumptive enhancer fragment can be coinjected without constructing individual ligated clones (Muller *et al.* 1997). Thus, the simplicity and rapidity of this enhancer screening method provides a certain advantage to this animal model when studying transcriptional *cis*-acting elements. Moreover, recently developed transposon technology facilitates the genome wide enhancer trap approach (Asakawa *et al.* 2008). When hsp:egfp is inserted stably into the genome near an endogenous enhancer, EGFP expression occurs in various patterns depending on the enhancer activities. Specific DNA expression itself can be useful in tracing dynamic cell behavior, and can be used as ectopic expression tools in combination with the GAL4-UAS system.

Laser activation of EGFP protein in hsp:egfp transgenic embryo can be used for studying cell lineages, which is one of the central issues of developmental biology. There are several methods used for lineage labeling in zebrafish embryos, which are selected based on accessibility of the target cells. For example, direct dye injection is preferable for large size cells in the early blastula stages, but it is difficult in deeper

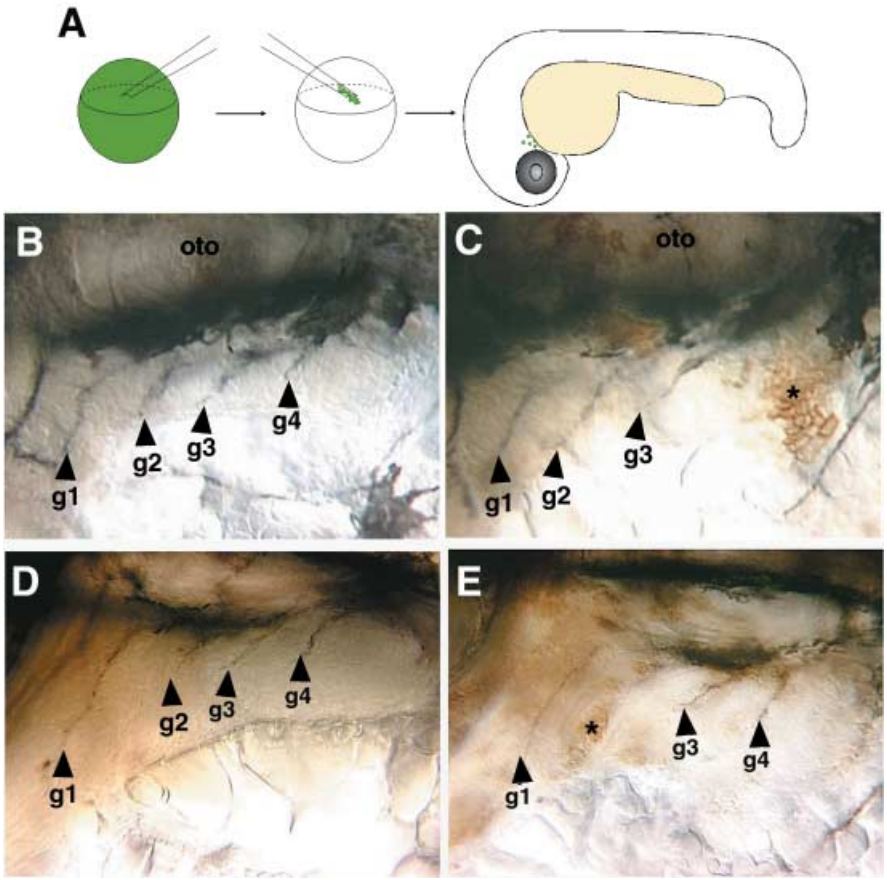

Fig. 5. Outgrowth by wild-type host gill motor axons was inhibited by Sema4E expressing cells transplanted from hsp:gfp-sema4e donor into non-transgenic hosts. (A) Schematic showing how cells were transplanted from hsp:gfp-sema4e embryos into non-transgenic embryos. (B and D) In control mosaic embryos that contained no green fluorescent protein (GFP)-positive cells within the arches, the gill motor axons (g1–g4) have extended normally into their respective gill arches. (C) In a mosaic embryo, a group of Sema4e-GFP expressing cells (asterisk) transplanted to the exit point for the gill 4 motor axons inhibited the host g4 but not the g1–g3 motor axons from exiting the hindbrain. (E) In another mosaic embryo, several small groups of Sema4e-GFP expressing cells (asterisks) transplanted to the pathway of the g2 motor axons inhibited the host g2 but not g1, g3, or g4 axons from exiting the hindbrain. The host embryos were heat induced at 28 and 35 h postfertilization (hpf), and the gill motor axons (anti-SV2) and location of the Sema4e-GFP expressing cells (anti-GFP) were analyzed at 48 hpf. Bar, 40 μm (Xiao *et al.* 2003).

tissues of later stages. Another method for lineage labeling is using DMNB-caged fluorescein, which is injected in fertilized eggs and is activated in individual cells by laser irradiation. This method enables labeling of small cells at stages later than gastrulation. Laser induction of GFP protein in hsp:egfp transgenic zebrafish can be substituted for the caged-fluorescein method, and this has several advantages. Cells in hsp:egfp embryos express GFP only in those cells and the progenies whose nucleus has been irradiated, and thus prevents unexpected activation. In addition, GFP protein is stable and its fluorescence can enable whole cell visualization for many days, whereas caged chemicals often give rise to background-like punctuated fluorescent spots within the cells. However, an advantage of the caged fluorescein method is its sensitivity to laser irradiation, which is much higher and easier to activate many cells in embryos; therefore, concomitant use of both these methods can be considered effective as well as reliable for lineage labeling (Sato & Yost 2003).

Future perspective

A potential disadvantage of using hsp promoter in transgenic zebrafish is that its sensitivity toward laser irradiation differs among strains (Halfon *et al.* 1997; Hardy *et al.* 2007). This probably reflects the copy number of integrated transgene and/or the position effects because of chromosomal location. To overcome this potential disadvantage, introduction of a GAL4/UAS system would be helpful. If an appropriate hsp:gal4 strain for laser activation becomes available, a standardized and more accessible protocol for targeted gene expression would be established for any UAS strains in the future.

In the past several years, new techniques such as the Cre/lox and Tet repressor systems have been successfully used in zebrafish (Huang *et al.* 2005; Sato *et al.* 2007). Because of heat conductivity in aquatic animals such as zebrafish, the use of hsp promoter helps retain some advantages in transgene expression. Furthermore, a combination of these new

methods and hsp promoter would provide a more powerful tool for *in vivo* manipulating of transgenic genes (Lee *et al*. 2007). This can also be facilitated by the Tol2 transposon system, which can help establish transgenic zebrafish strains.

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