Applications of transgenic technology in ornamental fish

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SUMMARY: The transgenic technology has been widely used in biotechnology, from generation of genetically modified (GM) foods to production of pharmaceutical proteins. Although fast-growing transgenic fish using exogenous growth hormone gene have been successfully produced, the marketing of such GM food fish remains to face an uphill battle. In the present study, we attempted to use the transgenic technology to develop novel varieties of ornamental fish. So far, we have succeeded in generation of several colourful transgenic zebrafish by transferring a jellyfish *green fluorescent protein (gfp)* gene and an anemone *red fluorescent protein (rfp)* gene. These transgenic zebrafish displayed green, red, yellow or orange fluorescent colors under both daylight and uv light. Thus, our work demonstrated the feasibility to generate novel varieties of ornamental fish by the transgenic techniques. Currently we are conducting mating behaviour experiments using these transgenic zebrafish. We are also aiming at development of biomonitoring transgenic fish by using some inducible gene promoters that can respond certain environmental pollutants.

KEY WORDS: BFP, CFP, dsRed, elastase, epidermis, EST, fluorescent, GFP, keratin, muscle, myosin light chain, pancreas, skin, RFP, YFP

INTRODUCTION

The transgenic technique has been applied to the zebrafish for more than a decade.¹⁾ However, the pace of transgenic zebrafish is rather slow at the beginning because of the lack of zebrafish gene promoters for analysis. As a results, essentially all early transgenic zebrafish works employed gene promoters from non-fish species.^{2,3,4)} In recent years, following the successful demonstration of the faithful expression of transgenic GFP under a zebrafish *gatal* promoter,⁵⁾ increasing number of transgenic zebrafish works has been reported using zebrafish gene promoters, including those from α -actin and β -actin,⁶⁾ *islet1*,⁷⁾ *hsp70*,⁸⁾ *Huc*,⁹⁾ β -tubulin¹⁰⁾ and *robopsin*.¹¹⁾ In all of these works, the *gfp* gene isolated from a species of jellyfish, *Aequarie Victoria*,¹²⁾ was used to express in transgenic zebrafish under several zebrafish promoters and dynamic patterns of GFP expression were observed in a non-invasive way. In all cases the transgenic expression mimics the expression of the endogenous genes from which the promoters were derived.

In the present communication, we will briefly summarize our progress in generation of *gfp* transgenic zebrafish under several different tissue-specific promoter isolated from zebrafish and discuss the potential applications of these *gfp* transgenic zebrafish.

SELECTION OF TISSUE-SPECIFIC ZEBRAFISH CDNA CLONES

Our transgenic work was originated from our EST (express sequence tag) project. In the past few years, our laboratory has generated about 3,000 zebrafish EST clones from several zebrafish cDNA libraries, mainly from an embryonic library, an adult library, a testis library and an ovary library.^{13,14)} About half of these clones can be identified with confidence by sequence homology search. From our collection, we have found tissue-specific clones representing essentially all major tissues and organs. The summary of representative tissue-specific and inducible cDNA clones from our EST collection is shown in Table 1. The tissue specificity of expression of many of these clones have been confirmed by northern blot analysis and in situ hybridization.

ISOLATION OF ZEBRAFISH GENE PROMOTERS

Selected tissue-specific cDNA clone were used to isolate their promoter regions. In our laboratory, in general, two methods have been used to isolate the promoters. One is linker-mediated PCR and the other is PCR navigated genomic library screening. The former has been described in detail in our previous publication.¹⁵⁾ Here we just describe the

second method. Briefly, an amplified genomic library (usually with $>2 \times 10^6$ primary clones) was first subdivided into over 20 sublibraries, each of which has about 70,000 clones. A pair of PCR primers was synthesized based on 5' sequence of a cDNA clone and used for PCR screening. A positive sublibrary would be further divided with fewer clones and screened by PCR. The subdivision approach would be repeated until a PCR-positive sublibrary is small enough (usually <100 pfu per plate) to apply PCR to individual clones for identification of a single positive clone.

Table 1 Example of tissue-specific and inducible zebrafish cDNA clones

Tissue specificity/inducibility	Encoded proteins
Central nervous system	Vimentin, AGFP
Eyes	Crystallins
Heart	Cardiac α -actin
Erythrocytes	Globins
Liver	Transferrin etc.
Pancreas	Elastases, Trypsin etc.
Intestine	Fatty acid binding protein etc.
Kidney	Nephrosin
Ovary	ZP2, ZP3 etc.
Testis	Testicular tectin
Skeletal muscle	MLC2f (Mylz2), CKM
Skin	Keritins etc.
Estrogen-induced	Vitellogenins
Heat shock/heavy metal induced	Hsp25, Hsp70, Hsp91 etc
Ubiquitous	EF1 α , ribosomal proteins

By the two methods, we have so far isolated six different zebrafish gene promoters. These promoters displayed various tissue specificity such as in skin (*keratin8* or *krt8*), muscle (*creatine kinase muscle* or *ckm*, and *myosin light polypeptide 2* or *mylz2*) and pancreas (*elastase A* or *elaA*). Some of them have potential inducibility such as by heavy metals (*heat shock protein 25* or *hsp25*) or by estrogenic compounds (*vitellogenin* or *vg*). One of them (*acidic ribosomal phosphoprotein P0* or *arp*) functions ubiquitously. All of these promoters have been linked with the *gfp* reporter gene. Some of them were also linked with a recently isolated *rfp* reporter gene.¹⁶⁾

GENERATION OF LIVING COLOR TRANSGENIC ZEBRAFISH

Previously, by transient expression in zebrafish embryos and fry, we have demonstrated the faithful expression of three of these promoters: *krt8* (previously named *ck* for *cytokeratin*), *ckm* (previously named *mck* for *muscle creatine kinase*) and *arp*.¹⁷⁾ The two muscle specific promoters, *mylz2* (previously named *MLC2f* for *myosin light chain 2, fast muscle isoform*) and *ckm*, were also tested in adult fish by direct injection of naked DNA into trunk muscles in the zebrafish.¹⁸⁾ Recently, transgenic works using the *mylz2*, *elaA*, *vtg* and *hsp25* promoters were also initiated or carried out.

In general, successful generation of transgenic fish can only be claimed after stable transgenic lines are established. Usually, the founder transgenic fish are mosaic and thus variable transmission rate from $<10\%$ to $>50\%$ can be observed in F1 offspring. If a stable line is obtained, F2 offspring from transgenic F1 should follow standard Mendelian ratios; i.e. when a heterologous (or hemizygous) transgenic F1 is crossed with a wild type fish, about 50% of F2 offspring should be transgenic. Based on this rule, we have obtained stable *gfp* transgenic lines for three of our transgenic DNA constructs: *krt8-gfp*, *mylz2-gfp* and *elaA-gfp*. In addition, we have also generated a stable transgenic line by insertion of two transgenic DNA constructs: *krt8-gfp* and *mylz2-rfp*.

A. *krt8-gfp* transgenic zebrafish

We have obtained five stable transgenic zebrafish lines with the chimeric construct, *krt8-gfp*. In all examined transgenic lines, GFP is expressed specifically in the outmost layer of skin epidermis and the innermost layer of epithelia in pharynx, esophagus and foregut, but not in the mid-gut and hind-gut. This pattern of expression is essentially identical to that of the endogenous *krt8* expression. Thus, the exogenous *krt8* promoter is faithfully activated in the transgenic zebrafish system.

B. *mylz2-gfp* transgenic zebrafish

mylz2 is a muscle specific gene and it is specifically expressed in fast skeletal muscles.¹⁹⁾ Two lines of *mylz2-gfp* transgenic zebrafish have been generated. In both lines, GFP expression is restricted to fast skeletal muscles but not to the slow muscles, which are located in the superficial layer of myotomes and can be distinguished by staining with a monoclonal antibody, F59, specifically against a slow myosin isoform.²⁰⁾ After 48 hpf, GFP expression was also observed in head muscles including eye, jaw and gill muscles. This pattern of expression is identical

to that of endogenous *mylz2* expression.¹⁸ Thus, this work again demonstrated the faithful expression *mylz2* promoter in transgenic zebrafish. Moreover, in both transgenic lines, GFP expression is so strong that green fluorescence can be easily observed from transgenic zebrafish even under a normal daylight. This observation provides a basis for generation of fluorescent ornamental fish as new varieties using the *gfp* reporter gene.

C. *ela4-gfp* transgenic zebrafish

Elastase A is synthesized and secreted from exocrine cells in pancreas. The transgenic zebrafish containing *ela4-gfp* express GFP specifically in exocrine cells but not in endocrine cells of the pancreas, indicating the high specificity of the *ela4* promoter. This transgenic fish line will be useful to trace pancreas development and to purify pancreas cells for in vitro culture and for establishment of cell type specific cDNA library for detailed study of gene expression profile.

D. Two-color transgenic zebrafish

Recently, we have generated two-color transgenic zebrafish by injection of a mixture of DNA constructs, *krt8-gfp* and *mylz2-rfp*. In this transgenic line, GFP is expressed in skin cells while RFP is expressed in muscles. Consistent with the observation for *mylz2-gfp* transgenic lines described above, the transgenic line containing *mylz2-rfp* express RFP strongly and a red color is observed, indicating the feasibility to develop multiple transgenic color fish by using different color genes under different tissue-specific promoters.

APPLICATIONS OF GFP TRANSGENIC ZEBRAFISH

gfp transgenic zebrafish has many applications in both basic research and biotechnology. In basic research in developmental biology, expression of a fluorescent protein gene under a tissue-specific promoter can be used to recapitulate gene expression program. This has been repeatedly demonstrated in both our studies and in the studies by others. Using *gfp* transgenic zebrafish, it will be easier to trace cell lineage and cell migration. An excellent example is the *gfp* transgenic zebrafish generated under a heat shock inducible promoter and using this transgenic line certain neuronal cell lineage has been traced after activation of GFP expression by a laser beam.⁸⁾ In addition, *gfp*

transgenic zebrafish can be used for cell and nucleus transplantation experiments, as GFP expression and *gfp* transgene are good cellular and genetic markers for this type of experiments. It is particularly useful in nuclear transplantation if GFP expression is targeted to nucleus by addition of a nuclear localization signal to the transgenic DNA construct. Furthermore, GFP expression can be used to purify specific types of cells by fluorescence activated cell sorting (FACS). This is especially important if a specific type of cells is too small in number or too difficult to visualize and to isolate without a proper labeling. Finally, *gfp* transgenic zebrafish under tissue-specific promoters may serve as an excellent system in generation and characterization of mutants for the targeted tissues as the restricted expression of GFP may greatly facilitate the screening and analysis of mutants for the targeted tissue/organs.

In biotechnology, as we show here, *gfp* and *rfp* transgenic fish may be used for generation of novel varieties of ornamental fish. Different color genes may be expressed in different tissues/organs by using different tissue-specific promoters. Currently, more mutant forms of *gfp* genes for at least three more colors of fluorescent protein are commercially available: YFP for yellow fluorescent proteins, BFP for blue fluorescent protein and CFP for cyan fluorescent protein (Clontech). More colors can be obtained by crossing the fish of two different colors. For example, we have obtained orange fluorescent transgenic zebrafish by crossing the *gfp* and *rfp* transgenic fish.

To evaluate the ecological consequence of *gfp* transgenic ornamental fish, the survival rates of transgenic fish were assessed from the offspring of crosses between *mylz2-gfp* fish and *mylz2-rfp* fish. In general, there is no apparent difference in survival rates among the four genotypes: wildtype, *gfp*, *rfp* and *gfp/rfp*. To evaluate reproduction success, a population of equal number of wild type and hemizygous *gfp* transgenic fish was maintained for breeding for a week, the expected ratio (43.75%) of transgenic offspring based on Mendelian genetics was observed (43.60%, 1158 out of 2656). Similar observation was also obtained for *rfp* transgenic zebrafish. Thus, there is no indication that the fluorescent transgenic fish would have a reproductive advantage and pose a threat to the wild population. This is in contrast to the recently proposed Trojan gene hypothesis that growth hormone transgenic fish would be favourably selected in mating because of their larger body size and escape of such transgenic fish would cause eventual extinction of wild population.²¹⁾

Furthermore, fluorescent transgenic fish may be used for biomonitoring of environmental pollution. For example, *gfp* or *rfp* can be engineered under an inducible promoter and the expression of the transgene can be activated only under environmental stimulations such as by estrogenic compounds or by heavy metals. In such case, the fluorescent color will be an indicator of environmental pollution. Some feasible promoters for these purposes include those from *vitellogenin*, which is inducible by estradiol, and *hsp25*, which can be activated by heavy metals as well as by heat shock (our unpublished data). Currently we are introducing these promoter/color gene constructs into zebrafish for development of transgenic biomonitoring zebrafish.

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