Transgenic Fish Research

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Progress and Potential

A transgenic animal is defined as an animal that has become transformed following the introduction of novel DNA into its genome. Gordon et al. (1980) revolutionized the procedure for producing transgenic animals based on the microinjection of cloned DNA into the pronucleus of fertilized eggs at the one-cell stage. Attempts to produce transgenic fish first began in the mid-1980s. Maclean and Talwar (1984) reported microinjection of cloned DNA into rainbow trout (*Oncorhynchus mykiss*) eggs. Zhu et al. (1985) microinjected fertilized eggs of goldfish with metallothionein promoter fused with the human growth hormone gene.

Following these initial successes, transfer of cloned DNA was reported in a number of other fish species; e.g., common carp (*Cyprinus carpio*), catfish (*Clarias gariepinus, Ictalurus punctatus*), salmon (*Salmo salar*), rainbow trout, tilapia (*Oreochromis niloticus*), goldfish (*Carassius auratus*), loach (*Misgurnus fossilis*), medaka (*Oryzias latipes*), zebrafish (*Brachydanio rerio*), northern pike (*Esox lucius*), rosy barb (*Barbus conchonius*), swordtail (*Xiphophorus*) and gilthead seabream (*Sparus auratus*). Current research on transgenic fish research targets growth promotion, temperature, tolerance, developmental genetics and gene regulation. These and other potential applications of transgenic fish technology have been reviewed by Fletcher and Davies (1991) (Box 1).

Methods of Gene Transfer

The most popular technique used for gene transfer in fish is microinjection. Other gene transfer techniques include electroporation, electrofusion, high velocity microprojectiles, blastula chamber injection, direct gene transfer into fish muscles in vivo, embryonic cells, sperm binding, retroviral integrase protein and chromosome-mediated gene transfer. Electroporation, electrofusion, high velocity microprojectiles, sperm binding and chromosome-mediated gene transfers are examples of mass gene transfer techniques by alternative techniques to conventional microinjection. The two most commonly used techniques, microinjection and electroporation, are featured here.

Microinjection

Microinjection involves the use of an injection pipette, the dimensions of which depend on the target species; e.g., pipettes of inner diameter of 3-5 µm are used for tilapia. Although microinjection is time consuming, laborious, species-specific and technically demanding, it remains the most widely used method for gene transfer in fish. Its advantages are that it is a well established procedure, formerly found to be effective in mice (Brinster et al. 1985) and that it can give high gene transfer efficiency (Inoue 1992).

The site of injection varies from species to species. In fish eggs, the nucleus or pronucleus cannot be seen using conventional light microscopy, mainly because of the opaqueness of the chorion and/or the cytoplasm. Hence, most transgenic fish studies have opted for cytoplasmic injection of DNA, following fertilization. The target is the thin layer of ooplasm under the chorion or developing blastodisc. The injection pipette must penetrate the chorion (which is often thick and opaque, except in some species such as catfish and medaka, which have transparent and thin chorions) and the membrane of the fertilized egg. Several methods of pretreatment have been reported, including a two-step method which involves piercing the chorion with a broken pipette before microinjection into its ooplasm (Chourrout et al. 1986), dechorionation (Zhu et al. 1985), and prevention of chorion hardening (Inoue et al. 1991). Others have reported microinjection through the micropyle (Brem et al. 1988), and microinjection before hardening of the chorion (Maclean et al. 1987).

The possibility of damaging eggs during microinjection is high and this technique requires a great deal of skill. The survival rates of different species of transgenic fish produced by this method have been reported to range from 5 to 90%.

Electroporation

Electroporation is a technique based on exposing a cell membrane to high intensity electric field pulses. As a result, specific regions of the cell membrane are temporarily destabilized. During this destabilization period, the cell membrane is highly permeable to exogenous molecules present in surrounding media (Chang et al. 1992). Although this method is simple, it remains unpopular because of its low gene-transfer efficiency. The technique has been tested on medaka, zebrafish, common carp, catfish and loach.

Preparation of Injected DNA

Many studies on producing transgenic fish have used injected DNA consisting of mouse metallothionein, Rous sarcoma virus (RSV) and Simian Virus 40 (SV40) promoters and viral enhancers. So far, only a few investigators have used piscine regulatory regions. The transcribed regions often consist of growth hormone and/or reporter genes. The function of the transcribed regions is to monitor promoter function.

The growth hormone genes used have originated mainly from humans, cattle and rats, but rarely from fish. The transcriptional activity of growth hormone genes is difficult to assay. The expression of growth hormone genes in putative transgenic fish is normally measured phenotypically; for example, by comparing the growth rates of putative transgenic fish and controls. For this reason, reporter genes are ideal for expression assays. The widely used reporter genes for transgenic fish studies are chloramphenicol acetyltransferase (CAT), β-galactosidase and luciferase. Besides growth hormone and reporter genes, other genes have also been used for expression studies; these include neomycin (Yoon et al. 1990), hygromycin (Stuart et al. 1990), winter flounder anti-freeze protein (Fletcher et al. 1988), chicken δ-crystallin (Ozato et al. 1986), carp α-globin

Box 1. Future applications of fish technology: a "wish list" from Fletcher and Davies (1991).

Better understanding of developmental, growth, gene regulation and reproductive processes in fish

Improved economics of fish culture

- Improve feed conversion efficiencies
- Improve cold resistance
- Improve freeze resistance
- Improve disease resistance
- Improve broodstock fecundity
- Utilize low cost diets (carbohydrates as opposed to protein)
- Control smolting and reproduction
- Reduce aggression

Production of fish tailored to specific models

- External appearance; food fish for exotic tropicals
- Flesh color, flavor, texture
- Fatty acid composition

Use of fish as 'bioreactors'

- Production of medically important compounds
- Production of commercially useful non-medical compounds

(Yoshizaki et al. 1991), tyrosine (Matsumoto et al. 1992) and fish melanoma oncogene (Cavari et al. 1993).

Detection of Transgenes

Introduced genes in fish cells have been detected in various ways. The methods summarized here are slot, southern blot, northern blot, *in situ* hybridizations, polymerase chain reaction (PCR) and expressions assays of reporter gene products.

Most studies on transgenic fish have used hybridized slot (Maclean et al. 1992), southern blot (Penman et al. 1991) and northern blot techniques (Zhu 1992) to detect transgenes. southern blot hybridization (Southern 1975) is the most widely used method. In this method, fragments of DNA generated by restriction digestion, are subjected to agarose gel electrophoresis. The separated fragments are then transferred to a nitrocellulose or nylon membrane by a blotting technique. The DNA of interest can be detected by hybridizing the membrane to a radioactive probe, which bears the same homology as the DNA. In addition to detecting gene rearrangements, it can also be used to identify structurally related genes in the same species and homologous genes in other species. e.g., DNA fingerprinting. northern blot is based on the same principle as southern blot, but RNA is used instead of DNA. It measures accumulation of RNA transcripts and it is extremely useful in studies of gene expression. The difference between slot and southern blotting is that slot blotting does not require the genomic DNA to be cut with restriction enzymes prior to transfer to a nylon membrane or nitrocellulose filter. Analysis of degraded DNAs and of multiple samples are possible with slot blotting. However, slot blot hybridization is not as informative as southern blot hybridization because it does not indicate integrations or rearrangements involving the transgene.

Western blotting is used for identifying and characterizing specific gene products. It involves the transfer of proteins from acrylamide gels to nitrocellulose membrane by electrophoresis. The membrane is then probed with an antibody to detect the protein of interest. An example of a study using this technique in transgenic fish research is that of Davies et al. (1990).

In situ hybridization is the hybridization of nucleic acids within cytological preparations. This method shows the localization of transgenes (Ozato et al. 1986).

PCR amplification is based on repeated cycles of denaturation, annealing of oligonucleotide primers complementary to the gene, and primer extension by Taq polymerase (Saiki et al. 1985). The amplified fragment can then be recognized as a discrete fragment on a gel or on a southern blot. Its application in transgenic fish research continue to grow (Du et al. 1992). However, strenuous precautions must be taken to avoid contamination.

Expression assays are aimed at detecting the presence of reporter gene products such as CAT and β-gal in host cells. The two methods used for detecting CAT are the two phase partition assay (TPP) and thin-layer chromatography method (TLC). TPP utilizes [^{14}C] or [^{3}H] acetyl CoA as acetyl donor, and relies on the diffusion of labelled acetyl chloramphenicol into a water-immiscible liquid scintillation technique. Despite its simplicity and convenience, the method has not been widely used in transgenic research. TLC is so far a more popular method for measuring CAT than the TPP. The presence of β-gal in fish cells can be detected with either in situ X-gal staining or fluorometric determination techniques. X-gal staining is used for detecting the product of transgene expression, β-gal, in fish tissue. In the presence of β-gal, X-gal in the stain is cleaved to form localized deposits of indigo dye. Fluorometric determination, on the other hand, measures the hydrolysis of 4-methylumbelliferone-β-D-galactoside by β-gal. The hydrolysis can be measured quantitatively on a fluorometer since methylumbelliferone is linked to a fluorogen.

Conclusions

Transgenic fish research is being as actively explored as other transgenic studies. There is no doubt that transgenic fish research will continue to contribute to our understanding of fish genetics, especially developmental genetics. The ultimate aim will be to produce a better quality fish for food to benefit humans. However, the question of whether or not transgenic fish will eventually reach practical utilization will depend on public confidence on the results of performance tests and ecological risk analyses.

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AQUABYTE NEWS ITEMS

Red Coloring from *Euglena sanguinea*

The red coloring of the water in a Chinese carp farm observed by Dr. R. Peyghan and Ms. S. Dehghan (Naga, ICLARM Q. 18(1):29) is a common phenomenon in dominated polyculture ponds by *Oreochromis niloticus* in northeast Thailand (17.3°N, 102.5°E). During intensive studies on a particular pond in 1992-93 (Roos and Riise-Eriksen 1994), an increasing red coloring was observed during a phytoplankton bloom ending up with a phytoplankton crash after one month. The phytoplankton community during the bloom was dominated by *Euglena sanguinea (approximate length, 100 μm)*. When the bloom had peaked after 14 days, the *Euglena* in the surface water formed non-motile resting spores. According to Pringsheim (1956) *Euglena* sp. are typically found in hypereutrophic ponds, and the red coloring occurs because *E. sanguinea* contains red carotenoid pigments, which are significantly increased when the environment becomes unfavorable. If light and nutrients support sustained growth, *E. sanguinea* is green and relatively small, but if growth becomes limited by light or nutrients, the cells enlarge, turn red and finally they encyst producing non-motile spores sinking to the bottom (Pringsheim 1956). The red coloring clearly appeared before the phytoplankton community crashed and the coloring did not disappear until the crash was over and the community was reconstituting. Red coloring of pond surface water is thus a good indicator of limiting growth conditions for a *Euglena*-dominated phytoplankton community before a crash occurs.

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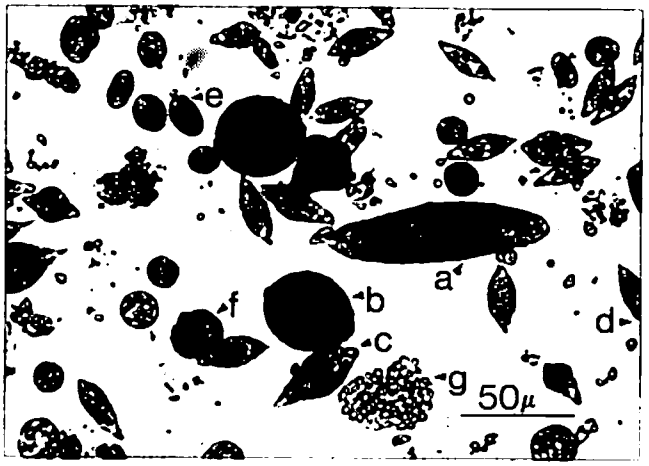
Source: J. Christian Riise, Fisheries Department, FAO Headquarters, Viale delle Terme di Caracalla, 00100 Rome, Italy.



Algae from the surface water of a red-colored pond. Three species of Euglena *dominate the phytoplankton bloom: a) vegetative cell of* Euglena sanguinea*; b) resting spore of* E. Sanguinea*; c-d)* Euglena *spp.; e-f) spores of* Euglena *spp; and g) colony-forming cyanobacteria.*