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Transgenesis and its Applications in Aquaculture

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Abstract

The main transgenesis techniques which are successful in introducing exogenous genes into fish are microinjection and electroporation. Genes are introduced into one-celled embryos as well as oocytes. Microinjection is the more established method but it depends on individual treatment of the egg. Electroporation, on the other hand, is a mass method only recently shown to be effective. Other transfer techniques, such as those using sperms, liposomes, microprojectiles, embryonic stem cells and retroviruses as the vehicle, have also been reported to produce transgenic fish. Most transgenic experiments are at present conducted on finfish, and hardly any on other aquatic organisms like invertebrates except for sea urchins and, more recently, abalones. The genes with potential transgenic applications in aquaculture which have been shown to be effective in fish are the growth hormone and the anti-freeze genes. The potentials of manipulating reproduction, sex, coloration, disease resistance and other phenotypes are discussed. Studies on eco-logical, social and ethical issues of transgenic research and applications to animals in general and to fish in particular, are also reviewed.

Introduction

Transgenesis may be defined as the introduction of exogenous DNA into the genome such that it is stably maintained in a heritable manner. Transgenesis or gene transfer is the process of *in vitro* transfer of exogenous and usually heterologous genes or recombinant gene constructs into the genome of organisms. The first successful gene transfer was demonstrated in the 1980s on mice (Gordon et al. 1980). Five years later, in 1985, this technology was used on farm livestock. The first transgenic farm animal produced was with a metallothionein growth hormone fusion gene (Hammer et al. 1985).

Transgenesis utilizes the technologies of recombinant DNA, embryo manipulation and embryo transfer. Developments in *in vitro* fertilization and embryo culture techniques have contributed to the success of transgenesis. The availability of micromanipulation apparatus has made it possible to conduct microinjection into single cells and embryos. The arrival of recombinant DNA technology has made it possible to isolate and clone particular genes of interest. All these three developments have made it possible to manipulate single

genes and to conduct gene transfer experiments. Its application to aquaculture is only just beginning.

Aquaculture is the farming and husbandry of aquatic organisms such as plants, finfish and shellfish, under controlled or semi-controlled conditions. It provides protein to human beings, and feed to farm animals; it serves to stock public waters for sportfishing, to enhance commercial fish stocks, and to reduce the pressure on endangered species. The aquaculturist, like the farmer on land, farms the water and, like land farmers, also manipulates the genetic composition and source of the farmed organisms. Recent advances in transgensis have added yet another powerful tool to the existing armory of genetic selection and manipulation techniques for animal breeders.

Genetic gains through traditional and conventional selective breeding programs by natural matings have been slow due to the difficulty of separating the beneficial traits from the undesirable traits. The biological barrier that prevented inter-species breeding has also prevented the genetic transfer of beneficial characteristics from one species into another. Transgenic technology now offers the possibility of introducing single foreign genes into the farmed animal's genome, thus increasing the repertoire of genes for breeding and selection and overcoming traditional barriers.

Most transgenesis studies on aquatic organisms published so far have been on finfish. The applications of transgenic technology in finfish did not begin until 1985.

Overviews on transgenesis in fish are given in Hew (1989), Ozato et al. (1989), Chen and Powers (1990), Chourrout et al. (1990), Maclean and Penman (1990), Hew et al. (1991), Houdebine and Chourrout (1991), Powers et al. (1992a), Chourrout (1993) and Jiang (1993). Pandian and Marian (1994) dealt with the problems and prospects of transgenic fish production. A book consisting of publications only on transgenic fish is by Hew and Fletcher (1992). The journal, *Molecular Marine Biology and Biotechnology* (1992) published a special issue on transgenic fish.

To date, commercialization of transgenic fish species has not begun, but the efficacy and the potential applications of gene transfer technology in finfish have been demonstrated and discussed by Maclean and Penman (1990) and Chen et al. (1991, 1993). This paper describes the development of transgenesis, the general protocols and the main methods of gene transfer in fish. Their potential applications in aquaculture are discussed followed by a brief review on safety and ethical aspects.

Protocol and Methods of Transgenesis

The primary aim of gene transfer is to introduce a novel gene such that it will integrate into the genome of the organism so that every cell in the whole organism will have the gene, and this gene will be transmittable to subsequent generations. The usual procedure is as follows: 1) identify and prepare the gene of interest; 2) find a suitable method of transfer; 3) conduct detection and monitoring analyses to identify the transgenic individuals to rear and use in a breeding and selection program.

For research testing of efficacies of promoter and newly isolated structural genes, techniques of transfer and methods of monitoring, the commonly used reporter or marker genes in eukaryotic studies are those which code for chloramphenicol acetyltransferase (*cat* gene; Gorman et al. 1982), firefly luciferase (*Luc* gene; de Wet et al. 1987), β-galactosidase (*lacZ* gene; Hall et al. 1983); resistance to neomycin/G418 (*neo* gene; Yoon et al. 1989; Duch et al. 1990) and human growth hormone (*hGH* gene; Selden et al. 1986). Most of these have commercially available test kits.

Khoo (1993) utilized recombinant plasmids containing the *cat*, *Luc* or the *hGH* genes to study the efficacies of transgenic zebrafish production by microinjection, electroporation and sperm-mediation. Sekkali et al. (1994) also made a comparative study on the efficacies of the *cat*, *Luc* and *lacZ* reporter genes. The luciferase gene was shown to be the best reporter for gene transfer experiments because of its high sensitivity. The enzymatic assay is easily and rapidly performed in a luminometer or a scintillation counter and non-destructive tests on live animals can be conducted, aiding both in the rapidity of screening and in selecting at an early stage the potential transgenic founders for rearing to maturity (Patil et al. 1994). The criteria to consider when choosing a reporter for transgenesis studies are: 1) the availability and sensitivity of the test for the expression products and; 2) the gene and its protein products should be distinct from the host's own genes and proteins, and it should not be toxic or interfere with the functioning of the host.

Many methods of gene transfer developed for mammalian systems have been applied to fish (Table 1). The main method commonly used to produce transgenic fish is by microinjection. Electroporation, sperm-vector, gene-gun and liposome-mediated methods have also been shown to be effective in transferring DNA into the genome of fish.

Microinjection

Microinjection is a well established and accepted method of gene transfer in fish. The theory and practice of microinjection are described by Proctor (1992); while information on micromanipulators and micromanipulation is given by El-Badry (1963). Essentially the process is straightforward but technically tedious: 1) number of oocytes or fertilized embryos are collected from natural spawning or from *in vitro* fertilization; 2) the individual oocyte or egg is held in position by an especially designed apparatus; then with a fine glass needle, a small volume of DNA solution is deposited into the oocyte or embryo which is then left to grow. There are, however, variations in the procedure depending on the species of fish involved and the laboratory from which the protocol originated.

For microinjection into the oocyte, foreign DNA solution is microinjected into the fish oocyte nuclei prior to ovulation. This method has been used only on medaka (*Oryzias latipes*) where the chorion is soft and the oocyte nucleus can be seen clearly near the animal pole of the immature oocyte. It, however, has an additional step which requires that the injected oocyte be cultured to maturity *in vitro* after injection. Ozato et al. (1986, 1992a, 1992b, in press), Inoue

Table 1. List of transgenesis methods and the corresponding fish species experimented on with the sources of reference.

	Transgenesis methods	Transgenic fish species	References
1.	Microinjection into germinal vesicle of oocyte	Medaka (*Oryzias latipes*)	Ozato et al. (1986, 1992a, 1992b, in press); Tamiya et al. (1990); Inoue (1992); Matsumoto et al. (1992); Sato et al. (1992) and Inoue and Yamashita (1993)
2.	Microinjection of one-celled embryo through the micropyle	Arctic char (*Salvelinus alpinus*)	Shears et al. (1992)
		Atlantic salmon (*Salmo salar*)	Fletcher et al. (1986); Hew et al. (1991); Du et al. (1992a, 1992b) and Shears et al. (1992)
		Brown trout (*Salmo trutta*)	Shears et al. (1992)
		Rainbow trout (*Oncorhynchus mykiss*)	Shears et al. (1992) and Maclean (1993)
		Blunt-nose bream (*Megalobrama amblycephala*)	Xia et al. (in press)
		Channel catfish (*Ictalurus punctatus*)	Dunham et al. (1987, 1992); Zhang et al. (1990); Chen and Powers (1990); Chen et al. (1992); Powers et al. (1990, 1992b) and Hayat et al. (1991)
		Tilapia (*Oreochromis niloticus*)	Brem et al. (1988); Rahman and Maclean (1992) and Maclean (1993)
3.	One-step direct cytoplasmic microinjection into embryo without dechorionation	Common carp (*Cyprinus carpio*)	Zhang et al. (1990); Chen and Powers (1990); Chen et al. (1991, 1992); Powers et al. (1992b); Xia et al. (in press) and Cavari et al. (1993a)
		Goldfish (*Carassius auratus*)	Guise et al. (1992)
		Medaka (*Oryzias latipes*)	Chong and Vielkind (1989); (1992); Vielkind (1992) and Hong et al. (1993)
		Northern pike (*Esox lucius*)	Gross et al. (1992); Guise et al. (1992) and Moav et al. (1992)
		Seabream (*Sparus aurata*)	Cavari et al. (1993b)
		Swordtail (*Xiphophorus* sp.)	Winkler et al. (1992)
		Walleye (*Stizostedion vitreum*)	Moav et al. (1992)
		Zebrafish (*Danio rerio*)	Stuart et al. (1988, 1990); Liu et al. (1990); Powers et al. (1992b); Vielkind (1992); Moav et al. (1992); Khoo et al. (1993) and Patil et al. (1994)
4.	Two-step cytoplasmic microinjection into embryo with manual dechorionation	Atlantic salmon (*Salmo salar*)	Rokkones et al. (1985, 1989) and McEvoy et al. (1988)
		Rainbow trout (*Oncorhynchus mykiss*)	Rokkones et al. (1985, 1989); Chourrout et al. (1986); Penman et al. (1990) and Maclean et al. (1992)
		Zebrafish (*Danio rerio*)	Stuart et al. (1988, 1989)

Continued

Table 1. Continuation.

Transgenesis methods	Transgenic fish species	References
5. Two-step cytoplasmic microinjection into embryo with enzymatic dechorionation	Rainbow trout (*Oncorhynchus mykiss*)	Inoue et al. (1993) and Yoshizaki et al. (1991)
	Common carp (*Cyprinus carpio*)	Zhu et al. (1985) and Zhu (1992)
	Crucian carps (*Carassius auratus auratus* and *Carassius auratus gibelio*)	Zhu et al. (1985) and Zhu (1992)
	Goldfish (*Carassius auratus*)	Yoon et al. (1989, 1990) and Yamaha et al. (1986)
	Loach (*Misgurnus anguillicaudata*)	Zhu et al. (1985) and Zhu (1992)
	Sea bream (*Sparus aurata*)	Knibb and Moav (in press) and Knibb et al. (1994)
	Zebrafish (*Danio rerio*)	Stuart et al. (1990); Culp et al. (1991); Lin et al. (1994a) and Sekkali et al. (1994)
6. Electroporation of fertilized eggs without dechorionation	Common carp (*Cyprinus carpio*)	Powers et al. (1992b)
	Channel catfish (*Ictalurus punctatus*)	Powers et al. (1992b)
	Loach (*Misgurnus anguillicaudata*)	Xie et al. (1989); Ge et al. (1992)
	Medaka (*Oryzias latipes*)	Inoue et al. (1990); Lu et al. (1992)
	Zebrafish (*Danio rerio*)	Buono and Linser (1992); Powers et al. (1992b)
7. Electroporation of fertilized eggs with dechorionation	Red crucian carp (*Carassius auratus*)	Xie et al. (1993)
	Rosy barb (*Barbus conchonius*)	Müller et al. (1993)
	African catfish (*Clarias gariepinus*)	Müller et al. (1993)
	Loach (*Misgurnus anguillicaudata*)	Xie et al. (1993)
	Zebrafish (*Danio rerio*)	Müller et al. (1993)
8. Electroporation with miniaturized electrodes	Medaka (*Oryzias latipes*)	Murakami et al. (1994)
9. Electroporation of oocytes	Common carp (*Cyprinus carpio*)	Powers et al. (1992b)
	Channel catfish (*Ictalurus punctatus*)	Powers et al. (1992b)
10. Electroporation of sperms	Chinook salmon (*Oncorhynchus tshawytscha*)	Sin et al. (1993, 1994); Symonds et al. (1994, in press)
	Common carp (*Cyprinus carpio*)	Müller et al. (1992), Powers et al. (1992b)
	African catfish (*Clarias gariepinus*)	Müller et al. (1992)
	Channel catfish (*Ictalurus punctatus*)	Powers et al. (1992b)
	Tilapia (*Oreochromis giloticus*)	Müller et al. (1992)
11. Sperm-mediated transfer without electroporation	Zebrafish (*Danio rerio*)	Khoo et al. (in press)
12. Microprojectile	Rainbow trout (*Oncorhynchus mykiss*)	Zelenin et al. (1991)
	Loach (*Misgurnus anguillicaudata*)	Zelenin et al. (1991)
	Zebrafish (*Danio rerio*)	Zelenin et al. (1991)
13. Liposome-mediated	African catfish (*Clarias gariepinus*)	Szelei et al. (1994)
14. Embryonic stem cells	Zebrafish (*Danio rerio*)	Collodi et al. (1992); Lin et al. (1992)
15. Retroviruses	Zebrafish (*Danio rerio*)	Burns et al. (1993); Lin et al. (1994b)

(1992), Matsumoto et al. (1992), Sato et al. (1992) and Tamiya et al. (1990) have microinjected DNA into the germinal vesicle of medaka oocytes. The injected oocytes are subsequently cultured in suitable media until maturity when the follicles are then removed and the oocytes inseminated. This procedure requires accurate knowledge of the species' oocyte development. Since this information is available for medaka, it is possible to determine the correct oocyte stage for microinjection. The injected eggs are then incubated *in vitro* until maturation. A major obstacle of this method is the limited time available for microinjection which is usually carried out when the germinal vesicle has moved to the oocyte periphery. Using media containing hormones to enhance oocyte maturation, Inoue and Yamashita (1993) showed that it is possible to introduce foreign genes into oocytes collected earlier than before. In fish species where the timing of oocyte maturation is not that well documented, this culture method may be used, thus opening the way for oocyte microinjection experiments to be conducted in species other than medaka.

Microinjection into the pronucleus of fertilized eggs is a more common procedure for mammals, one however, which is not possible in fish where the egg nucleus is not visible through the opaque chorion. Moreover, the yolky nature of most fish eggs tends to mask the visibility of the pronuclei. Thus the alternative is to introduce the DNA as close to the pronuclei as possible. Microinjecting through the micropyle will achieve this objective since the pronuclei tend to lie below the micropyle.

The micropyle is the opening through which the fertilizing sperm enters (Szollosi and Billard 1974; Kuchnow and Scott 1977; Riehl 1980). The fertilizing sperm nucleus remains in the ooplasm immediately under the micropyle and located close to the female pronucleus. The micropyle provides the site on the egg surface to microinject and it guides the micropipette and the release of the injected DNA to the area of the male and the female pronuclei. Atlantic salmon (*Salmo salar*), rainbow trout (*Salmo gairdneri = Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and Arctic char (*Salvelinus alpinus*) eggs have been injected through the micropyle to produce transgenic fish (Shears et al. 1992). Micropylar microinjection was also conducted on tilapia (*Oreochromis niloticus)* by Brem et al. (1988) and Rahman and Maclean (1992); on Atlantic salmon by Fletcher et al. (1986), Hew et al. (1991) and Du et al. (1992a, 1992b). Locating the micropyle to inject through, however, requires much time, patience, keen eyesight and good micromanipulation skill. Since microinjection usually has to be completed before first cleavage of the egg, it must be conducted quickly as well. In order to increase this pre-cleavage time period, Lu et al. (1992) slowed down the rate of embryonic development in medaka eggs in a cold water bath (4-6°C) immediately after natural spawning.

Because of the difficulties of locating the micropyle in most fish species, microinjection into the cytoplasm of the one-cell embryo was found to be an effective alternative. Several variations of the cytoplasmic microinjection procedure have been reported. In fishes with thin chorion, DNA was microinjected directly through the chorion and the vitelline membrane directly into the center of the germinal disc of the fertlized egg. This was conducted for goldfish *Carassius auratus* (Guise et al. 1992) and northern pike *Esox lucius* (Gross et al.

1992; Guise et al. 1992). Cytoplasmic injections into one-cell, two-cell and four-cell embryos of common carp (*Cyprinus carpio*) were performed by Chen and Powers (1990), Zhang et al. (1990), Chen et al. (1991, 1992), Powers et al. (1992b), Xia et al. (in press) and Cavari et al. (1993a). Cytoplasmic injection into zebrafish *Brachydanio* (=Danio) *rerio* embryo was conducted by Stuart et al. (1988, 1990), Liu et al. (1990), Powers et al. (1992b), Vielkind (1992) and Patil et al. (1994).

Chong and Vielkind (1989), Winkler et al. (1992), Vielkind (1992) and Hong et al. (1993) used the method on medaka. Winkler et al. (1992) used it on swordtail (*Xiphophorus*); Cavari et al. (1993b) on sea bream (*Sparus aurata*); Dunham et al. (1987, 1992), Zhang et al. (1990), Chen and Powers (1990), Chen et al. (1992), Powers et al. (1990, 1992b) and Hayat et al. (1991) on channel catfish (*Ictalurus punctatus*); Xia et al. (in press) on blunt-nose bream (*Megalobrama amblycephala*) and Moav et al. (1992) microinjected cytoplasmically into northern pike, walleye (*Stizostedion vitreum*) and zebrafish eggs.

Khoo et al. (1993) described the procedures and requirements for microinjecting into zebrafish. The microinjection was administered at the vegetal pole diametrically opposite the animal pole, that is, through the chorion and then the yolk, which is easier to puncture with the needle, before penetrating into the cytoplasm of the germinal disc. This procedure also allowed the germinal vesicle to be better targetted since the egg tended to roll around in the large chorionic space. Maclean (1993) gave details on microinjection into salmonids and tilapia.

In some fish species, the tough chorion of the fertilized egg tends to present a barrier to microinjection. One of the solutions was to mechanically drill a hole into the chorion to allow the fine glass needle containing the DNA to reach the egg membrane. Rokkones et al. (1985, 1989) conducted this two-step cytoplasmic microinjection with dechorionation using a small metal suture needle to make a tiny hole in the zona radiata at the animal pole of the egg. The micropipette was introduced into this hole in experiments on Atlantic salmon and rainbow trout. Chourrout et al. (1986) manually drilled over the animal pole of rainbow trout eggs with a broken micropipette of 50 μm diameter resulting in a 100 um hole through which a 10 μm pipette was driven down to the ooplasm. Penman et al. (1990) and Maclean et al. (1992) used a small needle to pierce the chorion of rainbow trout eggs. McEvoy et al. (1988) used a similar two-step process on Atlantic salmon embryos. Stuart et al. (1988, 1989) manually dechorionated the hard chorion of zebrafish eggs.

Enzymatic removal of the chorion was an alternative to manual dechorionation. Goldfish *Carassius auratus* eggs were dechorionated by a 4-6 minute incubation in 0.2-0.25% trypsin in Holtfreter's solution, 5 minutes after fertilization (Yoon et al. 1989, 1990). Yamaha et al. (1986) determined the optimum levels of enzymes for dechorionation in goldfish. The hard chorions of zebrafish eggs were dechorionated by digestion with 500 $\mu g \cdot ml^{-1}$ pronase (Stuart et al. 1990; Culp et al. 1991; Lin et al. 1994a; Sekkali et al. 1994). The egg chorion of the crucian carp (*C. auratus auratus*), loach (*Misgurnus anguillicaudata*), common carp, varieties of mirror and red carp and silver cru-

cian carp (*C. auratus gibelio*) were removed with 0.25% trypsin (Zhu et al. 1985; Zhu 1992). The chorion was monitored under a stereomicroscope (10-20x); and when the chorion became thinner and more transparent but not completely digested, the trypsin solution was replaced with fresh Holtfreter's solution. The process took about 5 minutes.

Yoshizaki et al. (1991) and Inoue et al. (1993) prevent the hardening of the chorion by soaking rainbow trout eggs in 0.5 mM glutathione (reduced form) made to pH 10 with NaOH. After swelling in glutathione, DNA solution was microinjected into the cytoplasm of the dechorionated eggs. Knibb and Moav (in press) and Knibb et al. (1994) used calcium ion-free seawater as well as seawater with 2 $mmol \cdot L^{-1}$ glutathione to inhibit chorion hardening of sea bream eggs.

Electroporation

Mass methods of gene insertion are needed if this technology is to be useful in aquaculture. Electroporation is a mass technique for treating large numbers of eggs (Zhao et al. 1993). It uses short electrical pulses to permeabilize the cell membrane thus allowing the entry of macromolecules into the cell (Zimmermann 1986). Essentially the method is to place the fertilized fish eggs or embryos in a solution containing the DNA of interest in between two electrodes, and to pulse them with electricity set at certain field strengths (V/cm) and capacitance (microfarad). The number of times they are pulsed has also been shown to be important. The advantages of electroporation over the microinjection method are that it is simple and rapid and allows for treatment of many embryos at any one time.

The first successful gene transfer by electroporation was demonstrated on medaka fertilized eggs by Inoue et al. (1990). Integration and germ-line transmission was shown but a low level of gene transfer efficiency of about 4%. Buono and Linser (1992) reported the first use of an exponential decay electroporation system to introduce foreign DNA into fertilized zebrafish embryos. It was shown that 65% of the surviving hatchlings carried the foreign construct. Transient expression was also observed. Lu et al. (1992) also used an exponential decay electroporator to introduce rainbow trout recombinant growth hormone gene into medaka, but obtained 20% integration.

Powers et al. (1992b) observed that electroporation tended to produce a greater number of transgenic individuals than microinjection for zebrafish, common carp and channel catfish. Electroporation-mediated DNA transfer into fertilized eggs of African catfish, zebrafish and rosy barb (*Barbus conchonius*) were improved by using multiple square pulses on dechorionated eggs (Muller et al. 1993). Dechorionation was in 10 $mg \cdot ml^{-1}$ pronase enzyme in Holtfreter solution 5-10 minutes after fertilization. Dechorionation improved DNA uptake.

Xie et al. (1989) electroporated loach eggs but gene transfer efficiency was low. The low level was attributed to the chorion and the distance of the egg membrane from the chorion. Xie et al. (1993) repeated the experiments on trypsin dechorionated loach and red crucian carp eggs and obtained higher gene transfer efficiency and transgene copy number per genome; these were

positively correlated to the voltage, duration and capacity of the pulse. Ge et al. (in press) also used the electroporation method on the loach.

Using miniaturized electrodes, Murakami et al. (1994) applied a localized electric field to the animal pole of fertilized medaka egg before first cleavage, and showed a higher ratio of gene introduction than the conventional electroporation method. Expression and integration of the introduced gene were also demonstrated. This technique, however, has a disadvantage in that it requires that the animal pole of the egg be oriented in between the microelectrodes, an additional step to the process. Its mass gene transfer advantage is thus obviated by this need.

Electroporation of germ products also successfully produced transgenic fish. Powers et al. (1992b) electroporated the oocytes and sperms of common carp and channel catfish. The procedure yielded a higher percentage of transgenic successes than electroporation of embryos. Muller et al. (1992) used electroporated sperm cells to produce transgenic common carp, African catfish and tilapia. Sin et al. (1993, 1994) and Symonds et al. (1994, in press) used electroporated sperms to produce transgenic chinook salmon (*Oncorhynchus tshawytscha*). Electroporated sperm-mediated transfer appears to be quite successful in a number of fish species.

Successful sperm-mediated gene transfer without electroporation, however, was first demonstrated in fish by Khoo et al. (in press). This technique has previously been shown to be successful in rabbit, cattle, mice and chicken (Khoo et al., in press). More recently in the fourth European Congress of Cell Biology in Prague, 26 June-1 July 1994, successful simple sperm-mediated gene transfers in sheep, Xenopus, rabbit, mouse, bovine and pig were again reported (pers. comm.). Khoo et al. (in press) showed that homologous sperms of zebrafish can be used as a vehicle to carry DNA into the embryo by simple incubation of the sperms in a solution of plasmid DNA containing a CAT reporter gene. Transfer of DNA and germ-line transmission were demonstrated, but expression was not observed. This was attributed to rearrangement of the introduced DNA and the extrachromosomal persistence of the introduced gene in the embryonic cells. Simple DNA incubation of sperm is advantageous in that it is simple to use, does not require expensive equipment and is also a mass method for treating and producing large numbers of fish. Negative results using similar techniques, however, were obtained in common carp, African catfish and tilapia by Muller et al. (1992); in rainbow trout by Chourrout and Perrot (1992); and in chinook salmon by Sin et al. (1993). The concentration of DNA used influenced the efficiency of the transfer (Sin et al. 1994). This could have been the reason for discrepancies in the differing results obtained. Khoo et al. (in press) used high concentrations of DNA (100-500 $\mu g \cdot ml^{-1}$). However the differences could be due to species differences. Uptake of DNA by carp, catfish and tilapia sperms, nevertheless, was observed by Müller et al. (1992).

Another interesting mass gene transfer method is the microprojectile or "gene gun" technique (Zelenin et al. 1991). Fertilized eggs of loach, rainbow trout and zebrafish were bombarded with high-velocity tungsten microprojectiles covered with DNA. Transgenic individuals were obtained. This method, however, though simple, requires expensive apparatus.

Other techniques have been tried to overcome some of the difficulties associated with microinjection, its low efficiency, the mosaicism of the fish produced and the technically tedious nature of the method. Szelei et al. (1994) demonstrated liposome-mediated gene transfer into African catfish (*Clarias gariepinus*) embryos. *In vitro* fertilized eggs were dechorionated by protease and the two- to four-cell stages were treated with liposome suspension. Very efficient DNA uptake has been indicated. Advantages of the method include the relative simplicity of the treatment, extended shelf-life of the liposomes, and the ease of using large constructs of DNA and its use for treating large numbers of eggs at a single time. Disadvantages are the lengthy liposome preparation, lack of integration, and problems associated with the dechorionation of eggs.

Embryonic Stem Cell

This is a powerful strategy for transgenesis. Cells are removed from developing blastocysts and are grown in culture in an undifferentiated state. Foreign DNA is introduced by electroporation, transfection or microinjection into the stem cells, and these cells are then reintroduced into the blastocyst which are then allowed to develop to term. If some of the transgene-containing cells develop into germ cells, then subsequent breeding will produce transgenic individuals. Research in this area conducted by Collodi et al. (1992) and Lin et al. (1992) have obtained positive results. This method allows for the pre-selection of cells as well as increasing the concentration of cells which contain the integrated exogenous DNA before introducing them into the blastocyst, thus increasing the chances of producing transgenic individuals. For example, selection can be conducted by fusing the gene of interest with a *neo* gene, which then allows the cells that incorporate this recombinant gene to grow in media containing G418 which is normally toxic to cells not possessing the gene.

Retroviruses and Other Viral Vectors

Retroviruses efficiently integrate their genetic materials into the genome of the infected cells by a precisely defined mechanism (Powers et al. 1992a). Only a single copy of the provirus is inserted at a given chromosomal site, and rearrangements of the host genome are not induced unlike other methods such as microinjection where foreign gene insertion may be in multiple copies (Chong and Vielkind 1989) or rearranged as in sperm-mediated transfer (Khoo et al. , in press). The foreign gene is incorporated into the viral genome which is then transferred to the host by viral infection. To date no fish retrovirus has been isolated. The retrovirus is often species-specific, thus murine and avian retroviruses, which are available and well characterized, have been assumed to be ineffective on fish. Burns et al. (1993) and Lin et al. (1994b), however, recently showed that a pseudotyped pantropic retroviral vector originally developed for human gene therapy was able to infect zebrafish cells and embryos, respectively. The latter microinjected a retroviral vector pseudotyped with the envelope glycoprotein of the vesicular stomatitis virus into the blastula-stage and eight out of 51 founders transmitted proviral DNA to their progeny. The advantages of retroviral technology can therefore be applied to fish. The use of

retroviruses for transgenesis is probably confined to developmental studies and is not used to produce fish for human consumption.

High efficiency gene transfer mediated by adenovirus coupled to DNA-transferrin (Zatloukal et al. 1992) and DNA-polylysine (Curiel et al. 1992; Curiel 1994) complexes into eukaryotics cells have been demonstrated. Its use and equivalent in fish have yet to be shown. Its use is limited by the potential safety hazards of using viral genetic material, however, its high efficiency makes it useful for research purposes.

Monitoring for the Presence of the Transgene

After transferring the gene using one of the above methods, the next step is to identify individuals which are transgenic. This is usually conducted using dot and Southern blot analyses (Sambrook et al. 1989). Essentially the methods involve extracting genomic DNA from whole or parts of the putative transgenic individuals, transferring them onto nylon membranes which are then probed for the presence of the inserted gene using labelled homologous probes. For Southern blot, DNA samples are electrophoresed on agarose gels before the transfer. Recently a more sensitive and rapid method of detection has been used and this is the Polymerase Chain Reaction (PCR) method (Erlich 1989; Wright and Wynford-Thomas 1990; Innes et al. 1990; Palumbi et al. 1991; Rolfs et al. 1991). To demonstrate that there is germ-line transmission of the gene insert, DNA extracts from F_1 and F_2 progenies would have to be shown to be positive for the presence of the transgene.

Monitoring for Gene Expression

If the foreign genes together with their promoters are integrated properly into the genome of the host, their expression products are expected. Assays for each of the translation products of the common reporter genes are well-documented: luciferase (Brasier and Ron 1992); human growth hormone (Selden et al. 1986); chloramphenicol acetyltransferase (Crabb et al. 1989) and lacZ (Lin 1994a).

Potential Applications of Transgenesis to Aquaculture

Once gene transfer technology is shown through basic research to be practical and efficacious for a species, it can be transferred for use in aquaculture. The aims of gene transfer technology in aquaculture would be similar to that of any genetic selection and gene transfer programs for farm animals, which is to produce the most efficient animal suitable for a particular environment so as to eventually benefit human beings. Phenotypic changes in potentially useful transgenic animals include the following areas of interest: (1) metabolic rates (accelerated growth using growth hormone genes); (2) tolerance to physical factors such as cold using antifreeze gene; (3) behavioral modifications effected through changes in the regulation of endocrine compounds such as those involved in reproduction, maturation and sex control, life history modifications, as

well as production of sterile individuals; (4) resource use: more efficient use of feed, elimination of dietary requirements by modifying particular biochemical pathways; disease resistance: resistance to parasites, pathogens or predators; (5) modification of body composition, for example, less fat and less non-edible parts such as skeletal parts; and finally (6) modification for increased production of useful pharmaceutical products such as carageenin in seaweeds (Roschlau 1991; Ward et al. 1991; Wall et al. 1992).

The applications of transgenic technology for increased growth in fish and their freezing tolerance are the two main areas of research to-date (Hew et al. 1991). A number of laboratories have successfully transferred growth hormone (GH) genes into eggs of a number of fish species and showed integration, expression and imheritance of the foreign GH gene (Chen et al. 1991; Moav et al. 1992; Pandian and Marian 1994). Most transgenic fish produced so far for enhanced growth, however, utilized either human, mouse, rat or bovine growth hormone genes as a recombinant with non-fish promoters such as the mouse metallothionein, the LTR promoter of the rous sarcoma virus and the simian virus 40 genes (Zhu et al. 1985; Chourrout et al. 1986; Dunham et al. 1987; Rokkones et al. 1989; Zhang et al. 1990).

Fish containing genes which are not of fish origin may eventually develop consumer preference problems. The metallothionein promoter was considered unsuitable for aquaculture because it is associated with heavy metals, a metabolic poison required for inducing the gene during culture which might contaminate the fish product. The association of viral genes with tumor-inducing sequences makes them a potential health hazard if consumed. All-fish gene constructs using structural genes and promoters of fish origin are therefore preferred for human consumption.

Promoters isolated and characterized from fish are limited. One is the rainbow trout metallothionein B gene (tMTb) (Zafarullah et al. 1988; Gedamu et al. 1990) which was linked to several reporter genes such as *cat* and *lacZ*, and tested on medaka by Hong et al. (1993). Liu et al. (1990) studied the efficacy of carp β-actin gene promoters on zebrafish. They developed two expression vectors, FV-1 and FV-2, which contain the proximal promoter and enhancer regulatory elements of the carp β-actin gene and the polyadenylation signal from the chinook salmon growth hormone gene. This promoter was also tested on walleye and northern pike (Moav et al. 1992). The ocean pout antifreeze polypeptide promoter was developed and tested on Atlantic salmon by Du et al. (1992a, 1992b).

As for structural genes, a number of growth hormone cDNAs from different fish species have been isolated and characterized (Table 2). These are from Atlantic salmon (Lorens et al. 1989), chum salmon (Sekine et al. 1985), coho salmon (Nicoll et al. 1987), sockeye salmon (Devlin 1993), chinook salmon (Hew et al. 1989; Du et al. 1993; Song et al. 1993), two from rainbow trout (Agellon and Chen 1986; Rentier-Delrue et al. 1989a), barramundi, *Lates calcarifer* and black bream *Acanthopagrus butcheri* (Knibb et al. 1991), red sea bream (Momota et al. 1988), bluefin tuna (Sato et al. 1988), yellowtail (Watahika et al. 1988), carp (Chao et al. 1989), eel (Saito et al. 1988), tilapia (Rentier-Delrue et al. 1989b), northern pike (Schneider et al. 1992), yellowfin porgy, *Acanthopagrus latus* (Tsai et al. in press),

Table 2. List of fish species from which growth hormone genes have been cloned and characterized and references.

Fish species	References
Atlantic salmon	Lorens et al. (1989)
Chum salmon	Sekine et al. (1985)
Coho salmon	Nicoll et al. (1987)
Sockeye salmon	Devlin (1993)
Chinook salmon	Hew et al. (1989); Du et al.(1993); Song et al. (1993)
Rainbow trout	Agellon and Chen (1986); Rentier-Delrue et al. (1989a)
Barramundi	Knibb et al. (1991)
Black bream	Knibb et al. (1991)
Red sea bream	Momota et al. (1988)
Bluefin tuna	Sato et al. (1988)
Yellowtail	Watahika et al. (1988); Nakashima et al. (1993)
Carp	Chao et al. (1989)
Eel	Saito et al. (1988)
Tilapia	Rentier-Delrue et al. (1989b)
Northern pike	Schneider et al. (1992)
Yellowfin porgy	Tsai et al. (in press)
Hardtail	Nakashima et al. (1993)
Flounder	Nakashima et al. (1993)

yellowtail *Seriola quinqueradiata*, hard tail *Caranx delicatissimus* and flounder *Paralichthys olivaceus* (Nakashima et al. 1993).

Hew et al. (1991) and Du et al. (1992a, 1992b) developed and tested two all-fish gene constructs consisting of the structural growth hormone gene from the chinook salmon linked to the ocean pout anti-freeze protein promoter (opAFP-csGHc and opAFP-csGHg). Up to a 13-fold increase in size and a four-fold increase in growth rate over a 100-day period was observed in the transgenic Atlantic salmon produced. Another all-fish construct consisting of the chinook salmon growth hormone gene linked to the common carp β-actin gene proximal promoter and enhancer regulatory elements was tested on northern pike (Gross et al. 1992; Moav et al. 1992). Cavari et al. (1993a) also developed three all-fish expression vectors: ptMTa-gbsGHcDNA and ptMTb-gsbGHcDNA (consisting of rainbow trout metallothionein a/b) and the gilthead sea bream growth hormone cDNA were tested on common carp. The third construct developed was pcAb-gsbGHcDNA which consisted of the carp β-actin and gilthead sea bream GH cDNA.

The most dramatic "superfish" produced was by Devlin et al. (1994) who inserted an "all-salmon" gene construct (pOnMTGH1) into coho salmon (*Oncorhynchus kisutch*) and Atlantic salmon. Both the metallothionein promoter and the type 1 growth hormone gene were from sockeye salmon. Growth of the transgenic coho salmon by over 11-fold and up to 37 times greater than the non-transgenic controls was observed.

The insulin-like growth factor (IGF) genes from coho salmon and rainbow trout (Cao et al. 1989; Shamblott and Chen 1992, respectively), common carp (Liang et al. 1994) and black sea bream (*Acanthopagrus schlegeli*; Wu et al. in press), have been isolated. IGFs are mitogenic peptide hormones that play an important role in the growth and differentiation of vertebrates. Transgenesis in fish using these genes, however, has not been conducted yet. Control of growth using these genes may be an alternative to manipulation of growth hormone genes.

The production of transgenic fish with increased growth rates is therefore technically feasible; but their commercial culture requires consideration of several factors such as: the risks to the natural populations if the transgenic individuals were to escape from the aquaculture facilities; the economics of such culture may not be as good; the transgenes may not be efficient feed converters or may be more susceptible to diseases thus incurring more costs; and also their taste may not be acceptable to consumers.

The antifreeze gene is another gene that can endow a fish with beneficial characteristics for aquaculture. The antifreeze protein gene (AFP) from winter flounder (*Pseudopleuronectes americanus*) has been isolated (Scott et al. 1985; Fletcher et al. 1986; Hew et al. 1991). Researchers (Hew et al. 1991) attempted to make Atlantic salmon more freeze-resistant by transferring antifreeze protein genes from the winter flounder to the genome of the Atlantic salmon. The levels of AFP expression are still too low to protect against freezing, but the potential for aquaculture in the cold temperate is good if commercial fish species like the Atlantic salmon and trout can be made to tolerate winter conditions.

One of the fish genes which has been isolated and studied and is potentially beneficial for fish culture are the DNA sequences that produce prolactin and somatolactin. Both are pituitary hormones implicated in osmoregulation and electrobalance (Ono et al. 1990). These genes are potentially useful for the development of saline-tolerant freshwater fish or vice versa for aquaculture.

In any aquacultural genetic program, the ability to tag individual fish and follow their genealogy would be most useful. Harris et al. (1991) has suggested the use of fingerprinting to assess inbreeding rates, as genetic markers to identify individuals and family groups, and to label stocks especially broodstock to authenticate ownership. A tag that is germ-line transmissable and easily identifiable would save much time and work. Production of transgenic fish would permit the development of fish with genetic markers consisting of inactive DNA sequences lodged in non-transcribed regions such as the introns. This would enable a breeding program to track genealogy (Maclean and Penman 1990). The technique could also be used to prove that certain individuals of a protected species are bred specimens and not poached wild individuals (for example, in the case of arowana [*Centropages formosus*]). There are many possibilities for the manufacture of oligonucleotides with specific sequences which can be targetted to the least harmful part of the fish's genome.

Control of sex is commonly practiced in the culture of fish which show differences in growth characteristics between the sexes. At the moment, sex ablation can be produced by chromosome manipulation. Such individuals grow fast besides being unable to breed with wild stocks if they happen to escape from aquaculture farms. Transgenesis provides an alternative to chromosome manipulation in the area of sex control and reproduction. Genetic ablation can occur by controlling the expression of sterility-inducing genes to tissues responsible for reproduction (Maclean and Penman 1990; Devlin and Donaldson 1992). Tissue-specific promoters can be used to drive the expression of a cytotoxic gene product such as diptheria-toxin that destroy specific targeted cells. The protamine gene promoters which are already well characterized for salmonids (Gregory et al. 1982; Aiken et al. 1983.) can be used for targeting to the testis.

Another strategy for the control and prevention of gonadal maturation is the use of anti-sense constructs to disrupt the reproductive hormones located in the pituitary and the hypothalamus. Genes that express gonadotropins which control gonadal maturation have been isolated from carp (cGTH-b; Chang et al. 1992) and chinook salmon (csGTHIIb; Hew and Xiong, in press), but these have yet to be used for gene transfer experiments for gonadal maturation control. The fish gonadotropin-releasing hormone genes, GnRH, have been cloned from a cichlid (*Haplochromis burtoni*) and Atlantic salmon. The potential use of the GnRH antagonist DNA sequence for control of sexual maturation is discussed by Alestrom et al. (1992).

Another area of great benefit to fish culture is disease resistance. Five classes of mammalian genes implicated in regulating disease resistance are currently seen as possible candidates for gene transfer experiments. These are the MHC, T-cell receptor, immunoglobulin, lymphokines and specific disease resistance genes. Similar genetic systems may be present in fish, however, none of these genes has been isolated in fish. The use of antisense RNA genes in fish viral diseases for protection against infection is another possibility (Hew et al. 1991).

Transgenic mice have been used in drug development and testing (e.g., in safety tests) (Harris et al. 1993). Transgenic fish produced with known DNA inserts sensitive to mutagens or carcinogens have the potential for use in monitoring mutagenic substances in the aquatic environment. This is similar to the muta™ mouse which is already available for *in vivo* mutagenic studies (Myhr 1991). The role of transgenic animals in toxicology and their application in genetic toxicology are reviewed by Goldsworthy et al. (1994) and Gossen et al. (1994), respectively.

Black pigmentation is controlled mainly by the tyrosinase enzyme. Matsumoto et al. (1992) transferred a mouse tyrosinase gene into the orange-red variety of medaka and obtained transgenic dark individuals with the active mouse tyrosinase enzyme. Melanization was observed in the amelanotic melanophores. Control of marking patterns using cell transplants of pigmented cells to genetically albino embryos have been conducted in zebrafish (Lin et al. 1992). In ornamental aquarium fish, which are appreciated for their beauty, and in food-fish, varieties with attractive colors and patterns have higher commercial value. The sea bream or red porgy, *Pagrus major* is a favorite fish in Japan partly because of its beautiful crimson color which serves also to decorate the table on festive occasions (Fujii 1993). *Oreochromis mossambicus* has a dark appearance and, prior to its hybridization to *O. niloticus*, did not have much commercial value in Southeast Asia. However, when a red hybrid variety (Maclean 1984) was produced, it became a popular and expensive restaurant fish in Singapore partially because of its more appealing color.

Modification of coloration and color patterns by gene transfer would result in the creation of fish with desirable hues and patterns. Such technology would have great potential for modifying ornamental fish. If identified and cloned, the genes responsible for chromatophore formation and differentiation, as well as the hormones (such as melanophore-stimulating hormone [MSH] and the melanin-concentrating hormone [MCH]) that regulate them can be useful in these respects. The MCH gene has been cloned and characterized in chum salmon

(Alestrom et al. 1992), and its protein product has been shown to cause contraction of the melanosomes in the melanophores of the dark stripes of zebrafish. In amphibians, a melanization-inhibiting factor (MIF) blocks the differentiation of melanoblasts into melanophores; if a similar factor exists in fish, then another way of controlling coloration can be explored and made available. For the expression of desirable colors or patterns, the genes from other species, not only within the same genus, but even from species belonging to other genera, families, orders, classes or even to other phyla, can be used (Fujii 1993).

Artificial markings that have no influence on swimming ability and behavior would be useful for stock assessment and migration studies of wild as well as hatchery fish stocks.

Transgenesis in Invertebrates and Non-fish Vertebrates

Gene transfer studies in the amphibian, *Xenopus laevis*, have been for the purpose of studying vertebrate development, and not for aquaculture purposes (Colman 1984; Kay and Peng 1991). Only two published studies were on aquatic invertebrates. Both were on the sea urchins, *Strongylocentrotus purpuratus* (McMahon et al. 1984; Arezzo 1989) and *Paracentrotus lividus* and *Arbacia lixula* (Arezzo 1989), but these were not aimed at aquaculture. There have been no published reports of gene transfer in crustaceans and molluscs (Benzie 1991). Recently, however, there have been conference reports of attempts to produce transgenic molluscs.

Traditional selective breeding has not been able to produce fast-growing abalones. Gene transfer technology may help to provide for faster selection by inserting IGF or growth hormone genes. The methodology for the production of transgenic abalone with the aim of increasing growth has been developed recently. Genetic engineering for a fast-growing strain of the red abalone *H. rufescens* has been reported (Powers et al. 1994). In the same meeting, Gomez-Chiarri et al. (1994) reported the cloning of an actin promoter isolated from the red abalone *H. rufescens*. Cadoret et al. (1994) also reported their first steps in the genetic manipulation of farmed invertebrates using microinjection, electroporation, liposomes and particle bombardment using CMV-β-gal and HSP-β-gal vectors.

Ecological, Socioeconomic and Ethical Issues of Fish Transgenesis

Ecological, socioeconomic as well as ethical issues have to be considered before transgenic animals can be allowed for culture or release. Such issues for transgenic animals in general were discussed at a workshop on transgenic animal research (Hopper et al. 1989). Andersson et al. (1992) reviews the ecological risk of transgenic organisms in Sweden. Ethics, values and animal welfare issues are analyzed by Sandoe and Holtug (1993) and Loew (1994). The ethics of human gene transfer is presented by Carmen (1992) and some of the criteria may be applicable for fish. Wohrmann and Tomiuk (1993) assessed the risks of deliberate release of transgenic organisms. Goodman (1993) discussed the use of transgenic

predators; while Adam et al. (1993) considered the effects of escaped transgenic animals. The food safety of transgenic animals is dealt with by Berkowitz (1990). Discussions on the ecological and social issues of transgenic fish are available in Kapuscinski and Hallerman (1990, 1991) and Hallerman and Kapuscinski (1990a, 1990b, 1992). Containment of genetically altered fish including transgenics is discussed by Devlin and Donaldson (1992). Biological containment such as sterilization, instead of physical containment, appears to be the best way to prevent ecological disruptions by transgenic fish released deliberately or inadvertently.

Future Prospects

The future of transgenesis of aquatic organisms for aquaculture, for direct human consumption and commercialization as well as for environmental monitoring and enhancement, is only just beginning. Its application in aquaculture is full of exciting prospects and possibilities limited only by human imagination and creativity.

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