Genetic Improvement of Fish for Aquaculture Through Foreign Gene Transfer

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Introduction

Aquaculture scientists are attempting to introduce novel genes to fish in the hope of rapidly improving commercial traits, particularly growth rate. There are indications that finfish respond to injections of exogenous growth hormone (Adelman 1977). Agellon et al. (1988) have reported successful results with a recombinant fish growth hormone. Other researchers are investigating recombinant DNA for production of vaccines for viral fish diseases (Leong et al. 1988); isolation of fish genes for important production traits (Kapuscinski 1989); cloning of growth hormone genes from fish species (Agellon and Chen 1986; Cavari et al. 1988; Gonzales-Villaseñor et al. 1988); construction of fish gene libraries (Johansen and Valla 1987; Maclean et al. 1987a) and refinement of gene transfer techniques (Penman et al. in press). Chen et al. (1987) have reviewed "genetic engineering" in fish.

Preparation of Foreign DNA

The gene constructs utilized in gene transfer to fish are presented in Table 1. Most researchers now use human (hGH) or rat (rGH) growth hormone genes, fused to a mouse metallothionein (mMT-I) promoter. A promoter is a DNA sequence which acts as a binding site for RNA polymerase and initiates transcription. Considerable skepticism has been voiced about the value of experiments using mammalian genes to create transgenic fish. However, whatever the origin of foreign gene sequences, a strong promoter increases the chances of good expression (Maclean et al. 1987a).

The preparation of foreign DNA relies on plasmids (Table 1). The term 'fusion genes' is used when regulatory sequences are bonded to the coding sequences of a gene (Penman et al. in press). The popularity of this fusion gene construct in fish probably derives from its successful use in mouse research. Important technical questions remain; for example, which genes are appropriate for transfer to which species? Will a gene sequence, when expressed, be beneficial to the transgenic animal in terms of performance? Methodological advances are therefore still being sought; e.g., the work of Yoon et al. (in press) with neomycin resistance as a selectable marker.

DNA Microinjection

Gene transfer can be accomplished through microinjection of copies of the foreign DNA into the nuclei of individual egg cells, using glass micropipettes or needles with an external tip diameter of 5-15 µm, controlled by micromanipulation apparatus. Most successful gene transfers in fish have been by microinjection into fertilized or unfertilized eggs (Table 2).

The following considerations are important: whether the foreign gene sequence is genomic or complementary DNA (cDNA); the form of the gene construct, whether linearized or circularized; whether the cloning vector should be included or not; the number of foreign DNA copies to be injected; and the stage of egg development and the location at which to inject the foreign DNA (Maclean et al. 1987a).

Genomic DNA copies incorporate all intron, promoter and some flanking sequences, both upstream and downstream from the coding sequence, whereas cDNA (which results from reverse transcription of messenger RNA) gen-

Table 1. Gene constructs used in the production of transgenic fish.

Species	Recombinant plasmid	Gene	Promoter	Reference
Oryzias latipes	pδC-1β	Chicken δ crystallin	Chicken δ crystallin	Ozato et al. (1986)
Salmo gairdneri	pSV507	human growth hormone/cDNA	Simian virus (SV40)	Chourrout et al. (1986)
Brachydanio rerio	pSV-hygro	hygromycin resistance	Simian virus (SV40)	Stuart et al. (1988)
Carassius auratus	pRSV-neo	neomycin analog G-418 resistance	Rous sarcoma virus (RSV)	Yoon et al. (in press)
Salmo gairdneri	pMGH	rat growth hormone	mouse metallothionein	Maclean et al. (1987b)
Salmo salar	pBR-met-β-gal	*E. coli* β-galactosidase	mouse metallothionein	McEvoy et al. (1988)
Oreochromis niloticus	MThGH[a]	human growth hormone	mouse metallothionein	Brem et al. (1988)

[a]This fusion gene contained a viral chromosome (a remnant from the genomic library from which the hGH gene was constructed) in addition to the gene and promoter.

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erally lacks these. To achieve strong expression, Maclean et al. (1987a) recommend the use of genomic copies. The successes shown in Table 2 compare well with those achieved in mice. Indeed Ozato et al. (1986) had even better percentage transformation. The high percentage transformation achieved by Chourrout et al. (1986) is not a clear cut 'success story' for the use of cDNA because the method used to estimate integration was scintillation counting on the dot blot and not conventional Southern blot analysis.

Most workers have employed linearized plasmids, prepared using 'restriction enzymes'. In rainbow trout *(Salmo gairdneri)*, linearized plasmids gave a higher transformation rate (75%) than circularized (40%) (Chourrout et al. 1986). In another study with circularized plasmids, the foreign DNA was not integrated into the genome but persisted in an extrachromosomal form (Penman et al., in press).

Maclean et al. (1987a) suggest that the 'cloning vector' should not be included in the injected gene sequence. The cloning vector is really just 'extra baggage' which could be discarded.

The number of DNA copies injected into fish eggs ranges from about 10^3 to 10^7, but not all studies state this numerically. Others have quantified the weight or volume of the injected DNA solution, making it difficult to compare values. About 10^6 copies of DNA per injected cell is generally regarded as sufficient (Maclean et al. 1987a, 1987b) but Penman et al. (in press) observed that more (10^8) copies of linear DNA significantly increased the percentage production of transgenic rainbow trout.

Microinjection studies in mice have demonstrated that integration of a foreign gene into the host genome is more efficient with nuclear than with cytoplasmic injection. The nuclei of fish eggs are usually very small and hard to visualize. Table 2 shows various approaches. Both cytoplasmic and nuclear injections have succeeded.

Very early embryos are probably the most suitable stages for stable incorporation of foreign DNA into the host genome. However, the work of Ozato et al. (1986) on medaka *(Oryzias latipes)* is interesting because this experiment gave the highest rate of integration when DNA was injected into oocytes. Probably, the optimal stage of egg development and location of DNA injection varies with species.

Transgenic rainbow trout have been produced by *in vivo* chromosome-mediated gene transfer (Disney et al. 1987). They fertilized albino rainbow trout eggs with gamma-irradiated brook trout *(Salvelinus fontinalis)* sperm and heat shocked the eggs to induce the retention of the second polar body. They successfully demonstrated stabilization, integration, expression and transmission of the foreign chromosome fragment.

Methods for Analyzing Integration and Expression

The Southern blot technique is the usual method employed to find evidence

Table 2. Summary of some important methodological considerations for foreign DNA microinjection into fish eggs. Experimental results are expressed in percentage transformation and presence or absence of foreign DNA expression.

Species	Injection site; Egg stage	Type; Form of microinjected DNA; Number of copies	Percent transformation	Presence or absence of expression	Reference
Salmo gairdneri	cytoplasm of fertilized eggs; before first cleavage	complementary; linear or circular; 200 pg	75%[a] or 40%	no	Chourrout et al. (1986)
Oryzias latipes	oocyte nuclei; before maturation	genomic; linear; $5x10^3$ - 10^4	16%	yes	Ozato et al. (1986)
Salmo gairdneri	perivitelline space of fertilized egg	genomic; linear; 10^6	4%	no	Maclean et al. (1978b)
Salmo salar	cytoplasm of fertilized ova	genomic; linear; $2x10^7$	---[b]	yes	McEvoy et al. (1988)
Brachydanio rerio	cytoplasm of embryos; at 1-, 2-, and 4- cell stage	genomic; linear; 300 pl	5%	no	Stuart et al. (1988)
Carassius auratus	center of germinal disc; before first cleavage	genomic; linear; 2 nl	7%	yes[c]	Yoon et al. (in press)
Oreochromis niloticus	germinal disc of fertilized eggs; at different stages	genomic; linear; 10^6	6%	no	Brem et al. (1988)

[a]Detection of foreign gene was done by scintillation counting on dot blots.
[b]Inconclusive since low DNA yield per ovum resulted in negative hybridization in the Southern blot analysis.
[c]Two out of four fish were positive with an RNA dot blot test.

for integration. This starts with extraction of DNA from the putative transgenic fish and subsequent digestion with one or several restriction enzymes. The resulting fragments are then separated through agarose gel electrophoresis and the banding pattern is transferred onto a nitrocellulose filter. Radioactively labeled copies of the injected sequence (DNA probe) are then applied to the filter for hybridization. The resulting autoradiograph of the filter will show specific radioactive band patterns corresponding to the discrete restriction fragments that are complementary to the injected DNA (Watson et al. 1988).

The following approaches have been used for transgenic expression assays: 1. detection of specific messenger RNA by Northern blots (Stuart et al. 1988, Yoon et al., in press) and 2. tissue biopsy with subsequent biochemical tests (Ozato et al. 1986, Maclean et al. 1987a). It is best to use blood constituents, either in cells or plasma, to avoid lethal sampling. If foreign gene expression is confirmed, whole organism bioassays (such as growth studies) could follow to compare transgenics with controls. The overall success rate of foreign DNA expression in putative transgenic fish is low and convincing demonstrations of improvements in commercial traits are still lacking.

Prospects for Applications in Aquaculture Industry

These results suggest that gene transfer to improve some commercially important traits in fish may be possible. Expression of a growth hormone gene for instance, might mean faster growth to marketable size, with attendant economic and management benefits. Gene sequences coding for specific enzyme activity might confer the ability to utilize non-conventional sources of food; for example, chitinase (to permit utilization of shrimp waste) and perhaps even cellulases to digest cheap plant materials. Another interesting possibility is the identification, cloning and insertion of disease-resistance genes. However, fish disease resistance probably has polygenic control which may preclude a simple gene transfer solution.

Before such ideas come to fruition several questions need to be answered: for example, how could a 100% transformation rate be achieved?, how could the integration efficiency of the introduced DNA sequence be improved for proper expression?, is foreign gene transmission stable through generations?

If fish gene transfer techniques are applied commercially, fish genes will become important commodities. Questions about the patenting of new breeds will crop up. Access to wild populations will become commercial and political issues. Who will benefit most from such developments - rich companies or struggling small fishpond owners? Are transgenic fish, particularly those with human genes, acceptable and safe for consumption?

The possible risks involved in implementation of this technology have not been studied yet. What happens when 'genetically engineered' fish are accidentally released in natural waters? What about the question of inbreeding if very few transgenics are used as broodstock? As in all gene transfer research, application to aquaculture is fraught with social and ethical issues. We should not condemn the approach out of hand. However, it is vital first to weigh with great caution the potential benefits and hazards and then decide on how to proceed.

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