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APPLICATION OF TRANSGENIC FISH TECHNOLOGY IN AQUACULTURE

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T T. Chen, C. M. Lin and K. Kight (1991) Application of transgenic fish technology in aquaculture. *Bull. Inst. Zool., Academia Sinica, Monograph* **16**: 375-386. The technique of injecting foreign DNA molecules into fertilized eggs has been used to generate a wide range of transgenic animal species, including fish, with a high degree of success. Studies conducted by Agellon *et al.* (1988c) and others (Gill *et al.*, 1985) showed that administration of biosynthetic rainbow trout (rt) or bovine growth hormone (GH) to juvenile rainbow trout or coho salmon resulted in a significant growth enhancement. Thene results point to the possibility of improving the growth rate of cultured fish *via* manipulation of growth hormone or its gene. In this paper we will summarize results from studies conducted in our own laboratory and others to demonstrate the effectiveness of improving the growth rate of cultured fish *via* manipulating GH genes by the transgenic fish technology.

Key words: Transgenic fish, Gene transfer, Growth hormone, Growth enhancement.

Traditionally, success in fisheries largely depended upon the natural population of fresh water and marine finfish, shellfish and macroalgae. However, due to increasing world consumption of fish products, accumulation of chemical pollutants in the aquatic environments, and poor restocking effort and management, the level of total world-wide annual harvest of fish products is rapidly approaching the maximal potential level of between 100 and 150 million metric tons, as recently reported by the U. S. Department of Commerce and NOA. In

fact, a number of regions have recently shown significant decline in the catches of important fish species such as salmon, striped bass, sturgeons, eels, Jacks, mullets, mackerel, kris, abalone, oysters and crabs (Fisheries Dept., FAO, 1986). As a result, fisheries have traveled farther to exploit more productive areas, switched to alternative species, and begun to employ sophisticated technology. This recent development has resulted in a significant increase of international fish prices. Since U. S. imports of fish products in recent years have risen almost twice as much as exports, such an increase in the international fish price has contributed significantly to the trade deficit. In fact, the U. S. trade deficit in fish products has escalated to 6.6 billion U. S. dollars per year in 1988. In order to cope with these problems, drastic measures for increasing fish production are required.

As a means of increasing the production of fish products, many countries have turned to aquaculture and mariculture. In 1985, the production of finfish, shellfish and macroalgae by aquaculture/mariculture reached 10.6 million metric tons, which was 12.3% of the world-wide catch tonnage generated by international efforts. Clearly aquaculture/mariculture has the potential to significantly increase the world production of fish products, and thus affect the trade balance of fish products.

Success in aquaculture and mariculture revolves around four key requirements: breeding, feeding, disease protection and management. Application of molecular biology and biotechnology can help to improve the first three requirements. These applications include enhancing growth rates, controlling reproductive cycles, improving feeds, developing vaccines, developing disease resistance and hardier genetic stocks, and many other possibilities. In collaboration with Rex Dunham of Auburn University and D. A. Powers of Stanford University, we are investigating strategies to increase growth rates of finfish and shellfish in aquaculture operations through manipulation of GH and growth factor genes. In this paper we will summarize results of our studies and others to enhance growth rates of cultured fish by the transgenic fish technology.

ENHANCEMENT OF GROWTH BY BIOSYNTHETIC FISH GROWTH HORMONE

Two genes encoding growth hormone polypeptides (GH1 and GH2) have been identifid and their respective cDNA isolated for rainbow trout, *Oncorhynchus mykiss* (Agellon and Chen, 1986; Agellon *et al.*, 1988a; Agellon *et al.*, 1988b). By expressing rtGH1 cDNA in *E. coli* cells, a large quantity of biologically active biosynthetic GH polypeptide was prepared (Agellon *et al.*, 1988c). This biosynthetic hormone is a fusion protein, containing 9 amino acid residues derived from the N-terminus of *E. coli* β-galactosidase and the entire sequence of the mature rtGH polypeptide. Agellon *et al.* (1988c) demonstrated in a series of studies that application of this hormone to yearling rainbow trout resulted in a significant growth enhancement. After treatment of yearling rainbow trout with the biosynthetic GH for four weeks at a dose of 1 μg/g body weight/week, the weight gain in the individuals of the hormone-treated group was two times greater than that of their controls (Fig. 1). Significant length gain was also evident in hormone-treated animals. The muscle chemical composition of hormone-treated fish was not significantly different from that of the controls (Table 1). When the same biosynthetic hormone preparation was administered to rainbow trout fry or small juveniles by immersing the fish in a GH-containing solution, the same growth-promoting effect was also observed (Table 2; Leong and Chen, unpublished results). These results are in agreement with those reported by Sekine *et al.* (1985) and Gill *et al.* (1985) that application of biosynthetic GH from other sources to rainbow trout, chum salmon or coho salmon also resulted in growth-enhancement. However, it is worth noting that the growth enhancement effect of the biosynthetic hormone was markedly reduced when more than 2 μg/g body weight of the hormone was applied to the test animals (Fig. 1). These results clearly suggest that when the total amount of GH exceeds the maximal threshold level, the homeostasis of the hormone will be disturbed, consequently affecting the performance of the animals.

Several years ago, Morse (1984) reported that bovine insulin and GH

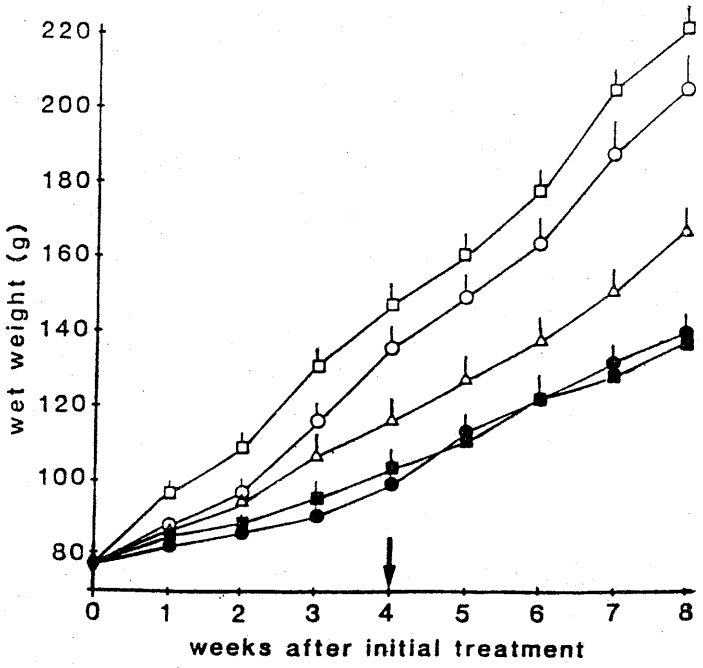

Fig. 1. Effect of biosynthetic trout GH on growth of yearling rainbow trout. Groups of yearling rainbow trout received intraperitoneal injection of biosynthetic GH or control extract for five weeks. Wet weights of GH-treated and control fish are shown (mean±SE). Open symbols, GH-treated fish: ○, 0.2 μg/g body weight; □, 1.0 μg/g body weight; △, 2 μg/g body weight. Closed symbols, control fish: ●, mock-treated fish; ■, untreated fish. The arrow indicates the time of the last hormone treatment. (Agellon *et al.*, 1988c; with permission).

Table 1
Percent of moisture, protein, lipid and ash in the muscle tissue of GH-treated and control fish (Agellon *et al.*, 1988c, with permission)

	Percent±SE (n=4)			
Treatment	Moisture	Protein	Lipid	Ash
Uninjected	78.58±1.4	16.54±1.0	3.58±0.5	1.31±0.1
Mock-treated	80.26±0.8	15.08±0.4	3.48±0.5	1.19±0.1
GH 0.2 μg/g)	78.91±0.9	15.98±1.0	4.04±0.6	1.17±0.1
GH (1.0 μg/g)	78.37±0.3	16.58±0.2	3.91±0.5	1.32±0.1
GH (2.0 μg/g)	79.02±0.9	15.99±0.3	3.89±0.9	1.24±0.1

Yearling rainbow trout were treated weekly for 5 weeks with control and GH-containing bacterial extracts. Four weeks after the withdrawal of hormone treatment, tissue samples were collected from the same region of the carcass of sacrified fish. Chemical composition analysis was performed after tissue lyopholization. Values shown are corrected to the initial wet weight of the tissue sample.

Table 2
Effect of GH treatment on the growth of rainbow trout fry
(Agellon *et al.*, 1988c, with permission)

Treatment	Weight (gm±SD)		
	Initial	Final	% Gain
Saline control	1.33±0.6**	3.94±1.8*	196
GH (50 μg/l)	1.29±0.7**	5.51±1.6***	327
GH (500 μg/l)	1.35±0.7**	5.30±1.3***	293

Groups of rainbow trout fry (n=15) were subjected to osmotic shock in the presence or absence of GH. Weight was measured prior to and 5 weeks post-treatment. Differences between mean weights of GH-treated and control groups were evaluated using Student's t-test. Mean weights were considered to significantly different if $p<0.01$.

* Significantly different from the GH-treated groups ($p<0.01$);
** No significant difference between these groups;
*** No significant difference between these two treatments.

enhanced the growth of California red abalone. Recently Paynter and Chen (1991) have observed that administration of biosynthetic rtGH polypeptide to spats of juvenile oysters (*Crassostrea virginica*) by the "dipping method" referred to above also resulted in significant increases in shell height, shell length, shell weight, wet weight, dry weight and oxygen consumption. These findings further suggest that the biosynthetic fish GH polypeptide can also be used to enhance the growth rate of shellfish under intensive culture conditions.

Although results described above clearly point to the possibility of enhancing growth rates of cultured finfish and shellfish by supplying optimal levels of biosynthetic GH, many studies are still required to delineate a number of basic and practical scientific issues. These issues include: (i) efficient methods for large scale production, purification and renaturation of biosynthetic GH polypeptide; (ii) effective means of hormone delivery; (iii) detailed dose regimens for each target fish species; (iv) the effect of both chronic and acute GH treatment; (v) nutrient requirements; and (vi) genetic and physiological regulation of GH action.

GENERATION OF TRANSGENIC FISH BY MICROINJECTING cDNA OF FISH GROWTH HORMONE

Although exogenous application

of biosynthetic GH results in a significant growth enhancement in fish, it may not be cost effective. The use of gene transfer technology to generate new strains of fish that express elevated but optimal levels of GH, as an alternative, would bypass many of those problems associated with exogenous GH treatment. Moreover, once transgenic fish strains have been generated, they would be far more cost effective than their non-transgenic counterparts because these fish would have their own means of producing and delivering the hormone, and could transmit their enhanced growth characteristics to their offspring.

Zhu *et al.* (1985), in China, reported the first successful transfer of human GH gene fused to a mouse metallothionein gene promoter into goldfish and loach. According to Zhu (personal communication), the F_1 offspring of these trangenic fish grew twice as large as their non-transgenic siblings. Although Zhu and his colleagues did not present compelling evidence for integration and expression of the foreign genes in their transgenic fish, many laboratories have successfully confirmed their work by demonstrating that GH gene and other gene constructs can be transferred into embryos of a number of fish species and integrated into the genomic DNA of the host fish (for review, see Chen and Powers, 1990b). While a few groups have demonstrated expression of foreign genes in trasgenic fish, Zhang *et al.* (1990) is the only group to document that a *foreign fish GH gene* is: (a) transferred to the target fish species; (b) integrated into the fish genome; (c) genetically transmitted to the subsequent generations. Furthermore, the expression of the foreign fish GH gene may result in enhancement of growth rates of both P_1 and F_1 generations of transgenic fish (Zhang *et al.*, 1990; Chen *et al.*, 1990a).

In gene transfer studies conducted by our laboratories (Zhang *et al.*, 1990; Chen *et al.*, 1990a), about 10^6 molecules of a linearized recombinant plasmid containing the long terminal repeat (LTR) sequence of avian Rouse sarcoma virus (RSV) and the rainbow trout GH1 or GH2 cDNA were injected into the cytoplasm of one-cell, two-cell and four-cell embryos of common carp and catfish. Genomic DNA samples extracted from the pectoral fin of individuals derived from these embryos were analyzed for the presence of RSVLTR-rtGH1-cDNA by PCR amplification (Fig. 2) and followed by Southern

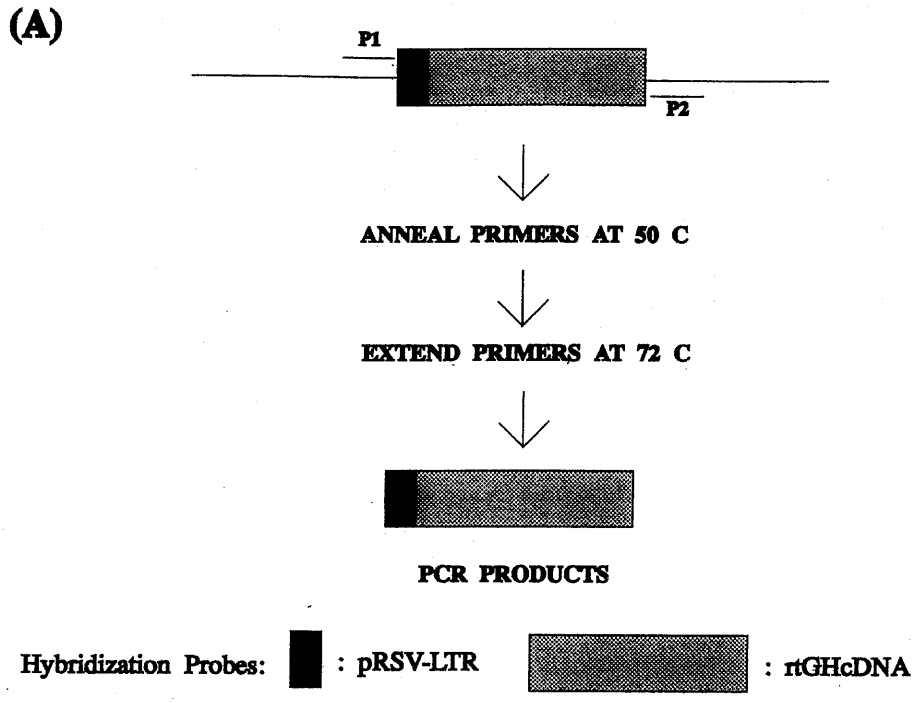

Fig. 2. Detection of rtGH cDNA transfer by Polymerase Chain Reaction (PCR). Reaction products (20 μl) from 30 cycles of amplification were analyzed by electrophoresis in a 2% agarose gel, transferred to a nylon membrane and hybridized to [^{32}P]-labelled probes. (A), Strategy of PCR amplification; (B), Analysis of the PCR amplified products. 1, pRSV-rtGH-cDNA; 2, 4, 7 : PCR products from positive transgenic catfish; 2, 5, 6 : PCR products from non-transgenic siblings.

blot hybridization of the genomic DNA samples to determine the pattern of foreign gene integration (Zhang *et al.*, 1990), using radio-labelled LTR of RSV and/or trout GH1 cDNA as probes. Results of

Table 3
Percent of hatching, survival and integration of carp embryos microinjected with pRSV-rtGH1-cDNA at different developmental stages (Chen *et al.*, 1990a, with permission)

Embryo stage	Embryos injected	Hatched (%)	Survival at 90 days	Fingerlings analyzed	Integration (%)
One-cell	1,034	39.3	52.7	217	9.9
Two-cell	331	33.3	70.6	77	5.6
Four-cell	381	33.0	58.4	73	1.4
Control (non-injected)	569	33.9			

these studies showed that above 35% of the injected common carp embryos survived at hatching, of which about 10% of the survivors had stably integrated the pRSVLTR-rtGH1-cDNA sequence (Table 3; Chen *et al.*, 1990a). A similar percentage of transgenic fish was also obtained when RSVLTR-csGH-cDNA construct was injected into catfish embryos (Table 4; Powers *et al.*, 1991). Southern blot analysis of genomic DNA samples of several transgenic fish revealed that a single copy of the RSVLTR-rtGH1-cDNA sequence was integrated at multiple chromosomal sites. In some cases, the *Hind* III site residing between RSVLTR and rtGH1-cDNA was modified. While foreign genes introduced into embryos by microinjection are usually integrated as a head-to-tail concatemer at a single chromosomal site and modified (Palmiter *et al.*, 1982; Dunham *et al.*, 1987), examples of single or low copy integrations of foreign gene sequences exhibiting deletions, modifications or rearrangements have also been observed (Gordon and Ruddle, 1985). Hence our results of RSVLTR-rtGH1-cDNA integration in transgenic carp agreed with those reported in other systems.

PRODUCTION OF TROUT GROWTH HORMONE IN TRANSGENIC FISH

It has been reported earlier that polyclonal antibodies to chum salmon GH reacted specifically with the GH polypeptide of rainbow trout (Allegon *et al.*, 1986). Hence these antibodies were used as probes for detecting the production of trout GH in transgenic carp by a quantitative dot blot immunobinding assay

Since these polyclonal antibodies showed a partial cross-reactivity with common carp GH, they were rendered specific to trout GH by extensive re-absorption with pituitary extracts of common carp. Trout GH was not detected in the circulation of P_1 transgenic carp, since the rtGH1-cDNA used in the gene transfer studies does not contain a signal peptide sequence. Instead, various levels of rtGH were detected in the red blood cell (RBC) extracts of transgenic individuals: ranging from 8.0 to 89.1 ng/mg RBC proteins (Zhang *et al.*, 1990; Chen *et al.*, 1990a). As expected, there was no correlation between the number of pRSVLTR-rtGH1-cDNA integrated and levels of rtGH expressed in RBC. These results are consistent with those in transgenic mice reported by Palmiter *et al.* (1982). Although there was considerable variation in size among the P_1 transgenic fish, they were 22%, on the average, larger ($p<0.05$) than their sibling controls.

INHERITANCE OF TROUT GROWTH HORMONE GENE IN TRANSGENIC FISH

To study patterns of inheritance of RSVLTR-rtGH1-cDNA sequence in transgenic carp, sperm samples collected from several sexually mature P_1 male transgenic fish (04R, 36L, 131L and 94R) were crossed to eggs collected from one non-transgenic female, and DNA samples extracted from resulting F_1 progeny were assayed for the presence of RSVLTR-rtGH1-cDNA sequence by dot blot hybridization. None of the F_1 progeny derived from transgenic male 04R received the RSVLTR-rtGH1-cDNA sequence from their father, suggesting that the RSVLTR-rtGH1-cDNA sequence was not integrated into the germ line of fish 04R. Although most of the F_1 progeny derived from fish 36L died, the four survivors inherited the injected DNA. Results obtained from F_1 progeny of fish 131L and 94R are most informative. About 31.3% and 42.3% of F_1 progeny derived from fish 131L and 94R respectively carried RSVLTR-rtGH1-cDNA sequence (Table 4). Since transgenic fish are independently derived by injecting the foreign gene at different stages of development, one would expect transgenic animals to be mosaic for RSVLTR-rtGH1-cDNA. The degree of mosaicism in transgenic animals determines whether a foreign gene will be present in the germ line and whether it will be transmitted to subsequent

Table 4
Percent of hatching, survival and integration of catfish embryos microinjected with pRSV-csGH-cDNA at different developmental stages (Powers *et al.*, 1991, with permission)

Embryo stages	Embryos injected	Hatched (%)	Survival at 10 wks.	Fingerlings analyzed	Integration (%)
One-cell	3,341	11.5	43.1	99	5.7
Two-cell	990	4.5	44.4	18	33.3
Four-cell	653	8.6	33.9	17	17.7
Control (non-injected)	325	9.7			

generations. Since nearly 50% of the progeny derived from fish 94R carry RSVLTR-rtGH1-cDNA, the transformed progenitor cells must be primordial to the entire germ-line. Conversely, the progeny ratios of 1 transgenic: 3 non-transgenic from fish 131L suggests this germ-line is mosaic. Similar patterns of inheritance of the foreign GH gene were also observed in P_1 transgenic catfish.

The body weights of F_1 progeny derived from fish 131L and 94R were measured at the age of three months, and their weights were about 20.8% ($p<0.05$) and 40.1% ($p<0.001$) larger than their non-transgenic siblings,

Table 5
Mean weight, range weight and percent inheritance at 90 days of progeny derived from transgenic fish 131L and 94R (Chen *et al.*, 1990a, with permission)

	Progeny of fish 131L		Progeny of fish 94R	
	Transgenic	Non-transgenic	Transgenic	Non-transgenic
N	31	65	11	15
% inheritance	32.3		42.3	
Mean weight (g)	120.6[a]	99.3[b]	206	147
SE[c]	17.4	14.7	45.2	48
Weight range	95-173[d]	65-129	115-283[e]	67-228
% difference	20.8[f]		40.1[g]	

a: $N=28$; b: $N=38$; c: SE=standard deviation
d & e: 32%[d] and 46%[e] of transgenic progeny were larger than largest control.
f & g: Transgenic progeny were larger than non-transgenic progeny at $p<0.05$[f] and $p<0.001$[g] respectively.

respectively (Table 5; Zhang *et al.*, 1990; Chen *et al.*, 1990a). Furthermore, these progeny were 32% and 46% larger than their largest non-transgenic siblings respectively. These results clearly demonstrated that the growth enhancement phenotype resulting from the presence of GH-cDNA sequence in transgenic fish 131L and 94R has been passed down to their respective F_1 progeny through the germ-line. In many subsequent studies with transgenic catfish, similar results of growth enhancement were observed in P_1 and F_1 fish harboring coho salmon GH cDNA sequence (unpublished results).

FUTURE PROSPECT

Although results from our own studies and others have shown that transfer, expression and inheritance of a foreign gene could be achieved in several finfish species with a relative ease, considerable amount of research is still required in order to develop strains of transgenic fish for commercial purposes. To achieve the goal of generating superior fish strains for aquaculture/mariculture by the gene transfer technology, several major breakthroughs are required. These include: (a) developing efficient mass gene transfer technologies; (b) identifying genes of desired traits; (c) developing rapid and convenient procedures for identifying presumptive transgenic individuals; (d) developing methods for targeting foreign gene integration into specific areas of the fish genome; (d) identifying suitable promoters to drive the expression of the foreign gene; (e) determining physiological, nutritional, immunological and environmental factors that will maximize the performance of transgenic individuals; (f) assessing safety and environmental impacts of transgenic fish.

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