TRANSGENIC FISH AND AQUACULTURE

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SUMMARY

A wide range of transgenic animal species, including fish, can be produced by transferring foreign DNA into developing embryos by microinjection or electroporation. This technology offers an excellent opportunity for modifying the genetic traits of commercially important finfish, shellfish and crustaceans speccies for aquaculture. Studies conducted in our laboratory (1,2) and those of others (3-5) showed that administration of recombinant fish or mammalian growth hormone (GH) to juvenile fish or oysters resulted in a significant growth enhancement. These results point to the possibility of improving growth rates of finfish and shellfish by manipulating GH or its gene. In this paper we will review results of our own studies as well as those of many others to determine the efficacy of improving the growth rates of finfish, shellfish and crustaceans by administering recombinant fish GH or producing fast-growing transgenic animals by means of the gene transfer technology.

INTRODUCTION

The world-wide harvest of fishery products traditionally depends upon the natural population of finfish, shellfish and crustaceans of fresh water and marine sources and macroalgae. Due to a rapid increase in consumption of fishery products as a consequence of population growth as well as over fishing activity and poor restocking effort and management, the level of total world-wide annual harvest of fish products has already approached the maximal potential level of about 150 million metric tons as forcasted by the US Department of Commerce and NOAA. In addition, accumulation of chemical pollutants in aquatic environments as a consequence of increasing industrial activities has detrimental effects A number of regions have recently experienced a significant decline in the catches of important fish species such as salmon, striped bass, sturgeons, eels, Jacks, mullets, mackerel, kris, abalone, oysters and crabs (6). Fishery fleets now travel great distances to exploit more productive areas. They have switched to alternative species, and begun to employ a variety of sophisticated technologies. These recent developments have caused a significant increase of international fish prices.

In the past several decades, many countries have turned to aquaculture/mariculture for increasing production of fishery products. In 1985, for instance, the world production of finfish, shellfish and macroalgae by aquaculture/mariculture reached 10.6 million metric tons, an amount equal to 12.3% of the world-wide catch tonnage of international fishery efforts. Thus, aquaculture/mariculture has the potential to resolve the pressing problem of meeting the world demand of fishery products. Production of fish by intensive culture depends on: (i) complete control of the reproductive cycle of the fish species in culture; (ii) excellent genetic background of the broodstock; (iii) efficient detection and effective prevention of disease infection; (iv) thorough understanding of the optimal physiological, environmental and nutritional conditions for growth and development; (v) sufficient supply of good quality water; and (vi) application of innovative management tewchnique. By improving some of these factors, the aquaculture industry has made impressive progress over the last several years. The application of molecular biology and biotechnology will further speed up the expansion of this industry. These applications include enhancing growth rates, controlling reproductive cycles, improving feed composition, producing new vaccines, and developing disease resistant and hardier genetic stocks. Over the last several

years our laboratory and others have been searching for innovative strategies to increase fish production by applying the contemporary technologies of molecular biology and biotechnology. In this paper, we will summarize results of our studies as well as those of many others to demonstrate the efficacy of applying techniques of modern biology, including transgenic fish technology, to increasing the production of cultured finfish, shellfish and crustaceans.

BIOSYNTHETIC FISH GROWTH HORMONE AND GROWTH ENHANCEMENT

In recent years, cDNA and the genomic sequence of GH have been isolated and characterized for several fish species (3, 7-11). Expression of rainbow trout (rt) GH1 cDNA in *E. coli* cells has resulted in the production of a large quantity of biologically active recombinant GH polypeptide (1). Agellon *et al.* (1) showed in a series of studies that application of this recombinant hormone to yearling rainbow trout resulted in a significant growth enhancement. After treatment of yearling rainbow trout with the recombinant rtGH for four weeks at a dose of 1 μg/g body weight/week, the weight gain among the individuals of the hormone-treated group was two times greater than that of the controls. Significant length gain was also evident in hormone-treated animals. When the same recombinant hormone was administered to rainbow trout fry (Table 1) or small juveniles by immersing the fish in a GH-containing solution, the same growth-promoting effect was also observed (1; Leong and Chen, unpublished results). These results are in agreement with those reported by Sekine *et al.* (5), Gill *et al.* (3) and many others (5, 12,13). However, it is important to mention 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 (1). These results suggest that when the total amount of GH exceeds the maximal threshold level, the homeostasis of the hormone will be disturbed, consequently affecting the growth performance of the animals.

Table 1. Effect of GH treatment on the growth of rainbow trout fry (1, with permission)

	Weight (gm±SD)		
Treatment	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 be 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.

Several years ago, Morse (14) reported that bovine insulin and bovine GH enhanced the growth rate of California red abalone. Recently Paynter and Chen (2) have observed that administration of recombinant 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 weight, wet weight, and dry weight (Table 3). Furthermore, they also showed that oysters treated with recombinant rtGH, native bovine GH or bovine insulin consumed more oxygen

per unit time than controls. These findings suggest that recombinant fish GH can be used to enhance the growth rate of shellfish under intensive culture conditions. It further suggests that growth in shellfish may also be regulated by hormonal factors similar to mammalian GH and insulin-like growth factors (IGFs).

Table 2. Effect of exogenously applied recombinanat rainbow trout growth hormone on oyster growth (2, with permission)

Treatment	Initial ht	Final ht	Total wt	Shell wt	Dry wt
Control	8.14 (0.25)	11.68 (0.27)	206 (11)	136 (8)	6.10 (0.66)
10^{-9} M	8.04 (0.27)	11.74 (0.23)	199 (9)	131 (6)	6.87 (0.66)
10^{-8} M	8.72 (0.18)	12.79 (0.27)[ab]	244 (20)	171 (11)[b]	9.42 (0.41)[ab]
10^{-7} M	8.65 (0,32)	13.00 (0.36)[ab]	252 (13)[b]	189 (13)[ab]	9.41 (0.74)[ab]

[a] Significantly larger than the control group (t-test; $P<0.05$).

[b] Significantly larger than 10^{-9} M treatment group (t-test; $P<0.05$).

Initial hight reprents mean size at the begining of the experiment aand final hight, total wt, shell wt, and dry wet are mean valuesdetermined after the five-week treatment cycle was concluded. Height (ht) was measured in mm from the umbo to the ventral shell margin; weight was measured in mg. Standard errors of the mean (SEM) are in parentheses.

FISH INSULIN-LIKE GROWTH FACTOR I & II

Mammalian insulin-like growth factors I and II (IGF I and IGF II) are single-chain polypeptides with 70 and 67 amino acid residues, respectively. At the amino acid level, IGF-I and IGF-II share about 70% homology between them. Since both growth factors share about 50% homology with proinsulin, together with insulin and relaxin, they all belong to the insulin gene family (15). By molecular cloning, the cDNAs of IGF-I and IGF-II have been determined for human, rat, mouse, bovine, porcine, bird, and frog (16, for review), but only an IGF-I cDNA sequence had been determined for coho salmon (17) when we began our efforts.

As part of our on going investigation on the regulation of growth in fish by GH, we have recently initiated studies to identify the presence of IGF I and IGF II in rainbow trout, and the involvement of these two hormones on somatic growth mediated by GH. By employing the polymerase chain reaction (PCR), an internal portion of IGF cDNA was amplified from the total cDNA of rainbow trout liver. Using this internal fragment of the IGF cDNA as a hybridization probe, several recombinant clones encoding IGF cDNA sequence were isolated from a rainbow trout liver cDNA library (18). Detailed characterization of these recombinant clones by digestion with different restriction enzymes and nucleotide sequence determination revealed the isolation of two distinct IGFs. On the basis of a 98.7% nucleotide sequence homology to coho salmon IGF I, one cDNA sequence was identified as rainbow trout IGF I. The second cDNA sequence shared 43.3% identity with trout IGF I at the predicted amino acid level and 53.6% identity with human IGF II, and was identified as trout IGF II. This is the first paper of a non-mammalian IGF II. Further analysis of RNA isolated from rainbow trout liver by reverse transcription (RT)/PCR analysis and nucleotide sequence determination of the PCR amplified products revealed the presence of multiple forms of IGF I mRNAs (IGF IEa-1, 1Ea-2, 1Ea-3 and 1Ea-4) (40). Only one form of the IGF II mRNA was observed by the same method, however. Quantitation of IGF mRNAs by the RNase protection assay revealed that all five IGF mRNA species are expressed in rapidly growing fish (40). The dependence of IGF I and II expression by GH has been determined

recently and the results showed that the expression of both IGFs are induced by growth hormone (Shamblott and Chen, in preparation) *in vivo*.

TRANSGENIC FISH HARBORING FISH GROWTH HORMONE cDNA

Although exogenous application of recombinant GH results in a significant growth enhancement in fish, it may not be cost effective. If new strains of fish producing elevated but optimal levels of GH can be produced, it would bypass many of those problems associated with exogenous GH treatment. Moreover, once these fish strains have been generated, they would be far more cost effective than their ordinary 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.

Gene Transfer Methodology

Animals into which a segment of foreign DNA has been introduced and stably integrated into the host genome are called transgenic. Since 1982, many transgenic animal species, including fish, have been constructed successfully (19-28). These animals play important roles both in basic research as well as in biotechnology application. Although various methods such as direct microinjection, retrovirus infection, electroporation, calcium phosphate precipitation, and particle gun bombardment have been used to introduce foreign DNA into somatic cells as well as germ-lines of mammals and other higher vertebrates, direct microinjection of DNA into the male pronuclei of the fertilized eggs has been the most prevalent method. This method has resulted in successful production of transgenic mice (19,20) and domestic animals including rabbits, sheep and pigs (34,35).

The microinjection method has also been employed to introduce foreign genes into several fish species in recent years. These include common carp (28,36), catfish (21,37,38), goldfish (29), medaka (25,32), rainbow trout (22), salmon (24), tilapia (27), and zebrafish (26). In general, gene transfer in fish by microinjection is carried out as follows. Eggs and sperm are collected into separate dry containers. Fertilization is initiated by adding water and sperm to eggs, with gentle stirring to enhance fertilization. Eggs are water hardened for various periods of time and then rinsed. Microinjection is done within the first two hours after fertilization, using a set-up which consists of a dissecting stereo microscope and two micromanipulators, one with a micro-needle for injection and the other with a micropipette to hold the egg in position during the injection. Since the male pronuclei of the fish embryos studied to date are not visible, the foreign genes are usually injected into the egg cytoplasm and the amount of the DNA injected into each embryo is in the range of one million copies or higher. Following injection, the embryos are incubated in water until hatching. Since natural spawning can be induced by adjusting photo-period and water temperature in zebrafish and medaka, precisely staged newly fertilized embryos can be readily collected from the rearing aquaria for microinjection. Within the first two hours after fertilization in medaka and zebrafish, the micropyl on the embryos is still visible under the microscope. Hence the DNA solution can be easily delivered into the embryos by a microinjection needle through this opening.

Although the microinjection method is successful in transferring foreign DNA into fish embryos, it is a very laborious and time-consuming procedure. There is a genuine interest in developing convenient mass gene transfer technologies for use in fish transgenesis studies. Among many of the mass gene transfer methods such as retrovirus-mediated gene transfer, liposome-mediated or sperm-mediated gene transfer, particle gun bombardment and electroporation, the method of electroporation has been shown to be the most effective means of transferring foreign genes into fish embryos. This method utilizes a series of short electrical pulses to permeate the cell membrane, thereby permitting the entry of DNA molecules into the embryos. Studies conducted by Lu *et al.* (32) showed that the rate of foreign gene integration in transgenic medaka produced by

electroportion was in the order of 20% or higher. Powers *et al.* (39) has recently reported a much higher rate of gene transfer in common carp and channel catfsh by using the same electroporator. Although the overall rate of transgene integration in transgenic medaka produced by electroporation was slightly higher than that of microinjection, the actual amount of time required for producing the same numbers of transgenic fish by this method is orders of magnitude shorter than that by microinjection.

Transgenic Fish Harboring Growth Hormone Gene

Zhu *et al.* (29), reported the first successful transfer of human GH gene fused to a mouse metallothionein (MT) gene promoter into goldfish and loach. According to Zhu (personal communication), the F_1 offspring of these transgenic fish grew twice as large as their non-transgenic siblings. Unfortunately, Zhu and his colleagues failed to present compelling evidence for integration and expression of the foreign genes in their transgenic fish studies. Recently, many laboratories throughout the world have successfully confirmed Zhu's work by demonstrating that human or fish GH and many other genes can be readily transferred into embryos of a number of fish species and integrated into the genome of the host fish (for review, see 30). While a few groups have demonstrated expression of foreign genes in transgenic fish, only Zhang *et al.* (28), Du *et al.*, (31) and Lu *et al.*, (32) have documented that a foreign GH gene could be: (a) transferred to the target fish species; (b) integrated into the fish genome; and (c) genetically transmitted to the subsequent generations. Furthermore, the expression of the foreign GH gene may result in enhancement of growth rates of both P_1 and F_1 generations of transgenic fish (28, 32).

In gene transfer studies conducted in common carp and channel catfish (28,33, 36-38), about 10^6 molecules of a linearized recombinant plasmid containing the long terminal repeat (LTR) sequence of avian Rous 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. Genomic DNA samples extracted from the pectoral fins of presumptive transgenic fish were analyzed for the presence of RSVLTR-rtGH1-cDNA by PCR amplification and followed by Southern blot hybridization of the amplified DNA samples, using radio-labelled LTR of RSV and/or trout GH1 cDNA as hybridization probes. In the case of transgenic carp studies (28, 33), about 35% of the injected embryos survived at hatching, of which about 10% of the survivors had stably integrated the RSVLTR-rtGH1-cDNA sequence. A similar percentage of transgenic fish was also obtained when RSVLTR-csGH-cDNA construct was injected into catfish embryos (37, 38). Southern blot analysis of genomic DNA samples of several transgenic carp revealed that a single copy of the RSVLTR-rtGH1-cDNA sequence was integrated at multiple chromosomal sites (28).

In the microinjection studies conducted in medaka by Lu *et al.* (32), a much higher rate of foreign gene integration (20-30% of the hatched individuals) than that in common carp or channel catfish was observed. This results suggest that DNA microinjected into the embryos via micropyle may have better access to the nucleus since the nucleus is situated beneath the micropyle.

INHERITANCE AND EXPRESSION OF FOREIGN GH GENE IN TRANSGENIC FISH

The patterns of inheritance of RSVLTR-rtGH1 cDNA in the transgenic common carp were studied by fertilizing eggs collected from non-transgenic females or P_1 transgenic females with sperm samples collected from several sexually mature P_1 male transgenic fish, and DNA samples extracted from the resulting F_1 progeny were assayed for the presence of RSVLTR-rtGH1-cDNA sequence by PCR amplification and dot blot hybridization (36). The percentage of the transgenic progeny resulting from nine matings were: 0, 32, 26, 100 (4 progeny only), 25, 17, 31, 30 and 23% respectively. If each of the transgenic parents in these 9 matings carries at least one copy of the transgene in the gonad cell, about 50 to 75 % transgenic progeny would have been

expected in each pairing. Out of these nine matings, two siblots, both control x P_1, gave transgenic progeny numbers as large or larger than expected ($P < 0.05$), and the remaining had lower than expected numbers of transgenic progeny. These results indicate that, though most of these P_1 transgenic fish had RSVLTR-rtGH1 cDNA in their germ-line, they might be mosaics. Similar patterns of mosaicism in the germline of P_1 transgenic fish have been observed in many fish species studied to date(23,25,26,28,32,38).

If the transgene carries a functional promoter, some of the transgenic individuals are expected to express the transgene activity. According to Zhang *et al.* (28) and Chen *et al.* (36), many of the P_1 and F_1 transgenic common carp produced rtGH and the levels of rtGH produced by the transgenic individuals varied about 10-fold. Chen *et al.* (36) recently confirmed these results by detecting the presence of rtGH mRNA in the F_1 transgenic carp using an assay involving reverse transcription (RT)/PCR amplification and RNA dot blot hybridization (36). Different levels of rtGH mRNA were detected in liver, eyes, gonads, intestine and muscle of the F_1 transgenic individuals.

GROWTH PERFORMANCE OF TRANSGENIC FISH HARBORING GH GENE

Since the site of transgene gene integration differs among the individuals in any population of P_1 transgenic fish, they should be considered as totally different transgenic individuals and thus inappropriate for direct comparison of growth performance among themselves. Instead, the growth performance studies should be conducted in F_1 transgenic and non-transgenic siblings derived from the same family. Recently Chen *et al.* (36) conducted studies to evaluate the growth performance of F_1 transgenic carp in seven families. In these experiments, transgenic and non-transgenic full-siblings were spawned, hatched, and reared communally under the same environment. Results of these studies showed that growth response by families of F_1 transgenic individuals in response to the presence of rtGH1 cDNA varied widely. When compared to the non-transgenic full-siblings, the results of these seven growth trials showed 20, 40, -27, 59, 22, -15 and -2% increase in growth. In three of the four families where F_1 transgenics grew faster than their non-transgenic full-siblings, the maximum and minimum body weights of the transgenics were larger than those of the non-transgenics. In the fourth family, the minimum, but not the maximum, body weight of the transgenics was larger than that of the non-transgenics. In two of those three transgenic families in which transgenics did not grow faster than their non-transgenic full-siblings, the maximum and minimum body weights of the transgenics were smaller than those of the non-transgenics. In the third family, however, one of the F_1 transgenics was the largest fish in the family. Since the response of the transgenic fish to the insertion of the RSVLTR-rtGH1 cDNA appears to variable, as a result of random integration of the transgene, the fastest growing genotype will likely be developed by utilizing a combination of family selection and mass selection of transgenic individuals following the insertion of the foreign gene. In the studies of transgenic medaka carrying chicken β-actin gene promoter human GH gene construct, the F_1 transgenic individuals also grew significantly faster than the non-transgenic siblings (32).

In an effort to study the biological effect of elevated levels of IGF I on somatic growth, transgenic medaka harboring trout IGF cDNA driven by carp β-actin gene promoter have been produced in our laboratory. Both P_1 and F_1 IGF I transgenic medaka hatched two days earlier than their non-transgenic controls. Furthermore, the P_1 transgenic individuals also grew faster than their non-transgenic controls.

GENERAL CONCLUSION AND FUTURE PROSPECT

Transgenic fish technology has a great potential in the aquaculture/mariculture industry. By introducing desirable genetic traits into finfish or shellfish, superior transgenic strains can be

produced for aquaculture. These traits may include elevated growth enhancement, improved food conversion efficiency, resistance to some known diseases, tolerance to low oxygen concentrations, and tolerance to sub-zero temperatures. Recent progress in our laboratory and those of others has shown that transfer, expression and inheritance of fish growth hormone transgenes can be achieved in several finfish species and the resulting animals grow substantially faster than their control siblings. This is a vivid example of the potential application of the gene transfer technology to aquaculture However, in order to realize the full potential of the transgenic fish technology in aquaculture or other biotechnological applications, several important scientific breakthroughs are required. These are: (i) developing more efficent mass gene transfer technologies; (ii) identifying genes of desirable traits for aquaculture and other application; (iii) developing targeted gene transfer technologies such as embryonic stem cell gene transfer method or ribozyme gene inactivation methods; (iv) identifying suitable promoters to direct the expression of transgenes at optimal levels during the desired developmental stages; (v) determining physiological, nutritional, immunological and environmental factors that will maximize the performance of the transgenic individuals; and (vi) assessing safety and environmental impacts of transgenic fish. Once these problems are resolved, the commercial application of the transgenic fish technology will be readily attained.

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