Application of Transgenic Fish Technology in Aquaculture

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Abstract

Organisms into which heterologous DNA (transgene) has been artificially introduced and integrated in their genomes are called *transgenics*. Since the mid 1980s, many species of transgenic fish have been produced by introducing desired foreign DNA into unfertilized or newly fertilized eggs by microinjection or electro-poration. More recently, transgenic finfish, shellfish, and crustaceans have also been produced by infecting newly fertilized eggs or the immature ovary/testis with pantropic, defective retroviral vectors carrying the desired foreign DNA. These transgenic fish serve as excellent experimental models for basic scientific investigations as well as biotechnological applications. In this chapter, we will review the current status of the transgenic fish technology and its potential application in producing fast growing transgenic fish by introducing fish growth hormone (GH) or insulin-like growth factor-I (IGF-I) gene into the developing embryos.

Introduction

The worldwide harvest of fishery products traditionally depends on the natural population of finfish, shellfish, crustaceans, and macroalgae of fresh water and marine sources. As a consequence of rapid increase in the worldwide consumption of fishery products, uncontrolled fishing activity, poor management and restocking efforts, the levels of the total annual harvest of fish products has already surpassed the maximal sustainable level of 98.1 million metric tons per year in 1992. In addition, rapid accumulation of chemical pollutants in aquatic environments, as a consequence of increasing industrial activities, has caused further decline of the world wide fishery productivity. A number of regions have recently experienced a significant decline in the catches of important fish species such as salmon, striped bass, sturgeon, eels, jacks, mullets, mackerel, kris, abalone, oysters, and crabs. The Food and Agriculture Organization (FAO) of the United Nations projected that while the worldwide demand for fishery products will increase to 120 million metric tons by year 2000, the wild catch will decrease to the level of about 85 million metric tons. As a result, aquaculture (fish farming) must increase from the 1994 level of 13.9 million metric tons to approximately 35 million metric tons in only six years in order to make up the difference. Projections showed that aquaculture production must further increase to 52 million metric tons by 2010 and 77 million metric tons by 2025.

To cope with the pressure of the worldwide demand of fishery products, many countries have turned to aquaculture/mariculture. Success of aquaculture depends on the following six factors: (i) complete control of the reproductive cycle of the cultured fish species; (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 pristine quality water; and (vi) application of innovative management skills. By improving some of these factors, aquaculture industry has already made impressive progress over the last decades. To sustain this growth, however, newly developed technologies in molecular biology and genetic engineering will have to be increasingly adopted by the industry. These technologies can be employed to enhance growth rates, control reproductive cycles, improve feed compositions, produce new vaccines, and develop disease resistant and hardier genetic stocks. In the last several years, we have been searching for strategies to increase fish production by manipulating fish growth hormone and growth factor genes. In this presentation, I will review results from studies conducted in my laboratory and those in other laboratories to demonstrate the importance of biotechnology in aquaculture.

Transgenic Fish Technology

Organisms into which heterologous DNA (transgene) has been artificially introduced and integrated in their genomes are called *transgenics* (1,2). Since 1985, a wide range of transgenic fish species have been produced (3-6) by microinjecting or electroporating homologous or heter-

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ologous transgenes into newly fertilized or unfertilized eggs. Several important steps are routinely taken to produce a desired transgenic fish (6), and these steps are: (i) selection of an appropriate fish species; (ii) preparation of a gene construct; (iii) introduction of transgene into fish embryos; and (iv) selection and characterization of the resulting transgenic fish.

Selection of Fish Species: Several different fish species including channel catfish, common carp, goldfish, Japanese medaka, loach, northern pike, rainbow trout, salmon, tilapia, walleye, and zebrafish (for review: 3,5,6) have been used as experimental animals for production of transgenic fish. Depending on the purpose of the transgenic fish studies, the embryos of some fish species are better suited for gene transfer studies than the others. For example, Japanese medaka (*Oryzias latipes*) and zebrafish (*Barchydanio rerio*) have short life cycles (3 months from hatching to mature adults), produce hundreds of eggs on a regular basis without exhibiting a seasonal breeding cycle, and can be maintained easily in the laboratory for 2 to 3 years. Eggs from these two fish species are relatively large (diameter: 0.7 to 1.5 mm) and possess very thin, semi-transparent chorions, a feature that permits easy microinjection of DNA into fertilized eggs. Furthermore, inbred lines and various morphological mutants of both fish species are available. Therefore, these fish species, are suitable candidates for conducting gene transfer experiments for: (a) studying developmental regulation of gene expression and gene action; (b) identifying regulatory elements that regulate the expression of a gene; (c) measuring the activities of promoters; and (d) producing transgenic models for environmental toxicology. However, a major drawback of these two fish species is their small body size that makes them unsuitable for some endocrinological or biochemical analyses. Channel catfish, common carp, rainbow trout, and salmon are commonly used as large body size model fish species in transgenic fish studies. Since the endocrinology, reproductive biology, and basic physiology of these fish species have been well worked out, they are well suited for conducting studies on comparative endocrinology and aquaculture applications. However, the long maturation time of these fish species and a single spawning cycle per year will limit research progress in this field. Loach, killifish, goldfish, and tilapia are the third group of model fish species suitable for conducting gene transfer studies since their body sizes are large enough for most biochemical and endocrinological studies. Furthermore, shorter maturation times, as compared to catfish, rainbow trout, or salmon allow easier manipulation of transgenic progeny. Unfortunately, the lack of a well-defined genetic background and asynchronous reproductive behavior of these fish species render them less amenable to gene transfer studies.

Preparation of Transgene Constructs: Trans-genes used in producing transgenic fish for basic research or application are recombinant gene constructs that produce gene products at appropriate levels in the desired tissue(s) at the desired time(s). Therefore, the prototype of a transgene is usually constructed in a plasmid to contain an appropriate promoter/enhancer element and a structural gene sequence. Depending on the purpose of gene transfer studies, transgenes are grouped into three main types: (i) *gain-of-function*, (ii) *reporter function* and (iii) *loss-of-function*. The *gain-of-function* transgenes are designed to add new functions to the transgenic individuals or to facilitate the identification of the transgenic individuals if the genes are expressed properly in the transgenic individuals. Transgenes containing the structural genes of mammalian and fish growth hormones (GH) genes or their cDNAs) fused to functional promoters such as chicken and fish β-actin gene promoters are examples of the gain-of-function transgene constructs. Expression of the GH transgenes in transgenic individuals will result in increased production of growth hormone and ultimate growth enhancement (7-11). Bacterial chloramphenicol acetyl transferase (CAT), β-galactosidase or luciferase genes fused to functional promoters are examples of transgenes with *reporter function*. These *reporter function* transgenes are commonly used to identify the success of gene transfer effort. A more important function of a reporter gene is used to identify and measure the strength of a promoter/enhancer element. In this case, the structural gene of the CAT, β-galactosidase or luciferase gene is fused to a promoter/enhancer element in question. Following gene transfer, the expression of the reporter gene activity is used to determine the transcriptional regulatory sequence of a gene or the strength of a promoter (12). The "*loss-of-function*" transgenes are constructed for interfering with the expression of host genes. These genes might encode an antisense RNA to interfere with the post-transcriptional process or translation of endogenous mRNAs. Alternatively, these genes might encode a catalytic RNA (a ribozyme) that can cleave specific mRNAs and thereby cancel the production of the normal gene product (13). Although these genes have not yet been introduced into a fish model, they could potentially be employed to produce disease resistant transgenic brood-stocks for aquaculture or transgenic model fish defective in a particular gene product for basic research.

Methods of Gene Transfer: Techniques such as calcium phosphate precipitation, direct microinjection, lipofection, retrovirus infection, electroporation, and biolistic bombardment have been used to introduce foreign DNA into animal cells, plant cells, and germ-lines of mammals and other vertebrates. Among these methods, direct microinjection and electroporation of DNA into newly fertilized eggs have been proven to be the most reliable methods of gene transfer in fish systems.

Microinjection of foreign DNA into newly fertilized eggs was first developed for the production of transgenic mice in the early 1980s. Since 1985, this technique has been adopted for introducing transgenes into Atlantic salmon, common carp, catfish, goldfish, loach, medaka,

rainbow trout, tilapia, and zebrafish (3,5,6 for review). In general, transfer of foreign DNA into fish by direct microinjection is conducted as follows. Eggs and sperm are collected in separate, dry containers. Fertilization is initiated by adding water and sperm to the eggs, with gentle stirring to enhance fertilization. Fertilized eggs are then microinjected within the first few hours after fertilization. The injection apparatus consists of a dissecting stereo microscope and two micromanipulators, one with a micro-glass-needle for delivering transgenes and the other with a micropipette for holding fish embryos in place. Routinely, about 10^6 - 10^8 molecules of a linearized transgene (with or without plasmid DNA) in about 20 nl is injected into the egg cytoplasm. Following injection, the embryos are incubated in water until hatching. Depending on the fish species, the survival rate of injected fish embryos ranges from 35% to 80% while the rate of DNA integration ranges from 10% to 70% in the survivors (Table 1; 3-6). The tough chorions of the fertilized eggs in some fish species, e.g. rainbow trout and Atlantic salmon, can frequently make insertion of glass needles in microinjection difficult. This difficulty can be overcome by any one of the following methods: (a) inserting the injection needles through the micropyle, (b) making an opening on the egg chorions by microsurgery, (c) removing the chorion by mechanical or enzymatic means, (d) reducing chorion hardening by initiating fertilization in a solution containing 1 mM glutathione, or (e) injecting the unfertilized eggs directly.

Electroporation is a successful method for transferring foreign DNA into bacteria, yeast, and plant and animal cells in culture. This method has become popular for transferring transgenes into fish embryos in recent years (9,10). Electroporation utilizes a series of short electrical pulses to permeate cell membranes, thereby permitting the entry of DNA molecules into embryos. The patterns of electrical pulses can be emitted in a single pulse of exponential decay form (i.e., exponential decay generator) or high frequencies multiple peaks of square waves (i.e., square wave generator). Studies conducted in my laboratory (9,10) and those of others (5) have shown that the rate of DNA integration in electroporated embryos is on the order of 20% or higher in the survivors (Table 1). Although the overall rate of DNA integration in transgenic fish produced by electroporation may be equal to or slightly higher than that of microinjection, the actual amount of time required for handling a large number of embryos by electroporation is orders of magnitude less than the time required for microinjection. Recently, several research groups have also reported successful transfer of foreign DNA into fish by electroporating sperm instead of embryos (14,15). Electroporation is therefore considered as an efficient and versatile massive gene transfer technology.

Although transgenes can be reproducibly introduced into various fish species by microinjection or electroporation, the resulting P_1 transgenic individuals possess mosaics germlines as a result of delayed transgene integration. Furthermore, these two gene transfer methods are not effective or successful in producing transgenics in marine fish and invertebrates. Recently a new gene transfer vector, a defective pantropic retroviral vector, has been developed (16). This vector contains the long terminal repeat (LTR) sequence of Moloney murine leukemina virus (MoMLV) and transgenes packaged in a viral envelop with the G-protein of vesicular stomatitis virus (VSV). Since the entry of VSV into cells is mediated by interaction of the VSV-G protein with a phospholipid component of the cell, this pseudotyped retroviral vector has a very broad host range and is able to transfer transgenes into many different cell types. Using the pantropic pseudotyped defective retrovirus as a gene transfer vector, transgene containing neo^R or β-glactosidase has been introduced into zebrafish (17) and medaka (18) (Table 1). Recently, the feasibility of using a pantropic pseudotyped retroviral vector for introducing genes into marine invertebrates and crustaceans

Table 1. Transfer of foreign DNA into medaka embryos by different gene transfer methods (6, with permission).

		Electroporation		Pantropic Retroviral Vector	
	Microinjection[1]	I[2]	II[3]	Electroporation[4]	Incubation[5]
Viability (at hatching)	50%	70%	90%	50%	70%
Integration rate[6]	20%	15%	25%	50%	70%
Transgene expression	yes	yes	yes	yes	yes
Efficiency (eggs/min)	1-2	200	200	200	200

[1]Injecting is carried out via micropyle prior to blastodisc formation.
[2]Exponential-decay impulse mode.
[3]Square wave impulse mode.
[4]Electroporation with square wave mode at 3.5 Kv.
[5]Fertilized eggs are exposed to a mixture of medaka hatching enzyme and pancreatin for 2 h. The dechorinated embryos are incubated with the pantropic pseudotyped retrovirus overnight at room temperature.
[6]Integration rate is calculated from the surviving embryos after gene transfer.

has been tested in dwarf surf clams (19) and crayfish (manmuscript in preparation), and satisfactory results have been obtained in both species.

Characterization Of Transgenic Fish

Identification of Transgenic Fish: The most time consuming step in producing transgenic fish is the identification of transgenic individuals. Traditionally, the presence of transgene in presumptive transgenic individuals is determined by dot blot or Southern blot hybridization of genomic DNA isolated from the test individuals. Although this method is expensive, laborious and insensitive, it offers a definitive answer whether a transgene has been integrated into the host genome. Furthermore, it also reveals the pattern of transgene integration if appropriate restriction enzymes are employed in the Southern blot analysis. In order to handle a large number of samples efficiently and economically, a polymerase chain reaction (PCR) based assay has been adopted (9,11). This assay involves isolation of genomic DNA from a very small piece of fin tissue, PCR amplification of the transgene sequence, and Southern blot analysis of the amplified products. Although this method does not differentiate whether the transgene is integrated in the host genome or remains as an extrachromosomal unit, it serves as a rapid and sensitive screening method for identifying individuals that contain the transgene at the time of analysis. In our laboratory, we use this method as a preliminary screen for transgenic individuals from thousands of presumptive transgenic fish.

Expression of Transgenes: An important aspect of gene transfer studies is the detection of transgene expression. Depending on the levels of transgene products in the transgenic individuals, the following listed methods are commonly employed: (a) RNA northern or dot blot hybridization; (b) RNase protection assay; (c) reverse transcription/polymerase chain reaction (RT/PCR); (d) immunoblotting assay; and (e) other biochemical assays to determining the presence of the transgene protein products. Among these assays, RT/PCR is the most sensitive method and only requires a small amount of sample. Briefly, it involves the isolation of total RNA from a small piece of tissue, synthesis of single-stranded cDNA by reverse transcription and PCR amplification of the transgene cDNA by employing a pair of oligonucleotide primers specific to the transgene product (11). The resulting products are resolved on agarose gels and analyzed by Southern blot hybridization using a radiolabeled transgene as a hybridization probe. Transgene expression can also be quantified by a quantitative RT/PCR method (20). Althgough this method is rapid and sensitive, it can be easily confused by transgene contamination in the reaction unless extra precaution is taken in the cause of setting up the reactions.

Patterns of Transgene Integration: Studies conducted in many fish species have shown that following injection of linear or circular transgene constructs into fish embryos, the transgenes are maintained as extrachromosomal units through many rounds of DNA replication in the early phase of the embryonic development. At later stages of embryonic development, some of the transgenes are randomly integrated into the host genome while others are degraded, resulting in the production of mosaic transgenic fish (for review, 5). In many fish species studied to date, multiple copies of transgenes were integrated in a head-to-head, head-to-tail or tail-to-tail form, except in transgenic common carp and channel catfish where single copies of transgenes were integrated at multiple sites on the host chromosomes (7).

Inheritance of Transgenes: Stable integration of the transgenes is an absolute requirement for continuous vertical transmission to subsequent generations and establishment of a transgenic fish line. To determine whether the transgene is transmitted to the subsequent generation, P_1 transgenic individuals are mated to non-transgenic individuals and the progeny are assayed for the presence of transgenes by the PCR assay method described earlier (9,11). Although it has been shown that the transgene may persist into the F_1 generation of transgenic zebrafish as extrachromosomal DNA (21), detailed analysis of the rate of transmission of the transgenes to the F_1 and F_2 generations in many transgenic fish species indicates true and stable incorporation of the constructs into the host genome (for review, 3,5). If the entire gremlin of the P_1 transgenic fish is transformed with at least one copy of the transgene per haploid genome, at least 50% of the F_1 transgenic progeny will be expected in a backcross involving a P_1 transgenic with a non-transgenic control. In many of such crosses, only about 20% of the progeny are transgenic (7,9,11,21-23). When the F_1 transgenic is back-crossed with a non-transgenic control however, at least 50% of the F_2 progeny are transgenics (Table 2). These results clearly suggest that the germ-lines of the P_1 transgenic fish are mosaic as a result of delayed transgene integration during embryonic development.

Application Of Transgenic Fish In Bio-Technology

Biosynthetic Growth Hormone and Growth Enhancement: In recent years, growth hormone (GH) cDNAs and genomic DNAs have been isolated and characterized for several fish species (for review: 80). Expression of rainbow trout or striped bass GH cDNA in *E. coli* cells results in production of a large quantity of biologically active recombinant GH polypeptide (24,25). In a series of *in vivo* studies, Agellon *et al.* (24) showed that application of the recombinant trout GH to yearling rainbow trout resulted in a significant growth enhancement. After treatment of yearling rainbow trout with the recombinant GH 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 (Fig. 1). Signifi-

Table 2. Effect of GH treatment on the growth of rainbow trout fry (24, with permission)

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

Values presented as mean ± SD. 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 (a=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.

among the individuals of the hormone-treated group was two times greater than that of the controls (Fig. 1). Significant length gain was also evident in hormone-treated animals. When the same recombinant hormone was administered to rainbow trout fry (Table 2) by immersing the fish in a GH-containing solution, the same growth-promoting effect was also observed (24). These results are in agreement with those reported by Sekine *et al.* (26), Gill *et al.* (27) and many others (28-30). However, it is important to mention that the growth enhancement effect of the biosynthetic hormone was markedly reduced when more than 2µg/g body weight of the hormone was applied to the test animals (24). Recently Paynter and Chen (31) have also observed that administration of recombinant trout GH 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 trout GH, native bovine GH or bovine insulin consumed more oxygen per unit time than controls. These results clearly suggest that exogenous application of recombinant fish growth hormone can enhance the somatic growth of finfish and shellfish.

Transgenic Fish Carrying GH or IGF-I Gene: Although exogenous application of biosynthetic GH results in a sig-

Figure 1. Effect of recombinant trout GH on growth of yearling rainbow trout. Groups of yearling rainbow trout received intraperitoneal injection of recombinant GH or control extract for 5 weeks. Wet weights of GH-treated and control fish are shown (mean + SE). Open symbols, Gh-treated fish: o, 0.2 ug/g body weight, 1.0 ug/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. (From Agellon et al., 1988).

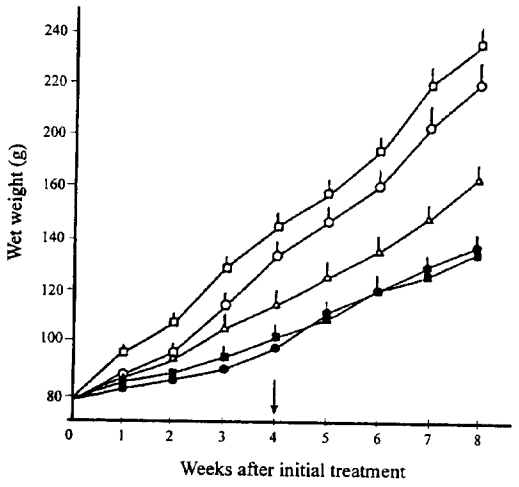

Table 3. Effect of exogenously applied recombinant rainbow trout growth hormone on oyster growth (31, with permission)

Treatment	Initial ht (mm)	Final ht (mm)	Total wt (mg)	Shell wt (mg)	Dry wt (mg)
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 ht represents mean size at the beginning of the experiment and final ht, total wt, shell wt, and dry wt are mean values determined after the five-week treatment cycle was concluded. Height (ht) was measured in mm from the umbo to the ventral shell margin; weight (wt) was measured in mg. Standard errors of the mean (SEM) are in parentheses.

Figure 2. Growth Hormones (GH) or Insulin-Like Growth Factor (IB\GF) I Transgenes

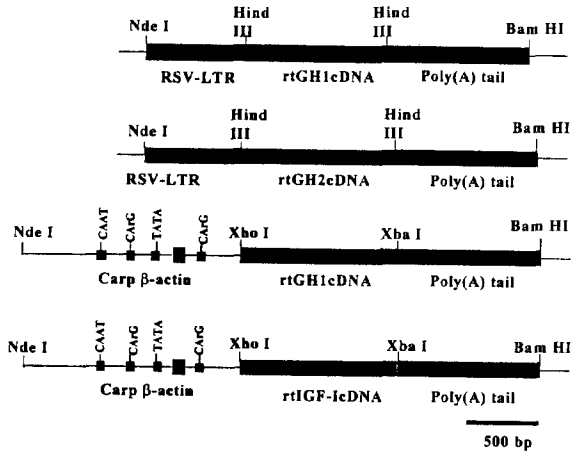

Fig 2. Growth Hormone (GH) or Insulin-Like Growth Factor (IGF) I Transgenes

nificant growth enhancement in fish, it may not be cost effective because of the following reasons: (a) the cost of producing a large amount of purified recombinant GH is too high; (b) treating individual fish with the hormone is labor intensive; (c) the optimal hormone dosage for each fish species is difficult to determine; and (d) GH uptake into fish from an exogenous source is inefficient. If new strains of fish capable of producing elevated but optimal levels of GH can be produced, it would bypass all of the problems associated with exogenous GH treatment. Moreover, once the transgenic fish strains have been generated, they would not only have their own means of producing and delivering the hormone but also be able to transmit the enhanced growth characteristics to their offspring.

Three aspects of fish growth characteristics could be improved for aquaculture: (a) improving initial growth rate allowing fish to reach maturation earlier; (b) enhanced somatic growth rate as adults to provide larger body size for market; and (c) fish with improved feed conversion efficiencies. Among these three, enhanced somatic growth rates via manipulation of GH or insulin-like growth factor (IGF-I) gene show considerable promise. Although there are numerous groups reported efforts of transferring human or fish GH genes into several fish species over the last 15 years (6), only Zhang *et al.* (7), Du *et al.* (8), Lu *et al.* (9) and Martinez *et al.* (32) have documented conclusively 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 (7-9,11,33).

In gene transfer studies conducted in common carp and channel catfish (7,11,33), 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 GH cDNA were injected into the cytoplasm of one-cell, two-cell and four-cell embryos (Fig. 2). 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 Southern blot hybridization of the amplified DNA products using radio-labeled LTR of RSV and/or trout GH1 cDNA as hybridization probes. In the case of transgenic carp studies (7,11), about 35% of the injected embryos survived at hatching, about 10% of which the had stably integrated the RSVLTR-rtGH1-cDNA sequence. A similar percentage of transgenic fish was also obtained when the RSVLTR-csGH-cDNA construct was injected into catfish embryos (33). Southern blot analysis of genomic DNA samples of several transgenic carp and catfish revealed that a single copy of the RSVLTR-rtGH1-cDNA sequence was integrated at multiple chromosomal sites (7).

The patterns of inheritance of RSVLTR-rtGH1 cDNA in 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. 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 (Table 4). 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 although most of these P_1 transgenic fish had RSVLTR-rtGH1 cDNA in their germ-line, they might be mosaics. Similar patterns

Table 4. Percent of F_1 progeny inheriting PRSVLTR-rtGH1 cDNA (11, with permission)

Family	Mating	N	Observed % inheritance	Expected % inheritance[a]
1	P_1 x control	17	0	50
2	P_1 x control	96	32	50
3	P_1 x control	26	42[b]	50
4	P_1 x control	4	100[c]	50
5	$P_1 \times P_1$	28	21	75
6	$P_1 \times P_1$	99	21	75
7	$P_1 \times P_1$	312	31	75
8	$P_1 \times P_1$	93	30	75
9	$P_1 \times P_1$	65	23	75

[a]All observed values are less ($p>0.05$) than expected except[b], which is not significantly different ($p>0.05$) than expected, and [c], which is greater ($p<0.05$) than expected (chi-square).

of mosaicism in the germline of P_1 transgenic fish have been observed in many fish species studied to date (7,9,21,33-35).

According to Zhang *et al.* (7) and Chen *et al.* (11), 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.* (11) further 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. They found that different levels of rtGH mRNA were detected in liver, eyes, gonads, intestine and muscle of the F_1 transgenic individuals.

Since the site of transgene integration differs among 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 the growth performance among these animals. 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.* (11) 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 carrying the rtGH1 cDNA varied widely. When compared to non-transgenic full-siblings, the results of four out of seven growth trials showed 20, 40, 59, and 22% increases in growth, respectively (Table 5 and Fig. 4). The same extent of growth enhancement was also observed in F_2 offspring derived from crossing the fast growing F_1 transgenics with non-transgenic controls. Similar results were observed when RSVLTR-csGHcDNA was transferred into channel catfish. Since the response of the transgenic fish to the insertion of the RSVLTR-rtGH1 cDNA appears to be 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.

More dramatic growth enhancement in transgenic fish has been obtained by introducing Chinook salmon GH cDNA driven by the promoter of ocean pout antifreeze protein gene into Atlantic salmon embryos (8). Some of these transgenic animals grew several times faster than their controls. In a series of recent studies conducted by Lu *et al.* (6) showed that both P_1 and F_1 transgenic medaka carrying a chicken β-actin gene promoter/human GH gene construct (9) or tilapia carrying carp-β-actin promoter-rtGH1cDNA (Figs. 2 and 3) exhibited a significant growth enhancement when compared with the non-transgenic sib-

Figure 3. Transfer of foreign genes into fish embryos by electroporation

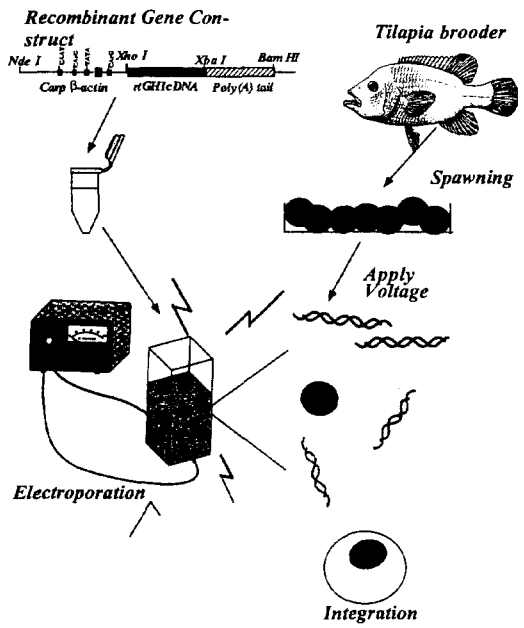

Table 5. Mean, standard deviation, coefficient of variation, and percent difference in body weight of transgenic common carp, Cyprinus carpio, and their nontransgenic full-siblings (16, with permission)

Family	Mating	Genotype	N	Mean Body Weight (SD)	Coefficient of of Variation	% Difference	Range in Body weight (g)
1	P_1 x control	T	31	120.6 (17.4)	14.4	20.8	95-173
		NT	65	99.3 (14.7)	14.8		65-129
2	P_1 x control	T	11	206.0 (45.2)	21.9	40.1	115-283
		NT	15	147.0 (48)	32.6		67-228
3	$P_1 \times P_1$	T	7	5.8 (3.4)	58.6	-26.6	1.8-11.3
		NT	21	7.9 (3.1)	39.2		3.3-17.9
4	$P_1 \times P_1$	T	28	66.1 (36.9)	55.8	58.5	18.5-338
		NT	65	41.7 (27.8)	66.6		8.3-141
5	$P_1 \times P_1$	T	17	14.7 (6.8)	46.3	21.5	6.5-30.4
		NT	82	12.1 (8.4)	69.4		3.9-56.1
6	$P_1 \times P_1$	T	97	114.2 (81.6)	71.5	-14.5	18.3-565.1
		NT	215	133.6 (83.6)	62.5		20.9-416.2
7	$P_1 \times P_1$	T	15	72.2 (58.0)	80.3	-1.5	7.1-214.4
		NT	48	73.3 (47.6)	64.5		8.7-203.3

T: transgenic; NT: nontransgenic; N=number of fish; SD: standard deviation.

Figure 4. Transgenic fish carrying growth hormone (GH) transgene. (A) Transgenic channel catfish carrying RSV-LTR-csGH cDNA. (B) Transgenic common carp carrying RSV-LTR-rtGH1 cDNA.

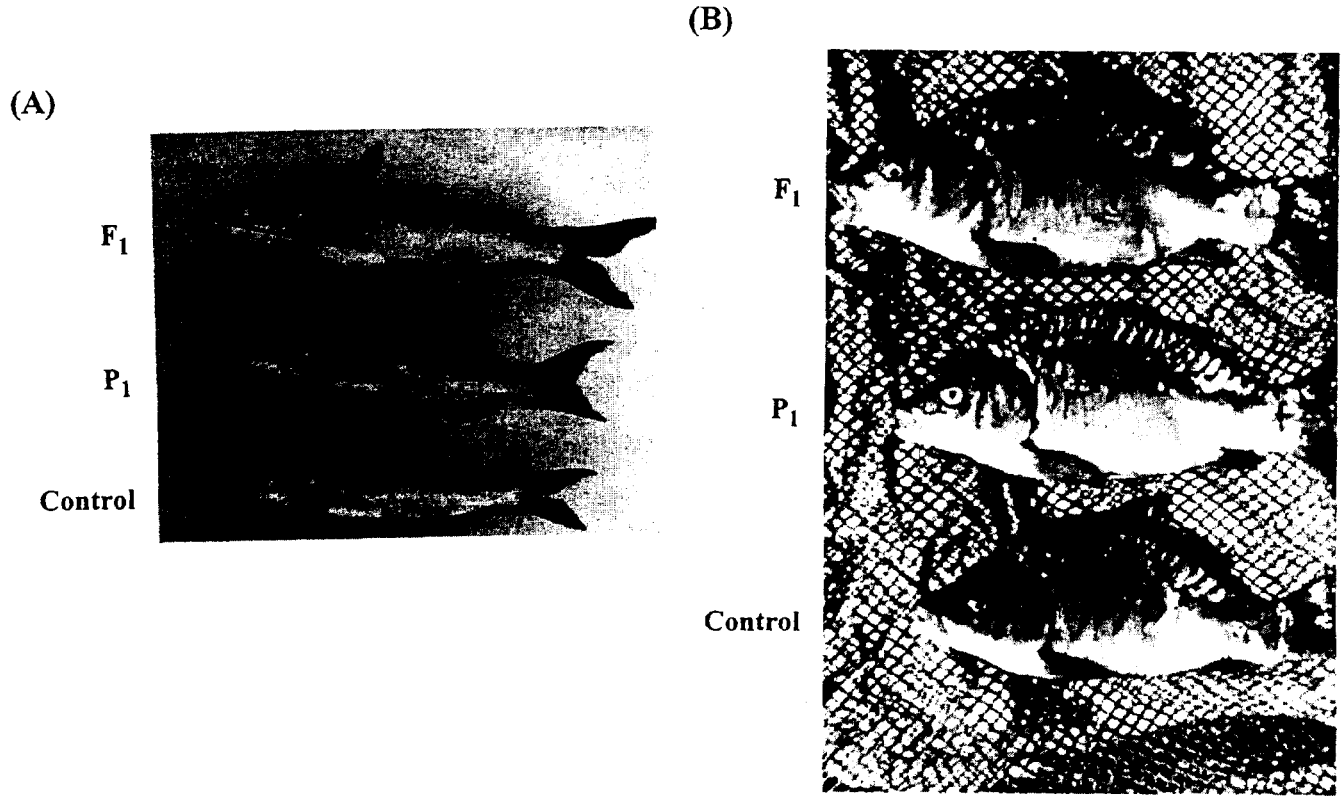

Figure 5. Weight distribution of P1 transgenic and nontransgenic Tilapia. (A) P1 transgenic, (B) P1 non-transgenic and (C) Body weight. D group, embryos electroporated about 2 hrs after fertilization and C group, embryos eletroporated 30 min after fertilization.

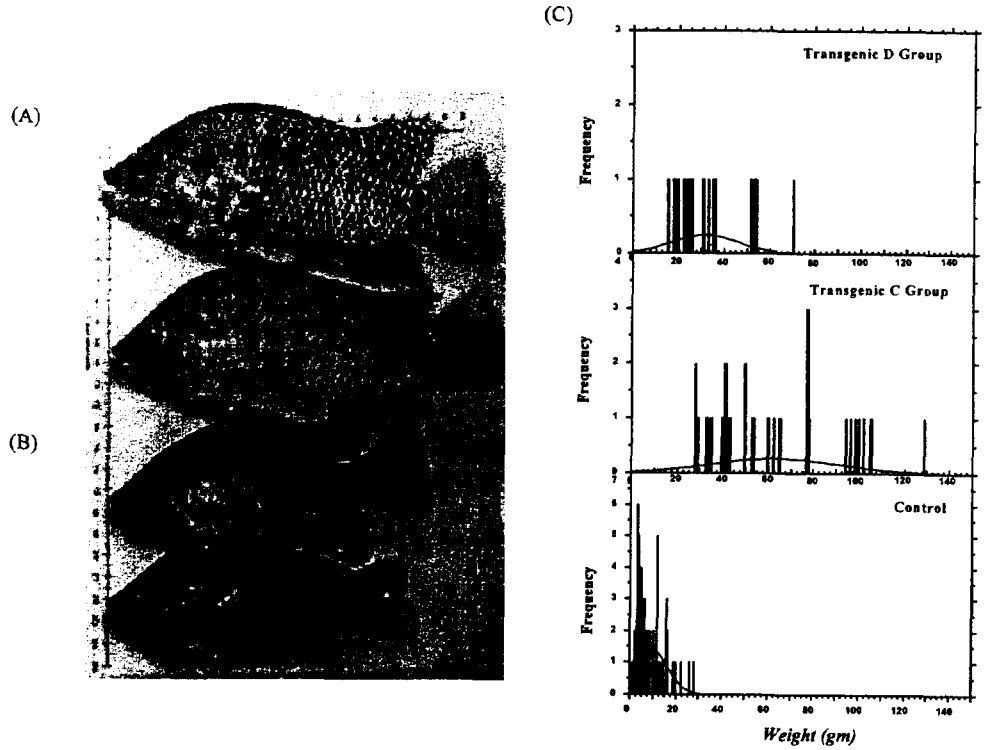

Fig 5. Weight distribution of P_1 transgenic and nontransgenic Tilapia. (A) P_1 transgenic, (B) P_1 non-transgenic and (C) Body weight. D group, embryos electroporated about 2 hrs after fertilization and C group, embryos eletroporated 30 min after fertilization.

lings (Fig. 5). Some of the P_1 transgenic tilapia grew several times faster than their controls.

The effect of IGF-I transgene on somatic growth has also been tested in medaka and tilapia recently. IGF-I transgenic medaka and tilapia not only developed faster during embryonic development, they also exhibited a significant degree of growth enhancement.

Future Prospective

Undoubtedly, transgenic fish technology has a great potential in revolutionizing the aquaculture industry. By introducing desirable genetic traits into finfish, shellfish, or crustaceans, superior transgenic strains with improved genetic traits 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 and IGF-I transgenes can be achieved in several finfish species and the resulting animals grow substantially faster than their control siblings do. This is a vivid example of the application of 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) identifying genes of desirable traits for aquaculture and other application; (ii) developing targeted gene transfer technologies such as embryonic stem cell gene transfer method or ribozyme gene inactivation methods; (iii) identifying suitable promoters to direct the expression of transgenes at optimal levels during the desired developmental stages; (iv) determining physiological, nutritional, immunological and environmental factors that will maximize the performance of the transgenic individuals; and (v) assessing safety and environmental impacts of transgenic fish. Once these problems are resolved, the commercial application of the transgenic fish technology in aquaculture will be readily attained.

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REFERENCES

1. Gordon, J.W.: Transgenic animals. Intl. Rev. Cytol. 1989; 155, 171-229.
2. Jaenisch, R.: Transgenic animals. Science 1990; 240: 1468-1477.
3. Chen, T.T. and Powers, D.A.: Transgenic fish. Trends in Biotechnol. 1990; 8: 209-215.
4. Fletcher, G.L. and Davis. P.L.: Transgenic fish for aquaculture. in "Genetic Engineering" (ed. by Setlow, J.K.), Plenum Press, New York, 1991; 13: 331-370.
5. Hackett, P.B. 1993. The molecular biology of transgenic fish. in "Biochemiostry and Molecular Biology of Fish" (eds. Hochachka, P and Mommsen, T), Elsevier Science Publishers B.V., 1993; 2: 207-240.
6. Chen, T.T., Lu, J.-K. and Fahs II, Richard: Transgenic fish technology and its application in fish production. In "Agricultural Biotechnology (ed. By Altman, A.), 1998. pp. 527-547, Marcel Dekker, Inc.
7. Zhang, P., Hayat, M., Joyce, C., Gonzales-Villasenor, L.I., Lin, C.-M., Dunham, R., Chen, T.T. and Powers, D.A.: Gene transfer, expression and inheritance of pRSV-Rainbow Trout-GH-cDNA in the carp, *Cyprinus carpio* (Linnaeus). Mol. Reproduc. Develop. 1990; 25: 3-13.
8. Du, S.J., Gong, G.L., Fletcher, G.L., Shears, M.A., King, M.J., Idler, D.R. and Hew, C.L.: Growth enhancement in transgenic Altantic salmon by the use of an "all fish" chimeric growth hormone gene construct. Bio/Technol. 1992; 10, 176-181.
9. Lu. J.K., Chrisman, C.L., Andrisani, O.M., Dixon, J.E., and Chen, T.T.: Integration expression and germ-line transmission of foreign growth hormone genes in medaka, *Oryzias latipes.* Mol. Mar. Biol. Biotechnol. 1992; 1: 366-375.
10. Chen, T.T., Vrolijk, N.H., Lu, J.K., Lin, C.M., Reinschuessel, R. and Dunhasm, R.A.: Transgenic fish and its application in basic and applied research. Biotechnol. Ann. Rev. 2: 205-236, 1996.
11. Chen, T.T., Powers, D.A., Lin, C.M., Kight, K., Hayat, M., Chatakondi, N., Ramboux, A.C., Duncan, P.L., and Dunham, R.A.: Expression and inheritance of RSVLTR-rtGH1 cDNA in common carp, *Cyprinus carpio*. Mol. Mar. Biol. Biotechnol. 1993; 2: 88-95.
12. Moav, Boaz, Liu, Z., Groll, Y., and Hackett, P.R.: Selection of promoters for gene transfer into fish. Mol. Mar. Biol. Biotechnol**.** 1992; 1, 338-345.
13. Cotten, M. and Jennings, P.: Ribozyme mediated destruction of RNA in vivo. EMBO J. 1989; 8: 3861-3866.
14. Symonds, J.E., Walker, S.P. and Sin, F.Y.T.: Development of mass gene transfer method in chinook salmon: optimization of gene transfer by electroporated sperm. Mol. Mar. Biol. Biotechnol. 1994; 3: 104-111.
15. Tseng, F.S., Lio, I.C. and Tsai, H.J.: Introducing the exogenous growth hormone cDNA into lach (Misgurnus anguillicaudatus) eggs via electroporated sperms as carrier. 3rd International Marine Biotechnology Conference, Tromso, Norway, 1994; 71.
16. Burns, J.C., Friedmann, t., Driever, W., Burrascano, M and Yee, J.K.: VSV-G pseudotyped retroviral vector:concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc. Natl. Acad. Sci, USA. 1993; 90: 8033-8037.
17. Lin, S., Gaiano, N., Culp, P., Burns, J.C., Friedmann, T., Yee, J.-K. and Hopkins, N.: Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. Science 1994; 265: 666-668.
18. Lu, J.-K., Jane C. Burns, and Chen, T.T.: Pantropic retroviral vector integration, expression and germline transmission in medaka (*Oryzias latipes*). Mol. Marine Biol. Biotechnol. 1997; 7: 289-295.
19. Lu, J.-K., Chen, T.T., Allen, S.K., Matsubara, T. and Burns, J.C.: Production of transgenic dwarf surfclam, Mulinia lateralis, with pantropic retroviral vectors. Proc.Natl. Acad. Sci. USA.1996; 93: 3482-3486.
20. Ballagi-Pordany, Andrea., Ballagi-Pordany, Andras and Funa, K.: Quantitative determination of mRNA phenotypes by polymerase chain reaction. Anal. Biochem. 1991; 196: 88-94.
21. Stuart, G.W., McMurry, J.V. and Westerfield, M.: Replication, integration, and stable germ-line transmission of foreign sequence injected into early zebrafish embryos. Development 1988; 109: 403-412.
22. Stuart, G.W., Vielkind, J.V., McMurray, J.V. and Westerfield, M.: Stable lines of transgenic zebrafish exhibit reproduction patterns of transgene expression. Development 1990; 109: 293-296.
23. Shears, M.A., Fletcher, G.L., Hew, C.L., Gauthier, S. and Davies, P.L.: Transfer, expression, and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*). Mol. Mar. Biol. Biotechnol. 1991; 1: 58-63.
24. Agellon, L.B.; Emery, C.J.; Jones, J.M.; Davies, S.L.; Dingle, A.D.; Chen, T.T.: Growth hormone enhancement by genetically engineered rainbow trout growth hormone. Can. J. Fish. Aqua. Sci. 1988; 45: 146-151.
25. Cheng, C.M., Lin, C.M., Shamblott, Mike, Gonzalez-Villasenor, L.I., Powers, D.A., Woods, C. and Chen, T.T.: Production of a biologically active recombinant teleostean growth hormone in *E. coli* cells. Mol. Cellu. Endocrinol. 1995; 108: 75-85.
26. Sekine, S.; Miizukzmi,T.; Nishi, T.; Kuwana,Y.; Saito,A.; Sato, M.; Itoh, H.Kawauchi, H.: Cloning and expression of cDNA for salmon growth hormone in *E. coli*. Proc. Natl. Acad. Sci. USA 1985; 82: 4306-4310.
27. Gill, J.A.; Stumper, J.P.; Donaldson, E.M.; Dye, H.M.: Recombinant chicken and bovine growth hormone in cultured juvenile Pacific salmon. Biotechnology 1985; 3: 4306-4310.
28. Sato, N.; Murata, K.; Watanabe, K.; Hayami, T.; Kuriya, Y.; Sakaguchi, M.; Kimura, S.; Nonak, M.; Kimura, A.: Growth-promoting activity of tuna growth hormone and expression of tuna growth hormone cDNA in *Escherichia coli*. Biotechnol. Appl. Biochem. 1988; 10: 385-392.
29. Moriyama, S.; Takahashi, A.; Hirano, T.; Kawauchi, H.: Salmon growth hormone is transported into the circulation of rainbow trout (*Oncorhynchus mykiss*) after intestinal administration. J. Comp. Physiol. B 1990; 160**:** 251-260.
30. Schulte, P.M.; Down, N.E.; Donaldson, E.M.; Souza, L.M.: Experimental administration of recombinant bovine growth hormone to juvenile rainbow trout (*Salmo gairdneri*) by injection or immersion. Aquaculture1989; 76: 145-152.
31. Paynter, K.; Chen, T.T.: Biological activity of biosynthetic rainbow trout growth hormone in the eastern oyster, (*Crassostrea virginica*). Biol. Bull. 1991; 181: 459**-**462.
32. Martinez, R., Estrado, M.P., Berlanga, J., Guillen, I., Hernandez, O., Cabrea, E., Pimentel, R., Moradle, R., Herera, F., Morales, A., Pinas, J.C., Abad, Z., Sanchez, V.,Melamed, P., Leonard, R., de la Fuente , J.: Growth enhancement in transgenic tilapia by ectopic expression of tilapia growth hormone. Mol.. Mar. Biol. 1995; 5: 65-70.
33. Dunham, R.A.; Ramboux, A.C.; Duncan, P.L.; Hayat, M.; Chen, T.T.; Lin, C.-M.; Kight, K.; Gonzalez-Villasenor, L.I.; Powers, D.A.: Transfer, expression and inheritance of salmonid growth hormone genes in channel catfish, *Ictalarus punctatus*, and effects on performance traits. Mol. Mar. Biol. Botechnol. 1992; 1: 380-389.
34. Ozato, K., Kondoh, H., Inohabra, H., Iwamatus, Y. and Okada, T.S.: Production of transgenic fish and introduction and expression of chicken ?-crystallin gene in medaka embryos. Cell Differ. 1986; 99: 237-244.
35. Powers, D.A., Hereford, L., Cole, T., Creech, K., Chen, T.T., Lin, C.M., Kight, K and Dunham, R.A.: Electroporation: a method for transferring genes into gametes of zebrafish (*Brachydanio rerio)* channel catfish (*Ictalurus punctatus*), and common carp (*Cyrinus carpio*). Mol. Mar. Biol. Biotechnol. 1992; 1: 301-308.