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Gene Transfer and Expression in Farmed Fish

**R.A. Dunham, B. Cavari, T.T. Chen, D.A. Powers,
G. Hulata, B. Funkenstein**

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Gene Transfer and Expression in Farmed Fish

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c. Abstract

Several growth hormone gene constructs were developed, and fused to a variety of promoters including RSV-LTR, carp β -actin and rainbow trout metallothionein. The β -actin promoter was modified in several ways resulting in regulatory sequences with a wide range of expression strength. Gene transfer efficiency was enhanced by evaluating the affect of microinjection at different developmental stages on integration rate and survival of embryos. Early in the one-cell and during the two-cell stage were the optimum developmental times for microinjection of common carp. Electroporation allowed greater production of transgenic individuals and higher integration rates. The RSV-LTR rainbow trout GH1 cDNA and the β -actin chinook salmon GHcDNA were successfully transferred to common carp. All parent and F_1 transgenic individuals produced rainbow trout GH. Expression level was variable, but was found in each tissue, liver, muscle, eye, red blood cell, intestine and gonad, tested. β -actin promoters allowed expression of foreign DNA in the fins of common carp. P_1 transgenic carp transmitted the foreign DNA to their progeny. However, progeny ratios indicated the P_1 individuals were mosaic. In most cases, F_1 transgenics transmitted the rtGH1 cDNA to their progeny in expected Mendelian ratios. In two-thirds of the families evaluated transgenic individuals grew 10-60% faster than their non-transgenic full-siblings. The variable response to the addition of rtGH1 cDNA among families is likely due to differences in copy number, level of expression, insertion site, genetic background and epistasis. Family selection will need to be combined with gene transfer to develop transgenic populations with optimum growth performance.

d. Objectives

The objectives of the project were:

1. To improve the efficiency of the insertion of CAT and GH genes into genome of developing carp by microinjection of DNA into zygotes.
2. To determine whether these introduced genes are expressed in the experimental fish.
3. To select for suitable promoters that allow the expression of these genes in fish.
4. To prove that the introduced genes are genetically transmitted to the F1 generation.

e. Results

Introduction

For the past decade, the prevalent method for introducing genes into the germ line of animals has been direct microinjection of DNA into the pronuclei of fertilized eggs. This method has been used successfully to introduce a host of foreign genes for generating transgenic mice (Gordon et al., 1980; Palmiter et al., 1982), cows, pigs, sheep, and rabbits (Hammer et al., 1985; Pursel et al., 1989). Transgenic individuals have also been generated in several fish species by microinjection; rainbow trout, Oncorhynchus mykiss (Chourrout et al., 1986; Maclean et al., 1987); Atlantic salmon, Salmo salar (McEvoy et al., 1988; Fletcher et al., 1988; Rokkones et al., 1990); loach, Misgurnus anguillicaudatus (Zhu et al., 1986); channel catfish, Ictalurus punctatus (Dunham et al., 1987); tilapia, Oreochromis niloticus (Brem et al., 1988); goldfish, Carassius auratus (Zhu et al., 1985); zebrafish, Brachydanio rerio (Stuart et al., 1988); and medaka, Oryzias latipes (Ozato et al., 1986). Because the pronuclei in most fish species are not visible, the

egg cytoplasm is microinjected, and the quantity of the foreign genes required for each injection is in the order of 10^6 molecules per egg or more. In general, eggs and sperm are collected from mature females and males and placed into separate dry containers. Fertilization is initiated by adding water and sperm to eggs, with gentle stirring to enhance fertilization and dispersal. Eggs are water-hardened for various periods (depending on the fish species) and then rinsed. Microinjection is usually carried out within the first few hours after fertilization (i.e., between one-cell to four-cell stages).

Because fish undergo external fertilization, the microinjected embryos do not require complex manipulations such as *in vitro* culturing of embryos and transferring of embryos into foster mothers-manipulations essential in mammalian systems. Another added feature in the fish system is that cytoplasmic injection is much easier and less injurious to development; therefore, survival rates of the injected embryos are generally higher in fish than in mammals. One exception to successful use of the cytoplasmic injection approach, however, is the medaka (Ozato et al., 1986), in which the pronuclei are visible. Depending on the fish species, the survival rates of the injected embryos range from 5 to 90%. Even though the foreign DNA is injected into the cytoplasm, the rate of foreign gene integration in the transgenic fish system is relatively high.

The tough chorion of many fish species has frequently presented problems in inserting glass microneedles into the eggs. This problem has been minimized by a number of strategies. In rainbow trout and salmon eggs, the opening in the eggs is made by microsurgery before the microglass needle is inserted (Chourrout et al., 1986; McEvoy et al., 1988). In Atlantic salmon and tilapia, a microneedle can be inserted through the micropyle (an opening

for sperm penetration at fertilization) (Fletcher et al., 1988; Brem et al., 1988). The chorion of zebrafish eggs can be removed manually with a pair of forceps (Ozato et al., 1986), and that of goldfish and loach by trypsin digestion (Zhu et al., 1985, 1986). Oshiro et al. (1989) reported that hardening of rainbow trout egg chorion can be prevented if fertilization takes place in a solution containing 1 mmol/L glutathione solution (pH, 8.0).

Integration rates range from 0 to 75% when fish embryos are microinjected with foreign DNA (Chourrout et al., 1986; Dunham, 1990a; Powers et al., 1990); however, typical integration rates are 5% or less (Dunham, 1990; Powers et al., 1990). Survival rates of microinjected embryos can be high (75-100%) for species such as rainbow trout (Chourrout et al., 1986) or low (0-10%) for species such as channel catfish (Dunham et al., 1987; Hayat, 1989; Powers et al., 1990).

Regardless of survival or integration rate, microinjection is a tedious procedure, and improved gene transfer technology for fish is needed. Mass transfer systems would be especially beneficial for recombinant DNA research in fish because of the high fecundity of fish (compared with mammals) and the high mortality rate of some fish species.

Electroporation is an excellent method for transfer of DNA into a large number of fish embryos in a short period. The first successful gene transfer utilizing electroporation yielded integration rates and survival of manipulated embryos similar to that of microinjection (Inoue et al., 1990). Electroporation, however, would be better than microinjection even if integration rates were equivalent because a much larger number of eggs can be treated per unit time with electroporation. Even if mortality was high for electroporated embryos, larger numbers of potentially transgenic individuals

could be produced, because electroporation is a mass transfer technique. This advantage is especially important for species, such as channel catfish, that have low survival rates when individuals are microinjected (Dunham et al., 1987; Hayat, 1989; Powers et al., 1990).

Electroporation utilizes short electrical pulses to permeabilize the cell membrane, thereby gaining entry of macromolecules into the cell. Utilization of this method for gene transfer has been proved successful in bacteria, cultured mammalian cells, and plant protoplasts (Shigekawa and Dower, 1988). Attempts have been made by several laboratories throughout the world to adopt this technique for gene transfer in fish systems. Although initial results with electroporation were not successful, recent attempts have been very promising.

We will present data that suggest electroporation is a useful method for mass transfer of growth hormone genes and other genes into experimental as well as commercially important fish species.

Additionally, survival and integration rates may vary with developmental stage. Apparently, survival and induction rates vary with developmental stage for other genetic manipulations such as polyploidy (Dunham, 1990b). Elucidation of developmental stages most conducive to high survival and integration rates for species and integration rates, is important to increase the number of experimental, transgenic fish. Another objective was to compare two species, channel catfish and common carp, *Cyprinus carpio*, for survival (hatching rate) and integration rate after microinjection with recombinant DNA at early, intermediate or late one-cell, two-cell, or four-cell stage to determine the most appropriate developmental stage for maximizing the production of transgenic individuals. Similar experiments will be conducted

utilizing electroporation.

Transgenic common carp, Cyprinus carpio, carrying the long terminal repeat (LTR) sequence of the avian Rous sarcoma virus (RSV) fused to the rainbow trout (rt) growth hormone (GH1) cDNA have been produced by microinjection (Zhang et al., 1990). In order to evaluate the expression and inheritance of the foreign gene and the effect of the transgene on growth in a larger number of the F_1 transgenic individuals, one non-transgenic female control x four P_1 (transgenic parental generation produced by microinjection), four P_1 x F_1 matings of transgenic carp containing RSVLTRrtGH1 cDNA were made. In this paper, we report the inheritance and growth performance in different families of F_1 transgenic fish. In addition, tissue distribution of expression of rtGH1 cDNA from one F_1 family is also reported.

Methods

Preparation of expression vectors

Liu et al. (1990a) cloned and mapped the carp beta-actin gene. A large number of gene constructs containing the beta-actin regulatory sequences and a reporter gene - chloramphenicol acetyl transferase (CAT) were prepared. Two pRSV-2-based plasmids (Gorman et al., 1982; McKnight and Tjian, 1986) containing the rainbow trout GH1 or GH2 cDNA sequences were constructed as follows. A BamHI-HindIII fragment, containing the respective GH cDNA sequences, was isolated from the plasmid, blunt-ended, and inserted into the Sall site of plasmid pRSV-2 (Gorman et al., 1982). The 5.23-Kb constructs, designated RSVLTR-rtGH1 cDNA and RSVLTR-rtGH2 cDNA, each contain 580 bp of the LTR sequence, 950 bp of the rtGH cDNA sequence, and 3.7 Kb of the pRSV flanking sequence. The 5' end of the GH cDNA sequence is linked to the RSVLTR promoter by a 13-bp fragment corresponding to the multiple cloning site.

Prior to electroporation, the recombinant clones were linearized by digestion with BAMHI.

During the third year of the project, "all-fish" gene constructs were made. One construct was ptMTA-gsbGHcDNA, which contains trout metallothionein promoter and the *Sparus aurata* GH cDNA. Five other constructs were made as follows: 1) ptMTb-gsbGHcDNA; 2) pc A-gsbGHcDNA; 3) pc A-csGHcDNA; 4) ptMTa-scGHcDNA; 5) ptMTb-scGH cDNA.

The first vector contains trout metallothionein promoter and the *Sparus aurata* GH cDNA. The second vector contains carp β -actin promoter and *S. aurata* GH cDNA. Vector 3 contains carp β -actin promoter (FV1 and FV2) and chinook salmon GH cDNA (Liu et al., 1990b). Vectors 4 and 5 contain the trout metallothionein promoters and silver carp GH gene.

Gene transfer

Common carp brood fish maintained at the Fisheries Research Unit, Alabama Agricultural Experiment Station, Auburn University, were spawned artificially by induction with carp pituitary extract (CPE) as described previously (Zhang et al., 1990). Eggs were stripped from a mature female into a dry Petri dish. Milt and water (18-20°C) were added to the eggs with gentle stirring to initiate fertilization. After water-hardening for 5 min, the fertilized eggs were rinsed with water and ready for microinjection.

Embryos were microinjected during the one to four cell stages with the aid of a dissecting microscope and a micromanipulator as described earlier (Zhang et al., 1990). About 20 nl of a DNA solution containing 10^6 molecules of linearized RSVLTR-rtGH1 cDNA transgene (Zhang et al., 1990) were delivered per embryo. Embryos were incubated in static water at about 20°C until hatching. All fish were heat branded, weighed and fin clipped.

Fertilized zebrafish eggs were collected directly from zebrafish tanks. Microinjection studies on zebrafish, common carp, and channel catfish were performed according to previously published procedures (Stuart et al., 1988; Zhang et al., 1990; Hayat et al., 1991).

A Baekon, Inc. electroporation device was used for the electroporation studies. Fertilized zebrafish eggs were electroporated with the RSVLTR-rtGH1 cDNA construct or with buffer alone. Channel catfish and common carp eggs and sperm were also electroporated with either of the fish growth hormone constructs or with buffer alone. In some cases, both the eggs and the sperm of representative channel catfish and common carp were treated by electroporation prior to fertilization, whereas in other cases, only fertilized eggs (embryos) were exposed to electroporation. In the first case (prefertilization), the unfertilized eggs were electroporated with either buffer alone (control group) or buffer containing RSVLTR-rtGH1 or RSVLTR-rtGH2 (experimental group). Sperm was treated similarly. The eggs and sperm were then mixed in appropriate beakers or petri dishes to maximize fertilization. In the other cases, the eggs were electroporated with either buffer alone or buffer containing RSVLTR-rtGH1 or RSVLTR-rtGH2 shortly after fertilization (early postfertilization) or substantially later (first cell division). The channel catfish eggs were electroporated 7 (early postfertilization) and 90 minutes after fertilization (first cell division), and carp eggs were treated 5 (early postfertilization) and 25 minutes (first cell division) after fertilization. These treatment times were chosen because they represented integration peaks for channel catfish and common carp embryos during previous microinjection studies (Hayat et al., 1991).

Fifty common carp eggs or 15 channel catfish eggs in 100 μ L buffer (TE,

0.088 mmol/L NaCl) were treated at the same time, using 2-mL cuvettes with electrodes set into the bottom. The majority of experiments involved a noncontact mode of electroporation, with the electrode 1 to 2 mm above the buffer. The number of cycles, burst time, and pulse width were held constant at 4 cycles, 0.8 seconds, and 160 us, respectively. Voltage was varied from 2.5 to 10 kV, and the number of pulses per cycle was varied from 2^6 to 2^{11} . A few experiments were performed with one of the electrodes touching the buffer (contact mode) and the following conditions: voltage = 1.06 kV; number of cycles = 1; burst time = 0.8 sec; pulse width = 160 us; and 2^6 pulses per cycle.

As with the noncontact procedure, these eggs were transfected with either buffer (TE, 0.088 mmol/LNaCl) or buffer containing 50 ug/mL of one of the DNA constructs (i.e., RSVLTR-rtGH1 or RSVLTR-rtGH2).

The following constructs were microinjected into crossbred carp (common carp - male, and koi female) in the first season (March-July

	<u>Form</u>	<u>Dates of microinjection</u>
1990).		
1) construct 4 + pHC6CSGH	Supercoil	19.4, 27.4, 8.6, 15.6
2) construct 4 + pRC6CSGH	Linear	19.6, 26.6, 9.7, 10.7
3) pRSVCAT + pRSV-2-rtGH	Supercoil	22.5, 22.8
4) pH3NCOCATGH3 + pH3NCOCSGH	Supercoil	28.5, 29.5, 22.8

Construct 4 (pRC6CAT) is identical to the pRC6CSGH except for the replacement of the CAT gene and SV40 intron and poly A site with the CSGH cDNA obtained from Choi Hew (Hew et al., 1989).

pRSV-2-rtGH is the rainbow trout GH cDNA (containing the secretory piece), driven by RSV LTR (obtained from T. T. Chen). pRSVCAT is the analogue CAT construct.

pH3NCO is the entire first intron plus a 1.1 Kb 5' element which is highly active in tissue culture (like construct 6). The SV40 polyadenylation site in construct 6 is replaced by the chinook salmon GH 3' to make it an all-fish vector (Liu et al., 1990c Moav et al. 1992).

Eggs and sperm were provided by Dr. G. Hulata at ARO Dor, and S. Rothbard in Gan Shmuel fish breeding institute. Microinjection was performed at the Tel Aviv University and at ARO Dor with full collaboration of B. Cavari's group of the IOLR, Haifa.

During the spawning seasons of 1991-1992, four constructs were microinjected into common carp. The first two constructs, ptMTa-gsbGHcDNA and the ptMTa-scGH, contain the trout metallothionein-a gene promoter and either the *Sparus aurata* GH cDNA or the silver carp GH genomic gene. Both constructs were used for transfection of carp epithelial cell line (EPC).

Fertilized carp eggs were also microinjected with two homologous constructs containing identical α -actin promoters with different structural genes: one contains the CAT gene (bacterial chloramphenicol acetyl transferase), and the other the chinook salmon growth hormone cDNA gene (CSGH).

Genomic DNA extraction

Genomic DNA was extracted from pectoral fin clips of 3-month-old fish when the zebrafish reached sexual maturity and when the common carp and the channel catfish reached a size between 5 and 10 gm, using the methods of Maniatis et al. (1982) with some modifications. These samples were analyzed by PCR. As part of a double-blind study, some individuals were randomly selected and fin-clipped once again when they were 5 months old to check PCR results by Southern blot analysis.

Pectoral fin tissue was lysed in 50 uL mmol/L Tris-HCl buffer (pH 8.0) containing 0.1 mmol/L EDTA; 1% SDS, and 20 ug proteinase K, and digested at 55°C for 15 hours. The mixture was diluted with distilled water to a final volume of 0.5 mL and boiled for 5 to 10 minutes to denature the proteinase K. Following centrifugation to remove the insoluble materials, the supernatant was extracted once with an equal volume of buffer, saturated phenol and chloroform, and the nucleic acid precipitated in ethanol. After ethanol precipitation, the DNA pellet was dissolved in 0.5mL TE buffer, and concentration was determined. This material was then used for PCR and Southern blot studies.

PCR analyses

Initial screening of presumptive transgenic fish was performed using PCR on crude genomic DNA extracts from fin tissue. A small amount of fin-clipped tissue from each fish was added to 50 uL lysis buffer consisting of 50 mmol/L Tris-Cl (pH, 8.0), 20 mmol/L NaCl, 1 mmol/L EDTA, 1% SDS, and 1 mg/mL proteinase K. The tissues were incubated in the lysis buffer for 15 minutes at 55°C, vortexed briefly, and incubated for an additional 15 minutes. After dilution to 500 uL with ddH₂O, the sample was boiled for 5 to 10 minutes and extracted once with an equal volume of TE-saturated phenol and once with an equal volume of chloroform. Insoluble materials were removed by brief centrifugation in microfuge, and the DNA was precipitated by bringing the solution to 0.3 mol/L NaOAc and adding 2.5 volumes of EtOH. The genomic DNA pellet was resuspended in 500 uL TE buffer, and 10 uL was aliquoted for PCR.

Each reaction was carried out in a volume of 100 uL consisting of 50 mmol/L KCl, 10 mmol/L TRIS-HCl (pH, 8.3); 1.5 mmol/L MgCl₂; 0.01% gelatin; 200 umol/L each dATP, dGTP, dGTP, and dTTP; 2 to 2.5 units of Taq polymerase

(Promega); and 75 pmol each primers P1 and P2. The primer sequences are complementary to pRSV2 vector sequence immediately adjacent to the multiple cloning sites as follows: P1-5'AAGTAAGGTTCTTCACAAA-3' and P2-5'TGCGGTATTTACACCGCAT-3'. The amplification cycle consisted of denaturation for 1 minute at 94°C. primer annealing for 2 minutes at 50°C. and synthesis for 3 minutes at 72°C. Thirty cycles were followed by an 8-minute extension at 72°C.

Twenty microliters of the reaction products were electrophoresed on an 0.8% agarose gel, transferred to nylon membrane, and probed with nick-translated ³²P-labeled RSVLTR-rtGH1 cDNA. Hybridization was performed for 12 hours in 6 XSSC. 25 mmol/L KPO₄ 0.1% SDS, 50% formamide at 42°C. After washing in 0.1X SSC 0.1% SDS at 65°C. the membrane was exposed to x-ray film for autoradiography.

Southern blot analyses

Southern blot analysis was performed on individuals, sampled at 5 months of age, that had been previously analyzed by PCR analysis. The technician who performed these analyses did not know the origin of the samples nor that they had been previously sampled.

For Southern blot analysis the genomic DNA was digested with HindIII or BamHI under conditions indicated by the supplier (Boehringer Mannheim) and electrophoresed on an 0.8% agarose gel. DNA samples in the gel were transferred to nylon membrane by diffusion. The membrane was soaked in 1.5 mol/L NaCl and 0.5 mol/L NaOH for 2 minutes, neutralized in 1.5 mol/L NaCl. 0.5 mol/L TRIS-HCl (pH, 7.2), and 1 mmol/L EDTA for 5 minutes. After air drying, the membrane was irradiated on a short wavelength UV transilluminator for 5 minutes. Subsequently, the membrane was probed with nick-translated

³²P-labeled RSVLTR-rtGHcDNA.

Copy number estimates were based on a haploid DNA content of 1.7 pg, using as standard the DNA content of one sea urchin sperm (0.98×10^9 bp). The molecular weights of fish genomic DNA and RSVLTR-rtGHcDNA were determined and used to estimate the amount of exogenous DNA (equivalent to 1 DNA molecule) present in 18 ug of each fish's genomic DNA. The total number of DNA copies was estimated from the optical density profile autoradiographs scanned under visible light.

The NdeI-HindIII and KpnI-HindIII restriction fragments isolated from the RSVLTR-rtGHcDNA construct were labeled with a [α -³²P]dCTP either by nick translation (Maniatis et al., 1982) or by random priming, then purified through a Sephadex G-50 spin dialysis column and used as probes. Prehybridization was performed in 20 mmol/L Tris-HCl (pH, 7.5), 0.1% SDS, 5x SSC, 10x Denhardt, and 100 ug/mL denatured calf thymus DNA at 42°C with constant shaking for 3 hours. Hybridization was performed in the same buffer containing denatured probe at 42°C with constant shaking overnight. Membranes were washed twice in a solution containing 2x SSC and 0.1% SDS for 15 minutes at room temperature, and twice in a solution of 0.2x SSC and 0.1% SDS for 15 minutes at 65°C.

RNA analysis

Total RNA was isolated from liver, muscle, eye, intestine and gonad following the acid guanidinium thiocyanate-phenol-chloroform method. Single-stranded (ss) cDNA was synthesized according to conditions described by Agellon and Chen (1986), using the above mentioned total RNA as templates. Trout GH and β -actin DNA sequences were amplified by PCR under the conditions described above using synthetic oligonucleotides specific to trout GH and -

amplification primers and ssDNA from different tissues as templates. The synthetic oligonucleotides specific for the amplification of trout GH are 5'-ATAGAAAACAACGGCTCTTC-3' and 5'-CTACAGAGTGCAGTTGGCCTC-3', and those for α -actin are 5'-TCACCAACTGGGGATGACATGG-3' and 5'-TCCACATCTGCTGCTGGAAGGTGG-3'. The PCR amplified products were resolved in 2% agarose gels, transferred to nylon membranes and hybridized to [32 P]-labelled rtGH1 cDNA and α -actin cDNA under conditions described earlier (Zhang et al., 1990).

For RNA dot blot hybridization, 18 ug of total RNA from different tissues was spotted onto nylon membranes, UV cross-linked and hybridized to [32 P]-labelled rtGH1 cDNA under conditions described by Zhang et al. (1990). The amount of hybridization was visualized by autoradiography.

Radio-immunobinding assay

Determination of rtGH polypeptide in the red blood cells of transgenic fish was carried out as described by Zhang et al. (1990) with some modifications, using an antiserum specific to chum salmon GH as a probe. This antiserum was prepared by extensive absorption of a polyclonal chum salmon GH antiserum with carp pituitary extract. Red blood cell extracts of transgenic individuals or controls (50 ug) were loaded on a Millipore Hybond nylon membrane. The membrane was blocked in a solution of Tris-buffered saline (TBS) containing 3% defatted powdered milk, and incubated overnight at 4°C in the same solution containing reabsorbed anti-chum salmon GH antibody. After washing 3 times in TBS/milk solution, the membrane was incubated for several hours in the TBS/milk solution containing [125 I]-labelled goat anti-rabbit IgG. The reaction between trout GH in the red blood cell extracts and chum salmon anti-GH serum was visualized by autoradiography.

Production of F₁ transgenic progeny

Transgenic individuals were identified and mated with other transgenic individuals or controls using the same artificial spawning procedures as described earlier. Eggs were hatched in either static or running water at 22 to 24°C. Each spawn with its transgenic and non-transgenic full siblings was kept in a separate experimental unit. Full-sib transgenic and non-transgenic fish were the same age, and were kept and evaluated in the same communal environment from the moment of fertilization. The common carp fry were fed with commercial fish feeds; first with salmon starter (50% protein) and then with catfish feed (30% protein).

Data analysis

Full-sibling transgenic and control common carp from each spawn were grown communally, one family per experiment unit. This design is advantageous since the transgenic and control individuals are the same age, exposed to the same environment from the moment of fertilization, and environmental effects and variation are minimized (Dunham et al., 1992). Individuals were weighed, genotype determined and mean body weights calculated. Mean body weight of transgenic and negative controls was compared with Students't-test. The percent increase in body weight of all transgenic families was also compared to controls using Students t-test. Observed and expected progeny ratios from matings of transgenic individuals, percentage of deformities in transgenic and control individuals, integration rates and survival traits were compared using Chi-square.

Results

Gene transfer

One of the goals of the project was to improve gene transfer procedures

for commercial fish species. This was done by evaluating gene transfer by microinjection and electroporation at different developmental stages in carp and catfish (Hayat et al., 1991; Powers et al., 1992). Survival (hatching rate) of microinjected common carp embryos at the early, intermediate, and late one-cell, two-cell and four-cell stages was 88.9, 85.3, 91.5, 85.3, and 83.4% of the control. Hatching rate of the uninjected controls was 30.8%. No difference ($P>0.05$) in survival was observed among treatments or controls. The correlation between cell stage and survival was -0.52.

Channel catfish embryos were more sensitive to microinjection than common carp embryos. Survival of microinjected channel catfish embryos at the early, intermediate and late one-cell, two-cell and four-cell stages was 65.0, 43.6, 38.5, 27.4 and 35.9% of the control. Hatching rate of the control was 12.2%. Survival of the control was higher ($P<0.01$) than survival of all treatments. Survival of embryos microinjected early in the one-cell stage was higher ($P<0.01$) than survival at all other stages of development. The correlation between cell stage and survival was -0.81 ($P<0.05$). Relative survival of microinjected common carp embryos was twice that of channel catfish. Relative survival of microinjected rainbow trout (Chourrout et al., 1986) and goldfish (Zhu et al., 1985) were also high compared to channel catfish in this and previous experiments (Dunham et al., 1987). Rainbow trout, common carp and goldfish do not give parental care to their eggs and fry. Channel catfish males incubate and guard their eggs and fry until they reach swim-up. Channel catfish eggs may be less adapted to environmental fluctuations and manipulations than rainbow trout, common carp and goldfish because of the increased parental care. However, Brem et al. (1988) recently reported high survival of microinjected *Oreochromis niloticus* embryos, a

species with much parental care. Apparently, the degree of parental care is not the only variable affecting the inherent ability of microinjected embryos to survive.

Since all cell-stages evaluated for common carp had similar survival, the developmental stage that results in the highest integration rate will be the most efficient time for microinjection of common carp embryos. The integration rates for the early, intermediate, late one-cell, two-cell and four-cell stages were 8.3, 1.0, 0.0, 11.5, and 1.7% respectively, for common carp. Southern blotting confirmed that the foreign DNA was integrated for both common carp and channel catfish (Hayat, 1989).

Apparently, common carp have two peaks, early one-cell, and two-cell for developmental stage most likely to yield transgenic individuals. The observed integration rate, 11.5% and number of transgenic embryos produced per 100 injected 9.8, is slightly higher for the two-cell than early one-cell stage, 8.3% and 7.4%, respectively. Intermediate, late one-cell and four-cell stages yielded 0.9, 0.0 and 1.4 transgenic individuals per 100 injected.

The pattern of survival exhibited by the channel catfish embryos was different from common carp. The early one-cell stage had the highest survival. However, the pattern of integration rate among developmental stages for the channel catfish was also different from common carp. One peak of integration from the late one-cell through the two-cell stage was observed, and the period of highest survival did not correspond to the period of highest integration rate. The integration rates for the early, intermediate, late one-cell, two-cell and four-cell stages were 2.1, 1.5, 15.2, 12.5 and 7.7% respectively.

The late one-cell stage of channel catfish and the highest observed

integration rate, 15.2% and yielded more transgenic embryos per 100 injected 5.9, than the two-cell and four-cell stages, 3.4 and 2.8. Early and intermediate one-cell stages yielded 1.4 and 0.1 transgenic individuals per 100 injected.

Survival patterns of microinjected channel catfish and common carp embryos differed. Common carp had similar survival rates at all five developmental stages injected, but channel catfish had highest survival when injected at the early one-cell stage. The pattern of integration rates among developmental stages was different between channel catfish and common carp. Both species had a peak near the two-cell stage; that for the channel catfish actually highest at late one-cell and extending into the two cell stage. Common carp had a second peak of integration at early one-cell stage. For most efficient and maximum production of transgenic individuals, channel catfish should be microinjected during late one-cell and common carp during the two-cell stage. If injection during these developmental stages increases mosaicism and absence of foreign DNA in the germ line, it could negate the advantage of maximum production of transgenic individuals. However, mosaic individuals and individuals injected at the two-cell stage often have the foreign genes present in their germ-line (Hayat 1989).

By utilizing electroporation at specific developmental stages, we have vastly improved integration rates to 50-100% for manipulated carp and catfish embryos (Powers et al., 1992). Large numbers of transgenic individuals can be generated in this manner, decreasing the need for screening of constructs in tissue culture. The Israeli team has also developed improved procedures for several species including sea bream. We have transferred gene constructs into tilapia (Indiq and Moav, 1988), walleye and northern pike (Moav et al., 1992),

goldfish (Moav et al., submitted), zebrafish (Liu et al., 1990b), and *S. aurata* (Funkenstein et al., 1990; Cavari et al., 1991).

To identify the experimental parameters at which transfection is greatest, voltage, number of pulses per cycle, and timing of treatment were varied in the absence of DNA, and percent survival at hatching was noted. The optimal parameters were then used for subsequent transfections experiments with the plasmid constructs.

Table 1 is a comparison of the electroporation and microinjection procedures. It is clear that many more eggs can be treated per unit time with electroporation than with the tedious microinjection procedure. In fact, the electroporation procedure is as much as two orders of magnitude more efficient in the rate of transfection than microinjection (see Table 1). The survival rates were similar in both procedures, which is consistent with the findings of Inoue and colleagues (1990). The presence of the Rous sarcoma virus long-terminal repeat (RSVLTR)-rainbow trout growth hormone 1 (rtGH1) complementary DNA (cDNA) or RSVLTR-rtGH2 cDNA sequence in the presumptive transgenic fish was first determined using polymerase chain reaction (PCR) amplification of the RSVLTR-rtGH cDNA construct from genomic DNA isolated from the fin clips of a 3-month-old fish. Southern blot analysis indicated that the foreign gene was integrated into the animals high molecular weight DNA. PCR results were checked by Southern blot analysis of genomic DNA isolated from the fin clips randomly selected individual fish at 5 months of age. In a double-blind study, the 15 individuals analyzed to date by both PCR and Southern analysis have yield identical results. This double-blind test included both positive and negative individuals.

In zebrafish electroporation resulted in significantly greater numbers

of transgenic fish than micro-injection. Variations in the survival rate and fraction of fish that were transgenic was much more dependent on the skill of the operator when the microinjection technique was employed than when the electroporation method was used. In fact, once the machine's parameters have been optimized for a given species, the electroporation method is very reproducible from operator to operator and consistent results can be obtained. For transient expression studies during development, electroporation can usually yield positive results for 60 to 90% of the fish embryos. Because these fish grow for several months, however, the fraction of adult fish that retain the introduced gene drops significantly.

Table 2 illustrates the hatching rate of fish after treatment by one of these electroporation procedures described in the Materials and Methods section. At all stages of electroporation, the hatching rate is greater for the control groups (electroporation in the absence of DNA) than any of the experimental groups (see Table 2). Table 2 also indicates that electroporation, with or without DNA, of the carp embryos at the early postfertilization stage yielded better ($p < 0.001$) hatch than either the prefertilization or the first cell division stages. In contrast, electroporation, with or without DNA, of the catfish embryos at the first cell division stage resulted in lower ($p < 0.001$) mortalities than treatment at the early postfertilization or prefertilization stages.

Table 3 illustrates the fraction of fish treated by one of the three electroporation procedures that still carry the foreign gene 3 to 5 months after electroporation. Eventually, all the fish identified in Table 2 will be assayed for the presence of foreign DNA; however, on the basis of the results illustrated in Table 3, the prefertilization procedure yielded a higher ($p <$

0.025) percentage of transgenic individuals for the common carp (58.1%) than the early post-fertilization treated group. Five out of the six tested in the first cell division group were positive. There was no difference among in treatments for the catfish, but the sample size was small. It is encouraging that approximately half the 16 channel catfish assayed for the first cell division group were transgenic, and all the channel catfish analyzed for the earlier treatment stages were transgenic (65% of all catfish analyzed were positive). The highest integration rates for channel catfish achieved with microinjection were 33%, with a typical value of 5% (Dunham et al., 1987; Hayat, 1989; Powers et al., 1990; Hayat et al., 1991).

Use of electroporation for generation of transgenic fish has many advantages over microinjection. Electroporation is much faster and less demanding technically than microinjection. One to two orders of magnitude more eggs can be processed per unit time with electroporation than with microinjection. The electroporation procedure is less dependent on the skill of the operator than microinjection; therefore, results are more reproducible from operator to operator and laboratory to laboratory. Moreover, the survival of embryos is not significantly different between the electroporation and microinjection procedures. Even if the mortality were greater, however, the significantly enhanced ability to handle more eggs with electroporation than microinjection would outweigh even a substantial increase in mortality.

Survival and integration rates for common carp and channel catfish are different when utilizing either microinjection or electroporation for gene transfer. When using microinjection, survival rate was equal among common carp embryos microinjected at different developmental stages (Hayat et al., 1991); however, survival was best ($p < 0.001$) for common carp when

electroporated early after fertilization. Survival of channel catfish microinjected early after fertilization was superior to later treatments (Hayat et al., 1991), but electroporation at first cell division resulted in greater ($p < 0.001$) survival than at early postfertilization.

Integration rates were much higher when electroporation was used for gene transfer in common carp and channel catfish compared with microinjection. Mean and maximum integration rates were 41 and 100%, respectively, for electroporated common carp embryos, compared with 5 and 16% respectively, for microinjected individuals. Mean and maximum integration rates were 65 and 100%, respectively, for electroporated channel catfish embryos, and 5 and 33%, respectively, for microinjected individuals.

Although we have not yet analyzed all fish, we cautiously conclude that the electroporation procedure appears to be a very promising means for generating large numbers of transgenic fish in a modest amount of time. We have shown that genes can be transferred into three species of fish; a large fraction of the treated fish retain those foreign genes for 3 to 5 months after hatching, and the foreign DNA has been integrated into their genomes. The stability of that integration, however, must be established by appropriate transmission genetics studies. Until inheritance studies have been done, we shall remain cautiously optimistic about electroporation as a means of mass treatment of fish gametes for production of transgenic fish. In the unlikely event that the genes are not transmitted to progeny, this electroporation procedure can still be used to study transient gene expression and to assay specific genes and their promoters.

Integration

Our laboratories have transferred rainbow trout GH1 cDNA and GH2 cDNA

(Agellon and Chen, 1986; Agellon et al., 1988) to the channel catfish and to common carp (Zhang et al., 1990). The rainbow trout GH cDNAs are fused to the long terminal repeat (LTR promoter) of RSV. In these studies, 1-10 copies of the foreign GH genes were integrated in either head-to-tail tandem arrays (Dunham et al., 1992), or single copy integrations at multiple sites (Powers et al., 1990). Junction fragments differed in size (Zhang et al., 1990) suggesting different integration sites in common carp. However, when several transgenic common carp (Zhang et al., 1990) and channel catfish (Dunham et al., 1992) were examined, many individuals appeared to have preferred integration sites.

The trout metallothionein *S. aurata* GH cDNA (ptMTa-gsbGHcDNA) construct was found to be integrated in 4 out of 17 fish tested when microinjected into fertilized carp eggs (Cavarai et al., 1991). Donaldson (personal communication) has shown that this metallothionein promoter shows strong activity even without any addition of heavy metals. In another experiment, various β -actin chinook salmon GH constructs were microinjected into carp. By using PCR, integration of the injected gene was found in 4 out of the 70 fish tested.

Integration of CAT gene into adult common carp was studied using specific oligodeoxynucleotides and the polymerase chain reaction (PCR) methodology (Figs. 1, 2). Nine of the 94 fish tested were PCR positive for the presence of the CAT gene either in blood DNA or in fin DNA. There is some indication that the positive fish found are all mosaics in nature; no CAT positives were found in sperm DNA of this fish group.

When an identical β -actin proximal promoter (Figs. 1, 2) was fused to CSGH cDNA containing a GH 3' polyadenylation site, and microinjected into carp

zygotes, three adult carp of the 77 tested showed a 300 bp band expected on the PCR test.

Using the proximal and the intron β -actin promoters fused to CSGH cDNA gene (containing a GH 3' polyadenylation site) (Fig. 1), we observed 12 adult carp of the 58 tested fish to show either the expected 1600 bp PCR band or the 490 bp CSGH internal PCR band. All the PCR GH positive DNAs were obtained from blood fins.

Expression

Constructs of β -actin fused to CAT reporter gene, and β -actin fused to chinook salmon GH gene, were microinjected into fertilized eggs of carp. High CAT activity was found in both cell lines and in the transgenic carp developed at the age of 12 months (Liu et al, 1990; Moav et al., 1992).

β -actin constructs were tested for their function by transfection into mouse L-cells (Liu 1990b and Moav et al., 1992). (Fig. 3).

The dissection of the beta-actin regulatory sequences by the various constructs and their activity in mouse cells indicated the presence of a positive transcription control element in the first intron that is orientation dependent. In addition, a negative control element was found in the upstream sequences. Two other negative control elements are found in the intron and in the 6th exon (see the scheme on Fig. 3).

The various beta-actin constructs (Fig. 3) were assayed for expression by transfection in fish EPC cells, a carp epithelial cell line. Results from transfection experiments are summarized in Table 4.

As in L cells (Liu and Moav et al., 1990b), expression in carp cells *in vitro* were stimulated by the presence of the intron (e.g. construct 6, Fig. 3) and inactivated when the intron enhancer was in an inverted orientation

(construct 9). The proximal promoter alone in EPC cells was inactive and needed a larger section of the 5' region for its activity (constructs 1, and 20).

Although preselection of constructs by expression in tissue culture is a necessary step for building an efficient fish expression vector, the final test is the fish species where transgenic expression is desired (Moav et al., 1992; Moav et al., submitted). We, therefore, pretested some of the constructs for potential high levels, medium levels and low levels of promoter/enhancer activities *in vivo* in the carp.

Initial results (constructs 6, 8, 9) indicated that construct 8, that was active in carp tissue culture cells, is also highly active in early carp development (Figs. 4, 5). While construct 4 is moderately active, construct 9 and 6 have low activity. These results were used for constructing fish vectors containing identical beta-actin regulatory units but instead of CAT they have chinook salmon growth hormone cDNA (CSGH).

The expression in early development represents initially transient expression due to the presence of thousands of copies of free introduced plasmids. Later on, expression is relatively lower per individual, and only where integration occurs does CAT specific activity remain high.

We have found a tendency for higher levels of CAT expression in fins of adult positive fish (Fig. 6), indicating either a higher rate of integration to precursors of fin cells in the embryo, or a higher rate of expression (transcription or translation) in fin cells, or both.

The ptMTa-gsbGHcDNA construct was tested in carp cell line and expression was detected by Northern blot analysis when induced with zinc ions (Cavari et al., 1992).

Previous studies by transient expression of a reporter gene indicated that the carp epithelial cell line (EPC) is highly suitable for efficient transfection and optimal expression directed by the two fish MT promoters in our conditions. Therefore, this cell line was chosen for this study. There is a band of ca. 1 kb as analyzed on Northern blots which corresponds to the gsbGH mRNA. This band was not visible in the RNA samples from untreated cells. In addition to this, two unexpected bands, for both tMTa- and tMTb-gsbGH, were detected. These bands are approximately 8 and 12 kb, and there is a positive correlation between the occurrence/intensity and Zn-treatment. Moreover, these "extra" bands were much stronger than the "authentic" one. It is possible that the bands resulted from the two or three rounds of run-off transcription of the circular plasmid DNA. Because of the correlation of their appearance with Zn and their absence in the mock-transfected sample (a plasmid containing only the GH gene without any promoter sequence), nonspecific hybridization or DNA contamination is excluded. The authentic band was not clearly visible when the filters were subjected only to low-(1x SSC-1% SDS for 1 h at 60°C) or moderate-stringency washing (0.2 x SSC-1% SDS for 1 h at 60°C), but after washing in 0.1 x SSC-1% SDS for 1 h at 65°C, the band expected from the correct transcription is clearly observed. These results may be indicative of an inefficiency of the transcriptional terminator of gsbGH plasmid.

With the ptMTa-/b-scGH, the extra bands associated with the gsbGH constructs did not occur. The scGH (2.5 kb) was efficiently transcribed and correctly processed, resulting in an RNA band of about 1.1 kb as expected. Again, a Zn-inducibility was observed.

Surprisingly, the carp β -actin promoter-containing construct of the

gsbGH did not give rise to any detectable signal.

Since the rtGH cDNA construct used in our initial gene transfer studies does not contain the signal peptide sequence (Zhang et al., 1990), we did not detect rainbow trout GH polypeptide in the circulation (serum) of the P_1 (produced by microinjection) transgenic carp carrying pRSVrtGH1cDNA sequence. Instead, the expression of rainbow trout polypeptide was observed in the red blood cells of the transgenic individuals (Zhang et al., 1990). The levels of trout GH polypeptide detected in the red blood cells of transgenic fish ranged from 8.9-89 ng/mg of red blood cell protein.

Our expression data for catfish parallel that for carp. In all transgenic catfish and carp evaluated, rainbow trout GH and mRNA was detected, and rainbow trout GH was found in the red blood cells of P_1 catfish.

The production of rtGH in transgenic fish was determined by the radio-immunobinding assay described previously (Zhang et al., 1990). All P_1 (Zhang et al., 1990) and F_1 transgenic common carp sampled produced rtGH, and the levels of rtGH varied about 10 fold in the F_1 generation as well. In F_1 transgenic fish, different levels of rtGH were detected in the red blood cells, liver, eyes, gonads, intestine and muscle. To confirm these results, the presence of rtGH mRNA was determined by the methods of reverse transcription/PCR (RT/PCR) amplification and RNA dot blot hybridization assay. Different levels of rtGH and mRNA were detected in liver, eyes, gonads, intestine and muscle of F_1 transgenic individuals. Normally, GH is produced in the somatotrophs of the anterior pituitary gland, secreted into the circulation and delivered to the target tissues. Apparently, the constitutive promoter, RSVLTR, allows production of the salmonid GH where it is not normally found.

RSVLTR also allowed expression of rtGH or coho salmon GH in the P_1 transgenic channel catfish (Dunham et al., 1992). This constitutive promoter has also been shown to drive the expression of transgenes in northern pike, *Esox lucius* (Schneider et al., 1989), goldfish, *Carassius auratus* (Yoon et al., 1990) and appears to be more consistent in driving the expression of transgenes than the mouse metallothionein gene promoter (McEvoy et al., 1988; Rokkones et al., 1989; Chourrout et al., 1986) and SV40 (Guyomard et al., 1988; Indiq and Moav, 1988) in fish.

Reproduction and inheritance

Both transgenic male and female P_1 common carp were fertile, and their relative reproductive potential compared to controls was not discernably different. Although some transgenic F_1 males were sexually mature at 12 months of age, the percentage of precocious transgenic males did not appear high compared to control fingerlings. Several studies reported to date have demonstrated that transgenic fish are fertile. These include several fish species, such as common carp (Zhang et al., 1990), channel catfish (Dunham et al., 1992), medaka (Lu et al., 1992), zebrafish (Stuart et al., 1989, 1991), Atlantic salmon (Shears et al., 1991), and rainbow trout (Guyomard et al., 1988) with a variety of foreign gene constructs. Dunham et al. (1992) demonstrated that channel catfish containing RSVLTR-rtGH1 cDNA or RSVLTR-csGH cDNA possessed normal reproductive ability under artificial conditions, however no studies have been conducted to determine their reproductive ability under more natural conditions.

To study the pattern of transgene inheritance in subsequent generations, one non-transgenic control female x four P_1 (parental transgenic common carp produced by microinjection), four P_1 x P_1 and one P_1 x F_1 mating of transgenic

individuals containing RSVLTR-rtGH1 cDNA were made and the DNA samples isolated from the fin tissue of progeny were analyzed for the presence of RSVLTR-rtGH1 cDNA by PCR amplification and southern blot analysis of the amplified products. The percentages of transgenic progeny resulting from these 9 matings were: 0, 32, 42, 100 (4 progeny only), 25, 17, 31, 30 and 23% respectively (Table 5). If each of the transgenic parent in these 9 matings carries 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 $\times P_1$, 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 indicated that though most of these P_1 transgenic fish had RSVLTR-rt GH cDNA in their germ-line, they might be mosaics, and at least one P_1 transgenic individual did not have the foreign gene in the gonadal tissue.

The progeny ratios observed in the four $P_1 \times P_1$ matings in this study, 21 to 31%, were consistent with at least two scenarios: (i) a mating of a mosaic individual with the RSVLTR-rtGH cDNA in 1/4 of his or her gametes with an individual without the RSVLTR-rtGH1 cDNA in the germ-line, or (ii) a mating between two mosaic individuals with RSVLTR-rtGH1 cDNA in 1/8 of their gametes. However, in the case of the cross $P_1 \times F_1$ the expected progeny should have been at least 50% rather than the observed 23%. Differential mortality of transgenic gametes or progeny may account for these results.

The majority of P_1 transgenic fish produced by microinjection to date have been shown to be mosaics (Ozato et al., 1986; Dunham et al., 1987; Stuart et al., 1989; Zhang et al., 1990; Shears et al., 1991; Dunham et al., 1992). Studies conducted by Stuart et al. (1989) indicated that DNA microinjected

into zebrafish embryos replicated autonomously for many cell divisions prior to its integration into the host genome. After many cell divisions, the foreign DNA in some cells is integrated while in others it is degraded, and thus may result in mosaics even for embryos injected with foreign DNA in the one-cell stage.

The F_1 transgenic zebrafish (Stuart et al., 1989; 1991) and Atlantic salmon (Shears et al., 1991) have been shown to contain the foreign gene construct in all cells, and to transmit the foreign gene in expected Mendelian ratios. However, one F_1 transgenic carp in the current study transmitted RSVLTR-rtGHi cDNA at 1/3 of the expected ratio indicating differential mortality of the transgenic gametes or embryos.

Growth performance of F_1 transgenic common carp

The growth performance of F_1 transgenic carp was evaluated in seven families. In these experiments, transgenic and non-transgenic full-siblings were spawned, hatched and reared communally, and were always in 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 20.8, 40.1, -26.6, 58.5, 21.5, -14.5 and -1.5% increase in growth (Table 6). 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, one of the F_1 transgenics was the largest fish in the family. In the current studies, the response of the transgenic fish to the insertion of the RSVLTR-rtGH1 cDNA appears to be variable. This may be a result of overall genotype, the site of the foreign insertion, copy number of the foreign gene and levels of the expression. Therefore, 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.

The coefficient of variation for body weight was smaller for F_1 transgenic fish than their non-transgenic full-siblings in families where the mean body weight of the transgenic individuals was larger than the controls (Table 7). These results could be accounted for by the following hypothesis. The production of the trout GH might cause the total growth hormone levels in the transgenic individuals to be more uniform and/or epistatically mask the genetic variation at other loci responsible for growth, and hence more transgenic fish will approach a maximum allowed growth rate. This would result in more uniform growth among individuals in a transgenic population, and this hypothesis should be evaluated. On the other hand, a larger size variation in the transgenic full-siblings than that of the non-transgenics was observed in families where the non-transgenics grew as fast or faster than their transgenic siblings (Table 7). If the production of rtGH was more variable in these families, some of the transgenic individuals may produce hyper-levels of rtGH which may reduce the growth rate of the fish as reported by Agellon et al. (1988). This portion of the transgenic population would be expected to be smaller, thereby decreasing the mean size of the population.

to test this hypothesis, matings involving two F_1 transgenics individuals from two different families that grow slower than their non-transgenic controls should be conducted. By evaluating the size variations, the genotype of the transgenic individuals, and levels of rtGH in the progeny, the hypothesis can be confirmed.

Overall the population variation was large for both transgenic and non-transgenic sub-populations in most families (Table 7). This type and quantity of variation in growth rate is typical for common carp. Several individuals, both transgenic and non-transgenic, were "jumpers", much larger than average fish in the population. The phenomenon of "jumpers" in a population usually occurs when individuals have slight environmental or genetic advantage and are able to magnify that advantage which could have significant effects on the results of transgenic research on fish species such as common carp. This also illustrates the importance of evaluating several families and large numbers of fish in transgenic fish experiments because natural variation in fish populations can be large.

Rates of deformities and survival traits of transgenic individuals

Although external deformities were observed in some families of common carp containing transgenic individuals, the percentages of deformities observed for transgenic and non-transgenic full-siblings were equal. Additionally, no tumors have been observed in transgenic or control common carp or catfish containing RSVLTR-rtGH1 cDNA or RSVLTR-csGH cDNA (Dunham et al., 1992). Transgenic common carp and their non-transgenic full-siblings were equally susceptible ($P < 0.05$) to short term exposure to lethal levels (200 ppm) of $KMnO_4$, showing 58 and 78% mortality, respectively.

Transgenic family 7 from a mating between transgenic P_1 and F_1 had a

large number of both transgenic and non-transgenic individuals exhibiting the deformities of incomplete operculum or diphead (cranial area posterior and dorsal to the eyes was more concave than normal which also made the fish appear to have an elongated snout). Although the body weight variation was high, the observed means of deformed transgenic individuals were lower than those for deformed non-transgenic full-siblings (Table 7).

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Table 1. Comparison of eggs treated per minute, percent survival, and percent transgenic individuals for embryos zebrafish (*Brachydanio rerio*), common carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*) when microinjected or electroporated with foreign DNA.

Treatment	Number of Eggs	Percent	Percent
	Treatment per Minute	Survival	Transgenic
Electroporation			
Zebrafish	200	72	35-75
Common carp	100	45	0-100
Channel catfish	35	5	50-100
Microinjection			
Zebrafish	3	89	
Common carp	1	45	0-16
Channel catfish	1	5	0-33

Table 2. Hatching percentage for common carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*) from three electroporation treatments.

Stage of Development Electroporation	Common Carp ^a	Channel Catfish ^b
Prefertilization		
Experimental group	5.8% (300)	2.5% (240)
Control group	15.6% (45)	13.5% (15)
Early postfertilization		
Experimental group	24.5% (1300)	1.5% (135)
Control group	31.7% (270)	11.1% (45)
First cell division		
Experimental group	1.0% (600)	11.1% (90)
Control group	11.1% (90)	26.7% (15)

^a Mean hatch difference among common carp treatments ($p < 0.001$, χ^2).

^b Mean hatch difference among channel catfish treatments ($p < 0.001$, χ^2).

Table 3. Percent transgenic fish from three electroporation treatments for common carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*).

Stage of Development at Electroporation	Common Carp ^a (N)	Channel Catfish ^b (N)
Prefertilization		
Experimental group	58.1% (62)	100% (6)
Control group	0% (10)	0% (10)
Early postfertilization		
Experimental group	33.8% (133)	50% (10)
Control group	0% (10)	0% (10)
First cell division		
Experimental group	83% (6)	50% (16)
Control group	0% (10)	

^a Mean percent transgenic different between prefertilization and early postfertilization for common carp ($p < 0.025$, χ^2).

Table 4. Function of carp beta-actin regulatory sequences in carp epithelial cell line.

Construct	Relative CAT expression	Construct	Relative CAT expression
1	100	13	15
2	35	14	6
3	33	15	2
4	4	16	180
5	3	17	13
6	320	18	340
7	16	19	4
8	230	20	180
9	3	21	20
10	5	22	30
11	3	23	10
12	110	24	10

Table 5. Percent of F_1 progeny inheriting RSVLTR-rtGH1 cDNA sequence.

Family	Mating	N	Observed % inheritance	Expected % inheritance ^a
1	$P_1 \times \text{Control}$	17	0	50
2	$P_1 \times \text{Control}$	96	32	50
3	$P_1 \times \text{Control}$	26	26	50
4	$P_1 \times \text{Control}$	4	100 ^c	50
5	$P_1 \times P_1$	28	25	75
6	$P_1 \times P_1$	99	17	75
7	$P_1 \times P_1$	312	31	75
8	$P_1 \times P_1$	93	30	75
9	$P_1 \times F_1$	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 the expected, and ^c which is greater ($P < 0.05$) than expected, Chi-square.

Table 6. Mean (g), standard deviation (SD), coefficient of variation and percent difference in body weight of transgenic common carp, Cyprinus carpio, and their non-transgenic full-siblings.

Family	Mating	Genotype	N	Mean body weight (SD)	Coefficient of variation	% Difference	Range Body weight
1	P ₁ X Control	T	31	120.6 (17.4)	14.4	20.8	95-171
	P ₁ X Control	NT	65	99.3 (14.7)	14.8		65-124
2	P ₁ X Control	T	11	206.0 (45.2)	21.9	40.1	115-283
		NT	15	147.0 (48.0)	32.6		67-228
3	P ₁ X 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 X 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 X 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 X 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 X P ₁	T	15	72.2 (58.0)	80.3	- 1.5	7.1-214.4
		NT	48	73.3 (47.0)	64.5		8.7-203.3

T, transgenic; NT non transgenic; N, number of animals; SD standard deviation.

Table 7. Mean body weight (g) and standard deviation for transgenic common carp, *Cyprinus carpio* containing RSVLTR-rtGH1 cDNA and their non transgenic siblings both exhibiting deformities (incomplete operculum or diphead).

Deformity ^a	Genotype	N	Mean body weight (SD)
incomplete operculum	Transgenic	4	20.0 (8.1)
	Non-transgenic	15	35.2 (15.0)
Diphead	Transgenic	3	133.7 (57.6)
	Non-transgenic	6	198.6 (221.8)

^a Incomplete operculum - The anterior half of the operculum was present.

Diphead - the cranial area posterior and dorsal to the eyes was more concave than normal. This also made the fish appear to have an elongated snout.

Fig. 1: Constructs used for microinjection

- Oligo 1: 5' - ATCACTGGATATACCACCGTTG - 3'
- Oligo 2: 5' - CCTGCCACTCATCGCAGTACTG - 3'
- Oligo 3: 5' - GCGTCTCAGCCTCACTTTGAG - 3'
- Oligo 4: 5' - CACTCGGGTCAGCTGTTGCTG - 3'
- Oligo 5: 5' - GTCTCTGCTGAGTGCCACACC - 3'
- 5' - flanking sequence
- exon
- intron
- poly A of SV40
- poly A of Chinook salmon

Fig. 2: PCR screening for transgenic carp:

- (a) Screening for CAT integration using oligo 1 and oligo 2.
Lane 1 - Mw marker; lanes 2,9 - positive control;
lanes, 5,8,12 - positive fish.
- (b) Screening for GH/β-actin integration using oligo 3 and
oligo 4. Lane 5 - positive control; lanes 2,3 - positive fish.

Fig. 3.

CONSTRUCT 4

CONSTRUCT 6

CONSTRUCT 7

Fig. 4

CONSTRUCT 8

CONSTRUCT 9

pH3NCOCATGH3'

Fig. 5

Fig. 6: Tissue distribution of CAT activity in transgenic carp

f. Description of the Cooperation

The history of the fruitful cooperation and collaboration between the U.S. and Israeli fish genetics teams was initiated almost 25 years ago and has been ongoing since that time. In 1969, Dr. R. Moav, followed by Dr. G. Wohlfarth, visited Auburn University and assisted Dr. R. O'Neal Smitherman in establishing a catfish genetics research program and a graduate level course in fish genetics and breeding. Then in the late 1970's Dr. Smitherman, Dr. W. Shelton and R. Dunham of Auburn University collaborated with Dr. Wohlfarth and Dr. G. Hulata on selective breeding of farmed fish. Trips were made to each country, scientific ideas and techniques exchanged, experimental protocol exchanged, tilapia germplasm exchanged, and improved fish developed. This collaboration continued into the 1980's with similar joint research among the U.S., Israeli and Egyptian partners in a trilateral arrangement.

The U.S.-Israeli collaboration evolved in the late 1980's to include Dr. Dunham, Dr. T. Chen and Dr. D. Powers of the U.S. collaborating with Dr. Hulata, Dr. B. Cavari and Dr. B. Moaz jointly researching gene transfer in farmed fish on this BARD project. Again the cooperation included joint visits to both countries with the exchange in both directions, scientific ideas, data, technology and training in the cooperating laboratories, joint research planning, and the construction and exchange of different gene constructs.

g. Evaluation of Research Achievements

Our first objective was to improve the efficiency of gene transfer in fish. This objective was accomplished. Typically, gene integration was 1-5% in original gene transfer research for common carp. We increased integration rates to 50% or better by determining the optimum developmental stage for microinjection and by developing improved electroporation procedures for gene

transfer in fish. Since transfer of isolated genes is the first critical step for gene transfer, the improvement of gene transfer efficiency is of great benefit for gene transfer research and aquaculture of fish.

Our second objective was to determine if introduced genes were expressed in the experimental fish. The second objective was accomplished. Every transgenic fish that was evaluated expressed foreign (rainbow trout) growth hormone.

The third objective was to select suitable promoters for the expression of exogenous genes in fish. This objective was accomplished. RSV-LTR and carp β -actin both allowed expression of foreign genes in fish. A series of carp β -actin promoters were developed by making a series of modifications, deletions, insertions and rearrangements. These different forms of β -actin promoter had widely varying strength of expression. This is very important because different levels of expression may be optimum for different genes.

Our fourth objective was to demonstrate that introduced genes are genetically transmitted to F_1 transgenic carp. Not only did we demonstrate this, but also transmitted rtGH1 cDNA to F_2 generation carp.

Additionally, we demonstrated that most families of transgenic carp exhibit 10-60% growth improvement, and initiated the first government approved outdoor tests of transgenic fish (carp) in the world.

This research demonstrated more thoroughly than any previous research the feasibility of improving fish for aquaculture through gene transfer.

Additionally, we are very close to having beneficial transgenic fish ready for utilization in and improvement of aquaculture.

h. List of Publications

1. Chen, T. T., Lin, C. M., Zhu, Z., Gonzalez-Villasenor, L.I., Dunham, R. A. and Powers, D. A. (1990). Gene transfer, expression and inheritance of rainbow trout and human growth hormone genes in carp and loach. In: UCLA Symposium on Transgenic Models in Medicine and Agriculture. Los Angeles, CA, USA, Wiley-Liss Inc., pp. 127-139.
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