# Chromosomal Manipulation in Fish (Part III)

Isolation and construction of genes responsible for desirable traits and their transfer into the germ line of broad stock may produce a quantum leap over traditional breeding and selection methods for the production of fast growing fish. Many growth hormone (GH) cDNA and full length genes of mammalian, avian and piscine origin have been transferred into fish eggs to accelerate growth. However, expression of the transgene depends mainly on the right choice of regulatory elements. The expression of these heterologous genes was found unsatisfactory for one or other of the following reasons.

(a) Undesired tissue specific expression (b) side effects leading to impairment of other functions. Hence an attempt was made to construct a transformation vector with a unique promoter Zp (Zona pellucida gene) of piscine origin to drive maximum expression of the fish growth promoting genes, yellow porgy growth hormone (Yp GHc DNA) and rainbow trout growth hormone (rt GHcDNA).

## What is Transgenic Fish

A transgenic fish is one which carries one or more foreign genes. The foreign genes a selectively incorporated by microinjection into the egg with a view to produce transgenic fish lines carrying such foreign genes.

The progress was made in genetic engineering to isolate eukaryotic genes is 1970s and by 1980s. It was possible to microinject such genes into animal eggs to produce transgenic animals. The techniqu was

applied to fish much later. Some significant progress was made which had potential for application to fisheries. More than dozen transgenic fish were produced by 1989. Some examples include:-

1. Transgenic Atlantic Salmon to which antifreeze protein gene of polar flounders was transferred. This was done to promote resistance to low temperature of ice waters in the salmon so that the habitat of salmon may be extended to polar water.
2. Transgenic food fishes trout, tilapia, cat fishes etc. to which growth hormone gene of human, rat or other mammals was transferred with a view to enlarging production of such fishes.

Fish transgenics is comparatively difficult because of the tough egg chorian which impeded microinjection. A prior puncture or use of micropile (An opening in the egg surface for sperm entry during fertilization) or, local trypsin digestion has to be made for microinjection. If gene transfer by microinjection may be accomplished, integrations have been rarely achieved without which transgenics is not complete.

## Transgenic Technology

Transgenic technology involves an appropriate method of gene transfer. Popular methods hitherto used for gene transfer in fish are:

1. Micro injection
2. Incubation of sperm
3. Electroporation of eggs
4. Electroporation of sperm

Accordingly to Marian, electroporation is more promising method for gene transfer. It is possible to electroporate unfertilized eggs of just fertilized eggs, but the latter is shown to be ideal, as it result in higher frequency of transgenics and low percentage of deformities. Dunham and °mither Mas describe elctroporation is an alternate method in fishes like the silurids in which sperm can not be stripped. A second objective of the present study is to subject just fertilized eggs of Zera fish to electroporation with one of the two recombinant transformation vectors ZpBpGH and ZpBrtGH.

## Genes and Transgenic Fish

1. Zhu was the first to inject human insulin gene into cracian carp and loach eggs. Human growth hormone gene was the first gene that was successfully used to produce such transgenic animals.
2. The growth hormone genes of rainbow trout and salmon were

sequenced and became available after 1986. The success with mammalian hormone is only 50% expression. Therefore there is an urgent need for constructing transgenes of fish origin.

3. Bacterial gene transfer into fish has become a powerful tool to study gene expression in vivo and in vitro CAT B-gal (Chloramphenical acetyl transferase, B, Galacto sidase).
4. The fish transgenic technology is applied to increase growth rate of food fish, freeze resistance genetic regulation of the development process using Zebra fish and Medaka.
5. In Asia, disease resistant and drought resistant transgenic fish production is the prime need.

TABLE 11.1 : ADVANTAGES AND LIMITATIONS OF USING FISH EGG AS A MODEL FOR MANIPULATION

| Advantages | Disadvantages |
|---|---|
| Fertilization external, artificial, stripping of eggs and milt possible, maturation of gametes artificially inducible. Egg are large, numerous and easily maintained after fertilization; provide maximum scope for expression of variation. Faster embryonic development lian pronucleus. | In many species, Zygote nucles of eggs are very small and not visible hence, nucleoplasmic microinjection into oocyte nucleus of oviparous and viviparous is possible only in some species. The oocyte nucleus is over 1000 times larger than the mammals. |
| Fish eggs are amenable for ploidy induction, Hence possible to generate hundred of clones from a single parent. This effectively saves one generation in the selection of homozygous mutants | Egg coat (Chorion) is very tough and resistant. |
| Relatively higher totipotency of fish cells render the production of pure transgenic line a much easier task than in mammals. | |

## GENE MANIPULATION

For gene manipulation, physical and manual techniques a employed. Such technique requires, microneedles with less than 0.1 tip, micropipette and holding pipette, that too the ir_strumet is

equipped for correct microincision and microinjection with proper slide adjustment. Micro injection of Bovine and rat growth hormone gene in Tilapia egg has been successfully carried out. (Fig. 9.1) Tilapia egg is highly opaque and the chorion is very hard, hence it can not be pierced through even with very fine micro needle. Therefore, foreign DNA is injected into the germinal disc through the micropyle before the starting of cell division with a micro manipulater. Microinjection of growth hormone gene in Zebra fish egg has been done directly by injecting into the cytoplasm before the first cleavage. Chinese have also taken trials in nuclear transplantation in crucian carp. Considerable progress has been made in the field of cloning and processing of recombinant DNA in virus, bacteria, algae, insects, sea urchins, amphibians, mammals etc. In fishes only preliminary attempts are reported in case of gold fish, medaka, loach, tilapia, trout, salmon, Zebra fish etc. However, gene manipulation, will take upto new horizon of aquaculture in passage of time and research in gene investigations.

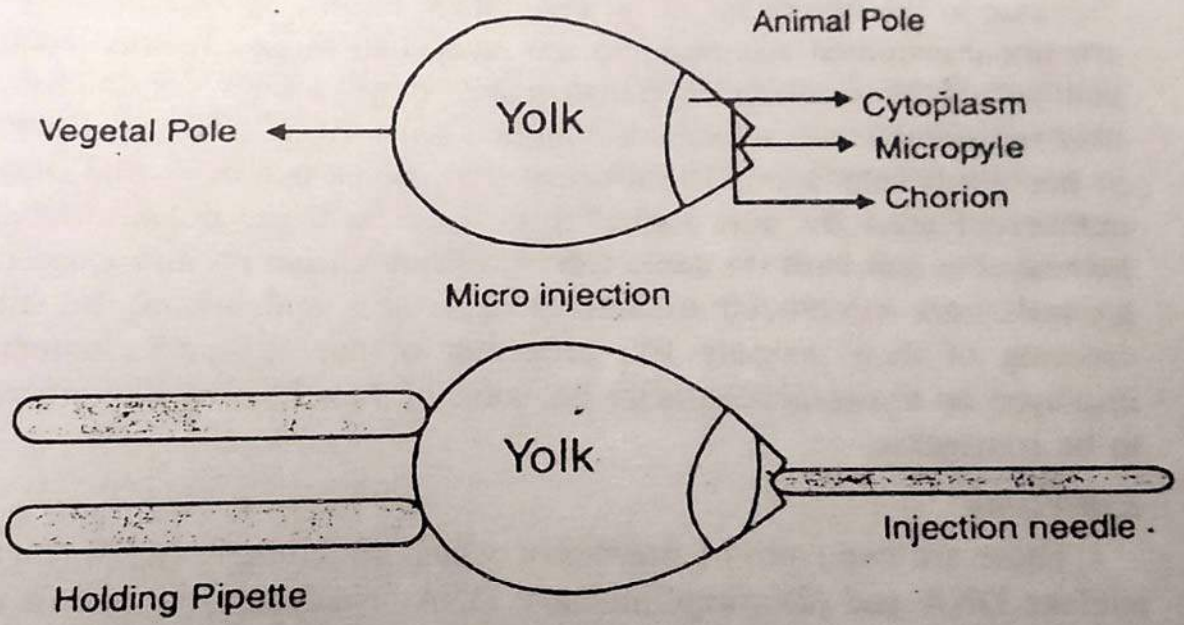


*Figure 11.1. Micromanipulatin in tilapia egg.*

Types of microinjection for fish

a. Human or rat gene for growth hormone.
b. Chicken-gene for delta-crystalline protein. c. E-coligene for B galactosidase. d. E-coligene for neomycine resistance.
e. Winter flounder gene for anti freeze protein. f. Rainbow trout gene for growth hormone.

## Method of Gene Transfer

In fishes common methods are electroporation, sperm mediated

transfer, high velocity micro projectile bombardment and micr"injection which are the most-prefered thod. Other methods adopted are drilling of chorion, manual removal enzymic digestion with trypsin injecting through micropyle. Some of the problems faced are digestion of the injected DNA by nuclear DNA and the rotation of the egg if perivitelline space is broad. In transgenic fishes mosaicism can be done by either the cytoplasmic introduction of the transgene or by the microinjection of egg even after the preblastoderm stage and in another method the inherent totipotency of fish eggs. The time available for microinjection is first 25 minutes and that too between fertilization and first cleavage. In forty minutes adequate cytoplasm is formed only after 15 minutes of fertilization.

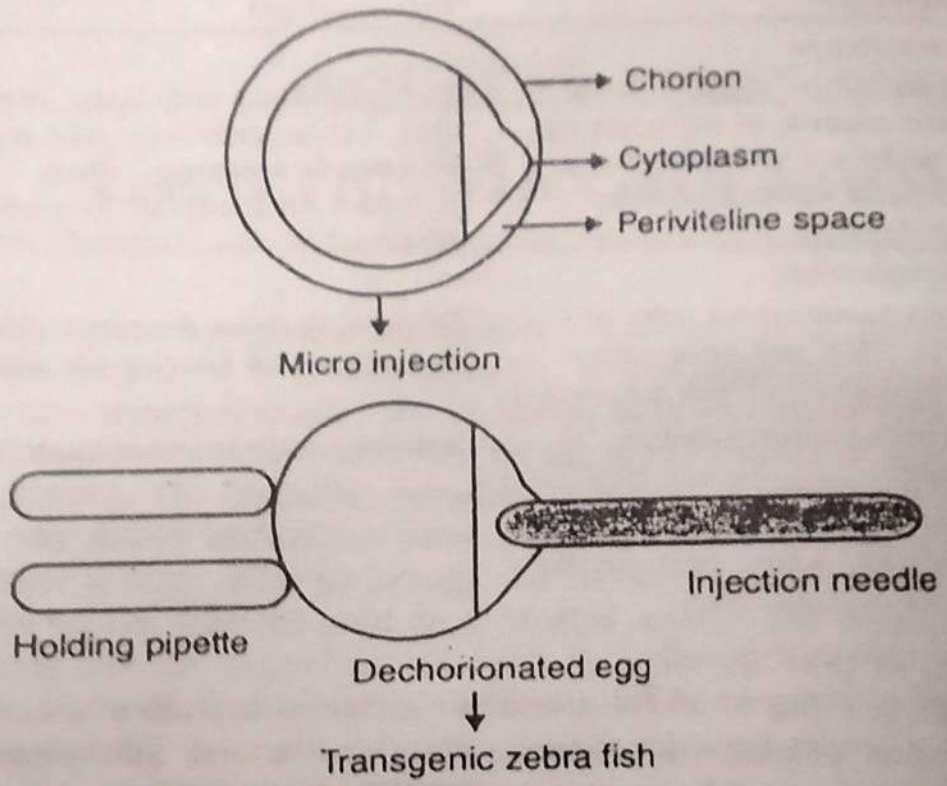


*Figure 11.2. Gene manipulation in zebra fish.*

The quantity of injected gene varies from human GH (Growth hormone) from 2.9 to 9.4 Kb. The largest gene introduced is chicken crystalline 14.5 kb. The volume of DNA solution is 0.2 to 20 ml. -10-15 ug/ml, which is toxic if more than 15 ug/ml is used. Number of the copies of the transgenes to the minium is 50,000. About 1-2 million copies are contained in 10-20 ml. with 10-15 ug/ml.

There are certain reasons for sensitivity of carps and cat fishes for micro injection which includes (1) Removal of chorion (2) diameter

of the needle used (3) The quantity of injected DNA (4) The quality of eggs (5) Time required for micro injection. Procedures are not fully time consuming but laborious too, at the same time it is specific and technically demanding. The production • of transgenic fish by this procedure is slow and serves a bottleneck to the explanation. Explanation and realization of the full potential of this powerful technology by this method the electroporation results in 760% efficiency, although the rate of survival is low. About one million copies are required for sperm mediated transfer. Many million sperms are required to ensure fertilization.

TABLE 11.2. ADVANTAGES AND LIMITATIONS OF USING DIFFERENT METHODS FOR GENE TRANSFER IN FISH TRANSGENES

| Advantages | Disadvantages |
|---|---|
| **Microinjection** | |
| Permits precise injection at the desired location of the target egg i.e. nuclei's. Cytoplasm permits quantitative estimation of the injected gene. | Time consuming techniques, requires skill, Limited embryonic time restricts injection to fewer eggs which introduce variations due to needle injury. |
| **Electroporation** | |
| Permits simultaneous entry of foreign DNA and brings about man production of fish transgenesis. | Difficult to assess the actual quantity of foreign gene entering the cells. |
| Does not requires technical expertise. | Sustains relatively higher mortality. |
| A more suitable method in some fish species, whose eggs are too small for microinjection. | |
| **Sperm mediated transfer** | |
| Absence of acrosome in fish sperm affords a greater scope for sperm | Its usefulness is claimed in birds but dispstued in fish and mammals. mediated gene transfer. |
| **High velocity micro projectile** | |
| Bombardment permits simultaneous entry of foreign DNA and thus brings about man production of fish transgenesis. | Difficult to assess the actual quantity of foreign gene entering the cells. |
| | Not used much because little is known about the mechanics of the process. |

*Integration*

As the injected DNA undergoes amplification there is degradation by DNA ase. Some extra chromosomal persistence of the transgene lead to mosaicism which is due to unequal distribution among daughter cells. There are large difference in the persisting copy number of the injected gene in tissue of transgenic trout. This is mainly due to delivery of the DNA later to two cell stage. But the integration frequency in <20% with fish genes and 740 with mammalian genes. However, the expression of the fish transgene is higher as compared to mammalian genes and 740 with mammalian genes. However, the expression of the fish transgene is higher as compared to mammalian gene. But the differences in the integration rate may be due to the tailoring of the selected gene, for hGH (human Growth Hormone) it ranged between 2.9 kb to 9.4 kb. The integration frequency was found to be 75% with 6.3% kb. and 20% with 2.9 kb.

*Transmission*

Due to the mosaic nature of integration, the transgenic characters are not transmitted according to the mendelian ratios. It was found that out of 20 presumptive transformants crossed with normal fish, only one female was transgenic mosaic. The foreign DNA contained in her germ cells was 20% at about 100 copies per cell. The copy number of hGH not only varied from tissue to tissue but also from individual to individual. In Zebra fish, F, offspring from positive founder, parents were crossbreed to untreated partners and subsequent out crossing of their progeny F1, progenies of the 4 founder parents displayed no transgenic character but some of their F, offspring proved to be transgenic.

*Expression*

These are two types of transgenes which are cloned (1) full length nuclear DNA and (2) complimentary DNA. Nuclear gene consist of coding sequence, promoter/enhancer sequence and natural spaces. There are three types of promoters/encliancers (1) enhancers from normal cellular genes active in most tissues (metallothionein-1: B-actin er! ncer-promoter complexes) (2) enhancers active only in specific tissues (3) Viral enhancers active in many tissues, especially those normally injected by the virus. These marker genes (Luciferase), presence of mRNA by northern blot or the protein by immunoblotting.

***Generalization on expression***

The viral promoters ensure more definitive expression than others

like metallothionein RtGH sequence comprising Rous sarcoma virus promoter ensured expression in carp, medaka and seabream. However MT or RSV transgenic fish are not fit for human consumption. The high level of expression is observed when all fish Chimeric GH gene construct is used. The presence of adequate number of copies of the injected gene in the desired tissue organs, determines the level of expression. The heterologous genes expression is unsatisfactory due to (a) undesired tissue specific expression (b) side effects leading to impairment of other functions.

## Application

Use of transgenics in selecting superior desired traits, also helped in production of super fish or super males and females. These forms owe their gaint size to the introduction and incorporation of such anabolic growth promoting genes as bovine growth hormone gene, human growth hormone gene. China has produced gaint loaches similar to gaint mice and gaint sheep produced by micro injection of human growth hormone gene.

This aspect has great fishery application from fish culture stand point. Somatic growth of farm fishes may be prolonged and made faster by introduction of bovine or human growth hormone gene.

The goal of transgenics is to produce an "ideal cultivated fish form" which may be envisaged as one having the desirable and superior traits. Such a dream fish "may be thought to combine the following traits with taste and food value.

1. Fast growth.
2. Fatty make up.
3. Greater longevity.
4. Relatively omnivorous feeding habit.
5. Higher fecundity.
6. Greater adaptability at developing stages.
7. More hardy.
8. More resistant to diseases, biocides, and pollutants.
9. Lack of fish bones and other undesirable features.