## PRODUCTION AND BREEDING OF TRANSGENIC SALMON

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Transgenic technologies have been developed in a wide variety of organisms, providing useful model systems for genetic studies and new genetic variation for practical applications that would otherwise be unavailable. The development of genetically modified plants for agricultural purposes is well under way, and the production of transgenic animals including fish for aquacultural purposes is also being pursued. Transgenesis offers great promise in aquaculture by providing new phenotypes and improving the efficiency of production. Some commercially important processes that are amenable to manipulation in transgenic fish for aquaculture are listed in Table 1.

Table 1. Biological processes and potential genes for use in transgenic fish for aquaculture.

| Process | Genes |
|---|---|
| Growth Enhancement | Growth Hormone<br>Insulin-like growth factors<br>Growth hormone receptor<br>Growth hormone release factor<br>Somatostatin (antisense)<br>Thyroid hormones (deiodinases) |
| Reproduction | Gonadotropin subunits<br>Gonadotropin release hormone<br>Steroidogenic enzymes and receptors<br>Protamine genes (ablation) |
| Health and Fitness | Stress genes (hsp)<br>Environmental tolerance (antifreeze genes)<br>Detoxification enzymes (P450IA1)<br>Viral Proteins<br>Nonspecific Immunity (ie. lysozyme)<br>Carbohydrate metabolism |

The production of transgenic fish has been attempted in a large number of species (Chen and Powers, 1990; Fletcher and Davies, 1991), including ornamental fish as well as species with potential for aquaculture. Early studies focused on the development of efficient methods for DNA delivery to fertilized eggs, a problem made difficult in many species due to the large size and opacity of their eggs. In salmonids, most transgenesis has been accomplished via microinjection of linear DNA into eggs shortly after fertilization. Although each species is unique in its requirements for microinjection, a variety of successful methods have been developed including predrilling access holes through the hardened chorion (Chourrout et al., 1986), needle insertion through the micropyle (Fletcher et al., 1988), or

injection into the germinal disc region of eggs immediately after transfer to fresh water (Penman et al., 1990) or in a solution of glutathione to inhibit hardening of the chorion (Yoshizaki et al., 1992). Studies examining electroporation as a gene transfer methodology have recently been described (see Powers et al., 1992). We have found that microinjection of eggs maintained in isotonic saline to prevent egg activation and chorion hardening provides an efficient method for producing transgenic Pacific salmonids. All of these methods are most likely delivering DNA through the vitelline membrane into the egg cytoplasm, and ultimately into the pro- or zygotic nuclei when their membranes have disassociated prior to the first cell division. Although the production of mosaic $P_1$ transgenic individuals implies that DNA insertion can occur after the first cell division, the exact mechanism remains unknown.

In salmonids, DNA retention has been demonstrated for several species. Several different gene constructs have been employed, including those containing antifreeze protein genes (Fletcher et al., 1988), alpha-globin genes (Yoshizaki et al., 1991), reporter genes (McEvoy et al., 1988; Yoshizaki et al., 1992), and, most commonly, growth hormone genes from mammalian (Chourrout et al., 1986; Guyomard et al., 1989; Rokkones et al., 1989; Penman et al., 1990 and 1991) or piscine (Du et al., 1992) sources. Many of these studies have shown the persistence of injected DNA sequences, although the frequency of DNA retention is dependent on the developmental stage of analysis after microinjection as well as the injection methodology and timing (Penman et al., 1990; Hyat et al., 1991). Some studies have shown by Southern blot analysis that DNA remaining late in development appears to be integrated into the host chromosomes (Fletcher et al., 1988; Guyomard et al., 1989; Penman et al., 1991). Using Southern, dot blot, or PCR analysis (Shears et al., 1991), the frequency of transgenesis in salmonids appears to be approximately 5%. We have developed a series of salmon transgenic expression vectors (Figure 2) with different promoters from sockeye salmon (Chan and Devlin, 1994). A sockeye salmon GH1 gene (Devlin, 1993) was inserted into the MT salmon vector and microinjected into fertilized salmonid eggs. Using this construct, and pOPAFPGHc (Du et al., 1992), we have found frequencies of DNA retention (determined by PCR) to be in this same range (4%) for coho salmon, and somewhat higher for rainbow trout. These frequencies are similar to those observed for transgenic mice and other non-salmonid fish (Breitman et al., 1987; Fletcher and Davies, 1991). Because the frequencies of gene retention are relatively low and the biochemical test methods are slow and expensive, development of fish expression vectors with dominant phenotypic marker systems would considerably ease the identification of transgenic fish from a population of treated individuals.

The form of the DNA insertion present in transgenic fish can be evaluated by Southern blot analysis. As with other vertebrate transgenics, the DNA construct can be present at more than one copy per insertion position, and are organized as concatemers (Dunham et al, 1987; Guyomard et al. 1989), in some cases in all possible head-tail configurations (Guyomard et al., 1989). This type of organization presumably reflects processes occurring to the DNA prior to insertion, including intramolecular ligations and rolling circle replication. The consequence of this variable insert structure is that individual transgenic $P_1$ fish will have different copy numbers and organizations for the introduced DNA construct at each unique chromosomal site of insertion.

Where analyzed, it appears that individual transgenic $P_1$ individuals can possess more than a single insertion site in their chromosomal DNA, and because multiple inserts can segregate to the same $F_1$ individual, these events must have occurred in the same embryonic cell. This result suggests that the insertion of DNA is stochastic, and that while some cells may receive multiple insertions, other cells may receive none. This random process leads to the production of mosaic $P_1$ transgenic individuals that may only be transgenic in a proportion of their cells, including their germline (Figure 3).

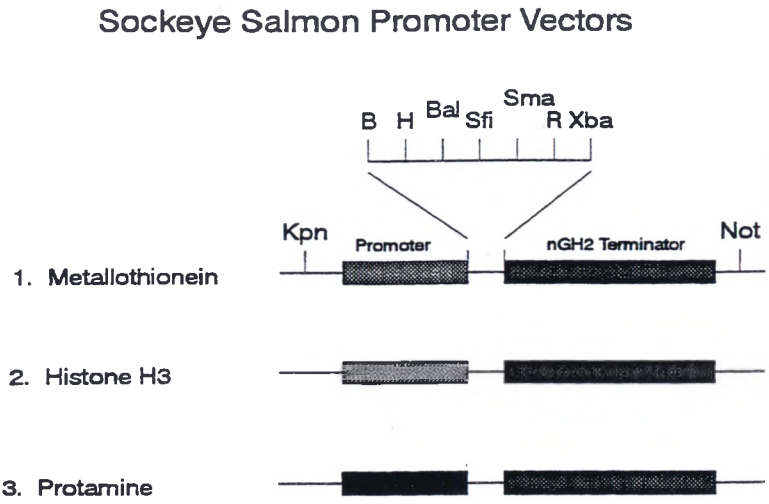


Figure 2. All-salmon transgenic fish expression vectors.

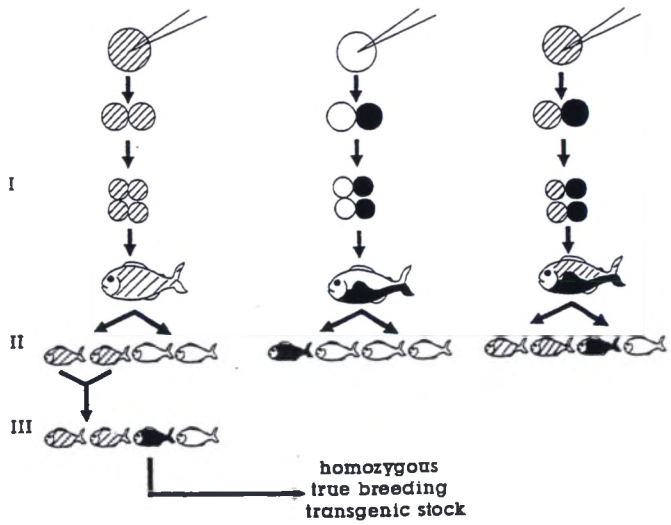


Figure 3. Non-mosaic (left), mosaic (middle), and multiple insertion (right) transgenic fish from integration events (shaded or filled cells) occurring before and after the first mitotic division.

In one of our studies, five transgenic male coho salmon sexually matured precociously at two years of age rather than the normal 3 years for this species. Of these five mature males, only four passed the introduced DNA through their germline to sperm cells. The fish which failed to transmit this new gene was a confirmed mosaic, with the gene present in blood but not fin tissue. Transmission of transgenic inserts in salmonids has also been observed in rainbow trout (Guyomard et al., 1989; Penman et al., 1991) and Atlantic salmon (Shears et al., 1991), however, mosaicism in testicular or ovarian germ cells can lead to transmission of an inserton to $F_1$ progeny at frequencies lower than the expected 50%. These results are similar to the frequencies of transmission to $F_1$ progeny that have been observed with other fish species (Stuart et al., 1988; Dunham et al., 1992; Lu et al., 1992; Chen et al., 1993). Consistent with these observations, coho salmon transgenic for growth hormone gene constructs can transmit the acquired DNA to their offspring, however in each case the frequencies were less than 50%, ranging between 2 and 20 %. These results again imply that mosaicism is common among the parental generation of transgenic fish. To avoid the difficulties associated with segregation of multiple insertions to $F_1$ progeny, it may be possible to generate strains of transgenic fish that are homozygous for the same insertion sites using late gynogenesis (suppression of the first mitotic division) or androgenesis (Figure 4). This methodology, coupled with sex control techniques, could shorten the generations required for producing true breeding strains.

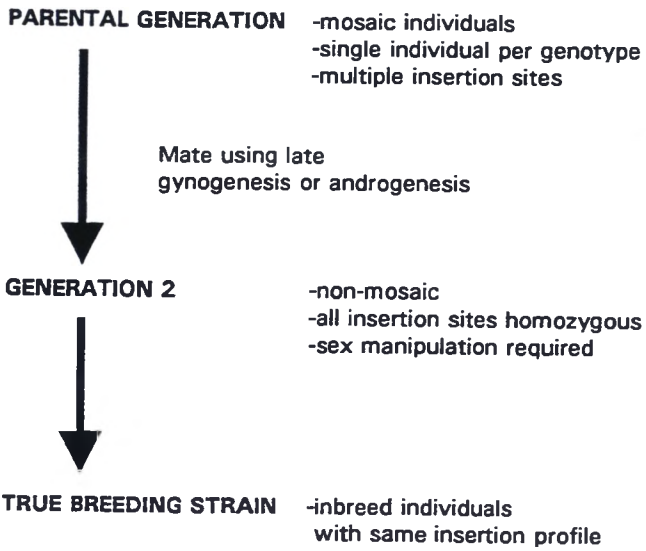


Figure 4. Incorporation of androgenesis or gynogenesis into transgenic fish breeding programs.

The expression of introduced DNA has been observed in several fish species (see Fletcher and Davies, 1991, and Moav et al., 1993 for reviews). In transgenic salmonids, expression has been observed in $P_1$ individuals using the Rous Sarcoma Virus (RSV) promoter driving the expression of reporter genes (Inoue et al., 1991; Yoshizaki et al., 1992) and with the mouse metallothionein promoter producing either beta-galactosidase or human growth hormone (McEvoy et al., 1988; Rokkones et al., 1989; but see Guyomard et al., 1989 for lack of expression with the same promoters), and in $P_1$ and $F_1$ individuals with the intact antifreeze gene from ocean pout (Shears et al., 1991). In most cases, it is difficult to estimate absolute levels of expression from the foreign DNA. In the case of Atlantic salmon transgenic for ocean pout antifreeze protein genes, the expression is considerably higher resulting in serum protein levels varying between 2 and 50 ug/ml. The level of expression of foreign DNA in injected individuals can be affected by a number of factors relating to a gene construct's structure, such as promoter type or the presence of introns. Expression can also be affected by copy number and organization within an insert, as well as the chromosomal domain in which the insert is situated. It should be noted that positive results obtained from $P_1$ samples may arise from transient expression and may not correlate with results obtained with the same promoter from older $P_1$ fish or $F_1$ individuals that contain only integrated copies of the introduced genes. To date, only the antifreeze promoter from winter flounder has been shown to be active in confirmed transgenic $F_1$ salmonids (Shears et al., 1991).

The expression of foreign DNA can also be inferred from effects on the phenotype of transgenic individuals. Growth hormone genes have been used most extensively in transgenic fish experiments, primarily because of the potential for improving growth performance and feed conversion efficiencies for aquacultural purposes. Several studies have shown that growth stimulation in transgenic fish is feasible, however, the magnitude of the response can be quite different depending on the gene construct and species employed, and on the developmental stage when the size determinations were made. Zhu (1992) and Enikolopov et al. (1989) reported that loach transgenic for mMThGH constructs were between 2- and 5-fold larger than controls, and resulted in up to a doubling in size in carp (Zhu, 1992). In medaka, Lu et al. (1992) showed that both the MMT or chicken actin promoters fused to hGH were capable of increasing the size of transgenic individuals by between 20 and 60% relative to controls. The RSV promoter fused to salmonid GH sequences stimulates growth in transgenic strains of carp by 20-40% (Zhang et al., 1990) and channel catfish by 26% (Dunham et al., 1992). However, it is interesting that transgenic fish with this promoter can show either increased or decreased growth relative to controls (Dunham et al., 1992; Chen et al., 1993), and as suggested by these authors it appears that different insertions and genetic backgrounds can markedly influence expression. Gross et al. (1992) have produced transgenic northern pike using either the carp actin or RSV promoters driving the expression of bovine or chinook GH, and in one experiment with RSVbGH, a 25% increase in the size of male fish was observed over controls.

It is well documented that salmonids possess a remarkable ability to be growth stimulated by treatment with exogenous GH protein (McLean and Donaldson, 1993). Our "all-salmon" GH gene constructs (derived from the vectors shown in Figure 1) have been designed to express sockeye salmon GH1 in a variety of tissues. These constructs should produce elevated levels of circulating GH both by allowing expression in a large number of tissue types, and by removing GH gene expression from the negative feed back regulation that normally limits the production of GH from the pituitary gland. Using these constructs, we have produced transgenic coho salmon with an average size over 10-fold greater than control fish (see Figure 5).

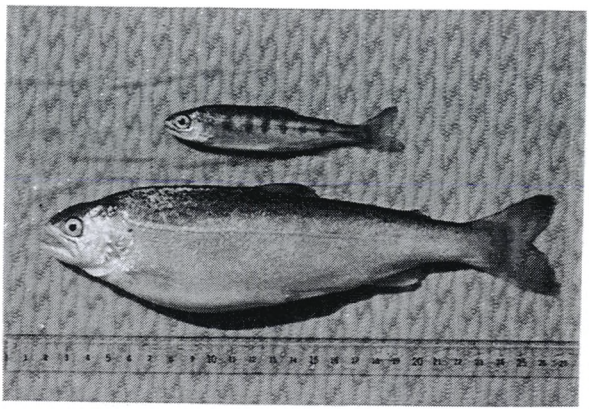

Figure 5. Transgenic fish (bottom) containing salmon pOnMTGH1 construct and control sibling of the same age (top). Note loss of parr marks in transgenic fish characteristic of smolt transformation.

These transgenic fish show growth enhancement throughout their development, and undergo smoltification (adaptation to sea water) in the fall of the year, approximately 6 months prior to the normal season. The physiological changes associated with these transgenic fish are presumably arising from overexpression of salmon GH from extra-pituitary tissues.

Application of transgenic fish technologies to aquaculture will require careful evaluation of the strains produced both in terms of product quality and safety for human consumption, and from the perspective of protection of the wild stocks of fish that escaped transgenic fish might interbreed with. In this regard, effective physical and biological containment measures (Devlin and Donaldson, 1992) need to be co-developed with transgenic fish to ensure that safe implementation can be achieved.

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