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Biotechnology in Aquaculture, with Special Reference to Transgenic Salmon

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Introduction

Almost 10% of the fish consumed in the world are produced by aquaculture. This regular supply of cultured fish is important to the food industry (Slinde, 1992). The aquaculture industry is expanding at a rapid pace, much of this growth coming from the increasing culture of salmonid species. Since its inception in the 1970s, the production of cultured salmon has grown to over 250 000 tons. The red colour, high quality, uniform size (approximately 4 kg) and year-round availability give cultured salmon its marketing appeal. Salmon represent a valuable source of ω-3 and ω-6 fatty acids, which may prevent 'lifestyle diseases' (Skjervold, 1992), and increased consumption of fish is recommended.

AQUACULTURE OF SALMON

As an anadromous species, Atlantic salmon (*Salmo salar*) spends the juvenile part of its lifecycle in fresh water, migrates to the sea where the major growth phase occurs, and returns to spawn in the freshwater stream where it was born. Spawning occurs in the brisk currents of shallow cold waters. The female deposits eggs into an indentation in the river bed, and the male releases sperm over the eggs. In aquaculture, a broodstock is kept for production of eggs and sperm. A few months after fertilization, the fry hatches and 'start-feeding' commences (*Figure 1*). The fingerlings grow until

Abbreviations: ACTH, adrenocorticotropin; AFP, anti-freeze protein; ATPase, adenosine 5'-triphosphate hydrolase; cAMP, adenosine 3'5'-monophosphate; cDNA, complementary DNA; CNS, central nervous system; CREB, cAMP response element binding protein; ES cells, embryonic stem cells; GH, growth hormone; GnRH, gonadotropin releasing hormone; GRF, growth hormone releasing factor; IGF-I, insulin-like growth factor I; PCR, polymerase chain reaction; Pit-1/GHF-1, pituitary-specific transcription factor I; SS, somatostatin.

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the parr marks disappear and smoltification occurs. This change from fry to smolt requires an enormous physiological adaptation to the impending rise in osmotic pressure. Smolts develop a silvery colour caused by subcutaneous deposits of guanine. The minimum size at which a salmon will smolt is 15 g, depending on genetic and environmental factors, although seawater survival is better at 30 g. The seawater grow-out phase is carried out in enclosed inlets, submerged cages or tanks.

Salmon growth is influenced by aquaculture production conditions. Salmon along the western Norwegian coast require over 4 years in freshwater streams before smolting. By raising the water temperature during egg incubation and the post-hatching period, 1-year-old smolts (i.e. fry that smolt in the spring of their second year) can be produced at commercial hatcheries. Totland *et al.* (1987) compared the growth rate of Atlantic salmon (2 kg, 56 cm, 17 kg fish m^{-3}) in a commercial scale swimming raceway (length 20 m, depth 4.5 m, width 4 m; streamsetters giving water speed of 28 cm se^{-1}) with salmon raised in normal sea-cages. Raceway-cultured fish gained 40% more weight than siblings raised in the ordinary sea pens. The difference was primarily due to the size distribution of the muscle fibres: white muscle fibres were hypertrophied, while the inner red fibres were smaller. Commercially it was found that the raceway contained 9.2% more 'superior quality' fish, a visual classifica-

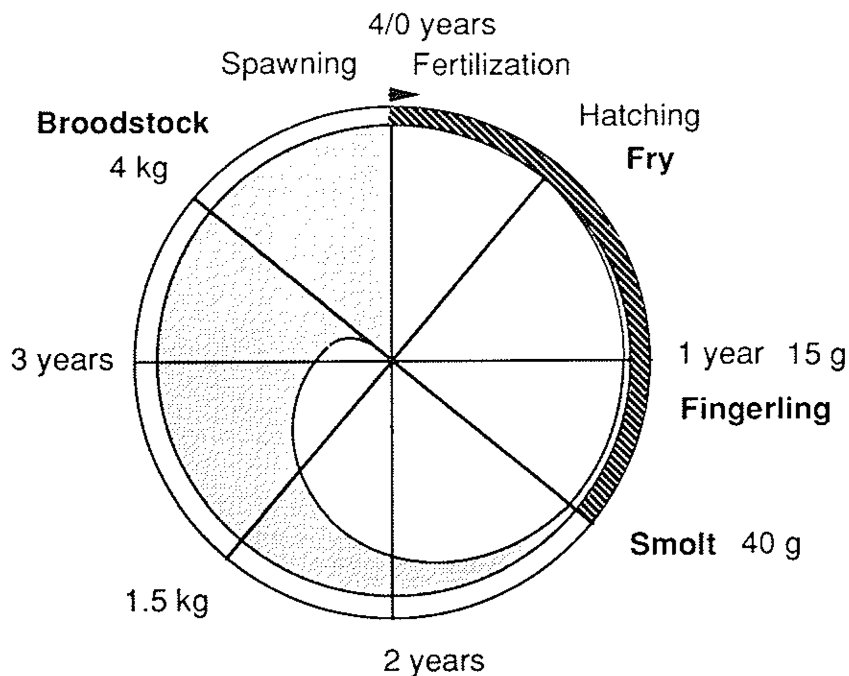


Figure 1. Schematic of Atlantic salmon life-cycle. In aquaculture of Atlantic salmon in the northern hemisphere, fertilization of eggs occurs in November–December and the eggs hatch in the following spring (April–May). The fry are kept in fresh water until the smolt stage is reached (1 year later for a large proportion of the fry). The smolt (30–50 g) are transferred to sea water and grow rapidly and may reach 6–8 kg after 2 years. Hatched area indicates freshwater phase. Usual growth rate is indicated by the shaded area.

tion (based, for example, on fin damage, lacerations) relating to market appeal. Optimal, controlled culturing conditions are necessary both to conduct reproducible experiments and produce high-quality fish for market. Continued improvement of culturing conditions, including refined feed, better caging and cautious selection of suitable aquaculture facility locations, are important future endeavours.

Aquaculture requires large capital investments in buildings, cages, equipment, broodstock, labour and feed. In Norway, smolt culture and the seawater grow-out phase are generally two separate operations. Expenditures related to mortality, inferior quality and disease are highly variable; however, feed quality is paramount to the economic survival of an aquaculture installation. Fish feed must contain all the necessary nutritients for growth, and maintain taste, size and shape in an aquatic environment. This is determined by water absorption, texture and buoyancy factors. The feed must also be acceptable to the fish at different stages of their development. During the seawater grow-out phase, feed comprises a third to a half of the total operating cost. Hence, improving feed utilization represents a major goal for aquaculture biotechnology.

A unique feature of salmon is the 'salmon-red' muscle colour. This is due to the presence of astaxanthin (3,3′-dihydroxy-β-β-carotene-4,4′-dione). Obtained in the wild from shrimps, it is today chemically synthesized and added to fish feed (Bernhard, 1990). Synthetic astaxanthin is expensive, adding 10–15% to the total feed cost. Astaxanthin produced by an algae (Burbrick, 1991) or a yeast such as *Phaffia rhodozyma* (Johnson and An, 1991) represent alternative natural sources.

Diseases caused by different fungi, algae, protozoa, parasites and crustacea create significant problems in aquaculture. Vibriosis, caused by *Vibrio anguillarum* (Knappskog *et al.*, 1993), has inflicted substantial losses on a variety of fish species. In salmon aquaculture, furunculosis (*Aeromonas salmonicida*; McCarthy and Roberts, 1980), bacterial kidney disease (*Renibacterium salmoniarum*; Sanders and Freyer, 1980) and viral diseases (e.g. from infectious pancreatic necrosis virus) can devastate an aquaculture farm. Adequate feed, high hygienic standards, reliable diagnostic methods and efficient pharmaceutical and chemical treatments minimize disease-associated problems. Efficient vaccines are available against different bacterial diseases, but currently no commercial vaccines exist to pre-empt viral diseases. Expenses incurred by fish diseases are substantial and further vaccine development is required.

BREEDING OF ATLANTIC SALMON

Traditional breeding of domesticated animals for phenotypic qualities like growth and disease resistance has been quite successful. Atlantic salmon have been in captivity for only 20 years and traditional breeding has only recently addressed specific traits. The traits targeted by contemporary breeding programmes include: a rapid growth rate, low feed conversion ratio (i.e. dry feed mass used to produce 1 kg of fish), tolerance of high stock densities (i.e.

kg fish per m^3 tank volume), disease resistance, late sexual maturation, large egg size, high fecundity and high market quality of the meat.

Atlantic salmon siblings demonstrate a tremendous (20-fold) growth variation, partly abolished in commercial production by frequent sorting. However, this reveals the potential for enhanced growth. Photoperiod manipulation may improve growth rate and delay sexual maturation of cultured salmon (Stefansson *et al.*, 1991; Hansen, Stefansson and Taranger, 1992). Breeding using specific genetic markers associated with a desirable phenotype is also possible, as illustrated by the specific trypsin isoenzyme marker recently linked to enhanced growth (Torrissen, 1987, 1991). Modern biotechnological techniques for the manipulation and transfer of single genes has engendered the possibility of controlled production of domesticated animals, including fish, with altered or new phenotypes.

Development of transgenic salmon in aquaculture

Transgenic animals have proved important in the study of gene structure and function. A range of species have been employed including several fish species. Various aspects of the production of transgenic fish were recently reviewed (Hew and Fletcher, 1992). Fish are especially suitable for transgenic study, as most have external fertilization and generate a large number of eggs. The rearing of the eggs is similarly simple, and virtually thousands of potentially transgenic embryos may be produced at relatively low cost. Most experiments with transgenic fish have been conducted in the interest of basic research; however, several applied aspects important to aquaculture, including disease resistance, control of sexual maturation, increased growth rate and feed utilization, may benefit from transgenic study. The development of transgenic animals for food production dictates several limitations regarding the choice of methods and their application. It is not our intention to evaluate the marketing of food produced from transgenic animals, but to consider the scientific basis and criteria for the commercial success of transgenic fish (*Table 1*). Further discussion is therefore limited to the use of genes, promoters and methods for DNA transfer which may meet these criteria.

TECHNICAL REQUIREMENTS AND CHOICE OF METHOD FOR INJECTION

Eggs from most fish must survive outside the mother and are therefore equipped with a robust outer egg shell (chorion or zona radiata), which must be penetrated or removed before microinjection. Fish eggs are usually relatively large in size and filled with a yolk mass that often obscures the pronucleus or zygote (*Figure 2*). Some researchers have succeeded in nuclear microinjection of medaka (*Oryzias latipes*) pre-ovulated oocytes followed by artificial fertilization (Ozato, Inoue and Wakamatsu, 1992). Generally, fertilized eggs are microinjected in the cytoplasm. After fertilization, the chorion hardens and may be difficult to penetrate with a microinjection needle. This problem has been circumvented by various methods such as mild trypsinisation (Zhu, 1992), drilling or cutting of a small hole (Rokkones *et al.*,

Table 1. Criteria for production of transgenic fish in commerical aquaculture

	General considerations	Requirements	
		Ecological	Physiological
Gene construct	Available gene sequence from same or closely related species Employ suitable promoter/enhancer Well characterized *in vitro* Stable when incorporated into genome Possible to detect *in vitro* and *in vivo* Avoid retroviral sequences Remove all cloning vector sequences prior to gene transfer Fulfils legislative criteria	Non-virulent Non-transposable Preferably encoding phenotypes which might arise by natural mutation	Use optimal promoter/enhancer to obtain expression in desired organ/tissue
Gene product	Non-toxic Effects of protein must be fully understood	Understanding of repercussions if passed to natural stocks	Non-toxic to animal Regulation and consequences must be fully understood
Transgenic fish	Fulfil legislative criteria Possible to detect transgenic individuals Commercially desirable traits	Comprehensive evaluation of the effects on natural stocks following inter-breeding Development of biological containment strategies (e.g. sterility)	Ethically acceptable changes Produces healthy fish in good condition Genetically stable and inheritable phenotype

1989; Chourrout, Guyomard and Houdebine, 1986) or by physically removing the egg shell with a pair of forceps (Ozato *et al.*, 1986). Salmon eggs are large, typically 5–6 mm in diameter. The pronuclei are not visible due to large amounts of fat droplets (*Figure 2*). The micropyle, an orifice for sperm docking, is visible and readily penetrated with a fine glass needle (Shears *et al.*, 1992; Lossius *et al.*, in press). This technique also ensures that the DNA solution is injected in close proximity to the nuclear area or blastodisc, located just beneath the micropyle after fertilization (*Figure 2a*). It is our experience that a skilled person can inject 100–150 eggs with this technique in a few hours. Normally, 0.1–0.5 μl is injected containing up to 10^6 copies of the DNA construct (2 to 3-fold more DNA than in the zygote), without influencing the survival rate. Several alternative methods for introducing foreign DNA into cells have been developed for use in cell cultures and their potential for production of transgenic fish is discussed in other reviews (Chen and Powers, 1990; Houdebine and Chourrout, 1991; Zhu, 1992). Gene transfer using electroporation has proven successful with eggs from medaka (*Oryzias latipes*: Inoue *et al.*, 1990; Ozato, Inoue and Wakawatsu, 1992) and loach (*Misgurnus anguillicaudatua*: Zhu, 1992), both of which produce relatively small eggs (1 mm). In salmon, however, this technique is limited by the relatively large egg size. An exciting technique which may prove valuable in the future is the culture of totipotent fish embryonic stem (ES) cells, derived from blastocysts. Genetically altered ES cells may be reintroduced into blastocysts to produce viable chimeric animals (Evans and Kaufman, 1981). Another alternative is nuclear transplantation and replacement of the egg pronucleus as discussed by Zhu (1992). These encouraging methods could facilitate rapid, large-scale testing for the recombinant products in cell cultures, a prerequisite for the detection of homologous recombination events in insertional mutagenesis (Thomas and Capecchi, 1986). The technique of gene transfer in ES cells is only reported in cells of mammalian origin; however, blastocysts from zebrafish (*Brachydanio rerio*: S. Lin *et al.*, 1992) and rainbow trout (Nilsson and Cloud, 1992) may be transferred to recipient eggs and contribute to the germ line.

Evidence from several experiments with transgenic fish have revealed that injected DNA can survive, replicate and be expressed extrachromosomaly (see Vielkind, 1992 and references therein). The DNA is apparently incorporated into the genome at a later developmental stage, resulting in a mosaic animal (Guyomard *et al.*, 1989; Fletcher and Davies, 1991). Success in incorporating injected DNA varies between species and researchers from 1 to 75% (Chen and Powers, 1990; Houdebine and Chourrout, 1991). The injected DNA seems to form large concatenates of end-to-end linked molecules (Maclean, Penman and Zhu, 1987; Stuart, McMurray and Westerfield, 1988; Chong and Vielkind, 1989; Guyomard *et al.*, 1989; Winkler, Vielkind and Schartl, 1991). The production of large molecules of non-integrated DNA may have resulted in an overestimation of the number of transgenic fish in some cases. An interesting observation in our laboratory is that protamine mixed with DNA at a 2 : 1 w/w ratio prior to microinjection may increase the rate of transgenesis (Lossius *et al.*, 1993). Injected super-

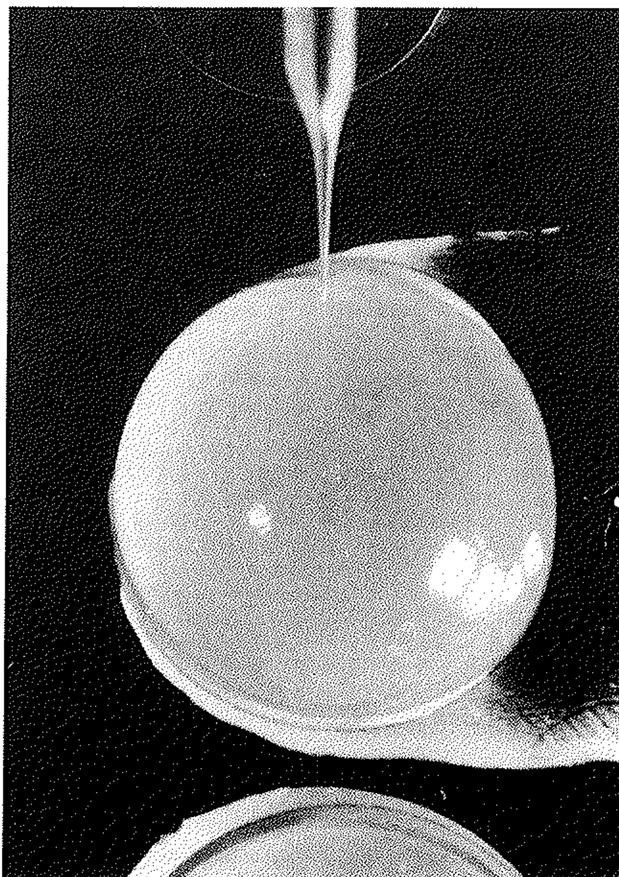


Figure 2. Microinjection in Atlantic salmon eggs. The eggs were fertilized 1–4 h prior to microinjection and kept in tap water on ice. Microinjection was performed through the micropyle using an ~10 μm glass needle connected to a micromanipulator. Approximately 10 nl containing 10^6 copies of Atlantic salmon GH-I gene was injected.

coiled plasmids are transiently expressed but less efficiently incorporated in the genome (Chong and Vielkind, 1989; Penman *et al.*, 1990; Winkler, Vielkind and Schartl, 1991).

The promoter/enhancer utilized in the gene construct dictates under what conditions and in which cell type(s) the gene will be expressed. It is critical to use promoter constructs recognized by the transcriptional machinery of the host organism, either by isolation of the promoter sequences from the same or a closely related species, or by using a strong promoter of viral origin competent to direct transcription in a broad variety of species and cell types. The supply of such sequences for fish systems is presently limited. Fortunately, this will be overcome in the future as more fish genes are characterized. A recent search in GenBank (May 1993) for teleost sequences revealed 1040 records, of which 116 had been added in the last 6 weeks.

To ensure expression from genes transferred into fish embryos, different promoter/enhancer constructs have been tested (reviewed by Chen and

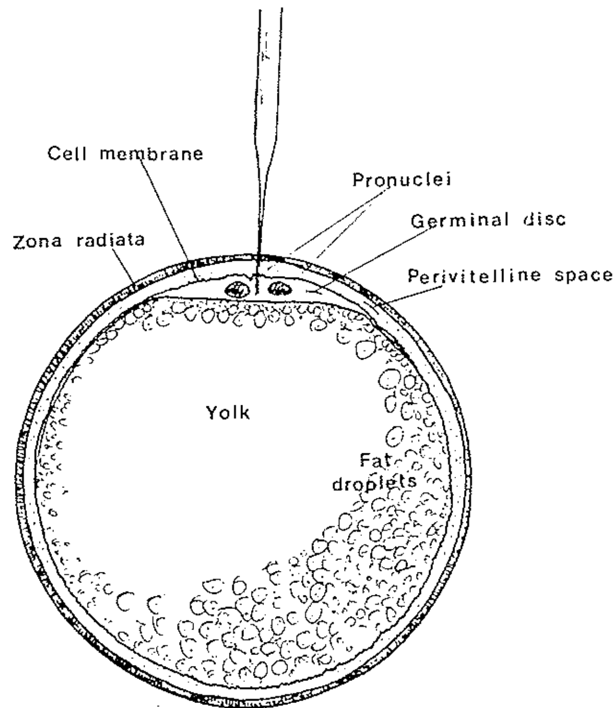


Figure 2a.

Powers, 1990; Moav *et al.*, 1992). Suitable fish cell culture models have recently been developed for analysis of promoters and enhancers (Friedenreich and Schartl, 1990; Collodi *et al.*, 1992; Sharps *et al.*, 1992). Furthermore, cell type-specific expression may be studied transiently *in vivo* in order to identify requisite promoter elements (Rinder *et al.*, 1992; Westerfield *et al.*, 1992; Gong, Hew and Vielkind, 1991). Most transgenic studies have been performed with non-fish promoters/enhancers, usually from animal viruses, which drive efficient gene expression in fish cells. Some recent reports describe transgenic fish which express injected gene constructs controlled by promoters from fish genes, including carp (*Cyprinus carpio* β-actin (Liu *et al.*, 1990; Moav *et al.*, 1992), Winter flounder (*Pseudopleuronectus americanus*) anti-freeze protein (Shears *et al.*, 1991; Fletcher, Davies and Hew, 1992) and ocean pout (*Macrozoarces americanus*) anti-freeze protein (Du *et al.*, 1992). Typically, the expression of injected gene constructs has varied between transgenic individuals, conceivably the result of integration within active or inactive regions of the genome. Preliminary assessment of gene constructs in cell culture models and rapidly growing teleosts (Zebrafish/medaka) is especially important for salmon, which have a long generation time (*Figure 1*).

Introduction of novel salmon phenotypes by genetic engineering

Several fish species are protected from freezing in ice-laden sea water by high levels of systemic anti-freeze protein (AFP: DeVries, Komatsu and Feeney, 1970; Hew and Fletcher, 1985; Davies, Hew and Fletcher, 1988). Salmon lack these proteins and may freeze if kept in sea water below –0.5 to –0.8°C. To improve the winter resistance of Atlantic salmon, the AFP gene from Winter flounder was microinjected into fertilized eggs (Shears *et al.*, 1991; Fletcher, Davies and Hew, 1992). The researchers used approximately 10^6 copies of an AFP construct in a plasmid (pUC9) as linear DNA and screened with a polymerase chain reaction (PCR) assay for transgenic salmon. Approximately 3% were found to carry the gene construct in full blood with 40% of these expressing the gene. Crossing of transgenic males with wild-type females generated F1 progeny carrying the gene construct below the expected Mendelian frequency, indicating that the transgenic male was mosaic for the AFP gene (Fletcher, Davies and Hew, 1992). Expression of the AFP gene was detected, albeit 100-fold less than that considered necessary for efficient protection at low temperatures. Furthermore, the AFP protein was not processed to remove the *N*-terminal precursor, as in the flounder. These observations indicated that Atlantic salmon lack the necessary proteolytic processing mechanisms for the AFR protein and possibly specific transcription factor(s) needed for efficient transcription of the flounder AFP gene.

MANIPULATING EXISTING GENES: REGULATION OF SEXUAL MATURATION

Early sexual maturation of cultured salmon incurs substantial losses in commercial aquaculture. It has been suggested that manipulation of gonadotropin releasing hormone (GnRH) may postpone or even totally inhibit sexual maturation in fish (Alestrøm *et al.*, 1992). GnRH is delivered by the hypothalamic portal system to the pituitary and stimulates the release of the pituitary gonadotropins which regulate gametogenesis and reproductive capability. Both cDNA and genomic clones of Atlantic salmon GnRH have been characterized (Klungland *et al.*, 1992). This facilitates the development of GnRH antagonists and gene constructs directing expression of these peptides to the hypothalamus to compete with native GnRH for receptor (Alestrøm *et al.*, 1992). Alternatively, sterile fish may be produced by hormonal sex reversal or by inducing tetraploidity using shock treatment of fertilized eggs with elevated temperature or pressure (Lincoln and Bye, 1989; Pepper, 1990 and references therein). These methods are relatively simple and applicable to eggs from cultured transgenic salmon to avert breeding of escaped farmed fish with natural salmon populations.

ENHANCEMENT OF SOMATIC GROWTH

The majority of transgenic fish experiments have been conducted with the growth hormone gene to enhance the growth rate, since it is both a convenient marker and a commercially important phenotype (Chen and

Powers, 1990; Houdebine and Chourrout, 1991; Moav *et al.*, 1992). The physiological and molecular regulation of growth have been studied intensely in mammals, but also in fish. Thus, manipulation of the growth rate of fish on an aquaculture scale may be accomplished in the near future. However, very few (if any) of the studies reported meet the limitations for use of transgenic fish in aquaculture outlined in *Table 1*. To fulfil these requirements, given that somatic growth is a highly regulated process, ramifying to other physiological processes, it is necessary to evaluate the current understanding of growth regulation and evaluate possible strategies for growth enhancement through gene transfer and the potential consequences of such manipulation.

The regulation of growth hormone

Growth hormone (GH), a pituitary polypeptide hormone, plays a key role in the somatic growth of vertebrate organisms. Growth hormone is synthesized by somatotrophic cells located in the anterior lobe (adenohypophysis) of the pituitary gland. Growth hormone secretion is under the dual control of opposing hypothalamic hormones: growth hormone releasing factor (GRF) and the GH-release inhibitor, somatostatin (SS: *Figure 3*). In mammals, the adenohypophysis is vascularized with a hypophyseal portal system, which delivers neuropeptides directly from the hypothalamic neurosecretory neurons. Although several fish species may control pituitary function by direct innervation (Ball, 1981), a study of the Atlantic salmon pituitary suggest that the brain utilizes neurovascular links to convey neuropeptides (Friedberg and Ekengren, 1977). Recent studies have revealed mammalian-like hypophyseal portal systems in other teleost species (Baskaran and Sathyanesan, 1992). Teleost GRF- and SS-hypothalamic neurosecretory pathways have been identified by immunohistochemistry (Pan, Liu and Bancroft, 1985; Marivoet, Moons and Vandesande, 1988; Olivereau *et al.*, 1984). Both human and a recently purified carp GRF stimulate GH release *in vitro* (Luo *et al.*, 1990; Le Bail *et al.*, 1991; Vaughan *et al.*, 1992) and *in vivo* (Peter *et al.*, 1984). Somatostatin has been characterized from a number of teleost species, including salmon (Conlon, 1990). Somatostatin inhibits GH secretion *in vitro* and *in vivo*, and auto-immunization against SS increases GH levels and somatic growth rates (Fryer, Nishioka and Bern, 1979; Luo *et al.*, 1990; Hall, Harvey and Scanes, 1986 and references therein; Le Bail *et al.*, 1991).

Mammalian GH secretion profiles are characterized by episodic bursts (Tannenbaum and Ling, 1984; Plotsky and Vale, 1985; Jansson, Edén and Isaksson, 1985), and pulsatile GH secretion was recently described in trout (Le Bail *et al.*, 1991). Episodic GH regulation is generated by the alternating secretion of GRF and SS: SS withdrawal sets the timing and GRF determines the magnitude of GH release (Plotsky and Vale, 1985; Kraicer *et al.*, 1988). This ultradian secretory pattern is critical to effective growth (Robinson and Clark, 1987). Pulsatile infusion of GRF, in contrast to continuous treatment, is required to achieve effective growth stimulation in rats (Clark and Robinson, 1985).

Control of the GH-regulating neuropeptides by the central nervous system

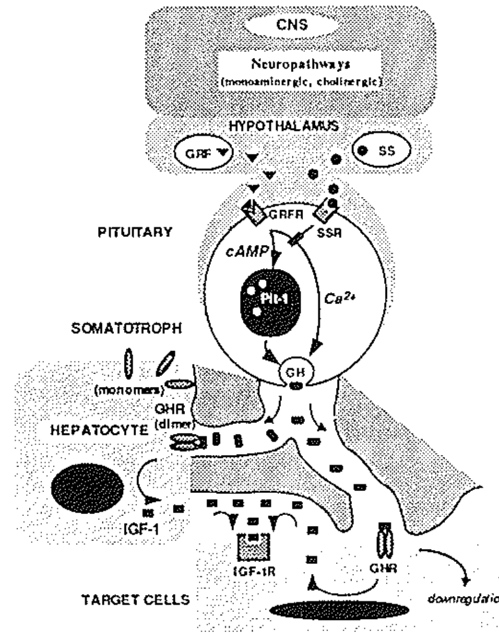


Figure 3. Schematic of growth hormone regulation and its action. Physiological stimuli, both external and internal, which influence GH levels in the body (top) act via neuropathways in the central nervous system (CNS). These neuropathways modulate hypothalamic neurosecretory neurons, which synthesize growth hormone releasing factor (GRF) and somatostatin (SS) (Rawlings and Mason, 1989). For example, cholinergic control of SS release is connected to peripheral feedback pathways mediated by glucose and GH (Page, Dieguez and Scanlon, 1989). The central adrenaline system exerts dual GH control: α_2-adrenoreceptor activation stimulates GRF secretion, while α_1-receptors exclusively stimulate SS release (Cella *et al.*, 1987). GRF and SS secreted into the hypothalamic–pituitary portal system act on GH-producing cells (somatotrophs) in the pituitary. GRF binds to its receptor, a member of the classical G-protein-coupled, seven-helix transmembrane domain protein superfamily (C. Lin *et al.*, 1992; Mayo, 1992). GRF cell-signalling operates by raising cAMP levels through the G_s-protein-linked receptor/adenylate cyclase pathway (Barinaga *et al.*, 1985). The rise in intracellular cAMP serves to stimulate GH secretion and synthesis. In addition, GRF cues proliferation of the somatotrophic cell lineage (Billestrup, Swanson and Vale, 1986). GRF stimulates GH synthesis independent of GH secretion, via the cAMP-dependent activation of the pituitary-specific transcription factor, Pit-1 (McCormick *et al.*, 1990; Chen *et al.*, 1990). Pit-1 interacts at the GH promoter with other ubiquitous transcription factors to enhance GH transcription. Somatostatin binding to its G_i-coupled receptor inhibits Ca^{2+}-influx through voltage-operated calcium channels (Rosenthal *et al.*, 1988), blocking exocytosis of storage vesicles containing Zn^{2+}-coordinated GH dimers (Cunningham, Mulkerrin and Wells, 1991). Once in the systemic circulation, GH is borne as a monomer by serum binding proteins. GH binds to its cell-surface receptor, located on several cell types. GH binds in a sequential manner to two GH receptor monomers (Cunningham *et al.*, 1991). Dimerization of the GH receptor generates the signalling mechanism, resulting in the synthesis and secretion of IGF-I in hepatocytes. Hepatic IGF-I will in turn circulate inducing proliferation at several target tissues. In addition, GH-induced IGF-I secretion may act in an autocrine fashion inducing proliferation and differentiation of different cell types. Feedback pathways exist at all levels: for example, GRF and SS stimulate each other's secretion while down-regulating their own release; both GH and IGF-1 decrease somatotroph GRF responsiveness while stimulating SS release; and there is down-regulation of the GH receptor on target tissues (Hall, Harvey and Scanes, 1986; Page, Dieguez and Scanlon, 1989; Gluckman and Breier, 1989). In addition, each of these different modes of GH regulation are subject to modulation by other factors, exemplified by the GH synthesis-enhancing effects of glucocorticoids and thyroid hormones and estradiol induction of hepatic high-affinity GH binding sites (Gluckman and Breier, 1989).

(CNS) appears to represent the major level of GH regulation. Physiological stimuli that influence GH release, such as changes in blood metabolites, stress and photoperiod, in most cases act via receptors in the CNS which activate brain neuropathways controlling hypothalmic function (Rawlings and Mason, 1989).

In the pituitary, GH synthesis and release are independent events, mediated through distinct signal transduction mechanisms (Barinaga *et al.*, 1985; outlined in the legend to *Figure 3*). GRF stimulates GH transcription through a cAMP-dependent pathway (Barinaga *et al.*, 1985). The transcriptional effects of cAMP are mediated by a family of transcription factors, cAMP response element binding proteins (CREBs) (Habener, 1990; Karin, 1989) activated by protein kinase A phosphorylation. Transgenic mice expressing a non-phosphorylatable CREB mutant resulted in a dwarf phenotype, clearly implicating CREB in the activation of GH transcription (Struthers *et al.*, 1991).

The key regulator of GH transcription is the pituitary-specific transcription factor Pit-1/GHF-1 (Karin, Castrillo and Theill, 1990), recently described in salmonids (Ono and Takayama, 1992; Elsholtz *et al.*, 1992). Pit-1 represents a major nuclear target of the cAMP-dependent pathway and Pit-1 transcription is induced by CREB. Once 'turned on', the Pit-1 gene remains active throughout the cell life-span and cooperates with other transcription factors at the GH promoter to induce GH transcription (Karin, Castrillo and Theill, 1990).

The effects of growth hormone

The immediate metabolic effects of GH administration in mammals resemble those of insulin (Press, 1988). Following this acute response, GH stimulates adipocyte lipolysis and hepatic carbohydrate catabolism. This insulin-antagonistic effect apparently balances the increased insulin secretion. Growth hormone is also an important protein anabolic hormone, increasing amino acid transport, nitrogen retention and stimulating protein synthesis. The metabolic effects of GH in fish are less well understood, although where investigated they are congruent with the mammalian studies. For example, GH treatment stimulated lipid mobilization and liver lipolytic enzyme activity in coho salmon parr (Sheridan, 1986).

The growth-inducing (mitogenic) effects of GH are largely mediated by insulin-like growth factor I (IGF-I). Originally, the liver was considered the target organ of GH action leading to the production of systemic IGF-I (Daughaday *et al.*, 1972). This 'somatomedin hypothesis' designated IGF-I as the effector molecule for GH, a model in concert with other pituitary hormone pathways. Indeed, IGF-I treatment leads to enhanced growth (Schoenle *et al.*, 1982; Guler *et al.*, 1989) and transgenic mice strains over-expressing IGF-I display a 1.3-fold increase in weight (Mathews *et al.*, 1988). However, it became clear that GH receptors are present on multiple cell types, also capable of IGF-I synthesis stimulated by GH binding (see Waters *et al.*, 1989; Humbel, 1990). Howard Green and colleagues have

recently proposed the concept of a dual effector system to describe the growth-inducing effects of GH (Green, Morikawa and Nixon, 1985). The theory predicts that GH creates the preferred target cells of IGF-I action through differentiation of precursor cells into mature, IGF-I responsive cells: GH was shown to promote the differentiation of precursor pre-adipocyte cells (Zezulak and Green, 1986). The young, differentiated adipocytes become sensitive to the mitogenic effects of IGF-I. Other cell types have also been implicated in this scheme: chondrocytes (Isaksson, Jansson and Gause, 1982), granulocytes (Hochberg *et al.*, 1989) and T-lymphocytes (Geffner *et al.*, 1990). It therefore would appear that GH regulates IGF-I production at two levels: (1) by generation of endocrine IGF-I from the liver, creating a large reservoir of serum IGF-I, bound to binding proteins; and (2) locally produced IGF-I, acting in a autocrine/paracrine fashion. The relative contributions of these two sources of IGF-I are still debated (see Humbel, 1990).

The role of IGF-I was recently addressed in salmonids. Recombinant mammalian IGF-I was shown to have a growth-enhancing effect in coho salmon only when constantly infused into animals exhibiting submaximal growth rates (McCormick *et al.*, 1992). Skyrud *et al.* (1989) did not observe enhanced growth following weekly (bolus) injections of mammalian IGF-I. This study revealed a dramatic IGF-I-induced hypoglycaemic and hypoaminoacidaemic effect, previously encountered in mammalian studies, possibly due to saturation of insulin receptors. Recently, several salmonid IGF-Is have been cloned, displaying 80% identity with the mammalian protein (Cao *et al.*, 1989; Shamblott and Chen, 1992; Duguay *et al.*, 1992). Cao *et al.* (1989) also demonstrated a transcriptional enhancement of hepatic salmon IGF-I following GH treatment, consistent with data from mammalian systems (Mathews, Norstedt and Palmiter, 1986).

The growth-promoting effects of GH in teleosts are well studied (Donaldson *et al.*, 1979; Weatherley and Gill, 1987). Pickford showed 40 years ago that administration of mammalian GH restores the effect of hypophysectomy and stimulates body weight and length in normal teleosts (Pickford, 1954). Several studies with partially purified fish hormones have documented a general growth-promoting effect (see Donaldson *et al.*, 1979; Weatherley and Gill, 1987). The availability of recombinant mammalian and later fish GH facilitated more extensive studies (Gill *et al.*, 1985; Sekine *et al.*, 1985; Agellon *et al.*, 1988). Gill *et al.* (1985) demonstrated that intraperitoneal injections of natural and recombinant chicken or bovine GH resulted in dramatic dose-dependent increases in growth. The GH-treated fish were longer, a consequence of accelerated bone growth (Takagi *et al.*, 1992), and demonstrated a greater feed conversion efficiency. Similar results were obtained by Agellon *et al.* (1988) using recombinant rainbow trout GH. Interestingly, the GH-treated fish retained their growth increase several weeks after the final GH treatment, growing to twice the size of control fish.

Several transgenic fish studies have assessed the effects of GH overproduction in fish (see Chen and Powers, 1990). In a recent study, Du *et al.* (1992) fused chinook salmon GH cDNA to an anti-freeze protein gene promoter. Salmon shown to carry the microinjected gene construct displayed

between 2- and 6-fold enhanced growth rate. Hence, our current understanding of GH action in fish indicates that many of the effects found in mammals are present. In addition, there are interesting fish-specific actions of GH. Growth hormone was recently shown to regulate the production of anti-freeze protein in Winter flounder (Idler *et al.*, 1989), and to participate in the seawater adaptation of several salmonid species (Barron, 1985). Growth hormone also enhances the activity of gill Na^{+}/K^{+}-ATPase and the steroidogenic capacity and ACTH responsiveness of the inter-renal gland, indicating both a direct and indirect role for GH during smoltification (Young, 1988; Richman and Zaugg, 1987).

The cellular effects of GH are mediated through interaction with membrane-associated receptors. The GH receptor was recently cloned from several mammalian species (see Mathews, 1991). The GH receptor belongs to the haematopoietic receptor superfamily, a group of receptors generally involved in cell growth and differentiation (Bazan, 1990). Recently, the three-dimensional structure of the extracellular domain of the human GH receptor bound to GH was determined (de Vos, Ultsch and Kossiakoff, 1992). A single GH molecule binds two receptor molecules, in a sequential manner, at two different regions of the GH molecule (Cunningham *et al.*, 1991). The nature of the intracellular signal from the GH receptor is still unknown, but requires the formation of a receptor dimer (Fuh *et al.*, 1992). At high GH levels, receptor binding is predominatly monomeric, attenuating the biological response (Fuh *et al.*, 1992). This could explain the lack of correlation between plasma GH levels and growth observed by Du *et al.* (1992) – individuals possessing the highest levels of GH showed no increase in growth compared with non-transgenic controls.

In general, the physiology and molecular biology of GH appear evolutionarily conserved between teleosts and mammals. Hence, the recent surge of understanding of GH actions and regulation will greatly aid aquaculture applications.

EVALUATION OF CANDIDATE GENES AND PROMOTERS FOR MANIPULATION OF GROWTH

The generation of transgenic salmon and their utilization in commerical aquaculture entail conflicting interests. The use of transgenic animals in food production is highly regulated and subject to public concern (Hallerman and Kapuscinski, 1992). This argues for a conservative approach in the design of gene constructs. The use of salmonid genes and promoters/enhancers for the generation of transgenic salmon would represent a *de facto* gene copy number increase and/or modest adjustment of existing physiological processes and would, presumably, be acceptable.

Injection of genes under control of their native promoters would ensure expression exclusively in the natural organ/cells and conceivably maintain a near normal physiological regulation. Such a ‘natural’ construct, as with all gene constructs, may be unduly influenced by the integration vicinity in the chromosomal DNA. Considerable proportions of chromosomal DNA in

somatic cells are not actively transcribed and the chance of integration in a silent area are substantial. Indeed, integrated test constructs driven by strong viral promoters are often not expressed in the F1 generation (Culp, Nüsslein-Volhard and Hopkins, 1991). Hence a large number of transgenic fish would have to be produced and tested for at least two generations to obtain the desired phenotypic effect. The effectiveness of a promoter may be manipulated once detailed knowledge of the transcription factor recognition sites (short DNA sequences required for specific interactions) is obtained. For instance, addition of multiple Pit-1 recognition sites to reporter constructs enhances transcription several fold in Pit-1-expressing cells (Ding *et al.*, 1991; Yan and Bancroft, 1991). The removal of repressor sequences from the rat GH promoter resulted in a 3- to 4-fold increase in activity (Pan, Liu and Bancroft, 1990). However, most experiments have utilized promoters that direct expression of the gene to an organ/cell-type which does not normally synthesize the protein (see Chen and Powers, 1990).

Salmon parr (pre-smolts) treated with GH for short periods maintained their growth advantage relative to untreated siblings (Agellon *et al.*, 1988). This could reduce the relatively long freshwater phase, as size is a major determinant of smoltification. Transgenic fish which express higher GH levels during this critical life-stage may later benefit. Thus a gene promoter primarily active at this stage would be useful. One candidate is the prolactin promoter. In salmon, prolactin has been associated with freshwater osmoregulation prior to smoltification (Barron, 1985). This may imply that the prolactin promoter is mainly active prior to seawater entry, but this must be confirmed experimentally.

Many of the factors involved in the regulation of somatic growth are potential targets for transgenic manipulation of growth. GRF, a key regulator of GH, is an obvious candidate. Transgenic mice over-expressing GRF exhibit enhanced somatic growth (Hammer *et al.*, 1985). Vaughan *et al.* (1992) recently reported the carp GRF peptide sequence. Even so, targeted expression to the cognate hypothalamic neurons will require characterization of the corresponding gene sequence. Pancreas is capable of expressing and processing the active GRF peptide; in fact, GRF was originally isolated from pancreatic neoplasias of acromegalic patients (Rivier *et al.*, 1982; Guillemin *et al.*, 1982). Hence, promoters from genes expressed in the endocrine pancreas could be employed. Supraphysiological GRF levels induce somatotrophic cell hyperplasia and could potentially damage adjacent hormone-secreting cells (Asa *et al.*, 1984; Hammer *et al.*, 1985; Billestrup, Swanson and Vale, 1986). The mammalian GRF receptor was recently cloned and characterized (C. Lin *et al.*, 1992; Mayo, 1992). Over-expression of the receptor in a transgenic strain, if not strictly limited to somatotrophic cells, could induce undesirable side-effects, as the cAMP signalling pathway mediates a variety of effects in different cells. However, mice carrying a cholera toxin gene construct under control of the GH promoter, generating somatotroph-specific cAMP elevation, resulted in gigantism (Burton *et al.*, 1991). The GRF receptor and GH are both regulated by the transcription factor Pit-1 (C. Lin *et al.*, 1992). But as

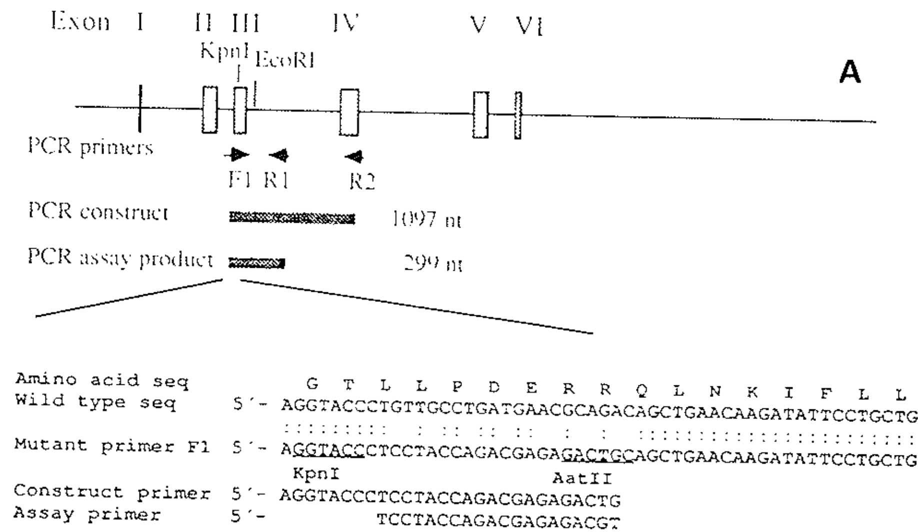
Pit-1 also regulates lactotroph and thyrotroph development (Li *et al.*, 1990), it may not be easily manipulated in transgenic animals.

Growth hormone has been the favourite reporter gene in transgenic fish studies; GH is a well-characterized system (see above), inducing an easily scorable phenotype, and many teleost genes are available. Several laboratories have generated transgenic fish exhibiting spectacular growth enhancement (Chen and Powers, 1990; Du *et al.*, 1992; Zhu, 1992). Growth hormone expressed in non-somatotrophic cells escapes hypothalamic regulation and feedback down-regulation (with the exception of the GH receptor; *Figure 3*). Constitutively high GH levels do not necessarily result in enhanced growth (Du *et al.*, 1992). Indeed, high GH levels could antagonize growth by sequestering the GH receptor in non-productive monomeric interactions (Fuh *et al.*, 1992).

The gene encoding the GH receptor could potentially represent a viable target for transgene manipulation. The gene is only characterized in mammals (Leung *et al.*, 1987; Mathews, 1991) and would require cloning from salmon. The GH receptor may be a limiting step in somatic growth regulation; however, the signal transduction mechanism is still unknown. As with the GRF receptor, over-expression may cause unacceptable effects in heterologous cells. The use of the GH receptor gene promoter or possibly the IGF-I gene promoter could avert this problem. The final effector of somatic growth, IGF-I, is probably produced in all GH-responsive cells. Thus IGF-I remains a promising target for gene transfer, but as pointed out above, expression would have to be fine-tuned to avoid cross-reaction with the insulin receptor. The conflicting results of IGF-I as a growth enhancer necessitate further study of the physiological effects of high levels of IGF-I before it may be useful in aquaculture.

MONITORING TRANSGENIC ANIMALS

Rapid and reliable methods for the identification of fish harbouring injected DNA are a prerequisite for large-scale transgenic studies. The polymerase chain reaction (PCR) (Saiki *et al.*, 1985) is ideal: it is specific and sensitive, requiring only minute tissue samples; a large number of samples can be assayed at relatively low cost; and it is amenable to automation. Various applications of PCR in screening transgenic fish have been reviewed by Davies and Gauthier (1992). The traditional PCR technique will not discriminate between integrated and extrachromosomal DNA and may therefore only be employed in preliminary screenings. Progeny from putative transgenic fish must be shown to carry the construct. Furthermore, this F1 generation should manifest the desired phenotype. The injection of native gene constructs presents a special problem; namely, how to differentiate between the endogenous and transferred genes. We solved this problem for Atlantic salmon GH-I gene transfers by modifying the gene sequence slightly to provide construct-specific PCR primers, without disturbing the encoded amino acid sequence (Lossius *et al.*, 1993). This strategy is outlined in *Figure 4*. With this technique, only a minute blood sample was required for detection



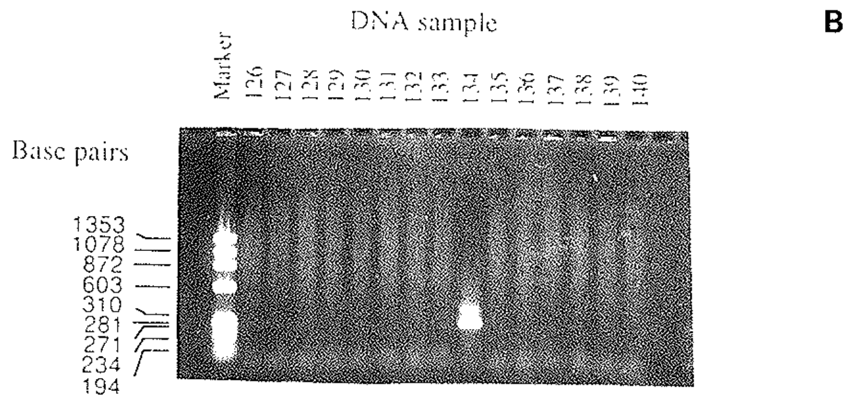


Figure 4. Mutagenesis and detection of Atlantic salmon GH gene in transgenic fish.
(A) The Atlantic salmon GH-I gene (Male *et al.*, 1992) was modified using PCR. The reaction was initiated with a 52 nucleotides (nt) primer from exon II containing a unique Kpn I restriction enzyme site followed by 20 nt selected to obtain maximum divergence from the wild type sequence, while conserving the encoded amino acids sequence, and flanked by 24 nt identical to the wild type GH-I sequence. The PCR reaction was supplemented with the reverse primer R2 from exon IV and a primer composed of the first 28 nt of F1 (construct primer). The resulting PCR fragment was cleaved with Kpn I and Eco RI and exchanged with the cognate wild type sequence. The resulting modified GH-I gene was injected into fertilized Atlantic salmon eggs as described (Lossius *et al.*, 1993).
(B) Analysis of 15 PCR reactions from individual fish. Two-year-old fish (n=140) injected with the GH-I construct shown in (A) were analysed by PCR using blood samples. One positive individual was identified. The assay primer and a reverse primer in intron III were employed to generate a construct-specific 299 bp DNA fragment. *Methods*: Blood (3 µl) from individual fish was mixed with 100 µl of 100/mM NaOH, boiled for 3 min, and centrifuged briefly (3 min, 12 000 × g); 1.5 µl from these samples were assayed in standard 50 µl PCR reactions (Innis *et al.*, 1991), incubated for 5 min at 94°C and run for 35 cycles: 94°C, 49.6°C, 72°C each for 1 min. The PCR reaction products were resolved in a 1.2% agarose gel and visualized by ethidium bromide staining.

(salmon erythrocytes are nucleated), although any tissue (e.g. fin clips) may be used.

Concluding remarks

The technical prerequisites are available for establishing transgenic salmon strains suitable for commercial aquaculture. The acquculture of transgenic species not only requires a thorough evaluation of applicable gene constructs, but raises ecological and ethical considerations, discussed in detail in several recent reviews (Tiedje *et al.*, 1989; Ferguson, 1990; Kapuscinski and Hallerman, 1990; Hallerman and Kapuscinski, 1992). Salmon are usually cultivated in submerged sea-pens, and escapees are inevitable, allowing the interaction of transgenic salmon with wild populations. The ecological implications of this interaction depend foremost on the fitness of the particular phenotype, but also on the number of escaped fish and their ability to enter spawning streams. A biological control to reduce or eliminate the reproductive potential of transgenic fish in aquaculture would at least partly alleviate these concerns. Physical and biological containment of transgenic fish has recently been reviewed by Devlin and Donaldson (1992). The production of sterile fish has the additional benefit of eliminating early sexual maturation, a substantial economic burden in the commercial production of salmon.

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