Transgenic fish. The future of fish with novel genes

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Introduction

Making transgenic lines of vertebrates represents a rather new technology. While transgenic fish have been introduced in the laboratory since the mid eighties, it has also become a reality in aquaculture since the early nineties. There are many reasons why people want to create genetically modified organisms (GMO) and there are many strategies to make "fish with novel genes". As such it has generated high expectations in society and directed the interests of researchers, but has also raised concerns about environmental and consumer safety, and generated numerous ethical and legal discussions.

A GMO can be defined as a "new" organism which contains an "intergeneric combination of genetic material" (HALLERMAN and KAPUSCINSKI, 1990a) or as an organism which contains "genetic material which has been modified *in vitro*".

I will first explain the technological aspects of generating transgenic fish. The implications for aquaculture are highlighted with the transgenes promoting growth, lowering freezing temperature and inducing disease resistance. I will also discuss environmental and consumer related aspects. In conclusion the future of GMOs is presented.

Aims of making transgenic fish

The transfer of DNA sequences and genes in fish is being done for three main purposes:
(1) to understand the molecular genetics of development
(2) as a model for mammalian, read human genetics
(3) to enhance aquaculture production
The first reason is motivated by fundamental research and is driven amongst others by the desire to study the molecular biology of the early development of zebrafish (ROSSANT and HOPKINS, 1992) and medaka (ISHIKAWA *et al.*, 1997). Progress is such that a growing number of housekeeping and cell-specific genes are being isolated, mapped, and the resulting phenotype characterised (GAIANO *et al.*, 1996a).

The second reason is influenced by the limitations of mammalian models to study human diseases. The reproductive biology of mice sets serious limits to the experimental study of early development, including the regulation of gene expression. ROSSANT and HOPKINS (1992) nicely review the capabilies of zebrafish in this perspective. In general, fish fertilise their eggs externally, have numerous progeny, are easy to culture, and have short generation times. It is possible to make isogenic lines in a single generation and to screen for mutants in haploid progeny (POWERS, 1989). An example of a promising application in medicine is the targeted study of circulatory and cardiac defects in zebrafish.

Finally, transgenesis offers perspectives for the acceleration of selection. Indeed, the domestication of organisms with an economic interest has been going on for tens of centuries. The process has been achieved through careful observation, directed breeding, culling and strain management. But fish has a rather short domestication history and thus the selection of strains and lines is limited. Common carp has been selected in China since 2000 B.P. (KOMEN, 1990), while the selection of rainbow trout started only last century and Atlantic salmon fourty year ago. Tropical fish such as tilapia and catfish have been submitted to directed selection for less than 10 year. A limiting factor is that selection progress is

strongly dependent on generation time; for example bacteria double every hour or so, while Atlantic salmon has a generation time of at least 3 year. As such, transgenesis could speed up the generation of lines with specific phenotypic traits: disease resistance, enhanced growth, colour mutation, and so on.

Methodology

Regardless whether transgenic research is fundamental or applied, three major steps have to be taken to generate transgenic fish. First a recombinant expression vector has to be made (1), second the construct has to be introduced into the germ line (2) and third, the desired phenotype has to be functioning properly (3) (HACKETT, 1993).

The construction of recombinant expression vectors for fish

Vectors

Expression vectors are recombinant DNAs that carry the transgene of interest and the regulatory sequences that determine where, when and at which level the transgene will be expressed (HACKETT, 1993). They include a vector consisting of a plasmid or viral sequence, regulatory sequences and the gene of interest.

Most vectors used are modified *E. coli* plasmids (*e.g.* AMSTERDAM *et al.*, 1995; GIBBS *et al.*, 1994; VOLCKAERT *et al.*, 1994), which can be replicated in *E. coli* cells such that high amounts of recombinant DNA can be obtained.

Retroviral vectors represent an alternative and promising strategy (BURNS *et al.*, 1993). The genome of the Moloney Murine Leukemia Virus (MLV) was modified with the envelope protein of

Vesicular Stomatitis Virus (VSV). The MLV/VSV pseudotype retroviral vectors showed enhanced success of integration although seriously limited by the low viral titers. A new generation of MLV/VSV pseudoptyped retroviral vectors has viral titers up to $1x10^9$ colony forming units/ml with an integration success of 83 % (GAIANO *et al.*, 1996b).

Recombinant DNA

Regulatory elements, promoters, enhancers and silencers

About 2,000 genes permit each cell to perform its specialised function in a particular tissue. Consequently most of these genes are non-functional most of the time and require precise signals to be activated (HACKETT, 1993). Therefore genetic engineering requires knowledge of the elements which initiate, modify and terminate transcription, splice mRNA and initiate protein synthesis. Regulation of transcriptional initiation is the major control in gene activation and is mediated by a number of regulatory modules each composed of a short DNA sequence and the proteins that specifically interact with them. It is the combination of various regulatory modules, each providing a response (tissue-specific expression, induction by a hormone, by a metal ion, by a heat-shock protein, etc) that cause the fine-tuning of gene expression. All the transcription factors bound to their DNA sequence and stabilised by protein/protein interaction, anchor to the promoter of the RNA polymerase II and are included in a large complex with general transcription factors. Reviews have been written amongst others by HACKETT (1993) and IYENGAR *et al.* (1996).

Numerous fish genes have been isolated, sequenced and a lesser number have been characterised, but the number of regulatory elements isolated and sequenced is rather limited. Therefore the number of piscine sequences tested *in vitro* (in a transiently or stably transfected cell system) or *in vivo* (in a transient or stable transgenic system) is limited.

The ubiquitously regulated metallothionein (MT) promoter contains Metal Responsive Elements (MRE) which are DNA sequences that mediate expression in the presence of Zn^{2+} and Cd^{2+} (ZAFARULLAH

et al., 1989; FRIEDENREICH and SCHARTL, 1990; HONG *et al.*, 1993). The interest of this promoter lies in its putative use as a monitor of heavy metal pollution (KINOSHITA *et al.*, 1994).

The housekeeping gene β-actin has been characterised by LIU *et al.* (1990) and MOAV *et al.* (1993) in carp and TAKAGI *et al.* (1994) in medaka. The latter authors developed an expression vector from the medaka β-actin gene for use in medaka. The medaka β-actin promoter showed variable but sometimes ubiquitous transient expression in medaka fry (TAKAGI *et al.*, 1994). Functional analysis of the carp β-actin promoter in fish cells was used to construct two expression vectors for use in transgenic fish (LIU *et al.*, 1990). Expression is species-specific and interactions were observed between the motifs in the proximal promoter and first intron in zebrafish (MOAV *et al.*, 1993).

The type II and type III-Antifreeze Protein (AFP) promotor has been functionally analysed *in vitro* and transiently *in vivo* (GONG *et al.*, 1991). Multiple positive and negative regulatory elements were detected in this promoter which could be useful in controlling gene expression of temperate species during cold temperature periods or low temperature expression in tropical species.

The hormone prolactin I regulates various functions in fish such as osmoregulation (freshwater adaptation in saltwater fish and calcium homeostasis in both saltwater and freshwater fish), Ca^{2+} metabolism, mucus secretion, hatching, general metabolism and behaviour. *In vitro* analysis of the regulatory elements of the prolactinI gene of tilapia (*Oreochromis mossambicus*) and rainbow trout (*Oncorhynchus mykiss*) include several binding sites for a pituitary-specific protein (called *Pit-1*), as well as positive (enhancers) and negative (repressors) regulatory elements (ARGENTON *et al.*, 1996; PONCELET *et al.*, 1996). A glucocorticoid responsive element-like motif is also present close to the second and third *Pit-1* binding sites (ARGENTON *et al.*, 1996). The transient expression of a fusion gene between the tilapia prolactin promoter and a reporter gene in epithelial carp cells is dependent on the co-expression of the pituitary-specific *Pit-1* factor (PONCELET *et al.*, 1996). A transient *in vivo* study of deletion mutants of the tilapia prolactin promoter revealed the presence of enhancing and

repressing sequences. The location of maximal and minimal expression *in vitro* and *in vivo* coincided to a limited degree.

Transgenes

Transgenes refer to the genes which are expressed in a transgenic system, either *in vitro* or *in vivo*, either transiently or stably. Their proper expression is regulated by homologous or heterologous promoter sequences. Three groups of transgenes can be discerned. (1) Transgenes which represent a gain-of-function lead to expression of a new protein in the cell. Examples are the growth hormone and the anti-freeze protein. (2) The use of selectable markers such as the gene conferring resistance to neomycin facilitate the selection and identification of transgenes. Reporter genes such as those encoding β-galactosidase, luciferase and green fluorescent protein permit the examination of regulatory elements. (3) The expression of loss-of-function transgenes interferes with the normal functioning of endogenous genes (HACKETT, 1993). A gene encoding antisense mRNA that would prevent GnRH protein synthesis is under development.

Reviews on this topic have been published amongst others by GONG and HEW (1995), HACKETT (1993) and IYENGAR *et al.* (1996).

Given the observation that in general "all-fish-constructs" perform the best in transgenic fish (BETANCOURT *et al.*, 1993), the currently used gain-of-function transgenes are virtually all of piscine origin and derive from the gene proper (gDNA) or are cDNA sequences with intron sequences and the polyadenylation signal added. There are a number of well documented cases, of which transgenics for growth hormone and antifreeze protein will be discussed later on.

The Xmrk oncogene encodes a receptor tyrosine kinase which causes melanomas and is located on the sex chromosome (SCHARTL, 1990). Injection of the CMVTk/Xmrk construct into medaka embryos induced embryonic tumour formation in tissues normally expressing the Xmrk proto-oncogene (WINKLER *et al.*, 1994). The system is used as a successful model for oncogenesis.

Gonadotropin releasing hormone (GnRH) has been cloned in a number of fish including zebra fish, tilapia, rainbow trout and catfish. It is responsible for sexual maturation and as such draws a

lot of interest in its potential to induce sterility (ALESTRÖM *et al.*, 1992).

Other transgenes of interest include lysozyme, an antibacterial enzyme found in various tissues of vertebrates. Current interest traces its origin to its isolation from rainbow trout by DAUTIGNY *et al.* (1991). The gene encoding the pituitary expressed hormone prolactin has been cloned amongst others from tilapia, it has been characterised *in vitro* by SWENNEN *et al.* (1992) and stably transferred to rainbow trout, in which its phenotype is under investigation (BRETON, pers. comm). The insulin-like growth factor-1 (IGF-1) is regulated by GH and is implicated in the growth promoting effects of GH. It has been cloned, sequenced and characterised amongst others in Atlantic salmon (DUGUAY *et al.*, 1992).

The second group of transgenes, the reporter genes, are meant to facilitate the study of regulatory elements because they are relatively easily and reliably detected. They can also be used in large screens of mutants by gene-trapping (identification of unknown gene products) and enhancer-trapping (identification of unknown promoter sequences) (GAIANO *et al.*, 1996a). They are usually of non-fish origin and carry characteristics which makes them relatively easily detected.

lacZ originates from *E. coli* and encodes the enzyme β-galactosidase which is detected *in vivo*, colorimetrically, histochemically, immunohistochemically or luminometrically (MACGREGOR *et al.*, 1990). It is the most often used reporter gene, is highly dependable and can be used under a variety of conditions (see ALAM *et al.*, 1995; LIN *et al.*, 1994; SEKKALI *et al.*, 1994). False positives can be obtained because of staining of the eye lens and endogenous β-galactosidase activity in the stomach.

Chloramphenicol acetyltransferase (*CAT*) is detected radioactively with a high degree of sensitivity in transgenic tissue extracts and immunohistochemically (see STUART *et al.*, 1990). However the need to work radioactively and the long protein half-life of 50 h (VOLCKAERT *et al.*, 1994) represent major drawbacks.

Luciferase (*luc*) originates from *Photinus pyralis* (firefly) and encodes the enzyme luciferase which can be detected with photon

acquisition systems (scintillation counter, luminometer, X-ray film and CCD camera) and immunohistochemically (see GIBBS *et al.*, 1994; MAYERHOFER *et al.*, 1995; VOLCKAERT *et al.*, 1994). It is currently the most sensitive and one of the most simple systems to use. Advantages include the absence of endogenous background activity, the simple polypeptide structure of luciferase, the linearity over a range of 10^6 and the half-life of 3h (SEKKALI *et al.*, 1994).

Green fluorescent protein (GFP) originates from the jellifish *Aequorea victoria* and produces a bright green fluorescent product under UV light (AMSTERDAM *et al.*, 1995). It does not seem to be harmful to the transgenic fish and does not require exogenously added product. Moreover, spectrally shifted mutants have been isolated (DELAGRAVE *et al.*, 1995).

The tyrosinase enzyme which is involved in pigmentation, has been introduced in mutants of medaka (MATSUMOTO *et al.*, 1992). The melanin concentrating hormone (MCM), also involved in pigmentation is another candidate reporter gene (ALESTRÖM *et al.*, 1992).

Selectable markers such as neomycin phosphotransferase (*neo*), an antibiotic resistance gene, can be used to select fully transgenic animals (see attempt by ZELENIN *et al.*, 1991). However, mosaic transgenic fish (the most common case in parental populations) will also be killed despite *neo* expression in a limited number of cells. This makes *neo* less suitable for transgenic research.

The third group of transgenes, called loss-of-function genes, can be divided in two: transgenes whose product interferes with the normal expression of another gene and transgenes that handicap the normal transcription of a gene through DNA insertion (enhancer-trap and gene-trap). Although this approach has not been used that much in fish for various reasons, there are a few cases. Transgenes that produce antisense mRNA of GnRH to block the normal expression of GnRH belong to the first strategy (ALESTRÖM *et al.*, 1994).

BAYER and CAMPOS-ORTEGA (1992) developed a zebrafish line with preferential expression in primary sensory neurons, which showed all the characteristics of an enhancer-trap. More recent is the insertional mutagenesis in chemically mutated zebrafish which facilitates the cloning of genes corresponding to a specific

phenotype (GAIANO *et al.*, 1996a). Although the latter does not fully correspond to the gene-trap qualification, it sets a standard for this kind of research.

Germline transmission

A second important phase in achieving stable transgenic fish is the permanent establishment of the transgene in the host genome. The transgene has to be incorporated in germ cells to be heritable. It has to be mentioned that with current practices transiently transgenic fish are the most likely outcome of any attempt to insert transgenic constructs. In such case plasmid-like structures amplify in the nucleus but outside the chromosomes during early embryonic development and disappear sometimes later during embryonic development (GONG and HEW, 1995; IYENGAR *et al.*, 1996; VOLCKAERT *et al.*, 1994). Reviews on the techniques to induce transgenes have been prepared amongst other by CLOUD (1990), GONG and HEW (1995), HOUDEBINE and CHOURROUT (1991), MACLEAN and RAHMAN (1994) and OZATO *et al.* (1989).

The first developed, most efficient and still most commonly used technique is micro-injection. Essentially, a micropipette is introduced in the oocyte or the newly fertilised zygote and a small amount of a physiological solution containing the DNA construct is injected. In the first case the target is the nucleus (see OZATO *et al.*, 1989); in the second case the target is the cytoplasm because the polar body can not be localised (WINKLER *et al.*, 1991). Usually about 10^4 to 10^7 copies of the construct are introduced; lower amounts jeopardise transformation and higher amounts tend to be toxic (10^7 construct copies is about equivalent to the DNA content of the nucleus). Fish eggs are relatively large ranging from 1 mm (zebra fish) to 7 mm (salmon), and show various degrees of transparency due to the presence of yolk. The cortical reaction influences the penetration of the chorion to various degrees. Therefore injection occurs in the blastodisc or through the micropyle shortly after fertilisation (VOLCKAERT *et al.*, 1994), or in dechorionated eggs (CULP *et al.*, 1991). DNA should ideally be injected into the nucleus or pronucleus of the egg so that integration

in the host genome is facilitated. If integration occurs during early development (blastula), all cells have a chance to be transgenic, although this depends also on the nature of the construct.

Sperm incubation might seem to be the easiest way to transfect organisms, but the results are variable (CHOURROUT and PERROT, 1992; KHOO *et al.*, 1992). It is thought that DNA might adhere to the cell wall and be introduced in the egg during fertilisation. An alternative is the electroporation of the sperm (MÜLLER *et al.*, 1993; SYMONDS *et al.*, 1994). Short electrical pulses permeate the cell membrane temporarily and thus facilitate the entry of macromolecules such as DNA into the cytoplasm. It allows to treat numerous eggs simulataneously, is highly reproducible and eliminates skilled labour.

Gene bombardment involves the bombardment of cells by high-velocity microprojectiles of small tungsten particles coated with a DNA construct (ZELENIN *et al.*, 1991). Although highly succesful in plant tissues, the absence of a impermeable cell wall of cellulose seems to inhibit transformation.

The inclusion of DNA in lipophylic particles (lipofection) which fuse with the cell membrane efficiently transfects bacteria and eukaryotic cells. It is suitable for use with fish sperm and dechorionated embryos. Transient expression of lipofected eggs of African catfish has been documented by SZELEI *et al.* (1994).

Retroviral infection has been reported above. Most remarkable is that its efficiency in transfecting is several orders higher than the previously mentioned methods. A drawback is that the modified retrovirus has to be injected.

However in all these methods, integration of the transgene in the host genome occurs at random. Homologous recombination, mostly aiming at selective disruption of a target gene or insertion of a reporter gene, is routinely achieved in mice because of the availability of totipotent embryonic stem cells (ES). In a first step, a targeting vector containing about 8 kb of the gene of interest interrupted by sequences encoding a selectable marker is introduced into ES cells. Selection and genomic DNA analysis identify ES cell clones with the appropriate integration event: these are injected into blastocysts to generate chimaeric animals.

Method	Ease of protocol	Parental expression	Stable inheritance
Bombardment	++	YES[1]	NO
Electroporation	++	YES[2]	NO
Lipofection	+	YES[3]	NO
Micro-injection	++	YES[4]	YES[5]
Retroviral infection	+++	YES[6]	YES[6]
Sperm incubation	+	YES[7]	NO

Table 1
The efficiency of various methods to deliver DNA as reported in the literature for ease of protocol, parental expression and stable inheritance. [1] ZELENIN *et al.*, 1991; [2] MÜLLER *et al.*, 1993; [3] SZELEI *et al.*, 1994; [4] Numerous reports, including VOLCKAERT *et al.*, 1994 and WINKLER *et al.*, 1992; [5] STUART *et al.*, 1990 and OZATO *et al.*, 1986; [6] GAIANO *et al.*, 1996b; [7] SYMONDS *et al.*, 1994.

If these have germ cells derived from the recombinant ES cells, they will transmit the genotype to their progeny. No totipotent ES cells are available yet in fish systems that would allow homologous recombination, but several laboratories are investigating exactly that. To this purpose cell transplants among zebrafish blastulae and the production of germ-line chimaeras (LIN *et al.*, 1992), embryonic stem cell culture (COLLODI *et al.*, 1992; HONG and SCHARTL, 1996) and suitable vectors for site-specific integration (IZSVAK *et al.*, 1995; BEARZOTTI *et al.*, 1992) are under development.

Expression of the transgene

Correct expression of the transgene is of course the major aim of transgenic integration. However, it is labour intensive to identify stable transgenic fish with reliable transgenic expression because of the high level of chimaeras observed. Several strategies have been developed to facilitate selection: *in vivo* detection of stable integration on genomic DNA extracted from skin tissue of progeny after proteinase K digestion (KAWAKAMI and HOPKINS, 1996). *In vivo* detection of expression by assaying enzymatic activity of Luc

(MAYERHOFER *et al.*, 1995), tyrosinase (MASTUMOTO *et al.*, 1992) and lacZ (LIN *et al.*, 1994) and by assaying fluorescence of GFP (AMSTERDAM *et al.*, 1995). Other screening methods such as fluorescence *in situ* hybridisation and histochemical staining to verify expression are rather time-consuming.

The fate of the DNA construct is remarkable immediately after introduction (transient replication) and corresponds more to the general expectations in the long term (heritable or stable integration).

Transient replication

The fate of the DNA upon introduction in the transgenic fish system behaves differently from mammals. Soon after being introduced in the cell, the circular fusion genes form head-to-tail concatemers while the linearised fusion genes form random arrangements of head-to-tail, tail-to-tail and head-to-head concatemers in the nucleus (IYENGAR *et al.*, 1996; VOLCKAERT *et al.*, 1994). During the rapid cleavage stages of early development the fusion DNA is quickly replicated at the time of rapid DNA synthesis in the developing embryo up to the 10^{th} or 11^{th} cell division. However after the midblastula transition, degradation rates exceed replication rates and DNA fragments of 200 bp long are formed. The pattern is similar to other lower vertebrates such as *Xenopus* (PRIOLEAU *et al.*, 1994).

Transient expression is in some cases tissue-specific (promoter of the carp myosin heavy chain isoform in zebra fish and catfish (IYENGAR *et al.*, 1996)) while not in others (for example the prolactin promoter in catfish (personal data) and the ubiquitous promoters (ALAM *et al.*, 1996)). However, transient expression seems to be highly influenced by the embryonal tissue. The yolk syncitial layer shows the highest level of expression and thus may influence transient expression in studies using quantative comparative analysis (WILLIAMS *et al.*, 1996).

The long term fate of introduced DNA

Exogenous DNA may have integrated into the host's chromosomal DNA after surviving the degradation process of the cell during early development. The chances of parental integration vary from absent, over poor (most cases, *e.g.* INOUE *et al* (1992) and SHEARS *et al.* (1991)) to 80 % (GAIANO *et al.*, 1996a). The site of integration is thought to be aspecific; often multiple sites of integration are observed (IYENGAR *et al.*, 1996). Moreover transgene expression is often impaired by the sequences flanking its integration site (the so called position effect) since most genomic DNA is considered to be transcriptionally inactive heterochromatin. Another important factor silencing expression is *de novo* methylation of DNA (GIBBS *et al.*, 1994; IYENGAR *et al.*, 1996). Methylation of cytosine residues at CpG sites in the promoter seem to be occurring. Genetic background of the host genome, species-specific developmental dynamics and environmental influences on gene expression may also play a role (KAPUSCINSKI and HALLERMAN, 1991). Stable integration is proven by means of F_1 (a 50 % inheritance is expected) and F_2 (a 100 % expected inheritance is expected) test crosses, Southern blotting of transgenic gDNA, selective amplification by the polymerase chain reaction (PCR) of the introduced sequence or fluorescence *in situ* hybridisation of the karyotypes.

Transgenic fish in aquaculture

Sometimes the novelty of the genetic engineering of transgenic fish has generated exaggetrated expectations in regards to its applications among scientists, fish farmers, administrators and the general public. Delayed maturation, enhanced freezing tolerance, disease resistance and especially enhanced growth of transgenic fish have caught the imagination. The following paragraphs will focus on three cases. In the next chapter the environmental, consumer and ethical aspects of transgenic fish are highlighted.

Growth enhancement

The growth hormone (GH) gene has been cloned in at least 17 fish species (WALLIS, 1996). It encodes for the important circulating peptide growth hormone, secreted in the pituitary and influencing amongst others metabolism and seawater adaptation. It draws special interest because of its growth promoting action when provided exogenously or transgenically. Initial attempts were marred by poor inheritance (an exception is INOUE *et al.*, 1990) and expression, which has been related to the nature of the construct, the mammalian origin, methylation or the absence of regulatory intron sequences. Presently an "all-fish" construct of chinook salmon GH under control of the ocean pout AFP promoter has induced dramatic growth increases (DEVLIN *et al.*, 1994b, 1995a, 1995b). On average the transgenic fish were 10 to 30 times heavier than the control group at 15 months of age. At present, it is not clear to what extent the endocrinology (*e.g.* the titer of growth hormone) and growth rate correlate, nor how the excess GH binds to the receptors. Transgenic coho salmon underwent precocious parr-smolt transformation during their first fall, about 6 months in advance to their non-transgenic siblings. It has to be mentioned that the unregulated overproduction of GH induces side effects such as craniofacial abnormalities (DEVLIN *et al.*, 1994a), colour change (DEVLIN *et al.*, 1995b), excessive fat deposition, and so on. Similar but less dramatic effects have been documented with growth hormone driven by the metallothionein promoter in coho salmon (DU *et al.*, 1992) and pike (GROSS *et al.*, 1992).

Cold tolerance

Arctic fishes synthesize Antifreeze Protein (AFP) which lowers the freezing point of the plasma. AFP is synthesised in the liver and then released in the circulatory system. The winter flounder type I AFP gene is seasonally regulated and contains a well characterised promoter (GONG *et al.*, 1991). Commercial fish with the AFP gene would be in a commercially advantageous position if they tolerated water temperatures below -0.7°C. Attempts are under way to transfer this gene into Atlantic salmon (SHEARS *et al.*, 1991) and

goldfish (WANG *et al.*, 1995) such that their tolerance to freezing can be lowered. About 3% of Atlantic salmon embryos injected with the AFP gene expressed AFP, although at low levels (10 to 50 µg/ml instead of 2-5 mg/ml). Inheritance has been shown, but further research is required before a commercial system is available. To the contrary F_2 and F_3 progeny of goldfish had a lower cold tolerance.

Disease resistance

An important aim of transgenic fish could be to create a barrier against viral and bacterial infections. One only has to think of the impact of furunculosis infections by the bacterium *Aeromonas salmonicida* causing high fish mortalities or Infectious Hematopoetic Necrosis Virus (IHNV), a rhabdovirus affecting Northwest Pacific salmonids. Several strategies are possible: either a protein is produced transgenically for vaccination, either genes are incorporated that eliminate viral or bacterial infections. Lysozyme has been shown to be a potent inhibitor of gram negative bacteria. Its antibacterial activity can be enhanced by protein engineering (IBRAHIM *et al.*, 1994). A rainbow trout lysozyme cDNA has been isolated (DAUTIGNY *et al.*, 1991) and spliced to the ocean trout AFP promoter. Integration in the Atlantic salmon genome is in progress (IYENGAR *et al.*, 1996).

Biosafety

Concerns have surfaced in conjunction with the high expectations from fish with novel genes. Researchers, administrations and the public soon discovered that the new opportunities offered by fish with new traits included as well postive as negative elements. Comments in the scientific literature and press focus on environmental issues, ethical issues and consumer concerns.

Central in the environmental concerns is the question whether escaped transgenic fish will transfer their special genes to wild and natural populations and whether these genetically modified organisms (GMOs) will be selected for. Will GMOs establish themselves permanently outside the confined environment of the aquaculture facility? The question is the more crucial as a growing number of wild species, including fish, go extinct due to habitat destruction, pollution, introductions, hunting and fishing. The accidental or conscious release of transgenic organisms may influence this process and pose a real threat to the natural ecosystem.

Evidence for the introgression of transgenics are not available yet and according to some authors the few transgenic fish released are most unlikely to be selectively favoured for (KNIBB, 1997). Transgenic fish have been documented to be released in nature. Well protected land-based hatcheries are operational for transgenic Atlantic salmon in Canada and Scotland. Some of the environmental concerns refer also to accidental escapes since transgenics are usually generated from domesticated fish and thus may be held in various kinds of containment facilities (land-, ocean- or lake-based). Containment may involve different options; biological containment refers to strategies such as sterilisation and monosex populations, chemical containment refers to the treatment of effluents with chlorination/dechlorination while physical containment varies from minimal options (a fish pen in the ocean) to maximal options (indoor research laboratories with discontinuous outflow) (KAPUSCINSKI and HALLERMAN, 1990). However it is and never will be perfect; the weakest link will determine the chances to escape. Escapes of Atlantic salmon off Norway have reached levels where the domesticated stock surpasses several times the wild stock, but legal pressure has curtailed these historic levels of escapees. Hybridisation between native and domesticated Atlantic salmon in a monitored river system in Ireland has revealed that wild animals and crosses between wild females with farmed fish have a high survival and fitness (CROSS, *pers. com.*). Farmed fish are often less adapted to the local situation, although their numeric dominance may make resources unavailable for the local stock. An interesting example of reduced fitness is the higher risk to natural predation of growth hormone treated fish because of the higher levels of activity

(JÖNSSON *et al.*, 1996). If introgression will happen, genes are likely to pass through the domesticated males. In general, escapes of transgenics draws similarity to the introduction of exotics, especially if a non-native species or strain is involved.

Another method to estimate the effect of transgenics is modelling based on established species (GLIDDON and GOUDET, 1995). Fitness traits, gene flow, fixation of alleles and behaviour affect the chances that a transgenic escapee breeds with local stock. It is clear that each transgenic line has its own traits and thus will have to be dealt with case by case. In addition, the stability of recombinant DNA in the natural environment is influenced by selection since in the absence of selection the insert will disappear due to the costs of carriage and expression of the recombinant (LENSKI and NGUYEN, 1988).

There may be other ways of reducing the risks of transgenic fish by switching on the transgenic genes "on demand". Promoters / enhancers are such that transcription is only initiated and terminated in selected conditions. An example is the antifreeze promoter which is activated at low temperatures (GONG *et al.*, 1991).

If recommendatations are made towards genetically modified transgenics (GMO), the following aspects should be considered:

(1) Is the phenotype sufficiently characterised? Is another trait induced than anticipated? Has the molecular behaviour of the promoter/enhancer and the gene been anticipated? How many copies are integrated? Does the DNA construct induce novel gene combinations? Is inheritance stable? Is the retrovirus sufficiently specific?

(2) Are there sufficient physical, chemical and biological barriers present in the experimental facility such that escapes are virtually impossible. Otherwise said, is the risk sufficiently assessed? What is the weakest link in the system (HALLERMAN and KAPUSCINSKI, 1995)?

(3) If sufficient information has been collected on the GMO in step (1), has it been part of a quantitative breeding program such that the novel trait can be further characterised and selected for? Inbreeding of GMO's is of concern.

(4) Does the customer run a risk when eating transgenic fish?

In general policies are under review amongst others in the USA, Canada, the European Union and Norway (HALLERMAN and KAPUSCINSKI, 1995).

Ethical aspects and consumer acceptance of GMO's intertwine and pose a serious pressure on the fish industry. In general the public is not in favour although the response depends on the socio-cultural background of the customers. A British survey of customers views on the acceptability of transgenic fish in fish farming showed that 36.5% had no opinion, 34.8% thought it was not acceptable and 28.7% thought it was acceptable (PENMAN *et al.*, 1995). The Canadian industry has reacted with the utmost care such that only one hatchery on the East Coast has taken the challange to breed transgenic Atlantic salmon. Food safety will have to be addressed to avoid that non-desirable side-effects are propagated (BERKOWITZ and KRYSPIN-SORENSEN, 1994; CHATAKONDI *et al.*, 1995).

Patenting of transgenic fish has been envisaged (HALLERMAN and KAPUSCINSKI, 1990b). If successful patents are developed, the fish food industry will likely be dominated by a few companies able to invest in the considerable R&D costs.

The future

Transgenic fish have entered the international forum and will stay there. It is clear that their value as research tool to study the regulation of gene expression, including the molecular biology of development and molecular endocrinology is extremely valuable. Several genes have been under scruteny thanks to this approach and many more are awaiting. It is only the question how many of the 10,000 housekeeping genes and 2,000 or so regulated genes will be studied to a certain level of detail.

Some of the genes isolated will be suitable for applications, either in a biomedical or an agricultural (including aquaculture) context. GH

has proven some potential, but because of imperfect knowledge of its regulation and action, much more research remains to be done. Other applications such as fish as bioreactor (to produce selected proteins in bulk) have potential, although not much has been proven at the moment. The current DNA constructs are surely not optimal. Further fine tuning of the promoter/enhancer region to control expression and the flanking regions to enhance specificity of integration are urgently needed. The research community is targeting this topic (IYENGAR *et al.*, 1996).

Another aspect which requires attention is the introduction of the DNA construct. The aim is specificity, but the lack of fish embryonic stem cells for homologous recombination has meant a serious drawback. Recent progress with modified proviruses looks promising (GAIANO *et al.*, 1996b) but suffers from a poor understanding of fish virology. Future research will likely be redirected towards this field.

An issue which has been somewhat neglected is the introduction of GMOs in current selection programmes. Producers will surely want to continue to make progress with selection of traits such as growth, food conversion, disease resistance and so on. How the transgene will be introduced in the population and maintained remains to be proven. Hybridisation is one option, the production of pure lines another one. Also, the question of multi-locus interactions of DNA inserts and environment-transgene interactions have not been addressed.

Environmental and consumer issues will likely dominate the public forum in the coming years. GMO's are new and society doesn't necessarily have a clear answer to such new issues surfacing. Legislation is under continuous review (reflecting the dynamic state of GMOs) by national and multinational organisations including the Organisation for Economic Cooperation and Development (OECD), the International Council for the Exploration of the Sea (ICES), the Food and Agriculture Organisation (FAO) and the United Nations educational, scientific and cultural organisation (Unesco).

Most likely economic factors and consumer acceptance of GMOs will determine large scale developments. At the moment progress is mostly driven by a scientific push, but interest by the economic community is growing steadily.

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