Review. Fish as biofactories: inducible genetic systems and gene targeting

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Abstract

The production of recombinant human proteins for pharmaceutical products in transgenic animals is progressing slowly. Compared to mammals, fish offer easier genetic manipulation since they produce hundreds of eggs per female and because of the independent development of fish embryos. Traditional methods in the past have employed fertilized egg injections with transgene constructions containing non-specific promoters and random genome integration. In fish, these methods have resulted in mosaicism and extrachromosomal expression. Inducible systems to control the temporal and tissue specific expression and gene targeting methodologies to increase efficiency and control of the locus of insertion of the transgene are, therefore, desirable. All these techniques incorporate stem cell manipulation, classification, selection and/or nuclear transplant of cell lines or stem cells. Some of these systems initially developed for mammalian cells are now available to be tested in fish. A large part of this field still remains unexplored.

Key words: aquaculture, transgenic fish, biopharmaceutical products, genetic manipulation.

Resumen

Revisión. Peces como biofactorías: sistemas genéticos inducibles y de inserción dirigida

La producción de proteínas recombinantes con interés farmacéutico está progresando lentamente en mamíferos transgénicos. Comparados con mamíferos, los peces son más fáciles de manipular genéticamente debido a la producción de cientos de huevos por hembra y al desarrollo embrionario independiente de la madre. Hasta ahora los métodos de obtención de animales transgénicos han empleado la inyección de huevos fecundados con transgenes bajo el control de promotores inespecíficos e integración al azar del transgén en el genoma. En peces dichos métodos han dado lugar a expresión en mosaico y extracromosomal de los transgenes introducidos. Por todo ello, es deseable la introducción de promotores inducibles para un mejor control de la expresión temporal y de promotores específicos de tejidos para localizar la expresión espacial de los transgenes. Además sería deseable el poder insertarlos en sitios definidos del genoma para aumentar el control sobre su expresión. Todas estas técnicas pasan por la manipulación, caracterización, selección y/o transplante nuclear de líneas celulares o células madre. Algunos de dichos sistemas inicialmente desarrollados para células de mamíferos, están ahora disponibles para ser ensayados en peces. La mayoría de ese trabajo está aún por explorar.

Palabras clave: acuicultura, peces transgénicos, productos biofarmacéuticos, manipulación genética

Transgenic animals as biofactories

Transgenic animals offer an alternative way to produce recombinant proteins of pharmaceutical interest. These animal producers of human proteins are called biofactories. The use of fish as biofactories has several advantages such as the large number of eggs produced and their development outside the female, which does not occur in mammals (Rocha et al., 2001).

If progress is to be made in obtaining selected pharmaceutical products, the technological design of transgenic biofactories must be improved. Work carried out to date, both in fish and in other transgenic animals, have revealed the need for more control in transgene expression. It is recommendable to control the time (inducible systems), the space such as the tissue (specific promoters) and the site of insertion in the geno-

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me (gene targeting) to achieve a better efficacy of transgene production and to minimize undesired effects in the transgenic animal.

**New strategies using inducible transgenes, specific promoters and gene targeting**

The conventional strategy of obtaining transgenes uses non-specific continually active promoters, usually viral ones, to increase transgenic expression. The transgene is injected into the nucleus of fertilized eggs where it randomly integrates into the fish genome with a low efficiency (maximum of 10-20% in mice). The potentially transgenic animals obtained are crossbred with non-transgenic animals to confirm whether genetic transmission of the transgene has occurred.

Although conventional transgenic strategies are quite successful, especially in mice, they have certain drawbacks that reduce their value both in basic science and their potential use as biofactories. The most important of these are that: i) the transgene is continually expressed resulting in accumulation of the transgenic protein, ii) the transgene is expressed in all the tissues giving rise to physiological problems in the transgenic animal and iii) the site of integration of the transgene in the genome, which has marked effects on the expression level, cannot be controlled (position effect) producing unexpected effects and gene silencing (Caldovic and Hackett, 1995; Liu *et al.*, 1990). Improvements in this technology have, therefore, focused on regulating the temporal expression of transgenes by incorporating inducible systems, regulating spatial expression by incorporating specific tissue promoters and developing methods to insert the transgenes in specific *loci* in the genome.

These strategies have mostly been used in fertilized eggs but can also be used in gametes before fertilization and in stem cells or in cell lines together with cell transplants to premature embryos or nuclear transplants to enucleated ovules (Rudolph, 1999). After obtaining the transgenic animals desired, these must be classified and assessed to identify their most suitable use, either as biofactories or for other purposes.

In mammals, these methods are restricted to the use of stem cells owing to the low number of eggs fertilized. In fish, however, the selection techniques can be directly applied to fertilized eggs i.e. these can be genetically manipulated in mass and then selected (Hackett and Alvarez, 2000).

In the strategy that uses inducible genes (homologous or heterologous), the switch or inducer that permits control of the temporal expression of the transgene is a molecule that acts on a transcription regulator protein (Figure 1) (Ristevski, 2001). The ideal induction system should permit a rapid and reversible control in the desired time (Gao *et al.*, 1999). The drawbacks that have arisen during the use of these systems in mammals include: expression in the absence of inducer, cellular toxicity and the lack of an inducer response.

In the strategy that uses specific tissue promoters, more fish promoters of the many that exist in each fish species or tissues must be studied so as to be able to clone and use them, since the promoters used to date have been limited to those obtained from animal viruses.

In the gene targeting strategy, several systems have been developed by genetic manipulation of embryonic stem cells *in vitro* followed by transplantation of the selected stem cells into other embryos. Afterwards, the embryos must be selected not only on the basis of their having the transgene but also on their ability to transfer it genetically. Introduction of genes by gene targeting into stem cells is done by increasing recombination either by using large genomic inserts which include the transgene and its flanking regions or by using recombination controlled by sequence specific enzymes (Figure 2).

![Figure 1. Diagram of the regulation of inducible systems.](image-url)
Inducible expression by homologous systems

The first inducible systems used genes of the same species or endogenous genes, also called homologous systems. Many homologous inducible systems developed several years ago in mammals use inducers such as heat, heavy metals or gamma-interferon (Ryding et al., 2001). More recently, homologous systems such as those based on cytochrome P-450 (Exon and South, 2000) or on steroid hormones (Amersham, Pharmacia) have been developed.

Cytochrome P-450 detoxifies hydrophobic compounds. It is only expressed in the presence of its substrate. For example, cytochrome CYP1A1 levels in mammals that metabolize aryl-hydrocarbonated compounds are increased 10,000 times in the presence of these compounds. Although these compounds are usually toxic, some are found in plants and can be used as natural inducers (Exon and South, 2000). A gene homologous to CYP1A1 has been found in fish (Williams et al., 2000).

Steroids have also been used for the development of inducible commercial systems. For example, the trans-
genes introduced in vector PMSG (Amersham-Phar-
macia) are only expressed in the presence of dexame-
thesone.

One advantage of homologous compared to hetero-
logous systems, is that they do not require the gener-
ation of doubly transgenic animals since they endoge-
nously have the regulator gene. However, their disad-
vantages include the fact that they induce ex-
pression of many genes at the same time, they present 
high levels of basal expression without inducer in se-
veral tissues at the same time or relatively low levels of induction (Gao et al., 1999).

Inducible expression by heterologous systems

This class of inducible systems has been developed in other non-vertebrate animal species. The Table 1 
summarizes some of the commercial inducible systems 
we briefly describe below.

One of the most common methods to produce indu-
cible expression of transgenes is that of tetracycli-
e (Schultze et al., 1996). The expression of the gene 
for tetracycline resistance (t) isolated from Escheri-
chia coli, is inhibited by the tetracycline repressor (tR), 
a protein that interacts with the t promoter (Gossen and 
Bujard, 1992). The addition of tetracycline follows its 
reaction with the repressor tR that stops its interaction 
with the promoter, inducing expression of the t gene. 
Some modifications have been introduced into this 
system to make it more controllable. For example, tR 
has been converted into a transcriptional activator 
(tTA) by fusion with a viral protein (VP16). The tTA 
inhibits transcription of the gene in the presence of te-
tracycline (Tet-off). By site-directed mutagenesis, tTA 
is converted into rtTA that is now activated in the pre-
sence of tetracycline (Tet-on). In both cases, the acti-
vation achieved by adding tetracycline, can reach 
10,000 fold. Other variations have been designed in 
this system such as alterations in the promoter to in-
crease expression. A related system, that of strep-
tomyycin, has been used in combination with tetracy-
cline, permitting a double induction (Fux et al., 2001).

Another system used is that of ecdysone. Ecdysone 
is a steroid hormone that induces metamorphosis in in-
sects. It interacts with its receptor to induce the ex-
pression of a set of genes in insects. This system was al-
so altered to include a viral TA and to reduce the possible interference with endogenous receptors (Hoppe et al., 
2000; No et al., 1996). New variations of this system 
continuously appear owing to the advantages it offers 
(Palli, 2001). For example, an in vitro comparison of 
systems controlled by ecdysone and by tetracycline have 
shown that the system controlled by ecdysone pro-
duces a smaller basal expression and greater speed.

One of the oldest inducible systems is that of poly-
merase T7. Polymerase T7 is specific to bacteriophaga 
T7 acting on its specific promoter (Fuerst et al., 
1986). Induction of the transgene controlled by the pro-
motor of T7 can be obtained by controlling the ex-
pression of polymerase T7. The T7 system has been 
used to obtain transgenic zebra fish (Verri et al., 1997) 
and as an assay to control viral cellular fusion in mam-
malian (Nussbaum et al., 1994) and fish (Lopez 
et al., 2001) cells. Difficulties have been encountered when 
these systems have been used in fish owing to the dif-
ferent optimum temperatures required for the activity 
of polymerase T7 (37°C) compared with the optimum 
temperatures required for protein expression in fish 
cells, usually below 20°C (unpublished).

Another system, based on the yeast promoter GAL4, has been manipulated molecularly to make it inducible by mifepristone (Table 1). The commercial 
vector pGene/V5-His contains a promoter of GAL4. 
Without the influence of additional factors this pro-
moter is inhibited. To activate its transcription, a reg-
ulator protein is used that interacts with the GAL4 
promoter in the presence of mifepristone. A GAL4

Table 1. Some commercially available gene inducible systems

<table>
<thead>
<tr>
<th>Name</th>
<th>Original species</th>
<th>Control vector</th>
<th>Transgene vector</th>
<th>Inducer</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>E.coli</td>
<td>pTet-On/Off</td>
<td>pTRE2</td>
<td>doxycycline</td>
<td>Clontech</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>E.coli</td>
<td>pcDNA6/TR</td>
<td>pCD4A/TO</td>
<td>doxycycline</td>
<td>InvitroGen</td>
</tr>
<tr>
<td>Ecdysone</td>
<td>Insects</td>
<td>PVgRXR</td>
<td>PIND</td>
<td>Ecdysone</td>
<td>InvitroGen</td>
</tr>
<tr>
<td>GAL4</td>
<td>Yeast</td>
<td>PSwitch</td>
<td>pGene/V5-His</td>
<td>Mifepristone</td>
<td>InvitroGen</td>
</tr>
<tr>
<td>IPTG</td>
<td>E.coli</td>
<td>PCMVLacI</td>
<td>POPRSVI</td>
<td>IPTG</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

The control and transgene vectors (plasmids) carry an antibiotic for selection of the plasmid in bacteria (Ampicillin, Kanamycin, etc) and an antibiotic for selection of transformant cells (mycophenolic acid, neomycin, zeocin, hygromicin, blasticidin, etc.).
system was used for transgenic expression in zebrafish (Koster and Fraser, 2001).

IPTG is an inducer of the lactose operon that has also been used to induce transgene expression in mammalian cells since it does not produce any adverse effects (Lin et al., 1994b; Sin et al., 1993). The commercial vector pCMVLacI, for example, produces the repressor that blocks transcription of its specific promoter in the absence of IPTG (Ryding et al., 2001). It has not been used in fish.

**Gene targeting systems**

Some systems of gene targeting translocate the transgene from a plasmid to a specific sequence (target site) in the genome. Recombination is induced by the action of a specific enzyme introduced simultaneously in the cell. For the transgene to be translocated to target sites within the genome it must be flanked by sequences recognized by the enzyme. Examples of these systems (name of system/name of target site) are that of the bacteriophage Cre/loxP, that of the yeast Flp/FRT, those of bacterial or fish transposons (Tn/tir) and of retrovirus pseudotypes (V/LRT). While the target sites lox or FRT do not exist in the eukaryotic genomes, tir or LRT are abundant.

Specific enzymes (recombinases, integrases, transposases, etc.) catalyse the recombination between genes flanked by the specific sequences and the target sites in the genome. This action results in the deletion, duplication, integration, inversion or translocation of genes depending on the number and direction of the specific sequences and of the target sites. Therefore, the use of these techniques for the insertion of a gene, requires the incorporation of specific sequences (lox, FLP, tir or LTR) flanking the transgene, incorporation of target sites in the genome (when they are not naturally found) and expression of a recombinase, integrase or transposase enzyme.

Production of the enzymes required can also be controlled spatially and temporally by using inducible promoters (Ryding et al., 2001). The combination of gene targeting with inducible systems is today the most powerful way to control transgene expression (Gao et al., 1999).

The recombinase Cre of a bacteriophage directs recombination between sites flanked by target sequences lox (13 bp of inverted repeats separated by 8 bp). The mice strains «Cre» and «floxed» are conserved in collections (Nagy and Mar, 2001).

The recombinase Flp of yeast directs the recombination between specific FRT and target FRT sequences. In a commercial system (Table 2), the transgene is inserted in the pOG45 vector that contains an FRT and an antibiotic resistance gene. The recombinase Flp is expressed by cotransfection of pOG45 and pOG454 (an expression plasmid of recombinase Flp). Recombinase Flp induces recombination between the FRT of pOG45 and genomic FRT.

The transposase of the transposons (Tn) of bacteria (Tn5 or Tn7) or fish (SB, «sleeping beauty») (Ivics et al., 1997; Izsvak et al., 2000) direct the integration of genes flanked by terminal inverted repeats (tir) into small target sequences in the genome (Coll, 2001). The system can be used to insert markers, promoters and/or a wide variety of control elements. In controlled conditions, there can be a single insertion per target molecule but there can also be more than 1000 per genome (Ivics et al., 1997; Izsvak et al., 1997).

The expression system based on the PCMV murine retrovirus uses optimized vectors to introduce genes in pluripotent cells and/or cell lines. This system uses long terminal repeats (LTR) of the PCMV around the transgene. The virus used does not have the envelope protein and must be produced in packing cells that ex-

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
<th>Transgene vector</th>
<th>Enzyme vector</th>
<th>Target</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre/loxP</td>
<td>Bacteriophage</td>
<td>Many</td>
<td>Many</td>
<td>34 bp</td>
<td>DuPont/Harvard</td>
</tr>
<tr>
<td>Flp</td>
<td>Yeast</td>
<td>pCDNA5/FRT</td>
<td>POG45</td>
<td>FRT</td>
<td>InvitroGen</td>
</tr>
<tr>
<td>FLP</td>
<td>Yeast</td>
<td>pOG45</td>
<td>PNeoGAL</td>
<td>FRT</td>
<td>Stratagene</td>
</tr>
<tr>
<td>Tn7</td>
<td>E. coli</td>
<td>pGPS3</td>
<td>TnsABC</td>
<td>5 bp</td>
<td>NewEngland BioLabs</td>
</tr>
<tr>
<td>Tn5</td>
<td>E. coli</td>
<td>PMOD</td>
<td>E2::Tntrasp</td>
<td>19 bp</td>
<td>Epicentre</td>
</tr>
<tr>
<td>Tn SB</td>
<td>Salmon</td>
<td>pT/BH</td>
<td>pCMV-SB</td>
<td>tir</td>
<td>Univ. Minnesotta</td>
</tr>
<tr>
<td>Retrovirus</td>
<td>Mice</td>
<td>PMSCV</td>
<td>PMSCV</td>
<td>LTR</td>
<td>Clontech</td>
</tr>
</tbody>
</table>

These systems operate throughout enzyme-mediated site-specific recombination.
press the G protein of the virus of vesicular stomatitis. The virus produced in this way can infect a greater number of cell types but cannot replicate. This system has been used to generate numerous insertional mutants in zebrafish (Gaiano and Hopkins, 1996; Lin et al., 1994a).

**Generation of transgenic animals incorporating new systems of temporal and spatial control**

To generate transgenic animals in the case of inducible systems, two must be generated independently, although one doubly transgenic animal can also be obtained. One of the transgenic animals bears the transgene under control of the inducible promoter and the other bears the regulator gene under a constitutive promoter. Crossbreeding of both transgenic animals generates a proportion of offspring with transgene regulation (Schultze et al., 1996).

In the case of gene targeting systems, in addition to requiring the transgene and the insertion site to be flanked by target sequences, expression of the corresponding enzyme is also required. The use of specific tissue promoters also permits expression of the enzyme to be restricted to a specific tissue. However, since expression of the promoter depends on the genomic context where it is integrated, selection must always be made to obtain the most appropriate transgene. To detect transgene expression, bidirectional promoters can also be used which simultaneously control expression of the transgene and of a marker gene, thus permitting a simpler selection of transgenic animals (Baron et al., 1995; Yamamoto et al., 2000).

Only tetracycline in inducible systems and Cre/lox in gene targeting systems have been used to obtain transgenes in a more controlled manner than those traditionally obtained in mammals, especially in mouse.

**Biopharmaceutical products in transgenic fish**

The production of human recombinant proteins in the milk of transgenic animals offers a renewable source of pharmaceutical products difficult to obtain by other means (Houdebine, 2000; Lubo and Palmer, 2000; Rudolph, 1999). In fish, in addition to other advantages, there is the possibility of these products being secreted in mucus or eggs (the milk substitute in mammals) and the advantage of a reduced probability of contamination by human pathogens. However, fish have not yet been used as biofactories (Chen and Powers, 1990; Hackett and Alvarez, 2000; Lin, 2000).

Genetic transfer to fish embryos has been successfully done since 1985 in several species: trout, salmon, carp, medaka tilapia, zebrafish and catfish (Pinkert, 1999). Survival of fish embryos with transgenes injected into their cytoplasm is reasonable and 1 to 5% of transgenic fish reach maturity. It has been demonstrated that many transgenic fish are mosaics, i.e. some of their tissues are transgenic and others are not. Moreover, some transgenic fish have been obtained (Inoue et al., 1990; Murakami et al., 1994) that do not have the transgene incorporated into their genome but that pass the transgene from one generation to the next extrachromosomally (Hackett y Alvarez, 2000; Niiler, 2000). This behavior has not been observed in mammals. According to some authors, this could possibly be used to obtain a better control of transgene expression (Houdebine, 2000; Houdebine and Chourrout, 1991). However, maintenance of transgenic zebrafish in which the transgene is extrachromosomal is too complex unless new technologies are developed to control it. Therefore, to ensure that a transgenic animal is really transgenic, it must be demonstrated in each case that the transgene is intrachromosomal and that there is persistence, integration in the genome, transmission to the descendents and expression of the genes introduced.

Aquaculture has returned with some success to traditional transgenic technology to obtain genetically modified animals with improved properties associated with the growth rate (Dunham, 1999; Izsvak et al., 1997) and resistance to cold or to diseases (Gong and Hew, 1995; Hew et al., 1995). For example, the transgenic Atlantic salmon obtained with the growth hormone gene of Chinook salmon presents a 1,000% increase in bodyweight compared to the non-transgenic animal (Hackett and Alvarez, 2000).

However, to apply induction or gene targeting technologies to fish, stem cells must be obtained and developed. It would also be desirable to have syngeneic «breeds» to increase the probability of successful transplants between embryos, as it occurs in mice. The first line of omnipotential stem cells from fish was obtained from zebrafish and like mammal stem cells, required the presence of feeder cells to be maintained (Sun et al., 1995a, 1995b, 1995c). Afterwards, stem cell lines derived from medaka fish (Hong et al., 1998)
Aquaculture in Spain has been growing over the last few decades, starting with the development of mussel breeding and culture (traditional technology) and of trout (imported technology) (Espinosa de los Monteros et al., 1999). This has been followed by the development of its own specific technology (gilt-head seabream, turbot and seabass farming) which it would be desirable to exploit as fully as possible. The saturation of the markets with some species for human consumption and the large investment made in R&D encourage the search for new technologies such as those that have been reviewed in this work.

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References


Table 3. Research in human biopharmaceuticals in fish

<table>
<thead>
<tr>
<th>Product</th>
<th>Species</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VII</td>
<td>Tilapia</td>
<td>Aquagene (USA)</td>
</tr>
<tr>
<td>Insulin</td>
<td>Tilapia</td>
<td>Philippine Council for Aquatic &amp; Marine Research &amp; Development (USA-Canada)</td>
</tr>
<tr>
<td>Collagen</td>
<td>Unknown fish</td>
<td>Meanwhile Shida Canning Co Ltd (Japan)</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Salmon</td>
<td>DiverDrugs (Spain)</td>
</tr>
</tbody>
</table>

Data modified from Bostock (1998).


NIILER E., 2000. FDA, researches consider first transgenic fish. Nature Biotechnol 18, 143


