Spanish Journal of Agricultural Research (2003) 1 (3), 3-11

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 farmaceútico 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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Received: 05-04-02; Accepted: 07-05-03.

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 embrionary 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).

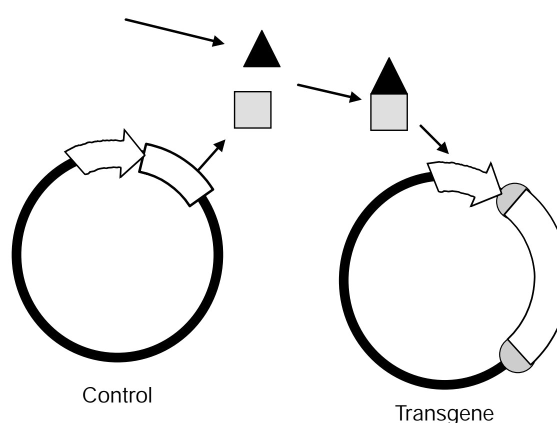

Figure 1. Diagram of the regulation of inducible systems. In the plasmid containing the transgene, transcription of the transgene (▭) is induced by the regulator protein (□) activated by the inducer (▲) acting over the transgene promoter (⇨). The control plasmid contains the regulator gene under a constitutive promoter. Both plasmids also contain an antibiotic gene for selection in eukaryotic cells and other antibiotic gene for selection in bacterial cells.

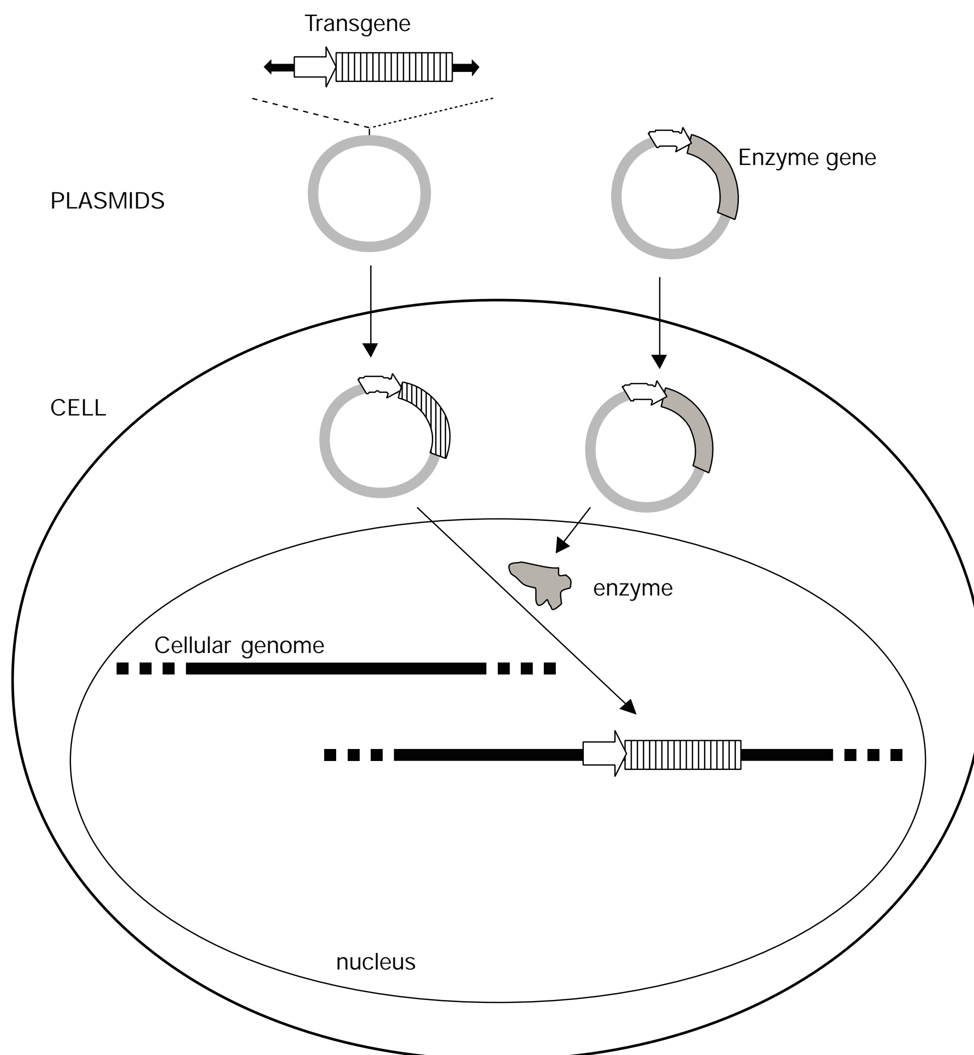

Figure 2. Diagram of a procedure to integrate transgenes in cells using gene targeting techniques. The system requires two plasmids. In one of them a recombination enzyme (▭) and in the other a transgene (▥) flanked by special target sequences (➧). Two plasmids are contransfected in one cell. The enzyme is expressed under a constitutive promoter and then integrates the transgene in the target sites in the genome. If the gene of interest is included together with an antibiotic selection gene, we can then select for resistance to the antibiotic. Only cells with the gene integrated in the genome will survive long enough. Since the enzyme gene plasmid will be lost during cell divisions the integration will be stable.

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-Pharmacia) are only expressed in the presence of dexamethasone.

One advantage of homologous compared to heterologous systems, is that they do not require the generation of doubly transgenic animals since they endogenously have the regulator gene. However, their disadvantages include the fact that they induce expression of many genes at the same time, they present high levels of basal expression without inducer in several 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 inducible expression of transgenes is that of tetracycline (Schultze *et al.*, 1996). The expression of the gene for tetracycline resistance (t) isolated from *Escherichia 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 tetracycline (*Tet-off*). By site-directed mutagenesis, tTA is converted into rtTA that is now activated in the presence of tetracycline (*Tet-on*). In both cases, the activation achieved by adding tetracycline, can reach 10,000 fold. Other variations have been designed in this system such as alterations in the promoter to increase expression. A related system, that of streptomycin, has been used in combination with tetracycline, permitting a double induction (Fux *et al.*, 2001).

Another system used is that of ecdysone. Ecdysone is a steroid hormone that induces metamorphosis in insects. It interacts with its receptor to induce the expression of a set of genes in insects. This system was also 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 produces a smaller basal expression and greater speed.

One of the oldest inducible systems is that of polymerase T7. Polymerase T7 is specific to bacteriophage T7 acting on its specific promoter (Fuerst *et al.*, 1986). Induction of the transgene controlled by the promoter of T7 can be obtained by controlling the expression 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 mammalian (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 different optimum temperatures required for the activity of polymerase T7 (37ºC) compared with the optimum temperatures required for protein expression in fish cells, usually below 20ºC (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/VS-His contains a promoter of GAL4. Without the influence of additional factors this promoter is inhibited. To activate its transcription, a regulator protein is used that interacts with the GAL4 promoter in the presence of mifepristone. A GAL4

Table 1. Some commercially available gene inducible systems

Name	Original species	Control vector	Transgene vector	Inducer	Company
Tetracycline	*E.coli*	pTet-On/Off	pTRE2	doxycycline	Clontech
Tetracycline	*E.coli*	pcDNA6/TR	pCDA4/TO	doxycycline	InvitroGen
Ecdysone	Insects	PVgRXR	PIND	Ecdysone	InvitroGen
GAL4	Yeast	PSwitch	pGene/V5-His	Mifepristone	InvitroGen
IPTG	*E.coli*	PCMVLacI	POPRSVI	IPTG	Stratagene

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.).

Table 2. Some available gene targeting systems

Name	Origin	Transgene vector	Enzyme vector	Target	Company
Cre/loxP	Bacteriophage	Many	Many	34 bp	DuPont/Harward
Flp	Yeast	pcDNA5/FRT	POG44	FRT	InvitroGen
FLP	Yeast	pOG45	PNeoßGAL	FRT	Stratagene
Tn7	*E.coli*	pGPS3	TnsABC	5 bp	NewEngland BioLabs
Tn5	*E.coli*	PMOD	E2::Tntrasp	19 bp	Epicentre
Tn SB	Salmon	pT/BH	pCMV-SB	tir	Univ. Minnesotta
Retrovirus	Mice	PMSCV	PMSCV	LTR	Clontech

These systems operate throughout enzyme-mediated site-specific recombination.

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-

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 (Inonue *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 intrachromasomal 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 resistence 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 medakafish (Hong *et al.*, 1998)

and gilt-head seabream were developed (Bejar *et al.*, 1999). These last two lines do not require the presence of «feeder» cells to remain undifferentiated, have a typical stem cell morphology (small size, large nuclei, capacity to form colonies, etc.) and a stable karyotype (Hackett and Alvarez, 2000). Moreover, they can be kept in liquid nitrogen and transfected (Bejar *et al.,* 1999; Hong *et al.*, 1998). The potential of these lines to produce fish composed of cells of the receptor embryo and the donor cell line is variable depending on the fish species used and is lower than when the embryos are injected with blastomeres (Hackett and Alvarez, 2000). In medaka, the donor stem cells contribute to the formation of numerous organs and are the first example of hybrids or chimeras obtained with this technology (Bejar *et al.*, 1999; Hong *et al.,* 1998). Chimeras have been obtained in salmonids of the genus *Oncorhynchus* (rainbow trout) by injecting the blastomere in embryos (Nilsson and Cloud, 1992), and it, therefore, seems logical that these can very probably be obtained with stem cells. Although much research is required into stem cell lines in fish, the fact that these have been obtained from such diverse species as zebrafish, medaka and gilt-head seabream suggests that these could also be obtained from other fish species.

Fish offer a unique and little explored opportunity to be used as biofactories of pharmaceutical products and several research projects have been started in this area (Table 3). The use of transgenic fish as biofactories has been developing in the US for several years. For example, the firm PCRMAD is collaborating with Canadian firms to develop transgenic tilapia to produce human insulin and the firm MSC in collagen production also from fish species (Bostock, 1998). In the European Union a network has been set up called the «Fish Biofactory Network» to coordinate research in this area (http://www.inia.es).

Table 3. Research in human biopharmaceuticals in fish

Product	Species	Company
Factor VII	Tilapia	Aquagene (USA)
Insulin	Tilapia	Philippine Council for Aquatic & Marine Research & Development (USA-Canada)
Collagen	Unknown fish	Meanwhile Shida Canning Co Ldt (Japan)
Calcitonin	Salmon	DiverDrugs (Spain)

Data modified from Bostock (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.

Acknowledgements

We would like to thank J. Perez Coll for typing the manuscript. This work was carried out using funding from the project CICYT ACU01003, from projects INIA CPE03-016-C4, SC00046 and RTA03217, Spain and from CCAA OT02003.

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