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# Application of Inducible and Targeted Gene Strategies to Produce Transgenic Fish: A Review

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**Abstract:** Compared to mammals, fishes offer easier transgenic technology because each female produces hundreds of eggs, the manipulated embryos do not need to be incubated inside the mother, and the probability of their harboring human-related pathogens is lower. In the last 15 years, traditional methods using injections of fertilized fish egg's and strong viral promoters have resulted in the generation of many transgenic fish species; however, they showed random genome integration with some mosaicism and episomic expression. The use of inducible gene systems that control temporal and tissue expression and of gene-targeting methodologies based on homologous recombination is desirable to control the expression, efficiency of insertion, and locus of incorporation of transgenes into fish genomes. A variety of systems developed for mammals are now available to be tested in fishes. The use of such systems would require further development of stem cell or nuclear transplant technologies in fish. Most of that work remains to be explored.

**Key words:** inducible gene, targeted gene, transgenic fish, stem cells.

## Transgene Strategies

### Conventional Strategies

The conventional transgenic approach uses very active promoters to drive the expression of a transgene. A transgene seems to have less chance to be expressed efficiently if it contains nonanimal sequences, is devoid of introns, and is integrated in multiple copies. Therefore, all these elements should be included or controlled in the DNA construct. The DNA construct is then injected into the nucleus of single-celled fertilized eggs, where it integrates randomly into the genome with a level of efficiency depending on the species. The founder animal that develops is then crossed with wild-type animals to ensure germline transmission. The conventional approach might also be used in gametes before fertilization (Relloso and Esponda, 1998; Sarmasik et al., 2001).

Despite their great successes, conventional transgenic approaches have some limitations that diminish their usefulness such as low-level production of the recombinant protein, position-dependent expression of the transgene (position effect), and ectopic expression (expression outside the target tissue thought to be due to positional effects). Position-independent expression of transgenes in zebrafish can been obtained by inclusion of border elements (for instance, matrix attachment regions, or MARs)

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in the DNA constructs (Caldovic et al., 1999). However, because the site of chromosomal integration and the sequences around it (Gutierrez-Adan and Pintado, 2000) have a profound effect on the level of expression (Liu et al., 1990; Caldovic and Hackett, 1995), many transgenic lines may have to be screened to identify a suitable one.

## Inducible and Targeted Gene Strategies

Manipulation of stem cells (cell transplant) or adult cells (nuclear transplant) made it possible to introduce inducible and targeted gene strategies in mammals (Rudolph, 1999). A variety of systems have been developed that share the manipulation and selection of stem cells and their subsequent transplantation to early embryos to make chimeras. Chimeras are then used to develop new, germline-modified strains. Another possibility that remains largely uninvestigated is the application of these techniques from either adult or stem-manipulated fish cells to nuclear transplant.

In the inducible (homologous or heterologous) approach, the switching on or off of a target transgene depends on a specific inducer acting over a transcription factor that binds the target promoter (Lewandoski, 2001; Ristevski, 2001) (Figure 1). The constitutive expression of some toxic or deleterious transgenes to the host is thus avoided, and the time and place of the expression can be controlled. A prerequisite for inducible systems is that the inducer or controller be innocuous and that the possibility of other loci being activated is inconsequential. The ideal inducible system should also allow transgene expression to be switched on and off, rapidly, reversibly, in a dosedependent manner, and only in the desired time and tissue (Gao et al., 1999). Limitations that have arisen during the use of these systems in mammals include leaky expression, cellular toxicity, unstable transcripts, and insensitivity to the inducer. In the targeted gene approach, genes are introduced into stem cells via homologous recombination by incorporating long genomic inserts or recombination sequences (Lox, Tn, Flp, LTR) (Figure 2). The combination of gene targeting with inducible gene systems constitutes the highest possible control of gene expression currently available (Gao et al., 1999).

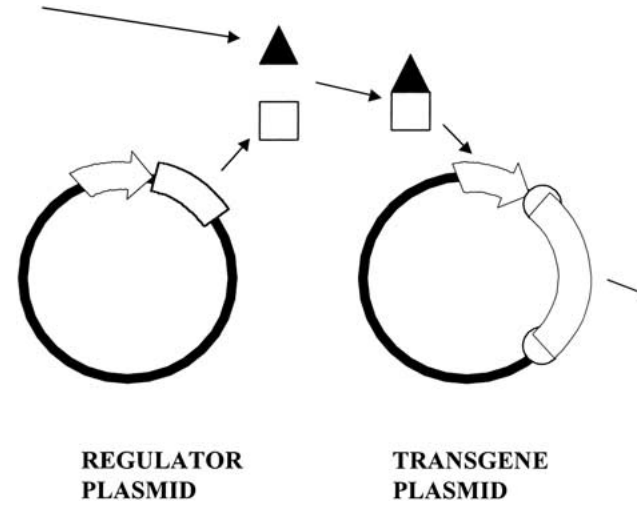


**Figure 1.** Scheme of regulation of gene expression by inducible systems. Transgene transcription is regulated by an inducible promoter activated or repressed by a transcriptional factor (activator or repressor) codified by another gene. The inducible promoter contains RNA polymerase, activator, and repressor binding sequences. To activate transcription by the bound RNA polymerase, both binding of activator and release of repressor are needed. The regulator plasmid contains the transcription factor gene (▭, left plasmid) (activator or repressor) under a constitutive promoter. The transcription factor binds a low molecular weight inducer either to activate (on) or repress transcription (off) (▲). The transgene plasmid contains the transgene gene (▭, right plasmid) introduced into a multiple cloning site (half circles), driven by the inducible promoter ( → , □). Both plasmids also contain antibiotic genes for selection in eukaryotic cells and antibiotic genes for amplification and selection in bacterial cells.

## Homologous-Inducible Expression Systems

Earlier inducible gene systems used endogenous homologous mammal genes. They showed responsiveness to inducers such as heat (shock proteins induced by 42°C), heavy metal ions such as zinc and cadmium (methallothioneins), or immunomodulators such as interferon gamma (Ryding et al., 2001). More recently, better homologous systems have been found and applied such as cytochrome P-450 and steroids.

Cytochrome P-450 enzymes detoxify lipophilic foreign compounds in the liver, their expression being induced by the presence of those substrates. For instance, cytochrome CYP1A1 can be induced 10,000-fold in a dose-dependent manner by aryl hydrocarbons. The main inconvenience of this system is that most aryl hydrocarbons are exogenous compounds that have deleterious effects in vivo, however, indole-3-carbinol can be used as a safe inducer because it is a natural plant aryl hydrocarbon (Exon and South, 2000). The finding of a CYP1A1 gene in fish (Williams et al., 2000) theoretically could make this approach available.

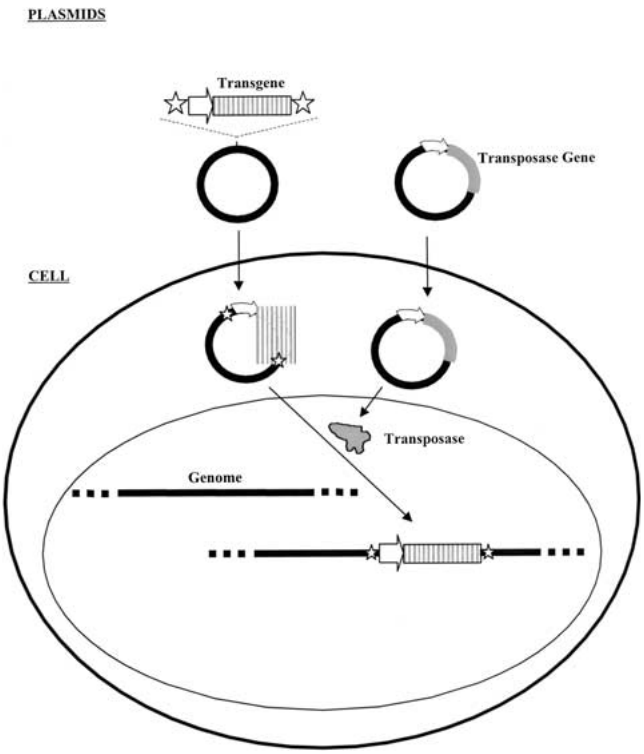


**Figure 2.** Scheme of a procedure to integrate transgenes into the cell genome using transposon techniques. The system requires two plasmids: a plasmid codifying the transposase (hatched rectangle, right plasmid) and a plasmid codifying the transgene (hatched rectangle, left plasmid) flanked by specific short tir sequences (*). The two plasmids are cotransfected in one cell. The transposase is expressed under a constitutive promoter (open arrow, right plasmid) and integrates the transgene into specifically recognized short sequences in the genome. If the transgene of interest is included together with an antibiotic gene, then only the cells with the transgene integrated into their genome will survive long enough in the presence of the antibiotic. Since the transposase plasmid will be lost throughout the cellular divisions, the integration will be stable.

In the pMSG steroid-inducible mammal vector (Amersham-Pharmacia), the transgenes inserted into its multiple cloning site (MCS) are expressed from the mouse mammary tumor virus long terminal repeat (MMTV LTR) promoter, which is inducible by dexamethasone. The pMSG vector contains the *Escherichia coli* xanthine-guanine phosphoribosyltransferase gene under the SV40 promoter to select the transformed cell for mycophenolic acid resistance. However, this system has limited use because of the high levels of unstimulated activity and only a 10- to 20-fold induction on stimulation.

The use of endogenous mammal gene regulatory systems suffers from the simultaneous activation of many genes, the high level of constitutive expression in many tissues, and the relatively low degree of transgene induction (Gao et al., 1999). They can avoid the requirement for doubly transgenic animals needed for most of the heterologous systems. However, heterologous systems are preferred because they do not overlap with mammal genes.

## Heterologous Inducible Expression Systems

Heterologous inducible systems have been developed in species other than mammals and therefore are less affected by some of the problems discussed above (Table 1).

The tetracycline (Tet) resistance gene from *E. coli* is constitutively repressed by the tetracycline repressor (TetR), a protein that binds to the Tet promoter (Gossen and Bujard, 1992). When Tet is added, it binds to the TetR, the Tet-TetR complex separates from the promoter, and the gene is expressed. The TetR has been converted to a transcriptional activator (TetTA) by fusion with a virally coded protein (VP16). The TetTA activates transcription in the absence of Tet (Tet-Off). By mutations, TetTA has been converted to rTetTA, which is now activated in the presence of Tet (Tet-On). Activation can be as high as 10,000-fold. The two systems are functionally equivalent when transferred into mammals. Doxycycline is generally used instead of Tet because it is active at 100-fold lower concentrations.

The major disadvantage of the Tet systems is that control of the expression is often leaky because of strong positional effects. This requires the generation of several transgenic animal strains to identify those that express the transgene not constitutively, but in an inducible manner. Recent reports described improvements of the Tet systems such as transactivators with less toxicity, different ligand sensitivities, bidirectional Tet minimal promoters, use of streptogramin for dual regulated expression (Fux et al., 2001), reduced leakness (Jaisser, 2000), and combination with adenovirus-based vectors (Clontech). The Tet system is one of the most commonly used to produce transgenic mammals (Schultze et al., 1996).

Ecdysone is a steroid hormone that triggers insect metamorphosis. It binds to its heterodimeric receptor EcR-USP to induce expression of a set of insect genes. The system was also altered to include a viral TA and to reduce cross reactivity with the endogenous mammal receptors. Because mammal RXR, the homologue of insect USP, functions also with the insect EcR (No et al., 1996), ectopic expression is possible. To avoid ectopic expression, EcR

**Table 1.** Human Biopharmaceuticals in Fish[a]

| Product | Species | Company | Target organ |
|---|---|---|---|
| Factor VII | Tilapia | Aquagene (U.S.A.) | Mucus |
| Insulin | Tilapia | Philippine Council for Aquatic & Marine Research & Development (U.S.A.–Canada) | Unknown |
| Collagen | Unknown fish | Meanwhile Shida Canning Co Ltd (Japan) | Unknown |
| Human calcitonin | Salmon | Diver Drugs (Spain) | Mucus |
| Pleurocidine | Salmon | Diver Drugs (Spain) | Mucus |
| Human defensins | Salmon | Diver Drugs (Spain) | Mucus |

[a]Data modified from Bostock (Bostock, 1998; Coll, 2000).

from other insect species that do not require RXR might be used (Hoppe et al., 2000). New variations are being developed continuously, such as bicistronic plasmids carrying both receptors (Palli, 2001; Wyborski et al., 2001). There remain technical difficulties in establishing stable ecdysone-inducible strains, such as leakage or low or lack of induction of the transgene expression. It is therefore advantageous to first establish a strain or cell line that stably expresses the receptor proteins and then test the induction of the transgene by using transient expression systems (Wakita et al., 2001).

T7 RNA polymerase is specific for bacteriophage T7 and it acts over a specific promoter (Fuerst et al., 1986). Plasmids expressing the T7 polymerase are available in the scientific community (Lopez et al., 2001), whereas the T7 promoter is commercially available. Difficulties have been found in the use of the T7 system in fish because of the difference between the optimal temperature for T7 polymerase activity (37°C) and the optimal temperature for protein expression in fish cells, which is generally lower than 30°C (for instance, salmon cells grow optimally at 20°C; not shown). The T7 system has been used to produce transgenic zebrafish (Verri et al., 1997).

The commercially available vector pGene/VS-His provides the hybrid promoter GAL4-E1b, consisting of binding sites for the yeast GAL4 and for the adenovirus E1b factor (In VitroGen Life Technologies). Without additional factors the GAL4-E1b promoter is transcriptionally silent. To activate its transcription, a regulatory protein is expressed from a thymidine quinase promoter on the plasmid pSwitch. The regulatory protein contains a mifepristone-binding domain and a transcription activation domain. Mifepristone binds to the protein and activates transcription from the GAL4 promoter. The GAL4-E1b activator and its GAL4-E1b-dependent transgene can be also placed on a single expression vector. Furthermore, GAL4-E1b can drive the expression of two or more transgenes from the same construct, resulting in simultaneous coexpression of the genes. A modification of this system, GAL4-VP16, is capable of driving expression of green fluorescence protein (GFP) in zebrafish embryos (Sadowski et al., 1988). This expression system works not only in zebrafish but also in *Xenopus*, chicken, mouse, and human cultured cells (Koster and Fraser, 2001).

IPTG, an inducer of the Lac operon, has also been used to induce expression in mammal and fish cells (Sin et al., 1993; Lin et al., 1994b). The commercially available pCMVLacI vector (Stratagene) produces the Lac repressor (LacR) that blocks transcription by binding to the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter of vectors carrying the transgene (pOPI3CAT or pOPRSVI/MCS). IPTG, which has no adverse effect in eukaryotic cells, decreases the binding affinity of LacR for the regulatory sequences, triggering transcription and expression of the transgene.

## Gene Targeting Systems

For over the last 15 years numerous experiments using traditional microinjection have shown that the expression of a transgene is highly unpredictable. Among the major limitations of microinjection are the low level of transgene expression and the random and low rate of transgene integration. In addition, transgene copy number is difficult to control. Usually integration is a singular event with multiple copies integrated in tandem. A high copy number often results in a gene-silencing effect, most probably because repetitive DNA sequences induce organization into transcriptionally inactive heterochromatin.

**Table 2.** Some Commercially Available Gene Inducible Systems[a]

| Name (species) | Regulator plasmid | Transgene plasmid | 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 |

[a]The transgene plasmids include an MCS to clone the transgene. The regulator and transgene plasmids carry a prokaryotic antibiotic for selection and amplification in *E. coli* (ampicillin, kanamycin) and a eukaryotic antibiotic for selection of transformed cells (mycophenolic acid, neomycin, zeocin, hygromicin, blasticidin). The ecdysone system also uses other more effective inducers such as the ponasterone A, muristerone A or GS-E.

One type of gene-targeting system includes larger genomic sequences in the constructs to be transfected, because low expression might be due to the fact that promoters, enhancers, and other signals are not included in the constructs based on cDNA transgenes (vanDen Velden et al., 2001). To improve expression, the alternative consists in using long genomic DNA fragments (100 to 300 kb) contained in yeast artificial chromosome (YAC) or in bacterial artificial chromosome (BAC) vectors (Fujiwara et al., 1999a, 1999b; Stinnakre et al., 1992). These long genomic DNAs, as opposed to cDNA-derived genes plus heterologous promoters and regulators, are expected to contain all the native elements required to obtain satisfactory transgene expression (gene insulators, chromatin openers, matrix attachment regions, enhancers, introns, and unknown signals). In addition, to contain all those native regulatory sequences, long genomic DNAs will be targeted by homologous recombination so that the percentage of insertion into the genome at random sites will decrease. YAC technologies, owing to their capacity to stably propagate fragments of genomic DNA longer than 2 Mb, provide an ideal basis from which to obtain those complex transgenics. However, large DNAs are difficult to handle, to microinject, and to integrate with high efficiency, even though some of these problems could be overcome by incorporation of antibiotic resistance markers followed by transfection into stem cells and selection. Few results using any of these methods, especially in fish, have yet been reported (Houdebine, 2000).

Another type of gene targeting is based on mobilization of a gene or genes from a plasmid to a site-specific position in the cell genome by the action of DNA-cutting and -pasting enzymes (recombinase, integrase, and transposase). Such recombination-insertion events result in deletion, duplication, integration, inversion, or translocation of sequences. Both the transgene to be translocated and the target position are generally flanked by small sequences (approx. 40 bp) recognized by the responsible enzyme (Table 2). Examples of these systems are the bacteriophage Cre/lox, the yeast Flp/FRT, the bacterial or fish transposons (Tn/tir), and the pseudotyped retroviral system (LTR). Whereas the lox or FRT target sites do not exist in eukaryotic genomes, the Tn or LRT sequences are abundant in those genomes.

The use of these techniques sometimes requires the incorporation of two target sites flanking the transgenes (lox, Flp, tir, or LTR flanked genes) and the expression of a recombinase, integrase, or transposase under a promoter in those cells in which one wants the exchange to occur (Figure 2 shows an example using transposase). Genes would be inserted with a higher frequency in those sites in the genome that either spontaneously or by manipulation would contain the specific target sites, which may or may not be identical to the flanking sequences. By using inducible promoters, the timing of the enzyme induction could also be controlled (Ryding et al., 2001). Mosaicism and ectopic expression can be problems in the application of these techniques.

The recombinase Cre of the bacteriophage P1 directs recombination between sites that have flanking lox target sequences (13-bp inverted repeats separated by an 8-bp spacer). Two vectors are generally used, but a single vector containing Cre and lox has also been described (Kaczmarczyk and Green, 2001). Transgenic Cre and ''floxed'' mice strains are available in a collection (*http://www.mshri.on.ca/nagy/cre.htm*) (Nagy and Mar, 2001).

The Flp integrase from *Saccharomyces cerevisiae* (ATCC 9763) directs recombination between unique FRT gene and target sites. In a commercially available system, the transgene is inserted into the pOG45 targeting vector, which

consists of an FRT, an antibiotic resistance gene, and a MCS. The integration is obtained after cotransfection of the transgene in the pOG45 and the FLP recombinase expression plasmid pOG454.

The transposons from bacteria or from fish (''sleeping beauty'', or SB) (Ivics et al., 1997; Izsvak et al., 1997) direct integration of genes flanked by terminal inverted repeat (tir) sequences to sites that have small target sequences in specific sites in the genome (Coll, 2001b). The bacterial transposon (Tn) systems permit random insertion with very high efficiency of a gene flanked by transposase recognition sequence. Tn7 or Tn5 integrates randomly into the target DNA; however, because of target immunity there is only one insertion per several kilobases most of the time. The Tn SB integrates in specific small sequences in the genome, the number of integration events being restricted by the size of the insert. The transposon systems can be used to insert into the target sequences different selectable or screenable markers, regions of homology, tags, promoter regions, or a wide variety of structural and control elements.

The murine stem cell virus retroviral expression system uses vectors optimized for introducing genes into pluripotent cells or any mammalian cell line. They employ flanking LTRs from the murine stem cell virus PCMV. The coat protein–less virus is pseudotyped in a packaging cell line that introduces the protein G of vesicular stomatitis virus. This system has been used extensively for insertional mutagenesis in zebrafish (Lin et al., 1994a; Gaiano and Hopkins, 1996). Combination of retrovirus-mediated gene insertion with the ecdysone-inducible system has been reported in mammalian cells (Stolarov et al., 2001).

## Use of Transgenic Technology in Fish

Microinjection of linear DNA constructs into eggs has been reported since 1985 in fish species including trout, salmon, carp, tilapia, medaka, goldfish, zebrafish, loach, and catfish (Lin, 2000). Currently the number of genetically manipulated species is higher for fish than for the rest of all vertebrate species (Pinkert, 1999). Most fish embryos survive injections, and 1% to 5% of them may be transgenic. After years of development there are a few examples of how to overcome technical difficulties in the aquaculture of transgenic fish.

Faster-growing fish have been obtained by incorporating growth hormone genes of humans or other fish species, or both, in carp, catfish, loach, tilapia, pike, and Atlantic and Pacific salmon. Among all these, the ''all-fish'' gene constructs displayed higher expression efficiency (Du et al., 1992). Second-generation transgenic Atlantic salmon containing a general tissue fish promoter linked to the salmon growth hormone gene expressed growth hormone in all tissues, not only in the pituitary gland. Those transgenic salmon (Devlin et al., 1994) entered seawater almost a year earlier than their nontransgenic siblings (Hew et al., 1999). Transgenic salmon brood stock are now being developed at Prince Edward Island, Canada, by A/F Protein Canada, but there are serious concerns about safety and environmental impacts (Reichhardt, 2000).

A different example was the transfer between fish species of the antifreeze protein (AFP) gene to confer freeze tolerance, since not all the commercially important fish contain AFP genes (Cheng, 1996; Zhong and Fan, 2002). However, in this case, although salmon has been produced with integrated, expressed, and inherited flounder AFP gene, the level of AFP expression was low and insufficient to confer freeze resistance (Hew et al., 1992). Control of gene dosage, stronger promoters, and different AFPs will have to be investigated further to boost AFP levels (Devlin et al., 1995; Cheng, 1996).

Compared with mammals, fish offer important advantages for the production of transgenics because of the larger number of eggs laid per female (for example, the zebrafish *Danio rerio* produces 200 eggs per female; the salmon *Salmo salar*, 10,000 per female; the carp *Cyprinus carpio*, 100,000 per female), out-of-mother embryonic development, and lower probability of carrying human related pathogens (Chen and Powers, 1990; Moffat, 1998; Hackett and Alvarez, 2000; Lin, 2000). After injection, however, the percentage of integration can often be low, and most transgenics are mosaics. That is why as an alternative to injection, several mass gene transfer techniques such as electroporation, bombardment, or sperm incubation with DNA are also in development. In addition, in some transgenic fish (Inonue et al., 1990; Murakami et al., 1994) the transgene did not incorporate into the genome, but it replicated independently (episomically) to pass from generation to generation (Hackett and Alvarez, 2000; Niiler, 2000). This behavior, which has not been observed in mammals, is taken by some people as an added difficulty, while others consider it an easier way to regulate the expression of a transgene because positional effects in the chromosome are avoided (Houdebine and Chourrout, 1991; Houtebine, 2000). In the opinion of some researchers, the availability of reliable episomal vectors would

**Table 3.** Some Commercially Available Gene-Targeting Systems[a]

| Name (species) | Enzyme vector | Tansgene vector | Site | Company |
|---|---|---|---|---|
| Cre/lox (bacteriophage) | Many | Many | 34 bp | DuPont |
| Recombinase Flp (yeast) | pOG44 | pcDNA5/FRT | FRT | InvitroGen |
| Recombinase Flp (yeast) | pNeoβgal | pOG45 | FRT | Stratagene |
| Tn7 (*E. coli*) | TnsABC | pGPS3 | 5 bp | NewEngl.BioLabs |
| Tn5 (*E. coli*) | E2::TnTransp. | pMOD | 19 bp | Epicentre |
| Tn SB (salmon) | pCMV-SB | pT/BH | tir | Univ. Minnesotta |
| Retrovirus (mice) | pMSCV | pMSCV | LTR | Clontech |

[a]These systems operate throughout enzyme-mediated site-specific recombination. The transgene vectors include a MCS to clone the transgene between site-specific sequences.

simplify the generation of controllable transgenic animals in all species (Houdebine, 2000). Fish seem to have those already. The maintenance of episomic transgenic fish strains would be quite complicated, which would also argue in favor of greater safety (Attal et al., 1997). Therefore, fish offer unique and often unexplored opportunities for use of transgenics as biofactories for important products to treat human diseases (Coll, 2001a; Rocha et al., 2001).

### Biopharmaceuticals in Transgenic Animals and Fish

Biopharmaceuticals in high concentrations have been produced in mammalian cells, bacteria, and yeast. Since the demand for biopharmaceuticals is expected to rise considerably in the future, alternative production systems should be evaluated. Among them, the production of recombinant human proteins in transgenic animals offers a safe, renewable source of difficult-to-obtain pharmaceuticals (Rudolph, 1999; Lubo and Palmer, 2000). However, whereas a few transgenic animals synthesizing detectable amounts of transgenes are sufficient for most research projects with the traditional techniques currently available for production of biopharmaceuticals a much higher level of expression is required. The milk of transgenic dairy animals has been the target of most studies in this area since high concentrations of recombinant proteins in a relatively simple mixture can be obtained. Species such as cow, goat, pig, rabbit, or sheep have been reported to produce recombinant human lactoferrin, $\alpha_1$-antitrypsin, growth hormone, anti-colon-cancer monoclonal antibody, tissue-plasminogen activator, factor VIII, protein C, calcitonin, superoxide dismutase, erythopoietin, insulin-like growth factor 1, interleukin 2, factor IX, and fibrinogen (Rudolph, 1999).

Fish might be the other alternative. Table 3 lists some of the worldwide ongoing projects to express factor VII, insulin, collagen, calcitonin, pleurocidine, and defensins in tilapia and salmon (Bostock, 1998; Coll, 2000).

## Conclusions

Practical use of most of the systems mentioned above to generate more controlled transgenic animals would require the generation of two independent transgenic strains. As an alternative, one could use a single construct containing the regulatory gene and the transgene in one unique plasmid to generate a single, doubly transgenic strain. This approach is less time-consuming and avoids segregation of regulatory sequences during breeding (Schultze et al., 1996) but is more rigid.

In the case of inducible systems, the first tests would include the introduction of the inducible-promoter transgene by transfection into a cell line and the choice of stable clones by antibiotic selection. Stable clones could then be tested for expression by an inducer over a control gene introduced by a transient plasmid. Alternatively, the control gene might be introduced stably into the host and the transgene induction would be tested by transiently introducing the corresponding plasmid. To check for expression, an alternative approach would be to use bidirectional promoters to drive simultaneous expression of both the transgene and a reporter gene (Baron et al., 1995; Yamamoto et al., 2000). Two corresponding transgenic strains would be then generated similarly. Crossing the two transgenic lines would generate some progeny with both transgenes, allowing the generation of a double transgenic, with the transgene under the inducible promoter. Although

the tetracycline system has been widely used to obtain inducible transgenics, examples using the other systems described above are not common (Ryding et al., 2001).

In the case of the targeted gene systems, the genomic target first must be flanked by the specific sites to increase specific incorporation. The many transgenics that result should be screened to find the most adequate. The Cre-lox system has been used to obtain transgenics with precisely defined gene deletions, but use of other systems is not frequent (Houdebine, 2000).

To apply most of the techniques described above, stem cells are needed. To increase chances of generating transgenics in mice, syngenic strains are often used for the stem cell technology. It is not yet known if syngenic fish strains might also be required to apply stem cell technology in fish. The first fish stem cell line described was from zebrafish (Sun et al., 1995a, 1995b, 1995c). Later on, fish stem cell lines were derived from medaka (Hong et al., 1998) and sea bream (Bejar et al., 1999). Some of them need no cell feeders, have a stable karyotype, can be frozen and transfected. Their capacity to form chimeric fish when transplanted to early embryos is variable depending on the fish species (Hackett and Alvarez, 2000). Since chimeric fish have been obtained by injection of blastomers in early embryos in fish species (Nilsson and Cloud, 1992), this should be not a problematic technology to develop.

Stem cells, nuclear transplants and inducible and targeted gene systems are as yet mostly unexplored opportunities to be used in the design of transgenic fish.

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