Bull. Inst. Zool., Academia Sinica, Monograph **16**: 341-356 (1991)

BIOTECHNOLOGY IN AQUATIC SCIENCES: IMPROVED FREEZING TOLERANCE AND ENHANCED GROWTH IN ATLANTIC SALMON BY GENE TRANSFER

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C. L. Hew, S. J. Du, Z. Gong, G. Fletcher, M. Shears and P. L. Davies (1991) Biotechnology in aquatic sciences: Improved freezing tolerance and enhanced growth in Atlantic salmon by gene transfer. *Bull. Inst. Zool., Academia Sinica, Monograph* **16**: 341-356. Gene transfer technology is a novel and a powerful approach used to manipulate the genetic makeup and phenotypic characteristics of both animals and plants. The production of transgenic fish, which has direct application in aquaculture is actively pursued in many laboratories worldwide. Due to the presence of cold and icy waters, salmon farming in Canada is handicapped by slow growth rate and inability of the fish to survive in icy seawaters. To overcome these difficulties, we have attempted to tranfer the antifreeze protein gene and the growth hormone gene separately into the Atlantic salmon by direct microinjection. The presence and expression of these transgenes were detected by polymerase chain reaction and immunoblots respectively. The incorporation of transgene is approximately 3% with 40% of the positives expressing the proteins. In the case of antifreeze protein gene transfer, the highest protein level produced in the plasma by the salmon is 50 μg/ml, and the transgene is stably inherited in F_2 generation.

The transgenic salmon, with growth hormone gene insert, exhibits a dramatic growth. The average size increase is approximately 5 to 6 fold larger than the injected, nontransgenic controls. Research is now in progress to increase the antifreeze level in the transgenic salmon for the effective freezing protection and to evaluate the growth performance of the growth hormone-transgenic salmon under optimized growth conditions.

Key words: Freezing tolerance, Enhanced growth, Gene transfer, Atlantic salmon.

The ability to incorporate "novel" genes to improve the genetic and phenotypic characteristics of both animals and plants has already made a major impact in many industries. For the past several years, significant progress has been made in the transgenic fish research, and several up-to-date reviews are available (Hew, 1989; Maclean & Penman, 1990; Fletcher & Davies, 1991). In the present communication, we will report our recent progress in producing transgenic salmon with enhanced growth and improved freezing resistance, as well as the development of an "all fish" gene cassette for gene transfer. Future directions will also be outlined.

GENE TRANSFER TECHNOLOGY

Fish, as an experimental model for transgenic studies, has several attractive features over mammalian animals. A single, mature female fish can produce several dozens to several thousands of eggs, providing a large number of genetically identical materials to start with. Its fertilization is external and can be readily controlled or delayed by experimental manipulations. Furthermore, it does not require the reimplantation of the fertilized, injected eggs into the recipient mother, thus making the transgenic fish technology less elaborate with a lower cost of operation, and more readily applied in many laboratories and hatcheries.

Fish eggs, however, depending on the species, may present a different set of problems. One of the major difficulties is the inability of most investigators to visualize the nucleus or pronucleus, due primarily to the opaqueness of the eggs. Some eggs, in addition, contain hard chorions, making it difficult to penetrate with glass needles. Several approaches have been tried to overcome these difficulties. These include the centrifugation of the eggs (Rok-

kones *et al.*, 1989), staining of the nucleus with fluorescent dye (Yamaha *et al.*, 1988), dechorionation (Zhu *et al.*, 1985), injection of the oocyte prior to ovulation (Ozato *et al.*, 1986; Inoue *et al.*, 1989), direct cytoplasmic injection (Dunham *et al.*, 1987; Chong and Vielkind, 1989; Zhang *et al.*, 1990), cytoplasmic injection *via* the micropyle (Fletcher *et al.*, 1988) and more recently, electroporation (Inoue *et al.*, 1990). Due to the variable degree of harshness in egg handling using these different techniques, and the relative accuracy in directing the injected DNA close to the nucleus, the mortality rate and the incorporation of the transgene varies from report to report (for review, see Fletcher and Davies, 1991).

In our laboratories, we have developed and adopted the microinjection technique *via* the micropyle of the salmonid eggs as a routine procedure. Approximately 1×10^6 copies of DNA was injected into the fertilized, preactivated eggs using very fine glass needle (2 to 3 μm external diameter). The injected eggs are then water activated and hatched. The number of eggs injected varies between 500 to 1,000 eggs per gene construct. The survival rate of 80% is identical between the injected and noninjected eggs. This procedure also provides a means to locate the pronuclei from the male and the female. The fertilizing sperm nucleus remains in the ooplasm immediately under the micropyle which is also close to the female pronucleus. Therefore, the injection of foreign DNA *via* the micropyle will deliver the DNA close to the pronuclei. The incorporation frequency of the transgene, measured by PCR and Southern blot using blood samples is approximately 3% (Fletcher*et al.*, 1988; Davies *et al.*, 1990; Shears *et al.*, 1991; Du *et al.*, 1991).

ANTIFREEZE PROTEIN GENE TRANSFER AND THE INHERITANCE OF THE TRANSGENE

The seawater temperature around the world fluctuates widely depending on latitude and seasons. In the polar and subpolar regions, the temperature in the winter could be as low as −1.9°C. Since most fish freeze at −0.6° to −0.7°C, many marine fishes inhabiting the ice-laden environment produce antifreeze proteins to protect them from freezing. In Atlantic Canada, many fishes are found to produce antifreeze proteins during the winter months. There is one type of antifreeze glycoprotein (AFGP)

and at least 3 types of antifreeze protein (AFP). The type I AFP are alanine rich, α helical peptides (3 to 5 K) from the right-eye flounders and sculpins, type II AFP are cystine-rich proteins (13 to 23 K) from sea raven, type III AFP are neither alanine nor cystine rich polypeptides (6 K) from the ocean pout and wolffish (Davies and Hew, 1990). All these AFP or AFGP lower the freezing temperature of the fish sera below that of the surrounding icy seawaters by inhibiting ice crystal formation, thus protecting them from freezing (DeVries, 1984; Yang *et al.*, 1988). The genomic structure and organization of these AFP genes have been characterized (Davies *et al.*, 1989). The AFP genes are multigene families, the number of gene varies from 15 copies in sea raven, 40-50 copies in winter flounder, to a high of 150 copies in ocean pout.

The Atlantic salmon lacks any of these genes and is vulnerable to freezing to death when cultured in sea pen culture. Earlier experiments by Fletcher *et al.* (1986) have demonstrated the direct application of AFP in the freezing protection of other fish species which are incapable of producing their own antifreeze proteins. Purified AFP from the winter flounder was injected into seawater acclimated rainbow trout. While the noninjected rainbow trout was incapable of freezing resistance, the injected animals could tolerate freezing temperatures as low as −1.6°C. The ability of injected rainbow trout to withstand the low temperature correlated with the amount of AFP in its circulation. It is concluded that the AFP alone is sufficient and effective in providing freezing tolerance. The acquisition and expression of the antifreeze protein gene by the Atlantic salmon might help to overcome this danger and expand many coastal regions in Atlantic Canada for salmon farming. As a part of the Canadian strategic grant program, we have undertaken the task in the AFP gene transfer.

The project was initiated in 1982. Early experiments have focussed on the techniques in gene transfer and handling of the salmon eggs (Fletcher *et al.*, 1988). Different AFP gene constructs from the genomic sequences of winter flounder, the ocean pout and wolffish AFP were used. The flounder AFP gene, 2A-7 (Fig. 1) was injected in 1985 and in subsequent years. Only the results initiated in the 1985 injection series with the flounder AFP gene will be discussed.

From the 1985 series, 4 transgenic

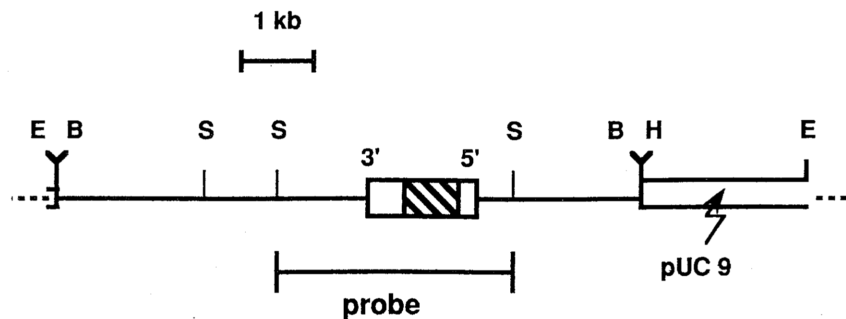


Fig. 1. A schematic diagram of flounder AFP gene (2A-7) used in antifreeze gene transfer in Atlantic salmon. Plasmid 2A-7 linearized at its unique *Eco*-RI site (E), is shown integrated into salmon genomic DNA (broken line). The pUC-9 section of 2A-7 is indicated by double lines and the flounder DNA insert by a single line within which is located a rectangular box representing the 1-kb AFP gene. The open areas of the box are the exons and the hatched area is the intervening sequence. The cleavage sites of *Bam*-HI (B), *Sst*-I (S) and *Hind*-III (H) are marked. The 2.7-kb *Sst*-I fragment used as a probe is underlined. From Davies *et al.* (1990).

salmons expressing the antifreeze protein in the serum were detected by immunoblot using rabbit antiAFP antibodies. The antifreeze in salmon occurs as the large proAFP instead of the mature AFP. The Atlantic salmon apparently lacks the enzyme(s) necessary for the processing of the mature AFP. The proAFP is 70% as active compared to the native AFP (Hew *et al.*, 1986). However, the level of the antifreeze is insufficient to protect the salmon from freezing. We are now modifying the gene construct to increase the expression of AFP (~3 mg/ml will be needed).

These transgenic salmons, on the other hand, provide us with the opportunity to examine the inheritance of the transgene. Inheritance and expression of the AFP gene in F_1 and F_2 generations were established by polymerase chain reaction (PCR) and immunoblot. A more detailed description of using PCR for the screening of transgenics will be described in the later section. In this case, the PCR primers selected would amplify a 483 bp section of the gene, stretching from base 50 of

Table 1
Inheritance of AFP transgene in F_1 offspring

	Wild type Female 1	Wild type Female 2
#1469	24/137	15/100
#1542	64/100	ND

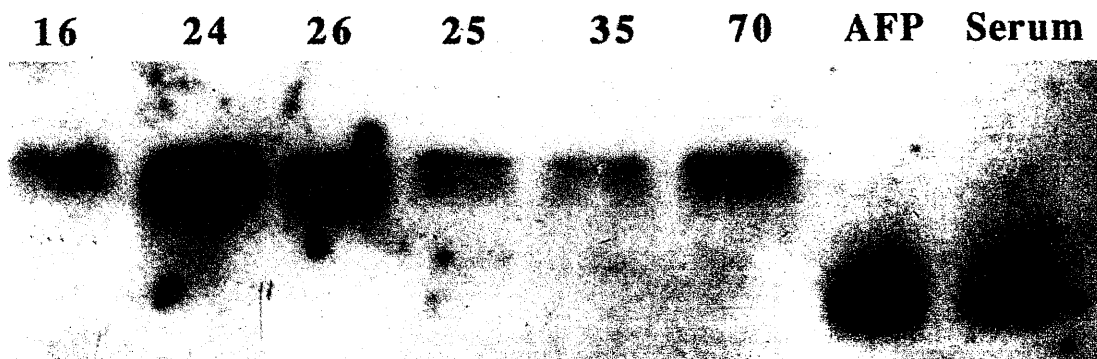


Fig. 2. Expression of proAFP in F_1 transgenic salmon by immunoblot. 3.5 μl of sera from transgenic F_1 #16, #28, #26, #25, #35, #70 were applied, AFP standard (13 ng) and 200 fold dilution of a winter flounder serum. After electrophoretic transfer, the immunoblot was carried out using specific antiflounder AFP antibodies and ^{125}I labelled protein.

5′ exon to base 532 of the intron (Shears *et al.*, 1991). In 1988, two of the transgenic males, #1469 and #1542 became precociously mature and produced milt. DNA was extracted from aliquots of the milt and was shown by Southern analysis to contain AFP transgene (Davies *et al.*, 1990). A cross between #1469 and #1542 with control female is shown in Table I. These results are consistent with the founder parents being germ-line mosaics for the incorporation of one or several copies of the flounder AFP gene (Shears *et al.*, 1991). The expression of the positive F_1 is shown in Fig. 2. The level of proAFP varies from a low of 0.07 μg to a high of 20 μg/ml. Subsequently, 50% of the embryos derived from F_1 transgenic salmon were positive for the AFP gene indicating the establishment of stable transgenic lines (Shears *et al.*, 1991). The availability of these transgenic offspring will enable us to examine the tissue-specific and seasonal expression of the AFP transgene and the production of high AFP-producing salmon by selective breeding.

DEVELOPMENT OF "ALL FISH" UNIVERSAL GENE CASSETTES FOR GENE TRANSFER STUDIES

One of the direct applications in gene transfer technology is the production of transgenic fish important for aquaculture. To be acceptable for human consumption, the gene constructs should be preferably derived from fish genes and the gene products safe. For example,

the mouse metallothionein promotor-human growth hormone gene construct widely used in many laboratories, although useful in experimental fish such as goldfish and zebra fish, is not suitable for direct application in aquaculture.

There are only a limited number of fish genes where their gene structure and promotors are well characterized. These include a protamine (Jankowski and Dixon, 1987), β actin (Liu *et al.*, 1990), metallothionein β gene (Zafarullah *et al.*, 1988) and several antifreeze protein genes (Davies and Hew, 1990; Gong and Hew, 1991). The protamine gene, due to its restricted testis-specific expression, may have limited use. The metallothionein promotor, due to the requirement of heavy metals for induction, may conceivably post some health hazards. The β actin gene, which belongs to a multiple gene family, encodes a major cytoskeletal protein. Each actin gene may be expressed only in a limited number of tissues. However, differential expression of individual members of actin genes is not yet characterized. We have, because of our interest in the biochemistry of the antifreeze proteins, explored the use of AFP promotors as a general and useful vehicle in gene transfer. The antifreeze promotor(s) has several distinct features which are attractive. First of all, the antifreeze protein gene is expressed predominantly in liver (Gong *et al.*, 1991a), a tissue ideally suited for the synthesis and secretion of many secretory proteins. The expression and secretion of exogeneous GH, growth hormone releasing factors and many other transgenic products in the liver of transgenic animals are well documented (Gordon, 1989). Secondly, the antifreeze protein genes have limited occurrence in only a small number of cold water marine species. Most of the economically important species, such as the salmonids, tilapias, carps etc. lack these AFP genes which eliminate the possibility of interference of endogenous gene expression because of the presence of homologous promotor (McGrane *et al.*, 1990). Thirdly, the absence of these genes in the host chromosomes makes the detection of these AFP-derived gene constructs easy and without any background contributed from the host DNA. Finally, there are at least three different types of antifreeze proteins (AFP) and one type of antifreeze glycoproteins (AFGP). All these genes have different *Cis* acting regulatory sequences modulated by a

variety of environmental or hormonal factors. For example, the AFP gene from the type I flounder antifreeze AFP is negatively regulated by GH (Fletcher *et al.*, 1989), and the AFGP gene from the Atlantic cod is cold-inducible (Fletcher *et al.*, 1987). Once these regulatory sequences are further defined, the AFP/AFGP gene family offers a variety of regulatable promotors to choose from. The development of the universal gene cassette is a logical extension. It can be designed with multiple cloning sites for easy insertion of various genes of interest, which can then be used directly in the transgenic studies.

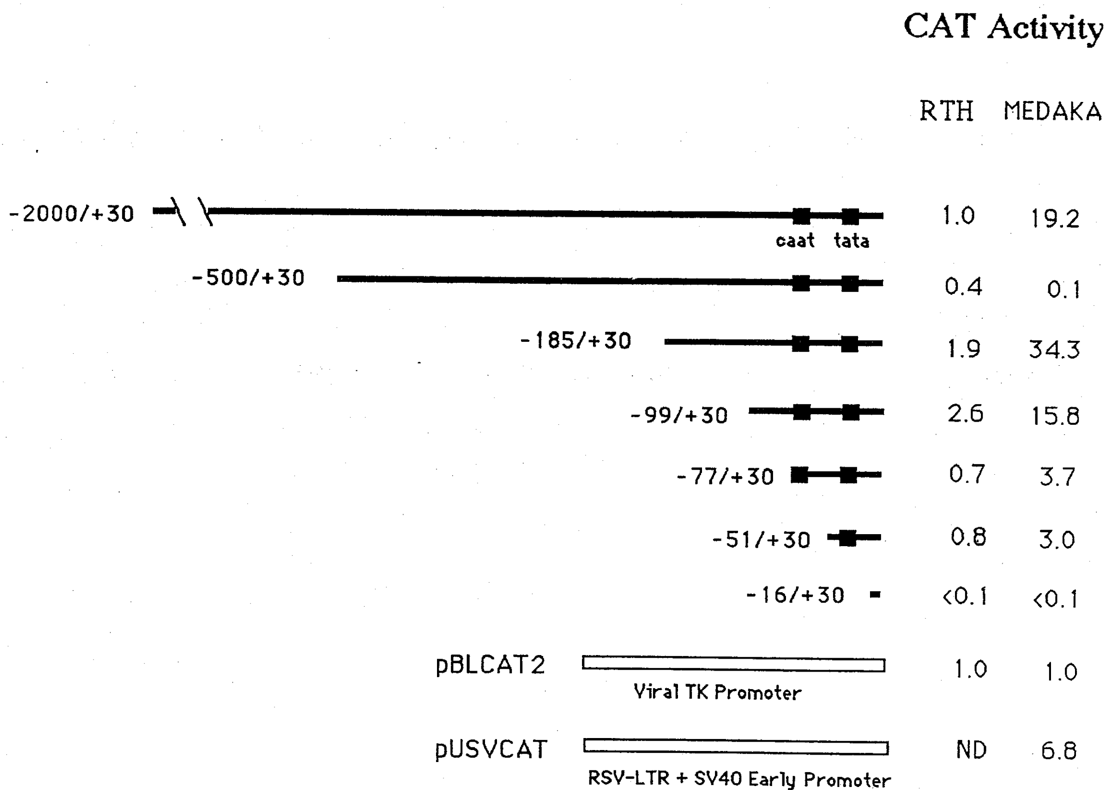


Fig. 3. Functional analysis of promotor regions from an ocean pout AFP gene. A series of 5′ deletions of promotor regions were fused to the bacterial CAT gene. The coordinates of the promotor regions are indicated at left of each construct. CAAT and TATA boxes are also indicated. Two viral promotor-enhancer CAT constructs, pBLCAT2 and pUSVCAT are also included in this study as controls. These constructs were then tested for CAT activity *in vitro* in the RTH cells and *in vivo* in the Medaka embryos. The promotor activity, indicated as CAT activity, is relative to that of pBLCAT2 and contains a viral thymidine kinase (TK) promotor.

To examine the fidelity of the AFP promotors, we have carried out an extensive gene construction with different lengths of the 5′ flanking sequences linked to the bacterial chloramphenicol acetyl transferase (CAT) (Gong and Hew, 1991). More than 60 AFP-CAT constructs from the three types of AFP genes were made. These AFP-CAT constructs were assayed *in vitro* in the RTH 149 (a rainbow trout hepatoma) cell line (Gong and Hew, 1991) and *in vivo* using the Japanese Medaka embryos (Gong *et al.*, 1991b). In the present communication, only the promotor from the type III AFP gene of the ocean pout will be used as an example. Fig. 3 summarizes the transient expression of the CAT activity of some of the opAFP-CAT constructs in the RTH 149 cell line and in Medaka embryos. All these investigations have clearly demonstrated that the ocean pout AFP promotor is active in both the rainbow trout hepatoma cell lines and the Medaka embryos. Our subsequent success in producing Atlantic salmon with promotor further confirm the usefulness of these AFP promotors in the transgenic fish studies.

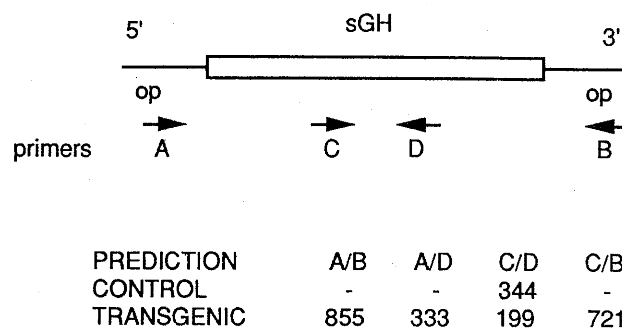
GROWTH HORMONE GENE TRANSFER AND THE APPLICATION OF PCR IN TRANSGENIC ANALYSIS

Since the pioneering investigation of Zhu *et al.* (1985) in producing transgenic goldfish with the human GH gene insert, many laboratories are actively pursuing these studies with the aims of creating a strain of faster growing and larger fish. The average size of the transgenic fish varies from a 20% increase reported by Chen *et al.* (1990) to the 2 to 3 fold larger reported by Zhu *et al.* (1985). However, as stated in our earlier discussion, most if not all of these studies utilize human GH gene and either the metallothionein promotor which requires heavy metal induction, the LTR promotor of the Rous Sarcoma virus, thus making these transgenic fish ill-suited to aquaculture.

Our success in demonstrating the fidelity of the AFP promotors in RTH 149 cell lines and Japanese Medaka embryos has encouraged us to use these promotors for GH gene transfer studies. An ocean pout AFP promotor (opAFP) was used and linked to either the cDNA (GHc) or the genomic GH clone (GHg) from the Chinook salmon isolated from

our laboratories (Hew *et al.*, 1989; Du and Hew, 1991). The resultant "all fish" gene constructs, opAFP-GHc and opAFP-GHg, were used to inject into the Atlantic salmon eggs *via* the micropyle. These studies were initiated in 1988, 1989 and 1990 during the salmon spawning seasons. Due to an accident in freshwater supply, the mortality of the 1988 injection series was extremely high and this series was not studied. For the 1989 injection series, blood samples were drawn from the frys in October 1990 and used the PCR analysis. Due to its ease of operation and sensitivity, PCR technology was adopted instead of the conventional Southern analysis to screen the large number of samples. The nucleated blood samples were treated with NaOH and used directly for PCR without any further manipulation (Du *et al.*, 1991). Fig. 4 shows a schematic diagram of the opAFP-GHc construct, and the strategy of performing PCR with a combination of 4 PCR primers. The position and direction of these primers, A, B, C, D are indicated. The use of these primers gives rise to 4 possible pairs, *i.e.* A/B, A/D, C/B and C/D with predictable PCR fragments (Fig. 4).

Because of the absence of the AFP gene in the Atlantic salmon, the use of A or B primer which is AFP-specific, either together or paired with other primers, will not generate any PCR fragment in the non-transgenic control samples. The transgenic samples, on the other hand, will generate single and unique PCR fragments for A/B, A/D and C/B pairs. The use of C/D primer, however, will be positive for both transgenic and nontransgenic fish because of the existence of the endogenous GH gene. However, due to the presence of a 145 bp intron (intron II) between primers C/D in



PREDICTION	A/B	A/D	C/D	C/B
CONTROL	-	-	344	-
TRANSGENIC	855	333	199	721

Fig. 4. A schematic diagram of opAFP-GHc and the positions of the PCR primers with the predicted size of PCR fragments listed below.

Primers C/D

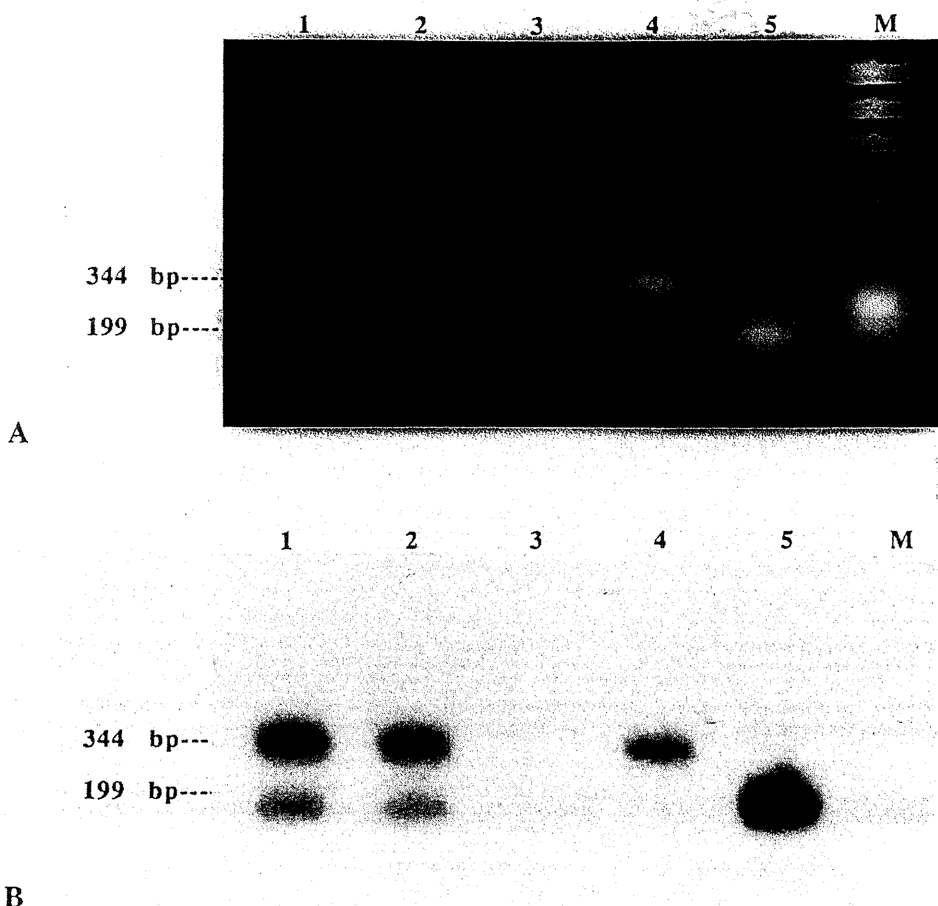


Fig. 5A. PCR analysis in transgenic salmon using the C/D primers. Lanes 1 and 2, Transgenic fish #28, #31; Lane 3, H_2O; Lane 4, Atlantic salmon DNA; Lane 5, opAFP-GHc; Lane 6, DNA molecular weight marker (ϕx-174-RF DNA *Hae*-III digest).

5B. Southern blot using GH specific oligonucleotide probe (+278 to +294 relative to the TATA box of the transgene opAFP-GHc).

the endogenous GH gene, all samples will produce a fragment of 344 bp. The transgenic samples which derive from a growth hormone cDNA gene lacking the introns will generate an extra smaller PCR fragment of 199 bp. All these predictions were confirmed experimentally, thus demonstrating the ease of detecting transgenic fish with these AFP promotor(s). Fig. 5 shows an example of the PCR results using C/D primers. The two transgenic fish (lanes 1 and 2) produce 2 distinct PCR fragments of 199 and

344 bp. The salmon DNA (lane 4) yields only the 344 bp while the opAFP-GHc (lane 5) produces the smaller 199 bp fragment. Lane 3 contains distilled water with no DNA. It indicates that there is no contamination. The identity of these fragments were confirmed by hybridization to the GH cDNA probe.

Based on these PCR results, 9 positive transgenic salmon were identified out of a total of 450 samples analysed. These were further confirmed by Southern analysis using the GH cDNA as probes. Five of the positive animals also exhibit enhanced growth, ranging from a fold to 13 fold increase in size. Similarly, these positive animals show a 4 fold increase in growth rate over a 100 day period (Du *et al.*, 1991). It is evident that the transgenic salmon with enhanced growth characteristics have been produced using these gene constructs. Research is now in progress to measure the circulating GH level in these transgenic salmon.

FUTURE DIRECTION

Optimized growth requirements for transgenic salmon

The dramatic growth of transgenic salmon fry with the growth hormone gene insert under laboratory conditions has raised an important issue of whether the observation can be maintained in adult fish or can be further enhanced under optimized growth conditions. The diet supplement, food utilization and energy conversion efficiency of these transgenic fish, their feeding behaviour, and economical implications will need to be examined in order to develop the best growth environment.

The physiology of excessive transgenic expression

One of the major consequences in transgenic studies is that the transgene of interest is produced in a different tissue from its normal tissue-specific expression. It is, therefore, no longer under similar physiological controls. For example, the expression of GH transgene, primarily in liver, is no longer regulated by the hypothalamus-pituitary axis. The deregulated expression of the GH transgene and its excessive level, has been known to be associated with several pathological conditions in several transgenic animals: hepatomegaly, glomerular sclerosis and female sterility in transgenic mouse, gastric ulcers, arthritis, cardiomegaly, dermatitis and renal disease in transgenic pig (Pursel *et al.*, 1989). It is important

to examine whether excess GH has any detrimental effect on fish health and reproductive ability or capacity. In this instance, it might be advisable to use a moderate/weak promotor to decrease the extent of transgenic expression, or the timing of its expression using regulatable promotors. For example, as pointed out by Pursel *et al.* (1989), these side effects may be eliminated by vigorous regulation of transgene expression to a duration of 1 to 2 months during the rapid growth phase. This will be analogous to a short term injection of exogenous GH which has not been known to cause any adverse effects (Gill *et al.*, 1985). Research is now in progress in our laboratories to investigate the control of the AFP and other fish promotors.

Safety containment

The issue of ecological impact should be addressed in the commercial farming of transgenic fish. Precautions must be taken to avoid potential ecological disturbance by ensuring that these fish will not escape from the containment. Regulations and international cooperation will be needed. It might be necessary to ensure that the transgenic fish cannot compete with the wild type or survive in the natural environment. The logical approach at present is to make these fish, except the brookstock, sterile by chromosome manipulation such as triploidy. Other novel approaches should also be explored.

Production of other transgenic fish

Except for experimental models using either the bacterial chloramphenicol acetyltransferase and β galactosidase, most investigations have focused on two specific genes, *i.e.* the growth hormone and the AFP gene with the aims in improving fish stock. The function of these two genes are well defined and the effects dramatic. With the development of the all fish gene cassettes and improvement in gene transfer techniques, one can now produce other transgenic fish without too much difficulty. The only requirements are the availability of gene of interest, and the knowledge of its DNA sequence. For example, we have already carried out prolactin gene transfer in rainbow trout. Other potential candidates include the recently identified pituitary hormone, somatolactin which is implicated in osmoregulation and electrobalance (Ono *et al.*, 1990), and insulin-like growth factor (Cao *et al.*, 1989). With the advent in using

antisense RNA, this approach can be used to inhibit the translation of undesirable gene products, such as the viral proteins, thus providing a novel approach to prevent viral or bacterial infection.

CONUCLSION

Although many problems remain to be solved, the transgenic fish has a bright and exciting future. It will have direct application in aquaculture. On a more basic level, the approach provides a means to study the physiology and genetics of the transgene, as well as a useful model to study vertebrate development.

Acknowledgements: This research is supported by MRC and NSERC Strategic Grant to CLH, GF and PLD. SJD is a recipient of a studentship from DFO, Canada.

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