GENE TRANSFER IN FISH

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Although conventional breeding programs in fish parallel those in chicken, unconventional techniques not available in chicken breeding, such as interspecific hybridization (Schwartz, 1981), ploidy manipulation and gynogenesis (Thorgaard and Allen, 1987), are used in breeding of fishes. Within the last five years, another unconventional means of genetic manipulation, gene transfer, has become available to animal breeders. Techniques utilized for gene transfer in fish are quite different from those utilized in chicken. In this report, the methods and status of gene transfer in fish are reviewed, with many aspects of progress in the field exemplified with results from our own work in the Minnesota Transgenic Fish Group.

Demonstration of dramatic growth in mice bearing introduced growth factor genes (Palmiter et al., 1982; Hammer et al., 1985) created widespread interest in the possibility of producing agricultural livestock exhibiting enhanced productivity through the action of introduced genetic constructs. Because of the possible value to aquaculture, gene transfer in fish has attracted a relatively large research effort. At least 23 laboratories (Table 1), nine in the U.S. and at least 14 in other countries, are involved in gene transfer in fish. The broad scope of the research effort has led to rapid development of protocols for gene transfer in fish, and the successful production of transgenic fish of at least 13 species (Table 2).

METHODOLOGY OF GENE TRANSFER IN FISH

Successful gene transfers in fish have been accomplished through microinjection of newly-fertilized fish eggs. Gene transfer protocols for particular fishes are variations upon a generalized series of steps, which is exemplified below by our protocol for production of transgenic goldfish.

Induction of spawning. Goldfish can be brought to spontaneous ovulation through appropriate manipulation of photoperiod and temperature, with injections of carp pituitary extract if necessary (Stacy et al., 1979).

Gamete collection. Gametes are readily collected from reproductively ripe individuals by gentle manual stroking of the abdomen toward the vent.
Fertilization. Eggs and semen are mixed in a petri dish. Activation of the gametes and fertilization are effected by addition of water.

Dechorionation. Removal of the chorion facilitates effective microinjection into the animal pole of the egg. Enzymatic digestion offers a practical means for rapid, en masse dechorionation of fish eggs. Dechorionation of goldfish eggs is carried out by a brief incubation of the water-hardened eggs in 0.25% trypsin solution (Yoon et al., in press).

Enzymatic dechorionation procedures have not, however, proven practical for all species, including certain species of aquaculture interest (Hallerman et al., 1988). Lack of a dechorionation protocol for eggs of rainbow trout led to use of a broken pipette to make a small hole in the chorion of the egg prior to microinjection (Chourrout et al., 1986). Other groups (Fletcher et al., 1988; Indig and Moav, 1988) have circumvented the problem of injecting through the chorion by microinjecting through the micropyle of the egg. For other species, such as walleye and northern pike, it has proven practical to microinject eggs immobilized by their natural stickiness onto agarose petri dishes.

Microinjection. Inserted promoter/coding region sequences are removed from plasmid vectors, purified, and resuspended in TE (10 mM tris, 1 mM EDTA; pH 7.4). The DNA solution is loaded into borosilicate glass needles (tip diameter approximately 2 microns). Microinjection is performed using either a simple system driven by oil pressurized by a syringe (Brinkman, 06-90-24) or using a sophisticated system driven by nitrogen pressurized by a unit regulating pressure and injection time (Medical Systems Corp., PLI-100). In either case, the aim is to inject DNA into the animal pole of the egg prior to first cleavage.

In a notable exception to the approach described, Ozato et al. (1986) microinjected mature oocytes dissected from medaka ovaries, thus introducing the novel genetic construct prior to fertilization.

Incubation. Goldfish eggs are incubated through hatch and yolk sac resorption in petri dishes. Embryos and larvae of other species such as northern pike, walleye, and rainbow trout, which require incubation in cool, moving water, are incubated in trays specifically designed for the purpose (Heath Techna Corp.).

Growout. Goldfish are grown out in aquaria until roughly 5 cm in length, and in large (approximately 300 l) troughs thereafter. Northern pike, walleye, and rainbow trout are grown out in cylindrical fiberglass tanks (approximately 500 l volume). The number of fish which can be grown out under laboratory confinement frequently limits the size of gene transfer experiments.
SUCCESS OF TRANSFER AND EXPRESSION OF INTRODUCED CONSTRUCTS

Transfer efficiencies. Transfer efficiencies reported for fish range from 3% (Brem et al., 1988) to over 70% (Z. Zhu, personal communication), with 20% of injected embryos more typically proving transformant.

Expression of introduced constructs. Most gene transfer experiments to date have aimed at production of faster-growing fish through introduction of growth-promoting genetic constructs. Zhu et al. (1985) first reported transfer of the human growth hormone gene into goldfish, and have since reported expression, germ-line transmission, and growth rate enhancement among transgenic goldfish and loach (Maclean et al., 1987b). Transfers of various growth hormone genes have subsequently been documented for several fish species (Table 2), and accelerated growth of transgenic carp (Zhang et al., 1988) and northern pike (Schneider et al., in press) have been reported.

Representing a contrasting approach to genetic improvement of fishes, the antifreeze protein of winter flounder has been cloned and transferred into Atlantic salmon (Fletcher et al., 1988). In winter flounder, this gene encodes a protein which depresses the freezing temperature of sera in the fish, allowing them to survive in supercooled waters. Results of early experiments indicated that levels of antifreeze gene expression were too low to provide protection to transgenic individuals. If sufficient levels of expression are ultimately achieved, the transferred antifreeze protein gene could expand the range of sites in which sea-cage aquaculture of salmon could be carried out.

Among gene transfers in fish to date, the levels of expression of introduced genes have not generally been high. Many groups have introduced genetic constructs containing the murine metallothionein promoter modelled after those used in the classic studies of transgenic mice. However, promoters from simian virus 40 (SV40) and Rous sarcoma virus (RSV) have produced higher levels of expression than the murine metallothionein promoter (Stuart et al., 1988). In avian systems, the RSV promoter seems to give rise to expression in muscle and connective tissues, the desired sites for expression of growth-promoting constructs. In northern pike (Schneider et al., in press) and goldfish (Yoon et al., in press), roughly one-third of transgenic fish showed expression of constructs bearing the RSV promoter.

FUTURE ADVANCES IN GENE TRANSFER IN FISH

Beyond the inclusion of additional species, future progress in gene transfer will center upon utilization of new expression vectors. Production of improved expression vectors will involve testing and practical utilization of new regulatory and protein-encoding genetic elements.

Regulatory elements. The success of any gene transfer is dependent upon the level, tissue-specificity, and ontogenetic stage-specificity of expression of the novel gene product, factors which are
determined by the function of the regulatory elements in the expression vector. The challenge is to find and utilize regulatory elements giving rise to patterns of gene expression appropriate to the purposes of the experiment. Hence, expression of growth-promoting factors would be optimized by use of a promoter which is highly active in muscle tissue throughout the juvenile stage, but which deactivates upon sexual maturity. In contrast, synthesis of antifreeze peptides would optimally be restricted to cold seasons of the year, perhaps with regulation mediated in response to levels of serotonin in the serum of the fish. The success of gene transfers aimed at elimination of nutritional requirements attributable to, say, lack of a particular enzyme, would depend upon constitutive levels of gene expression in all tissue types.

Future advances in gene transfer in fish are thus highly dependent upon discovery, characterization, and utilization of new promoter or enhancer elements. As development of such elements is and will remain a focus of research activity, it is not surprising that groups involved in gene transfer are generally reluctant to discuss experiments in progress.

Structural genes of economic interest. The second functional component of an expression vector is the coding sequence for the novel gene product. Anticipated progress in production of new expression vectors will center upon incorporation of additional growth promoting genes. The search for genes which promote growth of fish has intensified. Growth hormone genes of rainbow trout (Agellon and Chen, 1986), gilthead sea bream (Cavari et al., 1988), chum salmon (Sekine et al., 1985), chinook salmon (C. Hew, personal communication), coho salmon (Nicoll et al., 1987; Gonzalez-Villasenor et al., 1988), yellowtail (Watahiki et al., 1988), tuna (Sato et al., 1988), and red sea bream (Momota et al., 1988) have been cloned.

In higher vertebrates, growth is regulated by a cascade of growth factors, with hypothalamic growth hormone releasing factor triggering the release of pituitary growth hormone, in turn triggering the production of the insulin-like growth factor I in the liver, which ultimately binds to receptors in somatic cells to promote growth. The degree to which this endocrine cascade is paralleled in fish is currently unknown. For purposes of investigating this question, efforts will be devoted to cloning such piscine genes using mammalian sequences as probes, and to transfer of mammalian growth factor genes into fish, followed by observations for enhanced growth.

It is indeed an open question as to what other broad types of structural genes might be attractive candidates for gene transfer in fish. Chum salmon prolactin has been cloned (Song et al., 1988), and represents the start of efforts to clone other piscine peptide hormone genes.

Targetted integration. Because introduced constructs are integrated into the host genome at random locations, there is a considerable probability that such integration events will result in insertional deactivation of a host gene. Furthermore, gene expression is to some degree dependant upon the location of a gene within the genome. Hence, direction of integration of a novel construct to specific sites within the genome is important not only in gene therapy,
but in a broad range of applications.

Direction of integration in gene therapy is approached through inclusion of promoter and coding elements between flanking sequences homologous to the desired site of genomic integration; it is hoped that the cell's own DNA repair mechanisms will splice the introduced construct within the defective gene. Parallel gene transfer experiments have been initiated in fish, using flanking sequences corresponding to sequences repeated within the piscine genome.

MASS GENE TRANSFER

Microinjection is a tedious and time-consuming procedure. In our laboratory, the collective efforts of six or more people are required in order to microinject 10,000 fish eggs per day. The labor required to produce the large number of transgenic fish from which to select an optimal gene transfer event is thus a factor limiting the success of gene transfer experiments. Hence, there is interest in developing mass gene transfer technologies for use in fish so that a large number of eggs may be treated at one time, with use of a subsequent selection step to distinguish transgenic individuals. At least three approaches to mass gene transfer are being investigated:

Electroporation. Electroporation is the utilization of short electrical pulses to permeabilize the cell membrane, thereby gaining entry of vector DNA into the cytosol, and ultimately the genome, of target cells. The utilization of electroporation for gene transfer, which has proven successful in bacteria, cultured mammalian cells, and plant protoplasts (Shigekawa and Dower, 1988), has also been attempted upon fertilized, dechorionated fish eggs. Although we have defined conditions that do not kill goldfish eggs, we have yet to produce transformant individuals using this technique (Hallerman et al., in press).

Lipofection. Liposome-mediated gene transfer (Felgner et al., 1987) is based on the encapsulation of vector DNA into a synthetic phospholipid bilayer, which fuses with the cell membrane of the target cell, introducing the foreign DNA into the cell. For purposes of gene transfer in fish, the target cells would be dechorionated fish eggs. While several groups, including ours, are attempting this mode of gene transfer, no success has been reported.

Adsorption of vector DNA onto sperm. In preliminary experiments in our laboratory, upwards of 10% of radiolabelled plasmid DNA (suspended in semen extender) mixed with fish semen has remained adsorbed to sperm cells through three cycles of washing in semen extender and centrifugation. In fish species lacking the acrosome reaction, the entire sperm enters the egg during fertilization. These observations lead us to believe that DNA adsorbed onto sperm in this manner might transform embryos so fertilized. This procedure would have the potential to effect gene transfer upon many eggs with relatively little effort.
Selectable markers. Transfer efficiencies in mass transfer protocols are expected to be low, and so it is preferable that a selectable marker (perhaps neomycin resistance; Southern and Berg, 1982) or a gene that gives rise to a phenotypic marker be used. Such selection regimes for fish embryos are under development (Yoon et al., in press).

USE OF FISH AS MODEL SYSTEMS FOR VERTEBRATE GENE EXPRESSION

In studies of vertebrate gene expression using gene transfer methodology, mouse is the most widely used model system. However, comparison of their respective life histories reveals features which render fish more suited for gene transfer experiments:
--the fecundity of fish is much higher, increasing the number of eggs available for microinjection, and ultimately, the number of transgenic individuals produced,
--fertilization and incubation of fish eggs is external, circumventing the need for in vitro fertilization of mouse eggs and reimplantation into a surrogate mother,
--fish eggs are relatively large, facilitating handling,
--transformation frequencies of microinjected fish eggs equal or exceed those of mouse eggs, and
--the action of regulatory sequences in fishes parallels those in higher vertebrates.

We thus believe that piscine systems will be more widely used as model systems in future studies of vertebrate gene expression.

Goldfish. Because of wide availability, ease of culture, and ease of inducing spawning, goldfish may become an important model system. The drawback of goldfish is the long (one year) generation time relative to mouse or certain other fishes, lengthening the duration of studies depending upon germ-line transmissions of novel genetic constructs.

Zebrafish. With a four-month generation time, zebrafish are an attractive model system for studies requiring germ-line transmission. Additionally, maps tracing the fates of particular cells in the early embryonic stages have been developed (Kimmel and Warga, 1988), providing the basis for studies of ontogenetic gene expression and regulation.

USE OF TRANSGENIC FISH IN AQUACULTURE

Applications of gene transfer technology may have major impacts on aquaculture breeding programs. The successful production of growth enhanced recombinant fish strains might have wide application and large economic returns. Although rapid progress has been achieved in production of transgenic fishes exhibiting favorably altered phenotypes, several factors will delay the use of transgenic fish in aquaculture.
Development of improved sterilization techniques. Production of sterile fish is desirable for proprietary protection of transgenic broodstock and as a means of minimizing the environmental impacts consequent to escape of transgenic individuals from an aquaculture facility. Three techniques might be used to produce functional sterility in fish: polyploidization (Thorgaard and Allen, 1987), interspecific hybridization (Schwartz, 1981), and hormonal treatment (Donaldson and Hunter, 1983). Protocols combining two or more of these approaches might be developed, as some individuals escape sterilization using any one of these methods alone.

Breeding constraints. The transgenic individuals produced in gene transfer experiments are not themselves intended as production animals, but as progenitors of new genetic lines. Several generations of breeding will be required to bring the frequency of novel genes to high frequencies and to carry out performance evaluations before transgenic lines can be utilized in conventional aquaculture breeding programs (Figure 1). The time constraint introduced by breeding considerations is important, as the generation times in aquaculture species (e.g., three years in rainbow trout and channel catfish) are long.

Legal constraints. Both the natural environmental conditions required for attainment of sexual maturity in certain species and the normal culture conditions required for identification of high performance lines dictate the need for outdoor containment during development of transgenic lines. Controlled environmental testing of organisms altered through recombinant DNA technology is a controversial element of public policy. Development of animals bearing introduced DNA constructs is regulated under the Coordinated Framework for the Regulation of Biotechnology (Office of Science and Technology Policy, 1984; 1985; 1986). Provisional policy guidelines regulating outdoor testing of transgenic animals have been promulgated by the Agricultural Biotechnology Research Advisory Committee of the U.S. Department of Agriculture.

No outdoor testing of transgenic animals of any kind has yet been carried out, although a permit has recently been granted for outdoor release of transgenic carp (Ezzell, 1989).

Development of public policies regarding the ultimate practical utilization of transgenic animals is proving controversial, with many interest groups voicing their concerns. Policies regarding field testing, and ultimately, distribution and final use of transgenic fishes should reflect a fundamental difference between aquaculture fishes and traditional agriculture animals. Transgenic fishes can become feral, giving rise to environmental impacts directly through their altered phenotypes or by reproducing within natural populations of conspecifics. Practical utilization of transgenic fishes in the U.S. may thus be delayed by development of field testing data and analysis of such data within risk assessment models.
ACKNOWLEDGEMENTS

We would like to thank Program Chairman Ramakrishna Reddy for his help in arranging this presentation. Our funding support came from the Minnesota Agricultural Experiment Station, the Legislative Commission for Minnesota Resources, and from Minnesota Sea Grant.

LITERATURE CITED


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Table 2. Gene transfers in fish.

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¹ The genetic constructs introduced into fish are referred to as fusion genes, comprised of a promoter sequence which regulates expression of the gene, and a structural sequence which encodes the protein product of the gene of interest. Abbreviations for promoter sequences: mMT, mouse metallothionein; AFP, antifreeze protein; RSV, Rous sarcoma virus; SV40, simian virus 40. Abbreviations for structural genes: bGal, beta-galactosidase; AFP, antifreeze protein; hGH, human growth hormone; tGH, rainbow trout growth hormone; sGH, salmon growth hormone; neo, neomycin resistance, CAT, chloramphenicol transacetylase; bGH, bovine growth hormone, cCRY, chicken crystalline; rGH, rat growth hormone, and hyg, hygromycin resistance.

² Status designations: I, genomic integration; T, transcription; E, expression of protein; G, germ line transmission; R, rapid growth.
FIGURE 1. Development of transgenic lines of fish for utilization in conventional aquaculture breeding programs.

Original Generation
~20% carry new gene, mostly as 'mosaics'

- Non-Transgenics
- Not Transformed in Germ Line

First Generation
Half of offspring hemizygous for new gene

- Non-Transgenics

More Inbreeding

Performance Testing

Crossbreeding with Existing Aquaculture Lines
Question: D. Harwood

Are the fish oocytes microinjected with intact plasmids or restriction fragments containing only the promoter and structural gene sequences?

Response: E. Hallerman

We have microinjected intact plasmid, linearized plasmid, and inserted promoter/structural gene elements into fish eggs. Although our data are presently incomplete, the picture that is as follows:

- Highest levels of transient expression are observed following introduction of intact plasmid.

- Genomic integration seems more frequent using linearized form.

- Levels of stable expression of the introduced construct seem highest when only the promoter/coding elements are introduced.

Thus, I would advocate microinjection of the promoter/coding elements only for the purposes of most gene transfer experiments, when genomic integration and stable expression are desired.

Please note also that we inject the expression vector, in whatever form, into fertilized ova, rather than oocytes.