Problems and prospects of transgenic fish production

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The restricted scope of cytoplasmic introduction of transgene, and the limited availability of fish cDNA sequences and promoters, are the major hurdles in the production of transgenic fish. Although integration frequency is low, expression is usually detected in transgenic fish, into whose eggs transgene of fish origin is introduced. Long-term researches are desired to produce drought-resistant (aestivating) transgenic carps. To ensure survival and transgenicity of the fish, a volume of 10–20 nl with a DNA concentration of 10–15 μg/ml containing 1–2 million copies is usually injected into the fish egg at its 1-cell stage. Catfish and tilapias are too sensitive to microinjection and hence must be subjected to alternate methods of gene introduction like electroporation and sperm-mediated transfer. The quantity of transgene is decreased as functions of injection dose and age of the transgenic fish. A most common observation is the mosaicism in transgenic fish; the delayed delivery and/or integration of the transgene is implicated as the causative factor. Much of available information on transmission is based on zebrafish, whose generation time is short. The number of copies of the transgene inherited by the offspring belonging to F_1 and F_2 varies from tissue to tissue, and individual to individual. Though effective viral and metalothionein promoters are now giving way to fish promoter genes and 'all-fish' gene sequences comprising endogenous fish promoters (e.g. β-actin, AFP) have been shown to be more effective. The tedious procedures involved in Southern and Western blot techniques have made some scientists to use reporter genes, whose expression can be detected by simple and rapid methods. The use of sterile triploid eggs is recommended for initial production of transgenic fish. Techniques for cryopreservation of sperm and androgenesis are urgently required for many Asian fish species, whose wild strains have to be preserved.

In 1982 Palmiter and his colleagues[1] successfully produced transgenic mouse by introducing metallothioncin-human growth hormone fusion gene (hGH) into mouse egg, resulting in dramatic increase in growth. Their publication triggered a series of gene transfer studies involving growth hormone genes in economically important animals including fish. While much success has been achieved in ensuring faster growth in domestically important mammals such as rabbits, sheep and pigs[2], it has not yet been possible to produce a pure line of transgenic fish, which consistently display dramatic growth similar to that observed in Palmiter's transgenic mouse. The reasons for this failure may be listed as follows: (i) The pronucleus of the fish egg is not usually visible, as in the case of mouse; therefore the desired transgene, i.e. the foreign gene is generally introduced into cytoplasm. (ii) Only a few cDNA constructs of fish are available, e.g.: rainbow trout growth hormone gene (rtGH) and antifreeze protein gene (AFP); hence most workers are left with no option other than using cDNA constructs of mammalian sources. (iii) Relatively less is known about the characteristics of fish promotor for the expression of the introduced transgene. This paper comprehensively summarizes relevant information on the problems and prospects of producing a pure line transgenic fish.

Fish as a model system

Techniques for generating transgenic animals involve four successive steps: (i) introduction of a transgene into eggs, (ii) genomic integration, (iii) transmission to the subsequent generations (G_1, G_2 and so on) and (iv) expression. Table 1 presents a comparative account of the advantages and limitations of using the eggs of fish and mammals. As a system, fish eggs offer relatively more advantages than those of mammals. Therefore, it is likely that fish may serve as a better experimental model for the production of expressive transgenic animal (see also Zhu[3]).

Tropical fish such as medaka and zebrafish, which have short generation time may serve as better models, in comparison to commercially important food-fish like the cyprinids and salmonids, which have long generation times (see also Table 8). Considering these facts, Fletcher and Davies[4] recommended the cichlids, which grow faster and large enough to yield adequate plasma samples (50 to 100 μl) but have a short generation time, as one of the best potential model fish for transgenic studies. For reasons stated elsewhere (Table 6), tilapias are too sensitive to microinjection; hence the recommendation of Fletcher and Davies may prove good only when an alternate method of gene transfer such as electroporation is used.

Table 1. Advantages and limitations of using fish egg as a model for gene manipulation in vertebrates

Advantages	Disadvantages
Fish	
External fertilization; Artificial stripping of eggs and milt possible; Maturation of gametes artificially inducible. Eggs are large, numerous and easily maintained after fertilization; hence provide maximum scope for expression of variation; Faster embryonic development.	In many species, zygote nucleus of eggs very small and not visible; hence nucleoplasmic microinjection not possible; Microinjection into oocyte nucleus of oviparous (e g. medaka) and viviparous (e g. *Gambusia*) is possible only in some species. The oocyte nucleus is over 1000 times larger than the mammalian pronucleus.
Fish eggs are amenable for ploidy induction; hence it is possible to generate hundreds of clones from a single parent; this effectively saves one generation in the selection of homozygous mutant individuals.	Egg coat (chorion) is very tough and resistant.
Relatively higher totipotency of fish cells render the production of pure transgenic line a much easier task than that for a mammal.	
Mammals	
Microinjection into egg nucleus possible; Soft egg membrane	Eggs are small, fewer and difficult to procure. Requiers skilled work for *in vitro* culture and reintroduction into the reproductive tract of the recipient female.
	Mammalian eggs are not amenable for ploidy induction.

Genes and transgenic fish

Although Zhu claims to have conceived the idea of producing transgenic fish by injecting human insulin gene into crucian carp and loach eggs, human growth hormone gene was the first gene that was successfully used to produce such animals[5,6]. Subsequently, the growth hormone gene of cattle[7] and rat[8] was also used to produce transgenic pike and trout respectively. Only in 1985, the first fish gene sequence namely, the antifreeze protein gene of the winter flounder became available and was used for the production of freeze-resistant salmon[9]. The commercial need for producing fast-growing fish and the repeated attempts of some scientists to produce such fast-growing transgenic fish using mammalian growth hormone gene necessitated the cloning of fish growth hormone gene for transformation. Subsequently the growth hormone genes of rainbow trout and salmon were sequenced and became available after 1986 (see Hew[10]).

Table 2 summarizes the prokaryotic and eukaryotic genes so far used to produce transgenic fish. Remarkably, almost all workers, who used one or the other transgene of fish-origin, detected expression; however not more than half of them have detected the expression of injected mammalian gene sequence in the presumptive transgenic fish. This prompts us to suggest that there is an urgent need for constructing transgenes of fish-origin.

Transgenic fish produced by transfer of foreign genes, especially those of bacteria, into fertilized egg has become a powerful tool in the study of gene expression *in vivo* and *in vitro*. From this point of view several scientific groups have used bacterial genes such as CAT, β-gal, Neo, Lac-Z and hygro to understand the processes of integration, expression and transmission of foreign genes in fish. In the presumptive transgenic fish (e.g. Stuart *et al.*[87]) transfection of bacterial genes into selected cell lines of fish has been undertaken to study these processes *in vitro*. In most cases there was expression of the injected transgene though sometimes transiently. Another advantage of bacterial genes is their use as reporter genes (see Moav *et al.*[36]). An example is the chloroamphenicol acetyl transferase (CAT) gene, which is not present in eukaryotes, and whose activity can be detected by a simple and rapid assay (e.g. Khoo *et al.*[79]; Vielkind[89]). Briefly, these studies helped us to gain a deeper insight into the genetic regulation of developmental events.

Most research groups, from the West as well as tropical countries are attempting to develop suitable transgenic technology to increase growth rate of foodfish. Some Canadians and Americans are attempting to improve freeze-resistance in salmonids. Many western scientists use transgene technology to have a deeper insight into the genetic regulation of the developmental process using zebrafish and medaka as models. A field, which is being still neglected by these scientists, is the production of disease-resistant transgenic fish. China is again the first country, where attempts are being made to produce disease-resistant transgenic fish[90,91]. Here it must also be indicated that to withstand drought in tropical freshwater systems, many fish like the murrel *Channa striatus* undergo aestivation; almost all anurans are also known to aestivate. At present it is not known whether these aestivating fish and frogs produce anti-drought protein(s), as is the case with the flounder-synthesizing anti-freeze protein to withstand the freezing waters. Long-term researches must be supported to understand the genetic control of aestivation phenomenon. Production of drought-resistant transgenic carps will indeed be a great blessing to freshwater aquaculture in the drought-prone countries of Asia.

Methods of gene transfer

A survey of the methods used for introducing transgene into fish eggs indicates that most workers have chosen

Table 2. Gene sequences/constructs used for transgenic fish production

Gene		Species	Expression	References
Eukaryotic genes				
(i) Genes from homiotherms				
Human growth hormone	(hGH)	Loach, goldfish	+	Zhu *et al.*[5, 6, 11]
		Loach	+	Enikolopov *et al.*[12]
		Loach	+	Maclean *et al.*[13, 14]
		Loach	–	Xie *et al.*[15]
		Rainbow trout	–	Maclean *et al.*[16]
		Trout	+	Rokkones *et al.*[17]
		Trout	–	Chourrout *et al.*[18]
		Trout	–	Guyomard *et al.*[19]
		Atlantic salmon	+	Rokkones *et al.*[17]
		Carp	–	Alok & Khillian[20]
		Carp & loach	+	Chen & Powers[21]
		Carp	+	Tian[23]
		Carp	?	Wei *et al.*[24]
		Crucian carp	?	Xu *et al.*[25]
		Carp & loach	?	Zou *et al.*[26]
		Red carp	?	Li *et al.*[27]
		Goldfish	?	Zhou[28]
		Tilapia	–	Brem *et al.*[29]
		Catfish	–	Dunham *et al.*[30]
		Catfish	–	Powers *et al.*[31]
		Zebra cichlid	–	Ken *et al.*[32]
		Zebrafish	+	Khoo *et al.*[33]
		Blunt snout bream	+	Xia *et al.*[34]
Bovine growth hormone	(bGH)	Northern pike	+	Schneider *et al.*[7]
		Northern pike	+	Gross *et al.*[35]
		Walleye	–	Moav *et al.*[36]
		Trout	–	Chourrout *et al.*[37, 38]
Rat growth hormone	(rGH)	Rainbow trout	+	Penman *et al.*[8]
		Zebrafish	–	Pandian *et al.*[39]
Chicken δ crystallin	(pδC)	Medaka	+	Ozato *et al.*[40–42]
		Medaka	+	Inoue *et al.*[43]
(ii) Genes from fish				
Antifreeze protein	(AFP)	Atlantic salmon	+	Fletcher *et al.*[44, 45]
		Salmonids	+	Shears *et al.*[46, 47]
		Salmonids	+	Davies *et al.*[48, 49]
		Winter flounder	+	Huang *et al.*[50]
		Goldfish	?	Wang[51]
		Medaka	+	Gong *et al.*[52]
		Atlantic salmon	+	Hew *et al.*[53, 54]
Rainbow trout growth hormone	(rtGH)	Carp	+	Zhang *et al.*[55, 56]
		Carp & loach	+	Chen *et al.*[22, 57, 58]
		Gilthead seabream	+	Cavari *et al.*[59]
		Medaka	+	Inoue *et al.*[60, 61]
Chinook salmon growth hormone	(csGH)	Walleye & Nothern pike	+	Cited in Moav *et al.*[36]
		Atlantic salmon	+	Hew *et al.*[53]
		Atlantic salmon	+	Du *et al.*[62]
		Loach	+	Ge *et al.*[63]
		Goldfish	?	Zhang *et al.*[56]
Chum salmon melanophore concentrating hormone		Medaka	+	Ozato *et al.*[64]

Contd . . .

(Table 2. *Contd . . .*)

Gene		Species	Expression	References
Lates calcarifer growth hormone		Seabream	+	Knibb & Moav[65]
		Gilthead seabream	+	Cavari *et al*[66]
Melanin concentrating hormone	(MCH)	Red carp	+	Guo *et al.*[67]
		Zebrafish	?	Alestrom *et al.*[68]
(iii) *Insect gene*				
Firefly luciferase	(luc)	Medaka	+	Tamiya *et al.*[69]
		Zebrafish	+	Kavumpurath *et al.*[70]
		Zebrafish	?	Patil *et al.*[71]
		Zebrafish, medaka	+	Alestrom *et al.*[68]
		Medaka	+	Sato *et al.*[72]
Prokaryotic genes				
Chloroamphenical acetyl transferase	(CAT)	Medaka	+	Chong and Vielkind[73]
		Zebrafish	+	Stuart *et al.*[74]
		Goldfish	+	Liu *et al.*[75]
		Salmon	+	Liu *et al.*[75]
		Walleye	+	Liu *et al.*[75]
		Zebrafish	+	Liu *et al.*[75]
		Nothern pike	+	Liu *et al.*[75]
		Walleye	+	Moav *et al*[36]
		Nothern pike	+	Moav *et al.*[36]
		Goldfish	+	Hallerman *et al.*[76]
		Zebrafish	+	Vielkind *et al.*[77]
		Zebrafish	?	Ivics *et al.*[78]
		Medaka	+	Vielkind *et al.*[77]
		Zebrafish	−	Khoo *et al.*[79]
		Medaka	+	Winkler *et al.*[80]
β-galactosidase	(β-gal)	Salmon	+	McEnvoy *et al.*[81]
		Medaka	+	Ozato *et al.*[82]
		Loach & zebrafish	−	Zelenin *et al*[83]
		Zebrafish	?	Culp *et al.*[84]
		Gilthead seabream	+	Cavari *et al.*[66]
Neomycin phospho-transferase	*(neo)*	*Goldfish*	+	*Yoon et al.*[85]
		Goldfish	−	Guise *et al*[86]
		Rainbow trout	−	Zelenin *et al.*[83]
		Zebrafish	?	Ivics *et al.*[78]
Hygromycin	(hygro)	Zebrafish	−	Stuart *et al*[87]
Lac Z		Zebrafish	+	Culp *et al.*[84]
		Zebrafish	+	Bayer & Campos–Ortega[88]

+ = expression; − = no expression; ? = no information.

microinjection as the preferred method. Other methods adopted are: electroporation, sperm-mediated transfer and high velocity microprojectile bombardment. Table 3 lists some the advantages and limitations of using these gene transfer methods. Interestingly, Inoue *et al.*[60] and Kavumpurath *et al.*[70] made a comparative study on the efficiency of gene transfer using one or more of these methods. Judging from the frequency of integration and survival of the presumptive transgenic offspring, microinjection seems to be a better method for introducing transgene into fish eggs. Depending upon the biological need, microinjection apparatus has been modified to different levels of perfection. For instance, Shears *et al.*[47] improved the efficiency of the injector system by constructing a grass stimulator model 544 to supply power to the 'gene pusher' and to control the frequency and duration of the bursts of nitrogen pressure into the injection needle.

As indicated in Table 1, the presence of relatively small and non-visible nucleus surrounded by opaque yolk and tough chorion renders microinjection of transgene into fish eggs a difficult task. Since the chorion is too tough in salmonids, a slit (by drilling) is made

Table 3. Advantages and limitations of using different methods for gene transfer in fish transgenesis

Advantages	Disadvantages
Microinjection	
Permits precise injection at the desired geographic location of the target egg, i.e. nucleus, cytoplasm. Permits quantitative estimation of the injected gene	Time-consuming technique, requiring skill. Limited embryonic time restricts injection to a fewer eggs
	Introduces variations, due to needle injury
Electroporation	
Permits simultaneous entry of foreign DNA and thus brings about mass production of fish transgenesis	Difficult to assess the actual quantity of foreign gene entering the cells
Does not require technical expertise	Sustains relatively higher mortality
A more suitable method in some fish species, whose eggs are too small for microinjection	
Sperm-mediated transfer	
Absence of acrosome in fish sperm affords a greater scope for sperm-mediated gene transfer	Its usefulness is claimed by avian biologists but disputed by mammalian and pscine workers
High velocity microprojectile bombardment	
Permits simultaneous entry of foreign DNA and thus bring about mass production of fish transgenesis	Difficult to assess the actual quantity of foreign gene entering the cells
	Not used much because little is known about the mechanics of the process

prior to microinjection. In eggs with a broad perivitelline space, enzymatic digestion of the chorion by trypsin is possible; these eggs can also be manually dechorionated prior to microinjection. However, such procedures reduce the time available for injection and also lead to a higher mortality of injected eggs (see Tables 4, 6). Yet in order to secure adequate samples (number of eggs/embryos/young ones/adults) as many eggs as possible have to be microinjected within the short period of the early developmental stage. Many workers have chosen to introduce the desired transgene through the micropyle during the first cell division to maximize the chances of integration and to avoid digestion of the injected transgene by nuclear DNAase. In eggs of a few cyprinids, in which the perivitelline space is too broad, the egg proper frequently rotates itself rendering the location of the micropyle a difficult task (e.g. Zhang *et al.*[56]).

A comparative analysis of the processes of integration and expression of the injected transgene into fish and mammalian systems suggests a greater prevalence of mosaicism in transgenic fish. This can be traced to one or more of the following facts: (i) the cytoplasmic introduction of the transgene, (ii) the microinjection of egg even after its pre-blastoderm stage and/or, (iii) the inherent totipotency of fish eggs. If the duration of microinjection is to be restricted to a specific developmental stage (from fertilization to 2-cell stage), one has to take minimum time to successfully complete the microinjection process. This becomes critically important for tropical fish, whose early developmental stages are completed far too quickly than in their temperate counterparts. Figure 1 illustrates the early developmental sequence in relation to time in a tropical catfish *Heteropneustes fossilis*. It may be seen that adequate cytoplasm

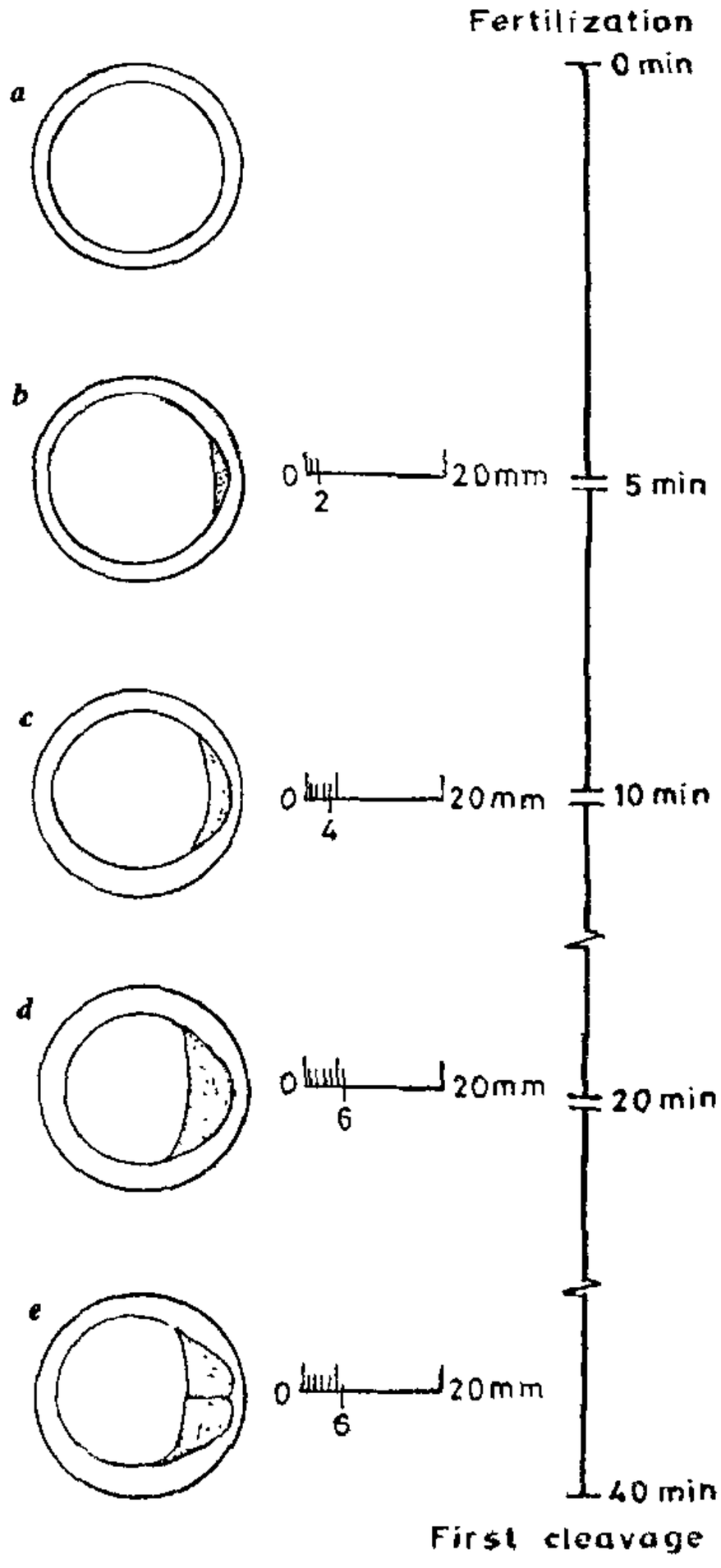

Figure 1. Early developmental stage in relation to developmental period in catfish egg. *a*, just fertilized egg; *b*, *c*, and *d* = 5, 10 and 20 min old egg; *e*, embryo (40 min after fertilization at first cleavage).

for microinjection accumulates only about 15 min after fertilization and the first cell division is completed at about 40 min after fertilization. Hence, not more than 25 minutes are available for completion of microinjection in this fish; this duration is comparable with that (25 min) reported for carps[3]. Though the time required for successful completion of microinjection of foreign gene into nucleoplasm or cytoplasm depends on factors like the efficiency of microinjection unit and injector as well as the toughness of the chorion, most workers have been able to complete microinjection within a short period of 0.02 to 2 min/egg (Table 4).

For some reason, most authors have not explicitly provided adequate information on quantitative aspects of the injected gene. Table 5 presents relevant information from the available publications. With regard to human growth hormone gene, the sizes of the plasmids used for microinjection reported by five research groups range from 2.9 to 9.4 kb. This is due to the fact that these authors have reconstructed the original gene into constructs of their own choice. The largest gene thus far introduced into fish eggs is the chicken δ crystallin gene of 14.4 kb.

The volume of DNA solution injected ranges from 0.2 to 20 nl. Figure 2 illustrates the approximate area covered by cytoplasm in eggs of selected fish. The catfish eggs appear to have relatively more cytoplasm than that of medaka eggs. It is not clear whether this is related to the highest mortality suffered by catfish following microinjection (see Table 6 also). For instance, the nucleus of medaka oocyte is large with a diameter of 135 μm[40]. The nuclear diameter of fertilized eggs of *Gambusia* and guppy is in the range of 150–200 μm (personal observation). The volume of the nucleus of these fish is about 1000 times larger than the male pronucleus of the mouse. Hence the nucleii of the eggs of medaka, guppy and *Gambusia* may be able to accommodate a larger volume of injected genes. Most workers have kept the concentration of the injected DNA between 10 and 15 μg/ml. Stuart *et al.*[87] showed that DNA concentration of more than 15 μg/ml can be toxic to zebrafish.

The number of copies of the transgene to be introduced depends upon the location of the site of introduction. For instance, a few hundred copies per egg is adequate, when injected into the male pronucleus of the fertilized eggs of mammals; more than 50,000 copies per egg are required to produce transgenic medaka in which microinjection is undertaken into the oocyte nucleus. However, as much as one to two million copies are required, when cytoplasmic injection is carried out. Briefly, to ensure the survival and transgenicity of the fish, it is recommended that a volume of 10–20 nl with the DNA concentration of 10–15 μg/ml containing 1–2 million copies may be injected into a fish egg up to 2-cell stage, i.e. the time lapse between fertilization and first cleavage.

Table 6 lists available data on survival of the control (non-injected) and injected eggs. The list provides comparative information for many species belonging to the groups, salmon, zebrafish, carp, tilapia and catfish, which have been used for microinjection experiments by various research groups working in laboratories situated at different geographical locations. Except tilapia[29] and catfish[30], the survival data are based on fairly large number of injected eggs. The reported values for survival at hatchability of the control eggs range from 10 to 95%, though most values fall between 78 and 86%. It is not clear why some authors have reported low survival (< 40%) for carps, tilapias and catfish. Tilapias (oral breeding) and catfish (e.g. *Ictalurus punctatus*) are known to take (parental) care of the eggs. It is likely that the procedures used for the artificial incubation of these eggs were not good substitutes for parental care, and consequently have led to high mortality.

A comparative analysis of the survival at hatching of the non-injected and injected eggs reveals that fish like medaka and salmonids (≈ 20% mortality of injected and control eggs) and carps (≈ 60% mortality of injected and control eggs) suffered almost no additional mortality

Table 4. Time required for microinjection in fish eggs

Species	Location and method of injection	Injection time required (min/egg)	Reference
Medaka	Oocyte injection	1.5	Ozato *et al.*[40]
Rainbow trout	Double prick	2.0	Chourrout *et al.*[18]
Atlantic salmon	Micropylar injection	1 0	Shears *et al.*[46]
Catfish	Cytoplasmic injection	1 5	Pandian *et al.* (unpublished)
Zebrafish	Cytoplasmic injection	1.0	Pandian *et al.*[39]
Zebrafish	Cytoplasmic injection	0.7	Stuart *et al*[87]*
Zebrafish	Cytoplasmic injection	0 3	Culp *et al.*[84]
Carp	Injection near II polar body	0.3	Zhu[3]*
Seabream	Germinal disc injection	0 02	Cavari *et al*[66]

*Does not include the required time for manual dechorionation or enzymatic digestion.

Table 5. Quantitative aspects of the injected genes into fish eggs

Species	Gene	Injected gene size (kb)	Injected volume (nl)	Concentration (μg DNA/ml)	Copy (No × 10^6)	References
Oocyte injection						
Medaka	PδC	14.4	0.2	10	0.005	Ozato *et al.*[40]
Cytoplasmic injection						
Medaka	CAT	–	0.5	50	–	Winkler *et al.*[80]
Medaka, zebrafish	Luc	–	–	–	1	Alestrom *et al.*[68]
Atlantic salmon	AFP	7.8	2–3	–	2	Fletcher *et al.*[44]
Atlantic salmon	AFP	–	–	–	1	Davies *et al.*[49]
Atlantic salmon	AFP	7.9	–	–	1	Hew *et al.*[53]
Atlantic salmon	AFP	–	–	–	1	Shears *et al.*[46]
Atlantic salmon	hGH	6.3	10	10	1	Rokkones *et al.*[17]
Atlantic salmon	β-gal	–	20	10	2	McEnvoy *et al.*[81]
Trout	hGH	–	20	10	–	Chourrout *et al.*[18]
Tilapia	hGH	4.0	–	–	1	Brem *et al.*[29]
Zebrafish	rGH	8.9	0.2	15	–	Pandian *et al.*[39]
Zebrafish	Hygro	5.2	0.3	15	–	Stuart *et al.*[87]
Zebrafish	CAT	–	–	–	2	Vielkind *et al.*[77]
Zebrafish	β-gal	–	–	25–35	–	Culp *et al.*[84]
Zebrafish	CAT/Neo	–	–	50	–	Ivics *et al.*[78]
Carp	rtGH	5.2	20	–	1	Zhang *et al.*[55]
Goldfish	hGH	9.4	1–2	–	1	Zhu *et al.*[5]
Goldfish	Neo-res	–	2	25	–	Yoon *et al.*[85]
Loach	hGH	7.9	1.2	30	–	Zhu *et al.*[6]
Loach	rtGH	–	–	–	1	Chen *et al.*[22]
Catfish	hGH	2.9	20	–	1	Dunham *et al.*[30]
Seabream	β-gal	–	300	100–200	1	Cavari *et al.*[66]

due to microinjection; the injected eggs of the carps also suffered almost no additional mortality despite being subjected to microinjection and enzymatic digestion of chorion. The second group comprising the zebrafish suffered about 40% additional mortality owing to microinjection and dechorionation; hence between medaka and zebrafish, the former appears better suited for experimental studies on transgenic biology. But the third group includes the cichlids, catfish and seabream, whose microinjected eggs suffered (total) mortality of 40 to 90%. Apparently these tropical catfish and cichlids are inherently too sensitive to microinjection and therefore render the task of producing transgenic fish more difficult. Unfortunately a comparative study on alternate methods of introducing transgenes has not yet been undertaken for the cichlids and catfish.

The possible reasons for the observed sensitivity of the carps, tilapias and catfish to microinjection may be listed as follows: (i) The need for removal of chorion manually or enzymatically prior to microinjection. (ii) The diameter of the needle used for microinjection; for instance, Brem *et al.*[29] and Phillips[92] observed 25 and 50% mortality in tilapia eggs, which were injected (via micropyle) with needles having diameter of 25 and 50 μm, respectively. (iii) The quantity of injected DNA; this idea leads to the new hypothesis of 'DNA load dependent-integration'; Fletcher and Davies[4] have pointed out that many salmonid eggs with large amount of DNA can successfully tolerate more than 200 pg DNA, whereas the zebrafish is sensitive to even 50 pg DNA. This interesting new hypothesis merits further research work. (iv) The egg quality; it has been repeatedly indicated by workers in this field that the survival of the uninjected (control) and injected eggs varies widely owing to egg quality, which appears to vary considerably from one spawn to another, and one female to another. For instance, the survival of the microinjected eggs ranged from 0 to 40% for the channel catfish[30], 0 to 50% for the Indian catfish (personal observations) and 16 to 72% for zebrafish[39]. (v) The microinjection time; the time required for successful completion of microinjection varies from 0.02 to 2 min/egg (Table 4); it is not clear whether the too short time taken for microinjection of a single egg (as in the case of the eggs of seabream) has led to a greater mortality (see Table 6).

At this juncture, it must be indicated 'that egg microinjection procedures are time-consuming, laborious, species-specific and in some cases technically demanding'. Consequently, transgenic fish production using this method is 'likely to be slow' and may prove to be a 'bottleneck to the exploration, expansion and realization of the full potential of this powerful technology'[4]. Unfortunately, very few comparative studies on different methods have been undertaken to test the efficiency of transfer of transgenes. The works of Inoue *et al.*[60], Zhu[3] and Kavumpurath *et al.*[70] are the ones, in which more than one

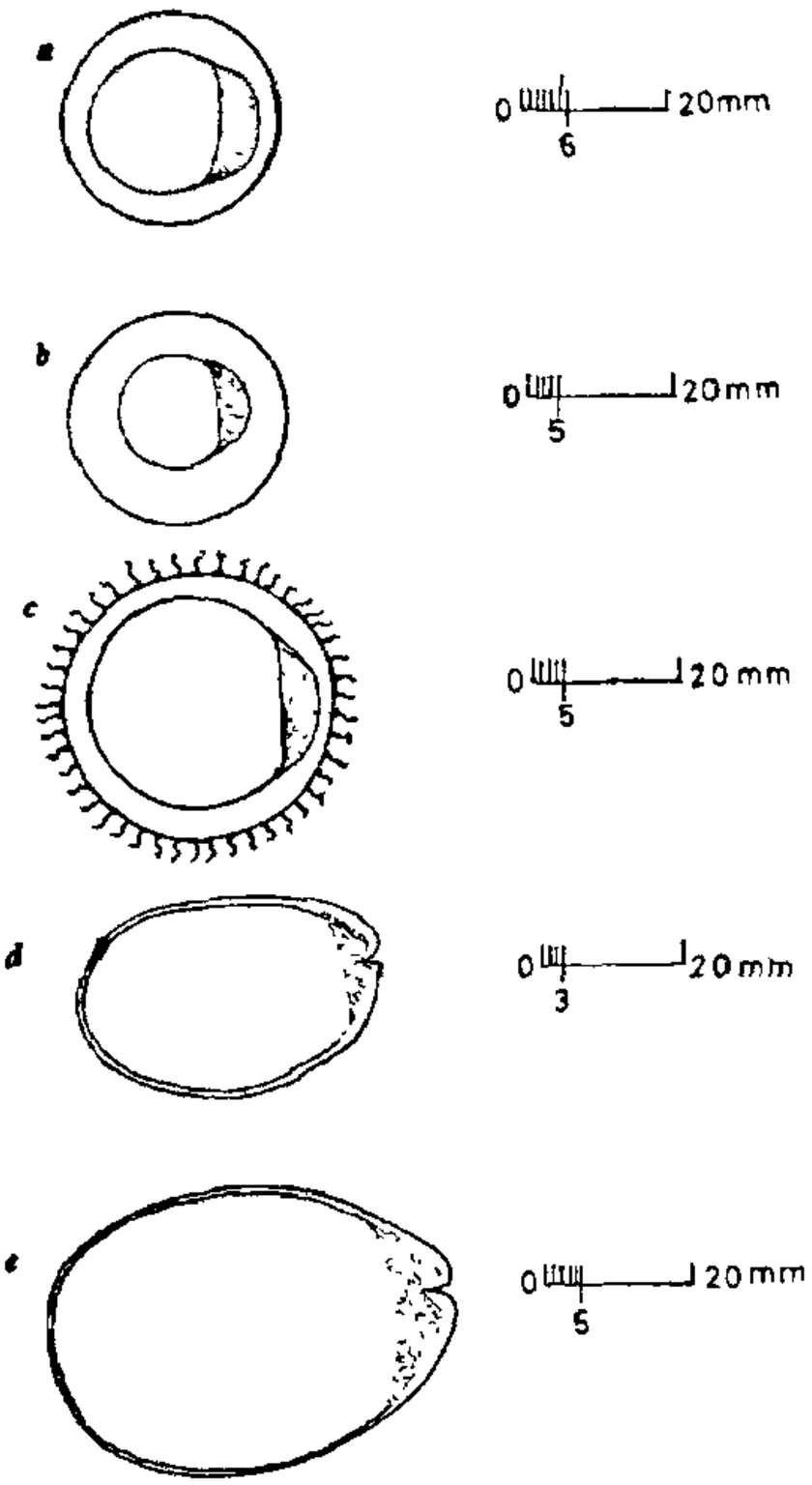

Figure 2. Approximate area covered by cytoplasm in eggs 20 min after fertilization of some fish. *a*, catfish; *b*, zebrafish; *c*, medaka, *d*, zebra cichlid and *e*, tilapia. In chichlid eggs micropylar injection is usually undertaken. Note the micropylar opening in the zebra chichlid and tilapia.

method of gene transfer has been tested in medaka, loach and zebrafish, respectively. A few others like Zou *et al.*[93] and Zhang *et al.* (cited in Chen *et al.*[58]) have used electroporation for the transfer of transgene in one or other carp species. Therefore, it is possible to compare the reported transfer efficiency values of these carps with those reported by others obtained for microinjection (e.g. Zhu[3]). Despite lower survival, electroporation ensures a higher (> 60%) transfer efficiency (e.g. Xie *et al.*[94]; see Powers quoted in Fletcher and Davies[4]). Regarding sperm-mediated transfer, it must be stated that such cytoplasmic introduction requires the transfer of 1 to 2 million copies (Table 5). 'Therefore it is difficult to conceive how one million copies of the transgene DNA could be transferred through micropyle by a single sperm. Besides, since many millions of sperm are required to ensure fertilization of a single egg, the proportion of sperm carrying the exogenous DNA would have to be high in order to attain the integration frequencies observed using microinjection'.[4] Yet more comparative studies on transferring transgene using electroporation

Table 6. Survival of microinjected fish eggs

Species	Injected eggs (No)	Survival at hatching (%)		References
		Non-injected eggs	Injected eggs	
Medaka	–	–	80	Ozato *et al.*[64]
Medaka	3000	78	70	Winkler *et al.*[80]
Atlantic salmon	–	–	75	Chourrout *et al.*[18]
Atlantic salmon	1800	80	80	Fletcher *et al.*[44]
Atlantic salmon	1000	80	80	Hew *et al.*[53]
Salmonids	10000	80	80	Shears *et al.*[46]
Salmonids	1227	86	78	Rokkones *et al.*[17]
Zebrafish	1002	–	43	Stuart *et al.*[87]
Zebrafish	1266	80	46	Pandian *et al.*[39]
Zebrafish	–	80	59	Culp *et al.*[84]
Carp	1746	35	37	Zhang *et al*[55]
Carp	1034	34	39	Chen *et al.*[22]
Tilapia	30	52	66	Brem *et al*[29]
Zebra cichlid	–	–	35	Ken *et al.*[32]
Catfish	80	95	13	Dunham *et al*[30]
Catfish	3341	10	12	Powers *et al.*[31]
Catfish	2400	65	14	Pandian *et al.* (unpublished)
Gilthead seabream	12000	–	10	Cavari *et al.*[66]

and sperm-mediated transfer techniques must be undertaken, when the mass production of transgenic fish are required to meet the aquacultural demands.

Integration

Table 7 shows several reports claiming genomic integration of one or the other transgene of either eukaryotic or prokaryotic source. The idea of introducing transgene into the cytoplasm of a 2-cell stage egg originates from the studies of *Xenopus laevis*[95,96], which poses common problems as described for fish (see Table 1). The transgene injected into the cytoplasm of 2-cell *Xenopus* egg persisted cytoplasmically not only in the tadpole but also in the adult. Not surprisingly many biologists working on transgenic fish did report almost similar findings, as those observed by Etkin and his colleagues. Consequently most of the claims regarding genomic integration or cytoplasmic persistence are based on results from dot blot or southern hybridization or polymerase chain reaction (PCR) of the DNA extracted from fry. Hardly a few contributors have based their claims on genomic integration/cytoplasmic persistence after using older or adult fish (e.g. Stuart *et al.*[87]; Rokkones *et al.*[17]). Therefore the following description should be considered with this background note of caution.

Most of the relevant studies have established that the transgenes undergo amplification and degradation simultaneously. Typically, the microinjected DNA is rapidly amplified after fertilization but only a small proportion of the replicated DNA is maintained after gastrula stage. For instance, the presence of the injected transgenes

in supercoiled, open circular, closed circular and multimeric forms has been detected almost throughout the embryogenesis in loach, goldfish, medaka and tilapia (e.g. Winkler *et al.*[80]). However, the sequences are rapidly converted into a high molecular weight form at hatching indicating that other forms had been degraded (e.g. Chong and Vielkind[73]).

During the corresponding embryonic (cleavage to gastrulation) period, the injected DNA also suffers degradation owing to the activity of DNAase. Fairly large number or replicated DNAs suffer fragmentation into dimers and multimers. The persisting concatemers are in most cases random polymers rather than tandem repeats, as found in mammals. As indicated elsewhere, eggs of salmonids can tolerate the introduction of fairly large amounts of transgene. Available reports show that salmonids have been injected with as small as 7 pg DNA[8] or as much as 500 pg DNA[19]. A comparative analysis suggests that (i) the percentage of transgenics decreased from about 74 to 7% with decreasing amount of the injected DNA, and (ii) it also decreased from 75% in the 2 1/2-month old individuals[17] to 40% in the 12-month old individuals[19]. A higher percentage (75%) of rainbow trout retained the linear form of the injected transgene than the supercoiled form (40%)[18]. Briefly, the percentage of transgenic individuals and the quantity of transgene in the transgenics decreased as functions of time and injection dose. A possible reason for this is the extrachromosomal persistence of the injected gene sequence. Such extrachromosomal persistence of the injected

Table 7. Cytoplasmic persistence and genomic integration of foreign genes in fish

Injected gene		Species	Genomic integration (%)	Cytoplasmic persistence (%)	Reference
Mammalian and Avian genes					
Human growth hormone	(hGH)	Rainbow trout	75		Chourrout *et al.*[18]
		Atlantic salmon		56	Rokkones *et al.*[17]
		Loach	50		Zhu *et al.*[5]
		Loach	40		Chen *et al.*[22]
		Loach	47		Xie *et al.*[15]
		Red crucian carp	42		Chen *et al.*[22]
		Crucian carp	52		Chen *et al.*[22]
		Minor carp	52		Chen *et al.*[22]
		Silver crucian carp	70		Chen *et al.*[22]
		Red carp	88		Chen *et al.*[22]
		Catfish	20		Dunham *et al.*[30]
		Zebrafish		64	Khoo *et al.*[33]
		Goldfish	43		Zhou[28]
		Tilapia	67		Brem *et al.*[29]
Rat growth hormone	(rGH)	Zebrafish	69*		Pandian *et al.*[39]
Chicken δ crystallin	(pδC)	Medaka	50		Ozato *et al.*[40]
Piscine genes					
Rainbow trout growth hormone	(rtGH)	Carp	6		Zhang *et al.*[55]
		Carp	10		Chen *et al.*[22]
		Catfish	6		Powers *et al.*[31]
Chinook salmon growth hormone	(csGH)	Atlantic salmon	2		Du *et al.*[62]
		Goldfish	22		
Antifreeze protein	(AFP)	Atlantic salmon	6		Fletcher *et al.*[44]
		Atlantic salmon	3		Shears *et al.*[46]
		Goldfish	20		Wang[51]
Prokaryotic genes					
CAT gene		Zebrafish	38		Khoo *et al.*[79]
β-Gal		Zebrafish	17		Culp *et al.*[84]

*Cumulative value of both genomic integration and cytoplasmic persistence.

gene sequence results in its unequal distribution between daughter cells, ultimately leading to the production of mosaic tissues, organs and individuals.

A most common observation made by almost all workers is this mosaicism. The spatial and temporal distribution of the injected gene, either at the cytoplasmic level[87] or genomic level[80] has been described in detail by various workers. Relatively large differences in the persisting copy number of the injected gene have also been observed in several tissues of presumptive transgenic trouts[19]. The cytoplasmic delivery of foreign DNA at later than the 2-cell stage has been traced as the main causative factor (see Houdebine and Chourrout[97]). If this is so, it is not clear why such mosaicism was observed by Ozato *et al.*[40], who introduced the δ crystallin gene into the oocyte of medaka; for instance, they estimated 50–100 copies in some embryos, and 1–3 copies in few other embryos. In fact 25% presumptive transgenic mice were observed to be mosaics by Palmiter *et al.*[1], who introduced the foreign gene into male pronucleus. Therefore it appears that any delay in genomic integration, prior to second cell division may lead to mosaicism; such delay may be due to delayed delivery and/or integration of the transgene, though it may be ultimately present in the system.

As stated earlier almost all the earlier researchers, who used any transgene of fish-origin, detected expression. Yet it may be noted that almost all these groups reported relatively very low integration frequency (< 20%) than those (> 40%) recorded for the integration of the transgenes of mammalian origin (Table 7). It may be pointed out that introduction of transgene of fish-origin facilitated a higher percentage of expression (Table 2), but ensured integration in a fewer injected eggs alone. This aspect merits further study. But it must also be indicated here that the integration frequency reported by Hammer *et al.*[2], who transferred the mammalian growth hormone gene into the male pronucleus of mouse, rabbit, pig and sheep, also ranged from 1 to 30%.

It was already pointed out that different research groups had differently tailored the selected gene sequence prior to injection. Therefore one has to be cautious in comparing the results reported by these authors. For instance, the hGH gene sequence used by five different research groups removed a shorter or longer fraction of the plasmid; hence it ranged from 2.9 to 9.4 kb (Table 5). Some of these reconstructs are illustrated in Figure 3. Unfortunately Zhu *et al.*[5] reported a value of 50% for integration frequency (see also Zhu[3]) in carp and loach of different age groups ranging from 'fry' to 'adult'. The other four groups made observations in comparable age groups (73–90-day old fingerling). Apparently the integration frequency decreased from about 75%, when the 6.3 kb sequence was chosen for introduction to 20%, when the injected sequence was as short as 2.9 kb.

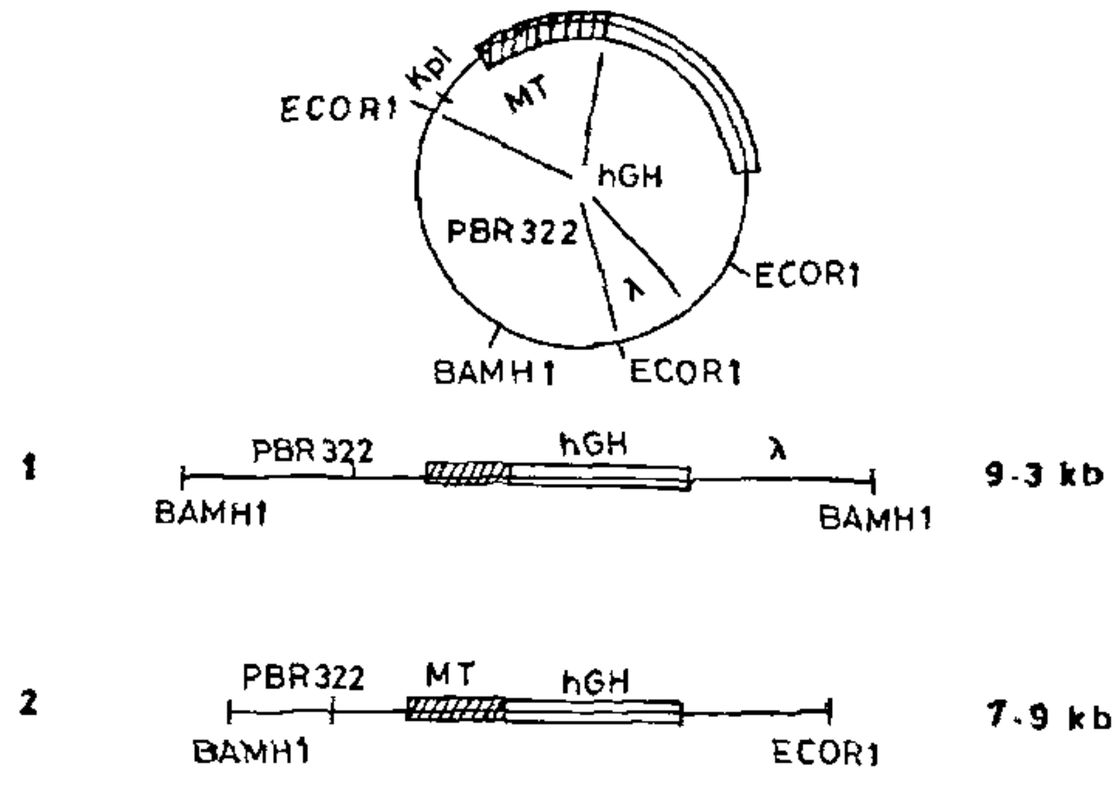

Figure 3. Reconstructs of human growth hormone (hGH) gene used for microinjection by 1. Rokkones *et al.*[17], 2. Zhu[3], 3. Brem *et al.*[29], 4. Dunham *et al*[30], 5. represents the probe used for hybridization studies.

However such a generalization requires confirmation from further work.

Transmission

Relatively very few research groups have published information on transmission of the introduced transgene. Their observations are listed in Table 8. The currently available information is meagre for food-fish like salmonids and cyprinids, which have a longer generation time[24]. In zebrafish, whose generation time is short (3–4 months), it has been possible to have some information on the mode of transmission of the transgene from founder parents to F_2 offspring. Most of the authors, who have followed transmission frequency of the transgene in zebrafish, have concluded that because of the mosaic nature of integration, the transgenic characters are not transmitted according to the expected Mendelian ratios. For instance, out of 20 presumptive transformants crossed with normal counterparts, only one female proved to be a transgenic mosaic. About 20% of her germ cells contained foreign DNA at about 100 copies per cell, but less than one copy per fin cell[87]. Wei *et al.*[24] have

Table 8. Transmission of foreign genes in fish

Species	Generation F_0	F_1	F_2	Reference
Atlantic salmon	+	+		Hew *et al.*[53]
Rainbow trout	+	+		Maclean *et al.*[13]
Trout	+	+		Guyomard *et al.*[19]
Trout	+	+		Penman *et al.*[8]
Carp	+	+		Zhang *et al.*[55]
Carp	+	+		Chen & Powers[21]
Carp	+	+		Chourrout *et al.*[18]
Carp	+	+	+	Wei *et al.*[24]
Zebrafish	+	+	+	Stuart *et al.*[87]
Zebrafish	+	+	+	Pandian *et al.*[39]
Zebrafish	+	+	+	Khoo *et al.*[79]
Zebrafish	+	+	+	Culp *et al.*[84]
Zebrafish	+	+		Bayer & Campos-Ortega[88]
Zebrafish	+	+		Kavumpurath *et al.*[70]
Medaka	+	+		Inoue *et al.*[60]
Medaka	+	+		Ozato *et al.*[64]
Catfish	+	+		Chen *et al.*[58]
Blunt snout bream	+	+		Xia *et al.*[34]

shown that the copy number of hGH gene not only varied from tissue to tissue but also individual to individual transformant carps. Khoo *et al.*[79] traced the genealogy of zebrafish by cross breeding F_1 offspring arising from the positive founder parents to untreated partners and subsequent outcrossing of some of their progenies. F_1 progenies of the 4 founder parents displayed no transgenic character but some of their F_2 offspring proved to be transgenic. Likewise, the results reported by Zhang *et al.*[55] for the transmission of rtGH gene into the F_1 carp offspring also confirmed the mosaic nature of the founder transformants.

Expression

In general two kinds of transgenes are cloned for use. They are: (i) full length nuclear gene and (ii) complimentary DNA (cDNA). A nuclear gene comprises different parts: the coding gene sequence, and promotor sequence and natural spacers (introns), which are not transcribed, but the cDNA contains only those sequences, which code for a specified protein. Consequently, the elements responsible for expression, i.e. the promotor/enhancer sequences may be absent in the cDNA. As promotors are not specific, they may be used for variety of coding genes (see Purdom[99]). Three types of promotors/enhancers have been utilized in transgenic studies: (i) enhancers from normal cellular genes that are active in most, if not all tissues (e.g. metallothionein-1 (MT-1)[100]; β-actin enhancer-promoter complexes[101, 102]); (ii) enhancers active only in specific tissues (e.g. the long terminal repeat (LTR) protein of the avian Rous sarcoma virus[103] and (iii) viral enhancers active in many tissues, especially those normally infected by the virus (see Moav *et al.*[36]).

Although it is generally known that a number of factors control expression of foreign gene in the transgenic animal, only a few detailed studies have been undertaken to understand the various aspects of expression.

Table 9 shows that the expression of a DNA has been detected by the presence of its mRNA using Northern blot analysis (e.g. Liu *et al.*[75]) and/or the corresponding protein by immunobloting (e.g. Rokkones *et al.*[17]) or radioimmunassay (e.g. Winkler *et al.*[80]). Those who used prokaryotic gene made a quantitative estimation of the enzyme (CAT; e.g. Vielkind *et al.*[77]). Phenotypic expression such as somatic growth has been regarded as a measure of the expression of injected genes. Very few authors have chosen more than one technique to confirm the expression of foreign genes (e.g. Rokkones *et al.*[17] and Zhu[3]). Analysis of presumptive transgenic fish by the extraction of DNA from the whole individual or blood cells (e.g. Moav *et al.*[36]), and Southern blot technique are tedious, laborious and expensive (see Hew *et al.*[104]). In most studies related to the injection of AFP gene (antifreeze protein gene), only the estimation of the level of AFP in serum was used to confirm the transgenicity; in these cases the need for DNA extraction for Southern blot analysis was eliminated. A few studies have chosen to use marker genes, such as luciferase and melanin concentrating hormone[68] to identify the positive transgenic fish. This has helped to eliminate the need for tedious technique as well as to avoid sacrificing the presumptive transgenics.

A survey of the available information on expression permits the following generalizations:

(i) Viral promotors ensure more definitive expression than others like metallothionine (MT). For instance, the rtGH sequence comprising RSV promotor ensured expression in carp[55]; medaka[61] and seabream[59]. The human growth hormone gene sequence with MT promotors ensured expression in loach and crucian carp[3] but not in tilapia[29] and catfish[30] (see also Table 2). Considering the potential health hazards and consumer acceptance, however, the use of viral or MT promotors is not advisable to generate transgenic fish for human consumption. Therefore, many workers have constructed 'all-fish' gene sequences comprising of an endogenous fish promotor, such as β-actin or antifreeze protein gene (e.g. Du *et al.*[98]). In this context, it may be interesting to note that Liu *et al.*[101, 102] analysed the β-actin proximal promotor using CAT gene as reporter sequence. The promoter includes CAAT, cArG and TATA boxes (Figure 4). Several constructs were made containing different fragments of the β-actin gene fused to the CAT gene. Liu *et al.*[101] for instance, observed maximum expression, when one or the other construct containing the TATA and cArG boxes was used. In general a high level of expression is observed, when 'all-fish' chimeric GH gene construct was used.

Table 9. Expression of foreign genes in fish

Species	Expression detected at the level of mRNA	Enzyme	Protein	Visible character	Reference
Medaka			+		Ozato *et al.*[40]
Medaka			+		Inoue *et al.*[43]
Medaka		+			Chong *et al*[73]
Medaka		+			Vielkind *et al*[77]
Medaka		+			Winkler *et al.*[80]
Medaka		+		+	Tamiya *et al.*[69]
Atlantic salmon		+		+	McEnvoy *et al.*[81]
Atlantic salmon	+		+		Rokkones *et al*[17]
Atlantic salmon			+		Davies *et al.*[48]
Atlantic salmon			+		Fletcher *et al.*[45]
Salmonids			+		Du *et al.*[62,98]
Salmonids			+	+	Hew *et al.*[53]
Rainbow trout			+		Penman *et al*[8]
Bluegill; salmon	+				Huang *et al.*[50]
Zebrafish	+				Liu *et al.*[75]
Zebrafish		+			Stuart *et al.*[74]
Zebrafish		+			Vielkind *et al.*[77]
Zebrafish			+		Shears *et al.*[46]
Zebrafish		+			Khoo *et al.*[79]
Zebrafish		+		+	Alestrom *et al.*[68]
Zebrafish		+		+	Kavumpurath *et al.*[70]
Goldfish			+		Zhou[28]
Goldfish		+			Yoon *et al.*[85]
Goldfish		+			Hallerman *et al.*[76]
Goldfish	+				Liu *et al.*[75]
Carps			+	+	Zhu[3]
Carps			+	+	Chen *et al.*[22]
Carps			+		Zhang *et al.*[55]
Carps			+	+	Tian *et al.*[23]
Red carp				+	Guo *et al.*[67]
Crucian carp; loach	+			+	Zou *et al.*[26]
Loach			+	+	Zhu[3]
Loach			+	+	Chen *et al.*[22]
Loach				+	Ge *et al.*[63]
Walleye			+		Moav *et al.*[36]
Northern pike	+				Liu *et al.*[75]
Blunt snout bream				+	Xia *et al.*[34]

(ii) The presence of adequate number of copies of the injected gene in the desired tissue/organ is another important criterion that determines the level of expression. When a strong promotor like virus or MT is not used, one must ensure the presence of adequate number of copies of the transgene, so that a cumulative expression of the injected gene results in the production of optimum quantity of the desired products. For instance, Fletcher *et al.*[44] injected large DNA fragments carrying the complete flounder AFP gene into the salmon egg. The AFP gene was expressed in the liver and secreted into the blood stream. Concentrations of the order of 5 ng AFP/ml serum, which is less than that required for protecting the fish in subzero waters were obtained. It is known that the winter flounder contains at least 40 AFP gene copies and many of these genes are expressed together to elevate the protein concentration to a level required for freeze resistance.

(iii) Another aspect that is receiving attention is the expression of homologous and heterologous genes (e.g. Friedenreich and Schartl[105]; Moav *et al.*[36]). The expression of heterologous genes proved unsatisfactory for two reasons: (i) the undesired tissue specific expression and (ii) the side-effects leading to impairment of other functions. Transgenic rabbits, pigs and sheep injected with human GH gene insert failed to grow at an enhanced rate[2]. Transgenic females of zebrafish were found to produce less number of eggs than the control[39]. Transgenic female mice expressing high levels of human GH gene were found infertile[1]. These studies have recommended the use of homologous transgene to avoid some of these possible complications. Therefore, considerable efforts have been made by Western and Chinese scientists to sequence the homologous GH gene of Chum salmon[106], Coho salmon[107], Chinook salmon[104], rainbow trout[108] and grass carp[109,110]. Regrettably, Indian and other Asian

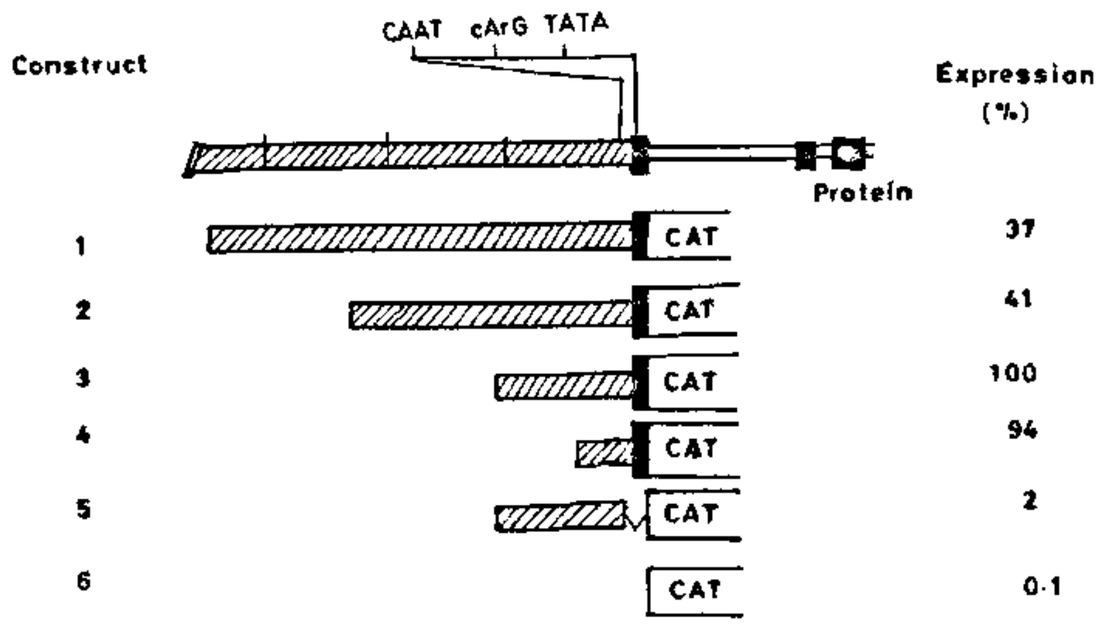

Figure 4. Expression of different constructs containing β-actin gene fused to CAT gene (from Liu *et al.*[101]). Construct 3 containing TATA and cArG boxes exhibit maximum expression.

scientists have not even commenced research work for the construction of homologous GH genes for the Indian carps and tilapias, whose dramatic growth could satisfy the needs of 'protein-hungry' Asians and Africans.

Containment and conservation

Containment

The need for containment of transgenic fish and regulations of their production and use are much discussed in Western countries and some of the aspects have been briefly summarized[111, 112]. The Canadian and Norwegian research workers prefer to use the tropical zebrafish for their studies on transgenic fish. As the thermal requirement of these fish is over 20°C, the adverse effects due to the escape of the presumptive transgenics into freshwater systems whose temperature never exceeds 20°C, will effectively be eliminated. In the tropical countries, where the rules and regulations regarding the transgenic organisms are neither formulated well nor implemented effectively, it is advisable to use triploid eggs for introduction of foreign genes, since such fish are usually sterile[113]. It has been shown that the procedure for production of triploid eggs is simple[114, 115]. Hence transgenic fish workers may not find it difficult to adopt this slightly modified procedure. In view of the fact that male triploids suffer only partial sterility[116], simple techniques have also been described for production of all female triploids[117] or all male triploids[118].

Conservation

Considering the immense potential for the production of new strains of economically important and other fish species, there is an urgent need for developing new techniques for conservation of wild strains. Cryopreservation of sperm and fertilized eggs has been suggested as a very useful technique for conservation. By virtue of the almost yolk-free (alecithal) nature of eggs, cryopreservation of fertilized eggs of mammals poses relatively less problems than the mesolecithal (amphibians) and telolecithal eggs of all other vertebrates; yet the scope for successful cryopreservation of fish sperm (e.g. Stoss[119]) and androgenesis (e.g. May *et al.*[120]) render the fish uniquely advantageous as these techniques can be used profitably to recover genotypes from cryopreserved sperm. Indeed the cryopreserved sperm may become 'the gene banks' for the wild strains that are to be conserved for future use. Whereas the techniques required for successful production of gynogens have been described for several species (see for a review Marian and Pandian[121]), those for androgenesis have so far been reported only for two salmonid species[120, 122]. Unfortunately, no method for inducing androgenesis in tropical fish is yet available. Hence the Asians are at a disadvantage for utilizing this very useful technique for conservation of their wild strains.

The doubling of paternal ploidy to obtain androgenetic diploid should lead to the production of both XX and YY individuals, as males are known to be heterogametic in many fish species[123]. Reports are available for several species to show that though low in numbers, YY-males are viable in medaka[124], goldfish[125], coho salmon[126], rainbow trout[122], tilapias[127, 128] and guppy[129]. It is possible to produce sex inversed male (XX)[117] and to preserve all X sperm, as well as to produce YY females[130]. Incidentally, all these procedures lead to the development of pure inbred lines.

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