Transgenesis in fish

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Abstract. Gene transfer into fish embryo is being performed in several species (trout, salmon, carps, tilapia, medaka, goldfish, zebrafish, loach, catfish, etc.). In most cases, pronuclei are not visible and microinjection must be done into the cytoplasm of early embryos. Several million copies of the gene are generally injected. In medaka, transgenesis was attempted by injection of the foreign gene into the nucleus of oocyte. Several reports indicate that the injected DNA was rapidly replicated in the early phase of embryo development, regardless of the origin and the sequence of the foreign DNA. The survival of the injected embryos was reasonably good and a large number reached maturity. The proportion of transgenic animals ranged from 1 to 50% or more, according to species and to experimentators. The reasons for this discrepancy have not been elucidated. In all species, the transgenic animals were mosaic. The copy number of the foreign DNA was different in the various tissues of an animal and a proportion lower than 50% of F1 offsprings received the gene from their parents. This suggests that the foreign DNA was integrated into the fish genome at the two cells stage or later. An examination of the integrated DNA in different cell types of an animal revealed that integration occurred mainly during early development. The transgene was found essentially unrearranged in the fish genome of the founders and offsprings. The transgenes were therefore stably transmitted to progeny in a Mendelian fashion. Southern blot analysis revealed the presence of possible junction fragments and also of minor bands which may result from a rearrangement of the injected DNA. In all species, the integrated DNA appeared mainly as random end-to-end concatemers. In adult trout blood cells, a small proportion of the foreign DNA was maintained in the form of non-integrated concatemers, as judged by the existence of end fragments. The transgenes were generally only poorly expressed. The majority of the injected gene constructs contained essentially mammalian or higher vertebrates sequences. The comparison of the expression efficiency of these constructs in transfected fish and mammalian cells indicates that some of the mammalian DNA sequences are most efficiently understood by the fish cell machinery. Chloramphenicol acetyl transferase gene under the control of promoters from Rous sarcoma virus, and human cytomegalovirus, was expressed in several tissues of transgenic fish. Chicken δ-crystallin gene was expressed in several tissues of transgenic fish. Rainbow trout growth hormone cDNA driven by the Rous sarcoma virus promoter was expressed in transgenic carps leading to a faster growth of these animals. The antifreeze protein gene from flounder was expressed in transgenic salmon. These data indicate that transgenesis in fish is relatively easy but that fish gene sequences must be preferably used to obtain a good expression of the transgenes. Fish is a good biological model, specially for developmental studies and it is an increasing part of human food. For these reasons, transgenesis in fish is most likely to be more and more practised in the coming years.
Key words. Gene transfer; fish.

Introduction

Fish represent a category of animals of great interest to biologists for several reasons[42]. The number of fish species is very large and they constitute a rich catalogue for adaptation and evolution studies. Embryos are in most fish species very abundant and their development takes place in simple experimental conditions. Fish cell lines are also available and their maintenance in culture conditions is easy. From a practical point of view, fish is one of the major human foods and fish aquaculture is amenable to a relatively rapid development throughout the world. Transgenesis may be a very efficient tool for all these studies. Gene transfer has been carried out successfully in all categories of animal species including mammals, birds, fish, insects and worms. The development of this technique in fish has become significant during the last five years. Although success still remains limited for these animals, the results which have been obtained by different groups working on various species are very encouraging. The present review aims at reporting the major experimental data established so far.

The methods of transgenesis in fish

In most fish species, external fertilization is a natural process and many embryos at a given stage of development can be obtained easily. For these reasons, fish is a biological material of choice for gene transfer. In practice, things are not so simple. In some species, embryos develop very rapidly and the first cell stage is too short to allow an easy gene transfer. In many fish eggs, the cell is surrounded by a chorion which becomes hard soon after fertilization. Pipets for microinjection cannot cross easily this barrier. Chorion can in some species be withdrawn mechanically or enzymatically, but this is not yet possible in some major species such as salmonids. Appropriate incubation medium may slow down hardening of the chorion and facilitate microinjection[57]. In eggs such

as those from salmonids a two-step process proved efficient to reach the embryo cell. The chorion was first pierced with a sharp piece of glass allowing micropipets to reach easily the cell membrane. Up to 60–80% vs 90% in the non-microinjected control embryos survived the manipulation and developed to hatching[9,33,44,59]. In the case of tilapia, injection of DNA through the micropyle soon after fertilization allowed introduction of the foreign material into the embryo cytoplasm[6]. Addition of 0.5% phenol red into the DNA solution facilitated the estimation of the microinjected volume[49]. Fish eggs are relatively large and their chorion is often poorly transparent. It is therefore impossible to routinely observe pronuclei and to microinject the foreign DNA into them.

In mammals, only microinjection into pronucleus or into nucleus of the two cells embryos proved to generate transgenic animals[5]. DNA injection into the cytoplasm of embryos or of cultured cells was in all cases inefficient. In mammals[45] and in birds retroviral vectors are able to carry foreign genes to the genome of embryo cells. No retrovirus has been identified unambiguously in fish and the use of such vectors is presently excluded in these species. Embryonic stem cells (ES cells) have been shown to be a good way to transfer foreign genes into mouse embryo[43]. No equivalent to mouse embryonic stem cells has been found and most likely stem cells have not even been looked for in lower vertebrates and this tool is not available for fish studies. The direct transfer of foreign DNA into spermatozoa before fertilization has been claimed to generate transgenic mouse[32] and sea urchin[1] at a high rate. This approach has been shown to be irreproducible in mouse and unsuccessful in trout (Chourrout et al., unpublished data). However, experiments carried out recently have demonstrated that chloramphenicol acetyl transferase and β-galactosidase genes are very efficiently transferred to chicken spermatozoa through a direct contact with pure DNA. The foreign DNA was integrated in the genome of adult chicken, expressed and transmitted to progeny (E. Revel, personal communication). The success in chicken seems to rely on the use of a particular buffer which stabilizes spermatozoa against inactivation and probably favors DNA uptake. Most of the transgenic chicken were mosaic. In chicken, as in fish and *Xenopus*, exogenous DNA is rapidly replicated in an early stage of embryo development. This phenomenon may have augmented the number of DNA molecules available for integration and contributed to generate a high proportion of transgenic mosaic animals as in fish. Similar experiments which are being performed in fish and mammals using the E. Revel solution should rapidly give an answer to these questions. In a lower vertebrate, *Xenopus*, experiments carried out several years ago showed that microinjection of DNA into cytoplasm of the early embryo was followed by a relatively long persistence of the foreign DNA sequences and to some expression[16,17]. These pioneer experiments suggested that a similar approach could also be efficient in fish. This happened to be the case and essentially all the experimentators microinject their gene constructs into the cytoplasm of early fish embryo[3,6,8,9,11,12,14,19,25,31,33,35,44,46,49,50,52,55,58,59]. Up to 20 nl containing 200 pg of DNA corresponding to 50 million copies of the gene are injected into the cytoplasm of the trout embryo. Higher amounts of DNA led to much lower survival of embryos[9]. Lower amounts of DNA do not enhance the number of surviving animals but tend to give less transgenic fish[9,41]. The buffer and the stage of development (one-cell vs two-cell embryos) did not appear to have a significant impact in salmonids. In carp, the embryos survived similarly when injected in the first stage or the two-cell stage whereas the channel catfish embryos were more resistant when injected in the one-cell stage[26]. In carp, early one-cell stage and two-cell stage proved to be the best period for integration of foreign DNA[26]. In channel catfish the late one-cell stage and the early two-cell stage were preferable[26]. Several groups observed that circular plasmids were systematically less readily integrated into fish genome[9,41]. The proportion of transgenic animals obtained with circular plasmids seemed however higher in fish than in mammals. One noticeable exception is medaka. In this species, microinjection into cytoplasm after fertilization showed poor efficiency. Foreign DNA was injected into the nucleus of oocyte leading to a good yield of transgenic animals. In these conditions, no more than 10,000 copies of the gene were sufficient to generate transgenic medaka[29,39]. Interestingly, electroporation carried out with whole early embryo also permitted gene transfer[30]. As many as 25% of the treated embryos survived to become adult animals and 4% of the hatched fry were transgenic. The foreign gene was transmitted to progeny. This technique, given its simplicity, may prove very helpful for species in which microinjection appears inconvenient.

The fate of the injected DNA

Experiments carried out in *Xenopus* have shown that soon after the cytoplasmic injection, the foreign DNA fragments are rapidly associated to each other forming large concatemers. In this structure DNA is rapidly replicated regardless of the origin or the sequence of the injected DNA. As the embryo development proceeds, a large part of the replicated DNA disappears and a small proportion is maintained in the form of free DNA or integrated into the host genome[2,16,17,36]. This replicative phenomenon seems to be induced by fertilization and to be dependent on the formation of nucleus-like structures containing the foreign DNA[4,21] which in this way may be protected against degradation. The rate of replication is also dependent on the topology and the size of the injected template. Supercoiled plasmids shows poor capacity to be replicated whereas the large concatemers are the most efficient[15]. It has also been postulated that

the fusion of the nucleus-like structures with the cellular nucleus during embryo development allows the foreign DNA to be integrated into the host genome. This phenomenon which takes place later than the first cell stage thus leads to a non-uniform integration and it generates mosaic transgenic animals.

Although studies have not been carried out in much detail, it seems that similar events take place in fish. In zebrafish early embryo, the microinjected DNA was amplified tenfold by 6–10 h after fertilization and only a small proportion of the replicated DNA was maintained after the gastrula stage[49,50]. Similarly, in loach[31], medaka[8] and trout[44], the microinjected DNA replicated rapidly and most of it disappeared progressively. This persistence of foreign DNA in an unintegrated and unstable form may in some cases have led to an overestimation of the number of true transgenic fish. In all fish studied, the foreign DNA appeared in the form of concatemers probably rapidly formed after the microinjection[8,25,31,44,49,50]. In zebrafish, concatemers could be observed even before replication of DNA started. The amplified DNA was progressively degraded generating 1 kb fragments whereas a small proportion of large polymers (>23 kb) persisted[49].

Southern blot analysis revealed that the concatemers were in most cases random end-to-end polymers rather than tandem repeats as found in mammals[8,25,31,44,49,50]. Unexpectedly, presumably free concatemers were found in blood cells of transgenic six-month-old trouts[25].

All fishes examined were mosaic and a relatively large difference of foreign gene copy numbers was observed between several tissues of the animals[25]. In no case, did a given tissue appear to contain systematically more foreign genes than the others, suggesting that integration was a random process. The fact that the animals were mosaic indicates that the integration step occurred later than the one-cell stage. Interestingly, red and white blood cells from transgenic trouts contained in all but one of the examined animals the same number of transgenes (Chourrout et al., unpublished data). The integration thus probably occurred relatively soon in the embryo development. Somewhat unexpectedly, in medaka, DNA injected into the nucleus of oocytes was most likely also integrated after the first cell stage since mosaic animals were obtained in all cases[39]. Interestingly, in *Xenopus*, all the transgenic animals examined were also mosaic[17].

The Southern blot analysis also revealed that the transgenes were essentially not rearranged. However, in addition to the major bands specific of the injected genes, minor fragments were in most cases observed. These bands may be junction fragments. The pattern of junction fragments was expected to be complex as soon as several independent integrations may have taken place in the various cells of a given tissue. Some of the bands may reflect a rearrangement as well. Whatever happens, rearrangement seemed a relatively minor event. Restriction of the cellular DNA with enzymes which did not cleave the injected DNA generated long fragments, suggesting that the foreign DNA was essentially integrated into the host genome[41].

Transmission of the foreign genes

Foreign DNA was found in spermatozoa of transgenic fish. A small proportion of transgenic zebrafish[49,50] and high proportion of transgenic trout[25] contained the foreign DNA sequence in their spermatozoa respectively. Fish ensuing from the fertilization of normal oocyte with sperm from transgenic fish were transgenic. However, in the F1 offspring, only 20–50% and 7–30% in zebrafish and trout, respectively, harbored the foreign genes[25,49]. The transgenes were also transmitted to medaka and carp offspring[30,58]. This low proportion of transgenic animals among the F1 fish confirmed that the progenitors were mosaic in their germ cells. The F2 offspring was transgenic with the proportion of 50% in zebrafish[49,51] and 50% or 75% in trout (Chourrout et al., unpublished data). The transgenes were thus transmitted following a Mendelian fashion. Surprisingly, the transgenic medaka generated by electroporation transmitted their transgenes at a very high frequency (100%) to their progeny and the F2 offspring obtained by mating transgenic F1 fish were transgenic in the proportion of 88%[30]. Several independent integrations may have occurred in the progenitors on different chromosomes.

Southern blotting analysis clearly showed that the transgenes were transmitted essentially unrearranged. In several cases, the pattern of DNA fragments appeared simpler in offspring than in founders, an observation compatible with the assumption that the F0 animals were mosaic. Several DNA fragments generated by restriction enzymes were specific of each fish line, suggesting that they represent junctions of the foreign DNA to the host genome[25]. Moreover, in trout the free concatemers found in blood cells were generally not transmitted as efficiently as the integrated DNA. In some trout lines however a long putative free DNA fragment was transmitted to the F2 offsprings at a 50% rate (Chourrout et al., unpublished data).

Expression of the transgenes

An examination of the expression of the transgenes evaluated by the presence of the transcripts and of the corresponding proteins showed unambiguous expression in several cases. Chloramphenicol acetyl transferase (CAT) gene under the control of Rous sarcoma virus enhancer and SV40 promoter was expressed in several tissues of tilapia, zebrafish and medaka[8,28,49–51]. Similarly, the same gene driven by the human cytomegalovirus early gene promoter was expressed in liver, muscle, gut, skin and blood cells of developing trouts (Chourrout et al., unpublished data). The neomycin resistance gene fused

to the LTR of RSV was expressed to a significant degree when transferred to goldfish[56]. Chicken δ-crystallin was expressed in transgenic medaka and preferentially in lens fiber cells in early development. Unexpectedly, at later stages, the gene was expressed in most tissues with no more specificity[29,39]. The flounder antifreeze protein gene was expressed in liver of transgenic salmons[12,19]. The bacterial β-galactosidase under the dependency of mouse metallothionein promoter was expressed in developing transgenic salmons[35]. A gene construct containing the RSV promoter and the trout GH cDNA was efficiently expressed in several tissues of transgenic carps. Both the mRNA and hormone were found in blood cells[58]. The hormone could not be detected in plasma. This fact was expected since the cDNA used did not contain the signal peptide and the synthesized hormone had very little chance to be secreted. Unexpectedly, however, the transgenic carps grew faster than the control animals and this property was transmitted to progeny. This surprising fact raises the fascinating question of whether growth hormone delivers its message from the inside of its target cells.

Bovine GH gene under the control of RSV LTR promoter was shown to be expressed in Northern pike[46], but not in trout (Chourrout et al., unpublished data).

The human GH gene fused to mouse metallothionein was expressed at a relatively higher rate in trout the days following microinjection. mRNA for hGH could be detected by Northern blot analysis and hGH was immunodetectable in the incubation water of embryos[44]. However, the expression of the hGH was not maintained as development proceeded. The size of the hGH mRNA was heterogenous suggesting that transcription was not correctly initiated in fish cells or that the pre-mRNA was not properly processed. The hGH gene under the control of metallothionein promoter was also expressed in carp[7] but not in adult trout[9].

Several other groups have claimed that their gene construct containing GH genes of mammal origin accelerated growth of transgenic fish[3,33,46]. These results must be considered cautiously. The evidence that the transgene is really expressed was generally not shown. On the other hand, the faster growth of some individuals is significant only if a relatively large number of fish are considered since social behavior has a significant impact on fish growth independently of transgenesis. In the case of trout, clearly none of the heterologous GH transgenes were expressed to a significant degree[25]. The poor efficiency of these transgenes in fish is probably not due to fundamental blocking events such as methylation, mutations or rearrangements. It is rather due to the fact that in almost all cases, mammalian genes and gene contructs used were optimized to work in mammalian but not in fish cells. It is conceivable, that the utilized promoters were not efficiently recognized by the fish cell transcription machinery. Alternatively, splicing of primary transcripts from mammalian genes might not take place in all cases in fish cells. Data obtained by several groups suggest that this might be sometimes the case. Moreover, the signal peptide found in mammalian proteins might not drive efficient secretion from fish cells.

The expression of foreign genes in fish cell lines

In vivo experiments suggested that most of the gene constructs used were not adapted to work efficiently in fish cells. In order to determine if the low expression observed in vivo was due to gene constructs per se or to the fact that they were integrated in the genome of transgenic animals, fish cells transfected with various genes have been used by several groups. Several cell lines are routinely utilized for purposes other than the study of gene expression. Among these cell lines are EPC (epidermal cells from carps), RTG (gonad cells from rainbow trout) and RTH (hepatoma from rainbow trout). These cells can be cultured essentially in conditions used for higher vertebrates cell lines. Lower temperatures specific of each fish species are to be kept. Conventional transfection methods using DEAE dextran, calcium phosphate, lipofectin, polybrene etc. proved to work as well in fish cells. Unpublished data obtained by our group demonstrated that genes giving resistance to neomycin or to puromycin led to the generation of clones after culture in the presence of antibiotics. Several promoters, early SV40, RSV, human cytomegalovirus (CMV), Xenopus β-actin, human herpes simplex thymidine kinase, avian erythroblastosis (AEV) and murine leukemia (MMuLV) fused to the neo^r gene gave rise to the generation of clones resistant to geneticin. The early SV40 promoter fused to puromycin resistant gene also permitted cells to survive and multiply in the presence of the antibiotic. The same promoters proved to direct the expression of the CAT gene in the three above-mentioned cell lines. RSV and CMV promoters showed very significant activity whereas SV40, *Xenopus* β-actin promoters were much less potent. When fused to bovine GH gene or cDNA, the RSV promoter allowed the hormone to be secreted out of EPC cells in a transient transfection assay and with stably transformed cells. The cDNA was more efficient, as in mammalian cell lines[40]. The CMV promoter fused to bovine GH gene was inactive, as judged by radioimmunoassay, and the early SV40 promoter fused to human GH cDNA[25] generated negligible amounts of the hormone. Xenopus β-actin promoter fused to human GH gene exhibited undetectable activity although it was fully active in CHO cells. The human heat-shock promoter (HSP 70) associated to CAT gene and to human GH cDNA[13] proved to work efficiently in the fish lines and in an inducible manner. The carp and the trout cells maintained for 2 h at 38 and 28 °C, respectively, were induced to express the two chimaeric genes. Low but significant amounts of human GH could be found in the culture medium after the heat-shock. Before and long after the

heat-shock the CAT and the hGH genes were expressed only at a low basal level.

The thymidine kinase promoter from herpes simplex associated enhancer from human cytomegalovirus, SV40 and metallothionein genes, showed high efficiency to drive the expression of the CAT gene after transfection in several fish cell lines [22].

The mouse and the salmon metallothionein promoter were able to direct the expression of CAT gene in fish cell lines. The promoters were further stimulated by the addition of Zn^{++} in the culture medium [22, 23].

These experiments clearly show that fish cell lines can be used to evaluate the efficiency of a promoter. The correlation between the activity of the promoters in the cells and in transgenic animals was rather good. These experiments also indicate that, as expected, no fundamental difference exists between fish and mammalian cell transcription machinery. However, most of the time, the efficiency of promoters from mammalian genes seems very significantly reduced in fish cells. Other problems may also come in some cases from post-transcriptional steps. Indeed, the human GH gene was unexpectedly poorly expressed when fused to the RSV LTR which was shown to be a strong promoter for the CAT gene (Chourrout et al., unpublished results). The same gene gave rise to unmatured mRNA in early developing salmon [44] and in fish A_2 cells [22]. Signal peptides present in mammalian proteins may also be less efficient than their fish counterparts in providing secretion from fish cells.

Essentially similar conclusions were drawn from experiments carried out in *Xenopus* [37]. The efficiency of a gene construct in fish cells is therefore largely unpredictable and each gene should be logically assayed in fish cells before being used to generate transgenic animals. Ideally, DNA sequences from fish genes should be used, although this may not be necessary in all cases.

Conclusion

Although a still small number of experiments have been carried out to generate transgenic fish, several features emerge from them. The foreign DNA can be microinjected into the nucleus of oocyte or more easily into the cytoplasm of one-cell embryos. The overall rate of transgenic fish is rather high in comparison to mammals. A relatively large number of embryos can be treated and the fact that electroporation was effective suggests that a massive production of transgenic fish (at least for some species) can be obtained at moderate cost if needed. This may be essential when fish of commercial interest are utilized. The transgenic fishes are mosaic and the foreign DNA is initially polymerized, then amplified, integrated and finally stably transmitted according to the Mendelian rules essentially without being rearranged. This is strikingly reminiscent of what is routinely observed in another lower vertebrate, *Xenopus*, but also in non-vertebrates such as nematodes [18, 47, 48], paramecium [24], and sea urchin [20, 27, 34]. These observations suggest that the capacity of early embryo to form nucleus-like structure and to replicate foreign DNA is broadly distributed in the animal kingdom. In this respect, mammals which behave differently might be more an exception than originally anticipated. From a practical point of view, these observations suggest that the methods defined for fish, including electroporation, might be used at a large scale to generate transgenic non-vertebrates of commercial interest such as molluscs and crustaceans. The fact that injected RNA can be retro-transcribed and integrated in the genome in goldfish [38] and in *Aspergillus nidulans* [60] offers perhaps interesting additional possibilities.

A high rate of expression has not been observed in most transgenic fish so far obtained. There is little doubt that this problem will be solved as soon as appropriate gene constructs containing fish DNA are used. More and more genes from fish are being cloned and studied. This should offer multiple possibilities in the near future.

Development of aquaculture is in part limited by the numerous diseases to which fish are subjected. The use of antisense and ribozymes RNA, the overexpression of viral genes and of specific monoclonal antibodies which is being attempted to neutralize pathogens in higher vertebrates through transgenesis may of course be extended to fish. The efficiency and the flexibility of methods of transgenesis in fish may significantly facilitate the progress of experimentation for this category of animals.

Gynogenesis and androgenesis can be obtained with high efficiency in several fish species [10, 53]. These techniques may contribute to accelerate fixation of new genetic traits introduced into fish through transgenesis.

Triploidization may also contribute to facilitate the study of transgenic fish. This procedure which sterilizes the animals may be helpful when reproduction of transgenic fish is not wanted.

Acknowledgment. This work was supported by the Bridge Programme of the European Community.

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0014-4754/91/090891-07$1.50 + 0.20/0

Production of transgenic birds

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Abstract. The avian embryo presents a tremendous challenge for those interested in accessing and manipulating the avian germ line. By far the most successful method of gene transfer is by retrovirus vector. The efficacy of retrovirus vectors has been demonstrated by germ line insertion of replication-competent retroviruses as well as the insertion of replication-defective retrovirus vectors carrying bacterial marker genes. Retroviral vectors have also been shown to be useful for the transfer and expression of genes in somatic cells. Further, germ line transgenesis has been reported in both the chicken and the Japanese quail. In addition, several alternative gene transfer methods are under development. These include transfection of avian sperm, development of germ line chimeras using primordial germ cells and blastodermal cells, and the development of embryonic stem cell lines. Potentially, basic research and the poultry industry will derive substantial benefit from this revolutionary technology.
Key words. Gene transfer; transgenic birds; retrovirus vectors.

Introduction

The ability to transfer genes into the germ line of birds has created new and revolutionary opportunities for both basic research and the poultry industry. Currently, there is much interest in using gene transfer technology to examine gene expression, evaluate genetic control elements, and to correlate these findings to developmental and physiological processes in birds. Equally exciting is the prospect for creating new and more efficient strains of poultry. Many investigators are transferring genes that may provide commercial birds with resistance to disease and improved feed efficiency and growth rates.
The development of gene transfer technology for avian species, however, has not progressed as rapidly as mammalian gene transfer technology. Progress in producing transgenic birds has been hampered, in part, by the avian reproductive and embryonic developmental system. The newly fertilized avian ovum is very large, fragile and yolk-filled. Embryonic development begins in the oviduct during egg formation. At the time the egg is laid, the blastoderm is composed of approximately 60,000 cells organized into a 1–2 layer disc[12, 22]. This has rendered the use of DNA microinjection into isolated early embryos, which has been used so successfully in mammalian gene transfer, ineffective. In fact, to date there have been no reports of transgenic birds produced by this method.
Thus, by necessity, avian gene transfer methodology has focused on several alternative approaches, most of which are in the early stages of development. As discussed in previous reviews[5, 8, 13, 14, 39, 43], successful gene transfer involves the identification, isolation and characterization of genes of interest, appropriate modification, and (if possible) preliminary testing in cultured cells. This paper will review the progress that has been made in producing transgenic birds including potential gene transfer methods that are currently under investigation. Applications of this technology to basic research and commercial poultry improvement will also be discussed.

Gene transfer methods

The ultimate goal for avian transgenics is to develop several methods for producing transgenic birds which will meet the needs of specific gene transfer applications. For example, retroviral vectors may be favored in situations where small genes are to be transferred and where regulated expression is not critical. Transfection (or injec-