Growth hormones and transgenic animals

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Introduction of foreign genes into animals at their very early stages of development is now becoming a powerful tool to produce transgenic animals with desired characteristics. Growth hormone genes have been very widely introduced to achieve faster growth in economically important farm animals. We review here the protein and gene structure of different growth hormones and the successes and failures of experiments aimed at producing transgenics with enhanced growth rate.

GROWTH hormone (GH) is one of the seven hormones secreted by the anterior portion of pituitary gland. It is secreted in response to a hypothalamic peptide, the growth hormone releasing factor (GRF). The secreted hormone is transported to the liver by the circulatory system, where it stimulates the production of a second set of hormones called somatomedins which are also known as insulin-like growth factors, viz. IGFI and IGFII. Somatomedins stimulate the growth of mesodermal tissues such as muscle, cartilage and bones which result in the overall growth of animals. Insufficient amounts of growth hormone lead to dwarfism and excessive amounts result in gigantism.

Structure of growth hormones

Protein structure

Growth hormones have been isolated, purified and studied in detail from a variety of animals including rat[1], ox[2], chicken[3], sheep[4], pig[5], fish[6], elephant[7] and human[8]. Growth hormone from sources analysed so far is made up of a single polypeptide with a molecular weight of about 22 kDa. The polypeptide chain is composed of 186–191 amino acids. The growth hormone is synthesized in all cases, as a prehormone with a signal peptide of 17–26 amino acids at the N-terminal end which is processed during secretion. The mature protein has four cysteine residues at positions around 52, 165, 182 and 188. Interestingly, the positions of the four cysteine residues are highly conserved in various growth hormones. The two disulphide bonds formed by these four cysteines in these proteins are essential for the biological activity of the hormone[9, 10]. The 11 different growth hormones studied by Ermacora and Rivero[11] have three long helical residues around the amino acids 20, 120 and 170. The strong amphiphilic character of these helices suggests that they might play an important role in folding and stability of the growth hormones. Growth hormones are essentially nonglycoproteins though glycosylation sites are found in some of the fish growth hormones[12–14]. The absence of glycosylation in majority of the growth hormones studied implies that glycosylation may not be functionally significant. Figure 1 compares the primary structure of growth hormones from different vertebrates. The primary structures of growth hormones show a high degree of homology among higher vertebrates. Though the growth hormones isolated from fishes also show a high degree of homology among themselves, yet the sequence homology between fishes and human is only about 30%. It is interesting to note that 30–35 amino acids of the C-terminal region are highly conserved in all the growth hormones studied.

Gene structure

The structural organization of growth hormone genes from various organisms has been studied using the cloned genes. As in other eukaryotic genes, the coding sequence of GH gene is interrupted by intervening sequences. The coding sequences of the GH gene is split into five exons with four introns in human, bovine, goat, ovine, porcine, chicken, rat and some of the fishes. The position of the introns is conserved in hGH, bGH and pGH and rGH genes. The size of the introns is also conserved between the animals except in rGH where the intron 2 contains repeated sequences[5]. The spliced mRNAs from human, bovine, goat, ovine and chicken code for 217 amino acids which include a 26 amino acid signal peptide. But the spliced mRNAs from rat GH and porcine GH genes code for 216 amino acids of which 26 amino acids form the signal peptide[1, 5].

Considerable amount of information is also available on the structure and organization of GH genes from a variety of fishes such as trout, salmons, eel, tuna and carp. Like the mammalian growth hormone genes, the fish GH genes also contain five exons and four introns except in rainbow trout and Altantic salmon in which the coding sequence is split into 6 exons with 5 introns. The existence of an additional intron (intron 5) in the trout and Atlantic salmon GH genes, is a notable

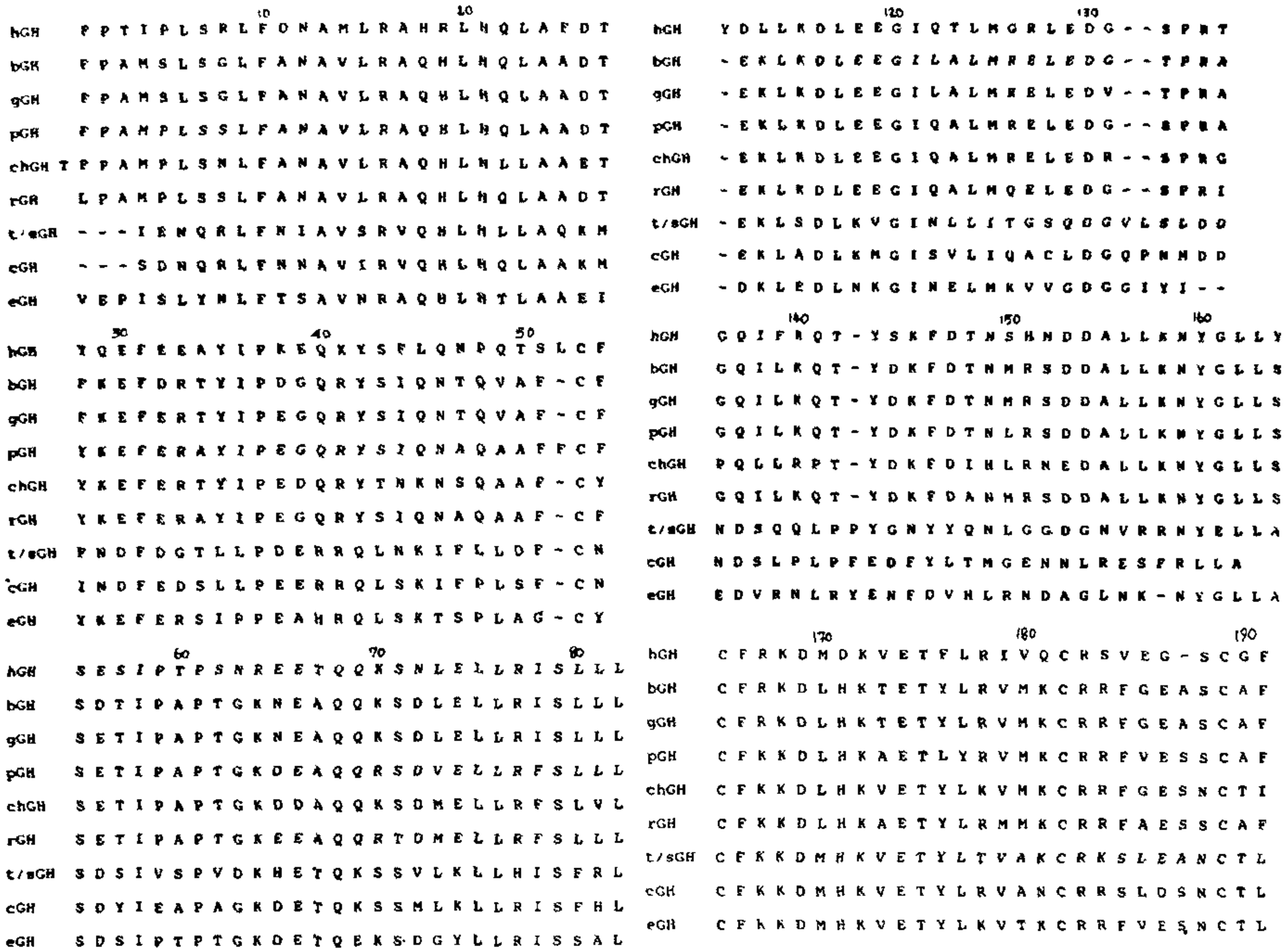

Figure 1. Comparison of amino-acid sequences of different matured growth hormones. Human growth hormone (hGH), porcine GH (pGH), chicken GH (chGH), rat GH (rGH), goat GH (gGH), trout or salmon GH (t/sGH), carp GH (cGH) and eel GH (eGH). Amino acids are indicated in one-letter notation. The amino acid sequences are aligned to maximize homology.

difference between the growth hormone genes of other fishes and mammals[15,16]. The spliced mRNAs of coho salmon, chum salmon and trout code for 210 amino acids which include the signal peptide of 22 amino acids[15,17,18]. Chum salmon and trout had identical amino acid sequences whereas coho salmon GH differed in 6 amino acids[18]. The cDNA from eel GH gene coded for 209 amino acids with a signal peptide consisting of 19 amino acids. The amino acid sequence of eGH showed 47%, 50%, 53% and 44% homology with salmon, chicken, rat and human growth hormones, respectively[13]. Sato *et al.*[19], have found that the cDNA of tuna GH gene coded for only 204 amino acids with a 17 amino acid leader peptide. The tuna GH showed 67% amino acid sequence homology with chum salmon, 90% homology with yellow tail and only 32% homology with hGH. The cDNA sequence of carp growth hormone was found to encode a polypeptide of 210 amino acids including a signal peptide of 22 amino acids. The carp GH presents a 63% amino acid sequence homology with the GH of salmon and rainbow trout[14]. The growth hormone from flounder, an edible flatfish, is the smallest one known with only 173 amino acids[20].

The 5′ and 3′ flanking sequences have also been studied for their role in regulating the expression of growth hormone genes. The upstream sequences of the GH genes of different mammalian species have been found to be highly similar to each other. Two regions (*tsr1* and *tsr2*) are known to be involved in tissue-specific expression of the human GH[16]. The flanking regions of GH genes are known to have both enhancer and silencer sequences which may play an important role in the expression of GH genes[21]. Figure 2 shows a typical GH gene with introns and exons.

Manipulation of growth hormone genes

Injections of pituitary extracts or growth hormones are known to stimulate growth in animals[22]. Therefore, to produce large quantities of growth hormone, recombinant DNA methods have been widely used. GH genes from various animal sources have been isolated, cloned and successfully expressed in prokaryotic as well as eukaryotic systems. This is essentially achieved by cDNA cloning strategy. But the synthesis of cDNA by reverse transcription lacks introns, promoter and flanking regulatory sequences. This problem is usually solved by using different expression vector systems having the promoter and other regulatory sequences for the successful expression of these cDNAs.

Salmon GH, eel GH and human GH have been cloned and successfully expressed in *E. coli* by using various promoters[13,17,23]. Sekine *et al.*[17], have recorded a very high level of expression of salmon GH in *E. coli* using *trp* promoter of *E. coli*. Chang *et al.*[23] have obtained a high level of secretion of human GH by using alkaline phosphatase promoter and a secretion signal coding sequence of the heat stable enterotoxin II from *E. coli*. Both the promoters were equally efficient and about 15% of the total cellular protein of *E. coli* was constituted by the growth hormones. Gary *et al.*[24] have used *Streptomyces* for production of bovine GH. The expression vector used, consists of the regulatory region of aminoglycoside 3′-phosphotransferase (aph) gene from *Streptomyces fradiae*, upstream of bGH structural gene along with the thiostrepton gene as selectable marker. The production of bGH in *Streptomyces* was very poor, as bGH constituted only 0.17% of total TCA precipitable proteins. The low level expression could be due to an inhibitory secondary structure of bGH mRNAs or due to lack of other regulatory sequences which might be required for the efficient expression of eukaryotic proteins in *Streptomyces*. Saito *et al.*[13], used the PL promoter from lambda phage to express eel GH in *E. coli*. But the eGH constituted only 5% of the total cellular proteins. Efficient and high-level secretion of authentic mature hGH was achieved in *Bacillus* by using the promoter and the signal sequence regions of a neutral protease. The organism secreted about 40 mg/l in a high density culture[25]. Over-production of tuna fish GH in *E. coli* was achieved using *tac* promoter. As much as 25% of the cytosolic proteins was constituted by the GH as inclusion bodies[19].

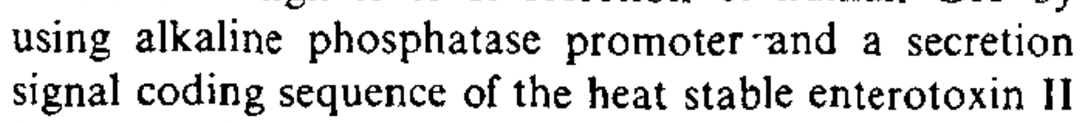

Figure 2. Structure of a typical mammalian growth hormone gene. ——— introns (1-4), ▬▬ exons (I V), ∿∿∿ flanking sequences. Approximate sizes of the introns and exons of growth hormone — Exon I-10 bp, II 160 bp, III-105 bp, IV 170 bp, V-210 bp; Intron 1-240 bp, 2 210 bp, 3 200 bp, 4 280 bp.

Growth hormones are also successfully expressed in mammalian cells using animal vectors. Pavlakis *et al.*[26], have reported synthesis and secretion of human GH in monkey cells infected with SV 40 recombinants. Expression of bovine GH using various eukaryotic viral promoters (such as from human cytomegalo virus (CMV), Simian virus 40 (SV40) and long terminal repeat (LTR) sequences from either Rous sarcoma virus or Moloney murine leukemia virus) in rat or mouse cell lines was studied by Pasleau *et al.*[27] and Gallardo *et al.*[28]. It was found that the CMV promoter and SV 40 early promoter were more efficient than the LTR sequences in these cell types. Pasleau *et al.*[29] also investigated the expression of the genomic bGH (containing the introns) and the cDNA clones (containing no introns) in cultured avian and mammalian cells. It is interesting to note that the bGH gene lacking the introns was expressed more efficiently than the genomic clones containing the introns.

Metallothionein (MT) based vectors have been constructed for the expression of a variety of genes in animal systems. These vectors are not tissue specific and are hyperinducible upon treatment with heavy metals such as Cu, Zn and Cd. Furthermore, these vectors have the potential to be used for the production of recombinant proteins in cultured mammalian cells and also for the gene expression in transgenic animals. Hybrid genes containing the hGH-cDNA and MT genes from mouse, synthesized MT-GH hybrid mRNAs in the mouse cells. The hybrid mRNAs were inducible by heavy metals such as cadmium[30]. MT promoters are also known to be functional with various genes. Figure 3 shows the gene construct used to produce transgenic mice by Palmiter *et al.*[1]. Such gene constructs containing rat GH gene and MT-promoter have been introduced into embryos of mice by microinjection. These authors have noticed a high level expression of the rGH gene resulting in the dramatic growth of the transgenic mice.

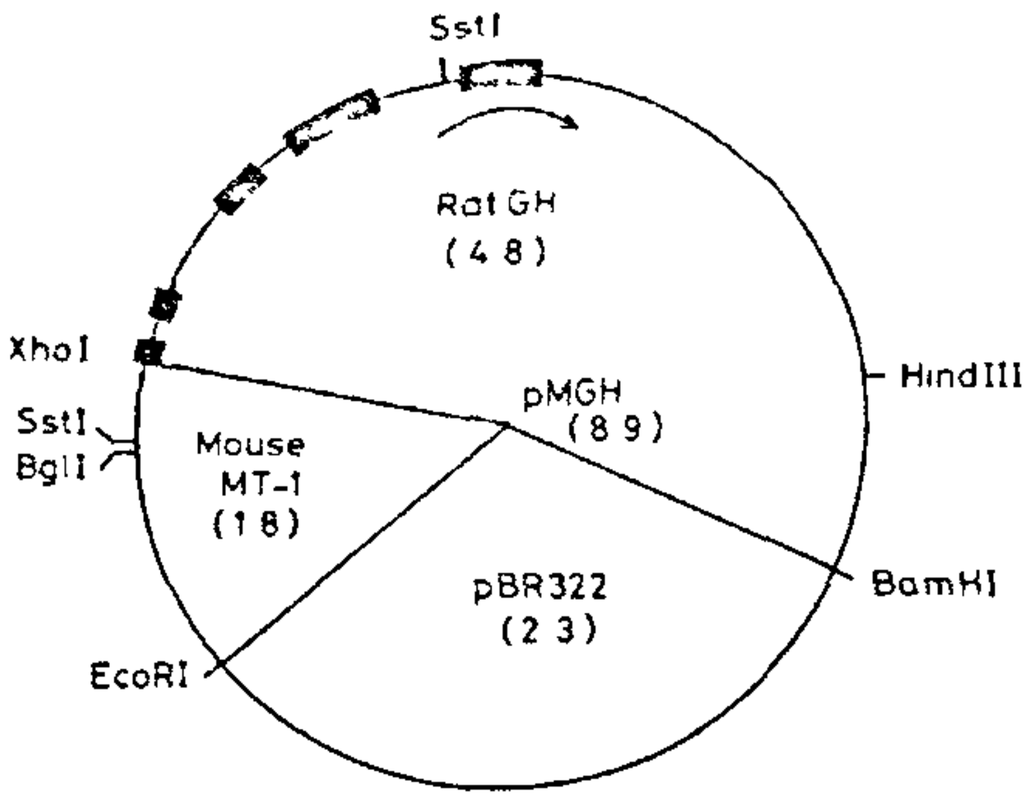

Figure 3. Gene construct pMGH (8.9 kb)[1] having mouse metallothionein promoter and rat growth hormone gene, used for production of transgenic mice and fishes

Transgenic animals with growth hormone gene

Traits such as growth in animals, lactation in dairy cattle, leanness of meat in pigs and disease resistance in poultry, are mainly dependent on their genotype, diet and environment. These traits are controlled by different genes. By introducing the corresponding genes it is possible to make the animals grow faster, produce more milk and survive better against a variety of diseases. For example, it has been reported that periodic injections of growth hormone, promotes growth in animals and milk production in dairy cattle. Instead of injecting growth hormones, attempts are now being made to directly introduce multiple copies of the gene into the fertilized eggs of animals. In the same way, other useful genes, such as disease resistance genes, can also be introduced. Thus the introduction of desired foreign gene(s) into the fertilized eggs of animals and analysis for their stable integration, expression and hereditary transmission have emerged as a powerful tool to improve the traits of farm animals. These transgenic animals, perhaps are the logical extension of conventional artificial breeds of farm animals.

Transgenic animals are produced usually by microinjection of desired gene(s) into the fertilized eggs of animals during their early stages of development. In microinjection technique, the linearized plasmid DNA containing the gene is used because the rate of integration is about five times higher than with the circular plasmids. A few nanoliters of the linearized plasmid DNA, which would contain few thousand copies of the gene, is microinjected into the pronucleus using the micromanipulator and the microinjected eggs are allowed to develop into offsprings. The integration and expression of the gene is analysed in the offsprings.

Some of the other techniques such as embryonic stem cell technology, retroviral vector systems, high speed particle gun and electroporation are also successfully used to produce transgenics. These techniques are briefly explained here.

Embryonic stem (ES) cell technology deals with gene transfer through ES cells into animals during their early stages of development[31]. Embryonic carcinoma (EC) cells, the stem cells of teratocarcinomas are tumors of gonads and they contain a wide range of differentiated cell types representing all three embryonic germ layers viz., the ectoderm, the mesoderm and the endoderm. Apart from differentiated cells, all teratocarcinomas contain a distinctive undifferentiated type of cells known as 'embryonal cells'. It is interesting to note that these cells are very similar to cells of the early embryo, in that they are also pluripotent. When the EC cells are injected into mouse blastocytes, they develop normally and their offspring display the expected chimerism, both for external and internal markers. Therefore, transferring desired genes to EC cells by using relatively simple retroviral vectors and then injecting the EC cells into early stages of development will produce desired characters in animals. Since the viral promoter LTR does not function in EC cells, an internal promoter from thymidine kinase gene has been used successfully in this technology[31].

Particle bombardment is a physical method for gene delivery which has received considerable attention recently[32]. This technique involves accelerating DNA-coated particles (microprojectiles) directly into cells and this technique has been used to transfer genes into a wide range of intact plant cells without removal of cell wall. Thus this method finds application not only in the production of transgenic crop species especially in maize and soyabean, but is also used for the introduction of DNA into organelles such as plastids and mitochondria of a wide variety of plants. This method has recently also been used for gene transfer in animal and bacterial cells. A noteworthy application of this method is the direct insertion of genes into the organs of living animals.

Transgenics are also produced by the method of electroporation. Electroporation is based on the observation that when cell membranes are exposed to short electric pulses of relatively high voltage, a number of pores are formed by a temporary cell membrane breakage. These transient pores allow the passage of macromolecules such as DNA into the cell. Subsequently the DNA gets integrated into the genome.

The microinjection method is very widely used for production of transgenics as it is easier and versatile in its applications. However, the microinjection technique has its own limitations. Poor rate of integration, poor expression of the integrated genes and absence of

hereditary transmission are some of the major problems yet to be solved to produce transgenics with desired characteristics. Some of the reasons for the above mentioned problems are discussed here and also by Maclean *et al.*[6].

Absence of integration

(a) In the fertilized eggs, if the nucleus is not readily visible, the DNA is usually injected into the cytoplasm. Since the cytoplasm is rich in a variety of endo and exonucleases, the DNA may get degraded even before it reaches the nucleus, resulting in poor or no integration at all.

(b) Other factors such as the location of injection, area, concentration of DNA, form of DNA, constituents of the buffer in which the DNA is suspended, nature of eggs and time of injection after fertilization, also play an important role in the integration of foreign DNA.

(c) In some instances, the injected DNA may persist as a discrete entity and is distributed to many tissues without getting integrated into the genome.

Absence of expression

In many cases it has been found that the DNA is integrated into the genome but is not expressed in detectable amounts in transgenic animals. One or more of the following reasons may explain the absence of expression of integrated DNA in transgenic animals. For example, when the injected DNA is partially degraded by nucleases before integration, the flanking regulatory regions may be lost, resulting in loss of expression, or when the injected DNA is integrated but not in the correct orientation, or when they do not have regulatory sequences, or when they have regulatory sequences but are not recognized by the organism, or when the sequences of the gene are rearranged during development of the organism, or when the DNA is inactivated by host methylation system.

Absence of hereditary transmission

The third problem in transgenic animals is the lack of hereditary transmission. This is mainly attributed to lack of integration of the DNA into the germ cells. When the injected DNA integrates after a few rounds of cell divisions, some tissues might be having the foreign DNA integrated in their genome while other tissues such as germ cells might not, leading to the absence of hereditary transmission.

Inspite of the above problems, serious efforts are being made to improve the efficiency of integration and expression of foreign genes in transgenic animals.

Palmiter *et al.*[1] have reported, for the first time, a dramatic growth of mice that developed from eggs microinjected with metallothionein-growth hormone fusion gene (Figure 3). One third of the injected mice carried the fusion gene and most of transgenics grew significantly larger than their littermates. Also it was found that these transgenic mice had extraordinarily high levels of the fusion mRNA in liver and very high concentration of growth hormone in the serum.

After Palmiter's success, a variety of farm animals have been used for producing transgenics. Chicken are very widely used for inserting beneficial genes such as disease resistance genes and growth hormone gene into the germ line. Souza *et al.*[3], found that a somatically introduced growth hormone gene led to the increased level of hormone production but had little influence on growth.

Vize *et al.*[33] have produced transgenic pigs by microinjecting a gene construct with human MT promoter and porcine GH gene. Out of six transgenic pigs, one grew at an increased rate as compared with its non-transgenic littermates. On analysis of the genome it was found that the gene sequences were rearranged in the transgenics which did not grow at a faster rate. Hammer *et al.*[4] have reported production of transgenic rabbits, sheep and pigs by microinjection. The gene construct used, carried the mouse MT promoter/regulator region and hGH structural gene. The frequency of integration was 12.8% in rabbit, 11.0% in pigs and only 1.3% in sheep. The hGH gene was expressed in rabbit and pigs but with no dramatic increase in body weight. This may not be surprising as the daily injection of bacterially produced hGH had no effect on the growth rates in these animals. Although the human GH gene was expressed in transgenic animals such as chicken, pigs, rabbits and sheep there was no significant change in their growth rates. Thus the biological ineffectiveness of growth hormones or other foreign gene products in transgenic animals poses another hurdle in transgenic technology. The biological ineffectiveness could be either because the proteins are not in the biologically active conformation or because the growth hormone is not recognized by the receptors in the target cells.

Integration and expression of foreign GH have been extensively studied to improve the growth rate of commercially important fishes. Furthermore, fishes being at a lower level of evolution among vertebrates, serve as experimental animals for the introduction of novel genes. Moreover, fishes have certain advantages over other animal systems. For example, fertilization of eggs is external and can be performed under controlled conditions. Eggs are numerous and in many species quite large (> 1 mm in diameter) rendering the injection of material and micromanipulation relatively easy.

(*Tilapia* produces 100-200 eggs of 2–3 mm size and zebra fish lays 500-600 eggs of 0.7 mm). Besides, eggs are easily maintained after fertilization and in many species the development is very rapid (the egg hatches within a week).

Zhu *et al.*[34] reported that when a foreign gene (human GH gene with MT-1 promoter) was microinjected into fertilized eggs of gold fish, the gene replicated during embryogenesis and 3 out of 6 fish examined had the gene integrated into the genome. A highly efficient integration (75%) was observed in rainbow trout by Chourrout *et al.*[35] and Maclean *et al.*[6] Zhu *et al.*[36] microinjected a mini-human GH gene with the MT-1 gene promoter into the eggs of loach, a small freshwater fish related to the carp family. These authors observed that the injected fish grew 3–4 times faster than the control group. But it was not established whether the faster growth was due to the expression of the injected gene.

An extensive analysis on the integration, expression and germ line transmission of growth hormone genes with various mammalian promoters have been done in rainbow trout by Guyomard *et al.*[37]. Though 50% of the injected fishes turned out to be transgenics, they could not detect GH in any of the 564 individuals analysed. Attempts to induce the GH (having the MT-1 promoter) by intraperitonial injection of zinc, were also not successful. These findings suggest that promoters and regulatory regions from a fish itself may play an important role in solving the problem of growth hormone gene expression in fishes.

Based on these observations, Liu *et al.*[38] have constructed an 'all fish expression vector' using carp β-actin regulatory elements (promoter and enhancer) and a polyadenylation signal from salmon GH gene. Though one of the constructs expressed well in fish cell lines, yet the expression in transgenic zebra fish was dramatically different. That is, the gene was expressed maximally after three days of microinjection but dropped after the fifth day. It is not clear whether any specific event that occurs during this interval is responsible for blocking the expression of the gene in this fish. Do *et al.*[39] have also constructed an 'all fish' chimeric GH gene with ocean pout antifreeze protein gene promoter and salmon GH cDNA coding region. The transgenic salmon with this 'all fish' chimeric gene construct, showed a dramatic increase in growth rate and the largest transgenic fish was 13 times that of the average non-transgenic fish.

Tilapia and zebra fish have also been used to produce transgenics by Pandian *et al.*[40], using Palmiter's gene construct (Figure 3). The survival rate of microinjected eggs was found to be about 60% in Tilapia and about 40% in zebra fish. Dot blot analysis has shown that the efficiency of integration was much higher in zebra fish (10/19) than in Tilapia (5/100). Stuart *et al.*[41] have found only 5% of the zebra fish microinjected had the foreign DNA integrated into their genome. But our results show about 50% integration in zebra fish. Stuart *et al.* have used a linearized vector containing hygromycin gene linked to SV 40 promoter in pUC 18 for microinjection. Pandian *et al.*[40] have also analysed the germ-line transmission of the gene into F1 and F2 generations in zebra fish. They found that the integrated DNA is successfully transmitted to the F1 and F2 generations in zebra fish. Although these authors have observed that some of the microinjected tilapia grew 2–3 times faster than the control group, it is not yet established that faster growth is due to the expression of the GH gene in these animals.

Conclusion

From the foregoing discussion it is becoming clear that the gene of interest and its regulatory regions from the same animal or closely related animal will express the gene and give desired effect(s) in transgenic animals. At the same time over-expression of particular gene(s) in transgenic animals may cause problems with fertility and health of the animals due to metabolic alterations and hyperplasia of tissues. In spite of these, transgenic animals with desired characteristics will play an important role and hold great promise in animal breeding in the near future.

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RESEARCH ARTICLE

Adequacy of thermosyphon cooling for an Indian PHWR

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The phenomenon of thermosyphon plays an important role in core heat removal in Indian pressurized heavy water reactors (PHWRs) following failure of primary coolant circulating pumps. To confirm the adequacy of thermosyphon cooling, tests were carried out on the first unit of Narora Atomic Power Station. This was the first time such a test was done in an Indian nuclear power reactor, even though such tests are carried out routinely in many countries. These tests were carried out during the commissioning of the reactor at power levels corresponding to decay heat following reactor shutdown. We describe here the thermosyphon tests conducted, the results obtained and the analysis of the data generated.

NUCLEAR reactors are designed for safety not only under normal operating conditions but also under off-normal and accident conditions. One such off-normal condition is the complete loss of coolant pumping power. Under this condition even though the reactor shuts down, the heat generated by decay of fission products needs to be removed to maintain fuel temperatures within safe limits. This is effected by coastdown of the circulating