TRANSGENIC LIVESTOCK
— Review —

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Summary

There are several gene transfer methods available to introduce foreign DNA into animals. The most common method at present is microinjection. However, the overall efficiency of producing transgenic livestock species is low compared to that of transgenic mice. One promising practical application of gene transfer technology to livestock species is the production of pharmaceuticals. Rare human proteins, which cannot be produced by microorganisms because of requirements for post-translational modification, can be secreted into milk of transgenic animals. Large amounts of biologically active protein may be obtained from transgenic farm animals using this system. Growth-related applications to livestock species using growth hormone genes or growth factor genes have been disappointing. There were many undesirable side effects noted in the transgenic animals. More sophisticated regulatory systems are needed to control expression of transgenes in the transgenic animals. Turning on or off transgene expression in response to endogenous or exogenous signals may allow for desired, positive effects while circumventing potentially harmful effects.
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Introduction

Since transgenic mice were first produced by DNA microinjection, the potential has been great for genetic engineering of animals through introduction of extra copies of cloned genes into the animal genome. Transgenic mice have provided powerful models for the examination of the regulation of gene expression in cellular and physiological functions. Also, transgenic mouse models have helped in the design of strategies for gene therapy. Other models have been generated for improved products or productivity of domestic animals and resistance to animal diseases.

In the last century, improvement in livestock efficiency has been achieved using genetic selection. However, there are several limitations in the use of selective breeding of livestock. Considerable time is required through several generations to fix genetic changes within a population. As there are limitations in the amount of genetic variation present within a particular species, the ability to effect change by genetic selection is also limited.

One way to overcome these limitations of time and genetic variability in livestock breeding is through the use of recombinant DNA and gene transfer. Using gene transfer, we can accomplish rapid genetic change in commercial breeding populations by the direct transfer of favorable genes to high performing stock. Therefore, new genetic variation can be established in those populations. Also, we can exchange genetic information between different species which is impossible by traditional breeding. Further, it is possible to target specific genes by homologous recombination to remove, replace or inactivate deleterious genes using embryonic stem cells. However, there are several obstacles for practical application of gene transfer technology to do-

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mestic animals. First, great expense is needed due to the relative low efficiency of the present procedures. Second, many genes are not identified for the potential use of practical application. Third, we have limited knowledge about the control of gene expression as well as physiological effects of transgenes in transgenic animals. In spite of these obstacles, transgenic cattle, sheep, goats, pigs and fish have been produced using present methods.

This review will be focus on recent methodologies and results with transgenic livestock including mammals, poultry and fish. Several authors have speculated about the application of gene transfer technology to livestock species (Petters, 1986; Land and Wilmut, 1987; Renard and Babinet, 1987; Rexroad and Pursel, 1988; Strojek and Wagner, 1988; Pursel et al., 1989; Ward and Nancarrow, 1991; Rexroad, 1992; Ebert and Schindler, 1993).

Gene Transfer Methods

1. Microinjection

Injection of cloned DNA into the pronuclei of fertilized embryos is currently the best way to make transgenic mammals. Exogenous DNA can be integrated into the host genome and passed from parent to progeny. The technique of pronuclear microinjection in the mouse has been described by several authors (Gordon and Ruddle, 1985; Hogan et al., 1986; Allen et al., 1987; DePamphilis et al., 1988). Using a micromanipulator, purified DNA is microinjected into one of the pronuclei of one-cell stage embryos. Just after injection or the following day, embryos that survive injection are transferred to the oviducts of pseudopregnant recipients. Integration of exogenous DNA in transgenic animals is determined by Southern blot analysis of DNA extracted from a tail biopsy. Transgenic founders are bred to produce offspring as a further confirmation of integration of the transgene and to establish a line. The efficiency of producing transgenic mice by microinjection varies among experiments and among laboratories. Under normal conditions, 50-70% of embryos survive microinjection. Of these embryos, 10-30% develop to term after transfer to recipients. Ten to thirty percent of pups born are transgenic (Brinster et al., 1985; Voss et al., 1990). However, the overall efficincy of transgenic farm animals is much lower. The efficiency of producing transgenic livestock species will be discussed in the section on transgenic mammals. Gordon et al. (1980) first produced transgenic mice by microinjection of plasmid DNA. Gorden and Ruddle (1981) found that this incorporated DNA can be transmitted to offspring in a Mendelian manner. Around 70% of the transgenic mice contained the transgene in all of their somatic and germ cells, and the remaining 30% showed some degree of mosaicism (Palmiter and Brinster, 1986). Thus, most integrations occurred prior to the first cell division. However, more recent data may suggest that mosaicism during development may be fairly high (Burdon and Wall, 1992).

In transgenic mice, injected DNA molecules are usually integrated at a single chromosomal site as a head-to-tail array (Palmiter et al., 1982). The copy number of integrated transgenes varies considerably from one to several hundred. The mechanism of DNA integration following microinjection is unknown, although several theories have been proposed (Brinster et al., 1985; Palmiter and Brinster, 1986; Gordon, 1989)

There are numerous factors that affect gene transfer efficiency (Brinster et al., 1985; Hogan et al., 1986; Allen et al., 1987; Walton et al., 1987; DePamphilis et al., 1987). A Tris buffer [10 mM Tris-HCl (pH 7.4), 0.1-0.25 mM EDTA] is usually used for microinjection. Higher concentrations of EDTA are toxic to embryos. About 1-2 pl of DNA solution is injected into the pronucleus of each embryo during microinjection. Injection of approximately 500 copies of DNA fragment (DNA concentration of 1-5 ng/μl) into each embryo was found to be efficient while a higher concentration of DNA was less efficient (Brinster et al., 1985). Up to 50 kb of DNA can be introduced into transgenic mice by microinjection (Constantini and Lacy, 1981). Linearized DNA was 5-fold more efficient than supercoiled DNA for the production of transgenic mice (Brinster et al., 1985). The shape of each end of DNA generated by restriction enzymes has little effect (Grosschedl et al., 1984; Brinster et al., 1985). Cytoplasmic microinjection of foreign DNA into mice and swine zygotes produced much lower transformation efficiencies when compared to pronuclear injection (Brinster et al., 1985; Hammer et al., 1986).

The presence of contiguous vector DNA sequences may affect the transgene expression in transgenic mice. Several cases were reported in which plasmid sequences severely inhibited the expression of such eukaryotic transgenes as β-globin (Chada et al., 1985; Townes et al., 1985), α-fetoprotein (Krumlauf et al., 1985) and actin (Shani, 1986). After the removal of plasmid sequences from the transgene, the β-globin gene was expressed in a tissue specific manner (Townes et al., 1985).

The culture medium is important to maintain the appropriate environment for the embryo during microinjection. M16 medium and M2 medium (Hogan et al., 1986) as well as CZB medium (Chatot et al., 1989) are common media for mouse microinjection. For microinjection, microneedles made from thin wall glass capillaries (outside diameter of 1 mm) are used. The DNA solution can be loaded into injection pipettes by capillary action or by aspiration of solution through the tip of the injection pipette. A tip size of 1 μm for the injection pipette is considered optimal for microinjection. Smaller openings in the tip may easily clog, while larger ones often damage the embryos (Walton et al., 1987).

The choice of mouse strain for producing transgenic mice is important. Strain variation in response to superovulation, pronuclear size and appearance, and survival following microinjection can affect the efficiency of transgenic mouse production. Brinster et al. (1985) reported that the efficiency of producing transgenic mice using embryos from C57BL/6 × CBA/J hybrid females was much higher than that using C57BL/6 inbred mouse embryos. However, to standardize the genetic background of transgenic mice, inbred zygotes should be used. Taketo et al. (1991) reported that the efficiency of producing transgenic mice and the survival rate after microinjection using FVB/N strain embryos were as high as those in other hybrid embryos. Zygotes from FVB/N females contain large, prominent pronuclei, which is an advantage for microinjection. In addition, the FVB/N strain has a larger litter size (average, 9.5 pups/litter) than many inbred strains of mice. Many researchers are using FVB/N zygotes for gene transfer experiments (Leonard et al., 1988; Yokoyama et al., 1990; Taketo et al., 1991).

2. Retrovirus-Mediated Gene Transfer

Genes can be transferred into mammalian embryos using retroviral vectors by injecting cells producing infectious virus into the blastocoel cavity of blastocyst-stage embryos (Jaenisch et al., 1981) or by injection into postimplantation embryos (Jaenisch et al., 1981; Stuhlmann et al., 1989) or by co-culture of zona-free embryos with cells producing virus (Jaenisch, 1976; Jaenisch, et al., 1981; Huszar et al., 1985; Rubenstein et al., 1986; Stewart et al., 1987). For cell lines, gene transformation can be achieved by incubation with medium conditioned by cells producing retrovirus. In chickens, virus stock or virus-producing helper cells can be injected directly into the blastoderm of eggs (Salter et al., 1986, 1987; Lee and Shuman, 1987; Shuman and Lee, 1988; Bosselman et al., 1989).

In contrast to microinjection of DNA, retrovirus-mediated gene transfer involves a single proviral copy insertion with few rearrangements of the host genome or the transgene, with the exception of a short duplication (4-6 bp) of host sequences at the integration site (Risser et al., 1983). Retroviruses can often infect a wide variety of cell types with high efficiency. As a biological delivery system, no special instruments are required for retrovirus-mediated gene transfer. However, there is a limitation on the size of the transgene; the maximum size is 10 kb (Gelinas and Temin, 1986). Additionally, there have been some problems with expression of transgenes when the long terminal repeat (LTR) served as the only promoter (Jaenisch, 1988). Another problem with retrovirus-mediated gene transfer is that infectious virus can be produced by recombination with helper virus, although the probability of this occurrence should be low.

Jaenisch et al. (1981) infected mouse embryos with Moloney leukemia virus and obtained transgenic mouse lines. Of 13 different strains (Mov 1 to 13) produced following embryo infection, each contained a single proviral insert in a different location within the genome. The provirus was stably integrated and transmitted to the progeny in a Mendelian manner. Subsequently, recombinant retroviral vectors have been used to make transgenic mice (Stuhlmann et al., 1984; Huszar et al., 1985; van der Putten et al., 1985; Rubenstein et al., 1986; Stuhlmann et al., 1989). These recombinant retroviruses are defective

for replication and yet retain their ability for infection. Such recombinant retroviruses were integrated into the host genome and eventually transmitted to a subsequent generation. Petters et al. (1987) injected cells producing an avian retrovirus into the blastocoel of pig blastocysts to provide a basis for retrovirus-mediated gene transfer procedures in farm animals.

Jahner et al. (1982) found that there was no expression in transgenic mice produced with retrovirus vectors, due probably to some mechanism of inactivation which prevents the proviral LTR from functioning properly as a promoter both in embryonic cells and later on during development. Soriano et al. (1986) produced transgenic mice using a recombinant retrovirus containing the human β-globin gene under the control of its own promoter. Though at a low level, this gene was expressed in hematopoietic tissues of transgenic mice. When Stewart et al. (1987) used an internal promoter in the recombinant retroviral vector, the gene whose regulation was independent of the inhibitory effect of the LTR was efficiently expressed in all the tissues examined in transgenic mice.

Retroviral infection of the developing mammalian embryo may results in mosaicism. Stewart et al. (1987) reported that while 35% of the mice born had retroviral DNA incorporated in their genome, germ line transmission varied from 0 to 27%.

The numerous discrete viral insertions carried by transgenic mice provide new chromosomal markers which may prove to be very valuable in cell lineage studies. A cell with a retroviral insertion and all its progenitors now have a genetic label which may afterwards be used for studies on cell lineage during embryonic development. The clonally labelled cells may be identified through the analysis of their DNA and the use of appropriate probes specifically recognizing the viral sequences (Robertson et al., 1986; Soriano and Jaenisch, 1986; Price, 1987).

For medical applications, retroviral vectors have been used to introduce genes into hematopoietic progenitors in several *in vitro* and *in vivo* studies (Hock and Miller, 1986; Kwok et al., 1986; Hogge and Humphries, 1987; Magli et al., 1987; Eglitis et al., 1988). Eglitis and Anderson (1988) described a simplified protocol for bone marrow gene transfer using a retroviral vector. Wilson et al. (1988) transformed adult rat liver cells by transfecting with a retroviral vector containing the β-glalactosidase gene. Liver dysfunction may be cured in the future using retrovirus mediated gene transfer. The production of transgenic poultry through the use of retroviruses will be discussed in a subsequent section on transgenic poultry.

3. ES Cell-Mediated Gene Transfer

Embryonic stem (ES) cells are pluripotent cells which can be derived from preimplantation stage embryos. They can be maintained in culture long enough for one to perform various *in vitro* manipulations. A standard technique for producing murine ES cells is to culture embryos *in vitro* up to and beyond a point at which they would normally implant in the uterus. This procedure is mimicked *in vitro* in mouse embryo culture technique through co-culture on fibroblast monolayers. The fibroblasts serve as substrate for maintaining undifferentiated ES cells enabling them to be harvested. The ES cells may be injected directly into the blastocoel of a host blastocyst which may then be transferred into a recipient female. In mice, about 10 to 30% of the live-born animals resulting from stem cell experiments contained tissue derived from the injected stem cells (Robertson, 1986; Capecchi, 1989).

The genome of ES cells can be manipulated *in vitro* by introducing foreign genes or foreign DNA sequences by techniques such as calcium-phosphate-mediated transfection (Bradley et al., 1984), electroporation (Mansour et al., 1988), microinjection (Lovell-Badge, 1987), or retroviral infection (Gossler et al., 1986; Robertson et al., 1986). After transformation, ES cells in culture can be cloned and then screened for incorporation of the foreign DNA. Use of a transformed clone will enable the production of a number of genetically identical mice with the transgene integrated at the same place in the genome. It may also be possible to screen for expression of the transgene prior to the production of transgenic animals.

The use of ES cells offers the best method for specific gene replacement. Exogenous DNA has been inserted by homologous recombination with the related endogenous gene in ES cells (Smithies et al., 1985; Doetschman et al., 1987;

Thomas and Capecchi, 1987). This allows replacement of the endogenous gene copy rather than the addition of a new copy Homologous recombination in ES cells can be used to alter the endogenous genes in transgenic animals either in an attempt to improve a normal gene product or to disrupt the expression of a normal gene by insertional mutagenesis (Hooper et al., 1987 ; Kuehn et al., 1987; Thomson et al., 1989). The frequency of cells containing a recombination event was low 1.4×10^{-6} but still selectable (Doetschman et al., 1987). Also, an easily detectable marker can make selection less difficult (Capecchi, 1989). The polymerase chain reaction (PCR) has been used to screen ES cells for those cells that have integrated the foreign DNA at a precise location via homologous recombination (Kim and Smithies, 1988).

Special reporter gene vectors, called "gene trap" or "entrapment" vectors, coupled with ES cell transfection can be used to identify *cis*-acting regulatory sequences of an endogenous cellular gene. If the vector is integrated nearby or within a gene, the reporter gene should be expressed. The requirement for reporter gene expression is integration of the gene within a transcriptionally active region. Using this method, tissue- or development-specific enhancers or promoters can be cloned (Skarnes, 1990; Rebertson, 1991).

The use of mouse ES cell mediated gene transfer may allow the replacement of existing genes affecting livestock productivity with specially engineered genes designed to improve production. This technique is preferable to simply adding a new gene into the genome at random. However, due to the lack of stable ES cell lines from livestock species, ES cell-mediated gene transfer techniques have not been applied to domestic species. Several groups are trying to isolate pig, cattle or sheep ES cells and characterize these cell lines (Evans et al., 1990; Notarianni et al., 1990; Piedrahita et al., 1990a, 1990b; Strojek et al., 1990; Notarianni et al., 1991; Sims and First, 1993).

4. Other Methods

Liposomes are micelles constructed using various lipids normally found in the cell and nuclear membranes. DNA encapsulated in liposomes has been used to successfully transform L-cells in culture (Schaefer-Ridder et al., 1982). The advantages of liposomes as carriers for introducing nucleic acids into cells are their simplicity of preparation, long term stability, low toxicity and ability to protect encapsulated nucleic acid from degradation. Purified genes, whole chromosomes, and intact nuclei can be encapsulated and introduced into cells (Mukherjee et al., 1978; Wong et al., 1980). Germ line transformation has not been reported using liposomes, but gene transfer into somatic cells of animals has been successful (Nicolau et al., 1983). Rottmann et al. (1985) microinjected liposomes containing the surface antigen gene of the hepatitis B virus fused to the LTR of the Moloney mouse sarcoma virus into the blastocoel of mouse blastocyst. Five out of 24 pups were transgenic with antigen and antibody found in the serum of these animals. Liposome-mediated gene transfer may increase the efficiency of production of transgenic animals (Reed, 1989; Minhas and Voelkel, 1989); however, further confirmation of this method is required.

The exposure of a cell to an electric field causes membrane components to become polarized and a voltage potential develops across the membrane. Subsequent to such treatment, the cells become permeable to exogenous molecules. Electroporation has been used for the transformation of a number of different cell types, such as mammalian primary cells (Toneguzzo and Keating, 1986; Tur-Kapsa et al., 1986) and mammalian ES cells (Thomas and Capecchi, 1987). Knutson and Yee (1987) transfected DNA up to 150 kb in size using electroporation. Nemec et al. (1989) reported production of transgenic mice through electroporation of mouse one-cell stage embryos. Recently, Inoue et al. (1990) reported successful generation of transgenic fish using electroporation.

A bacterial plasmid based vector, containing the origin of replication and large T antigen of polyoma virus, was shown to produce 100% transformation of mice following pronuclear microinjection (Rassoulzadegan et al., 1986). Founder mice and the subsequent generation transmitted the vector DNA to 80 to 100% of their progeny. The vector DNA was not integrated into the mouse genome, but was maintained as an autonomously replicating plasmid in all tissues. The vector was considerably rearranged and did not express the polyoma large T gene. Further research is needed to accomplish gene

transfer in animals using autonomous replicating vectors.

Sperm-mediated gene transfer was reported for mice and pigs (Lavitrano et al., 1989; Gandolfi et al., 1989). Sperm cells were incubated with foreign DNA and used for *in vitro* fertilization with oocytes. From liveborn mice, high frequencies of transgenics were produced. However, these reports could not be confirmed by others (Brinster et al., 1989).

Foreign DNA can be directly introduced into target tissues or organs in living animals by using DNA-coated microprojectiles (Williams et al., 1991). DNA-coated gold particles or tungsten particles (1 to 5 μm in diameter) were shot into tissues using a bombardment apparatus which was first used to transform plant tissues. Williams et al. (1991) reported that 10 to 20% of skin and liver cells expressed the foreign genes after introduction of plasmid DNA using this technology. This methodology may be useful to transform foreign DNA into a wide range of target cells or tissues of living animals.

Transgenic Livestock

1. Transgenic Mammals

1) Efficiency

The most common method used for gene transfer in mammals in microinjection into pronuclei of zygotes. There are several difficulties in applying this method to domestic animals. First, the pronuclei of rabbit and sheep embryos can be seen under the differential interference-contrast (DIC) microscope. However, DIC microscopy is not suitable to identify the pronuclei of pig and cattle embryos, because these zygotes have opaque cytoplasm that prevents observation of pronuclei (Wagner et al., 1984). Wall et al. (1985) used high-speed centrifugation of zygotes for 5 minutes to stratify the lipid constituents of cytoplasm and allow the observation of pronuclei for microinjection. Second, superovulation protocols may be difficult and lead to variable results. Third, *in vitro* culture systems for domestic animal embryos are not yet optimized which could compromise embryo viability. The net result is that the microinjection technique for gene transfer is less efficient in farm animals than in mice. Several groups reported the efficiencies of production of transgenic pigs at various stages during microinjection procedures (Brem et al., 1985; Hammer et al., 1985; Pursel et al., 1987; Ebert et al., 1988; Vize et al., 1988; Pursel et al., 1989; Wieghart et al., 1990; Shamay et al., 1991; Wall et al., 1991; Swanson et al., 1992). Microinjection itself causes some degree of embryo lethality (Williams et al., 1992). About ten percent of microinjected pig zygotes resulted in livebirths. Four to twelve percent of the live born piglets from microinjection were transgenic. Vize et al. (1988) reported the highest frequency with 6 transgenic pigs out of total 17 born. However, the average frequency was 6% transgenic rate among live born piglets (Wieghart et al., 1990). Shamay et al. (1991) reported the overall efficiency (no. transgenic pigs/no. embryos microinjected) was 0.9% to produce transgenic pigs containing mouse whey acidic protein gene. Overall, the efficiency of production of transgenic pigs is less than 1%.

In sheep, the overall rate of efficiency for transgenic production was variable: 0.099 4.5% (Hammer et al., 1985; Simons et al., 1988; Rexroad et al., 1989, 1991; Wright et al., 1991). Rexroad et al. (1991) reported the highest efficiency (11 transgenic sheep out of 247 embryos injected). Recently, Wright et al. (1991) produced 5 transgenic sheep from 543 embryos injected (0.91%). Ebert et al. (1991) produced two transgenic goats from 203 embryos injected and transferred (0.99%).

The overall efficiency of integration of foreign genes into bovine embryos is lower than that typically obtained in pigs and sheep: 0.02 1.5% (Biery et al., 1988; Bondioli et al., 1991; Krimpenfort et al., 1991). The lower efficiency of gene incorporation into bovine was due to the low proportion of injected embryos that produced fetuses (6%) and to the low proportion of collected fetuses in which the transgene was integrated (Bondioli et al., 1991).

The conditions used for microinjection into pig, sheep and cattle embryos were similar to those for producing transgenic mice which may not be optimal for all species. Adjustment of the conditions for microinjection is required for the improvement of overall efficiency of transgenic production in livestock.

Zygotes produced by *in vitro* maturation and fertilization of bovine oocytes are cost effective when compared to an *in vivo* source of pronu-

clear embryos. Krimpenfort et al. (1991) reported that transgenic cattle containing bovine alpha S_1-casein regulatory element fused to human lactoferrin were produced from such embryos. Of 19 offspring, one male and one female carried the transgene. Recently, Bowen et al. (1993) produced transgenic cattle from such embryos subjected to PCR. The procedures used in these experiments have shown savings in labor and expense for the production of transgenic cattle.

2) Growth performance in transgenic animals

GH or GRF transgenic pigs and sheep exhibited some positive phenotypic traits. Studies have demonstrated enhanced growth performance in transgenic GH pigs including significantly greater feed efficiency and lesser back fat in comparison to control littermates (Pursel et al., 1987, 1989, 1990; Ebert et al., 1988; Vize et al., 1988; Rexroad et al., 1989; Wieghart et al., 1990). Transgenic pigs and sheep containing GH genes exhibited similar responses to those of transgenic mice, where elevated serum levels of GH increased deposition of protein and muscle mass while decreasing fat deposition in muscle and subcutaneous regions (Ebert et al., 1988; Vize et al., 1988; Pursel et al., 1989; Rexroad et al., 1989; Ward et al., 1989). In transgenic pigs containing a hGH gene, high levels of hGH in plasma were observed, but transgenic pigs did not grow faster than non-transgenic litter mates. However, the elevated GH levels inhibited lipid storage and affected IFG-I levels. Endogenous porcine GH levels were also reduced probably due to negative feedback by transgenic GH on the hypothalamus and pituitary gland (Ebert et al., 1988). Human GRF did not increase pGH levels probably due to an inactive form of *hGRF* being produced due to improper processing (Pursel et al., 1989).

Thickness of backfat in transgenic pigs containing a GH gene was significantly lower than in control pigs and transgenic pigs were 16% more feed efficient than controls (Pursel et al., 1989; Wieghart et al., 1990). Transgenic pigs containing a GH gene had an enhanced growth rate (11-20% faster than controls) between 30 and 60 kg body weight with high protein diets (Vize et al., 1988; Pursel et al., 1989). Rexroad et al. (1991) reported that transgenic sheep containing either the mouse transferrin (Trf) enhancer and promoter fused to bGH or the mouse albumin enhancer and promoter fused to hGRF did not enhance growth rates.

Overproduction of GH in GH transgenic pigs and sheep resulted in detrimental side effects including altered endocrine profiles and metabolism, insufficient thermoregulatory capacity, joint pathology (lameness and arthritis), low libido, infertility and increased susceptibility to pneumonia (Pursel et al., 1987, 1989; Ebert et al., 1988; Rexroad et al., 1989, 1991; Ward et al., 1989; Pinkert et al., 1990; Wieghart et al., 1990). Rexroad et al (1991) observed a diabetes-like condition with weight loss and little subcutaneous fat in the transgenic sheep containing bGH or hGRF. Pursel et al. (1989) reported that about 30% of transgenic pigs died within 6 months of age and 20% of them died before weaning. It was suggested that the various abnormalities in metabolism and growth performance observed in GH transgenic pigs may be simply due to chronic exposure of transgenic animals to high level of GH released in a non-pulsatile manner (Miller et al., 1989) or inadequate dietary intake and altered metabolic requirements (Pinkert et al., 1991).

Another way to increase skeletal muscle is being investigated using the Ski gene which involves myogenic differentiation. Sutrave at al. (1990) showed that transgenic mice containing chicken Ski gene (cSki) exhibited a large increase in skeletal muscle with less fat. Subsequently, transgenic pigs containing mouse sarcoma virus LTR promoter fused to cSki DNA (Pursel et al., 1992). These transgenic pigs exhibited variable muscular hypertrophy between 3 and 7 months of age. However, some transgenic pigs exhibited muscular atonia and weakness in front and rear legs.

3) Modification of milk composition

To alter the composition of milk constituents, different proteins such as pharmaceuticals were targeted to the mammary gland using regulatory elements of beta-lactoglobulin gene, whey acidic protein or beta-casein (Pittius et al., 1988; Wilmut et al., 1990). The transgenic animal system may be more efficient than a mammalian cell culture system for the production of several types of human proteins (van Brunt, 1988). If transgene products are secreted in the milk of lactating

livestock species at high levels, the transgenic mammary gland system will be ideal to produce low cost pharmaceuticals. Pharmaceuticals being tested in this system are tissue plasminogen activator (t-PA), which is effective in dissolving the fibrin clots responsible for coronary occlusions, human alpha-l-antitrypsin which is required especially for genetically deficient patients who are at risk for life threatening emphysema, human anti-hemophilic factor IX and human interleukin-2. Also transgenic hemoglobin may be the pharmaceutical product which would be safer and cheaper than that from human blood. Simons et al. (1987) demonstrated that sheep beta-lactoglobulin was expressed in the milk of transgenic mice. Ebert et al. (1991) produced two transgenic goats containing a longer-acting glycosylation variant of t-PA under the control of murine whey acidic protein promoter. One female produced t-PA in her milk with enzymatic activity at low concentration (milligram level per liter), comparable to cell-derived material. The gene for human anti hemophilic factor IX was inserted into the 5' untranslated region of sheep beta-lactoglobulin gene, and this gene construct was used to produce transgenic sheep (Simons et al., 1988). Two transgenic ewes secreted the human anti-hemophilic factor IX into their milk at the very low level of 25 ng/ml (Clark et al., 1989). Wright et al. (1991) produced transgenic sheep carrying a modified genomic alpha-1-antitrypsin gene fused to the ovine beta-lactoglobin promoter. Milk from the lactating females contained alpha 1-antitrypsin with biological activity identical to human plasma-derived protein at levels of grams per liter. The pig also can be a bioreactor for large quantities of foreign protein in milk during lactation periods (Shamay et al., 1991; Wall et al., 1991). Transgenic pigs containing mouse whey acidic protein gene secreted high concentrations of non-porcine protein into milk. Swanson et al. (1992) produced functional human hemoglobin in transgenic pigs containing human β-globin locus control region (LCR) linked to human two α genes and a single β gene. The purified hemoglobin from transgenic pig blood exhibited similar oxygen binding characteristics to human control hemoglobin. Recently, Shamay et al. (1992) tried to induce lactogenesis in transgenic pigs with hormonal stimulation to detect transgene expression of a mouse whey acidic protein gene before the first lactation.

2. Transgenic Poultry

In contrast to the use of DNA microinjection methodology in mammalian and fish eggs, gene transfer in chickens has depended on the use of a retrovirus vector system. The anatomy and physiology of birds make it very difficult to manipulate and culture avian one-cell embryos *in vitro*. Also, the large dense yolk and multiple cells in a laid egg obscure visualization of the nuclear region of the developing egg for direct microinjection. As an alternative, retrovirus-mediated gene transfer has been developed in poultry. Fertile eggs are placed in egg cartons large end up to orient the blastoderm underneath the air cell. A small hole is drilled into the large end of the egg and then cells producing a retrovirus vector or retrovirus particles are injected into the blastoderm area. After sealing the hole, eggs are incubated in a standard egg incubator for hatching.

Attempts have been made to produce transgenic chickens by injection of helper cells producing vector virus (SNV vector) into developing follicles (Shuman and Shoffner, 1986). Souza et al. (1984) reported the first successful transformation of chickens using a retrovirus vector. A retrovirus containing the chicken growth hormone (cGH) gene was constructed from a Rous sarcoma virus. This vector was injected into 9-day old eggs to achieve infection and transformation. A portion of the chickens developing from the infected eggs had elevated levels of cGH in their plasma indicating that the cGH gene had been successfully transferred into their embryonic cells by the retrovirus vector. However, there was no observation of enhanced growth rates and germline transmission. Two retroviruses which have been investigated for use as gene transfer vectors are avian leukosis virus (ALV) and reticuloendotheliosis virus (REV) (Crittenden and Salter, 1986). Hughes et al. (1986) demonstrated that the *in vivo* oncogenic activity of ALV could be significantly reduced by replacing the LTR region of ALV with the LTR region of RAV-0. Salter et al. (1986, 1987) demonstrated the feasibility of achieving germline transmission of transgenes in retrovirus infected birds. First generation (G_0) males were produced by infecting newly laid eggs with several types of ALV. Second genera-

tion (G_1) and third generation (G_2) chickens were generated using provirus G_0 males that were viremic and mosaic. The frequencies at which these males transmitted the DNA to progeny ranged from 1 to 11%. However, when G_1 birds were used to produce G_2, the viral DNA was transmitted in a Mendelian fashion indicating that the retroviral genes had been integrated into the germline. Transgenic chickens produced by ALV transformation experiments (Salter et al., 1986, 1987) have subsequently been identified in which a subgroup A envelope glycoprotein was expressed. Males heterozygous for this trait were backcrossed with G_0 females. In fibroblast cell lines produced form the resulting embryos, five cultures were identified in which this antigen was expressed. These cell lines were approximately 5000-fold more resistant to subgroup A sarcoma virus (a common virus in commercial chicken) than were fibroblasts not expressing the insert. The resistance to subgroup A type sarcoma virus by embryonic cells as demonstrated in this experiment provides an example of the potential for use of retroviruses to transform poultry for enhanced disease resistance.

Recently, the avian REV, spleen necrosis virus (SNV), has been used to generate a defective retrovirus vector carrying the bacterial gene for chloramphenicol acetyl transferase (CAT) (Lee and Shuman, 1987). This vector has been shown to be effective for the transfer of the CAT gene into quail embryos (at least 20% integration). Lee et al. (1987) showed the expression of the CAT gene in quail embryos when placed under the control of the murine immunoglobulin kappa promoter. Subsequent experiments have demonstrated this SNV vector system effective for the integration of the CAT gene in chicken embryos (Shuman and Lee, 1988). The gene was expressed in 100% of the treated embryos after 5 days of incubation. Also, tissue-specific expression of the transgene was observed in adult. A similar con struct containing the hygromycin resistance gene under the control of the herpes simplex thymidine kinase promoter has been developed (Zhao and Shuman, 1988). This may be effective as a method for selecting vector virus breeding stock for use in generating transgenic poultry. Lee (1989) and Lee and Shuman (1990) produced transgenic chickens and quail containing the CAT gene in an SNV vector. About 50% of hatched chicks contained vector sequences. Germline trans mission rate of G_0 was around 2%. Expression of CAT gene in transgenics was detected in a tissue-specific manner (highest activity in intestine, muscle, skin and thymus). Bosselman et al. (1989) transferred the cGH into chicken embryos using a REV vector. DNA from thirteen (52%) out of 25 injected embryos hybridized to vector probe on 7 day of development. Of 30 injected embryos, 16 embryos (55%) exhibited 10 times higher levels of cGH in serum than control. Bosselman et al. (1989) also reported that a recombinant REV vector containing the neomycin resistance gene was integrated into chicken germline. Of 760 hatched chicks following injection, 173 chicks (22.8%) contained vector sequences. Thirty three out of 82 males carrying vector sequences contained vector sequences in their semen. Germline transmission rates of these males were 2 to 8%. Transgenic chickens containing this recombinant SNV vector actively expressed the transgene under the control of the SNV LTR (Briskin et al., 1991).

New methodologies being developed for the production of transgenic poultry include sperm-mediated gene transfer, transfection of early embryos and *in vitro* culture of pluripotent cells (Shuman, 1991). Recently, Petitte et al. (1990) and Brazolot et al. (1991) produced somatic and germline chicken chimeras using transplantation of blastoderm cells. The use of stem cell technology with retroviral vectors may allow further progress in the field of transgenic poultry.

3. Transgenic Fish

The most common method for producing transgenic fish is microinjection of DNA into fertilized eggs. A primary problem with this method is the inability to visualize the pronucleus or nucleus within fertilized fish eggs due to the dense cytoplasm and large yolk mass. The large size of the fish egg prevents the use of low resolution stereomicroscopy. As a result of these limitations, the gene transfer in fish has relied on microinjection of DNA into the cytoplasm of developing eggs near the center of the germinal disk. In contract to mammals, foreign DNA was incorporated at very high frequencies (up to 75 % in some batches of eggs) following cytoplasmic injection (Chen and Powers, 1990; McEvoy et al., 1992). Another unique characteristic of fish

eggs which makes microinjection difficult is the acellular external protective covering (the chorion) which undergoes a process called water-hardening at the time of fertilization. Water-hardened eggs such as those of salmonids are too rigid to puncture with a microinjection needle. Usually, a larger needle is used to puncture the chorion of these eggs. Chorions of some species such as catfish have a secretion at the time of water-hardening which is very sticky. Eggs can be deposited into a fertilization dish in a single layer by artificial spawning which facilitates microinjection of the eggs.

Vielkind et al. (1982) microinjected total genomic DNA from platyfish strains into the neural crest region of developing swordtail eggs. A low percentage of swordtail offspring which developed from the injected eggs showed a specific pigmented cell type which normally occurred only in the DNA donor strain of platyfish. Maclean and Talwar (1984) first attempted microinjection of DNA into fertilized rainbow trout eggs using a mouse metallothionein (MT) gene. Subsequently, Maclean et al. (1987) reported at least some of fish produced in this early experiment had the transgene incorporated into their germ line. Zhu et al. (1985) first reported successful gene transfer into 1-cell fish zygotes. Fertilized goldfish eggs were dechorionated with trypsin. A recombinant fusion gene containing the MT promoter and a human growth hormone minigene was microinjected into the center of the germinal disk. Three of six fish randomly selected from those developing had the foreign DNA integrated into their genome. Ozato et al. (1986) used the unique approach of removing unovulated eggs from the ovaries of medake (*Oryzias latipes*) females and microinjecting chicken delta crystallin DNA into the nucleus of the unfertilized oocytes. Then, the eggs were cultured to maturity in defined medium, inseminated and cultured extensively in water until seven days of age. Ten of 30 animals analyzed by *in situ* hybridization and immunohistological techniques contained the delta-crystallin genes and proteins. This was the first report of transgene expression in fish. Chourrout et al. (1986) demonstrated high integration rates by microinjection of fertilized rainbow trout eggs. Either a circular or linearized fragment of the plasmid pSV507 containing the human growth hormone gene and SV40 promoter was injected into the center of the germinal disk of the developing zygote. The rigid chorion was punctured manually with a broken micropipette to allow the delicate microinjection needle access to the vitelline membrane of the eggs. About 77% of the microinjected eggs survived to hatching. Transformation rates of fry were higher for microinjection of the linearized plasmid (75%) than the circular plasmid (40%).

Transgenic loach (*Misgurnus anguillicaudatus*) offspring containing a MT-hGH gene were 3 to 4.6 times larger than non-transgenic controls at 135 days of age (Maclean et al., 1987). Also, Maclean et al. (1987) reported that transgenic fish containing growth hormone gene grew larger and more efficient than controls without any detectable abnormalities. McEvoy et al. (1987) microinjected either MT-rGH or MT-beta-galactosidase (MT-GAL) into fertilized salmon eggs. About 20% of 3 month old fish expressed the beta-galactosidase gene in their tissues. Brem et al. (1988) microinjected MT-hGH gene into tilapia eggs. Out of 65 fish developing to the age of 90 days, four were transgenic. Stuart et al. (1988) observed that after microinjection of SV40 promoter sequences fused to the hygromycin resistance gene into zebrafish eggs, 10 day survival rate was 3 to 43% depending on DNA concentration. Of 547 fish at 4 months of age, 28 (5%) carried transgene sequences. Most of the founder fish were mosaic (about 20% germline transmission rates). Rokkones et al. (1989) produced transgenic salmon and rainbow trout containing a MT-hGH gene. High incorporation rates (75%) were observed at 70 days of age. Human growth hormone was detected at early embryonic stages. Hallerman et al. (1990) reported that transgenic goldfish [containing Rous sarcoma virus (RSV) long terminal repeat fused to chloramphenicol acetyl transferase (CAT) gene] expressed CAT activity in a tissue-specific manner (highest level in muscle). Zhang et al. (1990) reported that of 365 hatched carp following microinjecting RSV LTR fused to trout GH, 20 individuals were transgenic. The average body weight of the transgenic fish at 3 months of age was 20% larger than that of sibling controls.

Antifreeze protein (AFP) of winter flounder depresses the freezing temperature of the fish allowing them to survive at low temperature (Chen and Powers, 1990). Huang et al. (1990) confirmed that AFP gene may be useful to pro-

duce cold resistant transgenic fish using cell lines derived from rainbow trout, bluegill and salmon. Davies et al. (1990) produced transgenic salmon containing the AFP gene. However, the level of AFP expression was not sufficient to protect fish from low temperature.

Liu et al. (1990) developed an all-fish expression vector consiting of regulatory elements of the carp β-actin gene and the polyadenylation signal of the salmon growth hormone gene and a CAT gene as a reporter gene. The CAT gene was expressed in fish cell lines and zebrafish embryos. Gedamu et al. (1990) isolated and characterized rainbow trout metallothioncin genes. Du et al. (1992) produced transgenic atlantic salmon containing salmon GH fused to ocean pout antifreeze protein (opAFP). Those transgenic fish grew 2 to 6 fold faster than non-transgenic control in the first 4 month. All-fish transgenic systems which contain DNA elements that originated from the same species, i. e., fish without any heterologous genes and viral or mammalian regulatory sequences, may be useful for production of commercial transgenic fish (Guise et al., 1991).

Other methods which can be used to produce transgenic fish are sperm binding, electroporation and lipofection. Recently, Inoue et al. (1990) reported successful gene transfer in fish using electroporation. Of 3109 fertilized eggs treated with electroporation (750 V/cm, 50 μs, 5 times), 783 eggs hatched and 4% of them were transgenic. Even though the success rate was lower than for microinjection, simplicity of this kind of methodology may provide a greater potential of applications with transgenic fish.

Discussion

There are several gene transfer methods available to introduce foreign DNA into animals. The most common method at present is microinjection. Most of transgenic livestock were produced by this method. In the mouse, the overall efficiency is approximately 5%, but it is less than 1% in domestic animals. This difference may be due to suboptimal microinjection conditions for livestock species. Retroviruses have an unique life cycle which includes integration of its genome into host chromosomes. Retrovirus-mediated gene transfer has some advantages over microinjection. Retrovirus-mediated gene transfer has been accomplished only in mouse and poultry. Establishment of a variety of retroviral vector systems is needed. The most promising method is embryonic stem cell-mediated gene transfer. However, establishment of embryonic stem cell lines of livestock species is required. The reported overall efficiency of producing transgenic livestock species by the DNA microinjection method is too low. Improvement of the efficiency of transgenic animal production, through alternative methods or optimization of present methods, will accelerate practical application of gene transformation to livestock species. Also, improvements in embryo culture or transfer techniques for sheep, pigs and cattle may improve the efficiency of gene transfer in these species.

One promising practical application of gene transfer technology to livestock species is for the production of pharmaceuticals. Rare human proteins, which cannot be produced by microorganisms because of requirements for post-translational modification, can be secreted into milk of transgenic animals. Large amounts of biologically active protein may be obtained from transgenic farm animals using this system. Growth-related application to livestock species using growth hormone genes or growth factor genes has been disappointing. Although some of transgenic animals containing growth-related genes were stimulated in growth and enhanced in food conversion, severe detrimental effects on the transgenic animals were also observed (Pursel et al., 1989). The major limitation to the use of gene transfer technology for the improvement of production traits in domestic animals is our lack of knowledge concerning the genetic elements which control production parameters. The generation of useful transgenic animals requires the ability to target gene expression to a specific tissue and to control the timing and level of expression of specific genes. Considerable basic research is still needed for tissue- and developmentally- specific promoter elements and for controlled gene expression when fusion genes are constructed which contain novel combinations of enhancer, promoter and gene coding elements. Many sophisticated regulatory elements are needed to be able to turn genes on or off at appropriate times and in appropriate tissues in transgenic animals. Also, isolation and characterization of

more genes of practical interests are required for real targeting of economic traits in livestock species.

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