# GENE TRANSFER IN PIGS

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## SUMMARY

The production of transgenic livestock has extended and improved classical breeding techniques. The objects of manipulation are individual genes rather than the entire genome of an animal. At the moment DNA-microinjection into the pronuclei of zygotes is still the most efficient and in pigs the only available technique of transferring genes. Transfer of genes involves collection, manipulation, microinjection, cultivation, and transfer of early embryos on the one hand. On the other hand molecularbiological techniques allowing cloning of gene constructs, preparation of suitable injection solutions, and techniques allowing detection of integrated and expressed transgenes in transgenic animals have to be employed. Gene transfer in pigs and other domestic animals is usually less efficient than in mice and yields approximately 1% transgenics per injected zygote.
Growth performance and carcass composition were the first to be manipulated by using genes encoding hormones of the growth hormone cascade. But the effects already known from experiments in mice could not be reproduced in pigs. Therefore additional applications of transgenes had to be envisaged. Only a few number of animal disease resistance genes are known. One example is the influenza resistance gene Mx of mice. Influenza is an infectious disease rapidly gaining importance for pigs. We are currently investigating whether the transfer of the influenza resistance gene Mx of mice will yield disease-resistant pigs.
Expression of transgenes sometimes causes unwanted and deleterious side effects, which has been reported in many cases. Instability of integrated transgenes and variability of gene expression over many generations has also been observed. It is assumed that in approximately 5% of all primary transgenic animals integration of the transgene leads to the generation of insertion mutations. Animals carrying these mutation can not be used for breeding. Much more work will be necessary in future before we will be able to employ gene transfer techniques in practical breeding programmes.

## INTRODUCTION

Modern animal breeding has developed rapidly in the last 30 years with a number of new breeding techniques. Artificial insemination, and embryo transfer techniques, are now supplemented by an entirely new breeding technique, *i. e.* gene transfer. Transfer of genes and gene constructs allows the manipulation of individual genes rather than entire genomes. Single genes can be employed according to the breed ideal in a more or less directed manner. Genes, controlled by new regulatory elements, can be handled across species barriers.
Gene transfer techniques have evolved from the first successful gene transfer experiments carried out in mice in 1980 (GORDON *et al.*, 1980). These experiments have contributed to our knowledge of gene expression regulation and have provided an enormous body of information concerning the realization of genetic information into

phenotypically distinguishable traits.
Generation of transgenic rabbits, pigs, and sheep has been described as early as 1985 (HAMMER *et al.*, 1985; BREM *et al.*, 1985). It should be mentioned however that the successes achieved in gene transfer experiments in mice have no direct comparable counterpart with regard to farm animals. The more we learned about gene transfer in farm animals the more it became clear that there are still a number of unsolved problems.

## MATERIALS AND METHODS

### *Production of Transgenic Pigs*

Production of transgenic pigs was done as described (BREM *et al.*, 1985; 1988). Briefly, donor animals were superovulated by administration of gonadotropic hormones and inseminated twice. Zygotes were collected by surgery and flushed of the oviducts approximately 24 hours after fertilization, *i. e.* 60-63 hrs after HCG application. Because nuclear structures cannot be observed in porcine oocytes, they were centrifuged for three min. at 15,000 g. This separates cellular components, thus making visible the pronuclei in the centre of the oocyte.
Gene constructs required for injection were purified from the vector component by cleavage with the indicated restriction endonucleases, and dissolved in injection buffer, following extraction, precipitation, and washing. All solutions used for microinjections were filtered sterile to remove particulate matter. The concentration of the DNA solutions were adjusted to contain approximately several hundred copies of the gene construct per picolitre.
Zygotes were injected by fixing them with the holding pipette in a position which allows observation of the pronuclei. The injection pipette is filled with DNA solution and inserted through the zona pellucida, the cellular membrane and the nuclear membrane into the pronucleus. The successful injection of 1-2 picolitres of DNA solution can be seen by the swelling of the pronucleus. Upon short-term intermediate *in vitro* culture the injected oocytes were introduced into the oviducts of recipients which have been synchronized by hormonal treatment.

### *Analysis of Transgenic Pigs*

All standard procedures were done as described elsewhere (AUSUBEL *et al.*, 1987). Tissue samples were taken from blood or tails of piglets born after microinjection and were used for isolation of high-molecular weight genomic DNA. Southern or slot blot hybridizations were used to detect integration and copy numbers of the injected gene constructs (BRENIG *et al.*, 1989).
Expression of transgenes was analysed by standard RNA-, and protein-techniques. Porcine peripheral blood lymphocytes (PBLs) were isolated from blood of transgenic pigs by Ficoll gradient and cultured *in vitro* in RPMI containing 10% FCS. $10^6$ - $10^7$ cells were incubated with 1000 U of the appropriate IFNs per mL medium for 2.5 h.
The Mx mouse embryofibroblast cell line (BALB.A2G) was established at the Institute of Immunology and Virology, University of Zurich, Switzerland.
Recombinant murine type I interferon (muIFN I, Stratech Scientific Ltd., Lot. No. 82011) was a gift from O. Haller, Institute of Immunology and Virology, University of Zurich, Switzerland. Porcine type I/II interferon (pIFN I/II) induced by human erythromyeloma cells (K 562) in porcine peripheral blood lymphocytes was a gift from M. Büttner, Institute of Medical Microbiology and Infectious Diseases, LMU Munich, FRG. Polyclonal anti-mouseMx antibodies were made available by O. Haller.
Protein labeling was done in methionine-free medium containing 50 mCi/mL $^{35}$S-methionine for 1 h. Labeled cells were washed and then extracted in lysis buffer. Clarified cell extracts were incubated for 30 min. at 4 °C with polyclonal anti-mouseMx anti-

bodies. Immune complexes were collected on protein A-Sepharose. Precipitated immune complexes were boiled for 2 min. in SDS dissociation buffer. Released radioactive proteins were separated by discontinuous buffer electrophoresis and visualized by fluorography.
After transgenic animals were shown to express the transgens properly they were raised and used for matings. Offspring were again monitored for the presence and stable integration of the transgenes.

## RESULTS

### *Manipulation of Growth*

Growth is regulated by a complex hormonal cascade. On stimulation specific regions of the hypothalamus release small proteohormones, somatostatin (SRIF, GHIF, GHIH) or growth hormone releasing hormone (GRF, GRH, GHRF, GHRH), which are transported through the portal venous system to the pituitary, where they induce the synthesis and secretion of growth hormone (GH). The GH reaches individual organs or organ systems via the blood stream and exerts its growth-promoting actions. It also stimulates the synthesis of somatomedins (IGF-I, IGF-II) predominantly in the liver. Somatomedins are mitogenic polypeptides which are structurally and functionally related to insulin and which may stimulate *in vitro* a number of processes usually stimulated by insulin. Apart from its endocrine action, IGF-I also has pronounced autocrine and paracrine activities *in vivo*. All three classes of genes mentioned above have been isolated, although not all of them from pigs. The porcine GHRH has been cloned by BOEHLEN *et al.* (1983). While the GHRH-peptides are not very species-specific in their action, the growth hormones, polypeptides of about 190 amino acids, exhibit very high species-specificity. cDNAs and structural genes of growth hormones have been isolated (ROSKAM and ROUGEON, 1979; MILLER *et al.*, 1980; SEEBURG *et al.*, 1983).
In our experiments two gene constructs were used to produce transgenic pigs. The constructs consisted of the murine metallothionein I-promoter fused to either the human growth hormone gene (mMT-I/hGH) or a synthetic human growth hormone releasing factor cDNA fragment (mMT-I/hGHRH). These fusion genes were used to alter growth performance and to study the effects of the ectopic expression of growth regulating proteohormones. Out of 1014 injected and transferred embryos with the mMT-I/hGH construct (Tab. 1, A) 4 transgenic pigs and 4 transgenic fetuses developed. The mMT-I/hGHRH construct (Tab. 1, B) was microinjected into 1041 embryos and 6 transgenics were obtained. The results are summarized in Table 1.

Table 1 Success Rates in Different Gene Transfer Projects

| | Embryos transferred (n) | Pregnancy rate (%) | Survival rate (%) | Transgenic piglets (n) | Integration rate (%) | Efficiency* (%) |
|---|---|---|---|---|---|---|
| A | 1014 | 20 | 2.1 | 4 | 19 | 0.4 |
| B | 1041 | 55 | 5.4 | 6 | 11 | 0.6 |
| C | 1083 | 41 | 2.0 | 6 | 27 | 0.6 |
| D | 1629 | 40 | 4.7 | 8 | 10 | 0.5 |

* transgenic piglets/embryos transferred

Figure 1 shows a structural comparison of the GHRH gene construct and its endogenous counterpart.

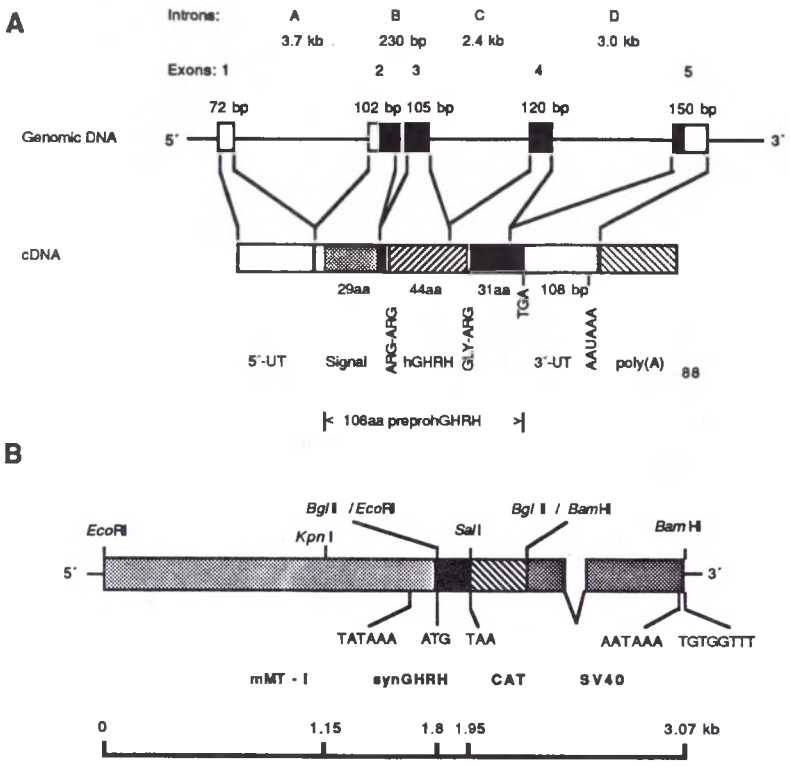


Figure 1 Structure of the GHRH transgene and the endogenous human GHRH gene. Panel A shows the genomic and cDNA organization of the human gene for growth hormone releasing hormone (for details see MAYO *et al.*, 1985). In panel B the structure of the transgene is depicted. Positions of restriction endonucleases and regulatory sequences are indicated. mMT-1: murine metallothionein-1 promoter; synGHRH: synthetic GHRH gene; CAT: part of the prokaryotic chloramphenicol acetyltransferase gene; SV40: parts of the small t-antigen of SV40 containing the splice sites.

As the expression of the growth hormone and growth hormone releasing hormone gene constructs had no influence on the phenotype of the transgenic pigs we wanted to isolate the last member of the hormonal cascade, *i. e.* the porcine IGF-I, to investigate the effects of this gene under the control of a homologous promoter. IGF-I is a polypeptide of 70 amino acids whose DNA sequence contains Exons 2 and 3 of a gene that habours at least five exons. These span a region of over 45 kb in the human genome (De PAGTER-HOLTHUIZEN *et al.*, 1986; ROTWEIN *et al.*, 1986). As the exact location of the transcription initiation site has not yet been determined, the gene may contain additional exons. The IGFs are produced by a differential splicing process. It has been assumed that biogenesis of IGF-I can be regulated at different stages, *i. e.* on the levels of transcription, RNA, and protein processing (BELL *et al.*, 1986).
The IGF genes of other species (rat, mouse) have already been isolated and show striking homologies to their human counterpart (SHIMATSU and ROTWEIN, 1987a; BELL *et al.*, 1986). This is also true for the porcine IGF gene (MÜLLER and BREM, 1990).

*Mx-transgenic Pigs*

Influenza infection in pigs is a acute and highly contagious disease. It leads to growth depression, loss of weight, and is fatal in approximately 1% of the afflicted animals. Therapy is currently not available so that prophylactic measures (hygiene and vaccination) must be taken. The causative agent belongs to the group of *orthomyxoviridae* (genus influenza virus type A) and plays a decisive role in influenza pandemics of man (A7 Hong Kong/68 H 3N1).
Several years ago an inbred mouse strain (A2G) was observed to be resistant against influenza A viruses infection (LINDENMANN, 1962; HALLER *et al.*, 1979; 1980a,b). LINDENMANN (1964) succeeded in allocating resistance to a single genetic locus on mouse chromosome 16 (STAEHELI *et al.*, 1986a), designated Mx. The inbred mouse strain A2G is homozygous for the dominant allele. *In vivo* and *in vitro* experiments have demonstrated that resistance is restricted to infections with influenza viruses and can be induced by α- and β-interferon, while γ-interferon was ineffective. Recently the coding regions of the gene have been isolated from a cDNA library (STAEHELI *et al.*, 1986b).
Our approach envisages construction of transgenic pigs carrying the Mx gene and analysis of such pigs for expression of the transgene. If transgenic pigs will express Mx protein it will be possible to investigate whether these pigs will have become influenza-resistant. Three constructs differing in their promoter region (human MTIIA-promoter, murine Mx-promoter, SV40 early promoter) were used with the murine cDNA coding for the Mx protein (Fig. 2).

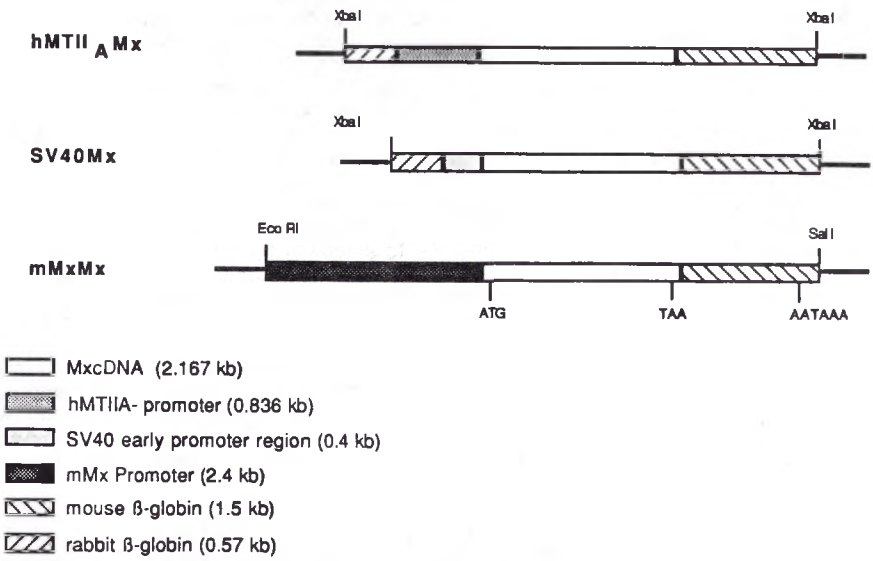


Figure 2 Structure of different Mx Gene Constructs

A summary of the data obtained from the transgenic pigs habouring these constructs is given in Table 1. No transgenics were detected with an intact SV40 Mx construct, probably due to a deleterious effect of the expression during embryogenesis. We also could not detect any expression in the pigs habouring the construct consisting of the human MTIIA promoter (Tab. 1, C) and the Mx gene. Only 2 founder animals containing

the construct with the murine Mx promoter (Tab. 1, D) expressed and transmitted the transgene. Table 2 summarizes the data from these transgenic pigs.

Table 2 Transmission and Expression of a mMxMx Gene Construct in Transgenic Pigs

| Founder animal | 4243 | | 4303 | | 4305 | | 4539 | 4682 | |
|---|---|---|---|---|---|---|---|---|---|
| Sex | f | | f | | f | | m | f | |
| Intact Copies/Cell (approx.) | 20 | | 30 | | 30 | | 10 | 20 | |
| Transmission | + | | + | | + | | + | + | |
| F1 (Transgenic), Individual | 13 (7), | 5645 | 7 (5), | 5660 | 15 (5), | 5617 | 43 (24) | 11 (3), | 5634 |
| F2 (Transgenic) | - | | 6 (3) | | - | | 7 (3) | 10 (7) | |
| Transcription of Transgene | + | | - | | - | | + | - | |
| after stimulation *in vitro* | | + | | - | | - | | | - |
| after stimulation *in vivo* | | + | | - | | - | | | - |

## DISCUSSION

A variety of gene transfer applications have been discussed recently for farm animals. Obviously gene transfer has been used for manipulation of traits which are due to the action of single, or a small number of genes. For example, growth, can be influenced by transfer of a single growth hormone gene. Our current knowledge about the effects of the foreign gene expression is still rather limited and although individual animals clearly show considerable variability in the extent to which they react it seems that single genes contribute to performance due to additive effects of gene action. Further investigations may broaden our knowledge of phenotypic expression caused by individual genes.
The most important factor in the application of gene transfer techniques to pigs is inheritance to progeny. This requires all or most of the germ line cells of primary transgenic pigs to contain the newly introduced transgene. Unfortunately little information is available about the molecular processes leading to integration of a newly introduced gene into the genome. Investigations of transgenic animals and experiments aimed at establishing transgenic offspring in particular have demonstrated that approximately 30 % mosaics may be obtained although the DNA had been introduced into the pronuclei of a fertilized zygote. These transgenic animals have only limited values for gene transfer programmes designed to establish pure transgenic lines.
In the experiments presented here all animals which had stably integrated the injected gene constructs passed on these transgenes to their progeny in a Mendelian pattern of inheritance. The constructs have been integrated at single chromosomal sites in tandemly arranged arrays.
Variabilities in the levels of gene expression were observed. The usefulness of transgenic animals is severely reduced if levels of gene expression are decreased (reduction of performance increase) or increased (negative effects on health due to over-expression). Therefore stable integration and expression of a transgene is the most decisive factor influencing the usefulness of gene transfer techniques for breeding programmes.

Our transgenic pigs habouring the GHRH gene construct did not show any altered growth performance. This is consistant with the results obtained from transgenic pigs (HAMMER *et al.*, 1985) which harbour and express the same gene construct used for the generation of transgenic giant mice (PALMITER *et al.*, 1982; 1983). These animals have shown even slightly reduced daily gains (PURSEL *et al.*, 1987). The most remarkable observation has been a reduction in back fat thickness from 18 to 7 mm. Moreover, the transgenic animals have shown a number of deleterious side effects (e. g. stimulation of the development of the mammary glands in male animals, lethargy, arthritis, incoordination of rear legs, susceptibility to stress, parakeratosis, anoestrus in gilts, lack of libido in boars). Only 2 out of 19 transgenic animals in which expression of the

transgene had been demonstrated reached the age of over one year. The main causes of death have been pneumonia, pericarditis, peptic ulcers (PURSEL *et al.*, 1987). These effects are not due to the use of a specific promoter or regulatory element, but appear to be unequivocally associated with the massively increased serum level of growth hormone.

Our own experiments with transgenic mMTI-hGH mice have demonstrated that the serum levels of growth hormone was as high as 2370 ng/mL and most animals had giant size. The absolute and relative weights of kidneys, liver, heart and spleen were significantly higher than those of control NMRI mice. Macroscopical examination revealed pale, yellowish-brown, swollen kidneys with most severe alterations of the glomeruli found upon histological examination. Comparable findings were described by DOI *et al.* (1988). Histological examination of the liver tissue demonstrated pronounced enlargement and the presence of polymorphous liver cells and cell nuclei.

Over-expression of growth hormone therefore appears to be responsible for severe side effects which may dramatically reduce the health and life span of the animals. As a corollary one should take into consideration the transfer of growth hormone genes only if further experiments would succeed in creating suitable gene control elements allowing levels of growth hormone to be controlled at low levels and regulated according to biological needs.

In the future gene transfer in pigs will focus on problems which have been addressed only unsatisfactorily with conventional breeding, such as reduced susceptibility towards infection and stress.

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