

CRITERIA FOR USEFUL GENE TRANSFER

H. KRÄUßLICH, WEST GERMANY
Institut für Tierzucht und Tierhygiene
Veterinärstr. 13, 8000 München 22

SUMMARY

In 1985, the first reports of the production of transgenic farm animals (rabbits, sheep and pigs) were published (Hammer et al., 1985; Brem et al., 1985). Thus, methods are available to change the genetic make-up of farm animals with a selectivity unapproachable by traditional genetic selection procedures. But our knowledge about the translation of the genotype into phenotype is very limited and we are not yet able to predict the consequences of genetic manipulations in the intact animal. The design, construction, and testing of useful genetic control sequences are the most important tasks in gene transfer.

In the paper the following aspects of gene transfer in farm animals are discussed: Utilization of identified and cloned genes for the manipulation of physiological processes which determine production characters (growth, lactation); possibilities to manipulate the immune response in mammals via gene transfer; identification and cloning of single genes of importance in animal breeding. The utilization of model systems, mainly transgenic mice, to find the most promising gene constructs for experiments with farm animals. The efficiency of gene transfer techniques in farm animals at the present stage and developments in progress which might improve the efficiency.

INTRODUCTION

A major achievement within the field of molecular biology is the development of procedures allowing the transfer of cloned genes into the germ line of individual animals. In late 1980, the first reports of the transfer of cloned genes into the germ line of individual laboratory animals were published (Gordon et al., 1980; Brinster et al., 1981; Constantini and Lacey, 1981; Wagner et al., 1981). Since then, injection of new genetic material into fertilized mouse eggs followed by development of the eggs into transgenic mice in foster mothers has become an almost routine tool in the analysis of a wide range of biological problems.

In 1985, the first reports of the production of transgenic farm animals (rabbits, sheep and pigs) were published (Hammer et al., 1985; Brem et al., 1985). Gene transfer into the germ line of individual farm animals allows the animal breeder to alter the genetic make-up of livestock species with a selectivity unapproachable by traditional genetic selection procedures. These include interspecific gene transfer, the introduction of novel functions such as the ability to alter a product

through external signals or to develop new products. The immediate significance of gene transfer will be the possibility to modify or delete gene function as a means of investigating the physiological consequences of genetic change. Wherever a strong link can be established between the action of a single gene and production efficiency there is a potential of improving livestock through transfer of cloned genes. The main prerequisite for the useful application of gene transfer in animal breeding is the prediction of the consequences of genetic manipulation in the intact animal or in other words the understanding of the translation of genotype to phenotype. Thus the immediate task of gene transfer experiments in animal breeding is to provide the necessary knowledge for the practical application of gene transfer in animal production.

SELECTION OF GENES

The design, construction, and testing of genetic control sequences in transgenic animals are the largest and most significant challenges facing those who wish to use gene transfer technology to improve livestock animals. At present there are few candidate genes for gene transfer experiments in animal breeding which can be grouped as follows:

- Cloned genetic sequences coding protein products which regulate or influence body growth, wool growth, lactation and reproduction.
- Cloned genetic sequences which regulate or influence immune response and resistance against diseases (for example genes of the major histocompatibility complex)
- Identification and cloning of single genes with major effects in farm animals. Most of the known major genes are affecting colour and morphology. But, there are exceptions as the Booroola high fertility gene in sheep and the halothane sensitivity - stress susceptibility gene in pigs and rare deleterious recessives in many populations.

It is important to keep in mind that present technology cannot replace "native" alleles, it can only add genes or inhibit the expression of genes by antisense RNA.

Another problem is the lack of control of the number of gene copies which are transferred. In mice experiments from 10 to 40 doses of the gene have been incorporated as tandem repeats. Thus, the effect of the new gene in transgenic animals on the phenotype cannot be studied in the genetic context which is provided for the "native" gene. A complete solution to the context problem would require replacing the "native" gene at its normal genetic locus in a germ line cell by the gene chosen for gene transfer. Such allele replacements have become routine genetic manipulations in prokaryotes and several fungi, but are not possible in mammals (Botstein and Shortle, 1985). In the following chapters some examples are given for selecting "useful" genes for animal breeding experiments.

Utilization of identified and cloned genes

1 Manipulation of physiological processes

Growth. Wagner et Jöchle (1985) give a comprehensive survey on the potential for improving growth in livestock by gene transfer. Palmiter et al. (1982) showed that transgenic mice harbouring gene constructs consisting of the mouse metallothionein-I (MT) promoter/regulator region fused to either the rat or human growth hormone (hGH) structural genes commonly exhibit high, metal-inducible levels of the fusion messenger RNA, substantial quantities of the foreign growth hormone in serum and enhanced growth. In addition the gene is stably incorporated into the germ line, making the phenotype heritable.

These results stimulated gene transfer experiments in farm animals using the fusion gene MT-hGH which resulted in transgenic rabbits, pigs and sheep (Hammer et al., 1985 Brem et al., 1985). As to be expected the levels of hGH found in the transgenic pigs did not increase body weight. A series of possible modifications for experiments with growth hormone fusion genes are discussed by Wagner et Jöchle (1985) and by Wagner (1985). Modifications concern either the promoter/regulator region or the structural genes. Selden et al. (1985) showed that a gene construct consisting of the human growth hormone structural gene and the glucocorticoid receptor binding site, ligated to the mouse metallothionein promoter sequences can be induced by glucocorticoids. The identification of the primary structure and expression of a functional human glucocorticoid receptor c-DNA allows an analysis of the molecular mechanism by which glucocorticoids regulate gene transcription (Hollenberg et al., 1985). These results indicate that it might be possible to use modified binding sequences, modified receptors and synthetic glucocorticoid analogues could be used as means of external regulation of transgenes. A further possibility are experiments with structural genes of growth hormones, growth hormone releasing factors and somatomedins of different species.

The following criteria for gene transfer experiments can be deduced from these examples:

- (1) Choice of the characters which can be improved (body growth in our example).
- (2) Integration of existing knowledge and expertise about these characters in animal physiology, biochemistry, endocrinology, pharmacology and in recombinant animal genetic technology in order to establish a model on the translation of genotype into phenotype.
- (3) Selection of structural genes and genetic control sequences which might positively effect the chosen character.
- (4) Tests in model systems such as mammalian cell culture and laboratory animals (mice).
- (5) Design of gene transfer experiments with farm animals. In agreement with Wagner and Jöchle (1985) it is stressed that the integration of existing knowledge and expertise of different disciplines in biology, is necessary.

Lactation. It has been shown that injections with bovine growth hormone produced by gene technology dramatically increase milk production per lactation (review Baumann et al., 1985). But, it is disputable whether gene transfer experiments in cattle with the bovine growth hormone structural gene in order to increase milk yield are useful.

Mercier (1985) discussed the potential application of gene transfer for improving the quality of milk both for nutritional and for industrial purposes. Special emphasis is laid on the possible reduction of the lactose content of milk to promote its consumption by lactose intolerant people and to facilitate the manufacture of lactose-free products. In principle, a decrease in lactose synthesis might be obtained by decreasing the amount of α -lactalbumin available in the Golgi apparatus or by altering its ability to interact with the UDP-galactosyltransferase. This cannot be accomplished by structural modification of the respective DNA sequences because it is not yet possible to replace a normal gene by an allele that has been altered in vitro. Mercier (1985) suggests two approaches to solve the problem:

- (1) Inhibition of α -lactalbumin gene expression by anti-sense RNA.
- (2) Secretion of an active β -galactosidase into milk by insertion of a fusion gene consisting of the SV 40 early promoter, the gene for a signal peptide of a milk protein and the β -galactosidase gene.

In the first model the inhibition of α -lactalbumin RNA production by duplex formation is expected in transgenic cows. In the second model, milk of the transgenic cows should contain an active β -galactosidase secreted concomitantly with milk proteins, and able to convert lactose into glucose and galactose, thus reducing the lactose content of milk. Another model suggested by Mercier (1985) is the creation of transgenic animals unable to synthesize β -lactoglobulin by using the antisense RNA methodology in order to allow the modification of cow's milk to simulate some characteristics of human milk. These examples demonstrate the possibilities of gene transfer to change the quality of animal products substantially in the desired direction.

2 Manipulation of immune response

MHC linked immune responsiveness has been mainly studied in mice, chicken and man, but results available up to now show the same mechanisms in our large farm animals (Vaiman, 1985). Two classes of cell surface molecules encoded in the major histocompatibility complex (MHC) genes are known to guide T cells of the immune system in their recognition of foreign antigens on cell surfaces. In general, cytotoxic T cells recognize foreign antigens in association with class I molecules, whereas helper T cells corecognize antigen and class II molecules.

Transgenic mice have been produced with cloned MHC genes and demonstrated that the introduced genes can be expressed in a functional way.

Freels et al. (1985) have introduced a porcine MHC class I gene into mouse germline DNA, all cells examined display the porcine class I molecule on the surface, and skin transplanted from the transgenic mouse to a normal mouse of the same strain is rapidly rejected.

The classical explanation for the maintenance of polymorphism is that it is caused by a selective advantage of heterozygotes over homozygotes. This refers especially to the MHC loci. Vaiman (1985) found that in swine SLA homozygosity quite often resulted in a decrease of the number of littermates. Transgenic animals might increase heterozygosity at the MHC loci in closed lines of high value.

Meur et al. (1985) and Yamamura et al. (1985) showed that class II antigens encoded by genes introduced into transgenic mice can function as T-cell restriction elements. The experiments suggest that it will be possible to endow mice with new MHC restriction elements that can function properly in the generation of an immune response. The relationship of MHC to immune responsiveness may become of increasing interest if synthetic vaccines are developed and used in farm animals. Such compounds will probably display a more limited range of epitopes compared to natural antigens, the immune response could be more dependent on the MHC's phenotype. Thus, an attempt could be made to utilize both of these approaches for the improvement of animal husbandry. A further possibility might be to isolate and clone resistance genes which turn up as mutants in mammalian cell cultures.

Identification and cloning of single genes of importance in animal breeding

1 Major genes

We have to admit that we simply do not know the genes with important effects on animal performance at this time. Major genes studied in animal breeding up to now have typically been those affecting colour and morphology. Examples are colour genes in many populations, polled genes in sheep and cattle, the double muscle gene in cattle, the Booroola high fertility gene in sheep, feather sexing, colour sexing, white feather colour, skin coloration and sex-linked dwarfism genes in chickens, the halothane sensitivity - stress susceptibility gene in pigs. None of these genes are identified on the DNA basis. As candidates for useful gene transfer experiments the Booroola gene and the double muscle gene might be of interest.

2 Deleterious genes

The eighth International Human Gene Mapping Workshop in Helsinki (August 1985) demonstrated rapid progress in allocating human genes to identified regions of individual chromosomes. In four years, the number of mapped genes has increased nearly three-fold to over 900. Sixteen mapped genes had been cloned four years ago; the present figure is 249 (Chapelle, 1985). Totally 808 DNA clones are listed (249 genes and 559 probes). This knowledge helps to clarify the molecular basis and pathogenesis of many mendelian disorders. The importance of mendelian disorders in populations of farm animals is presently underestimated.

Hopefully, similar progress might be achieved in gene mapping for farm animals. However, it is often much easier to isolate a gene than to find out what it does. Biochemists use mutations to probe the relationship between the structure and activity of proteins; cell biologists are using mutations to define the roles of particular proteins and protein assemblies in the cell; developmental biologists are using mutations to determine the logic and order of molecular events during differentiation and morphogenesis. Thus, isolated deleterious genes have to be considered as candidates for useful gene transfer experiments with laboratory animals and in special cases even with farm animals.

Changing cloned genes by in vitro mutagenesis

Botstein and Shortle (1985) recently reviewed strategies and application of in vitro mutagenesis. Methods for precise allele replacement are now routine for some microorganisms, including *S cerevisia* making the ideal mutation experiments readily achievable. It is possible to clone a gene from these organisms, make a single mutation, replace the normal gene in its proper genetic context by the mutant allele, and then study the consequences. It is hoped that methods for gene replacement will also be developed for higher eukaryotes in the near future, so that at that time the genes and genetic regulatory elements in the developmental pathway and phenomena of lactation, reproduction etc. may become amenable to systematic analysis with gene mutations.

EXPERIMENTAL APPROACH

The first step of gene transfer experiments is to define and to evaluate a "useful" gene construct. In chapter 2 some criteria for the selection of "useful" gene constructs are discussed. For the evaluation of the constructs model systems such as mammalian cell cultures and transgenic mice are available. The last step are experiments with farm animals.

The mouse as a model system

To work with large domestic animals rather than mice requires a considerable investment, particularly in the number of animals required. It seems likely that the rules governing expression of exogenous genes in mice will be generally applicable to other mammals. If this would be true we could test the suitability of particular gene constructs in transgenic mice before introducing them into farm animals. Thus, comparisons of expression mechanisms between mice and the domestic species are of great importance for the future development of gene transfer in animal breeding.

Gene transfer in farm animals

The animal used so far to develop methods for gene transfer in mammals has been the laboratory mouse. But, there are no substantial restrictions to utilizing these methods in farm animals. The method of micro-injecting recombinant DNA directly into the pronuclei of zygotes shortly after fertilization can be applied in rabbits, pigs and sheep (Hammer et al., 1985; Brem et al., 1985).

The efficiency of the method in the different domestic species is very important for useful gene transfer. Table 1 gives a survey on the expected results at the present status.

Table 1 Comparison of different mammalian species with regard to the production of transgenic animals

	Mice	Rabbit	Pig	Sheep	Cattle
Ovulations per animal without superovulation	6-10	8-12	10-15	1-3	1
using superovulation	15-30	20-30	30-40	4-8	4-7
Visualization of pronuclei and injection	+++	+++	++	+	+
Survivalrate (offspring/injected egg)	10-15%	8-12%	5-10%	5-7%	3-5% (?)
Integration frequency (transgenies/offspring)	15-25%	10-15%	8-12%	2-5%	?

Fortunately, it is likely that in the future mammalian gene transfer will be supplemented by viral systems. Elegant retroviral systems for the efficient transfer of cloned genetic sequences into mammalian cells and embryos are presently under development. Cone and Mulligan (1984) developed a recombinant retroviral gene transfer system that might be capable of introducing DNA sequences into a broad range of mammalian species including farm animals by developing a cell line that contains the viral protein and enzyme coding genes from the Moloney murine leukemia virus, but utilizes the viral envelope coding sequences from the amphotropic virus 4070 (Weiss, 1982). If this system can be directly applied to livestock species it may not be necessary to develop specific livestock retroviral gene transfer systems for each farm animal. The results published by Jaenish and co-workers make it probable that specific genetic sequences may be introduced into animal germ lines by zygote infection of chimeric retroviruses deficient in viral function. A very important criterion for the choice of the domestic species for gene transfer experiments is the efficiency of the method. At the present stage pigs offer advantages because they are multiparous, sheep are cheaper than cattle and rabbits are as well a laboratory animal as a farm animal. At the present stage of knowledge the aim of gene transfer experiments in farm animals is to understand the biology of development, production, reproduction and immune

response in domestic species. This is to be considered if breeds or lines are chosen for gene transfer experiments. The gene constructs selected should be introduced into a genetic context in which we hope to find the best answer to basic questions mentioned above. Much has to be done before gene transfer can directly be aimed for the improvement of breeds or lines.

Insertion Mutations

Gene transfer experiments with mice demonstrated that the foreign DNA can cause mutations by inserting itself into the recipients' own genes. Some of these insertions have resulted in developmental abnormalities (Marx, 1985). Insertion mutations are not detectable in the original transgenic animals because they would have the insert in only one of the two copies of any affected gene and would still retain a functional gene. It is thus necessary to produce homozygous animals with comparable inserts in both copies. Mintz (see Marx, 1985) estimates that incorporation of transferred DNA into one of the recipient animal's genes occurs in up to 20 percent of the animals that develop from injected eggs. That is a frequency which makes it necessary to perform breeding experiments needed to detect a deleterious insertion mutation before using transgenic farm animals for breeding purposes.

CONCLUSIONS

At the second World Congress in Madrid 1982 Allan Robertson made the following statement regarding gene transfer: "We may find that the methods are available before we know what to do with them." In the meantime methods are available. Admittedly these methods have a low efficiency, but they will be improved. Thus, the question what to do with gene transfer is of great importance for the future development of animal breeding. The following possibilities of useful gene transfer are of interest:

- (1) Selection of cloned genes which regulate developmental processes, physiological processes and immune response in animals; testing the chosen constructs in model systems; gene transfer experiments in farm animals with the most promising constructs. At the present stage of knowledge the main aim of such experiments is to understand the basic biology of farm animals which is necessary for a controlled application of gene transfer in animal breeding.
- (2) Identification and cloning of single genes with major effects in farm animals. Some of these genes may become candidates for gene transfer between breeds and between species.

- (3) Experiments aimed for the use of transgenic farm animals carrying novel gene constructs as production systems of biologically and clinically important peptides and proteins.
- (4) Investigation of similarities and differences of mechanisms which control genes between model systems (transgenic laboratory animals) and transgenic farm animals.

REFERENCES

- BAUMANN,D.E., EPPARD, P.J., DE GEETER, M.J. and LANZA,G.M., *J.Dairy Sci.* 68, 1352-1362 (1985)
- BOTSTEIN,D. and SHORTLE,D. *Science* 229, 1193-1201, (1985)
- BREM,G., BREINIG,B., GOODMAN,H.M., SELDEN,R.C., GRAF,F., KRUFF,B., SPRINGMANN,K., HONDELE,J., MEYER,J., WINNACKER,E.L., KRAEUSSLICH,H. *Zuchthyg.*20, 251-252 (1985)
- BRINSTER,R.L., CHEN,H.Y., TRUMBAUER,M.E., SENEAR,A., WARREN,R., and PALMITER,R.D., *Cell* 37, 367-379 (1981)
- CONE,R.D. and MULLIGAN,R.C., *Proc.Natl.Acad.Sci.* 81, 6349-6353, (1984)
- CONSTANTINI,F. and LACEY,E. *Nature* 294,92-94 (1981)
- DE LA CHAPELLE,A. *Nature* 317,472-473 (1985)
- FRELS,W.I.et al. *Science* 228,577 (1985)
- GORDON,J.W., SCANGOS,G.A.,PLOTKIN,D.J., BARBOSA,J.A. and RUDDLE,F. *Proc.Natl.Acad.Sci.* 77, 7380-7384 (1980)
- HAMMER,R.E., PURSEL,V.G., REXROAD,JR.C.E.,WALL,R.J., BOLT,D.J., EBERT,K.M., PALMITER,R.D.and BRINSTER,R.L. *Nature* 315, 680-63 (1985)
- HOLLENBERG,S.M., WEINBERGER,C., ONG,E.S., CERELLI,G., ORO,A., LEBO,R., THOMPSON,E.B., ROSENFELD,M.G. and EVANS,R.M. *Nature* 318, 635-641 (1985)
- MARX,J.L., *Science* 228, 1516-1512 (1985)
- MERCIER,J.C., CEC Seminar: Exploiting New technologies in animal breeding, Edinburgh, 19.-20.06.1985 (1985)
- SELDON,R.F., WAGNER,T.E., YUN,J.S., MOORE,D.P.and GOODMAN,H.(submitted for publication 1985; cited from Wagner (1985)

VAIMAN, M. 36th EAAP Meeting, Greece, 30 September-30 October (1985)

WAGNER, T.E., Polycopy, Genetic Engineering of Animals: An Agricultural Perspective, Davis California 9-12.09.85, (1985)

WAGNER, E.F., STEWART, T.A. and MINTZ, B. Proc. natn. Acad. Sci. USA, 78, 5016-5020 (1981)

WAGNER, T.E. and JÖCHLE, W; P.J. BUTTERY, editor, Butterworth, Press, London (impress) (1985)

WEISS, R., TEICH, N., VARMUS, H. and COFFIN, J. (Eds) Cold Spring Harbor, Laboratory (1982) YAMAMURA, K. et al. Nature 316, 67 (1985)