Genetically Engineered Food

Methods and Detection

Edited by
Knut J. Heller

WILEY-VCH GmbH & Co. KGaA

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Library of Congress Card No.: applied for
British Library Cataloguing-in-Publication Data. A catalogue record for this book is available from the British Library.

Bibliographic information published by Die Deutsche Bibliothek
Die Deutsche Bibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data is available in the Internet at <http://dnb.ddb.de>.

Printed in the Federal Republic of Germany.
Printed on acid-free paper.

Composition Hagedorn Kommunikation GmbH, Viernheim
Printing betz-druck GmbH, Darmstadt
Bookbinding Litges & Dopf Buchbinderei GmbH, Heppenheim

ISBN 3-527-30309-X

Preface

A new phase in the era of molecular biology was entered 30 years ago with the construction and successful transformation of the first recombinant DNA-molecule by Cohen and co-workers in 1973. This event marked the birth of genetic engineering which on one hand allowed a very thorough analysis of cellular functions and on the other hand provided the tool for targeted manipulation of the genetic material of cells and organisms. Supported by the development of the efficient chain termination method for DNA sequence analysis by Sanger and co-workers in 1977 and the polymerase chain reaction method for targeted amplification of DNA segments of choice by Mullis and co-workers in 1986, genetic engineering of prokaryotic organisms and later of eukaryotes became soon a task easily performed in many laboratories.

Very soon the potential of genetic engineering for food production was recognised and the first genetically engineered food organisms, the now famous "Flavr Savr" tomato with delayed ripening, was created and approved in the United States of America in 1994. Many other plants like rape, maize, and soy beans followed, and for these the introduction of herbicide resistance was the predominant genetic modification. The development of this new breeding technique initiated e.g. in Europe the introduction of new legislation like the "Council Directive (90/220/EEC) on the deliberate release into the environment of genetically modified organisms" and the "Regulation (258/97/EC) concerning novel foods and novel food ingredients". The rationale behind the introduction of this new legislation was to harmonise legislation concerning free trade, to protect public health and consumer rights, and to duly consider environmental aspects.

One consequence of these regulations was the development of detection methods to identify unambiguously foods produced with the aid of genetic engineering. Today, these methods must be capable of determining the amount of genetically engineered ingredients quantitatively at a level of 1% of the entire amount of the ingredient. This is necessary to differentiate between deliberate application and accidental contamination of the genetically engineered ingredient.

This book addresses in three parts the three different aspects of genetic engineering of foods: in part 1 current applications and future potentials of this breeding technique are discussed, in part 2 the legislation in Europe and the frame it sets for the application of this technique are presented, and in part 3 methods developed to detect foods produced with the aid of genetic engineering are presented

and the limits of detection are discussed. The book is by no means comprehensive. The focus concerning detection methods is clearly on those methods detecting DNA. Methods for the detection of protein e.g. are not described separately but are dealt with in different chapters of part 3 whereever it is appropriate. The issues food-safety and consumer-acceptance are not delt with deliberately. Food safety is not a specific issue for novel foods but an issue for food in general. Consumer acceptance for genetically modified foods is an issue of very controversial debates and often the arguements in these debates are not at all scientific. It is the feeling of the editor that taking up the consumer acceptance issue would be an obstacle obstructing the view onto the scientific data presented in the book.

All authors are established and active researchers in their fields. It is their expertise which makes me confident that this book will be a valuable work for anyone interested in novel foods and methods of detection. I am grateful to them for having contributed so excellently to this book.

I also wish to acknowledge the excellent cooperation of Karin Dembowsky and of Andrea Pillmann, both from WILEY-VCH, in the initial and in the finishing phase, respectively.

Finally, I like to thank my family Dagmar, Steffen, and Daniel for their patience and constant support especially during the finishing phase of the book.

Knut J. Heller,
Kiel, April 2003

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Part I
Application and Perspectives

1
Transgenic Modification of Production Traits in Farm Animals

Mathias Müller and Gottfried Brem

"Genetic engineering" is the umbrella term for procedures that result in a directed alteration in the genotype of an organism. The combined use of molecular genetics, DNA recombination and reproductive biology allows the generation of transgenic animals. For animals, the term "transgenic" originally referred to the stable introduction of new genetic material into the germ-line [1, 2]. This definition of transgenic animals must be extended with respect to two aspects. First, further developments of genetic engineering of animals allow not only additive gene transfer (gain of function) but also deletive gene transfer (knockout, loss of function) and replacement gene transfer (knockin, exchange of function). Second, in addition to germ-line integration of transgenes, somatic gene transfer approaches result in (mostly transient) gene expression, with the longest duration being a life span and no transmission of the transgenes to the progeny. Although somatic gene transfer experiments in farm animals for production purposes have been performed [3, 4], this technology in animal production is more beneficial for the development of DNA-based vaccines [5]. Here, we will mainly concentrate the discussion on germ-line transgenic animals. The production of transgenic farm animals was first reported in the mid-1980s [6, 7], since when the main progress in exploiting this technology has been made in the establishment of animal models for human diseases [8, 9], the production of heterologous proteins in animals (gene farming) [10], and the production of organs for xenotransplantation [11, 12]. In addition to these biomedical approaches, research has focussed on the improvement of the efficiency and quality of animal production by transgenic means (this review and Ref. [13]).

1.1
The Creation of Transgenic Animals

The main routes to transgenesis in mammals include: (i) microinjection of DNA into the pronucleus of a fertilized oocyte (zygote); (ii) integration of a (retro)viral vector into an early embryo; (iii) incorporation of genetically manipulated pluripotent stem cells into an early embryo; and (iv) transfer of genetically altered nuclei

into enucleated oocytes. For additional gene transfer methods, especially sperm-mediated gene transfer, we refer to other reviews [14–16].

1.1.1 Pronuclear DNA Microinjection

Microinjection of foreign DNA into the pronuclei of zygotes is the classic method of gene transfer into farm animals, and since its first reports [6, 7], this technique has accounted for production of the large majority of transgenic farm animals [9, 17–19]. DNA microinjection results in random integration of the foreign DNA into the host genome, and is therefore not suitable for targeted modification of genomes. Despite microinjections being performed at the 1-cell-stage, between 20 and 30 % of the founder animals are mosaic and therefore may not transmit the integrated gene construct to their progeny [17, 20]. Random integration of the gene constructs may cause alteration of one or more gene loci. An insertional mutagenesis is recessive and mostly characterized by a recombination event in the kilobase range at the transgene integration site [21]. In mice, approximately 5–15 % are affected by this recessive mutations [22]. Except for studies in transgenic rabbits [23, 24], few data have been published on the analysis of homozygous transgenic farm animals, this being mainly due to the long generation intervals. However, there is no reason to doubt the mutagenesis frequencies estimated for mice in other transgenic mammals generated by the same technology. In addition, random integration of the gene constructs may result in varying, aberrant or abolished transgene expression due to effects of the adjacent chromatin overcoming the transgene's regulatory sequences. One possibility of avoiding these integration site-dependent effects is the transfer of large DNA constructs, which are able to form functionally independent chromatin domains [25]. The first successful example of this strategy in livestock was the generation of transgenic rabbits harboring yeast artificial chromosomes (YACs) [26]. An alternative approach to protect transgenes from chromosomal position effects is the use of boundary elements (e. g., insulators, locus control regions, matrix attachment regions) in the gene constructs in order to achieve copy number- and promoter-dependent and position-independent expression of transgenes [27]. Although success following this strategy has been reported, the effects of the elements were not in all cases as expected.

The gene transfer efficiency (transgenic newborns/microinjected zygotes) in general is rather low, especially in large animals. One transgenic animal can be expected after microinjection of 40, 100, 90–110 and 1600 zygotes in mice, pigs, small ruminants and cattle, respectively [19]. Differences in efficiency emphasize fundamental differences in the reproductive biology of species. Hence, a high level of technical skill and experience in embryo collection and embryo transfer are critical for efficient transgenic production, though this applies equally to all gene transfer programs.

As mentioned earlier, the protocols for generating large mammals by DNA microinjection have remained basically unchanged for the past two decades, and little

improvement in DNA transfer efficiency has been achieved. Following DNA microinjection, embryos are transferred to synchronized foster mothers. Major progress has been made in the field of embyro transfer which, in all farm animal species, has been facilitated by the development of endoscopy-guided minimally invasive techniques, thereby reducing stress to the foster mothers and maximizing embryo survival and pregnancy rates [28–31]. This embyro transfer technique is also advantageous for the gene transfer methods discussed in the following sections.

1.1.2 Retroviral Vectors

The first germ-line transgenic mice were produced by retroviral infections of early embryos [32]. Retroviruses can be considered natural gene delivery vehicles to mammalian cells. Endogenous retroviruses (ERVs) are a subset of retroelements which represent up to 10 % of the mammalian genome [33]. The capability of ERVs to reintegrate into the genome through reverse transcription mechanisms results in continuous insertion of new ERVs into the host genome. The retroviral vectors were, until recently, not considered for farm animal transgenesis. This was due to biosafety concerns and the dependence of most retroviruses on dividing cells for integration into the host genome. Retroviral gene transfer therefore often results in genetic mosaics when developing embyros are infected. With the development of replication-defective retroviral vectors mainly for gene therapy purposes, a powerful tool for gene transfer in mammalian cells has been established [34]. To avoid mosaicism, Chan et al. [35] inoculated bovine oocytes in the final stage of maturation with retroviral vectors. These authors obtained a remarkably high transgenesis rate and, as expected, no mosaic transgenic cattle. A similar approach resulted in the generation of transgenic piglets [36]. One major limitation of retroviral vectors is their limited cloning capacity (<10 kb). However, gene constructs grow increasingly larger in order to omit variegated transgene expression (see above). A second problem with many retroviral vector-mediated transgenics relates to transcriptional shutdown of the transgenes [37]. Lentiviral vectors are a new generation of retroviral vectors which, in contrast to the above-mentioned oncoretrovirus-based vectors, do not undergo transcriptional silencing. In addition, lentiviruses are able to infect both dividing and nondividing cells. Recently, germ-line transmission and expression of transgenes delivered by lentiviral vectors to 1-cell embryos has been reported [38]. This technique of transgenesis is more efficient and cost-effective and technically less demanding than pronuclear injection. The obstacle of the limited size of the constructs to be transferred remains, however. As with gene transfer by pronuclear injection, retrovirus-mediated gene transfer can be only used for additive gene transfer, and also carries the danger of insertional mutagenesis.

1.1.3
Pluripotent Stem Cell Technologies

Pluripotent stem cells are capable of developing into many cell types including germ cells upon fusion with proimplantation embyros (morulae, blastococysts). Pluripotent stem cells can be maintained in tissue culture and genetically manipulated and selected *in vitro* prior to reconstitution of the embryo. In mice, the handling of pluripotent cells has become a routine method for targeted modification of the genome by homologous recombination, i. e., deletive or replacement gene transfers [39]. As yet, many efforts to establish pluripotent stem cells in species other than mice have failed, and possible reasons for this failure are discussed elsewhere [40, 41]. As nuclear transfer using transgenic donor cells (see below) has become an attractive alternative tool for targeted gene transfer, efforts to establish germ-line competent stem cells from farm animals have been reduced.

1.1.4
Nuclear Transfer using Transgenic Cells

Nuclear transfer technology – also known as cloning – comprises the transfer of a donor nucleus (karyoplast) into the cytoplasm of an enucleated zygote or oocyte (cytoplast). Initial nuclear transfer experiments in farm animals used early embryonic stages as nuclear donors [42] (see also Ref. [43] for a review). In breakthrough experiments with sheep it was demonstrated that *in vitro*-cultured differentiated fetal cells [44], and even cells derived from adult tissues [45], could serve as nuclear donor for the reconstitution of enucleated oocytes. Cloning by nuclear transfer has subsequently been achieved in cattle [46–48], goat [49], pigs [50–52], and rabbits [53]. For farm animal transgenesis, a novel tool became available in that cultured cells can be genetically modified by conventional transfection methods prior to their use for nuclear transfer. The first reports on this novel gene transfer technique were the generation of transgenic sheep and cattle by nuclear transfer using transfected and selected fetal fibroblasts [54, 55]. Transgenesis by nuclear transfer of genetically modified cells provides a number of advantages over the other additive gene transfer techniques:

- mosaicism is avoided and germ-line transmission is guaranteed, since all cells of the cloned animal contain the transgene;
- insertional mutagenesis and chromosomal positioning effects can be avoided, since integration and eventually transgene expression can be monitored *in vitro*; and
- the use of male or female cell lines predicts the gender of the transgenic animal [56, 57].

Most importantly, gene transfer by nuclear transfer provides the means for gene targeting in farm animal species [40]. Both the targeted disruption of genes by homologous recombination (deletive gene transfer, knockout) in sheep and pigs

[58–60] and the targeted integration of a gene of interest into a given locus (replacement gene transfer, knockin) [61] have been reported.

Despite these impressing reports and the intriguing advantages of the nuclear transfer technique for the generation of transgenic farm animals, the broad use is not an easy task because: (i) the primary fibroblasts presently used for gene transfers have a limited capacity to divide; (ii) homologous recombination is less frequent in somatic cells than in pluripotent stem cells; and (iii) cloning by nuclear transfer has a low yield which is still diminished when nuclear donor cells are previously cultured [62]. In addition, there is an ongoing debate whether it is possible to overcome abnormalities observed in cloned animals [63, 64]. The abnormalities are not restricted to transgenic cloned animals, suggesting that they originate from the nuclear transfer procedure or the *in vitro* culture conditions. Although healthy clones have been reported [65], improvements in the technology and further investigations of the effects of cloning are required [66].

1.1.5 Gene Transfer in Poultry

Depending on the developmental stage, various strategies are used for the generation of transgenic birds, including DNA microinjection of fertilized ova, retroviral infection of blastodermal cells, and genetic manipulation of primordial germ cells (PGCs) or embryonic stem (ES) cells. As in mammals, the first method developed to transfer genes into birds was through microinjection of DNA into the germinal disc of fertilized ova [67]. Although successful germ-line transmission has been reported [68], the method is labor-intensive, ineffective, and frequently results in mosaicism. Retroviral vectors are able to introduce transgenes into the genome at low but acceptable efficiencies. The first transgenic birds were produced using replication-competent vectors, and thus could not be used for a broad application [69]. The development of replication-defective vectors led to a wide use of this technique in the production of transgenic birds [70] and stable transgene expression [71]. As an alternative, chimeras bearing transfected pluripotent cells originating from the blastoderm, from PGCs or from ES cells have been reported, but have not yet yielded a transgenic bird with germ-line transmission [72, 73].

1.1.6 Gene Transfer in Fish

The techniques for gene transfer into fish have focussed on direct transfer of DNA into gametes or fertilized eggs, and include DNA microinjection, electroporation, retroviral vector infection and biolistic methods [74–77]. Stem cell-based technologies are not available in farmed fish. The creation of transgenic fish is distinguished from gene transfer in mammals or birds because: (i) fish generally undergo external fertilization and no culture or transfer of eggs into recipient females is required; (ii) the eggs of many fish have a tough chorion such that special methods are required to deliver the gene constructs; and (iii) DNA delivery (including

that by microinjection) is usually made into the cytoplasm. It is most likely due to the cytoplasmic nature of DNA delivery that a high number of founder transgenic fish are mosaic. Germ-line mosaicism seems also to occur because the frequency of transgene transmission to F_1 is clearly less than at Mendelian ratios. Transmission of the transgenes to later progeny occurs at Mendelian frequencies, indicating the stable integration of the transgenes.

1.2 Transgenes: Gene Constructs

The exogenous DNA integrated into the host genome usually is referred to as gene construct or transgene, and encompasses the elements controlling gene expression (5′ promoter region, 3′ control regions) and the sequences (cDNA, genomic DNA) encoding the transgene product. The various transgenic sequences used for the differing gene delivery methods and gene transfer programs are summarized in Table 1.1.

For additive gene transfer experiments by DNA microinjection or spermatozoa, the prokaryotic cloning vector sequences are removed from the gene construct. Prokaryotic sequences, and especially their CpG dinucleotide base pairs, may undergo methylation or heterochromatin formation in animal cells, which leads to transgene silencing. It is becoming increasingly clear that silenced transgenes have been recognized as foreign elements by host cellular mechanisms, as are retroviral and transposon sequences [78, 79]. As mentioned above, one obvious way to avoid transgene silencing or chromosomal positioning effects would be to use large gene constructs and the abdication of viral vectors. Therefore, an increasing number of transgenic animals carry gene constructs based on phage (PAC), bacterial (BAC) or yeast (YAC) artificial chromosomes [25]. For expression and replication, these large transgenes are dependent on integration into the host genome. In contrast, mammalian artificial chromosomes (MACs) provide both an independent transcription and replication unit. Studies that originated mainly from human gene therapy programs have resulted in the development of human artificial chromosomes based on episomal viral vectors [80] or engineered minimal chromosomal elements [81–83]. In the future, MACs may be also used in farm animal transgenesis.

Alternatively, gene transfer *in vitro* followed by reconstitution of embryos by nuclear transfer or stem cell technologies allows the targeting of transcription units in the host genome or *in vitro* analysis of the chromosomal integration site. These gene transfer techniques however require methods for identification of the genetically modified cells. The identification of transgenic cells is mostly based on (drug) selectable markers, e. g., antibiotic resistance genes, added to the gene constructs. In plants, the presence of marker genes in the genetically modified organism is the main topic of concern regarding biosafety [84]. By analogy, a genetically modified farm animal that is sold commercially should be free of such genes; this can be achieved by using site-specific recombinases to remove undesirable sequences

Table 1.1. Characteristics of transgenes used for different gene delivery methods and gene transfer programs in farm animals.

Functional consequence of gene transfer	*Methods of gene transfer*	*Sequences not related to the transgene per se*	*Composition and origin of transgene*	*Example (see below)*
Gain of function	Pronuclear DNA micro-injection; sperm-mediated; physical/chemical methods		• species-specific sequences • cross-species sequences • new combination of promoter and coding sequences (species-specific or cross-species)	• additional copies of casein alleles • humanized milk • mammary gland-specific expression of antibodies
	Artificial nonmammalian chromosomes	PAC-, BAC-, YAC-vector elements	See above	See above
	Mammalian artificial chromosomes	Chromosomal elements	See above	See above
	Retroviral vector	Viral sequences	See above	See above
	Cloning by transfer of genetically modified nuclei	Selectable marker genes[1]	See above	See above
Loss of function	Cloning by transfer of genetically modified nuclei	Selectable marker genes[1]	Deleted or nonsense-mutated species-specific sequences	Generation of PrPc gene-deficient ruminants
Exchange of function	Cloning by transfer of genetically modified nuclei	Selectable marker genes[1]	• Introduction of novel allelic variants • exchange of coding sequences in a transcription unit	• targeted alteration of milk protein genes • replacement of genes

[1] Unwanted sequences may be removed *in vitro* by site-specific recombinases.

after successful identification of the transgenic cells [85, 86]. However, it should be mentioned that these additional genetic engineering steps have not yet been conducted in farm animals.

Retroviral vector-mediated gene transfer methods bear the advantage of the transgene being actively delivered to the cells and integrated into the host genome. The disadvantage of the system is the above-mentioned transcriptional shutdown and heterochromatin formation of the transgenes due to the presence of the viral sequences.

1.3 Transgenic Animals with Agricultural Traits

A key element to the enhanced production of domesticated species is the development of genetically superior breeding stocks that are tailored to their maintenance conditions, and also to the marketplace. Characteristics that are generally desirable in all species include improvements in growth rates, feed conversion efficiencies, disease resistance, and a capacity to utilize low-cost or nonanimal protein diets. The attempts to improve productivity traits in farm animals by transgenesis can be divided into products designed for the consumer's consumption *per se* and for traits not affecting the food chain in the first place. The first area includes stimulation of growth rates, food conversion and alteration of carcass and milk composition. The second field aims at the improvement of fiber products, enhanced disease resistance and the introduction of novel biochemical pathways. Although the transgene product in this field is not meant to be used as food, the meat or milk of genetically modified animals could be considered for consumption.

Initially, it should be noted that progress on the manipulation of agricultural animal traits has occurred far more slowly than was originally envisaged during the early days of transgene technology. The first reason for this is the finding that most economically important traits are controlled by multiple genes, which are still largely unknown and hence not amenable to manipulation. Even in the case where all genes contributing to a complex trait have been identified, the genetic engineering of this trait would require multiple gene transfers. The second reason is that the low efficiency of gene transfer in farm animals (see above) renders research on trangenesis costly. The third reason is that the ability to regulate expression of transgenes is still far from adequate (see above). Finally, public acceptance of genetically modified organisms in the food chain is – at least in Europe – currently not given.

Compared with mammals and fish, gene transfer experiments in chicken are somewhat limited, though the aims of gene transfer into poultry are basically identical to those used in other farm animals (for reviews, see Refs. [87, 88]).

1.3.1 Improved Growth Rate, Carcass Composition, and Feed Efficiency

1.3.1.1 Transgenes in mammalian farm animals

Among the genetically determined factors regulating growth rate and feed conversion, the genes encoding polypeptides of the growth hormone cascade are of particular interest. The positive acting growth hormone-releasing hormone (GHRH, somatoliberin) and its antagonist, somatotropin release-inhibiting factor (SRIF, somatostatin) control the production of growth hormone (GH, somatotropic hormone (STH), somatotropin). The GH action is highly dependent on the metabolic situation of the organism: low blood glucose levels result in catabolic effects (lipolysis), and a positive energy balance causes anabolic effects which is mainly governed by insulin-like growth factor 1 (IGF-1, somatomedin C). Early studies on farm animal transgenesis were influenced by the results of Palmiter et al. [89] which indicated that mice expressing excess GH grew much faster and bigger than nontransgenic control mice. A number of GH transgenic pigs and sheep were produced with human, bovine, ovine, porcine or rat GH under control of several promotors [90, 91]. GH-transgenic pigs expressing the gene constructs at high levels were found to have faster growth rates and an increased feed efficiency. The most dramatic effect of elevated GH levels in pigs was the reduction in carcass fat as transgenic pigs approached market weight [13, 92], though the constitutive and/or high-level expression of GH in pigs was found to cause a variety of pathologic side effects [13, 93, 94]. Transgenic ruminants (cattle, sheep, goat) carrying growth-promoting genes have been also generated, though no positive effects on either growth performance or carcass composition have been reported [90, 95].

It has been recognized that tight regulation of transgene expression would be required to avoid deleterious effects from continuous exposure of mammals to elevated GH, and so far most efforts to use dietary inducible promoters have failed [90]. Two studies have reported the production of growth-promoting transgenic pig lines. A metal ion-inducible promoter linked to the porcine GH gene was introduced into pigs, and a large number of transgenic founder animals were produced. Transgenic pigs were tested for metal-induced transgene expression, and animals showing high basal levels of transgene expression or plasma GH levels outside the range of nontransgenics were excluded from the study. Following this strategy, negative side effects could be avoided [91]. However, due to the random integration of transgenes by DNA microinjection and a lack of shielding sequences, the transgenic lines showed a high degree of variegated gene expression. In a second study, the expression of IGF-1 was directed to muscle by using a skeletal muscle-specific expression cassette. By avoiding the systemic effects of GH, an increase in carcass leanness and no detrimental side effects were observed [96]. Interestingly, in the context of the use of growth hormone cascade transgenes, somatic gene transfer might suffice the demands for improved growth performance. A somatic gene transfer protocol employing a singular intramuscular injection and electroporation of muscle-specific expression vectors encoding a protease-