Genetically Engineered Food

Methods and Detection
Second, Updated and Enlarged Edition

Edited by
Knut J. Heller

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Contents

Preface

The first edition of this book was published in 2003. At that time one could not foresee its success, because admission of genetically engineered food into Europe had been suspended for several years. Since the lifting of the moratorium, however, applications, especially for admission of genetically engineered plants to be used as either food or feed, have again increased, showing that this is still a dynamic field of applied molecular biology.

The era of molecular biology entered a new phase thirty years ago with the construction and successful transformation of the first recombinant DNA molecule (Cohen SN, Chang AC, Boyer HW, Helling RB. Construction of biologically functional bacterial plasmids in vitro. Proc Natl Acad Sci USA 1973; 70:3240–3244). This event marked the birth of genetic engineering which enabled very thorough analysis of cellular functions and 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 (Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74:5463–5467) and the polymerase chain reaction method for targeted amplification of DNA segments of choice (Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol. 1986; 51:263–273), genetic engineering of prokaryotic organisms and, later, of eukaryotes became a task easily performed in many laboratories. The potential of genetic engineering for food production was very soon recognized and the first genetically engineered food organisms, the famous "Flavr Savr" tomato with delayed ripening, was constructed and approved in the United States of America in 1994. Many other plants followed, for example rape, maize, and soy beans. The development of this new breeding technique initiated in Europe the introduction of new legislation needed for harmonization of legislation concerning free trade, for protection of public health and consumer rights, and for consideration of environmental aspects. This legislation has been as dynamic as the science behind genetic engineering – for example, the famous "Regulation (258/97/EC) concerning novel foods and novel food ingredients" established only in 1997 was replaced by "Regulation (EC) No 1829/2003 on genetically modified food and feed" to encompass food produced with the aid of genetic engineering. To enforce this legislation, detection methods had to be developed enabling unambiguous identification of foods produced with

the aid of genetic engineering. Today, these methods must be capable of quantitative determination of amounts of genetically engineered ingredients at levels of 0.9% of the entire amount of the ingredient. This is necessary to guarantee that the accepted level of contamination of food with technically unavoidable genetically engineered material is not exceeded.

Because of the success of the first edition of this book, we have used the same structure in the second edition. Current applications and future potentials of this breeding technique are discussed in Part 1. Part 2 covers the current state of legislation in Europe; the framework it sets for application of this technique is presented. Methods developed for detection of foods produced with the aid of genetic engineering are highlighted in Part 3.

The book is by no means comprehensive. The focus of detection methods is clearly on detection of DNA. Issues of food safety and consumer acceptance are deliberately not dealt with. Whereas food safety is not a specific issue for novel foods but an issue for food in general, consumer acceptance of genetically modified foods is a very controversial topic of debate, and often the arguments in that debate are all but scientific. It is my feeling as editor that covering the consumer-acceptance issue would obstruct consideration of the scientific data presented in the book.

Last but not least, I wish to acknowledge the excellent cooperation of all the authors in updating their contributions to this second edition and to thank Waltraud Wuest, Wiley-VCH, for her support during the entire production phase of this edition.

Knut J. Heller
Kiel, June 2006

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Part I
Application and Perspectives

1
Transgenic Modification of Production Traits in Farm Animals

Gottfried Brem and Mathias Müller

1.1
Introduction

"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 enables 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 has to be extended for two reasons. First, further developments in the genetic engineering of animals enable not only additive gene transfer (gain of function) but also deletive gene transfer (knock-out, loss of function), replacement gene transfer (knock-in, exchange of function), and spatial–temporal gene transfer (conditional knock-out) [3, 4]. 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 have been performed for production purposes [5, 6] this technology in animal production is more beneficial for the development of DNA-based vaccines [7]. 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 [8, 9]. The main progress in exploiting this technology has been made in the establishment of animal models for human diseases, production of heterologous proteins in animals (gene farming), and the production of organs for xenotransplantation [10–12]. In addition to these biomedical approaches research has focused on improvement of the efficiency and quality of animal production by transgenic means (this review and other work [13, 14]).

1.2
The Creation of Transgenic Animals

The main routes to transgenesis in mammals include:

1. microinjection of DNA into the pronucleus of a fertilized oocyte (zygote);
2. integration of a (retro)viral vector into an oocyte or an early embryo;
3. incorporation of genetically manipulated pluripotent stem cells into an early embryo; or
4. transfer of genetically altered nuclei into enucleated oocytes.

For alternative gene transfer methods we refer to another review [15]. Sperm-mediated gene transfer, at least for pigs, has been proven to result in high-efficiency transgenesis ([16] and Refs. therein), although the method is not as broadly established as the methods described below.

1.2.1
Pronuclear DNA Microinjection

Microinjection of foreign DNA into pronuclei of zygotes is the classic method of gene transfer into farm animals. Since the first reports [8, 9] this technique, nearly unchanged, has accounted for most transgenic farm animals [15, 17]. DNA microinjection results in random integration of the foreign DNA into the host genome and is therefore not suitable for targeted modification of genomes. Although microinjections are performed at the one-cell-stage approximately 20–30% of the founder animals are mosaic and, therefore, may not transmit the integrated gene construct to their progeny [18]. Random integration of the gene constructs may cause alteration of one or more gene loci. Insertional mutagenesis is recessive and mostly characterized by a recombination event in the kilobase range at the transgene integration site. In mice approximately 5–15% are affected by this recessive mutation [19]. Except for studies in transgenic rabbits [20, 21], little has been published on analysis of homozygous transgenic farm animals. This is mainly because of the long generation intervals. There is, however, no reason to doubt the mutagenesis frequencies estimated for mice in other transgenic mammals generated by the same technology. Random integration of the gene constructs may also result in varying, aberrant, or abolished transgene expression, because of effects of the adjacent chromatin overcoming the transgene's regulatory sequences. One possible means of avoiding these integration site-dependent effects is the transfer of large DNA constructs, which can form functionally independent chromatin domains [22]. The first successful example for this strategy in livestock was the generation of transgenic rabbits harboring yeast artificial chromosomes (YACs) [23]. An alternative means of protecting transgenes from chromosomal position effects is the use of boundary elements (e.g. insulators, locus control regions, matrix attachment regions) in the gene constructs to achieve copy number-dependent and promoter-dependent and position-independent expression of transgenes [24]. Although success with this strategy has been reported, the effects of the elements were not always as expected.

Gene transfer efficiency (transgenic newborns and/or microinjected zygotes) is usually rather low, especially for 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 [25]. Differences in efficiency emphasize fundamental differences in the reproductive biology of species. Hence a high level of technical skills and experience in embryo collection and embryo transfer are critical for efficient transgenic production. This applies for all gene-transfer programs, however.

As mentioned above, the procedures for generating large mammals by DNA microinjection have remained basically unchanged and little improvement in DNA-transfer efficiency has been achieved. After DNA microinjection, embryos are transferred to synchronized foster mothers. Great progress has been made in the field of embryo transfer. For all farm animal species embryo transfer has been facilitated by the development of endoscopy-guided minimally invasive techniques, reducing stress to the foster mothers and maximizing embryo survival and pregnancy rates [26–29]. Methods are currently being developed with the objective of improving the *in-vitro* production (IVP) of embryos [30, 31]. Endoscopic embryo transfer and IVP is also advantageous in the gene transfer methods discussed next.

1.2.2 Retroviral Vectors

The first germ-line transgenic mice were produced by retroviral infection of early embryos [32]. Retroviruses can be considered natural vehicles for gene delivery to mammalian cells. Endogenous retroviruses (ERVs) are a subset of retro-elements 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. Until recently, retroviral vectors were not considered for farm-animal transgenesis, because of 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 embryos 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. They achieved a remarkably high rate of transgenesis 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). Gene constructs, however, grow larger and larger to omit variegated transgene expression (see above). A second problem with many retroviral vector-mediated transgenesis relates to transcriptional shutdown of the transgenes [37]. Lentiviral vectors are a new generation of retroviral vectors which, in contrast with standard oncoretrovirus-based vectors, are reported to escape transcriptional silencing. In addition, lentiviruses are able to infect both dividing and

nondividing cells (reviews are available elsewhere [38, 39]). Germ-line transmission and expression of transgenes delivered by lentiviral vectors to one-cell mouse embryos has been reported [40]. Subsequently lentiviral gene transfer has been successfully used in swine [38, 39] and cattle [41]. 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. In the same way as for gene transfer by pronuclear injection, retrovirus-mediated gene transfer can only be used for additive gene transfer and also bears the danger of insertional mutagenesis. Analysis of transgene expression in lentiviral transgenic pigs revealed that, in contrast with early reports, some transgenics showed epigenetic silencing [42].

1.2.3
Pluripotent Stem-cell Technology

Pluripotent stem cells are capable of developing into many cell types, including germ cells, on fusion with pro-implantation embryos (morulae, blastococysts). Pluripotent stem cells can be maintained in tissue culture and genetically manipulated and selected *in vitro* before reconstitution of the embryo. With mice, handling of pluripotent cells has become a routine method for targeted modification of the genome by homologous recombination, i.e. deletive or replacement gene transfer [43]. Numerous efforts to establish pluripotent stem cells in species other than mice have so far failed. Possible reasons for this are discussed elsewhere (Refs [44, 45] and references cited therein). Since nuclear transfer using transgenic donor cells (see below) became an attractive alternative tool for targeted gene transfer, efforts to establish germ-line-competent stem cells from farm animals have been reduced.

1.2.4
Nuclear Transfer Using Transgenic Cells

Nuclear transfer technology – also known as cloning – comprises 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 [46]. In breakthrough experiments with sheep it was demonstrated that *in-vitro*-cultured differentiated fetal cells and even cells derived from adult tissues could serve as nuclear donor for the reconstitution of enucleated oocytes [47, 48]. Cloning by nuclear transfer has subsequently been achieved in cattle [49–51], goats [52], pigs [53–55], rabbits [56], mules [57], horses [58], cats [59], dogs [60], and some wildlife species (a review is available elsewhere [61]). For farm animal transgenesis a novel tool has become available in that cultured cells can be genetically modified by conventional transfection methods before 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 [62, 63]. Transgenesis by nuclear transfer of geneti-

cally modified cells has several advantages over the other additive gene transfer techniques:

1. mosaicism is avoided and germ-line transmission is guaranteed, because all cells of the cloned animal contain the transgene;
2. insertional mutagenesis and chromosomal positioning effects can be avoided, because integration and, eventually, transgene expression can be monitored *in vitro*; and
3. the use of male or female cell lines determines the gender of the transgenic animal.

Most importantly, gene transfer by nuclear transfer provides a means of gene targeting in farm-animal species [64, 65]. Both the targeted disruption of genes by homologous recombination (deletive gene transfer, knock-out) in sheep and pigs [66–68] and the targeted integration of a gene of interest into a given locus (replacement gene transfer, knock-in) [69] have been reported. The sequential targeting of both alleles of two genes has been achieved in cattle [70].

Despite these impressive reports and the intriguing advantages of the nuclear transfer technique for generation of transgenic farm animals, widespread use is not an easy task because:

1. the primary fibroblasts currently used for gene transfer have limited capacity to divide;
2. homologous recombination is less frequent in somatic cells than in pluripotent stem cells; and
3. cloning by nuclear transfer has a low yield which is still diminished when nuclear donor cells are previously cultured [61].

There is also an ongoing debate about whether it is possible to overcome abnormalities observed in cloned animal [71, 72]. The abnormalities are not restricted to transgenic cloned animals, suggesting they originate from the nuclear transfer procedure or the *in vitro* culture conditions. Although healthy clones have been reported [73], improvement of the technology and further investigation of the effects of cloning are required [74].

1.3 Gene Transfer in Poultry

Depending on the developmental stage a variety of strategies are used for 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. In the same way as in mammals, the first method developed to transfer genes into birds was microinjection of DNA into the germinal disk of fertilized ova [75]. Although successful germ line transmission has been reported [76], the method is labor-intensive, ineffective, and frequently results in mosaicism. Retroviral vectors can introduce transgenes into the genome with low but acceptable efficiency. The first transgenic birds were produced using replication-competent vectors and thus could not be used for broad appli-

cation [77]. The development of replication-defective vectors led to wide use of this technique for production of transgenic birds and stable transgene expression [78, 79]. As an alternative, chimeras bearing transfected pluripotent cells originating from the blastoderm, from PGCs, or from ES cells have been reported (reviews are available elsewhere [80–82]).

1.4 Gene Transfer in Fish

The techniques for gene transfer into fish have focused on direct transfer of DNA into gametes or fertilized eggs and include DNA microinjection, electroporation, retroviral vector infection, and biolistic methods (Ref. [83] and references cited therein). Stem-cell-based technology is not available for farmed fish. The making of transgenic fish is different from gene transfer in mammals or birds because:

1. fish usually undergo external fertilization and no culture or transfer of eggs into recipient females is required;
2. the eggs of many fish have a tough chorion requiring special methods for delivering the gene constructs; and
3. DNA delivery, including by microinjection, is usually into the cytoplasm.

Probably because of the cytoplasmic nature of DNA delivery, many founder transgenic fish are mosaic. Germ line mosaicism seems also to occur because frequencies of transgene transmission to F1 are clearly less than at Mendelian ratios. Transmission of the transgenes to later progeny occurs at Mendelian frequencies, indicating stable integration of the transgenes. A variety of inducible and targeted transgene strategies developed for mammals are now available to be tested and explored in fish (a review is available elsewhere [84]).

1.5 Transgenes – Gene Constructs

The exogenous DNA integrated into the host genome usually is referred to as the gene construct or transgene. The different transgenic sequences used for the different gene-delivery methods and gene-transfer programs are summarized in Table 1.1.

For additive gene transfer experiments the gene construct encompasses the elements controlling gene expression (5′ promoter region, 3′ control regions) and the sequences (cDNA, genomic DNA) encoding the transgene product. For DNA microinjection or sperm-mediated gene transfer the prokaryotic cloning vector sequences are removed from the gene construct. Prokaryotic sequences – 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 have retroviral and transposon sequences [85, 86].

Tab. 1.1 Characteristics of the 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 the transgene	Example (see below)
Gain of function	Pronuclear DNA micro-injection; sperm-mediated; physical and/or 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
	Artificial mammalian 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*	See above	See above
Loss of function	Cloning by transfer of genetically modified nuclei	Selectable marker genes*	Deleted or non-sense-mutated species-specific sequences	Generation of PrPc gene-deficient ruminants
Exchange of function	Cloning by transfer of genetically modified nuclei	Selectable marker genes*	• Introduction of novel allelic variants • exchange of coding sequences in a transcription unit	• Targeted alteration of milk protein genes • replacement of genes
Reduction of function by RNAi	Pronuclear DNA micro-injection; sperm-mediated; physical and/or chemical methods; retroviral vectors	RNAi expression construct	RNAi expression construct gene(s)	Specific "knock-down" of target

* Unwanted sequences may be removed *in vitro* by site-specific recombinases

As mentioned above, one obvious way of avoiding transgene silencing or chromosomal positioning effects would be the use of large gene constructs and the abandoning of viral vectors. An increasing number of transgenic animals therefore carry gene constructs based on phage (PAC), bacterial (BAC), or yeast (YAC) artificial chromosomes [22]. For expression and replication, these large transgenes depend on integration into the host genome. In contrast, mammalian artificial chromosomes (MACs) provide independent transcription and replication units. Work originating mainly from human gene therapy programs has resulted in the development of human artificial chromosomes based on episomal viral vectors or engineered minimal chromosomal elements [87, 88]. MACs were recently used to generate "transchromosomic" cattle; this was achieved by introducing human artificial chromosomes *in vitro* into bovine cells which were subsequently used for cloning by nucleus transfer [89, 90].

Alternatively, gene transfer *in vitro* then reconstitution of embryos by nuclear transfer or stem-cell technology enables 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 the identification of the genetically modified cells. 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 a topic of concern about biosafety [91]. Analogous a genetically modified farm animal going on the market should be free from such genes. This can be achieved by use of site-specific recombinases to remove undesirable sequences after successful identification of the transgenic cells [92, 93]. Additional methods for site-directed genome modification are reviewed elsewhere [94, 95]. It must, however, be mentioned that these additional genetic engineering steps have not yet been conducted in farm animals.

RNA interference (RNAi) is the process in which dsRNA leads to gene silencing, by either inducing the sequence-specific degradation of complementary mRNA or inhibiting translation. RNAi has been very successfully applied as gene-silencing technology in both plants and invertebrates, but many practical obstacles need to be overcome before it becomes a versatile tool in mammalian cells. Greater specificity and efficiency of RNAi in mammals is being achieved by improving the design and selection of small interfering RNAs (siRNAs), by increasing the efficacy of their delivery to cells and organisms, and by engineering their conditional expression (reviews are available elsewhere [4, 96, 97]).

The power of this technology in transgenic animals has been demonstrated by production of mice constitutively expressing RNAi directed against a given transcript which show a similar phenotype as mice with homologous targeted disruption of the locus [98].

Retroviral vector-mediated gene transfer methods have 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, because of the presence of the viral sequences.