GENETIC ENGINEERING OF FARM ANIMALS: FROM TRANSGENESIS TO GENE MAPPING, WHAT DOES THE FUTURE HOLD?

GENETSKI INŽENJERING DOMAČIH ŻIVOTINJA: OD TRANSGENEZE DO MAPIRANJA GENA, ŠTO NOSI BUDUĆNOST?

C. A. Pinkert, D. L. Kooymans, M. J. Martin

Professional paper - Stručni članak
UDD: 036.2.3.4.536.05
Received - Primljen: 20. may - Svibanj 2002.

SUMMARY

Our ability to manipulate the genome of whole animals has influenced the sciences in a most dramatic fashion. In less than 15 years, manipulations of the genetic composition of transgenic animals have allowed researchers to address fundamental questions in fields ranging from production agriculture to biomedical research. In a host of transgenic animal models, basic research into the regulation and function of specific genes forged the way to in vivo genetic modifications that resulted in either the gain-of-function of a transferred gene or the ablation of an endogenous gene product. Pioneering efforts in transgenic animal technology have markedly influenced our appreciation of the factors that govern gene regulation and expression, and have contributed significantly to our understanding of the genetic bases of reproduction and development.

I. INTRODUCTION

The molecular biology and genetics of animal production have experienced a tremendous growth and diversification over the last 25 years. At times, it is difficult to distinguish if this is a result of new technologies or if new technologies were developed along the way. One need only look at the human genome project as an example of how technology and basic research have worked hand in hand to revolutionize biology. As is often the case, new technology is prohibitively expensive except for a select group of research entities. However, eventually the scientific community embraces the technology as utility and cost make it uniformly available.

A. Development of transgenic animals. The ability to introduce functional genes into animals provides a very powerful tool for dissecting complex biological processes and systems. Transgenic animals represent unique models that are custom tailored to address specific biological questions. Furthermore, classical genetic selection cannot engineer a specific genetic trait in a directed fashion. Thus, gene transfer in farm animals can surpass classical breeding practices where long life cycles slow the rate of genetic improvement.

Although the entire procedure for microinjection into living cells was described in the late 1960s, it took more than a decade before transgenic animals were actually created. Following the description of a microinjection process by T. P. Lin in 1966, the first

Carl A. Pinkert, University of Rochester Medical Center Rochester, New York; David L. Kooymans, Brigham Young University Provo, Utah; Michael J. Martin, Nextran Princeton, New Jersey USA.
technological shift toward production of a transgenic vertebrate occurred in 1977, when Gurdon transferred mRNA and DNA into Xenopus embryos and observed that the transferred nucleic acids could function. Then, in 1980, Brinster and his colleagues reported on similar studies in the mouse. They found that an appropriate translational product was produced following transfer of a specific messenger RNA (mRNA) into embryos. Sequentially, these studies laid the groundwork for the development of the first transgenic mammals. From late 1980 through 1981, six research groups reported success at gene transfer and the development of transgenic mice. In gene transfer, animals harboring new genes (foreign DNA sequences integrated into their genome) are referred to as transgenic - a term first coined by Gordon and Ruddle in 1981. As such, transgenic animals are recognized as specific strains or even species variants, following the introduction and integration of new gene(s), or transgenes, into their genome. More recently, the term transgenic has been extended to chimeric or knock-out mice in which gene(s) have been selectively removed from the host genome.

Production of transgenic mice marked the convergence of previous advances in the areas of recombinant DNA technology and the manipulation and culture of animal germplasm. Transgenic mice provide powerful models to explore the regulation of gene expression as well as the regulation of cellular and physiological processes. Experimental designs have taken advantage of our ability to direct specific (e.g., cell-, tissue-, organ-specificity) as well as ubiquitous (whole-body) expression in vivo. From embryology to virology, transgenic technology provides unique animal models for studies in various disciplines that would otherwise be all but impossible to develop spontaneously (see Hogan et al., 1994; Pinkert, 1994, 2002; Monastersky & Robl, 1995; Houbenbine, 1997; Pinkert et al., 1997b; Pinkert & Murray, 1998).

Some key terms at this juncture, will help in understanding some of the underlying technologies associated with genetic engineering efforts. DNA MICROINJECTION is a gene transfer technique where DNA constructs (transgenes) are directly injected (or microinjected) into pronuclei or nuclei of fertilized ova. DNA microinjection is the most commonly used gene transfer technique for creating transgenic mammals. In contrast, EMBRYONIC STEM (ES) CELL TRANSFER involves the transfer of pluripotent embryonic stem cells into a developing embryo. GENE TRANSFER can be conducted as one of a set of techniques directed toward manipulating biological function via the introduction of foreign DNA sequences (genes) into living cells. Today, a TRANSGENIC ANIMAL can be an animal either integrating foreign DNA segments into its genome following gene transfer, or resulting from the molecular manipulation of endogenous genomic DNA. A TRANSGENIC LINE is a direct familial lineage derived from one or more transgenic founders, characterized by the passing of the transgene(s) to successive generations as a stable genetic element. The line includes the founder and any subsequent offspring inheriting the specific germ-line manipulation. Lastly, in relation to mitochondrial genetics, mitochondria are found in the cytoplasm of eukaryotic cells and serve as centers of intracellular enzyme activity, producing the energy needed for cellular metabolism. In turn, HETEROPLASM refers to the coexistence of more than one form of mitochondria DNA (e.g., two or more mitochondrial genomes present) within a single cell or within cells that comprise an individual organism.

B. Applications Of Transgenic Animals. A number of methods exist for gene transfer in mammalian species (Table 1). Transgenic technology was reported in a variety of animal species including mice, rats, rabbits, swine, ruminants (including sheep, goats and cattle), poultry and fish. With advances in the characterization of factors that control gene expression (including promoter-enhancer elements and transcription-regulatory proteins), gene transfer technology has become a proven asset as a means of dissecting gene regulation and developmental pathways in vivo.

Normally, gene function is influenced by cis-acting elements and trans-acting factors. For transferred genes, the cis- and trans-activators in conjunction with the gene integration/insertion event within the host genome influence gene function. Using genes that code for reporter proteins (e.g., oncogene, lac Z/βgal or fluorescent protein gene, or GH gene constructs), analysis of
transgenic animals has revealed the importance of such factors in determining developmental timing, tissue distribution, and relative efficiency of gene expression. Additionally, transgenic animals have also proven quite useful in determining in vivo artifacts of other model systems or techniques.

**Table 1. Gene Transfer Methodologies.** Mouse modeling techniques evolved from procedures for non-specific (whole genome) transfer to the transfer of discreet genes and the modification of endogenous genes.

<table>
<thead>
<tr>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastomere/embryo aggregation</td>
</tr>
<tr>
<td>Teratocarcinoma cell transfer</td>
</tr>
<tr>
<td>Retroviral infection</td>
</tr>
<tr>
<td>DNA microinjection</td>
</tr>
<tr>
<td>Electrofusion</td>
</tr>
<tr>
<td>Nuclear transplantation</td>
</tr>
<tr>
<td>Embryonic stem (ES) cell transfer</td>
</tr>
<tr>
<td>Spermatozoa- and spermatogonia cell-mediated transfer</td>
</tr>
<tr>
<td>Particle bombardment and jet injection</td>
</tr>
</tbody>
</table>

There are a number of strategies in the development of transgenic mouse models, including systems designed to study: dominant gene expression, homologous recombination/gene targeting and the use of ES cells, efficiency of transformation of eggs or cells, disruption of gene expression by antisense transgene constructs, gene ablation or knockout models, reporter genes, and marking genes for identification of developmental lineages.

II. PRODUCTION OF TRANSGENIC ANIMALS

A. The mouse and other laboratory animal models. The relative importance of using particular strains or breeds of animals in gene transfer experimentation will vary dramatically according to the species under consideration. Probably the most complex system is encountered in the production of transgenic mice, simply because so much work has been done with this species. Well-documented differences in reproductive productivity, behavior, related husbandry requirements, and responses to various experimental procedures influence the efficiency and degree of effort associated with production of transgenic founder animals. A general discussion of these factors therefore serves as an appropriate starting point for understanding the many processes and procedures that must be evaluated and monitored when considering production of transgenic animals.

DNA microinjection, prior to nuclear transfer in domestic animals, was the most direct and reproducible method for producing transgenic animals (Fig. 1). Beyond the mouse model, other laboratory animal species may be necessary to study a particular biological phenomenon. The significance and critical importance of optimized protocols cannot be underestimated. In any given species, selection and management of donor females that respond well to hormonal synchronization and superovulation, embryo transfer recipients that are able to carry fetuses to term and then care for neonates appropriately, and the effective use of males in a breeding regimen will all add to the relative experimental efficiency that one might encounter. In turn, transgenic animal protocols developed in mice have been modified to accommodate production of other transgenic species.

B. DNA microinjection. DNA microinjection generally involves the use of mechanical or hydraulic micromanipulator systems to physically inject the DNA construct solution into embryos (Polites and Pinkert, 2002). Virtually any cloned DNA construct can be used. With few exceptions, microinjected gene constructs integrate randomly throughout the host's genome, but usually only in a single chromosomal location (the "integration site"). This fact can be exploited to simultaneously co-inject more than one DNA construct into a zygote; where the constructs co-integrate together at a single, randomly located, integration site.
The integration process itself is poorly understood, but it is apparently does not involve homologous recombination. During integration, a single copy or multiple copies of a transgene (actually as many as a few hundred copies of the particular sequence) are incorporated into the genomic DNA, predominantly as a number of copies in head-to-tail concatemers. Regulatory elements in the host DNA near the site of integration, and the general availability of this region for transcription, appear to play major roles in affecting the level of transgene expression. This "positional effect" is presumed to explain why the levels of expression of the same transgene may vary dramatically between individual founder animals as well as their offspring. It is therefore prudent to examine transgene expression in offspring from at least three or four founder animals in order to determine what might be a result of the integration location, and what might reflect the activity of the transgene.

Host DNA near the site of integration frequently undergoes various forms of sequence duplication, deletion, or rearrangement as a result of transgene incorporation. Such alterations, if sufficiently drastic, may disrupt the function of normally active host genes at the integration site and constitute insertional mutagenesis, wherein an aberrant phenotype may result. Such events are generally not purposefully designed, but have led to the serendipitous discovery of previously unsuspected genes and gene functions. Because DNA microinjection is usually accomplished in unicellular zygotes, transgene incorporation occurs in essentially every cell that contributes to the developing embryo. Incorporation of the transgene into cells that will

**Figure 1. DNA microinjection and ES cell transfer in mice**

**Slika 1. Mikropropunjavanje DNA i transfer ES stanica u miševa**

For DNA microinjection, an in vitro culture step is not required (left). DNA is injected directly into the male pronucleus of a fertilized one-cell embryo (zygote). Generally, if transgenic mice (represented by black mouse) are derived from DNA microinjection, all of their cells contain the new transgene(s). On the right, after clonal selection of transfected ES cells, one of two techniques is employed. ES cells are either injected directly into a host blastocyst or co-cultured with eight-cell to morula stage embryos. With blastocyst injection, transgenic offspring are termed "chimeric", as some of their cells are derived from the host blastocyst and some from the transfected ES cells (denoted by mice with black patches). Using co-culture and tetraploid embryos, one can obtain founder mice derived completely from the transfected ES cells (denoted as solid black mice). (Reprinted with permission, Pinkert et al., 1997b.)
eventually contribute to development of germ cells (sperm or ova) is a common occurrence with this method, and makes heritability of the transgene by offspring of founder animals likely within one generation. In such cases, the transgene has been said to be germ-line or the animals are referred to as germline-competent. However, integration of the microinjected DNA construct into the host’s genome occasionally may be inexplicably delayed. In such a case, if cells of the early embryo (blastomeres) undergo mitosis before the transgene-integration event occurs, some but not all of the cells will contain the transgene, and the founder animal, although still considered to be transgenic, will be classified as a mosaic or chimera.

C. Retrovirus-Mediated Gene Transfer. Transfer of foreign genes into animal genomes has also been accomplished using retroviruses (Chan et al., 1997). Although embryos can be infected with retroviruses up to midgestation, early eggs, from oocytes to 16-cell stage ova, are used for infection with one or more recombinant retroviruses containing a foreign gene. Immediately following infection, the retrovirus produces a DNA copy of its RNA genome using the viral enzyme, reverse transcriptase. Completion of this process requires that the host cell undergoes the S phase of the cell cycle. Therefore, retroviruses effectively transduce only mitotically active cells. Modifications to the retrovirus frequently consist of removal of structural genes, such as gag, pol, and env, which support viral particle formation. Additionally, most retroviruses and complementary lines are ecotropic in that they infect species-specific cell lines, limiting risk to humans in animal experimentation.

D. Embryonic Stem (ES) Cell Technology. Gene transfer has been used to produce both random and targeted insertion or ablation of discrete DNA fragments into the mouse genome (Fig. 1). For targeted insertions, where the integration of foreign genes is based on a recombinational gene insertion with a specific homology to cellular sequences (termed homologous recombination), the efficiency of DNA microinjection is extremely low (Capacchii, 1989). In contrast, the use of ES cell transfer into mouse embryos has been quite effective in allowing an investigator to preselect a specific genetic modification, via homologous recombination, at a precise chromosomal position. This preselection has led to the production of mice that incorporate novel foreign genes into their genome, carry modified endogenous genes, or lack specific endogenous genes following gene deletion or "knock-out" procedures. Technologies involving ES cells, and more recently primordial germ cells, have been used to produce a host of mouse models. Pluripotent ES cells are derived from early pre-implantation embryos and maintained in culture for a sufficient period for one to perform various in vitro manipulations. The cells may then be injected directly into the blastocoeel of a host blastocyst or incubated in association with a zona-free morula. The host embryos are then transferred into intermediate hosts or surrogate females for continued development. The use of ES cells to produce transgenic mice faced a number of procedural obstacles before it became competitive with DNA microinjection as a standard technique in mouse modeling. Within the last few years, the addition of coculture techniques involving tetraploid host embryos (8-cell stage to morulae) has resulted in founders that can be derived completely from the cocultured ES cells (Wood et al, 1993). Hence, the founders are no longer chimeras, as all the cells come from the same progenitor cells and the founder animals will breed true (and faithfully transmit the genetic modification in the first generation offspring).

Yet, while ES cell lines have been identified for species other than the mouse, the production of germline-competent ES cell-derived/chimeric farm animals has not been reported. With the advent of nuclear transfer-related technologies, the need to identify and use ES or primordial germ cells (PGCs) to effect genetic change, has in turn become of lesser consequence.

E. Production of Transgenic Domestic Animals. The success of transgenic mouse experiments led a number of research groups to study the transfer of similar gene constructs into the germ-line of domestic animal species. These efforts have been directed primarily toward three general endpoints: improving the productivity traits of
domestic food animal species, development of transgenic animals for use as bioreactors (i.e., producers of recoverable quantities of medicinally or biologically important proteins), and in transplantation-related modeling efforts. Since 1985, numerous studies have focused on transgenic farm animals created using growth-related gene constructs. Unfortunately, for the most part, ideal growth phenotypes were not achieved because of an inability to coordinately regulate gene expression and the ensuing cascade of endocrine events that unfolded.

Today, DNA microinjection, retroviral transfection and nuclear transfer procedures are the only methods used to successfully produce transgenic livestock (see Fig. 2 and 3). Although involved and at times quite tedious, the steps in the development of transgenic models are relatively straightforward. For either DNA microinjection or nuclear transfer, once a specific fusion gene is cloned and characterized, sufficient quantities are isolated, purified and tested in cell culture. If in vitro mRNA expression of the gene is identified, the appropriate fragment is linearized, purified, and readied for preliminary mammalian gene transfer experiments. In contrast to nuclear transfer studies, DNA microinjection experiments are first performed in the mouse. While the transgenic mouse model will not always identify likely phenotypic expression patterns in domestic animals, we have not observed any gene constructs that would function in a farm animal

Figure 2. Transgenic pig production by DNA microinjection. For microinjection into zygotes (or later-stage ova), visualization of the pronuclei (or nuclei) is necessary. This is accomplished by centrifugation of the ova to stratify the opaque lipids, making pronuclei or nuclei readily visible (reprinted with permission, Pinkert, 1994).

Slika 2. Transgenska proizvodnja svinja mikroinjektacijom DNA. Za mikroinjekciju u z igote (ili kasniji stadij jajašca) potrebna je vizualizacija pronukleusa (ili nukleusa). To se postiže centrifugiranjem jajašca radi stratificiranja / uslojavanja nepropusnih lipida, čime pronukleusi ili nukleusi postaju odmah vidljivi (pretiskano s dopuštenjem Pinkert, 1994.).

Flush fertilized eggs from superovulated donors
Ispрати олуђена яйца из superovuliranih donora

Centrifuge eggs, inject foreign genes into one pronucleus (or both nuclei of 2-cell eggs)
- Центрифугиране яйца, убрзгати две гене у jedan pronukleus (ili oba nukleusa od dvostaničnih jaja)

Transfer injected eggs into oviducts of synchronized recipient sows,
allow sows to farrow
Предијети убрзгана яйца у jajove odхронизираних крачка примателица, пустити да се крначе oпразн

Use slot blot or PCR analysis to identify animals harboring foreign genes
Испотржебити blot или PCR analizu za идентификане животinja koje nose đude гене

- Perform tissue biopsies - analyze foreign DNA integration, mRNA transcription, and protein production
- Провести биопсију ткива - анализирајте интеграцију тушних DNA, транскрипцију mRNA и произвођњу бијелачевина.

- Establish transgenic lines to study gene regulation in progeny - Uspostaviti transgenske linije radi прoučavanja regulacije гена у потомству

Figure 2.
when there had been no evidence of transgene-encoded expression in a pilot mouse model.

Five years ago, the successful cloning of a sheep, followed by the use of nuclear transfer to produce transgenic sheep and cattle, captured the imagination of researchers around the world (Wilmut et al., 1997). Within the next few years, these and subsequent technological breakthroughs should play a significant role in the development of new procedures for genetic engineering in a number of mammalian species. It should be noted that nuclear transfer, with nuclei obtained from either mammalian stem cells or differentiated adult cells, is an especially important development in species beyond the mouse model. This is because a technological barrier was surpassed that allowed for specific in vitro manipulations that lead to targeted genetic modifications in first generation (G0) animals. Previously, it was not possible to produce germline-competent transgenics in mammalian species (other than in mice), using any technique other than DNA microinjection (that only allowed for random and imprecise integration of transgenes in founder animals). Unfortunately, relative efficiencies for nuclear transfer experimentation still pale in comparison to conventional DNA microinjection. However, while nuclear transfer might be considered inefficient in its current form, major strides in enhancing experimental protocols within the next few years are envisioned, comparable perhaps to the early advances in DNA microinjection technology.

**E.1. Pig Models.** In contrast to gene transfer in mice, the efficiency associated with the production of transgenic livestock, including swine, is quite low (Martin and Pinkert, 2002). However, two advantages offered by swine over other domestic species include a favorable response to hormonal superovulation protocols (20-30 ova can be collected on average) and as a polytocous species, they have a uterine capacity to nurture more offspring to term.

An initial problem encountered during the creation of transgenic farm animal species concerned the visualization of the pronuclei or nuclei within the ova. As swine ova are lipid-dense, the cytoplasm is opaque and the nuclear structures are not discernable without some type of manipulation. Fortuitously, centrifugation of pig ova resulted in stratification of the cytoplasm rendering pronuclei and nuclei visible under the microscope.

**Figure 3. Cloning by nuclear transfer in sheep**

*Slika 3. Kloniranje nuklearnim transferom u ovaca*

Cells from blastocysts (e.g., inner cell mass cells) or other somatic tissues are obtained and propagated in culture. These cells are used as nucleus donors for transfer into enucleated oocytes. In contrast to DNA microinjection, a fusion step is needed to fuse the transferred nuclei and enucleated oocytes. Here, electrofusion is used to fuse couplets (transferred nucleus + oocyte) that are transferred to recipients for the remainder of gestation. Liveborn offspring are then evaluated for the genetic modification, (reprinted with permission, Pinkert, 2000).

---

Krmiva 45 (2003), Zagreb, 1; 47-62

53
developmental stage of ova injected, the duration of in vitro culture, synchrony of ova donors and recipients, the number of ova transferred, and donor age. Other factors, which have been shown to influence transgenic mouse production, including technician proficiency and embryo handling/transfer, pipette dimensions, DNA preparation, and the viability of manipulated ova - are all factors that readily influence transgenic production efficiency for all other species.

Following DNA microinjection, surgical embryo transfer is necessary. However, the mechanical insult to the embryos is severe and only 15-25% of them will still be viable 5 days after transfer. Therefore, 30 to 50 microinjected embryos are routinely transferred per recipient sow with the expectation that 50% of the recipient females will maintain pregnancy. While the number of embryos transferred may seem excessive, the basis is derived from classical studies that establish a requirement of 4 viable embryos at the time of implantation for a sow to initiate and maintain a successful pregnancy.

For studies targeting pigs, the use of outbred domestic pigs is the most practical way to produce transgenic founders. However, miniature or laboratory swine are now used with increasing frequency in biomedical research, where their well-characterized background genetics make them more suitable for human modeling studies (e.g., xenotransplantation research). Reproductive efficiency in miniature swine is low compared to commercial swine and is characterized by a low ovulation rate, low birth weight and small litter size. Average litter size is between 4 and 7 pigs at birth with each breeding sow producing 12 to 18 pigs per year. Estrous cycles and gestation length are similar to standard commercial swine, however sexual maturity in males and females occurs between 4 and 6 months of age in some breeds, which is sooner than observed in commercial swine.

E.2. Ruminant Models. In contrast to swine modeling, the relative experimental efficiencies associated with the production of transgenic ruminants (goats, sheep and cattle) are even lower (Rexroad and Hawk, 1994; Niemann et al., 2002).

While the different techniques from DNA microinjection to nuclear transfer require a large number of embryos to ensure success, other factors play significant roles in experimental yields and success. Such factors include the low rate of embryo survival following manipulation, uterine capacity (generally these species are monochorous - one to at most three offspring would be feasible), long generation interval, and relative cost of hormonal induction/ova recovery/ova transfer and animal maintenance, all negatively affecting experimental costs and efficiencies. In many laboratories, in vitro maturation (IVM), in vitro fertilization (IVF) and culture of ova (in surrogate hosts or incubators, although culture conditions are not optimal for embryo survival at this time) prior to final transfer aid in maximizing resources for production of genetically engineered ruminants. Many of these steps are timely, as the ability to biopsy individual blastomeres for analysis of specific genetic modifications can minimize the number of animals used and maintained in these studies.

For cattle, ova can be collected surgically (by laparoscopy or laparotomy, either by flank incision or transvaginally, and with or without the aid of ultrasonography) or from ovaries at necropsy. The most common procedure at this time is to collect large numbers of ova from slaughterhouse ovaries and subject them to IVM/IVF. If slaughterhouse ovaries are not available, standard superovulatory regimens using various gonadotropins can be employed to obtain oocytes, zygotes, or later stage ova (Rexroad and Hawk, 1994; Niemann et al., 2002). For sheep and goat studies, reproductive cycles and seasonality can limit the use of either males or females, and the selection of specific breeds to establish appropriate modeling should be carefully evaluated. Most sheep and goats are seasonally polyestrous, although there are breeds that can reproduce throughout the year, if photoperiod and temperature are controlled.

While sheep and goat ova are transferred surgically into the oviduct, cattle ova can be transferred surgically, or after culture to the morula or blastocyst stage, they can be transferred transvaginally or transcervically using established embryo transfer protocols. Lastly, with the rather long gestation lengths in ruminants, if blastomere
biopsy is not used prior to embryo transfer, then a number of techniques may help decrease the long experimental time lines. Early pregnancy diagnosis following implantation is routine today, and can be determined by onset of behavioral estrus, ultrasound, hormonal profiles (progesterone or placental hormones), or in cattle by palpation. In addition to pregnancy diagnosis, analysis of the specific genetic modification can be performed using a number of fetal biopsy techniques as well as determining if a transgene-encoded product is present (if appropriate to the particular modification).

III. EVOLVING TECHNOLOGIES

A. From Spermatozoa to Cloning. In contrast to progress in embryo manipulation, a completely different tact was taken with the advent of sperm-related transfer procedures. In 1989, sperm-mediated gene transfer was reported but hotly disputed when many laboratories around the world were unable to duplicate the outlined procedures. Yet, by 1994, the sperm-mediated story generated interest that resulted in the development of spermatogonial cell transplantation procedures as feasible alternatives for in vivo gene transfer (Brinster and Avarbock, 1994; Nagano et al., 2001). However, whole animal and somatic-cell techniques (including liposome-mediated gene transfer, particle bombardment, and jet injection), coupled with novel vector systems, will continue to evolve in order to genetically engineer animals in an efficient and effective manner.

B. From the Nucleus to the Mitochondrion. Until recently, in vivo mitochondrial transfer remained a technological hurdle in the development of mitochondrial-based gene transfer and therapies. In domestic animals, various production traits have been associated with specific mitochondrial populations. Cytoplasmic-based traits in domestic animals have included growth, reproduction and lactation. In addition, mitochondrial restriction fragment length polymorphisms (RFLPs) were identified and associated with specific lactational characteristics in a number of dairy cattle lineages (Brown et al., 1989; Koehler et al., 1991). In humans, metabolic and cellular pathologies exist due to mutations arising exclusively within the mitochondrial genome. Various diseases have been associated with mtDNA point mutations, deletions and duplications as well age-associated changes in the functional integrity of mitochondria. Therefore, for both agricultural and biomedical research efforts, the ability to manipulate the mitochondrial genome and to regulate the expression of mitochondrial genes would provide one possible mode of genetic manipulation and therapy.

We and others have initiated studies revolving around mitochondrial transfer and techniques to produce animals harboring foreign mitochondrial genomes (Pinkert et al., 1997a; Irwin et al., 1999, 2001; Levy et al., 1999; Marchington et al., 1999; Inoue et al., 1999; Sligh et al., 2000). The creation of heteroplasmic transgenic mitochondrial mice represents a new model system that will provide a greater understanding of mitochondrial dynamics, leading to the development of genetically engineered production animals, and therapeutic strategies for human metabolic diseases affected by aberrations in mitochondrial function.

Mammalian mitochondria contain between one or more copies of a double-stranded DNA that is bound to the inner mitochondrial membrane and is not associated with histones. The mtDNAs of all vertebrates are highly conserved and relatively small in comparison to the nuclear genome; the mitochondrial genome is approximately 16.5 kb in length.

Mitochondrial genes encode 37 genes including those responsible for 13 of the protein subunits that function in the mitochondrial oxidative-phosphorylation system, along with two ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs).

To make a transgenic mitochondrial animal model, the ability to manipulate normal and mutant mitochondria in vivo has been a critical and difficult first step. In vivo mitochondria modification remains a technological hurdle in the development of mitochondria-based gene transfer and genetic therapies and in the generation of experimental animal models for the study of mitochondrial dynamics and mitochondria-based traits. While transgenesis has been performed in a host of cell types and organisms, transfer of nuclear DNA until
recently has been the only demonstrable form of mammalian gene transfer to date. Initially, these models will serve to explore mitochondrial dynamics in an in vivo system. As such, pilot transmitter mitochondrial mouse models will be used to explore the role of the mitochondrial genome in other species.

To date, we have developed a method for mitochondria isolation and interspecific transfer of mitochondria in mice. Electron microscopic observations of the mitochondria preparations used for microinjection demonstrated intact mitochondrial vesicles with little microsomal contamination. Current studies to establish and further characterize our mitochondrial transfer-derived models, and follow-up studies in collaboration with Dr. Ian Trounce at the Genomic Disorders Research Center at the University of Melbourne will be discussed. The creation of these transmitter mitochondrial models is a first step in developing a greater understanding of mitochondrial dynamics, supporting advancement of technologies for genetically engineered production animals and therapeutic strategies for human metabolic diseases affected by aberrations in mitochondrial function.

C. Microarray Technology to Proteomics.
Another tool in establishing functional genomics technology includes microarray analysis (Fig. 4). Microarray analysis is essentially a method whereby the relative abundance of specific DNA or mRNA can be assessed within any given system (plant, animal, or microbe). A survey of relative DNA or mRNA levels for specific genes can be a powerful tool for helping to understand the significance of specific genes in terms of spatial and temporal importance. Indeed, a careful survey of mRNA expression patterns can provide powerful evidence of specific cell-type, tissue, or gene function. Additionally, microarray technology has a variety of uses. For instance, Ooi et al. (2001), described a microarray-based screen of yeast mutants. They used oligonucleotide arrays that hybridized to 20-nucleotide “barcodes” to screen for components of the nonhomologous end-joining (NHEJ) pathway. In other work, Zammatteo et al. (2002), designed a cDNA microarray system that they used to discriminate between 12 specific genes involved in anti-tumor immunotherapy. As such, microarray technology provides many uses in a variety of systems.

Figure 4. Microarray technology
Slika 4. Tehnologija mikroreda

Until recently, gene discovery was performed studying individual genes, one gene at a time. With microarray technology, standard molecular biological principals could be employed on an industrial scale, allowing one to generate fully quantitative and qualitative gene expression profiles for thousands of genes simultaneously. Gene expression profiles could then be used to decipher molecular mechanisms that underlie targeted biological perturbations or comparative development in various biological systems (figure courtesy of A.I. Brooks and the URMC Functional Genomics Center, 2002).

Microarray Technology and Data Acquisition - Tehnologija mikroreda i dobivanje podataka
A microarray experiment is generally divided into four basic protocols. For the purposes of this example we will describe a cDNA array experiment. First, a quantity of known cDNA originating in the representative tissue or genes that will be surveyed is generated. This is the base line from which relative expression will be assessed. Second, the cDNAs, sometimes referred to as expressed sequence tags (ESTs) are bound to a poly-L-lysine coated glass slide. Multiple slides representing multiple/known RNAs can be generated. Third, RNA is extracted from the microbe or tissue of interest and a cDNA library is made in which one of the nucleotides used in the reverse transcription step is labeled (generally with a fluorescent label). This library of labeled cDNAs is the test RNA pool that will be analyzed against the gene-specific slide previously prepared. In other words, the labeled cDNA library will be hybridized to the gene-specific cDNAs captured on the glass slide. Fourth, after the labeled cDNAs are hybridized to the captured ESTs, the relative fluorescence is detected and quantified. The significance of these data can be examined in the context of gene product and complex expression patterns. Hence, it is difficult to talk about microarrays without at least mentioning proteomics and bioinformatics.

The growth and diversification of molecular biology has also been the fodder for development of new fields such as proteomics and bioinformatics. As new technology becomes available and the price of that technology falls within the reason of many research laboratories the field of proteomics has exploded. Proteomics is the study of proteins and protein interactions to understand cell metabolism and behavior. More and more proteomic databases are becoming available to researchers today (see Hodges et al., 2002). It also covers the production of protein starting from DNA. Proteomics encompasses the study of all processes that involve proteins. The capability to study proteins in mass and in highly specific ways has revolutionized our ability to understand their roles (see Poon and Johnson, 2001). For example, modern proteomics technology can be used to determine all of the post-translational modifications that proteins undergo and potentially determine what differentiates a normal from a mutant protein resulting in an effect on a cell or organism. One recent paper illustrated the use of powerful proteome analysis techniques to identify pathogenicity factors in bacteria and potential vaccine candidates (Jungblut, 2001). In one sense proteomics goes hand in hand with microarray technology. The high throughput characterization of gene expression patterns along with the capability to closely examine the products of gene expression has been a successful marriage resulting in greatly enhanced understanding of cell/organism metabolism and behavior.

Of course, the keystone to these technologies is in relation to bioinformatics (see Demirev et al., 2001). Without the capability to amass significant amounts of data in performing experiments and analyzing the subsequent outcomes, these powerful technologies would be of little use. The capability to store and to use a genomic or proteomic database relies on bioinformatics infrastructure. In addition, the analysis and interpretation of data generated by new technologies are absolutely dependent on computer-generated algorithms. Such revolutionary enhancements are affecting and changing every field of biology and will surely have a profound impact on production agriculture in the near-term.

D. Experimental Considerations and Genome Mapping. Using DNA microinjection, the types of genes introduced into livestock species become important considerations. Pursel and Rexroad (1983) provided a comprehensive list of gene constructs used in the production of transgenic cattle, goats, pigs, and sheep that has not materially changed - a reflection of the enormous resources necessary for such endeavors. The major scientific limitations to the wide scale application of transgenic technology to improve farm animals have not basically changed over the last decade. Those limitations included a lack of knowledge concerning the genetic basis of factors limiting production traits, lack of temporal- and spatially-controllable or inducible sequences for use in developing gene constructs, expression vectors, and in gene targeting, a need to increase efficiency of transgenic animal production. Gene mapping of animals probably originated with the development by Sturtevant (1913) of a recombination map of the Drosophila X chromosome. Since then, concerted efforts have led
to the mapping of over 20,000 genes and markers in humans and greater than 15,000 in the mouse. Mapping of livestock genomes, however, currently lags far behind that of the human and mouse. As of 1998, the physical genetic map of the pig consisted of only 600 genes and markers while the linkage map contained over 1800 loci and 250 genes (Rothschild, 1998). Similar situations exist with regard to bovine (only about 200 genes are on the ordered genetic map; Ozawa et al., 2000) and ovine genomes (at least 477 loci have been mapped; Crawford et al., 1995).

The creation of detailed genomic maps for various livestock species has at least three important applications (Womack, 1996). The first of these entails the use of mapping data to increase our knowledge and understanding of mammalian evolution. As noted by Womack, mapping homologous genes in multiple species provides more information about chromosomal evolution than we can retrieve with today's best cytogenetic technologies. The use of comparative mapping between the bovine and hamster to demonstrate that human chromosome 21 evolved from a larger ancestral mammalian chromosome is but one example of livestock mapping's utility as an evolutionary tool (Threadgill et al., 1991). A second application involves the identification of DNA markers of alleles positive for various economic traits loci (ETL). These loci include quantitative trait loci (QTL), infectious disease loci (IDL) and other loci. Of greater importance is the use of mapping to identify markers or "candidate genes" that are directly responsible for the expression of a phenotypic trait. Knowledge of these markers and genes will allow one to perform marker-assisted selection for various phenotypic traits. This approach will maximize the efficiency of selective breeding. A third application of livestock mapping entails the identification, sequencing and cloning of genes that are directly responsible for ETL.

Of the applications cited above, the identification of markers for ETL and the cloning of genes responsible for ETL represent the greatest potential for economic benefit to agriculture. Great strides have been made in both areas especially with regard to the discovery of QTL. A partial list of QTL, IDL, candidate and major genes (individual genes that have a measurable effect on phenotype) that have been identified and/or located in livestock species is presented in Table 2.

Many of these QTL and candidate genes are being utilized in the agricultural industry today. One example is the estrogen receptor (ESR) gene. The B allele of this gene that occurs in Chinese pigs and Large White and Yorkshire breeds has been shown to have a significant effect on litter size (Rothschild et al., 1996). Subsequent analysis of 9015 litter records from 4264 Large White commercial sows genotyped at the ESR locus found that both the total number of pigs born (TNB) and number born alive (NBA) increased per favorable allele. More specifically, the additive effect per allele for both TNB and NBA was, on average, 0.4 pigs/litter in the first parity and 0.3 pigs/litter for subsequent parities (Short et al., 1997). When the study's authors examined the economic impact of fixing this allele in a 1000 sow herd, the results were surprising. Assuming an initial ESR B allele frequency of 0.25 in parent females, an additive effect of 0.4 pigs/litter per copy of the B allele, the production of 2.3 litters/sow/year and a marginal economic value of $30 per additional pig, the authors projected an economic benefit of $20,700 per year or over $20/sow/year.

Future discovery of economically important QTL such as ESR will depend upon our ability to create detailed comparative maps for the livestock species. This objective can be met by using one of several established approaches, such as radiation hybrid panel (RH) analysis and linkage mapping, or a relatively new high throughput strategy termed "comparative mapping by annotation and sequence similarity" or COMPASS. COMPASS is an extremely powerful tool for it allows one to map in silico the location of a randomly chosen sequence of DNA using human mapping data (Ma et al., 1998; Ozawa et al., 2000). Its success, however, depends upon the existence of detailed sequence and mapping data for a reference species and adequate comparative mapping information for the target species. COMPASS is less costly than (RH) or linkage mapping and it can be utilized in a highly selective manner to fill gaps in RH and comparative maps. The incorporation of COMPASS in livestock mapping should allow the creation of detailed comparative maps that will aid in the identification of additional ETL and the genes that influence them.
Table 2. A partial list of QTL, IDL, candidate and major genes identified in livestock  
Tablica 2. Djelomičan popis QTL-a,IDL-a, kandidata i značajnijih gena identificiranih u stoci

<table>
<thead>
<tr>
<th>QTL (Chromosome location) - QTL lokacija kromosoma</th>
<th>Swine - Svinje</th>
<th>Cattle - Goveda</th>
<th>Sheep - Ovce</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate - Stopa rasta (1, 4, 6, 7, 13)</td>
<td>Weaver disease / Milk production Čvorasta bolest / Proizvodnja mlijeka</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatness - Debljina (4, 6, 7, 13)</td>
<td>Somatic cell score Rezultat somatskih stanica (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meat quality - Kakvoća mesa (3, 4, 12, 15)</td>
<td>Milk fat yield Proizvodnja mlijeka (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litter size - Veličina legla (7, 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune response Imuna reakcija (1, 4, 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RYRI (porcine stress syndrome) - (sindrome svinjskog stresa)</td>
<td>CD18 (neonatal infection) CD18 (neonatalna infekcija)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K88AB (neonatal diarrhea) (neonatalni prolijev)</td>
<td>XLA/BTK (susceptibility to salmonella, leishmania) - (osjetljivost na salmonelu, leishmanije)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR (litter size) veličina legla</td>
<td>MXA - (susceptibility to influenza) MXA (osjetljivost na gripu)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RN (meat quality) RN (kakvoća mesa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIT (white coat color) KIT (boja bijele kože)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC1R (red/black coat color) - MC1R (crveno/crna boja kože)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IV. CONCLUSIONS AND FUTURE DIRECTIONS

A. Gene Transfer and Genetic Engineering

Today, the expertise and effort associated with gene transfer experimentation in animal biology are quite significant and challenging. Innovative technologies to enhance experimental gene transfer efficiency in different species are desperately needed. Such enabling techniques would not only bring the cost of individual projects into a reasonable realm but would also increase the likelihood of new innovations in many disciplines. The outlined efficiencies for the production of transgenic animals represent what is considered current state-of-the-art technology. It is envisioned that procedures will be modified and enhanced as new research breakthroughs are reported. As such, there are a number of specific achievements that would significantly enhance experimental productivity.

B. Future Directions. Much has been learned about various physiological processes in transgenic farm animals that were created to date. Even if a better production animal has not reached the marketplace as yet, the pioneering studies were not unsuccessful. Various transgenic animals have provided far reaching insights in redefining many
regulatory and developmental processes previously misunderstood in agricultural species. While studies of transgene expression in farm animals may not always correlate exactly, the utility of transgenic animal models to scientific discovery cannot be underestimated. The road to such successes will continue to be an arduous but exciting challenge for animal scientists in the 21st century.

REFERENCES


SAŽETAK

Naša sposobnost manipuliranja genomom čitavih životinja djelovala je na znanost na najdramatičniji način. U manje od 15 godina manipulacija genetskim sastavom transgenih životinja omogućile su istraživačima obraćanje temeljnim pitanjima na područjima od proizvodne poljoprivrede do biomedičkih istraživanja. U množstvu modela transgenih životinja temeljna istraživanja regulacije i funkcije određenih gena kršila su put do genetskih modifikacija in vivo, što je dovelo do postizanja funkcije prenesenog gena ili uklanjanja proizvoda endogenog gena. Pionirski napori u tehnologiji transgenih životinja značajno su djelovali na našu razumijevanje čimbenika što upravljaju regulacijom i izražavanjem gena i znatno su doprinijeli našem shvaćanju genetskih temelja reprodukcije i razvoja.

---

**TVORNICA STOČNE HRANE »VALPOVKA«**

**KOMBINAT VALPOVO**

**PROIZVODI 40 GODINA ZA VAS!**
- SVE VRSTE GOTOVIH KRMNIH SMJESA,
- SUPER KONCENTRATE - DOPUNSKE KRMNE SMJESE
- PREMIKSE I DODATKE STOČNOJ HRANI,
- BRIKETIRANU I RINFUZ STOČNU SOL

**STOČARI I POLJOPRIVREDNICI!**
TRAŽITE DIJETELINU SA ČETIRI LISTA
ZA DOBRO VAŠIH DOMAČIH ŽIVOTINJA

"VALPOVKA" =
- BRŽI PRIRAST
- JEFTINJA PROIZVODNJA
- BOLJA KAKVOĆA PROIZVODA