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Current State of Production, Research and use of Transgenic Laboratory Animals.

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Introduction

The use of transgenic animals, in particular mice, has over the last ten to fifteen years led to major breakthroughs in biological research areas such as genetics and gene expression, physiology, and embryology. Furthermore, it has added considerably to biomedical research into inherited and acquired genetic diseases. Here I will first review the most widely used technique for transgenesis: micro injection of DNA into the pronuclei of newly fertilized eggs. Next, the use of transgenic animals in biomedical research will be described and examples given of transgenic disease models. In the final sections I will discuss some promising future improvements in transgenic techniques.

Three methods for transgenesis

The first transgenic animals were made using infection with viral vectors as a way of introducing exogenous DNA into cells of early mouse embryos (Jaenisch and Mintz, 1974; Jaenisch, 1976). The use of retroviral vectors gives a high efficiency of gene transfer and a precise enzymatic insertion of single vector copies at random chromosome sites (van der Putten et al., 1985). However, a major disadvantage of the method has been the blockage of gene expression of inserted genes from sequences in the retroviral vectors (Teich et al., 1977; Loh et al., 1987; Feuer et al., 1989). Thereby, functional exploitation and analysis of the transferred genes have not been possible.

The next method to be developed, and the one that is very widely used today, is pronuclear injection (Gordon et al., 1980; Gordon and Ruddle, 1981). By this technique DNA is injected directly into one of the two pronuclei, the non-fused nuclei from sperm and egg in a newly fertilized egg. The DNA integrates at one or a few random sites in the embryo chromosomes and can be inherited stably through many generations.

The third method of producing transgenic animals is based on embryonic stem cells (ES cells) and can result in specific alterations of target genes in mice. Unique ES cell lines can be derived from the inner cell mass of mouse blastocysts, and grown under certain culture conditions without further differentiation. When such cells are introduced in the hollow of a host blastocyst, they will contribute to the developing tissues in the embryo, including germ tissue (Evans and Kaufman, 1981; Gossler et al., 1986). As a result, genetic alterations introduced during the culturing of the ES cells will reveal their effect in the mice produced. One commonly used alteration in ES cell lines is a »knock out« mutation of a predetermined target gene (Capecchi, 1994). This can result from the introduction into the ES cells of DNA pieces with sequence homology to a desired target gene. These sequences flank a central sequence that can block gene expression. The possibility of selecting the rare ES cell clones in which the DNA has become inserted by homologous recombination means that this technique is the only one able to target exogenous DNA to specific sites in the genome.

Unfortunately, gene targeting is feasible only in mice, as ES cells have not yet been derived for any other mammalian species. Thus at present the only method by which transgenic mammals other than mice can be produced is the pronuclear injection technique. Among the laboratory animals for biomedical re-

search, this method has been established for rats (Mullins et al., 1990), rabbits, and pigs (Hammer et al. 1985).

Using transgenic mice to understand differentiated gene expression

The invention of transgenic mice in the beginning of the 1980' gave a very powerful procedure which opened the avenue for analysing, what were at that time the virtually unknown mechanisms for differential gene expression of eukaryotic genes. There are major advantages for analysing gene expression in transgenic mice as compared to transient or stable expression in cultured cells of exogenous DNA. One is that the transgene is present in all the cells of a transgenic mouse, so in theory all kinds of cells can be subjeced to analysis (Krumlauf et al., 1985; Ornitz et al., 1985). It turned out that many important DNA regions for the differentiated regulation of genes are situated outside the protein coding sequence of the genes in the upstream or downstream DNA areas. In order to analyse these by the transgene technique, DNA constructs for pronuclear injection were built with variable parts of the regulatory regions linked to a suitable reporter gene that could facilitate identification of the transgene expression in tissue or cells. The regulatory mechanisms are similar for related species, for instance, human genes often show the typical differentiated expression when established in mice as transgenes (e.g. Neznanov et al., 1993).

By the use of transgenic and in vitro techniques, shorter functionally well defined sequences flanking the coding sequences of genes have been described. However, much in the way of their biochemical function is still a mystery. One fundamental and ubiquitous kind of element that functions as an individual locker-key combination, consists of a short DNA element and a corresponding nuclear protein. The binding of the corresponding protein to the element promotes folding or bending of the DNA and thereby opens the specific gene for transcription and expression, provided that the right combination of other nuclear binding proteins is present. Thus differential expression of genes in multicellular organisms seems primarily governed by the battery of different nuclear proteins and transcription factors that is present in a particular cell (reviewed by e.g. Mitchell & Tjian, 1989; Hernandez, 1993).

The Pronuclear Injection Method

The method of producing transgenic animals by pronuclear DNA injection was optimised during the early 1980' (Brinster et al., 1985; Palmiter & Brinster, 1986; Hogan et al., 1986). In our laboratory newly fertilized eggs are obtained about 10 hours post coitus (hpc) from the oviducts of sacrificed four weeks old females which were hormonally induced to ovulate. The one-cell embryos are cultured in vitro for a few hours until the diameter of the pronuclei has reached a size of about 1/5 of that of the egg cell (12 – 18 hpc). In a droplet of medium under a microscope, each embryo in turn is held by a blunt glass pipette and injected by another finely drawn pipette. A diluted solution of a linear DNA construct is delivered into one of the pronuclei, so that it swells about 50 percent in volume. The embryos are then cultured in vitro overnight and those that have cleaved are transferred surgically to the oviducts of pseudopregnant foster mothers. These have copulated with sterile males during the past 8 to 12 hours. After three weeks about 1/5 of the transferred embryos have developed to term and are born. After another three weeks tail-tips of the pups are clipped. Tail DNA is screened for integration of the injected DNA into the genomic DNA by using Southern blotting analysis. Roughly one out of five pups is transgenic, i.e., is positive for the injected DNA, and is bred with suitable mice in order to become the founder (G0) of a transgenic line.

Not all founders are equally successful in transmitting the transgene to their offspring. A transgene is usually inserted at a site in one member of a chromosome pair, and ma-

ny founders transmit the transgene to half of their offspring. However, about 1/4 of the founders transmit transgenes more rarely. These founders are mosaics for the transgene, that is to say the DNA became inserted at stages after the first cleavage division. Mosaic founders carry the transgene in only some of their somatic and germ cells (Wilkie et al., 1986). However, transgenic offspring from founders, the G1 animals, have the transgene in all cells and are more suitable than founders for a scrutiny of the effect of the transgene.

Using the above described procedure over a year, 111 transgenic mice (G0) were obtained after 25 days of injection with 19 different DNA constructs in our laboratory. To obtain one transgenic mouse a mean of 42 fertilized eggs had to be injected, of which 25 developed normally to the two-cell stage. About 30 two-cell stage embryos were transferred to each foster mother, that on average gave birth to four normal and one transgenic pups.

Disadvantages

The pronuclear DNA injection method of producing transgenic mice is successfully used within a broad field of biological research areas, yet it has a number of inherent disadvantages. These are believed to result from the insertion mechanism by which injected DNA enters at the sites of breaks in chromosomes.

The procedure is inefficient and 30-50 eggs must be injected in order to obtain one transgenic pup. There has been only moderate increased in efficiency over the more than ten years that the method has been used.

Approximately one out of 20 independently established transgenic lines carries a new mutation that segregates along with the transgene. Most often this has been uncovered through attempts to breed mice that are homozygous for the transgene (Meisler, 1992). In some instances altered phenotypes are seen, but most often the homozygous genotypes are lethal. In these latter cases the pronuclear injected constructs have become inserted into vital genes and have disrupted their functions. It is thus evident that insertion events take place at apparently random positions along the chromosomes and that the selecting of specific positions is as yet beyond the control of the experimenter.

The level of gene expression from insertions of a given DNA construct can vary considerably among independently derived transgenic mouse lines, but has a characteristic level in mice within the lines. The variation results from positive and negative influences on the transgene expression from the neighbouring DNA regions (Wilson et al., 1990; Eissenberg & Elgin, 1991). Due to this position effect the expression level of a given DNA construct must be analysed in five to ten independently derived transgenic lines. Attempts to overcome the position effects have been successful for constructs from number of genes by including locus control regions (e.g. Fraser et al., 1993).

The disadvantages that prevail by the pronuclear injection procedure could be alleviated by the design of DNA elements that would target the injected construct at a predetermined position in the chromosomes. At present such a perspective is considered with moderate optimism.

Molecular processes during DNA insertion

The molecular processes that injected DNA goes through before it integrates into the chromosomes can be deduced to some extent from the structure of the inserted DNA. The results suggest that enzymatic end-to-end ligation as well as recombination-like events take place. In transgenic mice the injected DNA constructs are often found as multiple copies at individual chromosomal inserts with the 5' ends facing the same way, i.e., in tandem. Co-injection with two constructs, sharing a minor homologous region can result in inserts where the two constructs are contiguous with only one version of the homologous region in between (Pieper et al, 1992). These observations demonstrate that

gans from animals to humans. As already mentioned, transgenic animals with overexpression of oncogenes or knock outs of tumour suppressor genes are more sensitive to carcinogens than normal mice. Mutagenic substances can be tested in other transgenic strains with increased sensitivity. Mice with transgenes of the bacterial genes lacZ or lacI can accumulate mutations in these genes as a result of mutagen treatment (Gossen & Vijg, 1993). DNA from such mice can be transferred to bacteria and individually mutated lac genes can be detected as single coloured colonies. The use of these sensitive new test-strains can probably reduce the number of animals used for these tests.

Organs from pigs are of a suitable size to be used as replacements for human organs. Transgenic pigs with compromised complement system are likely to be reported soon. Organs from such pigs, when transferred to humans will supposedly survive for a longer time before rejection than ordinary pig organs. This perspective is very promising and may have medical potentials in the future.

Improvements to Come

New technical advances in transgenic procedures may refine the use of transgenic laboratory animals. Improvements are on the way, e.g, the use of larger DNA constructs for pronuclear injection, the use of retroviral vectors that allow gene expression, and the use of site-specific recombination to control the expression from transgenes.

Larger constructs

Considerably larger constructs of hundreds of kb have already been successfully introduced by pronuclear injection or through uptake in ES cells of artificial chromosomes of yeast (Schedl et al., 1993; Jakobovits et al., 1993). There are still problems in handling these large constructs in terms of cloning fragments in and out, stability and rearrangements. However, the technique offers the possibility for transferring complete genomic genes, which are more likely to give normal levels of expression as well as a relief from position effects (Schedl et al., 1993). The technique may allow for several genes to be transferred at the same time, an attractive prospect for modelling polygenic diseases in transgenic animals. Larger constructs can also accommodate regulatory regions which may then co-ordinate the genes carried by the constructs.

Expression from retroviral vectors

The use of retroviral vectors as vehicles for introduction of transgenes was the first transgenesis technique to be used. However, upon transfection of early mouse embryos, sequences probably situated in the long terminal repeat enhancers and/or in the primer binding site for reverse transcriptase led to the inhibition of gene expression for the whole transgene (Loh et al., 1987; Feuer et al., 1989). Recently, retroviral vectors have become very important as vehicles in somatic gene therapy of humans with genetical diseases and as a result efforts to improve viral vectors have intensified. One positive result has been the design of vectors with altered primer binding sites that may exert less repression after introduction in EC cells (Petersen et al., 1991; Kempler et al., 1993; Lund et al., 1993). Thus, it seems profitable to explore the use of new vectors as expression vehicles for transgenic animals, and thereby take advantage of the precise and efficient gene transfer accomplished by virus vectors.

Control of transgene activity

With the purpose of controlling the activity of transgenes, much attention has been given over the past few years to site-specific recombination systems such as the Cre/lox from bacteriophage P1 (Sauer, 1993). The Cre recombinase mediates both excision and insertion of DNA fragments attached to lox sites in bacteria. In mammalian cells excision has been demonstrated (Sauer & Henderson, 1988) and circular DNA carrying a lox site has been inserted with the aid of Cre enzyme into a pre-existing chromosomal lox site

(Fukushige & Sauer, 1992). The latter procedure resulted in highly reproducible gene expression from the transferred DNA. It remains to be seen whether researchers can induce a similar process in newly fertilized eggs using pronuclear injection. One condition for this would be the establishment of transgenic strains with single lox sites from which to obtain the fertilized eggs. Cre mediated insertion of lox bearing circular constructs in chromosomes of such eggs could allow the production of many independent transgenic strains with the same or different transgenes inserted at the same chromosomal position. This would probably decrease the variation among transgenic strains due to position effects.

Excision of chromosomal DNA fragments flanked by lox sites as a way to control gene expression was recently demonstrated in transgenic mice (Lakso et al. 1992). In one transgenic mouse strain a stop sequence flanked by lox sites was placed between an eye lens-specific promoter and a SV40 T-antigen gene. Mice with this construct had normal eyes. When these mice were crossed to another transgenic strain with expression of Cre enzyme governed by the same lens-specific promoter, offspring carrying both transgenes had lens tumours. In these mice the stop fragment in the first construct had been removed and thereby nullified the stop signal for expression of T-antigen in the lens with the consequence of uncontrolled cell growth.

Recently, expression was abolished from the pol-beta gene in a single differentiating cell type in mice by excision of lox flanked sequences. The lox sites had been suitably installed in the pol-beta gene proper by a targeted substitution with a gene version carrying lox sites (Gu et al., 1994). It has previously been shown that when the pol-beta gene is knocked out in the standard way, offspring homozygous for the mutation could not be found in crosses between heterozygous mice. This is presumably because the functional lack of the pol-beta genes is lethal at early developmental stages. Thus, the question of whether the pol-beta gene product is dispensable for the rearrangement at lymphocyte antigen receptor genes in the maturing T cells was not answered by the previous experiments. In order to do so two new transgenic strains of mice were made by Gu and colleagues. The one already mentioned had a still active pol-beta gene equipped with lox sites and it was raised from ES cell clones where the gene had been substituted through a homologous integration process. This strain was crossed to another strain carrying a transgene where the expression of Cre enzyme was controlled by a promoter active only to the maturing T cells. In the majority of maturing T cells in double mutant offspring Cre mediated recombination deleted the lox carrying pol-beta gene, whereas this gene remained undeleted in cells of all other analysed tissues. In this way the lethal effect in early development of a gene knock out can be circumvented by activating the knock out at a later stage through expression of Cre enzyme in a stage or cell type-specific manner. This gene switch procedure will have important applications in biomedical research in the numerous cases where the gene-effect is on demand only in specific cells of a laboratory animal.

Summary.
Transgenic techniques have prompted major contributions to a number of research areas of biology and medicine. Transgenic mice are produced by three methods: transfection with viral vectors, DNA injection of egg nuclei, and from genetically altered embryonic stem cells. Most widely used in mice and the only available method for other laboratory mammals is that of DNA injection. By this method the genomic insertion of foreign DNA relies on enzymatic processes active at random breaks in chromosome. In addition, ligation and homologous recombination processes take place between injected DNA molecules in the nucleoplasm, resulting in multiple copies per insert. Gene expression from an inserted transgene varies considerably depending on its position in the genome, and it is only recently that attempts to overcome this has been successful. Animals showing symptoms known from human diseases can be used as models for the development of new therapies. Many transgenic animal models have been made for both dominantly and recessively inherited diseases, but still only a few for polygenic or

complex immunological and cardiovascular diseases, and for the acquired infection and cancer diseases. These models are used extensively in both basic and applied biomedical research. Future improvements in the transgenesis techniques can result from the use of more suitable retroviral vectors and from the use of larger DNA constructs for pronuclear injection. These could accommodate multiple genes as well as their genetic co-ordination. Finally, there will be wide applications for the current development of genetic switches for turning transgenes off and on by the use of site specific recombination.

Dansk resumé:
Transgene teknikker har givet store resultater indenfor en række biologiske og medicinske forskningsområder. Transgene mus kan laves på tre måder: ved transfektion med virale vektorer, ved injection af DNA i cellekerner på æg, og ved anvendelse af genetisk modificerede embryonale stamceller. Den mest anvendte metode for mus og den eneste mulige for andre laboratorie pattedyr er kerneinjektion af DNA. Ved denne metode sker indsættelsen af fremmed DNA ved enzymatiske processer, som er aktive ved tilfældigt opståede brud i kromosomer. Derudover udsættes de injecerede DNA molekyler for både ligerings- og homolog rekombinationsprocesser i kerneplasmaet og konsekvensen er multiple kopier per insertion. Genekspressionen fra de indsatte transgener varierer meget betinget af deres position i genomet. Det er først for nyeligt at forsg på at undgå dette er lykkedes. Dyr der udviser symptomer, som kendes fra sygdomme hos mennesket, kan anvendes som modeller til udvikling af nye behandlingsformer. Mange transgene sygdomsmodeller er blevet lavet for både dominant og recessivt nedarvede sygdomme, og noget færre for de polygene eller komplekse immunologiske og hjerte-kar sygdomme, samt for infektios- og kræftsygdomme. Disse modeller bliver meget benyttet i både basal og anvendt biomedicinsk forskning. Forbedringer af de transgene teknikker kan i fremtiden ske at anvende mere velegnede virus vektorer, eller større DNA konstruktioner til kerneinjektion. Disse kunne indeholde flere gener og samtidig en genetisk styring af deres koordination. Endelig vil der være bred anvendelse for den udvikling af genetiske »kontakter«, som nu er igang, til aktivering eller nedlukning af transgener ved hjælp af stedsspecifikke rekombinaser.

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