Transgenic Animals

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Transgenesis implies that a foreign DNA fragment is introduced into the genome of a multicellular organism and transmitted to progeny. Transgenesis therefore differs from gene transfer into cultured cells (transfection) or into the somatic cells of a patient (gene therapy).

Introductory article

Article Contents

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Introduction

Transgenesis has two distinct purposes: (1) the addition of a foreign gene to a genome and the specific replacement of a given gene by a foreign gene; and (2) the suppression of endogenous genetic information by replacing the foreign endogenous gene by a homologous inactive deoxyribonucleic acid (DNA) fragment. Both approaches are essential for biologists and biotechnologists.

In all cases, the foreign DNA must be present in a stable form in the embryo at an early stage of development in order to be transmitted into progeny.

Gene Addition

In a laboratory amphibian, *Xenopus*, the only efficient way of generating transgenic animals is to introduce the foreign DNA into isolated sperm previously treated with mild detergents to create pores in the membrane. The sperm, which is then loaded with the foreign DNA, will have lost their natural capacity to fertilize oocytes and must be injected into the oocyte cytoplasm to induce embryo development. This technique has also been used successfully in mice. In various species sperm incubated with isolated DNA can generate transgenic animals. This method has limited efficiency but is being improved. Sperm precursors can also be transfected by foreign genes and reimplanted into testis, leading to the generation of transgenic animals.

The method currently used to generate transgenic animals is based on microinjection of isolated genes into an embryo at the one-cell stage. In mammals, the foreign genes are microinjected into one of the pronuclei of the embryo (generally the nucleus supplied by the sperm). About 5000 copies of the foreign gene contained in 1–2 pL are injected into one of the pronuclei. This technique is rather laborious and of relatively low efficiency. In mouse, between one and five transgenic mice can be generated from an initial base of 100 embryos. Foreign DNA is highly mutagenic and lethal for many embryos: successful integration of a foreign DNA fragment occurs in no more than 10–20% of the cells.

The technique is basically the same for mouse, rat, rabbit, pig, sheep, goat and cow (**Figure 1**). In practice, the success rate of the method is much lower for larger animals. This is because they are much less prolific and because integration of foreign DNA occurs with lower frequency. Superovulation followed by *in vivo* fertilization is currently used in mammals. A specific protocol is used successfully in the cow. It involves the *in vitro* maturation of oocytes collected from ovaries in the slaughterhouse, *in vitro* fertilization, gene microinjection, *in vitro* development of embryos until the blastocyst stage, selection of putative transgenic embryos (using a coinjected reporter gene) and, finally, transfer into foster mothers.

In lower vertebrates and invertebrates, the pronuclei are generally not visible. Microinjections must be performed into the cytoplasm of one-cell embryos. This implies transferring a million copies of the gene into the embryo. This technique has been adopted to generate transgenic fish.

In birds, microinjection of conventional DNA fragments is difficult and inefficient. Several specific tools have been defined to reach this goal. Transposons and retroviral vectors carrying foreign genes are both capable of being efficiently integrated into genomes. Transposons have to be injected into the embryos, whereas retroviral vectors are capable of infecting embryonic cells. Retroviral vectors are injected in the vicinity of the primordial germ cells that are the precursors of gametes. The resulting transgenic animals are mosaic for the transgene. This means that a small proportion of the offspring is transgenic at the next generation. The transmission of the transgene in subsequent generations follows mendelian laws. Retroviral vectors have also been used to generate transgenic cows and monkeys after infecting oocytes before fertilization. Transposons are also efficient tools in generating transgenic invertebrates (*Drosophila*, silk worm, etc.).

Gene Replacement

When a DNA fragment is introduced into the nucleus of a cell or an embryo, it has a statistical chance of recognizing a short region of the host genome. Imperfect hybrids are thus formed between the foreign DNA and the cellular DNA

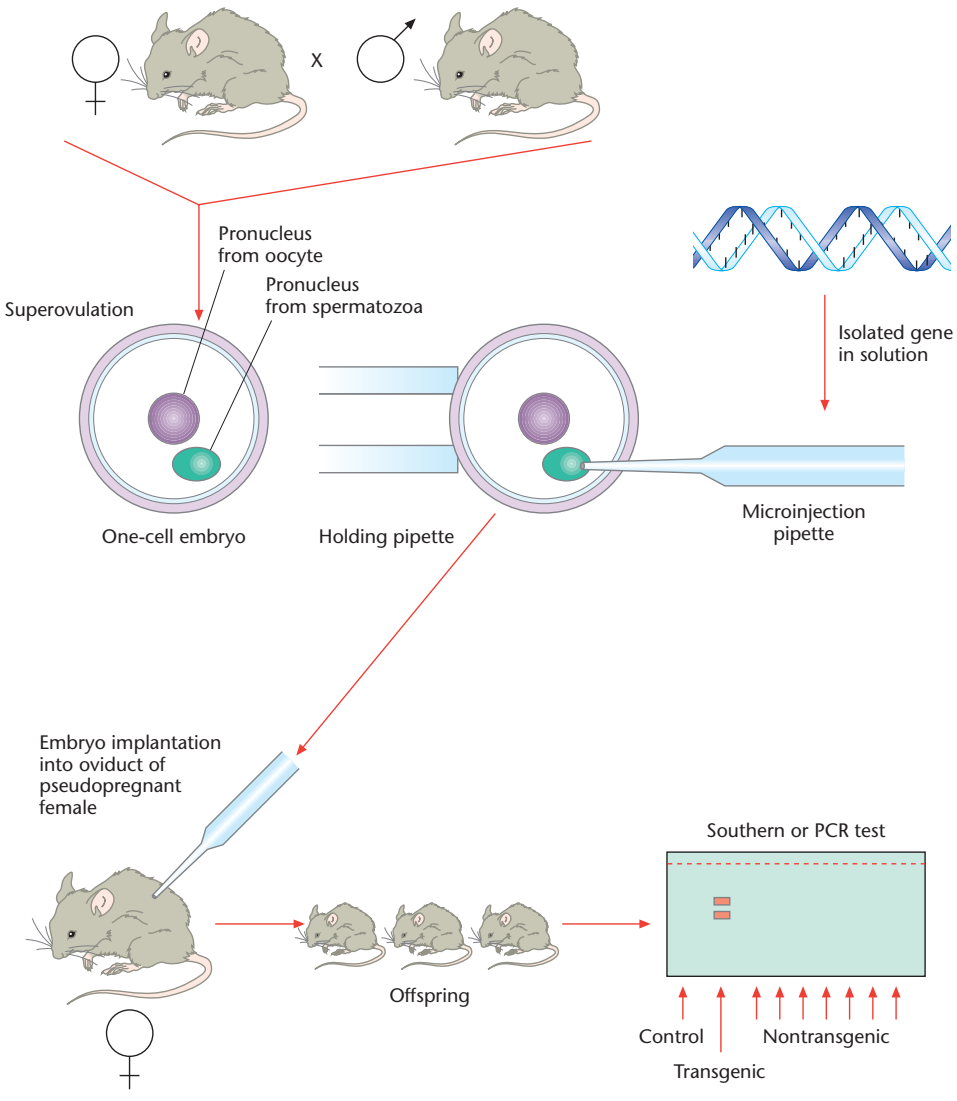

Figure 1 The generation of transgenic animals by gene microinjection. The embryos obtained by superovulation or by *in vitro* fertilization receive the foreign genes and are developed in foster mothers. Transgenes are detected and transmitted to progeny by normal reproduction. PCR, polymerase chain reaction.

(**Figure 2**). During cell multiplication, DNA is replicated. The complex formed by the foreign and the cellular DNA is recognized by the repair system of the cell. This leads to the foreign DNA being integrated into the host DNA. This process is considered to be illegitimate recombination. Only short DNA regions are involved in the formation of the hybrids. Hence, this kind of event can take place at many sites in the genome.

When long fragments of DNA (generally several thousands of nucleotides) with a sequence similar to a region of the host genome are introduced into a cell, homologous recombination may occur. This complex process leads to the specific and perfectly precise replacement of the host DNA region by the foreign DNA. If several DNA fragments with sequences similar to specific regions of the host genome are introduced simultaneously into the cell, independent homologous recombinations may occur. A nonhomologous DNA sequence surrounded by two homologous sequences can thereby be specifically introduced into a given site of a genome. This property is being used to replace targeted genes by foreign gene constructs (**Figure 3**). The most frequent experiments based on this phenomenon aim at inactivating a host gene. This experiment is known as gene knockout. The replacement of a gene by another active gene using the same protocol is known as knockin.

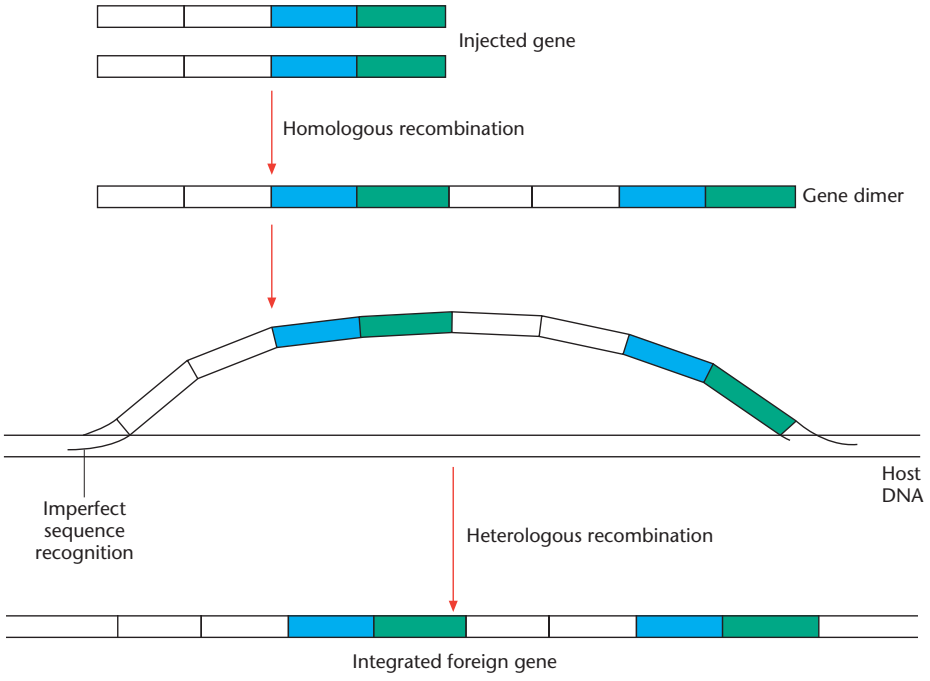

Figure 2 The mechanisms leading to the random integration of a foreign gene into an animal genome. The foreign DNA sequences recognize short and partially homologous regions of the genome. Reparation mechanisms integrate the foreign DNA. Before integration, a homologous recombination mechanism generates polymers of the foreign gene organized in tandem.

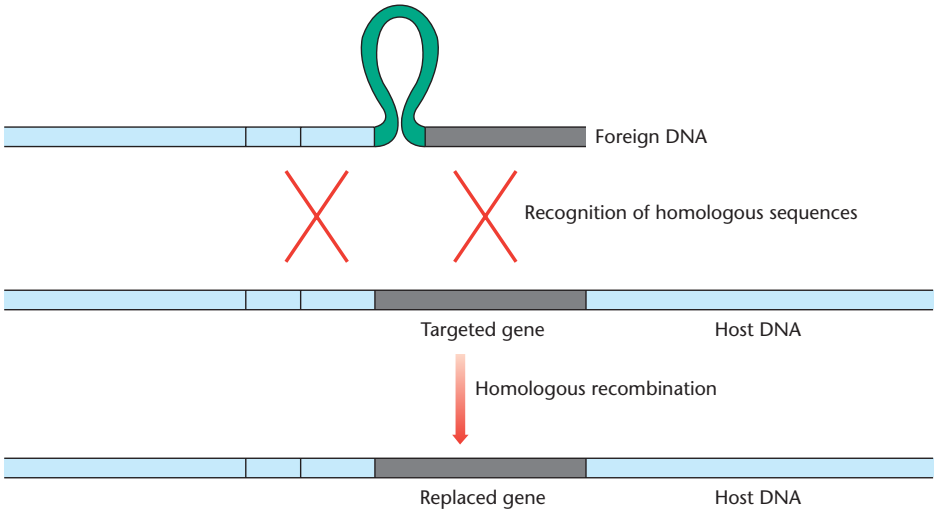

Figure 3 The experimental protocol leading to specific gene replacement. A gene construct containing two long regions strictly homologous to the targeted host gene and containing a foreign DNA region transferred to cells. The homologous sequences recombine and the targeted gene is replaced by the foreign gene. The cells in which gene replacement occurred are saved by double selection (not shown here).

The process of gene replacement by homologous recombination is a rare event. The illegitimate recombination described above is much more frequent, and cells in which gene replacement has occurred must be selected. To reach this goal, a double-selection system is commonly used. Two selection genes are introduced in the construct. One gene, conferring cell resistance to an antibiotic, is inserted within the gene between the two sequences

homologous to the targeted host gene. Another gene, generally the thymidine kinase from the herpes simplex virus, is added upstream or downstream of the homologous sequences. In the presence of the antibiotic, only the cells harbouring the foreign DNA will survive. This negative-selection step does not discriminate between cells in which homologous or illegitimate recombination has occurred. Negative selection is then performed by adding chemical compounds, such as ganciclovir. This drug is transformed into a cytotoxic substance by thymidine kinase. Thus, the cells previously selected by the antibiotic and harbouring the thymidine kinase gene are eliminated. Those in which homologous recombination has occurred do not harbour the thymidine kinase gene and they can survive up to the second selection. Homologous recombination occurs in yeast and in animal cells but only marginally in plant cells; gene replacement is therefore a common practice in yeast and in some animals but not in plants.

Since homologous recombination is a rare event, the selection of the cells in which it takes place is a relatively long process. It is observed only quite exceptionally in embryos after gene injection. Hence, the cells in which a gene has been replaced must still be able to participate in the development of an embryo and the generation of a living organism. The protocol defined approximately 12 years ago in mouse is based on the use of pluripotent embryonic cells. Cell lines derived from blastocysts have been established. These cells, reintroduced into another blastocyst, can participate in the development of the embryo to generate chimaeric animals bearing the genetic mutation previously introduced during the culture (**Figure 4**). Some of these cells are precursors of gametes and give birth to animals that have one of their genes replaced. Crossbreeding leads to animals homozygous for the mutation. This protocol is presently applied only in mouse since pluripotent cell lines capable of generating chimaera able to transfer their mutation to progeny have been established in no other species so far.

An alternative approach is the cloning technique used to generate 'Dolly the sheep' and other animal species. Skin fibroblasts from a fetus can be cultured for several weeks. During this period, gene addition and replacement have proved to be feasible in sheep, mice and pigs (**Figure 5**). The selected cells harbouring the mutation can be used to generate living animals after introduction into enucleated oocytes. Gene addition is more easily performed in this way than by microinjection. Gene replacement has become possible in virtually all species in which the embryo cloning technique can be applied.

A recently discovered mechanism revealed that long double-strand RNA is cleaved into small RNA (21–23 bp) known as siRNA (small interfering RNA). The siRNAs induce the specific degradation of RNA having similar sequences. This phenomenon, known as RNAi (RNA interference), is a protection mechanism against viruses and may participate in the control of cellular gene expression. Vectors capable of directing the synthesis of siRNA in cells and transgenic animals have been designed. This makes possible the specific inhibition of messenger RNAs (mRNAs). This new tool is extremely promising for basic and applied research. It is simpler and more flexible than knockout and it has allowed the generation of thousands of *Caenorhabditis elegans* lines, each of them having a specifically silenced gene at the mRNA level. The method is now being extended to *Drosophila* and mammals.

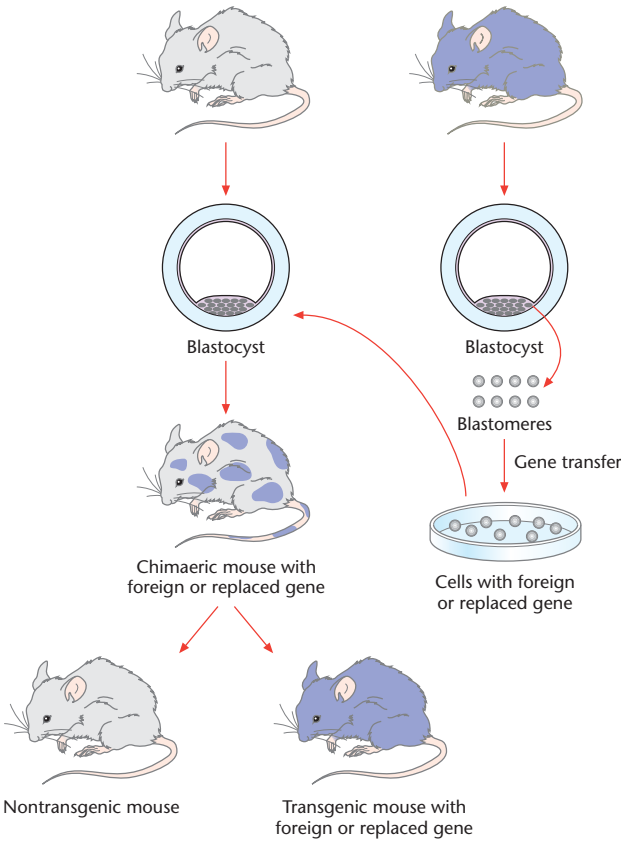

Figure 4 The transmission of a mutation by the generation of chimaeric animals. The mutipotent embryonic cells in which gene replacement occurred are transferred into a recipient embryo and participate in its development. The mutation can be transmitted to progeny.

Efficiency of Transgenesis

Up to 40% of founder animals obtained by DNA microinjection are mosaic for the transgene. Transmission of the transgene to progeny occurs with variable frequency or even not at all. In the next generation, the transgene is transmitted according to the mendelian laws at the expected frequency.

Experiments carried out in several species indicate that two, or even three, genes injected simultaneously are

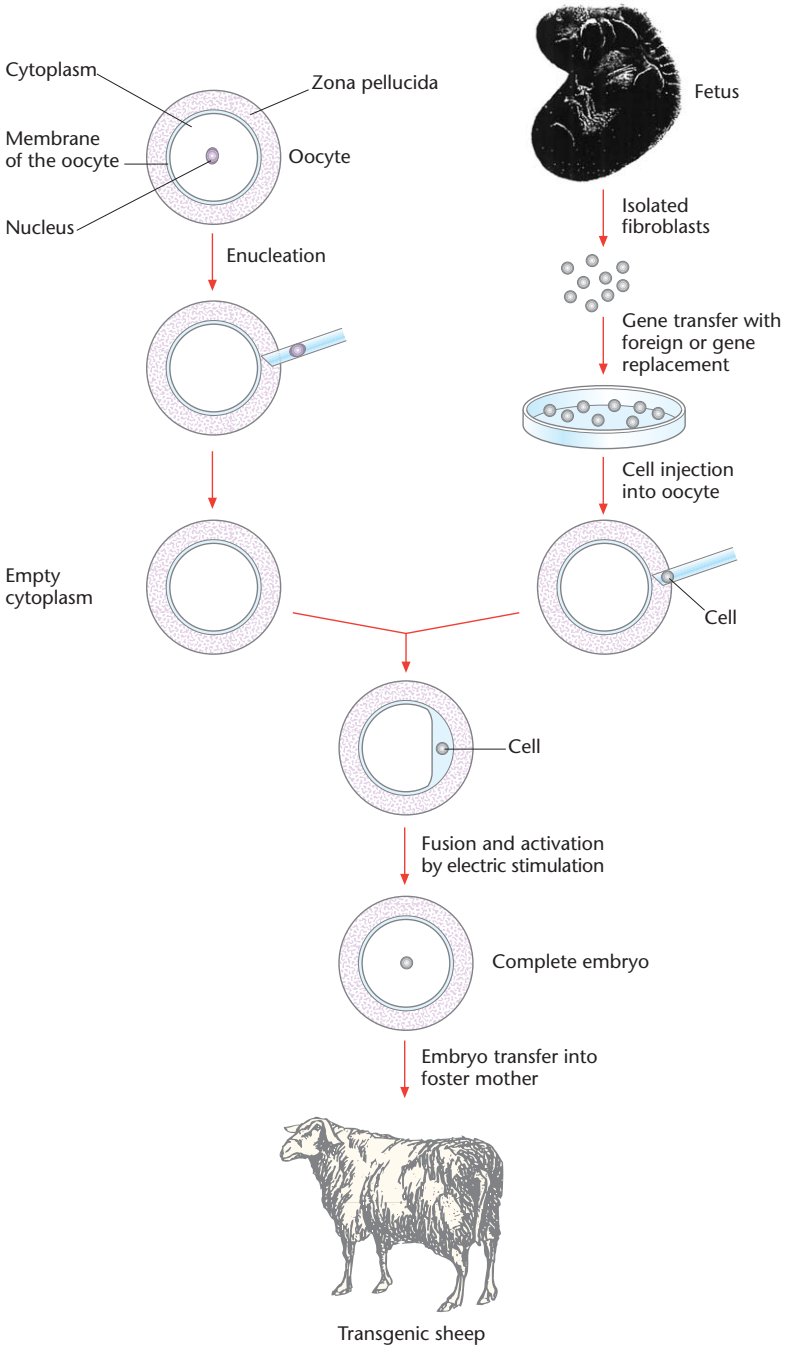

Figure 5 The transmission of a mutation by the cloning technique. The fetal cells in which gene addition or replacement occurred are used to generate living embryos after transfer into enucleated oocytes. The mutation is transmitted to progeny.

cointegrated with a frequency of about 70%. The presence of the reporter gene, easily identified by a noninvasive technique (e.g. the green fluorescent protein gene from jellyfish), indicates that the gene of interest is also likely to be present.

Expression of Transgenes

The expression of a gene is under the control of DNA sequences located upstream of the genetic message proper, called promoters. A certain number of promoters deter-

mine the expression of the associated genes specifically in a limited number of cell types. When used in transgenesis, promoters lose part of their specificity to variable degrees.

These unexpected and nonreproducible events depend on the position of the transgenes in the host genome. Regulatory elements involved in controlling endogenous genes and located in the vicinity of the transgenes are supposed to inactivate the transgenes in some cases or, in contrast, to stimulate their expression in an inappropriate manner. The reality is probably more subtle and the illegitimate expression of a transgene may also result from stimulation by neighbour host transcription enhancers.

The extinction of a transgene, or its irregular expression, is more frequently observed when the transgene is formed with a copy of the mRNA (complementary DNA, cDNA) instead of the native gene containing introns and other unknown signals. Multiple copies of the transgene also often have a negative effect on expression. Some DNA sequences, particularly those from bacteria, contribute to the inhibition of transgene expression. Cellular mechanisms exist to inactivate foreign sequences. This phenomenon protects genomes from invasion by transposons and retroviruses. The transgenes are thus inactivated and embedded into inactivated chromatin. This occurs more frequently when the transgene is integrated in the centromeric or telomeric regions of chromosomes that do not contain active genes.

Efficient expression of a transgene is generally obtained when it contains long sequences of native genomic DNA. These sequences probably contain insulators, which inhibit nonspecific stimulation by neighbour stimulators and prevent the silencing of the transgene. In a limited number of cases, such elements have been identified and their addition to transgenes allows for accurate expression.

Future Perspectives

Transgenesis is, more than ever, an essential tool for biologists. The study of human diseases relies to a great extent on the use of animal models. Gene addition and replacement are being used increasingly for this purpose. The mouse is, and will remain, the species in most demand. Others are also highly valued. This is the case for a laboratory fly, *Drosophila* and a worm (*Caenorhabditis elegans*), which represent simplified animal models. In some cases, transgenic rats, rabbits, pigs or monkeys are more likely to mimic human disease than mice.

Animals can be the source of recombinant proteins of high value for pharmaceutical use. Milk from transgenic animals is already the source of highly active human proteins that are produced at low cost. In the future, egg white, urine, pig seminal plasma or silk worm cocoon may also be sources of recombinant proteins for humans or animals. Pigs might become an essential source of cells and organs for transplantation to humans. The inactivation of pig genes coding for proteins involved in some rejection mechanisms is expected to occur in the next decade. This is already the case for α_1-galactosyltransferase gene, which generates many potent glycosylated antigens at the surface of pig cells.

Projects aimed at conferring disease resistance in farm animals are under way. Other projects are being developed to optimize the digestibility of food; for instance, in pig, to reduce rejection of polluting compounds such as phosphate. Gene transfer is also being used tentatively to improve the quality of sheep wool. Other projects will be developed gradually to improve the quality of animal products, for example milk.

These types of project raise limited environmental problems in most cases, although difficulties have been encountered. The possible uncontrolled spread in the sea of transgenic salmon with accelerated growth, for example, presently prevents their use at an industrial scale. Clearly, the utilization of transgenic animals will remain relatively slow in comparison to that of transgenic plants. This is due to the obvious difficulty and cost of creating transgenic animals but also to the time required to spread the interesting transgenes.

Further Reading

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