GENE TRANSFER IN ANIMALS

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ABSTRACT

Animals into which genes have been transferred are called "transgenic animals" .Several methods have been developed for gene transfer into mice: microinjection, infection with retroviral vectors and embryonic stem cell mediated gene transfer. Of these, microinjection has been the most widely used technique in mice, and to date the only method successfully applied to livestock animals. The ability to introduce foreign genes into the germ line and the successful expression of the inserted gene in the organism have allowed the genetic manipulation of animals on an unprecedented scale. The information gained from the use of the transgenic technology is relevant to almost any aspect of modern biology including developmental gene regulation, the action of oncogenes, the immune system, and mammalian development. Because specific mutation can be introduced into transgenic mice, it becomes feasible to generate precise animal models for human genetic diseases and to begin a systematic genetic dissection of the mammalian genome.

INTRODUCTION

The introduction of genes into the germ line of mammals is one of the major recent technological advances in biology. Transgenic animals have been instrumental in providing new insights into mechanisms of development and developmental gene regulation, into the action of oncogenes, and into the itnricate cell interactions within the immune system. Furthermore, the transgenic technology offers exciting possibilities for generating precise animal models for human genetic diseases and for producing large quantities of economically important proteins by means of genetically engineered farm animals (3).

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There are currently three methods for incorporating exogenous DNA into the genome to produce transgenic animals. These are (I) retroviral vectors, (2) embryonic stem cells and (3) pronuclear microinjection (Palmiter & Brinster, 1986; Scangos & Bieberich, 1987; Simons & land, 1987). At present most transgenic animals are produced by pronuclear microinjection of fertilized eggs. Fertilized eggs are obtained from superovulated donor animals and tens to hundreds of DNA molecules are injected through a fine injection pipette into one of the pronuclei. Surviving eggs are implanted into foster mothers ad proceed to term (S. Harris et. al., 1990) (Fig 1).

Transgenic offspring are identified by molecular analysis of their DNA for the presence of the injected DNA molecules. The integrated DNA is frequently present in every cell at a single chromosomal location, usually as multiple copies in a tandem array. In mice up to 5% of injected eggs may developed to be transgenic. In domestic animals, such as sheep or pigs, the average success rate is approximately 1%. This may reflect differences in both reproductive physiology and the technical demands of microinjection into more fragile and almost opaque eggs (Simons et al., 1988). In this article I will not a t temp to give a comprehensive review of the field but rather emphasize principles and recent developments. Several detailed review articles have been published over the last 7 years that cover the earlier work on transgenic animals.

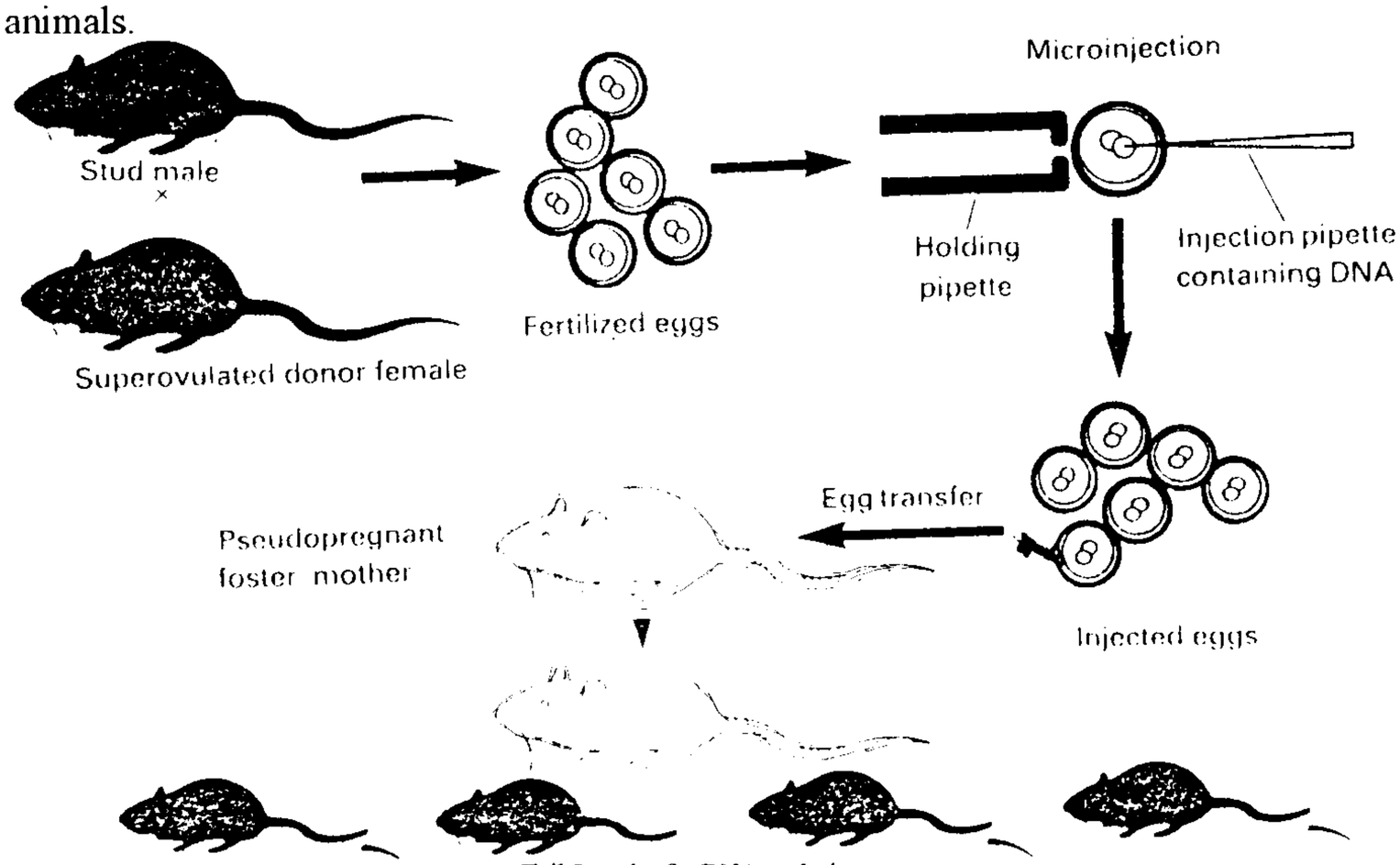

Fig. 1 : Production of Transgenic mice by pronuclear injection (see text for details).
Source : S. Harris & et al, J. Reprod. Fert. (1990) 88, 707-715)

METHODS OF INTRODUCING GENES INTO ANIMALS

Microinjection of DNA into Pronucleus. Microinjection of cloned DNA directly into a pronucleus of a fertilized mouse egg has been the most widely and successfully used method for generating transgenic mice. Typically, multiple DNA molecules arranged in a head-to-tail array integrate stably into the host genome. It is thought that he injected DNA molecules associate insert subsequently at a single chromosomal site. It has been proposed that random chromosome breaks, possibly caused by repair molecules, may serve as integration sites of the foreign DNA (7). Frequently, rearrangements, deletions, duplications (8), or translocations (9) of the host sequences occur at the integrate into the host genome. Bovine papilloma virus (BPV), for example, either integrates stably into the genome of transgenic mice or is maintained as an episome depending on the structure of the injected DNA (10). Episomal replication and transmission to the offspring have also been reported for a rearranged plasmid coding for the polyoma virus large T antigen, although the mechanism responsible for the episomal state has not been resolved (11).

The principal advantage of direct microinjection of recombinant DNA into the pronucleus is the efficiency of generating transgenic lines that express most genes in a predictable manner. However, one disadvantage of this method is that it cannot be used to introduce genes into cells at later development stages. Moreover, the cloning of the chromosomal insertion site may be difficult because of the multiple copy inserts and the host sequence arrangements.

Retrovirus infection. in contrast to microinjeted DNA, retroviruses integrate by a precisely defined mechanism into the genome of the infected cell. Only a single proviral copy is inserted at a given chromosomal site and no rearrangements of the host genome are induced apart from a sort duplication of host sequences at the site of integrations (15). Preimplantation stage mouse embryos can be exposed to concentrated virus stocks (8) or cocultivated on monolayers of virusproducing cells (16). Methods also have been devised to introduce virus into postimplantation embryos between days 8 and 12 of gestation (17). While this allows infection of cells from many somatic tissues, germ cells are infected with a low frequency. Similarly, when chicken embryos were exposed at the blastodisk stage to avian leukemia virus, infection of germ cells was inefficient (19). Because the chick pronucleus cannot be microinjected with DNA, retrovirus infection is probably the only feasible method for generating transgenic chickens.

The main advantage of the use of retroviruses or retroviral vectors for gene transfer into animals is the technical ease of introducing virus into the embryos at varous developmental stages. Furthermore, it has proved much easier to isolate the flanking host sequences of a proviral insert than those flanking a DNA insert derived from pronuclear injection. This is of considerable advantage when attempting to identify the host gene disrupted by insertion of the proviral DNA.

The main disadvantages of the use of retrovirues for gene transfer are the size limitation for transduced DNA and the unresolved problems of reproducibly expressing the transduced.

Embryonic stem cells. Embryonic stem cells are established in-vitro from explanted blastocysts and retain their normal karyotype in culture (20). When injected into host blastocysts, ES cells can colonize the embryo and contribute to the germ line of the resulting chimeric animal (9). Genes can be efficiently introduced into ES cells by DNA transfection or by retrovirus-mediated transduction, and the cell clones selected for the presence of foreign DNA retain their pluripotent character. By means of this approach, mice have been generated from cell clones that were selected in vitro for a specific phenotype (21). This opens exciting possibilities for deriving mouse strains carrying specific mutations. Although only a few laboratories have reported successful germ line contribution of ES cells to date, it is likely that this approach will receive increasing attention for the genetic manipulation of mice.

Genes have also been microinjected into rabbit, sheep, and pig embryos(23). The success rate of generating transgenic domestic farm animals is, however, much lower than that obtained with mice, in large part because of technical difficulties in visualizing the pronucleus in the embryos of these species. A human growth hormone gene successfully introduced into the pig in spite of these difficulties was nevertheless unable to increase growth, perhaps because the human hormone was not biologically active in pigs(23). Although the importance of genetic engineering for improving livestock has been questioned (4) it is likely that transgenic farm animals may become a source of economically valuable proteins. For example, medically relevant proteins, whose expression has been targeted to the mammary epithelial cells, may be harvested from the milk of transgenic cows as has been shown to be possible for transgenic mice(25).

Experimental Protocol as developed by J. Siman et al, 1988, for a successful gene transfer in sheeps is given below.

DNA for microinjection. pMK[15] was prepared for microinjection by BamHI digestion, phenolchloroform extraction and ethanol precipitation. BLG-FIX and BLG-IAT fusion genes were released from the plasmid vectors by digestion with SalI and XbaI, electrophoresed on 0.5% agarose gels and electroeluted onto dialysis membrances. Isolated fragments were purified on elutip D columns (Schleicher and Schull), (Fig 2).

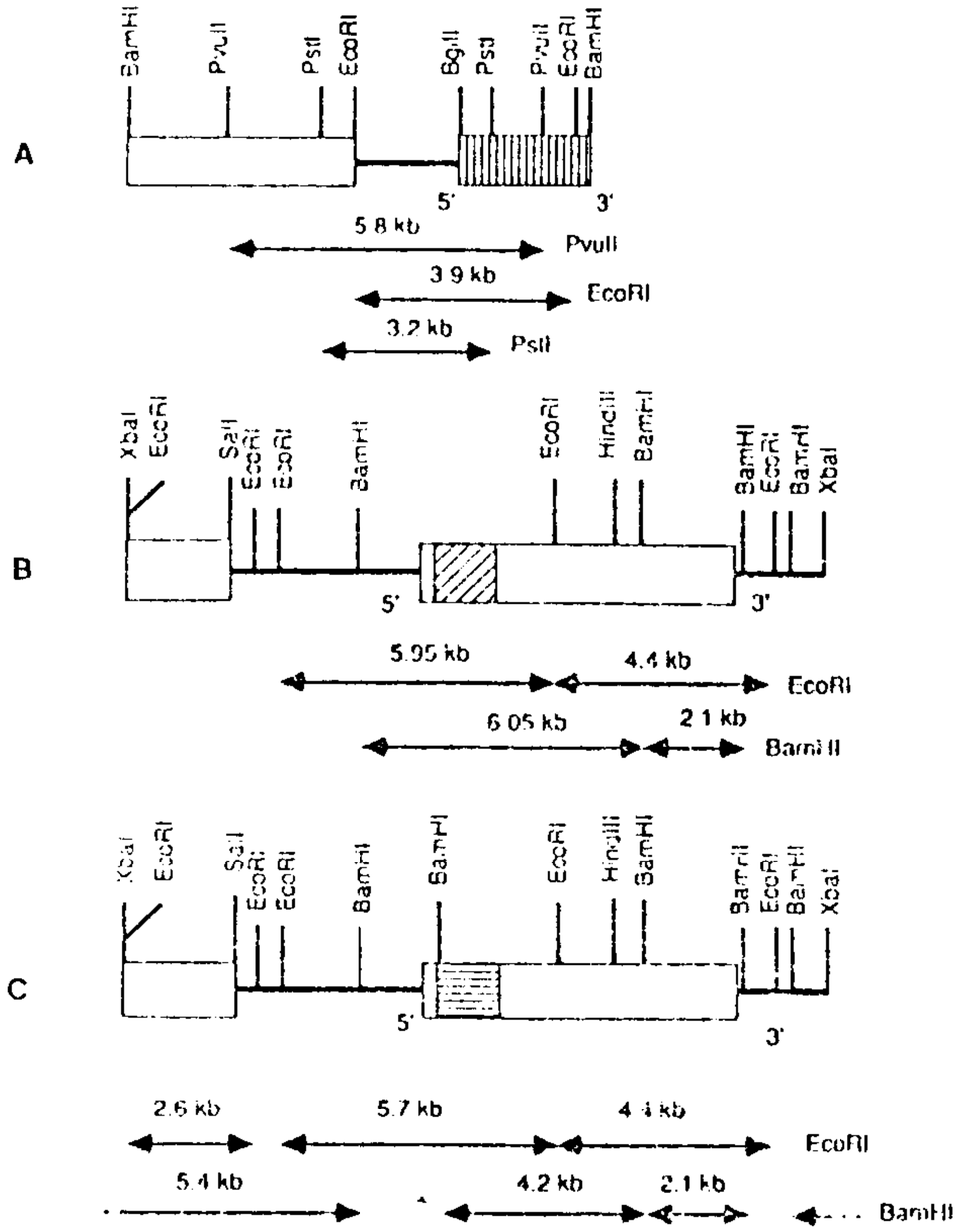

Figure 2. Restriction maps of microinjected constructs.(A). Plasmid pMK15. Stippled box: pBR 322 vector; solid line: mouse MT-I promoter segment, vertical shaded box: HSV TK gene. Fragments relevant to the analysis of the transgenic sheep are indicated below the map. PMK was injected after linearization with Bam HI. (B). BLG-FIX. BLG-FIX, the insert of pSS 1tgXS-FIX is a 10.5 kb XbaI-SalI fragment from BLG genomic clone SSI, comprising the 4.9 kb

from p5'G3'cVI, 1.55kb NheI-HindIII fragment) inserted into a PvuII site in the 5' untranslated region of the BLG gene. The predicted transcript of this fusion gene is a bicistronic mRNA 5'-FIX-BLG-3') Stippled box: vector pPoly1; open boxes: BLG transcription unit; solid lines: BLG flanking sequences; diagnonal shaded box: FIX cDNA sequences. Beneath the map, the origins of the BamHI and EcoRI fragmens detected (Fig.2b) with the FIX and BLG cDNA probes are shown. The fragment injected was the Xba-SalI insert. (C). BLG-αIAT. BLG-αIAT, the insert of pSS Itg XS-aIAT is analogous to BLG-FIX except that in place of the FIX cDNA a human αIAT cDNA was inserted (p8alppg (a gift of R. Cortese), 1.3 kb TaqI-BstNI fragment). Horizontal shaded box: alAT cDNA sequences. The fragment was injected as a mixture of XbaI-SalI insert and XbaI lineariezed pSSItgXS-alAt. Beneath the map, the origins of the BamHI and EcoRI fragments detected (Fig.2c) by probing with alAT and BLG cDNAclones are shown. (Source:J.P. Simonsand et αl., BIO/TECHNOLOGY 6:181,1988)

redissolved at about 100 μg.ml in 0.2 mm-filtered 10 mM Tris. Hcl, lmM EDTA (pH 7.5), and stored frozen. For microinjection, the DNA was diluted to 2 ug.ml (pMK) or 1.5 ug.ml (BLGFIX and BLG-IAT) in 0.2 μm-filtered double distilled water. Micropipette tips used to handle the DNA were rinsed thoroughly with filtered double distilled water to remove particles that could block microinjection pipettes. The DNA solution was centrifuged at 10,000 g for 30 min.; an aliquot withdrawn from below the surface was used to fill injection pipettes.

Collection of eggs. Animals used as egg donors were ewes of proven fertility of variety of breeds : Welsh Mountain, Scottish Blackface, Greyface (Scottish Blackface x Border Leicester), and Cheviot, Rams of proven fertility were of several breeds: Finnish Landrace x Dorset Horn, Scottish Blackface and East Friesland. Donor ewese treated with progestagen sponges (Veramix, Upjohn Ltd., Crawley, UK.) for 12-16 days to synchronize the time of estrus. Superovulation was induced by subcutaneous injection of equine FSH[24] in 2 equal doses of 1.75 or 2.15 mg, 30h before and at the time of sponge withdrawal (noon). Ewes were tested for the onset of estrus at 0800h, 1200h, 1600h and 2000. At estrus, each ewe was served by at least two rams. Eggs were recovered[25] by flushing oviducts with prewarmed ovum culture medium (Flow Labs. Irvine, UK) during mid-ventral laparotomy.

Microinjection of DNA and remplantation of eggs. Microinjection was performed using essentially standard techniques[26] Eggs were manipulated in cover-slip chambers in ovum culture medium supplemented with 20% fetal calf serum. The ends of the chambers were covered with Dow Corning 200/50 cs fluid.

Microinjection pipettes were very fine (<0.5μm) and tapered at a small angle (<5 dgrees), to minimise lethal damage to the eggs, They were pulled from thin wall borosilicate glass (1.0 mm O.D., Kwik- fill, Clark Electromedical Instruments, Pangbourne, Uk). Single-cell eggs had DNA injected into one pronucleus, 2-cell eggs into both nuclei (Season 1985/86) or into one nucleus(1984/85, or when only one was visualized, 19/86). Injection of 4 -cell eggs was into one, two or three nuclei (1985-86). When 2- or 4- cell eggs were injected more than once, injections were performed without repositioning the eggs on the holding pipette to avoid confusion over which cells remained to be injected. Successful injection was indicated by marked swelling of pronuclear or nuclear. Injected eggs were incubated for at least 30 min. before transfer, to allow damage to become apparent. Some eggs were centrifuged prior to injection at 10, 000 g for 10 min, or for 5 min. after 30 min, incubation in medium containing 0.1μg/ml colcemid. Egg recipients (Welsh Mountain ewes) were treated with progestagen sponges Veramix, Upjoh; crawley, UK.) for 12-16 days to synchronize their estrus cycles with hose of the egg donors. Injected eggs were implated as deeply as possible into the oviducts of recipients which had ovulated. 1-3 eggs were transferred per recipient (1985/86 or 1-4, 1984-1985 or when the recipients were one day out of synchrony 1985/86). The embryos were distributed between the oviducts.

DNA Analysis. Samples of DNA (10 mg) from peripheral blood lymphocytes were analysed by restriction enzyme digestion, gel electrophoresis (1% agarose), Southern transfer to nylon membranes (Hybond N, Amersham) and hybridization using standard procedures.

APPLICATION OF TRANSGENIC TECHNOLOGY

(a) Models for oncogenesis and diseases. The potentioal for using specific promoters or enhancers to direct expression of heterologous gene to a specific cell type has stimulated numerous attemps to change the physiology of an animal experimentally. The transgenic technology has ben particularly valuable for studying the consquences of oncogene expression in the animal (26). With the use of transgenic mice, problems can be addressed that cannot be approached satisfactorily in cell culture: for example, the spectrum of tissue that are suceptible to the

transforming activity of an oncogene, the relation between multistep oncogenesis and cooperativity of oncogenes, and the effect of oncogenes on grwoth and differentation.

(B) *In the study of Immune Systems.* Transgenic mice have also been important to the study of Ig gene expression. Several groups showed functionally rearranged Ig genes introduced into the germ line to be correctly activated and to alter the expression of the endogenous immunoglobulin repertoir (22). Recent avidence also obtained with transgenic mice suggests that the expression of the functional Ig genes can cause complex abnormalities in the immune system (27). Chicken or rabbit Iggenes in germ line configuration became rearranged in transgenic mice and form functional hybrid Ig molecule (28), suggesting that the production of interspecies monoclonal antibodies may be possible in genetically engineered mice. A detailed review discussing the implications of these results for our understanding of the development immune system has recently been published (74).

(C) *Lineage Marker.* A central issue in contemporary biology is the construction of fate maps to assess cell ancestry, cell location, and cell commitment in the developing embryo. Visual observation and injected lineage tracers have been used to study the early stages of mammalian dvevelopment because the preimplantation embryo is easily amenable to experimental manipulaton (30). However, the inaccessibility of the embryo once it has implanted in the uterus impeds the study of cell lineage at later stages and has prevented the use of direct lineage tracers. Therefore, the study of cll lineage in the postimplantation embryo necessitated the development of stable markers of individual progenitor cells that leave the embryo undisturbed.

The introduction of exogenous DNA into embryos after the one cell stage generates genetic mosaicism that may be used to analyze such lineage relations. In the first study of this type, preimplantation mouse embryos were infected with retrovirus that served as genetic markers for the progeny of an infected blastomere (18). Similar lineage studies were performed with genetically mosaic mice generated by microinjection of plasmid DNA into a pronucleus.

(d) Mutation in Transgeni Animals. The insertion of foreign DNA sequences into the cellular genome can cause mutational changes by disrupting the function of and endogenous gene. Most insertional mutations in transgenic mice are recessive and have induced by infection of embryo or ES cells with retroviruses or by microinjection of recombinant DNA into the pronucleus. The majority of the mutant strains have an embryonic lethal phenotype (11,22,31,32). Other phenotypes include defects in limb formation (12, 33), transmission distortion (34), or disturbance of kidney function (35). We have identified four retrovirus induced mutations after inbreeding of 70 transgenic mouse strains (31,32,35), and similar frequencies have been observed in other laboratories (36). Thus, the available data suggest that retroviruses induce mutations at an overall frequency of 5 to 6%; pronuclear injection of plasmid DNA may be slightly more mutagenic (37). This figure is likely to be an underestimate, because some mutations may have only subtle phenotypes that are not easily detected (R. Jaenisch 1990).

The generation of mutants by insertional mutagenesis is attractive because the introduced DNA can serve as a probe for isolating the integration site and the flanking host sequences. Flanking sequences have infact been cloned for a number of mutants but the mutated gene has been identified in only one case, the move 13 strain, which carries an M-mul V proviral genome in the first intron of the ∝ (I) collagene gene (31).

(e) Milk Proteins. A considerable amount of resarch has been devoted to isolating and characterizing genes coding for milk proteins. Bonsing and Mac kinlay (1978) reviewed information on the structure of casein genes from a range of mammalian species. Bovine ∝ slcasein (Nagao et al. 1988) and K-casein (Kang and Rechardson 1988) genes have been expressed in E.coli, and tissue specific expression of a rat β-casein genes appear to be highly conserved - the 5' noncoding region (involved in stabilisation of mRNA, perhaps), the leader sequence (for translocation of the protein into milk), and those coding for sequences that are phosophorylated by casein kinase (Rosen et al. 1986). The bovine BLG A gene has been cloned (Jamieson et al. 1987) as has the ovine BLG gene (ALI and Clark 1988); this latter gene was expressed in the lactating mammary gland of transgenic mice (simons et al. 1987). Subsequently, Simons and co-workers (1988) constructed a fusion gene consisting of the 5' putative regulatory regions of the BLG gene and the

structural gene coding for either human coagulation factor IX or human αl-antitrypsin. Transgenic sheep containing these fused genes in their genomes have been constructed, but evidence showing appropriate expression of the genes in the mammary tissue has not been reported yet.

CONCLUSIONS

The last few years have witnessed an extraordinary increase in the use of transgenic animals. Methods of manipulating embryos and transferring genes have been refined and now constitute standard procedures used for a variety of purposes. Each of the three methods for generating transgenic animals has distinct advantages for some and disadvantages for other applications. Pronuclear injection of recombinant DNA is the method of choice for obtaining expression of a foreign gene in almost any specific tissue. Retroviruses or retroviarl vectors are superior when genetic tagging of chromosomal loci, for example for insertional mutagenesis, or of cells for lineage studies are desired. Finally, the most recently developed method of generating transgenic animals from ES cells allows in principle the derivation of mice with any genetic or phenotypic characteristics for when in vitro screeing or selection methods are available.

ACKNOWLEDGMENT

The author is thankful to Prof. J.P. Simons, AFRC Institute of animal Physiology and Genetics Research, Kings buildings, West Mains Road, Edinburgh EH9 3JQ, UK, for providing literature and reprints.

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