Genetic manipulation of mammals and its application in reproductive biology

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Introduction

During the past decade, revolutionary new opportunities in biological research have been created by bringing together techniques developed in two very different areas of biology: molecular biology and mammalian embryo manipulation. A gene from one animal can be isolated, multiplied and modified in the laboratory and transferred to another animal of the same or a different species (for a general review, see Palmiter & Brinster, 1986). More recently, endogenous genes in mammals have been modified by methods of gene targeting (reviewed by Capecchi, 1989a, b).

Gene transfer and gene targeting pave the way for a deeper understanding of the roles of specific genes and their products in the development and physiology of intact organisms and of

the mechanisms that regulate gene expression. Recombinant DNA techniques have allowed the cloning and precise determination of the structure of numerous genes. The reader is referred to Alberts *et al.* (1989) for a review of gene structure and function and to a recent review in this journal (Stewart *et al.*, 1988), which covers some of the topics with particular reference to reproductive biology. Many of these techniques are descriptive and allow documentation of the changes in gene expression, but do not give insight into the underlying regulatory mechanisms.

Gene transfer into cells in culture has long been used in the study of gene expression. This approach has been very successful, but there are numerous problems. Sometimes no cell lines are available which retain or approximate the characteristics of the tissue from which they derive, and many processes (such as reproduction) are not amenable to study *in vitro*. There are examples of in-vitro studies which gave clear results, but which, when extended into transgenic mice, gave quite different results (Brinster *et al.*, 1988; Dente *et al.*, 1988; Lorès *et al.*, 1988).

The aim of this review is to stimulate use of the new methods of genetic manipulation of mammals in studies of reproduction and development. Others have reviewed the methods of gene transfer, their general application (Palmiter & Brinster, 1986; Jaenisch, 1988; Hanahan, 1989) and their particular value in livestock (Land & Wilmut, 1987; Pursel *et al.*, 1987, 1989) or in the creation of models of human disease (Hooper, 1990). On the other hand, gene targeting in mammals is a very new technique, and less has been achieved (methods are reviewed by Frohman & Martin, 1989; Capecchi, 1989a, b; Rossant & Joyner, 1989).

The paper is in three main sections. The first describes the methods of gene transfer and manipulation. As there has been little research in relation to reproduction, general strategies for the application of methods of genetic manipulation are reviewed before a consideration of research specifically concerned with reproduction.

Methods of genetic manipulation

Two types of genetic manipulation are considered in this review, transgenesis and gene targeting. Transgenic animals have been defined as 'animals that have integrated foreign DNA into their germ-line as a consequence of experimental introduction of DNA' (Palmiter & Brinster, 1985). Recent developments also allow the introduction of specific mutations into mice by 'gene targeting'; while such experiments do lead to the introduction of foreign DNA, their purpose is to mutate an endogenous gene.

All of the established methods used for the production of transgenic animals involve manipulation of embryos. The reason for this is that, by introducing the foreign genes early in development, they become incorporated into a large proportion of the cells of the adult and, if incorporated into cells of the germline, transgenic lines can be bred. Similarly, gene targeting experiments involve the manipulation of cultured embryonic cells, followed by their reintroduction into embryos. Most work on transgenic mammals has been performed in mice, but transgenic animals of other species have also been generated. All gene targeting experiments so far have been performed in mice.

Microinjection

The principal method for the introduction of foreign genes into mammals is by microinjection of naked DNA into one pronucleus of fertilized eggs (Fig. 1). This technique of gene transfer was first described by Gordon *et al.* (1980) and soon followed by others (Brinster *et al.*, 1981; Costantini & Lacy, 1981; E. F. Wagner *et al.*, 1981; T. E. Wagner *et al.*, 1981). Detailed methods are provided by Hogan *et al.* (1986) and Allen *et al.* (1987).

Fertilized eggs are collected from mated mice, usually after superovulation. When pronuclei are visible, the eggs are immobilized on a holding pipette and DNA is injected into one pronucleus with a fine glass micropipette. Each egg receives 1–2 pl of DNA solution, which typically contains tens of

hundreds of molecules of DNA. The injected volume is sufficient to induce marked swelling of the pronucleus, and this swelling appears to be necessary for the success of microinjection (Brinster *et al.*, 1985). The eggs which survive injection (50–85%) are reimplanted into the oviducts of pseudo-pregnant foster mothers either on the day of injection or after culture; 10–30% develop to term. DNA is usually prepared from a tail biopsy and used to determine whether the injected DNA has been incorporated; 10–30% of the live-born (generation 0 (G_0)) mice should carry the injected DNA and thus be transgenic. This technique may appear inefficient, since only a small proportion of microinjected eggs become transgenic mice; but it is easily possible to microinject >200 mouse eggs in a single day and this should yield several transgenic animals.

Fig. 1. Gene transfer by direct injection. Embryos are usually recovered from donor females at the pronuclear stage of development. Each embryo is secured upon a holding pipette by suction to allow the introduction of a fine pipette into one of the pronuclei. About 10^{-9} ml of DNA solution is expelled, sufficient to cause distension of the pronucleus. In mice the pronucleus can be seen readily, especially if differential interference contrast optics are used, but in sheep this is more difficult. In pigs and cattle, the embryo must be centrifuged to stratify the cytoplasm and reveal the pronuclei. Embryos which survive microinjection are transferred into foster mothers which are pseudopregnant or synchronized, as appropriate. Following birth of animals derived from injected embryos, DNA is prepared from a sample of tissue or blood and analysed for the presence of the injected sequences.

Genes incorporated into mice following microinjection are almost invariably integrated into a chromosome, as is evident from analysis of the structure of the incorporated DNA (Brinster *et al.*, 1981; Gordon & Ruddle, 1981), from in-situ hybridization to metaphase chromosomes (Lacy *et al.*, 1983; Michalova *et al.*, 1988) and from breeding studies. Usually, the DNA is integrated into a single chromosomal site, although, in some transgenic animals, the DNA is integrated in more than one location (Lacy *et al.*, 1983; Wilkie *et al.*, 1986). The number of copies of the DNA integrated is variable, ranging from less than one copy to several hundred copies (Palmiter *et al.*, 1982; Chada *et al.*, 1985; Swift *et al.*, 1984; Allen *et al.*, 1988), although very high numbers of copies are unusual. When multiple copies are integrated, Southern blot analysis shows that the copies are usually in a tandem array, all in the same orientation (head-to-tail) (Brinster *et al.*, 1981; Gordon, 1986), although head-to-head and tail-to-tail arrangements have been documented (Shani, 1985). Several instances of interspersion of transgene and endogenous DNA sequences have been found (Wagner *et al.*, 1983; Covarrubias *et al.*, 1986; Wilkie & Palmiter, 1987). The endogenous sequences do not necessarily derive from the site of integration, but may originate from elsewhere in the genome. The chromosomal DNA which flanks the site of insertion may also be subject to rearrangements (Woychik *et al.*, 1985; Covarrubias *et al.*, 1986; Wilkie & Palmiter, 1987), including deletions and duplications. However, as the most detailed analyses of integrated DNA have all been performed on lines of transgenic mice with abnormal phenotypes which were subsequently found to be associated with insertional mutation, such a sample may be very biased. If separate DNA molecules are co-injected into pronuclei, they are frequently integrated together (Lo *et al.*, 1988). There is no clear specificity in site of integration: for example, by in-situ hybridization, Lacy *et al.* (1983) and

Michalova *et al.* (1988) have demonstrated integration of the injected DNA in diverse regions of the mouse genome. There is only one documented case of integration of injected DNA at the site of the endogenous gene. When a large systematic study was conducted to correct a deletion in the major histocompatibility class II E_a gene, > 10 000 eggs were injected, yielding > 500 transgenic mice, among which only one had incorporated the injected DNA at the site of the endogenous gene (Brinster *et al.*, 1989a). Injected DNA is generally assumed to be integrated into random sites, although the frequency of recovery of insertional mutations may indicate that DNA is integrated preferentially into active chromosomal domains.

The mechanism of integration of DNA into the chromosome is not understood. Linear DNA is integrated much more efficiently than supercoiled circular molecules (Brinster *et al.*, 1985) and it is inferred that free DNA ends are important for integration. The predominance of arrays of head-to-tail tandem repeats of injected DNA in transgenic mice shows that the formation of the array frequently proceeds by a mechanism other than random ligation of DNA. It has been suggested that some of the injected linear molecules circularize and recombine with linear molecules to form a linear array (Brinster *et al.*, 1985); homologous recombination has been demonstrated between co-injected DNA molecules that have a region of homology (Palmiter *et al.*, 1985). Ligation must play a part in the formation of head-to-head and tail-to-tail arrays and in the co-integration of different DNA molecules injected together. Brinster *et al.* (1985) proposed that DNA is integrated at the site of chromosomal breaks. This is consistent with the requirement for swelling of the pronucleus, which may lead to chromosomal damage and thus provide sites for integration. This hypothesis is supported by lines of mice which have reciprocal chromosomal translocations; the translocated chromosomes always segregate with the transgene and it is likely that translocation and integration occurred simultaneously (Overbeek *et al.*, 1986; Mahon *et al.*, 1988; Gordon *et al.*, 1989).

Most G₀ transgenic mice transmit the transgene to ~ 50% of their progeny (Wilkie *et al.*, 1986). This is consistent with the gene being incorporated into one homologue of a pair of chromosomes. In ~ 30% of G₀ transgenic mice, the gene is transmitted to significantly < 50% of progeny and sometimes to none. In mice where transmission is poor, it is inferred that the DNA was integrated after the first round of DNA replication, so that the animals are mosaics made up of some cells that carry the transgene and others that do not. After passage of the transgene through the germline, the progeny carry the transgene in every cell of both the soma and germline. The integrated DNA is usually inherited in a Mendelian fashion, and homozygous transgenic animals may be bred, except where a recessive lethal insertional mutation has occurred. Patterns of inheritance indicative of sex linkage have been observed (Krumlauf *et al.*, 1986; Andres *et al.*, 1987; Simons *et al.*, 1987). Although it has not been thoroughly investigated, in most cases the gross structure of the integrated DNA appears to be stable through generations (Palmiter *et al.*, 1982; Lacy *et al.*, 1983; Shani, 1986). Exceptions are limited to modifications attributable to genomic imprinting (see below) and to a few lines of transgenic mice which are particularly prone to rearrangements. Shani (1986) described one mouse line (CV1) in which the integrated DNA is occasionally deleted or amplified relative to that of the parents.

Gene transfer has been accomplished in mammals other than mice, namely pigs (Brem *et al.*, 1985; Hammer *et al.*, 1985; Vize *et al.*, 1988; Miller *et al.*, 1989), rabbits (Brem *et al.*, 1985; Hammer *et al.*, 1985; Knight *et al.*, 1988), sheep (Hammer *et al.*, 1985; Simons *et al.*, 1988; Murray *et al.*, 1989), cattle (Biery *et al.*, 1988; Roschlau *et al.*, 1989) and rats (Mullins *et al.*, 1990); in all cases, this has been achieved by microinjection of DNA into pronuclei or nuclei of early embryos (1–4-cell). Gene transfer into species other than mice suffers from several disadvantages. In mice, it is possible to select strains which give high yields of robust eggs (Brinster *et al.*, 1985), but this is not feasible in large animals. Embryos are more difficult to recover from large animals; and from species such as sheep and cattle, which have small litters, large numbers are difficult to obtain. With current knowledge, precise control of the timing of ovulation and fertilization is not possible, and thus embryos are recovered at a variety of stages, not just the pronuclear stage. There are differences

between species in the ease with which it is possible to see pronuclei. Pronuclei and nuclei are readily visible in mouse and rabbit eggs (Hammer *et al.*, 1985); in sheep, pronuclei and nuclei are more difficult to see, but most can be seen with differential interference contrast microscopy (Hammer *et al.*, 1985; Simons *et al.*, 1988). In pigs and cattle, the eggs are centrifuged (typically at 15 000 *g* for 3 min) to visualize pronuclei and nuclei. This treatment stratifies the cytoplasm separating the pronuclei and nuclei from the optically dense material (Wall *et al.*, 1985; Wall & Hawk, 1988). Remarkably, the viability of pig and cattle eggs appears not to be reduced by centrifugation.

Retroviral vectors

To discuss the use of retroviral vectors, some background of the life cycle of retroviruses is required (for more detail, see Varmus, 1983). Retroviruses have a single-stranded RNA genome, which after infection of the host cell is reverse-transcribed into DNA and integrated into a host chromosome in a single copy; in this form, it is known as a provirus. Provirus have an identical sequence at each end, the long terminal repeats (LTRs), that flank unique-sequence DNA which encodes viral proteins. A short duplication of chromosomal DNA is found at each end of proviruses. To complete the life cycle, the provirus is transcribed from a promoter in the 5' LTR and the RNA transcripts are used both as messenger RNA for the synthesis of viral proteins and as the genomic RNA to be packaged into virions. Assembled viral particles are budded from the surface of the infected cell. Infection of embryos with a wild-type retrovirus can result in integration and germline transmission of the provirus (Jaenisch, 1976).

Many natural variant retroviruses are known in which viral genes have been replaced by nonviral genes (particularly oncogenes). Such viruses are said to be 'defective' as they have lost the capacity to synthesize the viral proteins required for progression through the life cycle. These proteins can, however, be provided in *trans* by wild-type 'helper' viruses, allowing propagation of defective retroviruses. Particular sequences are required in *cis* for propagation of defective retroviruses, to achieve synthesis of the genomic RNA, packaging of the RNA into virions, reverse transcription and integration. The *cis* sequences required are the LTRs and some of the unique sequences. Defective retroviral vectors have been constructed (e.g. Cepko *et al.*, 1984; Yu *et al.*, 1986; Belmont *et al.*, 1988) by deletion of nonessential viral sequences, allowing foreign sequences to be inserted. The defective vectors can be propagated given appropriate help, for example, by propagation of wild-type virus with the vectors or by the use of specially constructed helper cell lines (Mann *et al.*, 1983). These lines express retroviral genes without the sequences required in *cis* for packaging into virions, and thus allow the production of 'helper-free' viruses.

Typically, for the production of transgenic mice by retroviral infection, 4–8-cell preimplantation embryos are collected, the zona pellucida is removed and the embryos are co-cultured with virus-producing cells for 16–24 h, after which they are implanted into foster mothers (Fig. 2). Early experiments (Jähner *et al.*, 1985; van der Putten *et al.*, 1985) employed recombinant retroviruses with help provided by wild-type virus. Such experiments result predominantly in infection by the helper virus and less frequent integration of the defective virus; the transgenic animals are usually viraemic because of the presence of the helper. Transgenic mice can be produced by infection with helper-free virus stocks, but at a lower frequency (van der Putten *et al.*, 1985; Rubenstein *et al.*, 1986; Soriano *et al.*, 1986; Stewart *et al.*, 1987).

The efficiency of production of transgenic mice by retroviral infection is similar to that obtained with microinjection and does not require micromanipulation of embryos; indeed it is possible to treat large numbers of embryos simultaneously. Transgenic mice produced by retroviral infection are, however, almost invariably mosaic, because infection occurs after several cleavages. Some animals transmit the integrated retrovirus to a small proportion of progeny, others do not transmit. Other drawbacks of this technique are the (nontrivial) requirement to build a recombinant retroviral vector for each gene construct to be transferred and the limited capacity of retroviral vectors

Fig. 2. Gene transfer by retroviral vector. Cleavage-stage embryos from which the zona pellucida has been removed are cultured with virus-producing cells. The recombinant viruses infect the cells of the embryo and the viral RNA is reverse-transcribed into proviral DNA; the DNA sequences are then inserted into host-cell DNA. After overnight co-culture, the embryos are transferred into the reproductive tract of foster mothers. As the proviruses lack sequences that direct the production of new virus, the virus is not able to reproduce and spread to other cells. An alternative to co-cultivation of embryos with virus-producing cells is microinjection of concentrated virus or of virus-producing cells under the zona pellucida. Transgenic animals are identified by analysis of DNA. Essentially, all transgenic animals derived by this method will be mosaic; nonmosaic animals may be generated by breeding.

for inserted sequences: vectors of >9.4 kb in total length are usually unstable or inviable (Gélinas & Temin, 1986).

Expression of sequences introduced by retroviral vector depends upon the inclusion of an internal promoter (see Jaenisch, 1988). Early experiments employed the viral LTR as the promoter of the gene of interest, but expression was not obtained. More recently, transgenes introduced by retroviral infection have been expressed in an appropriate manner when controlled by internal promoters, rather than by the LTR.

The use of retroviruses in transgenic animals has been reviewed by Jaenisch (1988). While retroviral infection has some distinct advantages, pronuclear injection is more appropriate for most purposes.

Embryonal stem cells

Although the techniques described above have contributed enormously to our understanding, they have several limitations. They are restricted to the addition of DNA sequences, so that it is not possible to produce recessive mutations other than by the chance occurrence of insertional mutagenesis. Since this is a random process, the target gene cannot be chosen. Although one instance of homologous recombination has been reported (see section on microinjection), DNA generally integrates at a nonhomologous chromosome site and frequently does so in the form of multiple copies. The integration site and number of copies cannot be characterized prior to producing an animal. Also, the methods are not readily applicable to the study of developmental dominant lethal changes, unless they can be introduced in a conditional form. These limitations do not apply to a more recently developed technique for germline manipulation using embryonal stem (ES) cell lines. These cell lines, established from peri-implantation blastocysts (Evans & Kaufman, 1981; Martin, 1981; reviewed by Robertson, 1987), have many properties in common with embryonal carcinoma (EC) lines established from the malignant stem cells of teratocarcinomas, including the ability to contribute to the somatic tissues of chimaeric animals following injection into host blastocysts. However, they have important advantages over EC cells in that lines with normal karyotypes can be readily obtained which are capable of colonizing the germ-cell lineage of chimaeric animals (Bradley *et al.*, 1984). This raises the possibility of using the techniques of somatic cell genetics to isolate ES cells with any of a variety of genetic modifications and then introducing these modifications into the germline (Fig. 3).

The first ES cells to be isolated were of mouse origin and were maintained by growth on a feeder layer of fibroblasts treated with mitomycin C (Evans & Kaufman, 1981; Martin, 1981). The presence of feeder cells was necessary to prevent the ES cells undergoing spontaneous differentiation. However, the selective isolation of genetically modified cells is complicated by a feeder layer and was greatly facilitated by the discovery that the feeder cells can be replaced by medium conditioned by the Buffalo rat liver cell line BRL (Smith & Hooper, 1987). More recent work (Moreau *et al.*, 1988; Smith *et al.*, 1988; Williams *et al.*, 1988) has shown that the requirement can be satisfied by a purified factor, variously designated on the basis of its other activities as LIF (leukaemia-inhibiting factor) or HILDA (human interleukin for DA cells). This factor is produced by a number of cell lines, including embryonic fibroblast lines and a line derived from parietal endoderm. It is synthesized in both matrix-associated and diffusible forms: these are generated by use of alternative promoters (Rathjen *et al.*, 1990), a feature likely to be important in achieving the correct spatial and temporal pattern of differentiation in the normal embryo.

The isolation of ES cell lines is more technically demanding than their maintenance. ES cells are thought to be derived from a subpopulation of cells, the epiblast (often termed embryonic ectoderm, although this is a misnomer, since it gives rise during subsequent development to all foetal tissues and some components of the extraembryonic membranes) in the inner cell mass of the late blastocyst. In the original isolation procedures, two approaches were employed. First, blastocysts subjected to implantational delay by ovariectomy were allowed to attach and outgrow in culture to expose the inner cell mass, which was then picked off, disaggregated and subcultured on feeder layers (Evans & Kaufman, 1981). Secondly, inner cell mass cells were isolated by immunosurgery and cultures established on feeder layers in medium conditioned by an EC cell line (Martin, 1981). Subsequent work has shown that neither implantational delay nor the use of EC-conditioned medium is essential. Although modifications involving the use of small volumes of medium (Axelrod & Lader, 1983; Axelrod, 1984), primary fibroblast feeder layers (Doetschman *et al.*, 1985) and a combination of BRL-conditioned medium and feeder layers (Handyside *et al.*, 1989) have been reported to give improved results, the efficiency with which ES cell lines can be obtained is still variable. The availability of purified LIF should facilitate the solution of this problem and, indeed, it has recently been possible to isolate mouse ES cells capable of germline colonization by plating blastocysts, or epiblast cells derived from them by immunosurgery, directly into LIF-supplemented medium in the absence of feeder cells (Nichols *et al.*, 1990; Pease *et al.*, 1990). A cell line with particularly high potential for colonizing the germline has been isolated by the use of feeder cells stably transfected with a LIF expression vector (McMahon & Bradley, 1990).

On subculture in the absence of feeder cells, BRL-conditioned medium or LIF, mouse ES cells undergo differentiation to a broad spectrum of cell types. Conditions particularly favourable to differentiation are obtained by culturing aggregates of cells in suspension and then allowing outgrowth on a surface (Martin & Evans, 1975). This is valuable in allowing a preliminary assessment of developmental capacity before resources are invested in the production of chimaeras.

In species other than mice there has been less progress in ES cell isolation and there is no other species in which ES-cell-derived chimaeras have been reported. Cell lines have been isolated from embryos of golden hamster (Doetschman *et al.*, 1988b), pig (McWhir *et al.*, 1988; Piedrahita *et al.*, 1988, 1990a, b; Evans *et al.*, 1990; Notarianni *et al.*, 1990a, b; Strojek *et al.*, 1990), sheep (Piedrahita *et al.*, 1990b; Notarianni *et al.*, 1990a) and cow (Evans *et al.*, 1990). Since the only published assessment of the lines has been of their morphology and ability to differentiate in culture, with no evidence as yet that they are capable of multilineage contribution to chimaeras, it is difficult to be certain of their nature.

Cell lines from golden hamster embryos are similar in appearance and differentiation *in vitro* to mouse ES cells (Doetschman *et al.*, 1988b). By contrast, the cell lines isolated from agricultural species are of two types: some are similar in appearance to mouse ES cells while others are epithelial-like. The latter cells are flattened and, when grown to confluence, form structures resembling epithelial sheets. Attention has been drawn to differences between lines in the size of the cells

(Notarianni *et al.*, 1990b). Although some lines undergo differentiation *in vitro*, their development differs from that shown by mouse ES cells. It has been suggested that the variation in pattern of differentiation of these cell lines may reflect differences in development between the species (Evans *et al.*, 1990). It is not clear whether the poor success rate in species other than mice is due to fundamental biological differences between species or simply to technical factors associated with the optimization of culture conditions.

The ES cell approach to germline manipulation is particularly well suited to the introduction of recessive mutations into the germline. Because of two technical advantages, the first locus to be exploited in this way was the *hprt* locus, which codes for hypoxanthine phosphoribosyltransferase in the purine salvage pathway. First it is X-linked, so that male cells are hemizygous and HPRT⁻ mutants arise spontaneously and at relatively high frequency. Secondly, such mutants can be efficiently selected by resistance to the purine analogue 6-thioguanine. Three *hprt*⁻ alleles have been

introduced into the mouse germline: one was a spontaneous partial deletion (Hooper *et al.*, 1987; Thompson *et al.*, 1989) and the other two were produced by retroviral insertion mutagenesis (Kuehn *et al.*, 1987). To generalize this approach to genes which are neither X-linked nor counterselectable on the basis of expression of the gene product, a different strategy for obtaining ES cell mutants is needed.

A method which shows great promise is gene targeting by homologous recombination between a native target chromosomal gene and exogenous DNA (Smithies *et al.*, 1985; see recent reviews by Frohman & Martin (1989), Capecchi (1989a, b) and Rossant & Joyner (1989). Depending upon the design of the targeting DNA, it is possible to achieve either insertion into the chromosomal DNA (e.g. Fig. 4a) or replacement of a segment of chromosomal DNA (e.g. Fig. 4b). These manipulations may be carried out to abolish gene expression, modify the pattern of expression of the gene or change the gene product.

Although replacement events appear to require two separate crossover events, they proceed at frequencies similar to those of insertion events, which require only one (Thomas & Capecchi, 1987). Possibly they proceed via a single-strand exchange within one region of homology, followed by branch migration through the nonhomologous insert and then resolution at the second region of homology, so that a single crossover event suffices (Ellis & Bernstein, 1989; Fig. 5).

Gene targeting has been used to correct the partial deletion mutant *hprt*^{b-m3} allele in ES cells (Doetschman *et al.*, 1987), and to inactivate the *hprt* gene in ES cells (Thomas and Capecchi, 1987). In both studies, the targeting DNA was introduced into the cells by electroporation, and homologous recombinants were obtained at frequencies of the order of 10⁻⁶, so that selection on the basis of HPRT phenotype was necessary to isolate them. However, at least for genes expressed in ES cells, a cell population can now be enriched for the products of homologous recombination events using selective procedures which do not depend upon the particular properties of the target locus. One such approach is to incorporate into the targeting vector a selectable marker whose expression requires transcription from an active chromosomal promoter (promotorless *neo*:

Fig. 3. Strategy for germline manipulation using embryonal stem cells. Shading patterns are used to indicate mice or embryos of different genotypes or the introduction of genetic change. Blastocysts from an inbred mouse strain homozygous for recessive mutations which produce a light coat colour are isolated (1) and used to derive a male ES cell line (2). ES cells are modified by techniques such as gene targeting (3) and the cells with the desired change (shown stippled) are identified or selected to provide a population of cells each of which has the modification (4). These cells are then introduced into the cavity of a blastocyst of a dark coat-colour genotype (5) where the ES cells colonize the inner cell mass (6 and 7). The injected blastocyst is then transferred to the uterus of a foster mother (8) and allowed to develop to term, producing a chimaera (9). These animals have cells derived from two embryos: those from the strain with the dark coat and cells from the strain with the light coat from which the ES cells were derived and into which the genetic change has been introduced. Male chimaeras are tested for germline chimaerism by mating to a female of the strain originally used to derive the ES cell line (10). Three types of mice may be born. Progeny resulting from fertilization by host blastocyst-derived sperm show the dominant, dark coat colour (11) while those produced by ES-cell-derived sperm show the recessive light coat colour (12 and 13). Half (statistically) of all offspring derived from the ES cells have the modification on one of a pair of chromosomes. These individuals are identified by Southern blotting of DNA obtained by tail biopsy (13).

Because the same strain is used for ES-cell isolation and mating to the chimaera, heterozygous offspring are coisogenic with this strain, i.e. they carry the genetic modification on the same inbred background. Coisogenic homozygotes can be derived from the heterozygotes by standard animal breeding. In the special case of mutations on the X chromosome, all the first-generation female ES-cell-derived progeny of the chimaera are heterozygous and all males wild-type. A useful strategy in this situation is to mate female heterozygotes back to the chimaera, since progeny of the chimaera's ES-cell-derived spermatozoa can again be identified by coat colour and will, in the absence of any lethality, include homozygous mutant females and hemizygous mutant males.

Fig. 4. Examples of gene targeting. (a) Correction of a mutant *hprt* allele (Thompson *et al.*, 1989). The wild-type *hprt* gene has nine exons (i) and the mutant allele has a deletion encompassing exons 1 and 2, the *hprt* promoter and at least 10 kb of upstream sequence (ii). The correcting vector contains the missing exons and a region of homology, including exon 3, into which a restriction enzyme cut is introduced. Simple insertion of the vector by homologous recombination generates a functional corrected gene and a nonfunctional upstream copy of exon 3 (iv). The corrected cells used for germline transmission had undergone a deletion of the upstream copy of exon 3 while the corrected gene remained intact (v). Nonhomologous integration cannot generate a functional gene, so that cells which have undergone the homologous recombination event can be selected directly on the basis of HPRT activity, conferring ability to grow in HAT selective medium.

(b, c) Enrichment for cells which have undergone homologous recombination in a gene whose inactivation confers no selectable phenotype. The vector design (b(i)) allows selection to be applied for integration and against nonhomologous integration (Mansour *et al.*, 1988). It is designed to replace part of the target gene (b(ii)) with DNA containing a functional *neo* gene placed between two segments of homology with the target gene in such a way as to interrupt an exon. It also contains a copy of the *HSVtk* gene placed outside the region of homology. Expression of a *neo* gene renders cells resistant to geneticin, whereas *HSVtk* expression confers sensitivity to ganciclovir. Homologous integration (b(iii)) results in incorporation of the *neo* gene, but not the *HSVtk* gene, so that cells become resistant to geneticin, but remain insensitive to ganciclovir. However, random integration (c) in general occurs via the ends of the linearized DNA, so that both the *neo* and *HSVtk* genes are incorporated and the cells become geneticin resistant and ganciclovir sensitive. Selection in geneticin plus ganciclovir therefore results in enrichment for cells which have undergone homologous recombination.

Fig. 5. Single-crossover model for a replacement event. The two DNA strands of the replacement vector (a) and the target chromosomal gene (b) are shown separately. The event is initiated by a single-strand crossover within the left-hand region of homology (1). This is followed by a branch migration through the nonhomologous insert into the right-hand region of homology (2). Resolution by a second crossover in the same strand within the right-hand region of homology (3) yields heteroduplex vector (c) and chromosomal (d) DNA. Replication (not shown) of the heteroduplex chromosomal DNA then yields one targeted and one parental DNA molecule, which segregate into different daughter cells at mitosis.

Doetschman *et al.*, 1988a; Schwartzberg *et al.*, 1989; Charron *et al.*, 1990). Another is to use a vector which allows selective pressure to be applied simultaneously for an integration event and against nonhomologous integration (Mansour *et al.*, 1988; Fig. 4c). This approach, termed positive-negative selection (PNS), has been successfully applied to the targeting of genes that are not detectably expressed in ES cells (Johnson *et al.*, 1989). Such enrichment procedures can be made so efficient that it becomes feasible to screen for mutant cells by direct examination of the DNA by Southern blotting, although there are indications that the efficiency of the enrichment

procedure varies with the target site. An alternative approach is to detect small numbers of targeted cells in a treated population using the highly sensitive polymerase chain reaction (PCR; Kim & Smithies, 1988; Fig. 6) and to use the classical technique of sib-selection (Cavalli-Sforza & Lederberg, 1956; Fig. 7) to enrich progressively for these rare cells until they can be isolated by cloning. This method has been successfully applied to the *Hox* 1.1 gene after introducing the targeting DNA by microinjection, which results in a much higher frequency of integration events than does electroporation (Zimmer & Gruss, 1989). A combination of selective and PCR approaches was used by Joyner *et al.* (1989), who targeted the *En-2* gene by using electroporation to introduce the targeting vector, selecting for integration and then using PCR and sib-selection to isolate homologous integrants from the selected population. A similar approach was used by Zijlstra *et al.* (1989) and by Koller & Smithies (1989) to target the β -2 microglobulin gene.

Two methods are available for the production of ES cell chimaeras, namely microinjection of ES cells into the cavity of a host blastocyst and aggregation of ES cells with morulae (reviewed by Bradley, 1987). There have been two reports of germline transmission of ES cell markers from aggregation chimaeras (Wagner *et al.*, 1985; Nagy *et al.*, 1990), but most germline chimaeras have been produced by the blastocyst injection method, which, although it requires specialized equipment, presents fewer technical problems in embryo handling. The strain of host blastocyst has a marked effect on the proportion of chimaeras transmitting markers derived from ES cells. Much higher frequencies of transmission are obtained using blastocysts of the inbred strain C57BL/6 than using blastocysts of the outbred strains MF1 and CD1 (Schwartzberg *et al.*, 1989; DeChiara *et al.*, 1990). This may be related to the observation that, in aggregation chimaeras produced from C3H and C57BL/6 embryos, the cells derived from C57BL/6 competed poorly in the male germ cell lineage (Mintz, 1968). However, the difference in degree of germline chimaerism between chimaeras produced using C57BL/6 and F2(C57BL/6 $\times$ CBA) blastocysts is not nearly as marked (P. Laird *et al.*, unpublished), suggesting that the avoidance of outbred blastocysts may be what is important. ES cells carrying a spontaneous partial *hprt* deletion gave similar germline colonization frequencies to those obtained with wild-type cells (Hooper *et al.*, 1987).

Initial attempts to obtain germline chimaerism with mouse ES cells following gene targeting gave disappointing results, but successful germline transmission has now been achieved with a number of targeted alleles (Table 1). While the improvement has resulted partly from dissemination of expertise in ES cell handling and partly from the use of C57BL/6 host blastocysts (see above), it has also become clear that certain parameters of the targeting procedure itself require careful optimization to ensure the retention of germline-colonizing potential. In all the cases listed in Table 1, the DNA was introduced by electroporation. This has the advantage that a large number of cells can be treated, but it is relatively inefficient, particularly if treatments minimizing cell trauma are used. Selective methods are therefore difficult to avoid. Selective agents need to be chosen with care to ensure that surviving cells have not received sublethal doses of mutagenic agents. The positive-negative selection procedure of Mansour *et al.* (1988) involves the use of the drug ganciclovir to select against the integration of a copy of the herpes simplex virus thymidine kinase (HSV tk) gene built into a targeting vector (Fig. 4b, c). Ganciclovir is phosphorylated to a small degree by the cellular thymidine kinase and the triphosphate is incorporated into DNA (StClair *et al.*, 1987), and so exposure of cells to ganciclovir should be minimized to avoid the introduction of mutations compromising germline-colonizing potential. Care should also be exercised in applying the promotor-less *neo* selection procedures to target genes expressed at a low level in ES cells. If the target gene is more strongly expressed in differentiated derivatives of the ES cells, the selection procedure may enrich for these derivatives at the expense of the totipotent cells. The PCR/sib selection approach to mutant isolation has the theoretical disadvantage of requiring more cell multiplication. However, as PCR is such a sensitive technique, this can be contained within satisfactory limits. Numerous variations of the above procedure, such as the use of different selectable markers, have been successfully used to isolate targeted cells, but their compatibility with germline transmission has yet to be demonstrated.

Fig. 6. Use of the polymerase chain reaction to detect the presence of cells which have undergone homologous recombination. Synthesis of DNA by a thermostable DNA polymerase is primed by oligonucleotides A and B. A hybridizes uniquely to a sequence on the targeting vector and B to a sequence on the opposite strand of the endogenous target gene. Polymerase and an excess of A and B are added to DNA from the test population, and a series of cycles is carried out, each consisting of heat denaturation of the DNA, annealing to allow primer binding and then DNA synthesis. Where the primer binding sites are brought into proximity on the same DNA molecule by the homologous recombination event, the sequence between them is amplified exponentially (left panel), while in the case of random integration the corresponding sequence is amplified only linearly. After a large number of cycles the exponentially amplified DNA may be detected directly by staining after gel electrophoresis, or by hybridization. In principle, it should be possible to detect a single targeted cell against a background of 10^4 or more cells carrying nontargeted integrations.

Introduction of DNA by microinjection is labour-intensive and needs specialized equipment and expertise, but it produces much higher integration frequencies than does electroporation and may give a higher ratio of homologous to nonhomologous integration (see Zimmer & Gruss, 1989). This means that selectable markers are unnecessary, so that the amount of nonhomology between vector and target gene can be very short, which in turn would be expected, on the basis of the model depicted in Fig. 5, to increase the frequency of homologous recombination (cf. Letsou & Liskay, 1987). Since there is no need to include the sequences for a selectable marker in the vector, it is also feasible to use vectors designed to introduce minor changes to the structure of the gene, rather

Fig. 7. Use of sib-selection in conjunction with the polymerase chain reaction (PCR) to isolate cells which have undergone homologous recombination. A test population of cells is subcultured and one of the two replicate cultures is used to establish the presence of the targeted cells ($\blacktriangle$) by the PCR method. If targeted cells are found, the other replicate is grown and used to inoculate a series of sib cultures at such a density that only a fraction of the cultures will contain targeted cells. After further growth, each culture is sampled for PCR analysis. Some cultures give a negative result, while others give a positive result and are enriched for targeted cells. The process is repeated until the population is sufficiently enriched for the targeted cells to be isolated by cloning. Because PCR is such a sensitive technique, the amount of cell multiplication required is minimal.

than being restricted to the abolition of gene expression. However, the very efficiency of the microinjection process may be a disadvantage, since the likelihood of unwanted second-site integrations in the targeted cell may be higher, and there may be a risk of concatemerization of the DNA before integration, as well as the occurrence of rearrangements. Germline transmission has not yet been achieved using targeted ES cells produced by microinjection.

It would clearly be advantageous if mutant mice could be produced directly from targeted ES cells without an intervening chimaeric generation. Newborn mice derived entirely from ES cells can indeed be produced by aggregating ES cells with tetraploid 4-cell embryos, but none has yet survived more than 2 days after birth (Nagy *et al.*, 1990). Whether this reflects a fundamentally compromised developmental potential in ES cells, or simply technical problems, remains to be established. In species other than mice, an alternative means of using modified ES cells to colonize the germline may be possible by transferring ES cell nuclei into enucleated oocytes or 1- or 2-cell embryos. In mice, such an approach is unlikely to succeed with present techniques because nuclei transferred from morulae or inner-cell-mass cells are unable to support normal development

Table 1. References to studies of germline transmission of targeted genes

Reference	Gene	Selective system
Thompson <i>et al.</i> , 1989	<i>Hprt</i>	HAT*
Koller <i>et al.</i> , 1989	<i>Hprt</i>	HAT
Schwartzberg <i>et al.</i> , 1989	<i>c-abl</i>	promoter-less <i>neo</i>
Zijlstra <i>et al.</i> , 1989, 1990	β -2 microglobulin	<i>neo</i> /PCR†
Koller <i>et al.</i> , 1990	β -2 microglobulin	<i>neo</i> /PCR
A. Joyner <i>et al.</i> , unpublished	<i>En-2</i>	<i>neo</i> /PCR
DeChiara <i>et al.</i> , 1990	IGF-II	PNS‡
Stanton <i>et al.</i> , 1990	<i>N-myc</i>	promoter-less <i>neo</i>
C. Moens <i>et al.</i> , unpublished	<i>N-myc</i>	promoter-less <i>neo</i>
Thomas & Capecchi 1990	<i>int-1</i>	PNS
McMahon & Bradley 1990	<i>int-1</i>	PNS
P. Laird <i>et al.</i> , unpublished	<i>pim-1</i>	PNS

*HAT = hypoxanthine + aminopterin + thymidine.

†PCR = polymerase chain reaction.

‡PNS = positive-negative selection.

(McGrath & Solter, 1984a; Tsunoda *et al.*, 1989). However, in ruminants the loss of this capacity does not occur as early in embryogenesis (Willadsen, 1986) and young have been born following the transfer of nuclei from cells of the inner cell mass to enucleated oocytes (Marx, 1988; Smith & Wilmut, 1989).

While there are still problems to be solved in extending the range of genes which can be targeted and species from which ES cells can be isolated, recent success encourages the prediction that ES cells will find widespread application in germline manipulation, with substantial advantages over other techniques for the production of transgenic animals. However, where chromosomal integration site is of secondary importance, pronuclear microinjection will continue to be the method of choice for analysing the effect of introducing exogenous DNA sequences into the germline.

Spermatozoa as vectors

An alternative approach to gene transfer was described recently in which sperm were incubated with DNA before in-vitro fertilization (Lavitrano *et al.*, 1989). The authors described the uptake of labelled DNA by mouse sperm which had been incubated with DNA. Production of transgenic mice was reported by in-vitro fertilization with sperm after such incubation, followed by transfer of fertilized eggs into recipient mice. Of 250 progeny, about 30% were transgenic, although the authors gave no indication of the number of copies incorporated. Germline transmission and expression of the incorporated gene were also reported, although in many cases the incorporated DNA was rearranged. The same group published a preliminary report of the production of transgenic pigs by incubation of spermatozoa with DNA, followed by deposition of the spermatozoa in the oviduct during surgery (Gandolfi *et al.*, 1989). Many laboratories have attempted to repeat the production of transgenic mice by the use of spermatozoa as vectors, using in-vitro fertilization (Brinster *et al.*, 1989b) or artificial insemination (Al-Shawi *et al.*, 1990a), but with no success. The reason for the discrepancy is unclear.

Factors affecting the expression of transgenes

In early studies of gene expression in transgenic mice, genes were expressed aberrantly, or not at all (e.g. Lacy *et al.*, 1983). Many of the early experiments were concerned with the expression of globin genes and expression of these genes is severely inhibited by the presence of bacterial cloning vector DNA (Chada *et al.*, 1985). The α -fetoprotein gene is similarly influenced by vector DNA

(Hammer *et al.*, 1987), but the elastase gene is insensitive to this effect (Swift *et al.*, 1984). The mechanism by which vector sequences interfere with expression and the generality of the effect are unknown, but as a precautionary measure the DNA segments to be injected should invariably be released from and purified away from the cloning vector.

The level of transgene expression is generally similar in mice from a transgenic line, but usually differs between lines of transgenic mice which carry the same transgene (see Palmiter & Brinster, 1986). The differences in expression must reflect variation in the structure of the integration site and could be due to differences in the number of integrated copies, the precise arrangement of the integrated DNA, mutation or modification of the transgene, or the nature of the chromosomal segment into which the gene is integrated. In two cases, DNA sequence elements have been identified which overcome the effects of position or arrangement (Grosveld *et al.*, 1987; Lang *et al.*, 1988). These elements, from the β -globin locus and the CD2 gene, respectively, are known as 'dominant control regions', their inclusion in the transgene results in levels of expression which are proportional to the number of copies of the transgene. Transcription from each copy of the gene is approximately equal to that of the natural gene. The effects of the dominant control regions are tissue-specific. It remains to be determined whether similar elements are associated with other genes. In the absence of such elements, the variability of expression among lines dictates that several independent transgenic lines be characterized before general conclusions can be drawn regarding the activity of a transgene. The pattern of expression in different tissues is usually the same in all of the lines of mice carrying the same transgene, but there are exceptions (e.g. Al-Shawi *et al.*, 1990b; Archibald *et al.*, 1990).

Another factor which can have major effects upon the expression of transgenes is the presence of introns. This was shown particularly clearly in the study of Brinster *et al.* (1988), when a number of lines were created carrying the same transgene with or without introns. The level of expression in transgenic mice was consistently higher when introns were present, although there was little effect on expression in transfected cell lines. The mechanisms underlying these phenomena are not understood.

Choice of species

Most transgenic mammals are mice. Mice are excellent animals for studies on gene expression or immunology, for example, but for many other purposes they are not ideal. It is likely that studies involving physiological measurements will be performed in larger animals, and rats are likely to assume a particularly important role. While they are large enough to facilitate some surgical procedures which are difficult in mice and to allow collection of serial blood samples, the costs of experimentation are not prohibitive. Experiments in livestock species, with the current technology, are likely to be primarily limited to those involved in practical applications.

Assays for transgene expression

One practical consideration which is common to most studies on transgenic animals is the need to distinguish between expression of endogenous genes and of the gene which is introduced. This can be accomplished in a variety of ways. At the RNA level, Northern analysis is appropriate provided that the sequences of the transgene are significantly different from the endogenous genes; expression of more similar genes can be distinguished by RNase protection or S1 analysis. Reverse transcription followed by PCR can also be used and is of particular value when the amounts of material are limiting, but accurate estimation of expression levels with this method is much more difficult. In-situ hybridization to RNA in tissue sections is also used and can give information about the cell-type specificity of expression.

At the protein level, there are two ways to meet this need: a gene may be transferred that encodes either a protein with different electrophoretic properties or an unrelated protein which can be assayed in some way (e.g. by virtue of biological or enzymatic activity or by the use of specific

antisera) and which acts as a reporter. Common reporter genes are human (and other) growth hormone genes, oncogenes, bacterial genes encoding β -galactosidase (*LacZ*) and chloramphenicol acetyltransferase (CAT), and the thymidine kinase gene of herpes simplex virus (*HSV-tk*). The last three are used because they encode enzymes which are simple to assay; β -galactosidase is not only easy to assay, but can also be assayed *in situ*. Growth hormone expression results in larger mice and can be detected by in-situ immunostaining, while oncogene expression frequently results in predisposition to tumour formation in the organ where the gene is expressed. Tumours do not usually result from oncogene overexpression alone, but require at least one more event, probably somatic mutation.

Caution must be exercised in the interpretation of results obtained with reporter genes; in at least two cases, reporter gene sequences have been found to influence the tissue distribution of expression. Hybrid genes employing the human growth hormone (*hGH*) gene as a reporter are frequently expressed in pituitary gonadotroph cells. This is due to the presence of sequences within the 3' flanking region of the *hGH* gene (Low *et al.*, 1989). Similarly, unexpected expression of the *HSV-tk* gene is frequently seen in the testes of transgenic mice when it is used as a reporter (see section on spermatogenesis).

Research strategies using genetically manipulated animals

There are a number of research applications where germline manipulation has made uniquely valuable contributions. The introduction of a gene into the germline is the only reliable way of studying the mechanisms of developmental regulation of gene expression. In this way, it has been shown for a variety of genes that tissue-specificity of expression is dependent upon enhancer sequences which in some cases are close to the promoter and in others are separated from it by several kilobases and may be upstream or downstream of the gene or contained within an intron (reviewed by Jaenisch, 1988). A further level of complexity has been revealed in the case of the β -globin gene cluster, whose component genes code for embryonic, foetal and adult polypeptides. While enhancer elements at the 5' and 3' ends of the genes were sufficient for correct tissue- and stage-specific expression, absolute levels of expression were low and variable. These anomalies were rectified when ~20 kb of sequences from either side of the β -globin gene cluster were placed on either side of the β -globin gene in the injected DNA (Grosveld *et al.*, 1987). This work led to the identification of a 'dominant control region' within 6.5 kb of sequence at the 5' end of the upstream flanking sequence, which encompasses four erythroid cell-specific DNase-I hypersensitive sites (Talbot *et al.*, 1989). If the dominant control region and the structural genes for γ and β -globin are present in the same transgene, expression from the transgene undergoes switching from γ to β -globin in parallel with the developmental switch in expression of the endogenous genes. This suggests that temporal regulation results from competition between genes in the cluster for interaction with the dominant control region, this competition being influenced by *trans*-acting factors (Behringer *et al.*, 1990; Enver *et al.*, 1990).

Ablation of tissues

An elegant means of ablating a selected tissue in order to study its function is to introduce into the germline a toxin-coding gene under the control of a tissue-specific promoter (reviewed by Beddington, 1988). Diphtheria toxin synthesis under the control of the pancreatic elastase promoter has been used to ablate the pancreas, and under the control of the γ -2-crystallin promoter to ablate the lens. A model of pituitary dwarfism has been produced by using the promoter sequence of the rat growth hormone gene to target diphtheria toxin synthesis to the somatotrophic cells of the pituitary of transgenic mice (Behringer *et al.*, 1988).

An extension of this approach in which the tissue-specific toxicity can be controlled is the use of the *HSV-tk* gene under the control of the immunoglobulin κ light-chain promoter and μ heavy-chain

enhancer to confer on lymphocytes sensitivity to the drug 1 (2-deoxy-2-fluoro- β -D-arabinosyl)-5-iodouracil (FIAU). When mice transgenic for this construct were exposed to FIAU using an osmotic minipump, the spleen and thymus underwent atrophy and the numbers of circulating B and T lymphocytes were reduced (Borrelli *et al.*, 1988). Subsequent refinement of this approach using a modified transgene and substituting ganciclovir for FIAU led to depletion of >99% of B and T lymphocytes, but progenitor cells remained which could repopulate all the T-cell lineages within a week of drug removal (Heyman *et al.*, 1989). Mice transgenic for HSV-*tk* under the control of the rat growth hormone promoter show dwarfism, with depletion of somatotrophes and lactotrophes from the anterior pituitary, when FIAU treatment is initiated 5 days after birth or earlier, but not when it is delayed until 10 days after birth. On removal of FIAU, the pituitary is repopulated with somatotrophes and (more slowly) lactotrophes. These results are interpreted in terms of a bipotential stem cell (Borrelli *et al.*, 1989). However, a potential complication of the application of this method to cells in solid tissues is that cells in communication with target cells via gap junctions will receive toxic nucleotide by metabolic co-operation (Hooper, 1982) and will also be ablated.

Cell lineages

Exogenous genes provide tracers which may be used to follow cell lineages. Lo *et al.* (1987) produced, by pronuclear microinjection, mice mosaic for an insertion of multiple tandem copies of the mouse β -globin gene, making it possible to follow cell lineages in these mice by in-situ DNA-DNA hybridization. Germline transmission of the DNA insert was obtained, making available a line of transgenic mice that can be used to construct chimaeras for lineage analysis by this method. By counting the number of foci of hybridization in mouse trophoblast giant cells, it has been shown that the increase in DNA content which occurs in these cells is a result of polyploidy rather than polyploidy (Varmuza *et al.*, 1988). Beddington *et al.* (1989) produced a transgenic mouse strain carrying 6 copies of the β -galactosidase gene from *Escherichia coli* under the control of the rat β -actin promoter. Although expression of the transgene was variable after mid-gestation, β -galactosidase activity was histochemically detectable throughout the hemizygous early post-implantation conceptus. In post-implantation grafts of transgenic cells, the expression of the transgene was cell-autonomous, so that lineage analysis was possible in grafted embryos at mid-gestation by histochemical staining.

While the mosaicism inherent in the use of retroviral vectors for gene transfer is disadvantageous for germline transmission, it has been of some value in lineage studies (Soriano & Jaenisch, 1986). Retroviral and other markers may also be introduced into post-implantation embryos (see Beddington & Lawson, 1990).

Identification of new genes

The introduction of a transgene may disrupt an endogenous gene (insertional mutagenesis). About 5% of insertions produce recessive mutations with a readily detectable phenotype, the majority being embryonic lethals. This strategy is being used in a systematic manner as a means of identifying new genes (reviewed by Jaenisch, 1988). However, the analysis of the effects of such mutations, at least for those generated by pronuclear microinjection, may be complicated by complex chromosomal rearrangements involving many genes (see earlier discussion). Retroviral infection, on the other hand, leads to precise integration of a single copy of the vector, simplifying the analysis of insertional mutations.

An interesting extension of the use of transgenes for insertional mutagenesis is the use of transgenic mice carrying *E. coli* sequences coding for β -galactosidase (*lacZ*) to identify developmentally significant genes. The product of the transgene can be detected histochemically. In the first case studied, the construct was inserted into the *dystonia* locus. Embryos homozygous for this mutation die in mid-gestation because of neural tube defects. In the heterozygote carrying the *lacZ* construct, the β -galactosidase gene is expressed in the neural tube, probably under the influence of

dystonia regulatory elements, so that it can act as a reporter for the activity of the normal gene (Kothary *et al.*, 1988).

Following this chance observation, an elegant method has been established for production of insertions in developmentally significant genes (Gossler *et al.*, 1989; reviewed by Rossant & Joyner, 1989). Two types of reporter construct have been devised, one designed to detect the presence of an enhancer and the other to identify sites of active transcription (Fig. 8). The reporter construct is introduced into ES cells and the resulting cell lines are screened by injection into blastocysts and histochemical examination of midgestation chimaeric embryos. Cell lines resulting in tissue- and/or stage-specific activation of the reporter are then chosen for further analysis. This approach has a unique role in the identification of new genes with important functions in development; however, novel patterns of expression may be induced by the presence of the inserted sequences or by rearrangements occurring at the time of integration. The flanking sequences must be cloned, and developmentally regulated expression of the endogenous gene must be confirmed.

Modification of gene function

An alternative approach to interfering with the function of an endogenous gene is to introduce a transgene with a dominant negative effect. Where the gene product is a multimeric protein, the expression of a transgene producing a modified monomer may cause the normal gene product to be captured in nonfunctional aggregates, as occurs in mice transgenic for an $\alpha 1(I)$ collagen gene subjected to site-directed mutagenesis *in vitro*. These mice die perinatally and are a model for the human autosomal dominant condition osteogenesis imperfecta type II (Stacey *et al.*, 1988). A dominant negative effect operating by a different mechanism is that of a transgene coding for a β -subunit of the $\alpha\beta$ T-lymphocyte receptor with a deletion of a major part of the variable region. The deletion allele is capable of mediating allelic exclusion and so inhibits rearrangement of the endogenous β -subunit genes, blocking T-cell differentiation (Krimpenfort *et al.*, 1989).

Where the dominant effect of the gene product is lethal, transgenic lines cannot be established, so that each affected animal is the result of a different integration event (Stacey *et al.*, 1988). Two approaches can be used to circumvent this problem. One is to use the ES cell route to produce more than one animal from a single cell line with a characterized transgene integration site and number of copies. The other is to use a transgene whose expression is conditional. Conditional expression has been achieved in one case by use of a promoter whose expression requires a *trans*-acting factor produced only in a second transgenic line, so that the gene product is only made in F_1 hybrids between the two transgenic lines (Byrne & Ruddle, 1989).

Another approach is to use an antisense transgene whose transcript interferes with the function of the normal gene. Mice with the 'shiverer' (*shi*) mutation have a recessive mutation in the myelin basic protein. The phenotype was mimicked by making *shi*/+ heterozygotes transgenic for an antisense myelin basic protein construct (Katsuki *et al.*, 1988). This approach is, however, likely to be restricted to instances where complete abolition of gene function is not necessary to produce the phenotype.

The most powerful approach is to disrupt one allele of the resident gene by homologous recombination in ES cells and then, following germline transmission, breed the disrupted allele to homozygosity. In this way, mice lacking $\beta 2$ -microglobulin have been produced (Koller *et al.*, 1990; Zijlstra *et al.*, 1990). These animals have little if any MHC class I antigen on the cell surface, because $\beta 2$ -microglobulin is required for the assembly and cell-surface expression of class I proteins. In spite of this deficiency, the mice are healthy and fertile when maintained under pathogen-free conditions, although they are defective in cytotoxic T-lymphocyte activity.

Disease models

A further important application of germline manipulation is the production of animal models of human disease (reviewed by Hooper, 1990). Models of genetic diseases in which the affected

gene has been characterized can be designed by introduction of a similar genetic change into the germline of an experimental animal. In the case of other diseases, transgenic animals can be produced to model abnormal gene expression at particular stages of the disease, bypassing early events which may or may not be genetic in nature. For example, mice transgenic for oncogenes have been invaluable in elucidating the role of these genes in tumour production and in establishing that carcinogenesis is a multistage process (reviewed by Hanahan, 1989).

Other uses of oncogenes in transgenic animals

The use of hybrid genes in which an oncogene is the reporter may result in the discovery of new sites of expression of a gene. When transgenic mice were produced with a hybrid gene composed of the promoter of a mouse major urinary protein (MUP) gene linked to the large T-antigen gene of simian virus 40, the mice developed tumour-like nodules in the skin, due to enlargement of sebaceous glands (Held *et al.*, 1989). The gene from which the promoter was derived was previously believed to be expressed specifically in the liver, and possibly in the mammary gland, but MUP mRNA has since been found in the skin of nontransgenic mice.

Tumours in mice which express oncogenes can allow the establishment of immortal cell lines (Efrat *et al.*, 1988; MacKay *et al.*, 1988; Paul *et al.*, 1988). By expressing the oncogene under the control of a promoter that operates in differentiated cells, some selection for maintenance of differentiated functions in the cell line may result (Efrat *et al.*, 1988). Cell lines have been derived by this method from cell types of interest, for which there were no previously available cell lines (MacKay *et al.*, 1988).

Somatic gene therapy

It is unlikely that the techniques of germline modification described here will ever provide a desirable route to the elimination of genetic disease in humans, as there are more-effective alternative approaches. However, the information obtained from animal germline manipulation will provide a basis for devising strategies for somatic gene therapy (see reviews by Friedmann, 1989; Hooper, 1990). This approach requires the solution of additional problems, particularly that of ensuring delivery of the correcting DNA construct to sufficient cells in the target tissue. Most progress has been made with haematopoietic cells, since bone marrow cells can be removed from the animal, genetically modified during growth *in vitro* and then returned to the donor. Promising results are also being obtained with hepatocytes and endothelial cells. Even certain diseases of the central nervous system may be amenable to this approach.

Fig. 8. (a) Principle of the use of *lacZ* reporter constructs in embryonal stem cells to identify enhancer sequences or genes that are active in early development. The 'enhancer trap' construct contains a *neo*^r gene to allow biochemical selection of the transgenic cells, and the *lacZ* coding sequences linked to a minimal promoter (*P**). Expression of *lacZ* can be detected histochemically and will occur if integration takes place near to an enhancer element. The *lacZ* gene in the 'gene trap' construct has no promoter or translation initiation site, but has a splice acceptor site (SA). A *lacZ* fusion protein may be produced if the sequences are integrated within a gene that is actively transcribed and in a manner which results in an intergenic splice in which the reading frames of the two genes are in phase.

(b) Cell lines derived using enhancer-trap or gene-trap constructs may be introduced into blastocysts to produce chimaeric embryos. The pattern of *lacZ* expression reveals whether the enhancer or gene is developmentally regulated. From Rossant & Joyner, 1989; first published in *Trends in Genetics*.

Research in reproduction

Embryogenesis

Genetic control of development is believed to involve the sequential expression of a gene hierarchy in which some genes act by controlling the spatial and temporal pattern of expression of other genes (reviewed by Ingham, 1988; Rossant & Joyner, 1989). The tendency for effective mechanisms to be conserved has led to the use of evolutionarily related gene products for similar functions in superficially very different species. With this in mind, molecular probes obtained in studies of *Drosophila* development have been used to identify the related genes in mammals. Conversely, growth factors first identified in mammals have recently been shown to modify the pattern of development in lower vertebrates (Ruiz i Altaba & Melton, 1989). Until recently, molecular genetic analysis of mammalian development has been largely descriptive. The techniques of gene transfer and gene targeting are now being used for functional analysis.

Several families of genes have important roles in development (Dressler & Gruss, 1988). A hierarchy of genes involved in the regulation of development in *Drosophila* is summarized in Fig. 9. It is postulated that segmentation genes divide the egg into smaller units before the characteristics of the segment are determined by the homoeobox-containing gene family. Descriptive studies of the patterns of expression of these gene families are consistent with their having analogous functions in mammalian embryogenesis.

Fig. 9. Hierarchies of genes that regulate early development in *Drosophila*. The diagram shows expression of representative members of each of the different classes of gene controlling pattern formation along the antero-posterior axis of the embryo. As development proceeds, successive classes of genes are expressed in an increasingly complex pattern. The arrows indicate known regulation of the expression of the members of one gene class by those of another. From Biggin & Tijan, 1989; first published in *Trends in Genetics*.

Homoeobox-containing genes. The homoeobox is an evolutionarily conserved DNA sequence that encodes a 60-amino-acid protein domain (reviewed by Gehring, 1987). It was first identified by analysis of the genes concerned in early development in *Drosophila*. The homoeodomain adopts a helix–turn–helix conformation characteristic of many other DNA-binding proteins; it has been shown to bind to DNA (Biggin & Tjian, 1989).

The role of the gene product of the homoeobox-containing genes was first studied by analysis of patterns of expression along the antero–posterior axis (Ingham, 1988). Each gene is expressed in a unique temporal and spatial pattern and genes within a cluster have overlapping areas of expression. The pattern of expression in the central nervous system and the organization of the *Hox* group of genes are shown in Fig. 10. The similarity of the *Hox* gene cluster to the *Antennapedia* and *Bithorax* complexes of *Drosophila* suggests that they share a common ancestral gene cluster that was already distinct when the insect and vertebrate lineages diverged (Graham *et al.*, 1989). The structural and functional conservation of the groups of genes within a cluster in *Drosophila* and mouse is very marked and led to the postulate that this is a consequence of *cis*-acting regulatory elements being distributed throughout the cluster (Graham *et al.*, 1989). Any change within the cluster would be expected to cause fatal disruption to the co-ordinated pattern of expression.

Fig. 10. Organization and pattern of expression in the central nervous system (CNS) of four *Hox* gene clusters of mouse and the *Antennapedia* (ANT-C) and *Bithorax* (BX-C) complexes of *Drosophila*. There are strong similarities between these species in the organization and spatial patterns of expression of these genes. This strongly suggests that this organization and pattern of expression was established in the ancestral gene cluster, before the insect and mammalian lineages diverged. Filled boxes are genes whose pattern of expression in the mouse embryo has been determined by in-situ hybridization; my = myelencephalon, met = metencephalon, mes = mesencephalon and pro = prosencephalon. Numbers in parentheses after gene names are chromosome location of genes. From Rossant & Joyner, 1989; first published in *Trends in Genetics*.

It is postulated that the homoeobox genes act by regulating the expression of other genes, many of which are still unknown. There is evidence to support this hypothesis both from in-vitro transcription assays and from studies on transient expression of cDNA coding for the protein and

target DNA sequences (reviewed Biggin & Tjian, 1989). However, these assays are limited in that they occur in the absence of other factors that may regulate gene expression and consider only one or a few genes. Direct tests of the mechanisms of regulation and of the role of the gene product are possible either by overexpression of the product or by prevention of expression.

Two studies have provided direct evidence of an effect of homoeobox gene expression upon the pattern of development in mammals. When a modified murine *Hox-1.4* gene with a substantial region of 5' sequences was transferred to mice, the tissue-specific expression of the transgene in adult animals matched that of the endogenous gene, but the level of expression was lower (Wolgemuth *et al.*, 1989). By contrast, in the developing embryo the transgene was expressed at higher than normal levels in the central nervous system, lung and gut. In particular, the overexpression was most marked in the mesenchyme of the gut and led to the occurrence of megacolon. The mice had difficulty voiding faeces and ultimately died, a condition resembling Hirschsprung's disease in man. In a rather different study, ubiquitous expression of *Hox 1.1* was induced by transfer of *Hox 1.1*-coding sequences driven by the chicken β -actin promoter (Balling *et al.*, 1989). This pattern of expression led to death from multiple craniofacial abnormalities shortly after birth.

The recently established procedures of gene targeting will allow inhibition of gene expression and the study of development in homozygous mutant embryos. Many homoeobox-containing genes have been modified already and at least one targeted allele has been introduced into the mouse germline (Chisaka & Capecchi, 1991). Reports of other mutant phenotypes can be expected soon.

Genomic imprinting

One of the most intriguing recent observations in developmental biology is the phenomenon of genomic imprinting in mammals. It had been assumed that both parents contribute equivalent nuclear information to the embryo at the time of fertilization. Transfer of pronuclei between zygotes has demonstrated convincingly that this is not the case (reviewed by Surani, 1986). Embryos are able to develop to term only if they contain both a pronucleus that developed from a spermatozoon and one from the egg (Barton *et al.*, 1984; McGrath & Solter, 1984b). Most diploid embryos that lack chromosomes of maternal origin fail to form normal blastocysts, suggesting that some of the differences are expressed during preimplantation development (Surani, Barton & Norris cited by Surani, 1986). In addition, there are marked differences in postimplantation development (Fig. 11). Embryos that lack chromosomes of paternal origin develop by Day 10 to form small 25-somite embryos that have very poorly developed extraembryonic tissues (Surani & Barton, 1983; Surani *et al.*, 1984). By contrast, embryos that lack chromosomes from the mother form only small embryos with 6–8 somites, but with extensive trophoblast (Barton *et al.*, 1984).

It is postulated that during gametogenesis there are sex-specific modifications (termed 'imprinting') to certain genes or regions of the chromosomes. These differences persist through cell divisions, but are subject to reprogramming during the subsequent stage of gametogenesis. The methylation patterns of some transgenes vary in a manner consistent with imprinting, and transgenes are being used to investigate the molecular mechanism of imprinting (Surani *et al.*, 1988). In general, transgenes inherited from a mother are more heavily methylated than the same transgene inherited from a father (Reik *et al.*, 1987; Sapienza *et al.*, 1987; Swain *et al.*, 1987), although there are exceptions (Hadchouel *et al.*, 1987; Sapienza *et al.*, 1987). In a few cases, the differential methylation patterns of transgenes are accompanied by differential patterns of expression (Hadchouel *et al.*, 1987; Swain *et al.*, 1987; Allen *et al.*, 1990). An effect of oocyte genotype on the degree of methylation has also been shown (Sapienza *et al.*, 1989; Allen *et al.*, 1990). Differential methylation is a mechanism that could explain genomic imprinting, but confirmation of this awaits the discovery and analysis of imprinted endogenous genes. These observations provide an explanation for earlier observations of differences between chromosomal regions inherited from mother or father (see Cattenach, 1986) and for the effect of maternal or paternal inheritance upon a number of human genetic disorders (reviewed by Reik, 1989).

Fig. 11. Pattern of development of mouse biparental, androgenetic and gynogenetic eggs. Normal development only occurs if the egg has two pronuclei, one derived from an oocyte and one from a spermatozoon. When the egg has two pronuclei both derived from spermatozoa, the conceptus develops no further than the 6-somite stage, but there is extensive development of the trophoblast. By contrast, if the pronuclei are both derived from oocytes, the conceptus may reach the 25-somite stage, but development of the trophoblast is limited.

To facilitate the identification of imprinted genes, Mann *et al.* (1990) produced ES cell lines from androgenetic embryos obtained by replacing the maternal pronucleus of a fertilized egg with a second paternal pronucleus taken from another egg. These lines produced chimaeras of which many died soon after birth and the survivors showed skeletal defects, suggesting that normal development requires stage- and cell-specific expression of imprinted genes.

Hypogonadal mice and the GnRH gene

Reproductive function has been restored in hypogonadal mice by transfer of the gene encoding gonadotrophin-releasing hormone (GnRH) (Mason *et al.*, 1986). The *hypogonadal* mutation is a deletion encompassing the distal half of the gene for the common precursor of GnRH and GnRH-associated peptide. Mutant mice of either sex are sexually immature and cannot reproduce. The defect in both sexes has been cured by a transgene containing the mouse GnRH gene with 5 kb of 5' and 3.5 kb of 3' flanking sequence. The transgene shows appropriate tissue-specific expression and transgenic mice of both sexes reach sexual maturity and mate; females raise healthy litters. All assays of the effect of the transgene, including follicle-stimulating hormone, luteinizing hormone and prolactin concentrations, and production of GnRH in appropriate neurons of the hypothalamus, suggested that the animals were fully restored. These observations establish this system as a model for further studies of the regulation of GnRH expression and of the role of the GnRH-associated peptide.

Development of the reproductive tract

The mechanisms that regulate the regression of the Müllerian duct and the development of the Wolffian ducts to form the male tract have been investigated by transfer of a construct containing the mouse metallothionein-1 promoter and the coding sequences for Müllerian-inhibiting

substance (Behringer *et al.*, 1990). This hormone is normally produced by the Sertoli cells of the foetal testis and the granulosa cells of the postnatal ovary. Transgenic females lacked uteri and oviducts, but there were other effects upon the germ cells in the ovary. In transgenic females, there were fewer germ cells in the ovary at birth, and within 2 weeks these were lost and the somatic cells reorganized into structures resembling seminiferous tubules. Although most of the males were normal, some in which the concentration of hormone was high showed impairment of the development of the male ducts, the testes did not descend and there was some feminization of the external genitalia. These observations confirm the role of Müllerian-inhibiting substance in preventing development of the Müllerian ducts, but suggest that the glycoprotein has several other functions in the differentiation of the reproductive tract.

Lactation

In response to pregnancy, the mammary gland undergoes profound changes, including rapid cell proliferation, and changes in gene expression (see Neville & Daniel, 1987). Milk contains large amounts of protein, most of which is synthesized in the secretory epithelial cells of the mammary gland. The most abundant milk proteins are the caseins and the whey proteins α -lactalbumin, with either β lactoglobulin in ruminants or whey acidic protein (WAP) in rodents.

Expression of several milk-protein genes has been studied in transgenic mice by transfer of coding sequences with flanking regions of DNA. The sheep β -lactoglobulin gene, bovine α -lactalbumin and rat WAP genes were all expressed at levels comparable to those of the endogenous genes, predominantly in the mammary gland (Simons *et al.*, 1987; Rosen *et al.*, 1989; Vilotte *et al.*, 1989); rat β -casein, on the other hand, was expressed at no more than 1% of the level of the endogenous gene (Lee *et al.*, 1988). Studies on the regulation of milk-protein gene expression through pregnancy and lactation have found that the expression is regulated broadly as expected (Pittius *et al.*, 1988b; Lee *et al.*, 1989; Rosen *et al.*, 1989; Harris *et al.*, 1990), but with different kinetics in experiments using WAP sequences (Pittius *et al.*, 1988b; Rosen *et al.*, 1989). The sheep β -lactoglobulin gene is expressed specifically in the secretory epithelial cells of the mammary gland of transgenic mice (Harris *et al.*, 1990). The fidelity of expression of this gene, of which there is no counterpart in mice, indicates remarkable conservation of the intracellular mechanism regulating mammary-specific expression between species which have different hormonal control of lactation.

Clark *et al.* (1987) have suggested that gene expression in the mammary glands of transgenic livestock may be useful for the production of large amounts and easy harvest of specific proteins, particularly those used therapeutically to treat human disease. Several human proteins have been produced in the milk of transgenic mice (Gordon *et al.*, 1987; Pittius *et al.*, 1988a; Tomassetto *et al.*, 1989; Yu *et al.*, 1989; Archibald *et al.*, 1990; Meade *et al.*, 1990), rabbits (Bühler *et al.*, 1990) and sheep (Clark *et al.*, 1989). Levels of expression high enough for successful application have so far only been obtained in mice (Archibald *et al.*, 1990; Meade *et al.*, 1990); because yields of mouse milk are limited, high-level expression in large animals will be required. Other potential applications of gene transfer for the modification of milk are reviewed elsewhere (Wilmut *et al.*, 1990).

Spermatogenesis

Transgenic animals have been used to identify *cis*-acting control sequences responsible for stage-specific gene expression in spermatogenesis. The *Pgk-2* phosphoglycerate kinase gene is transcribed only in meiotic and haploid male germ cells and the protamine genes *mP1* and *mP2* only in haploid, round spermatids. The promoters of these genes share a number of common sequence motifs and, by deletion analysis in transgenic mice carrying reporter genes driven by promoter fragments, the sequences essential for tissue- and stage-specific expression of *mP1* and

Pgk-2 have been localized to a region of <500 bp including three of these motifs (Peschon *et al.*, 1987, 1989; Robinson *et al.*, 1989). An additional, translational level of control operates such that *mPl* mRNA is not translated until the elongating spermatid stage, about 1 week after its transcription. The sequences responsible for this temporal translational control have been localized to a 156-nucleotide stretch of 3'-untranslated sequences. Changes in the time of translation were accompanied by changes in the subcellular localization of the gene product (Braun *et al.*, 1989b). Another regulatory element was revealed when it was shown that a 185-bp fragment of the 5' flanking sequence of the metallothionein-I gene directed ornithine-transcarbamylase expression in male germ cells during the tetraploid, pachytene stage of meiosis when fused to the coding sequence of ornithine transcarbamylase (Kelley *et al.*, 1988).

Several known autosomal recessive mutations in mice and humans affect both neurological function and male fertility. A transgenic mouse line has been identified with a mutation that is allelic with one such known mutation, Purkinje cell degeneration (Krulowski *et al.*, 1989). Use of probes for the transgene to isolate the flanking sequences will facilitate the cloning of the affected locus and hence the elucidation of the nature of the gene product. Male sterility associated with the presence of a transgene has also resulted from faulty chromosomal synapsis at meiosis caused by a translocation at the site of integration (Gordon *et al.*, 1989).

Expression of the thymidine kinase (*HSVtk*) gene from herpes simplex virus in the testes of transgenic mice can lead to male sterility (Al-Shawi *et al.*, 1988; Braun *et al.*, 1990). The G_0 male transgenic mice are usually sterile, or if they are fertile they do not transmit the transgene to their progeny. The fertile G_0 males may be mosaics of transgenic and nontransgenic cells, with only the nontransgenic cells giving rise to functional sperm. Expression of *HSVtk* in the testes has been found in transgenic mice carrying the intact *HSVtk* gene (Kiessling *et al.*, 1986; Braun *et al.*, 1990) and in mice which carry hybrid genes in which the *HSVtk* structural gene was used as reporter for a variety of promoters (e.g. mouse major urinary protein, Al-Shawi *et al.*, 1988; mouse mammary tumour virus LTR, Ross & Solter, 1986 [see Al-Shawi *et al.*, 1988]; interferon- β , Iwakura *et al.*, 1988). In each of these cases, expression in the testes was unexpected; it was probably due to transcription from a cryptic promoter within the *HSVtk* gene, as expression was also observed from a disabled *HSVtk* gene with no cellular enhancer or promoter elements (R. Al-Shawi, personal communication). Within the adult testis, *HSVtk* has been localized by immunohistochemistry to round and elongating spermatids, but *HSVtk* activity has also been detected in the testes of young mice, prior to the initiation of meiosis (Braun *et al.*, 1990; R. Al-Shawi, personal communication).

Mice which express very high concentrations of *HSVtk* produce few, grossly abnormal spermatozoa while expression at lower levels results in the production of morphologically normal, but non-motile, spermatozoa (R. Al-Shawi, personal communication). Braun *et al.* (1990) noted a variety of ultrastructural abnormalities in the developing and mature spermatozoa: acrosomal defects, incomplete condensation of chromatin and abnormal axonemes in sperm tails. *HSVtk* expression in the testes does not always lead to sterility (Kiessling *et al.*, 1986; Braun *et al.*, 1990; R. Al-Shawi, personal communication), probably because expression is below a threshold level.

It is not clear why expression of *HSVtk* should be so disruptive. *HSVtk* has a broader range of substrates than the mammalian enzyme and it has been suggested that this may lead to the production of nucleotide analogues that interfere with nucleotide metabolism or to the depletion of ATP by a futile cycle of thymidine phosphorylation and dephosphorylation (Braun *et al.*, 1990).

Expression of interferons in the testes of transgenic mice has been reported to cause male sterility (Hekman *et al.*, 1988; Iwakura *et al.*, 1988). In both cases, expression of the interferon sequences was driven by the promoter from the mouse metallothionein-I (MT) gene. Hekman *et al.* (1988) introduced a metallothionein-I, interferon $\alpha 1$ hybrid gene into mice. Two G_0 males were sterile and one transgenic line was established from a G_0 female. The transgene was expressed exclusively in testes, and males were sterile, or fertile for a period before becoming sterile. Histological analysis revealed a spectrum of effects, from a diminution of spermiogenesis to complete

atrophy of testes with calcification. Iwakura *et al.* (1988) reported male sterility due to expression of interferon- β in the testes of transgenic mice. However, the construct employed also contained HSVtk sequences, and HSVtk was expressed in the testes of most mice. With hindsight, it is likely that in this case HSVtk was the cause of sterility, although effects of interferon- β cannot be excluded.

In one pedigree of mice transgenic for the HSVtk coding sequences under the control of the metallothionein-I promoter, males are fertile but do not transmit the integrated sequences to any of their progeny, i.e. there is segregation distortion (Palmiter *et al.*, 1984). In this pedigree, the integration event has been accompanied by considerable sequence rearrangement, and the effect has been interpreted as being probably due to disruption of a gene whose function is required postmeiotically, although an effect mediated by HSVtk expression cannot be ruled out (Wilkie & Palmiter, 1987). These authors cite unpublished results indicating that excision of the transgene can occur in cells of the male germline, allowing transmission of the chromosome which originally carried the transgene. However, it is not clear how segregation distortion can result from effects on postmeiotic gene expression, since the spermatids developing from a single spermatogonium remain connected by cytoplasmic bridges until after RNA synthesis ceases, so that the transcription products of haploid genomes are not segregated into individual spermatozoa. Braun *et al.* (1989a) studied mice heterozygous for a transgene consisting of sequences coding for human growth hormone (hGH) under the control of the protamine-I promoter. Although transgene expression, as shown by in-situ hybridization, was restricted to haploid spermatids, >90% of spermatozoa stained positively for hGH in the acrosomal compartment, and this proportion was identical to that seen in the homozygote. Some mechanism to prevent the sharing of certain gene products would therefore seem to be necessary to account for segregation distortion.

Conclusions

Together, the methods of gene transfer and gene targeting permit the introduction of a great range of genetic changes. These procedures are receiving widespread application in mice at present and will be exploited more extensively in other species as ES cells become available.

It is possible to isolate a gene in the laboratory and to transfer it into another animal of the same or a different species. Before the gene is transferred, it may be modified. Two common types of modification involve either linking the regulatory elements of one gene to the coding sequences of a different gene or the removal of segments of the gene. These changes may be made either to define the regulatory elements of the gene or to study the role of the gene product.

Gene transfer has several limitations. In particular, the level of expression of transgenes is variable because of the influence of the neighbouring DNA at the site of integration: the procedure can only add a gene, which must act in the environment created by the endogenous genes. These limitations may often be overcome by the use of ES cells. The ability to introduce small modifications to specific DNA sequences will permit the analysis of their role in the regulation of gene expression, while the role of a gene product may be studied either by abolition of gene expression or by the introduction of changes to the protein produced.

The experiments already completed confirm that genetic manipulation can provide new insight into many aspects of mammalian physiology, including reproductive biology. It seems probable that the techniques will become more precise, efficient and reliable and that as a result they will be established in many different laboratories. Exploitation of the techniques of molecular biology, including genetic manipulation, may increase our understanding of mechanisms that are already understood at a physiological level, but they can also be expected to solve a number of mysteries that have proved intractable to other approaches. Biologists studying reproduction may look forward to a very exciting period of research, for there are many surprises in store.

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