Minireview

Gene knock-out technology: a methodological overview for the interested novice

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Abstract

Gene targeting by homologous recombination is a powerful technique, generating mouse strains with defined mutations in their genome. These genetically modified, 'designer' animals allow us for the first time to ask simple questions about elaborate and complex biological systems. Dissecting the function of individual components of the immune system is a perfect application of this technology. Although the techniques involved in the generation of gene knock-out mice are increasingly well defined, to many immunologists the language and concepts are confusing. This review presents the essentials of the technology in a form digestible by the non-expert.

Keywords: Homologous recombination; Embryonic stem cell; Gene targeting; Immunology

1. Introduction

The introduction of defined modifications at a genomic level by gene targeting has become a widely used technique. It is now possible to design mice lacking single gene products or exhibiting changed regulatory properties. This new approach has already had a major impact, especially in immunology, where the disruption of a particular gene which is normally not essential for the animal during development, can be studied in vivo.

The possibility of homologous recombination between foreign DNA and existing homologous sequences in the mammalian genome was studied from the beginning of the eighties but ultimately, was demonstrated by Smithies and colleagues (Smithies et al., 1985). Parallel research over the same period demonstrated that pluripotent murine stem cell lines (Evans and Kaufman, 1981; Martin, 1981) have the capacity to contribute to the germ-line tissue of mice (Bradley et al., 1984), even after extended periods in culture. Mutations introduced into these cell lines are transmitted to the offspring (Doetschman et al., 1987; Gossler et al., 1986; Robertson et al., 1986). Thus, the concept of designer mutations became a reality.

The underlying concept of gene targeting by

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homologous recombination is relatively simple (Figs. 1 and 2). A targeting vector carrying a selectable marker (usually the neomycin resistance gene) flanked by sequences homologous to the genomic target gene is constructed and introduced by transfection into an embryonic stem (ES) cell line. The homologous flanking sequences enable targeted insertion into the
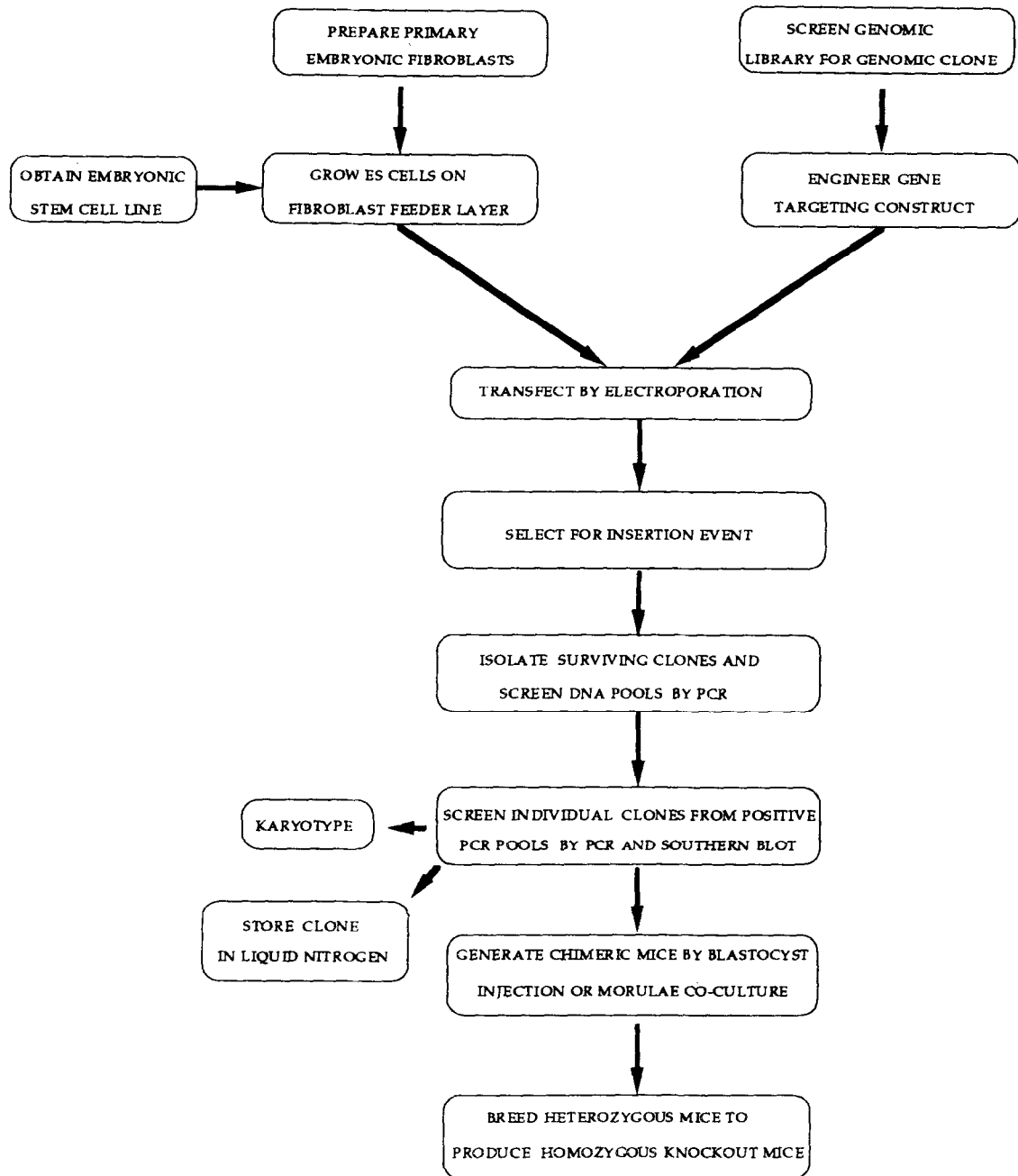

Fig. 1. Schematic representation of the procedures involved in the generation of gene targeted mutant mice.
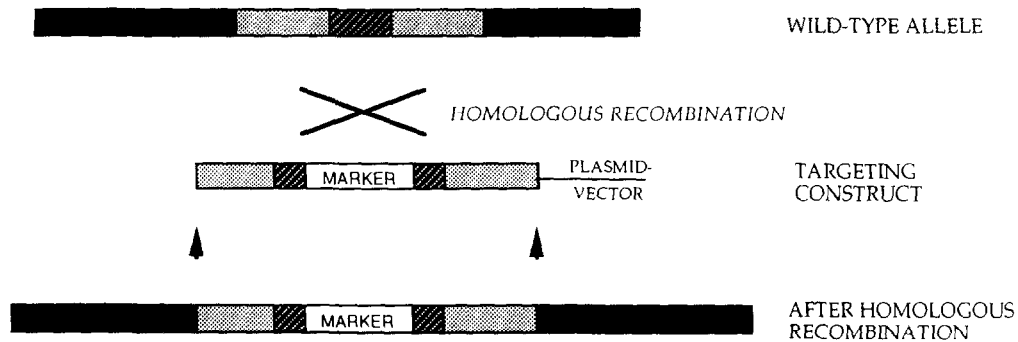
Fig. 2. Sequence replacement construct. A targeting construct is created by cloning a stretch of genomic DNA containing the gene (hatched) to be disrupted into a plasmid vector. Subsequently, a selectable marker is inserted in an exon of the gene flanked by stretches of homologous DNA (grey). The plasmid is linearised at a unique site prior to transfection. In undergoing homologous recombination, the plasmid construct is excised. Note that this occurs on one chromosome only, so the ES cell still carries one functional gene.

genome and the gene mutated by the selectable marker replaces the original wild-type sequence. Subsequently, the successfully targeted ES cell line is injected into blastocysts (3.5 day embryos; 32 cell stage) or co-cultured with morulae (2.5 day embryos; 8–16 cell stage) and contributes to the tissues of the developing animal including the germ line. A colour tag (e.g. ES cells derived

Table 1
ES cell lines

Name	Genotype	Mouse strain	Comment	Reference 1. Origin 2. Representative knock-out
J1	xy	129Sv		1. Li et al., 1992 2. DNA-methyltransferase; Li et al., 1992
D3	xy	129Sv	Available from ATCC	1. Doetschman et al., 1985 2. β_2-microglobulin; Zijlstra et al., 1990
CCE	xy	129Sv		1. Robertson et al., 1986 2. Invariant chain; Bikoff et al., 1993
AB1	xy	129Sv		1. McMahon and Bradley, 1990 2. IFN-γ; Dalton et al., 1993
E14TG2a	xy	129/Ola	Available from ATCC	1. Hooper et al., 1987 2. IL-2; Schorle et al., 1991
E14.1	xy	129/Ola	Subclone of E14TG2a	1. Kühn et al., 1991 2. IL-4; Kühn et al., 1991
BL/6 III	xy	C57BL/6		1. Ledermann and Bürki, 1991 2. Perforin; Kägi et al., 1994
Bruce 4	xy	C57BL/6		1. Köntgen et al., 1993 2. MHC class II I-A [a]; Köntgen et al., 1993
TT2	xy	C57BL/6 × CBA/JNCrj		1. Nada et al., 1993 2. Tyrosine kinase Csk; Nada et al., 1993

from mice with black coat colour injected into blastocysts from mice with white coat colour results in black and white spotted mice) is used to identify chimeric animals. Breeding is required to obtain homozygous animals exhibiting the phenotype of the inserted mutation in all cells.

While the underlying principles for generation of gene knock-out mice are straightforward and many aspects of the technique are standardized and well documented, there are many potential pitfalls. In this review we focus on the broader methodological issues involved in gene targeting, highlighting initial strategic approaches to the technology and typical problems that may be encountered.

2. Generating 'gene knock-out mice' – theory and practice

2.1. ES cell lines

ES cell lines (Table 1) are derived from pluripotent, uncommitted cells of the inner cell mass of pre-implantation blastocysts. These cell lines are cultivated in the presence of differentiation inhibiting factors (see below). ES cells are able to contribute to all tissues including germline tissue. The most common genetic background of ES cell lines is 129Sv, a mouse strain which tends to develop spontaneous teratocarcinomas and is widely used for studies of early embryonic development. In immunological terms, the 129Sv strain is poorly defined and thus, to avoid extensive backcrossing of the targeted mouse strain, ES cells of genetic backgrounds other than 129Sv have been derived, although are not yet as widely used as 129Sv ES cells (Table 1). Recently a gene knock-out using an ES cell line derived from (C57BL/6 × CBA/JNCrj) F1 mice has been developed and two lines of C57BL/6 background have shown successful germ-line transmission and produced knock-out mice. The C57BL/6 mouse strain has been extensively studied in the immunological context, in contrast to 129Sv, and many transgenic models have been established on this background.

2.2. Fibroblast feeder layers and leukemia inhibitory factor (LIF)

To maintain ES cells in an uncommitted state they are cultured in the presence of growth inhibited feeder cells which provide the appropriate signals to preserve this embryonic phenotype and serve as a matrix for ES cell adherence. For the normal expansion of ES cells, these feeder cells commonly consist of primary embryonic fibroblasts (see below) that are established from tissue of day 13–day 14 embryos of virtually any mouse strain. Heads and livers are removed and the remaining tissue is dissociated mechanically. The tissue homogenate is diluted in DMEM supplemented with sodium pyruvate, glutamine, β-mercaptoethanol, antibiotics and heat-inactivated fetal calf serum (FCS) (15%) and plated on dishes or in tissue culture flasks. Adherent cells are washed 24 h later and again fed with fresh medium. The fibroblasts are split (1:2–1:4) or stored frozen when the plates are confluent. Embryonic fibroblasts have only a restricted proliferative capacity (about five cell passages) and therefore, sufficient stocks need to be prepared and stored frozen in advance.

For the growth of transfected ES cell clones on embryonic fibroblasts, it is preferable although not essential, that the feeder layer be resistant to the selection medium (e.g. Geneticin to select for neomycin resistance). Mice transgenic for the neomycin resistance gene neo (Gossler et al., 1986; Stewart et al., 1987) or knock-out mice carrying this transgene as a selection marker are available and can be used to establish the necessary stock of feeder cells. Also, ES cells grown on a feeder layer of STO cells, a fibroblast cell line, have been used to establish germ-line tissue (Bradley et al., 1984). All feeder cells must be inhibited in their growth capacity by chemical treatment (mitomycin C; 20 μg/ml) or irradiation (Robertson, 1987). We have had considerable success with 3000 R γ-irradiation of fibroblasts. The irradiated cells are viable for up to 2 weeks in culture.

One of the differentiation-inhibiting signals provided by feeder cells has been identified and cloned (Smith et al., 1988; Williams et al., 1988).

The presence of this cytokine, termed leukemia inhibitory factor (LIF), is sufficient in itself without feeder cells for the maintenance of ES cells in an undifferentiated state. Moreover, germ-line transmission using LIF alone has been achieved (Pease and Williams, 1990). However, note that the LIF produced by primary fibroblasts is sufficient to preserve the uncommitted state of ES cells. ES cells seem to do better when grown on a feeder layer, rather than plastic, and the cost of recombinant LIF could be substantial if this alone is used. We routinely grow ES cells on neo^r fibroblasts with the addition of a small amount (200 U/ml) of LIF.

2.3. Tissue culture

The correct procedures for maintenance of ES cells in tissue culture are crucial for successful gene targeting. Conditions for ES cell tissue culture are well established and documented extensively (Abbondanzo et al., 1993; Robertson, 1987).

ES cell lines are usually cultured in DMEM (high glucose) supplemented with sodium pyruvate, glutamine, nucleotides, β-mercaptoethanol, antibiotics and 10% FCS. In general, ES cell culture requires the exclusive use of high quality reagents dissolved in ultra-pure water. Disposable pipettes are preferable and glass materials have to be rinsed extensively because ES cells are very sensitive to traces of residual detergents from dishwashers. The mitogenic ability and specific support for ES cell growth of particular batches of FCS has to be tested empirically. A surprisingly high number of FCS batches are not suited to this specialised tissue culture. Under normal conditions, ES cells proliferate rapidly and have to be split frequently.

2.4. Targeting construct design

The first step to construct design is the availability of a genomic clone containing the gene(s) of interest. If the exons which encode regions of a protein are defined, inactivation of the molecule can be achieved without further knowledge of the regulatory sequences controlling transcription of the gene(s) encoding the molecule. The procedures for obtaining such a clone are well outside the scope of this review, but are detailed elsewhere (Sambrook et al., 1989).

To target genes which are transcriptionally inactive, a gene cassette encoding the neo^r gene is frequently used which has been optimized for use in ES cells (Mansour et al., 1988; Thomas and Capecchi, 1987) The gene is under the control of a Herpes simplex virus thymidine kinase promoter and a polyoma virus enhancer. Additionally, the cassette encompasses a synthetic translation initiation sequence and a poly A addition signal. The neo^r cassette is used to disrupt transcription of a targeted gene and then to serve as a selectable marker using the antibiotic Geneticin (G418 sulfate). Geneticin is an aminoglycoside and inhibits protein synthesis in mammalian cells and also in yeast, fungi and bacteria. The neo^r gene encodes the bacterial protein aminoglycoside phosphotransferase which can be expressed in mammalian cells and confers resistance to aminoglycoside antibiotics. The widespread use of this marker gene cassette (commercially available from Stratagene) testifies to the reliability and reproducibility of the approach. The regulatory sequences used in this gene cassette ensure that a single copy of this marker gene expresses sufficient aminoglycoside phosphotransferase to guarantee the survival of the transfected cell. A range of combinations of this marker gene with different promoters and enhancers has also been tested successfully (see review, Koller and Smithies, 1992). Other marker genes used successfully for selection of homologous recombination events in ES cells are the hygromycin β gene cassette, another aminoglycoside (Te Riele et al., 1990), and the hprt gene cassette (Van der Lugt et al., 1991). Different marker genes can also be used to target both alleles in two consecutive rounds of gene targeting (Te Riele et al., 1990).

With few exceptions, the approach used to date for the inactivation of immunologically relevant genes is the *sequence replacement vector* (Fig. 2), where a selectable marker is inserted in an exon of a particular gene, flanked by stretches of homologous DNA. The marker gene serves as a mutagen, disrupting the coordinated transcription of the gene. Another approach, the *sequence in-*

sertion vector, does not rely on insertion of a marker gene in the region of homology. Rather, a stretch of homologous DNA is inserted in a plasmid carrying the selectable marker and this is opened at a unique site in the gene. Homologous recombination leads in this case to gene duplication and the marker gene as well as the plasmid DNA is inserted. While similar or higher gene targeting efficiency has been achieved using insertion vectors (Deng and Capecchi, 1992; Hasty et al., 1991b; Hasty et al., 1991c) this approach has not been adopted for knock-outs of direct immunological relevance.

There are some disadvantages to the replacement/insertion vector strategy such as the strong activity of heterologous enhancer/promoter combinations and the expression of foreign genes in situ and sophisticated techniques have been used to obviate these problems. These will be introduced later. For most purposes however, the widely used sequence replacement vector has proven adequate.

2.5. Factors influencing homologous recombination efficiency

In the years since homologous recombination in mammalian cells was described, it has become clear that homologous recombination is not necessarily a rare event compared to non-homologous random insertion, particularly if one uses appropriate targeting vectors. A syngeneic background (targeting vector derived from the same mouse strain as the ES cell) is an advantage and may improve the success rate. A second important parameter is the length of homology between targeting vector and genomic (ES cell) DNA.

The influence of both factors has been studied carefully. For example, it was demonstrated in one study that the use of isogenic DNA (129Sv-derived) versus non-isogenic DNA (BALB/c-derived) resulted in a 20-fold improvement in the targeting frequency when 129 ES cells were used. It was suggested that the length of uninterrupted perfectly matching sequence was the crucial factor rather than the existence of a low percentage of mismatched bases in general (Te Riele et al., 1992). In another study however, non-isogenic

DNA was found to perform equivalently and only minor differences could be demonstrated (Deng and Capecchi, 1992). The difference in these two studies could be due to the individual genes used for the experiments, being the retinoblastoma susceptibility gene in one case and the hprt gene in the other. In a few extreme cases the use of isogenic DNA has proven to be crucial (Wurst et al., 1994) but the fact that non-isogenic DNA has been used successfully for the generation of many gene knock-out mice demonstrates that synergy is not an absolute requirement.

The *length* of homology between targeting vector and genomic DNA is certainly of greater importance, as the frequency of homologous recombination is exponentially dependent on this. Early experiments showed that an increase in length of homology from 4 kb to 9.1 kb resulted in an augmentation of homologous recombination by 40-fold when a replacement vector was used (Thomas and Capecchi, 1987). Similarly, using different replacement and insertion vectors, Deng and colleagues demonstrated that a direct relationship exists between the length of homology and successful recombination (Deng and Capecchi, 1992). Comparable results were produced in another study which also suggested that the minimal length of homology necessary for recombination is about 2 kb. Interestingly, the length of individual flanking regions (see Fig. 2) seems to be unimportant (Hasty et al., 1991b) and the length of the stretch of DNA which is replaced by the selectable marker seems also of minor importance. In targeting the T cell receptor β chain for example, 15 kb was removed and replaced with the targeting construct (approx. 9 kb), without reducing the recombination efficiency substantially (Mombaerts et al., 1991). Also the transcriptional activity of the gene locus in ES cells seems to have no influence on the frequency of gene replacement (Johnson et al., 1989).

2.6. Enrichment of yield

A variety of strategies have been used to increase the number of clones positive for homologous recombination above the background of
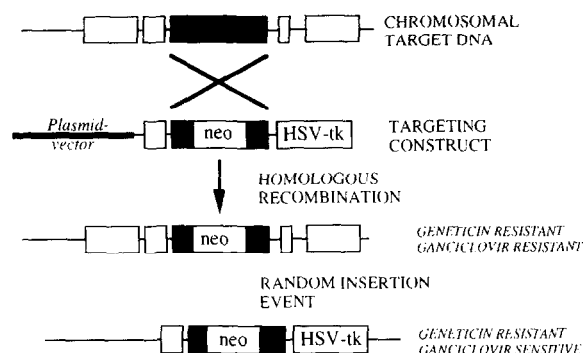
Fig. 3. Positive-negative selection. A targeting construct is designed containing the neomycin resistance gene cassette for positive selection, encoding for resistance to Geneticin. In addition, the construct contains an HSV-tk gene for negative selection, which is capable of converting Ganciclovir into toxic intermediaries. Cells that undergo the specific crossover event are Ganciclovir and G418-resistant. Cells which contain the randomly integrated construct will retain both the neomycin and HSV-tk genes and although G418-resistant, will be Ganciclovir sensitive.

clones surviving selection due to non-homologous (random) insertion of the selection marker (usually neo^r) into the genome. The most widely used of these, developed by Mansour and colleagues (Mansour et al., 1988) uses a second, *negative* selection step, in addition to the *positive* selection afforded by neo^r gene insertion (Fig. 3).

The transcriptionally active Herpes simplex virus thymidine kinase (HSV-tk) gene when present in eukaryotic cells is not in itself, toxic to the cells. However, in the presence of the synthetic guanine analogue, ganciclovir (9-(1,3-dihydroxy-2-propoxymethyl) guanine) or the uracil derivative FIAU (1-(2-deoxy,2-fluoro-β-δ-arabinofuranosyl)-5-iodouracil), toxic metabolites are produced by the viral enzyme, which interfere with cellular DNA metabolism and ultimately, kill the cell. Herein lies the strategy: an HSV-tk gene cassette is engineered to one or both sides of the DNA sequence (consisting of neo^r targeted gene and flanking sequences) expected to be inserted following homologous recombination. In the event of *homologous recombination*, the HSV-tk cassette is excised (Fig. 3). Thus, addition of ganci-

clovir or FIAU has no effect and the cell survives. If the targeting vector is *inserted randomly*, the HSV-tk gene is likely also to be included. Addition of selection drugs will kill the cell. Note that selection on the basis of neomycin resistance alone would not have distinguished between the random and homologous recombination events. A similar approach using other negative selection markers can be used, including the diphtheria toxin A fragment (Nada et al., 1993; Yagi et al., 1990) or the hprt gene cassette (Reid et al., 1990).

The enrichment factor using the positive-negative strategy varies from 2 to 2000 times depending on the targeted locus. Another contributory factor to success or failure of this strategy may be the level of endogenous cellular exonuclease activity which is responsible for destruction of the distal negative, but not the internal positive, selection marker leading to increased survival of cell clones after random insertion despite the selection pressure (Bernet-Grandaud et al., 1992). Note that many of the earlier knock-out mice used the HSV-tk strategy, but more recently as conditions become increasingly well defined, it has proved unnecessary in many cases.

2.7. Transfection

Methods for introducing the targeting construct into ES cells range from calcium phosphate DNA co-precipitation to retrovirus infection (Lovell-Badge, 1987). Today, electroporation is certainly the method of choice since it is both reliable and reproducible (Thomas and Capecchi, 1987). A wide variety of different plasmid DNA concentrations, field capacities (0.3–500 μF), voltages (220–800 V) and buffer compositions (mostly phosphate buffered saline) have been used. The best approach is to use different published conditions and choose empirically. We are using the Bio-Rad electroporator and find 220 V, 500 μF in a phosphate buffer to be optimal. Note that the transfection is carried out using DNA linearized at a unique restriction site in the plasmid vector to increase the targeting frequency (Valancius and Smithies, 1991a).

2.8. ES cell growth and selection

After transfection the cells are transferred onto a fresh feeder layer in normal growth medium. The next day (24 h after transfection) this is replaced with selection medium containing 200 μg/ml Geneticin (and/or other drugs of choice) and this continues on alternate days thereafter. The non-transfected cells should begin to die within 48 h while remaining clones grow within 8-12 days to aggregates of macroscopic size. To pick single clones, the medium is aspirated from the ES cell culture and replaced by PBS. A single clone is transferred to PBS in a 96 well plate (U bottom) after flushing it from the feeder layer. Trypsin/EDTA is added to the cell aggregates and the plate is incubated at 37°C for 5 min. After this incubation period, PBS is added and the ES cell clones can be dispersed. The cells can then be seeded on prepared feeder layers in 24 or 48 well plates and screened after growing to confluency.

2.9. Screening

Despite the relatively high proportion of homologous recombination events a construct can mediate, an appropriate screening strategy has to be employed to differentiate between the background of random insertion and the actual gene targeting event. PCR, Southern blotting and often, a combination of the two, have been used successfully to detect the replacement of the targeted gene.

Before any of these techniques can be used, genomic ES cell DNA of sufficiently high quality must be prepared. DNA is first isolated from individual clones. Individual DNA preparations or pools of these (eg from six clones) can then be screened. In the latter case, the presence of a positive necessitates re-testing of DNA from the individual clones used to create the pool, but if the pool is negative considerable time and reagents will have been saved. For PCR, cell lysis in distilled water (15 min, 80°C) can be used (Kim and Smithies, 1988). The lysate is digested subsequently with proteinase K to produce DNA of sufficient quality. A different method is required to achieve higher quality DNA preparations if a Southern blot strategy is applied. In our hands a simple and highly reproducible procedure involves cell lysis and digestion in lysis buffer containing SDS and proteinase K for several hours at
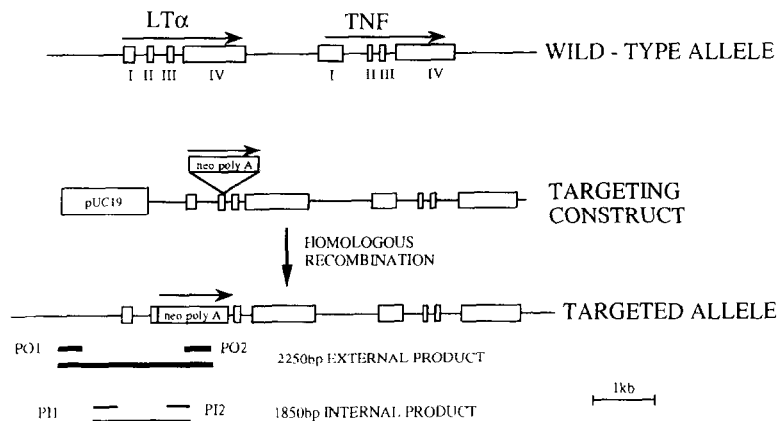

Fig. 4. Diagrammatic representation of the PCR strategy used to detect homologous recombination following replacement of the wild-type LTα gene with the same gene disrupted in exon II by the neomycin-resistance cassette. The external primers (PO) are used in the first round PCR. Only following homologous recombination are the two juxtaposed and able to generate a 2250 bp product. This acts as the template for the internal primer pair (PI), which will amplify a specific 1850 bp product in the second round PCR, observed by agarose gel electrophoresis.

56°C. The DNA is precipitated and dissolved in Tris-EDTA buffer (Laird et al., 1991; Köntgen and Stewart, 1993). Importantly, extraction with organic solvents is not required.

Generally it is desirable to develop a PCR strategy for detection of positive clones as this method, unlike Southern blot analysis, works with small amounts of low quality DNA, is fast and permits the screening of many clones in a short time. The usual strategy requires one primer in the genomic DNA outside the targeting construct, and one primer in the heterologous marker (e.g. *neo^r* cassette) DNA. Amplification is not dependent on gene expression and gives a unique product if homologous recombination occurs (Kim and Smithies, 1988). However, it can be difficult to obtain reproducible results from a PCR consisting of only one set of primers when amplifying from small amounts of template DNA (Kim et al., 1991). A nested set strategy can obviate many of these problems (Nitschke et al., 1993) and we have adopted this approach with good results. Here, an outer pair of primers gives rise to a larger amount of template DNA for a second round of amplification utilizing an internal pair of primers. The complete strategy for detection of successful targeting of the lymphotoxin (LT)-α gene is shown in Fig. 4, including the product size expected in the event of homologous recombination.

There are of course circumstances in which screening by means of Southern blot analysis may be the preferable alternative to PCR, such as the existence of long flanking regions (decreasing efficiency of PCR amplification) or repetitive sequences at the desired position of the primer. While the process is more laborious and time-consuming and DNA quality must be higher, the screening should nevertheless produce a clear result (Köntgen and Stewart, 1993) assuming appropriate restriction sites in the targeting construct are available to give a distinct difference in product size in the event of homologous recombination. A useful clue to the presence of successful homologous recombination as detected by Southern blot analysis is, in addition to a correct band size, that the intensity of the endogenous gene product after probe hybridization should be

similar to that of the targeted gene, given that each represents the gene on one chromosome.

2.10. Karyotyping

It is desirable to limit the number of passages of the ES cell prior to the actual generation of chimeric mice. The ability of ES cells to contribute to germ-line tissue is inversely proportional to the number of cell passages in vitro (Nagy et al., 1993). A fast screening procedure for example, is highly advisable otherwise clones should be stored frozen until recombinants have been detected. Karyotyping of the ES cells is also recommended at this stage in the procedure, particularly when the cells used for targeting are in higher passages. Cells contributing to the germ line have to be euploid (40 chromosomes in mouse) and it has been demonstrated that this normal set of chromosomes is retained for more than 15 cell passages (Robertson, 1987) but it is inevitable that cells kept in culture for a prolonged period accumulate mutations which result ultimately in the loss of chromosomes. However, while each cell line can comprise subpopulations which are exhibiting hypoploid (39 chromosomes) and hyperploid (41 chromosomes) sets the proportion of normal euploid cells should remain constant (about 65–80%; Ledermann and Bürki, 1991).

2.11. Production of chimeric mice

The determining factor in the production of a gene knock-out mouse strain is the generation of ES cell-embryo chimeras that transmit the desired alteration to subsequent generations. Until recently, chimeric mice were almost exclusively produced by injection of ES cells into the blastocyst (Bradley, 1987). This is a technically difficult task requiring the use of sophisticated micromanipulation apparatus which anchors the recipient blastocyst to one micropipette while an injection needle pierces the outer embryonic layer, on the opposite side, permitting deposition of the ES cells in the primary cavity of a blastocyst. The considerable investment in equipment and expertise with this technique places it beyond the real-

Table 2
Methodologically interesting gene knock-outs of immunological relevance

| Knock-out	Reference	Comment
T cell receptor β chain	Mombaerts et al., 1991	Targeted introduction of a genomic deletion of 15 kb encompassing the T cell receptor β chain locus
TGF-β1	Shull et al., 1992	Phenotype of generalized inflammation. No positive-negative selection, only Geneticin used
MHC class II I-A [a]	Köntgen et al., 1993	First published knock-out using a cell line of C57BL/6 background
IgH intron enhancer	Gu et al., 1993	Study of regulatory elements involved in B cell gene rearragement using the Cre-loxP system (see text)
Replacement of the κ light chain constant (C) region	Zou et al., 1993	Substitution of the mouse κ light chain constant (C) region gene with the human C κ gene. Humanized mouse antibodies
CD8	Nakayama et al., 1994	ES cells are targeted on both alleles and chimeric mice show the complete phenotype

istic means of many laboratories, especially those contemplating the generation of one or only a few knock-out lines. Thus, blastocyst injection is often the rate-limiting, or indeed, prohibitive step, in the production of knock-out mice.

An alternative approach to chimera production has been developed which does not require any sophisticated equipment and only basic embryo manipulation skills. This approach is based on the observation that ES cells (the same as those used for blastocyst injection) readily adhere to eight cell embryos (morulae) and, upon subsequent culture, are internalised to colonise extensively the blastocyst including the cells which will give rise to the germ line. There are two variations of this technique for bringing the ES cells and embryo into contact, termed coculture (Wood et al., 1993b) and 'darning needle aggregation' (Nagy et al., 1993). Both include the following steps: (i) collection of eight cell embryos, (ii)

removal of the glycoprotein sphere (the zona pellucida) surrounding the embryo by simple enzyme or acid digestion, (iii) aggregation of 'denuded' embryos upon a lawn of ES cells (coculture) or nestling with a clump of ES cells in a depression ('darning needle aggregation'), (iv) culture overnight to blastocyst stage, and (v) reimplantation into pseudopregnant recipient females. Savings are made in terms of both cost and time with this technique. First, in terms of equipment, only a flame drawn pasteur pipette and dissecting microscope are required and the use of superovulated random-bred mice, and not naturally mated inbred strains, significantly decreases animal costs. Second, much less time is required to acquire the basic skills of embryo manipulation and, on the day of the experiment, the attachment and uptake of the ES cells proceeds unaided and can be done en masse.

Both aggregation techniques have been used

Fig. 5. Cre-loxP recombinase mechanism for gene inactivation. Three LoxP sites are introduced along with marker cassettes into the mouse genome by homologous recombination. Cre-recombinase is transiently expressed and mediates recombination, resulting in the loss of marker cassettes and the gene of interest (grey) (type I) or marker cassettes only (type II). Type II recombinants are used to generate chimeric mice expressing the gene product flanked by two LoxP sites. Tissue-specific inactivation of the gene is then achieved by mating this mouse with a transgenic mouse expressing the Cre-recombinase enzyme under the control of a tissue-specific promoter. The progeny express a functional gene in all tissues except the selected tissue compartment.
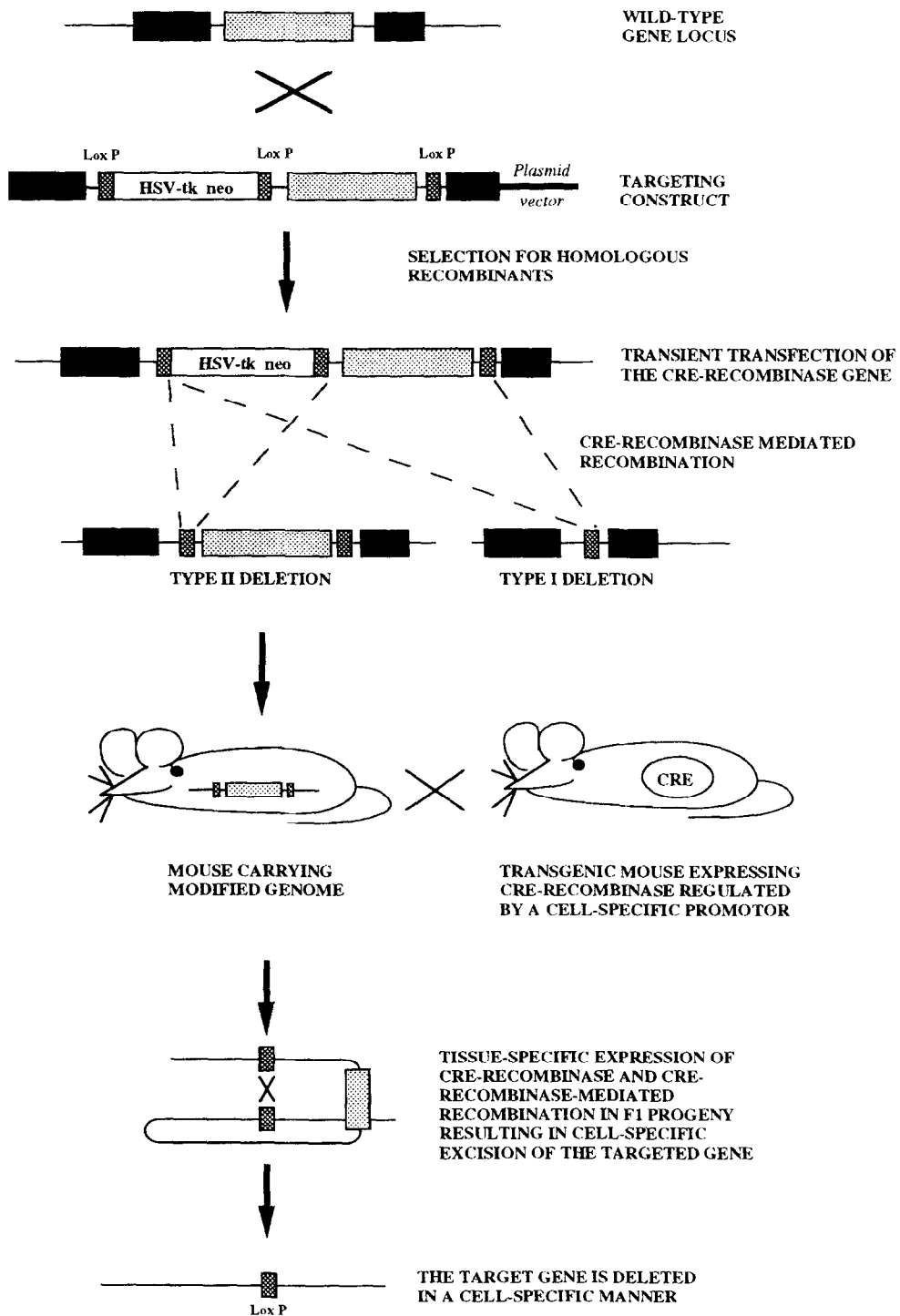
to produce germ-line chimeras (Nagy et al., 1993; Wood et al., 1993a; Wood et al., 1993b). In these experiments, the frequency of generating germ-line competent chimeras was approximately 5% of the blastocysts transferred (Wood et al., 1993a; Wood et al., 1993b). Interestingly, nearly all of these mice transmitted the mutated genotype to all of their offspring. Similar frequencies (2–10%) of production of germ-line competent animals have been reported following blastocyst injection (Schwartzberg et al., 1989; DeChiara et al., 1990; Mombaerts et al., 1992; Nagy et al., 1993). It should be noted however, that rates of up to 33% have been achieved by experienced blastocyst injectors (McMahon and Bradley, 1990). Although aggregation techniques have not as yet reached this level it should be emphasised that for the majority of knock-out experiments the generation of even one germ-line competent chimera is sufficient to establish a transgenic mouse line. With a little practice, the reimplantation of 100 embryos following aggregation represents one experiment and could well result in the establishment of a novel mouse strain.

2.12. Immunological impact

A review of current literature indicates that over 50 knock-out mice of significant potential interest to the understanding of immunological processes, have been published. It is not within the brief of this review to detail these and excellent overviews are already available (Fung-Leung and Mak, 1992; Pfeffer and Mak, 1994). However, it is of interest in the context of the methodology, to record how these were generated as an indication of the diverse protocols that successive reports of new knock-out mice have utilized. Details of six immunologically relevant gene knock-out mice generated using a methodologically novel aspect, are listed in Table 2.

2.13. Specialised and technically demanding approaches

As mentioned previously, for most purposes the standard replacement vector is an adequate strategy. However, following homologous recom-bination, large sequences of heterologous DNA (i.e. marker cassettes) containing functioning promoter and enhancer sequences, remain in the genome. While this is of no consequence in the production of a null allele, the presence of transcriptionally active foreign marker DNA makes it virtually impossible to study the effect of subtle mutations on genes and promoter/enhancer elements in situ. If such studies are desirable it is necessary to devise a strategy where marker cassettes inserted into the targeted gene are integrated into the target sequence then removed subsequently leaving only a subtle (but sufficient) mutation. The 'hit and run method' designed to achieve this (Hasty et al., 1991a; Valancius and Smithies, 1991b) has proven difficult due to the intrinsic problem of the low frequency of successful recombination events, and the need for extensive cell culture and screening if the genes are transcriptionally inactive in ES cells. To date, no immunologically significant mouse strain has been generated using this technique.

Another limitation of the original knock-out technique is that it is difficult to study genes which are essential for murine development and therefore, lethal if disrupted, because there is no specificity to the gene inactivation. This problem has now been overcome by the development of an elegant system in which such genes can be inactivated in a tissue or cell-type specific manner (Gu et al., 1993; Gu et al., 1994). This technique is based on the Cre-loxP recombination system of bacteriophage P1 (Sternberg et al., 1986). Cre-recombinase is an enzyme which catalyses the site specific recombination between 34-base pair motifs termed loxP sites. To utilise this in eukaryotic systems, a targeting vector is engineered by introducing three loxP sites to flank the gene segment of interest and the selectable marker gene cassettes (see Fig. 5). ES cells positive for this event are detected by screening for selection markers and are then transfected transiently with the gene encoding the Cre-recombinase enzyme. This has specificity for the introduced loxP sites. Three deletion mutants result; the first where both the selectable markers and the gene segment are removed following recombination (type I) and second, the desirable outcome, where the mark-

ers are deleted leaving the gene sequence flanked by two loxP sites (type II). The third outcome, deletion of the gene segment and a remaining selection marker, is lethal under selection pressure. The ES cells exhibiting type II mutations are used to generate chimeric mice carrying the modification which are bred until a homozygous offspring is produced. These mice still carry a functional gene. In parallel, separate transgenic mice must be generated that carry the Cre-recombinase gene under a tissue-specific promoter (Orban et al., 1992). The latter are crossed with those mice carrying the 'targeted' gene flanked by loxP sites. The result is viable progeny which lack the gene of interest in those specific cell types where tissue-specific promoter-driven production of the Cre-recombinase is occurring.

3. Conclusions

Employing the gene targeting technology reviewed here, from the simple sequence replacement vector approach to the highly sophisticated Cre-loxP system, it is now possible to explore the functions of genes by assessing the impact of the absence of products for which they encode. In immunological terms, this approach has proven to be of significant benefit in revealing the broader roles of molecules, which previously were not evident from in vitro studies. Spectacular examples include the inflammatory bowel diseases associated with the inactivation of the IL-2 (Sadlack et al., 1993) and IL-10 (Kühn et al., 1993) genes and the spontaneous and lethal multi-organ inflammation in TGF-β1 knock-out mice (Shull et al., 1992). There can be little doubt that novel gene knock-out mice will continue to offer insights into the function and regulation of complex biological systems of which the immune system is but one.

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