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Transposon-mediated generation of targeting vectors for the production of gene knockouts

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ABSTRACT

Vectors used for gene targeting experiments usually consist of a selectable marker flanked by two regions of homology to the targeted gene. In a homologous recombination event, the selectable marker replaces an essential element of the target gene rendering it inactive. Other applications of gene targeting technology include gene replacement (knockins) and conditional vectors which allow for the generation of inducible or tissue-specific gene-targeting events. The assembly of gene-targeting vectors is generally a laborious process requiring considerable technical skill. The procedures presented here report the application of transposons as tools for the construction of targeting vectors. Two mini-Mu transposons were sequentially inserted by *in vitro* transposition at each side of the region targeted for deletion. One such transposon carries an antibiotic resistance marker suitable for selection in mammalian cells. A deletion is then generated between the two transposons either by LoxP-induced recombination or by restriction digestion followed by ligation. This deletion removes part of both transposons plus the targeted region in between, leaving a transposon carrying the selectable marker flanked by two arms which are homologous to the targeted gene. Targeting vectors constructed using these transposons were electroporated into embryonic stem cells and shown to be effective in gene-targeting events.

INTRODUCTION

A 'gene knockout' refers to the targeted inactivation of a gene in a cell or an organism. The technology relies on the replacement of the wild-type gene on a chromosome (the target gene) by an inactivated gene on a targeting vector by homologous recombination. The targeting vector generally contains a selectable marker such as the neomycin resistance (*Neo*^{r}) gene flanked by two homology arms. The conventional way of constructing a targeting vector consists of multiple cloning steps, some of which involve the manipulation of large fragments which are frequently susceptible to DNA rearrangements. This makes the construction process technically demanding and time consuming.

To overcome these issues we developed a series of transposons which allow the random insertion of both selectable markers and restriction (or recombination) sites. Transposons are discrete DNA segments able to undergo random insertion events, a process referred to as transposition [for recent reviews, see (1–4)]. Such elements are present in both prokaryotes and eukaryotes. Bacterial transposons have been studied in detail and used widely in molecular biology. For example, some of them have been inserted into eukaryotic genes as well as bacterial genes for the purpose of insertional mutagenesis (5–11). They have also been utilized as a tool to insert primer-binding sites for sequencing (5,9,12). The mechanisms and modes of transposition vary for different transposons (1,13). Generally, transposition involves the recognition of the transposon ends by the transposon-encoded transposase(s), followed by recombination events between the transposon ends and the site of insertion. A number of *in vitro* transposon systems have been developed, where one or more purified transposase(s) catalyse the transposition of a mini-transposon to a recipient DNA molecule (14–20). In such systems, generally, the transposase(s) alone is sufficient and no accessory protein and DNA cofactors are required.

The current study reports a generic system for the generation of classical targeting vectors that can be used in any species, using bacteriophage Mu-based transposons. An added benefit of this system is that most steps of the process may be automated allowing for the high-throughput generation of targeting vectors.

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MATERIALS AND METHODS

PCR, DNA manipulation and cloning

PCR was carried out essentially as described previously (21). DNA manipulation and molecular cloning were performed according to Sambrook and Russel (22).

In vitro transposition

In vitro transposition reactions were carried out using the MuA transposase (Finnzymes, Espoo, Finland) following the manufacturer's instructions. Briefly, the transposon fragment was released from the plasmid vector by digesting with BglII, separated from the vector backbone by agarose gel electrophoresis and purified using the Qiaquick Gel Extraction Kit (Qiagen). Each 20 µl *in vitro* transposition reaction mixture contained 1× reaction buffer, 20 ng transposon fragment, 400 ng target DNA and 0.22 µg MuA transposase. The reaction mixture was incubated at 30°C for 1 h followed by 10 min at 75°C. One microlitre of the reaction mixture was electroporated into *Escherichia coli* DH10B, selecting for resistance to an antibiotic encoded by the transposon and resistance to another antibiotic coded for by the target plasmid.

Bacterial medium and antibiotics

Luria–Bertani medium was used for the growth of *E.coli* cells. Antibiotics were used at the following concentrations: ampicillin (Amp), 100 µg/ml; chloramphenicol (Cam), 35 µg/ml; kanamycin (Kan), 15 µg/ml; tetracycline (Tet), 15 µg/ml.

Screening for transposition event by PCR

Bacterial colonies derived from *in vitro* transposition were screened by PCR using a primer on the target gene and another primer located just inside the transposon end with its 3′ end facing outward of the transposon. Three parameters could be established by this PCR: whether the transposon was located within an amplifiable distance from the primer on the target gene and, if it was, the approximate location and orientation of the transposon.

Recombination between two LoxP sites by Cre recombinase

Recombination was performed by two means. The first was to electroporate the plasmid containing two LoxP sites to an *E.coli* strain expressing the Cre recombinase. Plasmid DNA was then isolated and the recombination event verified by PCR, restriction enzyme digestion and/or sequencing. The second involved the *in vitro* recombination using purified Cre recombinase (Invitrogen) as follows. Each 20 µl reaction mixture contained 1× reaction buffer, 100 ng plasmid DNA and 50 ng Cre recombinase. The reaction was incubated at 37°C for 20 min followed by 5 min at 65°C. One microlitre of the reaction mixture was used to transform *E.coli* DH5α. Individual colonies were screened by PCR, restriction enzyme digestion and/or by sequencing to identify the clones in which recombination had taken place.

Gene targeting in mouse embryonic stem cells

The targeting construct was linearized with AscI and electroporated into Bruce4 ES (embryonic stem) cells (23) selecting for resistance to G-418 (0.2 mg/ml). Ten days later, individual colonies were picked, expanded and split into two. One plate was frozen at −80°C in media supplemented with 10% dimethyl sulfoxide. The other plate was lysed and the DNA was extracted and screened by PCR using the Expand Long Template PCR System (Roche). Clones that gave the expected sized band were then confirmed at both the 5′ and 3′ ends by Southern analysis.

Transposons and BAC clones used in this study

Transposons constructed in this study included: TnCR1 (GenBank accession no. AY781408); TnCR2 (GenBank accession no. AY781399); TnCR3 (GenBank accession no. AY781400); TnCR4 (GenBank accession no. AY781401); TnCR7 (GenBank accession no. AY781405); TnCR8A (GenBank accession no. AY781406); TnCR8B (GenBank accession no. AY781407).

BACs used in this study were: RP23-225H9 for *emr1*, RP23-77M4 for TWEAK, RP23-351I24 for sperizin gene and RP23-461K18 for ubiquitin-specific protease 1 gene.

RESULTS

Construction of mini-Mu-based transposons

For the generation of deletions in cloned genes, three pairs of mini-Mu transposons were constructed (Figure 1) based on the transposon end sequence of Haapa *et al*. (20). The two transposons in each pair carried a different bacterial selectable marker which enabled selection of transposition events after transformation. One of the two transposons carried a *Neo*^{r} gene, which could be replaced with any suitable marker. The two transposons in each pair also carried either a LoxP site or complementary restriction endonuclease sites (Figure 1).

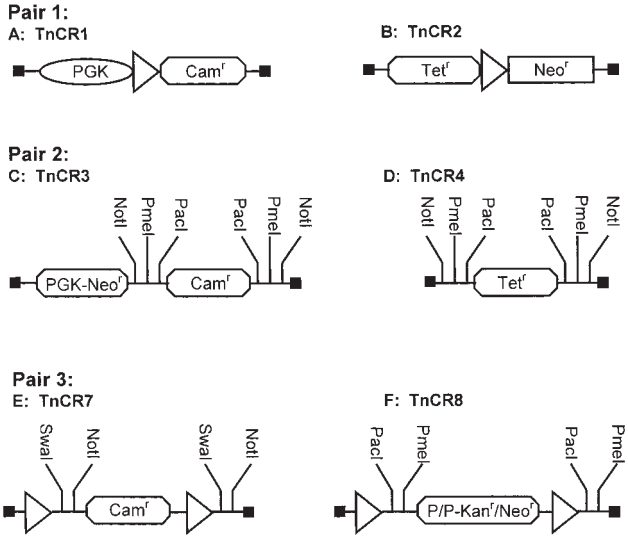

Figure 1. Mini-Mu transposons for the construction of targeting vectors. Each transposon has one or two antibiotic resistance genes flanked by transposon ends (filled black squares) with LoxP (open triangle) and/or restriction sites. PGK, the human PGK promoter; *Cam*^{r}, bacterial chloramphenicol resistance gene; *Tet*^{r}, bacterial tetracycline resistance gene; *Neo*^{r}, G-418 resistance gene without a promoter; PGK-*Neo*^{r}, G-418 resistance gene driven by the PGK promoter; P/P-*Kan*^{r}/*Neo*^{r}, eukaryotic/prokaryotic dual promoters driving the expression of kanamycin resistance in *E.coli* and G-418 resistance in mammalian cells.

Randomness of transposition event

Transposon events are generally considered to be random or nearly random (24) with some exceptions. However, given the novelty of our approach and the concern that the presence of extended palindromic sequences (from both the transposon ends and the LoxP sites), the randomness of the transposition events was assessed directly. TnCR7 (Figure 1) was used to test if the transposition was random in both insertion site and orientation. A plasmid was constructed by replacing the tetracycline resistance gene of pBR322 with a mouse DNA fragment containing the ubiquitin-specific protease 1 gene. This plasmid when digested with HindIII produces six fragments of various sizes. TnCR7 was transposed to this clone by *in vitro* transposition and plasmid DNA extracted from 46 randomly selected colonies was digested with HindIII. As there is no HindIII site on the transposon, any fragment of the plasmid which had acquired the transposon would produce a bigger band when subjected to electrophoresis on agarose gels. Among the 46 colonies tested, the number of insertions on each fragment is reported in Table 1.

It can be seen from Table 1 that the frequency of insertions in any particular HindIII fragment was directly proportional to the size of the fragment. An exception is the 3322 bp vector fragment which had less insertion than the smaller fragments. This was expected because transposon insertions to the ampicillin resistance gene and to regions essential for plasmid maintenance would be eliminated under the experimental conditions. To assess the orientation of the transposition events, 19 insertions on 3 fragments were tested to determine if there was any bias. This was done by PCR using a primer on the plasmid and another on the transposon. Table 2 reveals that 10 insertions were in the forward orientation and 9 in the reverse orientation, indicating that there is no orientational bias for the transposition events. These results indicated that mini-Mu-based transposons insert into plasmid DNA in a random manner unaffected by the presence of the extended palindromic sequences.

Generation of targeting vectors using Pair 1: TnCR1, TnCR2

TnCR1 contains the PGK promoter and the bacterial chloramphenicol (*Cam*^{r}) gene, separated by a LoxP site (Figure 1A). TnCR2 carries the bacterial tetracycline resistance gene, followed by a LoxP site and then the *Neo*^{r} gene without a promoter (Figure 1B). This pair of transposons was used to generate deletions in an 11 kb fragment containing part of the mouse *emr1* gene (25) (Figure 2).

TnCR1 was inserted into the *emr1* region by *in vitro* transposition. Among the 95 colonies screened by PCR, four clones produced PCR products of ~500 bp (Table 3). One was selected and confirmed by DNA sequencing to have TnCR1 inserted 466 bp upstream of exon 1. TnCR2 was inserted to this clone and 30 colonies were screened by PCR. Two clones were identified as having the TnCR2 located ~2 kb downstream of exon 1 and in the right orientation. One clone was selected and shown to have TnCR2 ~2 kb downstream of exon 1. The exact location was not determined because the transposon landed in a region for which the sequence was unavailable in the public databases. This highlights another advantage of this transposon-based strategy—the complete genomic sequence was not required to generate targeting vectors, i.e. exonic sequence information was sufficient. This clone was used to generate deletions between the two LoxP sites on the transposons, by two Cre treatments. First, DNA was electroporated into an *E.coli* strain expressing the Cre recombinase,

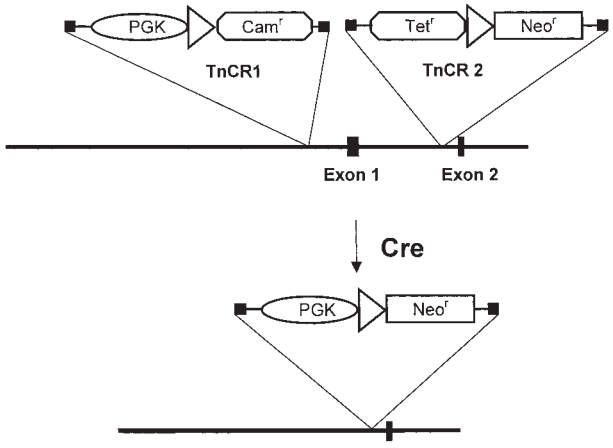

Figure 2. Schematic representation of generating targeting vectors using TnCR1 and TnCR2. TnCR1 is inserted into the target gene by *in vitro* transposition. TnCR2 is then inserted at the other side of the region to be deleted. Deletions are generated between the two LoxP sites by one of two Cre treatments. First, DNA is electroporated into an *E.coli* strain expressing the Cre recombinase. Second, the construct is treated with Cre recombinase (Invitrogen) and then transformed to *E.coli*.

Table 1. Randomness of the mini-Mu transposon insertion

Size of the HindIII fragment	Number of insertions
7580 bp	21
3322 bp (vector)	5
1430 bp	9
1355 bp	8
1038 bp	2
392 bp	1

Table 2. Random orientation of the mini-Mu transposon insertion

HindIII fragment	Orientation 1	Orientation 2
1355	4	4
1038	2	0
1430	4	5

Table 3. Generation of deletions in the *emr1* gene by TnCR1 and TnCR2

	Deletion generated 2.5 kb deletion	4.5 kb deletion
Frequency of ideal TnCR1 position by PCR	4/95	2/95
Frequency of ideal TnCR2 position by PCR	2/30	1/160
Efficiency of recombination in *E.coli* expressing Cre	5/5	10/10
Efficiency of recombination by Cre recombinase	12/18	4/10

selecting for ampicillin resistance. All five transformants selected were found to be sensitive to chloramphenicol and tetracycline, indicating that recombination had occurred. Restriction digestion of plasmid DNA showed digestion patterns corresponding to recombination between the two LoxP sites, generating a 2.5 kb deletion. Second, the same DNA carrying both transposons was treated *in vitro* using Cre recombinase, followed by transformation into *E.coli* with the selection of ampicillin resistance. Eighteen colonies were tested and 12 were found to be sensitive to chloramphenicol and tetracycline. A 2.5 kb deletion was subsequently confirmed by restriction mapping.

In another experiment also using *emr1*, 95 colonies were screened by PCR after transposition with TnCR1 and two clones produced PCR products of ~2 kb. One was selected and found, by sequencing, to have the transposon 2321 bp upstream of exon 1. After transposition with TnCR2, 160 colonies were screened by PCR. One clone was identified as having the second transposon located ~2 kb downstream of exon 1. This clone was used to generate deletions between the two LoxP sites on the transposons. After DNA was electroporated into the *E.coli* strain expressing the Cre recombinase, 10 ampicillin-resistant transformants revealed that they were all sensitive to chloramphenicol and tetracycline. Restriction digestion of plasmid DNA showed digestion patterns corresponding to recombination between the two LoxP sites, resulting in a 4.5 kb deletion. The same DNA carrying both transposons was treated *in vitro* using Cre recombinase, followed by transformation into *E.coli* with the selection of ampicillin resistance. Ten colonies were tested and four were found to be sensitive to chloramphenicol and tetracycline. A 4.5 kb deletion was subsequently confirmed by restriction mapping.

Generation of targeting vectors using Pair 2: TnCR3, TnCR4

TnCR3 contains the PGK- *Neo*^{r} construct and the bacterial *Cam*^{r} gene, the latter being flanked by sequences for three 8-base cutters: Not1, Pme1 and Pac1 (Figure 1C). TnCR4 carries the bacterial tetracycline resistance gene flanked by sequences for the same 8-base cutters (Figure 1D). A plasmid carrying 14.4 kb of mouse genomic DNA containing exons 1–5 of the TWEAK gene (26) was used to construct targeting vectors (Figure 3 and Table 4). TnCR3 was inserted into the TWEAK region by *in vitro* transposition and from the 96 colonies screened by PCR, three clones produced PCR products of the desirable sizes. Two clones, with the transposon at 854 and 1008 bp upstream of the ATG start codon, respectively, were selected to insert TnCR4. Ninety-six colonies were screened by PCR with seven clones having TnCR4 located in the ideal location and orientation. The DNA of all seven clones was digested with Pac1 and two of them gave a band of ideal size to be deleted. The digested DNA of these two clones was re-ligated and transformed to *E.coli* selecting for ampicillin resistance. One colony from each was tested and found to be sensitive to chloramphenicol and tetracycline. Plasmid DNA of both clones was sequenced to identify the deletion. Both deletions started at 1008 bp upstream of the ATG start codon. One deletion ended inside of exon 5 and the other ended 188 bp downstream of exon 5.

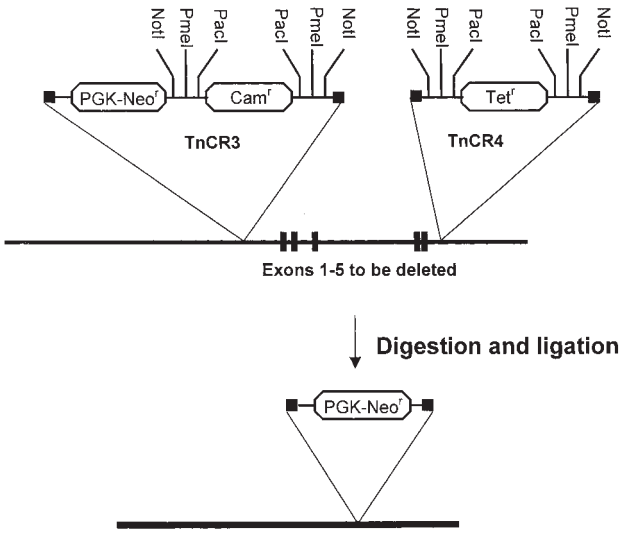

Figure 3. Schematic representation of generating targeting vectors using TnCR3 and TnCR4. TnCR3 is inserted into the target gene by *in vitro* transposition. TnCR4 is then inserted at the other side of the region to be deleted. Deletions are generated by digesting the DNA with one of the restriction endonucleases followed by ligation and transformation to *E.coli*.

Table 4. Generation of deletions in the TWEAK gene by TnCR3 and TnCR4

Frequency of ideal TnCR1 position by PCR	3/96
Frequency of ideal TnCR2 position by PCR	7/96
Frequency of clones with desirable size of deletion	2/7

TnCR3 was also used to generate a targeting vector for the mouse sperizin gene (27). *In vitro* transposition was carried out to a plasmid containing 11 kb mouse genomic DNA with this single exon gene. Thirty-two colonies were screened by PCR and seven generated PCR products of ideal size. Four were selected and their DNA sequenced. Two out of the four were found to have the transposon in an ideal position and orientation. One of them, with the transposon at 1376 bp upstream of the ATG start codon, was used to generate a targeting vector. Due to the presence of a Pac1 site 1459 bp downstream of the TGA stop codon, deletions were generated by digesting the DNA with Pac1 and re-ligation. Hence, only a single transposition event was required to delete the entire sperizin gene. After transforming to *E.coli*, 23 transformants tested were all found to be sensitive to chloramphenicol. One was sequenced to confirmed the integrity of the deletion.

Generation of conditional targeting vectors using Pair 3: TnCR7, TnCR8

TnCR7 carries the *Cam*^{r} gene flanked by a pair of 8-base cutters and LoxP (Figure 1E). TnCR8 carries the eukaryotic/prokaryotic dual promoter driving the expression of the *Kan*^{r}/*Neo*^{r} gene (Figure 1F). Two versions of the dual promoters were used. TnCR8A contains the human PGK promoter in front of the prokaryotic kanamycin resistance gene promoter (derived from Tn5) whereas TnCR8B uses

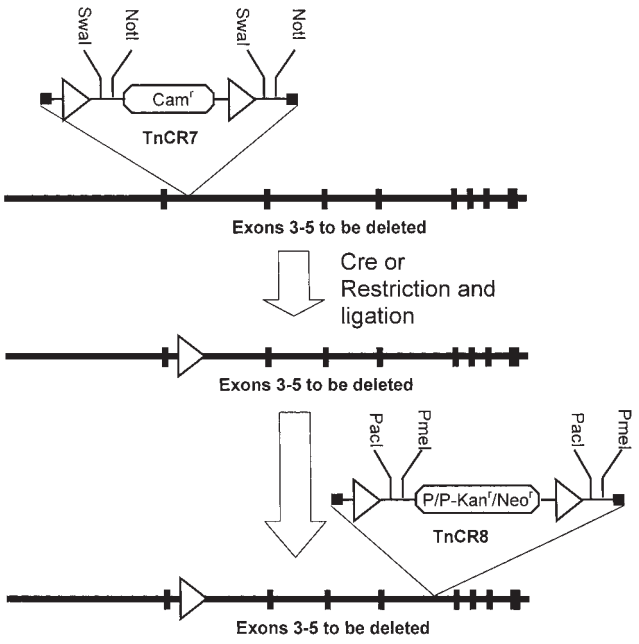

Figure 4. Schematic representation of generating conditional targeting vectors using TnCR7 and TnCR8. TnCR7 is inserted into the target gene by *in vitro* transposition. The *Cam^r* gene is deleted using either Cre or a restriction endonuclease, leaving a single copy of LoxP. TnCR8 is then inserted at the other side of the region to be deleted. The construct is now a conditional targeting vector.

the bacterial bla promoter followed by the viral SV40 promoter. The P/P- *Kan^r/Neo^r* cassette in TnCR8 is also flanked by a pair of 8 bp restriction sites and LoxP sites. These two transposons were specifically designed to generate conditional targeting vectors (Figure 4). A conditional targeting vector contains recombination sites such as LoxP in the introns flanking the exon(s) targeted for deletion. After a targeted event, the gene can still function because the position of the LoxP sites is selected to minimize the impact on gene expression, i.e. a non-coding region away from splice acceptor sites and regulatory elements is desirable. Upon expression of a recombinase gene such as *Cre*, however, the region between the LoxP sites will be deleted, resulting in an inactivated gene. TnCR7 should be first inserted at one side of the exon(s) to be deleted. The *Cam^r* gene and a copy of the LoxP site can then be deleted either by Cre-mediated recombination or by restriction digestion followed by ligation, leaving one copy of LoxP on the vector. TnCR8 is then inserted at the other side of the exon(s) to generate the conditional targeting vector. These transposons were used to generate conditional targeting vectors for the mouse ubiquitin-specific protease 1 gene (Table 5).

A plasmid with 12 kb mouse genomic DNA containing exons 2–9 of the ubiquitin-specific protease 1 gene was subjected to *in vitro* transposition by TnCR7 and 96 colonies were screened by PCR. Among six positive colonies, five had the transposons between 146 and 1576 bp upstream of exon 3. One clone, with the transposon located in intron 2 (265 bp upstream of exon 3), was selected and plasmid DNA electroporated into the *E.coli* strain expressing Cre selecting for ampicillin resistance. Twenty-five transformants were patched on a Cam plate and all were found to be sensitive to chloramphenicol,

Table 5. Generation of conditional targeting vectors in the ubiquitin-specific protease 1 gene by TnCR7 and TnCR8

TnCR7 (clones with PCR products)	Position in clone selected	Efficiency of deleting *Cam^r* by Cre recombination	Transposon (clones with PCR products)	Position in clone selected
6/96	265 bp before exon 3	25/25	TnCR8A (15/48)	536 bp after exon 5
			TnCR8B (10/48)	624 bp after exon 5

indicating the deletion of the *Cam^r* gene between the two LoxP sites.

One clone was selected and subjected to *in vitro* transposition of both TnCR8A and TnCR8B. Forty-eight colonies were screened by PCR from each transposition experiment and 15 produced products for TnCR8A and 10 for TnCR8B. One clone with the transposon located in intron 5 was selected from each transposition as the conditional targeting vector, with TnCR8A and TnCR8B located 536 and 624 bp downstream of exon 5, respectively.

Gene targeting in mouse ES cells using targeting vectors generated by transposons

The ability of the transposon-generated vectors to target genes was tested in mouse ES cells. Two of the constructs (one for spz-1 and the other one for TWEAK) were electroporated into Bruce4 ES cells, selected with G-418 and 10 days later 480 individual colonies were picked, expanded and split onto two plates. One plate was frozen down and the other plate was lysed and DNA extracted. All colonies were screened for targeting at the 3′ end by a PCR assay using a forward oligo in the *Neo^r* gene and a reverse oligo external to the 3′ arm. Targeting was subsequently confirmed by Southern analysis at both the 5′− and 3′− ends. Four and five correctly targeted clones were identified for the two genes, respectively, representing a targeting efficiency of ∼1% of the neomycin-resistant clones picked, which was comparable with other targeting vectors that we have used. This efficiency is relatively low because the vectors do not have a negative-selection marker (such as TK) to eliminate clones resulting from non-homologous integration events. However, negative-selection cassettes may be easily incorporated into the vector. These targeted cells were subsequently used to generate knockout mice.

DISCUSSION

We have developed procedures to construct targeting vectors using transposons. Compared with conventional methods for vector construction, our methods are rapid and technically simple. Multiple targeting vectors are routinely generated in ∼2 weeks. The procedure does not require direct cloning from genomic fragments and, hence, no restriction enzyme sites are required on genomic DNA. Furthermore, it is not necessary to amplify homology arms by PCR, thus eliminating the chance

for PCR generated errors. Such errors tend to reduce targeting efficiency (28).

Other variants of the transposons presented here may also be generated. For example, the *Neo*^{r} gene could be switched from TnCR2 to TnCR1, to be inserted between the PGK promoter and LoxP. Furthermore, it would be advantageous to have a series of transposons carrying different eukaryotic selectable markers including hygromycin and puromycin resistance. Although the *in vitro* transposon system was initially used, *in vivo* systems can also be developed where transposition is achieved by bacterial mating. We have successfully developed such a system to mutagenize bacterial genes by Tn5 insertion (7). This can potentially be adapted for the construction of targeting vectors. This would negate the need for any transformations which would result in further time savings. The mini-Mu transposons were selected in this study due to their high efficiency of transposition and lack of transposition immunity, thus allowing two transposons on the same target molecule (20). Other bacterial transposons may also be used but may be less flexible than mini-Mu.

The transposon-mediated procedure for generation of deletions will speed up vector construction as soon as a gene is cloned. In our method, the optimal size for the initial genomic clone is 10–15 kb. This may be derived directly from a lambda clone or any other large genomic clone. Two parameters should be taken into consideration when choosing the fragment size: (i) it should be short to facilitate easy manipulation and high efficiency of transposition to any desired region; (ii) at the same time, it should be long enough to leave homologous arms of sufficient length after a deletion event. Given the randomness of the transposon event, targeting a 1 kb region within a 20 kb clone should result in 5% of all clones screened being positive for the transposon. This efficiency would be reduced by half if a specific orientation was required. In our method, the picking of 96 clones for a PCR-based screen generally resulted in 1–5 clones with the desired transposon event. Occasionally, lower rates have been observed but this was easily overcome by either screening more clones or selecting a slightly different target for the transposition event.

We have tended to use regions of genes isolated from BAC (C57BL/6) clones which have either been generated by direct subcloning or by ET cloning (29,30). We do not use negative selection but this may easily be incorporated into the overall strategy, preferably by inserting the initial genomic clone into a vector carrying a negative-selection cassette (31). The system described here has a number of advantages over current methods. The most significant of these is the generic nature of the transposons which may be used directly without any requirement for modification. Speed and the ability to handle multiple genes simultaneously are other key advantages.

A number of technologies are currently available that accelerate the process of generating knockouts. ET cloning (32) is probably the most notable technique that has emerged for the generation of traditional knockout vectors. Compared with the transposon technology presented here, ET cloning is also rapid and highly flexible. An advantage that the transposons have over the ET technology is that the vectors are generic and may be used on any gene. ET cloning requires long PCRs which occasionally suffer from sequence-specifc technical difficulties; furthermore, the requirement for relatively long PCR primers add significant cost to the application of the ET technology. One advantage of the ET system is that the vectors constructed are precise rather than semi-random. Non-traditional strategies have recently emerged, which include indexed vectors (33) and the use of ES cells with a defective mismatch repair system (34,35). While these new approaches are able to rapidly generate knockout cells or animals they lack the precision of the traditional application of knockout vectors and as such have a limited application.

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