Embryonic stem cell gene targeting using bacteriophage \( \lambda \) vectors generated by phage–plasmid recombination

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ABSTRACT

Targeted mutagenesis is an extremely useful experimental approach in molecular medicine, allowing the generation of specialized animals that are mutant for any gene of interest. Currently the rate determining step in any gene targeting experiment is construction of the targeting vector (TV). In order to streamline gene targeting methods and avoid problems encountered with plasmid TVs, we describe the direct application of \( \lambda \) phage in targeted mutagenesis. The recombination-proficient phage vector \( \lambda 2TK \) permits generation of TVs by conventional restriction–ligation or recombination-mediated methods. The resulting \( \lambda TV \) DNA can then be cleaved with restriction endonucleases to release the bacteriophage arms and can subsequently be electroporated directly into ES cells to yield gene targets. We demonstrate that in \( \text{vivo} \) phage–plasmid recombination can be used to introduce \( \text{neo} \) and \( \text{lacZ–neo} \) mutations into precise positions within a \( \lambda 2TK \) subclone via double crossover recombination. We describe two methods for eliminating single crossover recombinants, \( \text{spi} \) selection and size restriction, both of which result in phage TVs bearing double crossover insertions. Thus TVs can be easily and quickly generated in bacteriophage without plasmid subcloning and with little genomic sequence or restriction site information.

INTRODUCTION

Targeted mutagenesis allows specific mutations to be engineered into the mouse germline via homologous recombination of exogenously altered DNA in embryonic stem (ES) cells (1,2). Using this technology, the function of any cloned gene may be examined by its disruption in mice. Thus gene targeting is a critical experiment in molecular medicine, enabling mimicry of human mutations in the mouse for generation of experimental therapeutic models (3).  

The original and still the most prevalent gene targeting approach, the knock-out’, uses a replacement vector to direct a positive selectable marker (i.e. neomycin resistance) into a specific chromosomal location via either double reciprocal exchange or gene conversion (4). However, many sophisticated variations on this original technique have become available, including the generation of point mutations, deletions and translocations and gene substitutions (5–9). Further, the application of cre recombinase from bacteriophage P1 allows additional genomic alterations at \( \text{loxP} \) target sequences following gene targeting so that mutations can be made tissue- or development-specific (10).

Although targeted mutagenesis provides a powerful tool for analysis of gene function, it is a complex and time consuming procedure. While methods of improving the efficiency of generating targeted ES cell lines (11) and mutant mice (12) have become available, little has been done to streamline construction of the targeting vector (TV). In general, two types of problems are encountered in construction of TVs. First, specific genomic regions undergo rearrangements in plasmid vectors and are difficult to clone either on their own or in combination with the \( \text{neo} \) and \( \text{tk} \) selectable marker genes. Second, the use of large genomic fragments in TVs often limits the choice of unique restriction sites available for inserting foreign DNA fragments, such as the \( \text{neo} \) or \( \text{lacZ–neo} \) gene cassettes, for modifying function of the test gene. Although both these problems may be alleviated by choosing a smaller genomic region on which to base the TV, the reduction in homology in the vector will likely lower the gene targeting frequency (13).

In order to obviate the difficulties with TV construction in plasmids, we describe the direct application of \( \lambda \) phage in targeted mutagenesis. Using a recombination-proficient bacteriophage, \( \lambda 2TK \), we are able to introduce \( \text{neo} \) and \( \text{lacZ–neo} \) mutations into precise positions within genomic subclones via double crossover recombination. In this approach the efficiency of TV construction is improved, as it is not dependent on restriction site availability. Furthermore, we demonstrate that bacteriophage TVs can be directly introduced into ES cells to yield targeted clones, thus avoiding the use of plasmid TVs altogether.

MATERIALS AND METHODS

Bacterial strains, bacteriophage and plasmids

\textit{Escherichia coli} strains MC1061 (\( \text{rec}^\circ, \text{sup}^B \)) and its P2 lysogenic derivative P2MC1061, as well as MC1061[P3], were kindly provided by Dr D.M.Kurnit (University of Michigan, Ann Arbor, MI). LE392 (\( \text{rec}^\circ, \text{sup}^E, \text{sup}^F \)) and its P2 lysogenic derivative P2392 were obtained from Stratagene (La Jolla).

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For routine cloning in phage, λ packaging extracts (Amersham) were split into thirds and either used directly or re-frozen on dry ice for later use. The gene targeting bacteriophage vector λ2TK (Fig. 2A) was preceded by construction of a λDash II (Stratagene) derivative containing thymidine kinase genes from Herpes simplex viruses 1 and 2 (HSVv1 and tk2; 12,14) in the left and right polylinkers respectively, between NoI and XhoI sites (not shown). A Sall fragment comprising the two tk genes and stuffer region was then transferred into λSyirn2A (15) to place the tk1 and tk2 genes adjacent to the short and long arms of the phage respectively. Finally, a XhoI fragment containing the polylinker and stuffer region of λGem11 (Promega) was shuffled into this derivative to yield λ2TK (Fig. 2A). The bacteriophage vector used for plasmid–phage recombination, λ2TK:CRABPI (Fig. 2B), was generated by inserting into λ2TK a 9.5 kb XhoI fragment encompassing the first and second exons of the murine cellular retinoic acid binding protein I gene (cRABPI; 16).

All of the plasmid constructs used in this study were harbored in the recA+ host MC1061. Plasmids bearing the supF gene were harbored in MC1061[3]. The P3 episome, which is Kanr, Ampr and Tetr, facilitates selection of supF in media containing kanamycin, ampicillin and tetracycline (17). Since all of the plasmids used in this study were ampicillin resistant, only tetracycline served to functionally select for the presence of supF. The plasmid pGam was generated by excising a 500 bp Sall fragment containing the gam gene coding sequence from bacteriophage λ (18) and inserting it into the Sall site of pBluescriptKS+ (Stratagene). supF (sensitive to P22 interference) activity (18,19) conferred by pGam was assessed initially by its inability to be grown in red–, gam+ recombinant λ phage plated on P2392.

Construction of the recombination plasmids used in this study (Fig. 3C) was preceded by the assembly of pCRABPIgSp, which is a small 400 bp BgIII–SpeI genomic subclone that encompasses the second exon of cRABPI. An Sall site which bisects this genomic fragment was converted to BamHI in order to facilitate cloning of supF-bearing cassettes of MC1neoA (4) and lacZ–MC1neo pA (5) in pCRABPI:neoF and pCRABPI:lacneoF respectively. pGamCRABPI:neoF is the pGam derivative of pCRABPI:neoF.

Phage–plasmid recombination

λ2TK:CRABPI phage (1 × 107 p.f.u.) were passaged via plate lysate over LE392 or MC1061[3], bearing either pGamCRABPI:neoF or pCRABPI:lacneoF, overnight at 37°C. Supernatant phage were collected the following day in phage dilution buffer and used to infect the indicator strain LE392, MC1061 or P2MC1061. In experiments using pCRABPI:neoF all phage–plasmid recomblicants (both double and single crossovers) were scored on MC1061, while double crossovers were scored on P2MC1061. Due to the size limitation of λ phage packaging, only double crossovers could be scored on MC1061 in experiments with pCRABPI:lacneoF. The structure of the resulting recomblicants was confirmed by restriction analysis of small scale phage DNA preparations.

Gene targeting

Phage targeting vectors were introduced into E coli cells by electroporation, as previously described for plasmid targeting vectors (4). Prior to electroporation, the arms of the λ targeting vector were removed by digestion with NotI and the DNA concentration was estimated on the basis of insert only. Targeted cell lines were enriched by positive–negative selection (11) using FIAU (1-[2-deoxy-2'-fluoro-1-[β-D-arabinofuranosyl]-5-iodouridine) instead of gancyclovir for negative selection (20). Clones bearing targeting events were identified by genomic Southern blotting using a probe which flanked the 5’-end of the TV (4). Integrity of the targeted locus was confirmed using a probe internal to the locus which spanned the site of the neo gene insertion.

RESULTS AND DISCUSSION

Bacteriophage gene targeting vectors

We have observed that during TV construction certain genomic regions are not amenable to cloning in plasmids, either on their own or in association with neo or tk selectable markers. This cloning difficulty is likely a combination of both the large homology requirement for the TV and addition of difficult regions bearing repetitive sequences. Although we have found that TV stability in plasmids can be augmented by shortening of the homology length or removal of certain difficult regions, we are concerned that either manipulation of the homology region could have serious consequences on targeting efficiency (4,13).

We have found, with little exception, that unstable plasmid regions can be maintained in bacteriophage λ. We and others have used λ phage previously to assemble TVs that are subsequently ‘rolled out’ of phage in plasmid form (21,22). In this approach λ and plasmid sequences occur on either side of phage arms such that following construction a plasmid TV is excised from the phage using rare restriction sites or cre recombinase. While this latter approach may be useful in some cases, the use of cre recombinase in TV construction may preclude its subsequent application at the genomic level following gene targeting. In this report we demonstrate that generation of plasmid TVs from phage precursors is redundant, as phage TVs may be used directly in gene targeting. With the elimination of plasmid sequences from the phage TV we demonstrate that double crossover phage–plasmid recombination can be used to direct gene targeting mutations, such as neo or lacZ–neo insertions into specific sites within λ phage vectors, without the use of restriction enzymes.

Strategies for generating targeting vectors via phage–plasmid recombination

Previously phage–plasmid recombination has been used to isolate λ phage from genomic libraries by recombination screening (15,17). In this method a λ genomic library (bearing amber mutations) is passaged over a recA+ bacterial strain bearing a small supF (amber suppressing) recombination plasmid. Homology in the recombination plasmid, usually derived from a cDNA sequence, directs the plasmid to integrate into the phage by single crossover, thereby generating supF-bearing phage recomblicants capable of growing on a suppressor-free (supF) host. Depending on homology length, the recombination plasmid can integrate at a frequency of ~10–2. While we considered the possibility that phage TVs could be constructed by single crossover recombination, we were concerned that condensation of this complex could result in reversion of any phenotype generated by the neo or lacZ–neo insertion. Therefore, we sought to introduce more stable changes in phage targeting vectors by selecting for double crossover events that introduce neo or lacZ–neo mutations by replacement.
Two strategies were tested to see whether double crossover events could be enriched by eliminating single crossover phage (Fig. 1). In the first approach a spi selection system was devised to eliminate phage that have integrated the entire recombination plasmid (Fig. 1A). λ phage that carry a functional copy of the g氨 gene are sensitive to P2 interference (spι+), and thus cannot be replicated on a P2 lysogenic host (18,19). Thus, if g氨 function could be maintained in a recombination plasmid, single crossover integrants bearing g氨 could be eliminated on a P2 lysogen. This approach was used to make a neo ‘knock-out’ TV. The second strategy took advantage of the size limitation in packaging λ phage (23; Fig. 1B). Thus, if a relatively large recombination plasmid was used in the reaction only double crossover phage could be propagated following recombination. This approach was used to make a lacZ-neo ‘knock-in’ TV.

λ2TK in phage targeting vector construction

λ2TK (Fig. 2A) is based on the recombination screening vector λSyrinx2A (15) but differs from that vector in having a gam+ stuffer fragment to enable spi selection of cloned inserts (18) and HSVtk1 and -tk2 genes adjacent to its small and large arms respectively. The tk1 and tk2 genes serve as negative selectable markers in gene targeting experiments (11). Otherwise, as with λSytinx2A, λ2TK is A3m, gam, S3m, requires the amber suppressor tRNA, supF, for lytic growth and is Rap+ for efficient recombination with the plasmid. The vector has unique XbaI and Xhol restriction sites for subcloning of relatively longer genomic pieces and can receive fragments ranging in size from 3.5 to 16.5 kb. Since the Xhol site is compatible with SpeI and AvrII, while Xhol can accommodate Sall, in addition to BamHI, BglII, BclI and Sau3A by partial fill-in of cohesive ends, there is a considerable amount of flexibility in subcloning of genomic regions.

Two basic approaches can be used to construct gene targeting vectors in λ2TK. One approach (not shown) relies on our observation that λ2TK, unlike plasmid TV constructs, are amenable to multiple partner ligations. Thus a TV is generated in one step by ligating a 5′ genomic fragment, a neo cassette and a 3′ genomic fragment into λ2TK. If supF derivatives of neo or lacZ-neo are used in ligation, selection of correct recombinants is facilitated by plating λ2TK phage on a supF- host, such as MC1061. In a second approach (see below) an 8–10 kb genomic fragment is subcloned into λ2TK and a neo or lacZ-neo cassette is converted into a specific site via in vivo recombination.

neo and lacZ-neo λ2TK TVs via double crossover recombination

Recombination experiments were preceded by construction of a λ2TK phage subclone and a number of test recombination plasmids (Fig. 2B and C). As a model for these experiments we used the gene cRABPI, which has been previously disrupted in mice (24). λ2TK-CRABPI (Fig. 2B) contains a 9.5 kb isogenic genomic fragment bearing the second and third exons of cRABPI (16). The homology region used in the neo and lacZ-neo recombination plasmids (Fig. 2C) was derived from a 400 bp SpeI–BglII genomic fragment bearing exon 2 of cRABPI. In pCRABPISpBgl, an SmI site which bisects this fragment within the coding sequence was converted to BamHI for convenient cloning of the supF-bearing cassettes of MCIneoA and lacZ-MCIneoA

For the recombination strategy which utilized λgam and spi selection to eliminate single crossover phage the homology and supF regions were subcloned in pGam to generate pGamCRABPI::neoF. pGam is a pBluescript derivative that carries a functional λgam gene and can confer a spi+ phenotype to recombinant phage. For the strategy involving size limitation of λ packaging pCRABPI::lameoF was constructed, which placed a lacZ-neo–supF cassette in-frame within pCRABPISpBgl. As this plasmid was 5.7 kb in length, it was theoretically too large to integrate into the vector by single crossover. The 5.7 kb lacZ-neo–supF portion, however, was sufficiently small to be converted into the λ2TK.CRABPI TV by double crossover.
Figure 2. Maps of recombination phage and plasmids. (A) \(\lambda\)2TK is a derivative of the recombination screening vector \(\lambda\)Syrinx2A (15). It carries amber mutations in the A, B and S genes and is Rap\(^+\), for efficient recombination with the plasmid. HSV\(tk\)1 and -\(tk\)2 cassettes (11,14) are adjacent to the small and large arms respectively, in divergent orientations. A red\(^+\), gam\(^+\) stuffer fragment was derived from \(\lambda\)Gem1. Restriction sites used for cloning (Xh, Xho\(I\); Xb, Xba\(I\)) or excising arms (S, Sal\(I\); N, Not\(I\)) are indicated. (B) \(\lambda\)2TK:CRABPI was constructed by inserting a 9.5 kb Xho\(I\)–Eco\(RI\) fragment from the cellular retinoic acid binding protein I gene (cRABPI; 16) into the Xho\(I\) sites of \(\lambda\)2TK. The position of the 400 bp SpeI–Bgl\(II\) fragment encompassing the region of homology used in the homologous recombination experiments is indicated. (C) \(\lambda\)GamCRABPI:neoF (panel i) is a plasmid subclone of this 400 bp fragment where a central Sst\(I\) site in the second exon has been converted to Bam\(HI\) for the introduction of neo–sup\(F\) and lacZ-neo–sup\(F\) cassettes. CRABPI:neoF (panel ii) is a derivative of this plasmid containing a 1.4 kb sup\(F\) cassette of MC1neoA (4). pgamCRABPI:neoF (panel iii) is identical to pCRABPI:neoF with the exception that the gam gene is included in this plasmid. pCRABPI:lacneoF (panel iv) is a derivative of pCRABPI:neoF that contains an in-frame fusion of a 5.7 kb sup\(F\) cassette of lacZ-MC1neoA (5).

\(\lambda\)2TK:CRABPI phage was passaged via plate lysates through MC1061[P3] bearing pCRABPI: neoF, pGamCRABPI:neoF or pCRABPI: lacZneoF, as well as a sup\(F^+\) control strain LE392. The resultant phage were plated on LE392, in order to estimate the titer of harvested phage, and on the restrictive host MC1061, to evaluate the titer of sup\(F^+\) recombinant phage. In addition, to confirm gam gene function in pGamCRABPI:neoF, the supernatants were plated on a P2 lysogen of MC1061. As shown in Table 1, the passage of \(\lambda\)2TK:CRABPI through LE392 resulted in no detectable sup\(F^+\) recombinant phage. In contrast, phage passaged through strains bearing the recombination plasmids did result in sup\(F^+\) recombinants, albeit at differing frequencies. Passage through MC1061[P3, pCRABPI:neoF] resulted in roughly 7% recombinants, as indicated by sup\(F^+\) phage grown on MC1061. Passage through MC1061[P3, pGamCRABPI:neoF] resulted in ~60% fewer recombinants on MC1061. This result was not unexpected, since gam creates a recBC phenocopy in E.coli (19) and phage–plasmid recombination is reduced in recBC\(^-\) mutants (25). spi selection appeared to be able to eliminate single crossover phage in this experiment as only 10% of these recombinants could grow on the P2 lysogen of MC1061. This result indicated that the occurrence of double crossovers between plasmid and phage is not a product of two independent single crossover events (i.e. \(10^{-2} \times 10^{-2}\)) but instead is a cooperative event.

Double crossover recombinants resulting from passage of \(\lambda\)2TK:CRABPI through MC1061[P3, pCRABPI:lacneoF] occurred at a frequency of ~5 \(\times 10^{-2}\). This number was found to be the same when plated on MC1061 and P2MC1061, indicating that, as with pCRABPI:neoF, the P2 lysogen did not affect the plating efficiency of recombinants not carrying the gam gene. Interestingly, when the distance between the homologous sequences is increased from
1.4 to 5.7 kb the frequency of double crossover recombination drops by almost two orders of magnitude. This phenomenon is different from what is seen in gene targeting of mammalian cells, where insert size appears to have little influence on gene targeting frequency (5). Perhaps in phage–plasmid recombination the cooperativity observed in double crossover is abolished by steric hindrance when longer distances occur between recombination sites.

To establish the authenticity of the apparent double crossover recombinants in both experiments, individual plaques were isolated from P2MC1061 plates and phage were grown on a small scale. Restriction analysis of 15 phage clones using *Bam*HI and *Xho*I indicated that in all cases a double crossover had indeed occurred (Fig. 3). For *neo–supF* the 2.2 kb *Xho*I–*Bam*HI fragment from λ2TK:CRABP1 is present as two bands as a result of the *Xho*I sites flanking the *supF* gene. The 5′ fragment which contains the *neo* gene is 2.3 kb in length, while the 3′ fragment has been shifted down to 0.9 kb. Similarly, for *lacZ–neo–supF* the 2.2 kb parental fragment is shifted to two bands; however, in this case the 5′ fragment is shifted up to 6.5 kb as a result of the large *lacZ–neo* insertion, while the 3′ fragment (0.9 kb) is common in both the cRABP1:neoF and cRABP1: lacneoF targeting vectors. The other common fragments (4.7, 1.8 and 1.5 kb) lie outside the recombination site and are common to λ2TK:CRABP1 and its neoF and lacneoF derivatives.

These experiments illustrate that flanking homologous sequences as short as 200 bp are sufficient to introduce heterologous sequences ranging from 1.4 to 5.7 kb into phage. We have also discovered that flanking homologous sequences derived from oligonucleotides as short as 25 bp are sufficient to direct double crossover recombination of *neo–supF* cassettes in phage TVs, albeit at a lower frequency of 10⁻⁷ (D.E. Rancourt, unpublished results). This approach, however, has failed to direct double crossover recombination of *lacZ–neo–supF* cassettes in phage.

**Gene targeting with phage replacement vectors**

To demonstrate that phage vectors could be used successfully in gene targeting experiments, double crossover clones bearing *neo* (λ2TK:CRABPI:neoF) and *lacZ–neo* (λ2TK:CRABPI:lacneoF) were grown on a large scale and prepared for electroporation into ES cells by excising the phage arms with *Not*I. Following electroporation cells were grown in medium containing G418 and FIAU (4,20). Individual clones resistant to both drugs were subjected to genomic Southern blotting analysis, using restriction enzymes and probes that distinguish random from homologous integration events. For λ2TK:CRABPI:neoF seven out of 96 cell lines had *neo* integrated within the *cRABPI* locus, while for λ2TK:CRABPI: lacneoF three out of 40 were positively targeted. Southern blot analysis of representative cell lines is shown in Figure 4. Parental ES cell DNA digested with *Eco*RI and hybridized with a 5′-flanking probe yielded a 4.9 kb fragment. In cell lines targeted with *neo* a portion of this hybridizing band representing the targeted locus was shifted down to 3.4 kb due to the presence of an *Eco*RI site in the *neo* gene cassette. With the *lacZ–neo* targets this hybridizing band shifted up to 7.8 kb because of *lacZ* sequences upstream of the *Eco*RI site in *neo*.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Restriction analysis of phage–plasmid recombinants. (A) DNAs from λ2TK:CRABPI (lanes C), putative neo–supF (lanes 1–8) and lacZ–neo–supF (lanes 9–16) recombinants were digested with *Bam*HI and *Xho*I, separated on a 0.5% agarose gel and stained with ethidium bromide. The migration distances of DNA standards derived from a HindIII digest of λ DNA are shown on the left. Shifted bands due to double crossover recombination are indicated on the right. Sample neo–supF #4 is absent due to degradation in this lane. (B) *Bam*HI and *Xho*I restriction map of λ2TK:CRABPI and the molecular weight of the corresponding fragments. Fragments resulting from integration of neo–supF or lacZ–neo–supF are shown underneath.

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<th>MC1061</th>
<th>P2MC1061</th>
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<tr>
<td>LE392</td>
<td>0</td>
<td>0</td>
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<tr>
<td>MC1061[P3, pCRABP:neoF]</td>
<td>7.1 × 10⁻²</td>
<td>7.1 × 10⁻²</td>
</tr>
<tr>
<td>MC1061[P3, pGamCRABP:neoF]</td>
<td>2.5 × 10⁻²</td>
<td>3.4 × 10⁻³</td>
</tr>
<tr>
<td>MC1061[P3, pCRABP: lacneoF]</td>
<td>5.1 × 10⁻⁵</td>
<td>5.1 × 10⁻⁵</td>
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λ2TK:CRABP1 (1 × 10⁸ pfu) was passaged over LE392 and MC1061[P3] bearing pCRABP1:neoF, pGamCRABP1:neoF or pCRABP1: lacneoF. Resulting phage lysates were then titrated and normalized on LE392 and then plated at various densities on the restrictive hosts MC1061 and P2MC1061. Recombination frequencies represent titer of phage on the restrictive host/titer of phage on LE392.
In addition to detecting targeted cell lines, analysis with EcoRI confirmed the integrity of the 5′-end of the targeted locus. To confirm that the 3′-end of each target was intact, DNAs from parental and targeted cell lines were digested with XhoI plus HindIII and probed with the internal SpeI–BglII fragment. In parental DNA digestion with XhoI and HindIII resulted in a 13 kb fragment which spanned the targeted region beyond the 3′-end of the TV. In both targeted cell lines this fragment shifted to a lower molecular weight due to the presence of XhoI sites flanking the supF gene at the 3′-end of both the neo and lacZ-neo insertions. On the 3′-end an 11.8 kb fragment resulted which was common to both the neo and lacZ-neo targeted cell lines. At the 5′-end a 2.4 kb band in the neo target contained the 5′-end of this fragment plus neo. In the lacZ-neo target a 6.5 kb fragment contained only the lacZ gene due to the presence of a HindIII site separating lacZ and neo.

In summary, we have demonstrated the ability to generate targeted ES cells using bacteriophage λ replacement vectors. Phage TVs have distinct advantages over plasmids and we describe methods for their generation, including novel procedures using phage–plasmid recombination. Recombination methods allow any desired mutation to be generated without dependence on restriction site availability. Using this approach TVs are easily and quickly generated and the overall efficiency of the targeted mutagenesis experiment is improved.

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