988–993 *Nucleic Acids Research, 1998, Vol. 26, No. 4*

Embryonic stem cell gene targeting using bacteriophage λ vectors generated by phage–plasmid recombination

Teruhisa Tsuzuki[1] and Derrick E. Rancourt*

Department of Medical Biochemistry, University of Calgary, Calgary, Alberta T2N 4N1, Canada and [1]Department of Biochemistry, Medical Institute of Bioregulation, Kyushu University, Higashi-ku, Fukuoka City 812-82, Japan

Received October 16, 1997; Revised and Accepted December 29, 1997

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 λ phage in targeted mutagenesis. The recombination-proficient phage vector λ2TK permits generation of TVs by conventional restriction–ligation or recombination-mediated methods. The resulting λTV 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 vivo* phage–plasmid recombination can be used to introduce *neo* and *lacZ–neo* mutations into precise positions within a λ2TK subclone via double crossover recombination. We describe two methods for eliminating single crossover recombinants, *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 *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 *neo* and *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 *neo* or *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 λ phage in targeted mutagenesis. Using a recombination-proficient bacteriophage, λ2TK, we are able to introduce *neo* and *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

Escherichia coli strains MC1061 (*rec*$^+$, *sup*^{0}) 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 (*rec*$^+$, *supE*, *supF)* and its P2 lysogenic derivative P2392 were obtained from Stratagene (La Jolla).

*To whom correspondence should be addressed. Tel: +1 403 220 2888; Fax: +1 403 283 8727; Email: rancourt@acs.ucalgary.ca

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 (HSV*tk*1and *tk*2; 11,14) in the left and right polylinkers respectively, between *Not*I and *Xho*I sites (not shown). A *Sal*I fragment comprising the two *tk* genes and stuffer region was then transferred into λSyrinx2A (15) to place the *tk*1 and *tk*2 genes adjacent to the short and long arms of the phage respectively. Finally, a *Xho*I 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 *Xho*I 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 *rec*$^+$ host MC1061. Plasmids bearing the *supF* gene were harbored in MC1061[P3]. The P3 episome, which is *Kan*^{r}, *Amp*^{am}, *Tet*^{am}, 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 *Sal*I fragment containing the *gam* gene coding sequence from bacteriophage λ (18) and inserting it into the *Sal*I site of pBluescriptKS$^+$ (Stratagene). *spi*$^+$ (sensitive to P2 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 pCRABPI$_{Bg/Sp}$, which is a small 400 bp *Bgl*II–*Spe*I genomic subclone that encompasses the second exon of *cRABPI*. An *Sst*I site which bisects this genomic fragment was converted to *Bam*HI in order to facilitate cloning of *supF*-bearing cassettes of *MC1neopA* (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 (1×10^3 p.f.u.) were passaged via plate lysate over LE392 or MC1061[P3], 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 recombinants (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 recombinants was confirmed by restriction analysis of small scale phage DNA preparations.

Gene targeting

Phage targeting vectors were introduced into ES 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 *Not*I 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 *tk* 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 *rec*$^+$ 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 recombinants capable of growing on a suppressor-free (*sup*^{0}) 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 *gam* gene are sensitive to P2 interference (*spi*$^+$) and cannot be replicated on a P2 lysogenic host (18,19). Thus, if *gam* function could be maintained in a recombination plasmid, single crossover integrants bearing *gam* 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 HSV*tk*1 and -*tk*2 genes adjacent to its small and large arms respectively. [The *tk*1 and *tk*2 genes serve as negative selectable makers in gene targeting experiments (11).] Otherwise, as with λSyrinx2A, λ2TK is A^{am}, B^{am}, S^{am}, requires the amber suppressor tRNA, *supF*, for lytic growth and is *Rap*$^+$ for efficient recombination with the plasmid. The vector has unique *Xba*I and *Xho*I 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 *Xba*I site is compatible with *Spe*I and *Avr*II, while *Xho*I can accommodate *Sal*I, in addition to *Bam*HI, *Bgl*II, *Bcl*I and *Sau*3A 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 *Spe*I–*Bgl*II genomic fragment bearing exon 2 of *cRABPI*. In pCRABPI$_{Sp/Bg}$ an *Sst*I site which bisects this fragment within the coding sequence was converted to *Bam*HI for convenient cloning of the *supF*-bearing cassettes of *MC1neopA* and *lacZ–MC1neo pA*.

For the recombination strategy which utilized λ*gam* and *spi* selection to eliminate single crossover phage the homology and *neoF* regions were subcloned in pGam to generate pGamCRABPI:neoF. pGam is a pBluescript derivative that carries a functional λ*gam* gene and can confer a *spi*$^+$ phenotype to recombinant phage. For the strategy involving size limitation of λ packaging pCRABPI:lacneoF was constructed, which placed a *lacZ–neo–supF* cassette in-frame within pCRABPI$_{Sp/Bg}$. As this plasmid was 8.7 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.

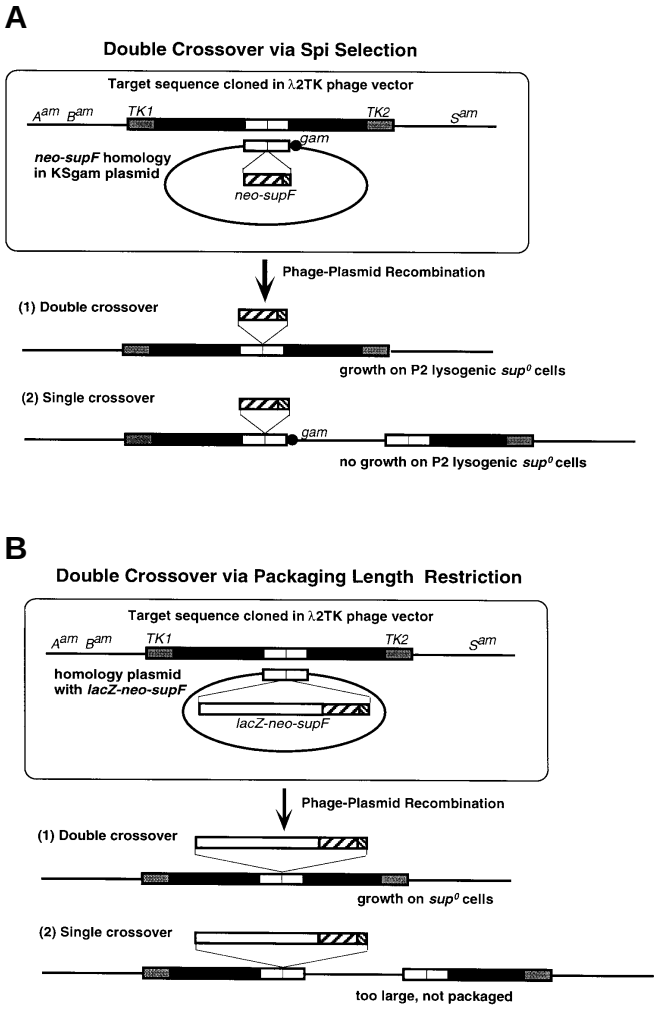

Figure 1. Phage targeting vectors via double crossover recombination with plasmids. Two strategies were used to target *neo* and *lacZ–neo* to specific sites within λ2TK TVs via recombination. Phage–plasmid recombination can occur either by single or double crossover, when recombinogenic phage are passaged over a *neo–supF* (A) or *lacZ–neo–supF* (B) split homology-bearing plasmid. Both recombination events are selected by integration of a *supF* cassette that suppresses amber mutations in the phage vector. However, both strategies selectively eliminated single crossover recombinants by virtue of plasmid integration into the phage. (**A**) In the first approach, used to construct a *neo* TV, the λ*gam* gene is incorporated in the plasmid so that single crossover recombinants are killed via *spi* selection. Double crossover recombinants are able to grow on a *sup*^{0} P2 lysogen, as these phage do not contain the λ*gam* gene. (**B**) The second method, used to make a *lacZ–neo* TV, takes advantage of the restricted size of phage packaging. If the recombination plasmid is sufficiently large, single crossover recombinants will fail to be packaged, whereas double crossover recombinants will not.

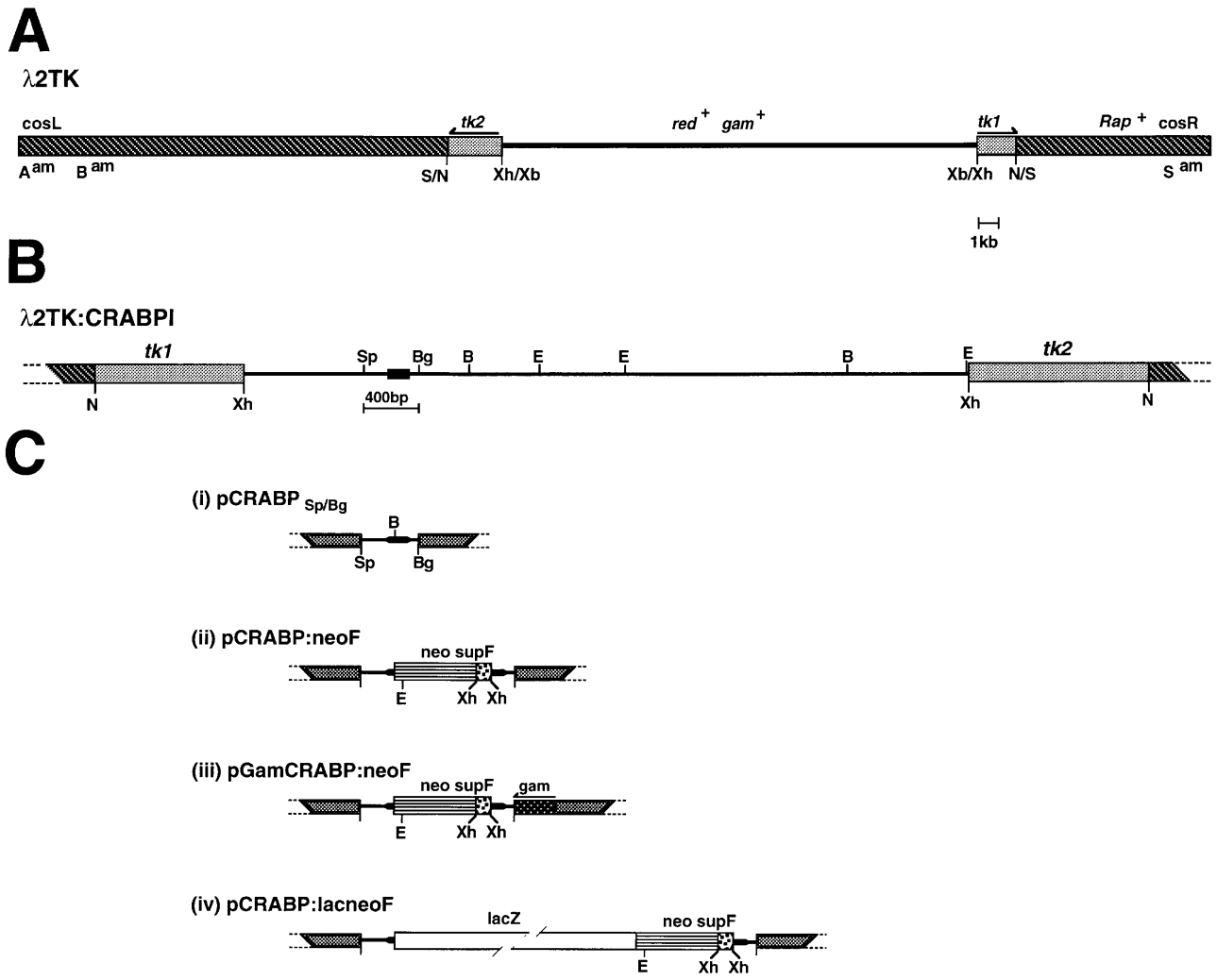

Figure 2. Maps of recombination phage and plasmids. (**A**) λ2TK is a derivative of the recombination screening vector λSyrinx2A (15). It carries amber mutations in the *A*, *B* and *S* genes and is *Rap*$^+$, for efficient recombination with the plasmid. HSV*tk*1 and -*tk*2 cassettes (11,14) are adjacent to the small and large arms respectively, in divergent orientations. A *red*$^+$, *gam*$^+$ stuffer fragment was derived from λGem11. Restriction sites used for cloning (Xh, *Xho*I; Xb, *Xba*I) or excising arms (S, *Sal*I; N, *Not*I) are indicated. (**B**) λ2TK: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 λ2TK. The position of the 400 bp *Spe*I–*Bgl*II fragment encompassing the region of homology used in the homologous recombination experiments is indicated. (**C**) pCRABPI$_{Bg/Sp}$ (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–supF* and *lacZ–neo–supF* cassettes. pCRABPI:neoF (panel ii) is a derivative of this plasmid containing a 1.4 kb *supF* cassette of *MC1neopA* (4). pGamCRABPI:neoF (panel iii) is identical to pCRABPI:neoF with the exception that the λ*gam* gene is included in this plasmid. pCRABPI:lacneoF (panel iv) is a derivative of pCRABPI$_{Bg/Sp}$ that contains an in-frame fusion of a 5.7 kb *supF* cassette of *lacZ–MC1neopA* (5).

λ2TK:CRABPI phage was passaged via plate lysates through MC1061[P3] bearing pCRABPI: neoF, pGamCRABPI:neoF or pCRABPI:lacZneoF, as well as a *supF*$^+$ 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 *supF*$^+$ 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 λ2TK:CRABPI through LE392 resulted in no detectable *supF*$^+$ phage in 10^6 that were plated. In contrast, phage passaged through strains bearing the recombination plasmids did result in *supF*$^+$ recombinants, albeit at differing frequencies. Passage through MC1061[P3, pCRABPI:neoF] resulted in roughly 7% recombinants, as indicated by *supF*$^+$ 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 λ2TK:CRABPI through MC1061[P3, pCRABPI:lacneoF] occurred at a frequency of ~5×10^{-5}. 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

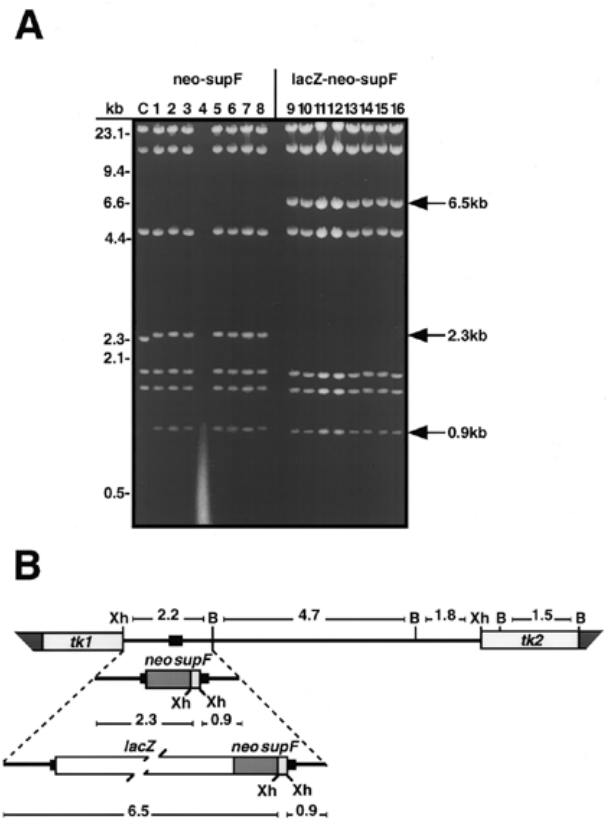

Figure 3. Restriction analysis of phage–plasmid recombinants. (**A**) DNAs from λ2TK:CRABPI (lane C), putative *neo–supF* (lanes 1–8) and *lacZ–neo–supF* (lanes 9–16) recombinants were digested with *Bam*HI and *Xho*I, separated on a 0.5% agarose gel and stained with ethidium bromide. The migration distances of DNA standards derived from a *Hin*dIII 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.

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:CRABPI 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 cRABPI:neoF and cRABPI: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:CRABPI 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*.

Table 1. Frequency of phage–plasmid recombination

Original passage	Recombination frequencies (secondary passage)	
	MC1061	P2MC1061
LE392	0	0
MC1061[P3, pCRABP:neoF]	7.1×10^{-2}	7.1×10^{-2}
MC1061[P3, pGamCRABP:neoF]	2.5×10^{-2}	3.4×10^{-3}
MC1061[P3, pCRABP:lacneoF]	5.1×10^{-5}	5.1×10^{-5}

λ2TK:CRABP (1×10^3 pfu) was passaged over LE392 and MC1061[P3] bearing pCRABPI:neoF, pGamCRABPI:neoF or pCRABPI:lacneoF. Resulting phage lysates were then titered 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.

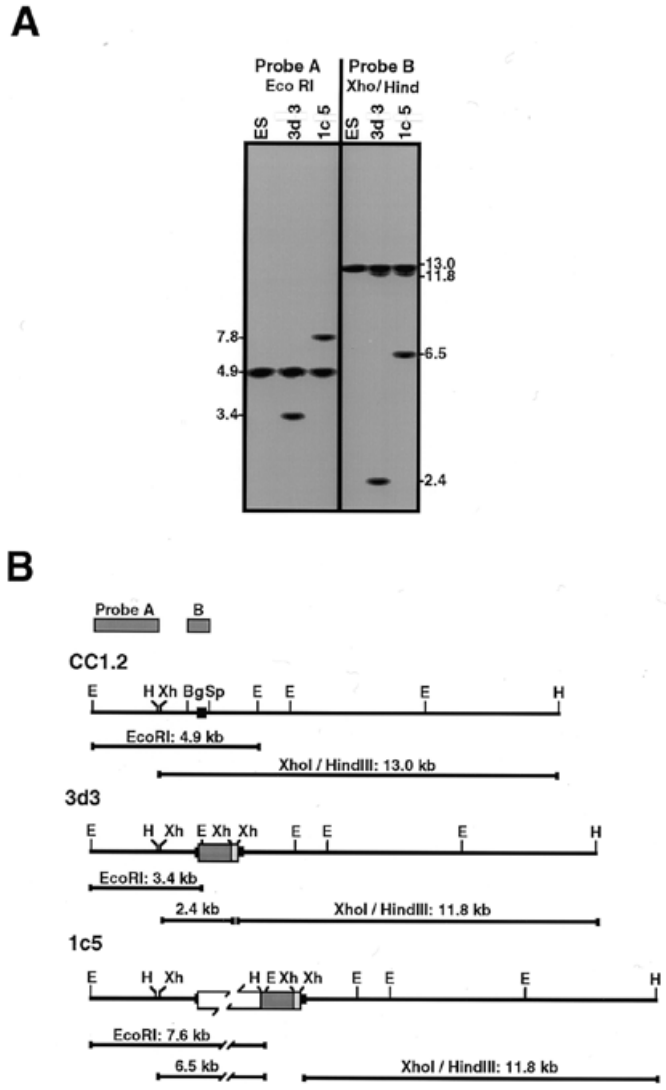

Figure 4. Southern analysis of gene targeting using phage vectors. (**A**) Genomic DNAs from parental (CC1.2), a *neo*-targeted (3d3) and a *lacZ–neo*-targeted (1c5) cell line were digested with *Eco*RI (probe A) or *Xho*I–*Hin*dIII (probe B) and blotted onto nylon filters and hybridized with the corresponding probes. Molecular weights of the hybridizing fragments are indicated. (**B**) Restriction maps of the *cRABPI* locus in parental and targeted cell lines are indicated, as are the sizes of hybridizing bands in each digest. The positions of the flanking probe A and internal probe B are indicated.

In addition to detecting targeted cell lines, analysis with *Eco*RI 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 *Xho*I plus *Hin*dIII and probed with the internal *Spe*I–*Bgl*II fragment. In parental DNA digestion with *Xho*I and *Hin*dIII 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 *Xho*I 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 *Hin*dIII 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.

ACKNOWLEDGEMENTS

We thank Dr Mario R.Capecchi for allowing us to initiate this research independently and acknowledge the technical support of Carol Lenz and Margarie Allen. We are grateful to Drs Gerard Bain, Kostas Iatrou and Karl Riabowol for critical reading of this manuscript. This work was supported, in part, by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan International Scientific Research Program (08044303), the Alberta Cancer Board and University of Calgary Research Foundation.

REFERENCES

1 Capecchi,M.R. (1989) *Science*, **244**, 1288–1292.
2 Capecchi,M.R. (1989) *Trends Genet.*, **5**, 70–76.
3 Humphries,M.M. *et al.* (1997) *Nature Genet.*, **15**, 216–219.
4 Thomas,K.R. and Capecchi,M.R. (1987) *Cell*, **51**, 503–512.
5 Mansour,S.L., Thomas,K.R., Deng,C. and Capecchi,M.R. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7688–7692.
6 Hanks,M., Wurst,W., Anson-Cartwright,L., Auerbach,A.B. and Joyner,A.L. (1995) *Science*, **269**, 679–682.
7 Hasty,P., Ramirez-Solis,R., Krumlauf,R. and Bradley,A. (1991) *Nature*, **350**, 243–246.
8 Mombaerts,P., Clarke,A.R., Hooper,M. and Tonegawa,S. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3084–3087.
9 Ramirez-Solis,R., Liu,P and Bradley,A. (1995) *Nature*, **378**, 720–724.
10 Tsien,J.Z., Chen,D.F., Gerber,D., Tom,C. Mercer,E.H., Anderson,D.J., Mayford,M., Kandel,E.R. and Tonegawa,S. (1996) *Cell*, **87**, 1317–1326.
11 Mansour,S.L., Thomas,K.R. and Capecchi,M.R. (1988) *Nature*, **336**, 348–352.
12 Nagy,A. and Rossant,J. (1996) *J. Clin Invest.*, **98**, S31–35.
13 Deng,C. and Capecchi,M.R. (1992) *Mol. Cell. Biol.*, **12**, 3365–3371.
14 Chisaka,O. and Capecchi,M.R. (1991) *Nature*, **350**, 473–479.
15 Lutz,C.T., Hollifield,W.C., Seed,B., Davie,J.M. and Huang,H.V. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4379–4383.
16 Wei,L.N., Chu,Y.S., Jeannotte,L. and Nguyen-Huu,M.C. (1990) *DNA Cell Biol.*, **9**, 471–478.
17 Seed,B. (1983) *Nucleic Acids Res.*, **11**, 2427–2445.
18 Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19 Haggard-Ljungquist,E., Barreiro,V., Calendar,R., Kurnit,D.M. and Cheng,H. (1989) *Gene*, **85**, 25–33.
20 Zheng,H. and Wilson,J.H. (1990) *Nature*, **344**, 170–173.
21 Nehls,M, Messerle,M., Sirulnik,A, Smith,A.J.H. and Boehn,T. (1994) *BioTechniques*, **17**, 770–775.
22 Rancourt,D.E., Tsuzuki,T and Capecchi,M.R. (1995) *Genes Dev.*, **9**, 108–122.
23 Umene,K., Shimada,K., Tsuzuki,T., Mori,R., and Takagi,Y. (1979) *J. Bacteriol.*, **139**, 738–747.
24 Gorry,P., Lufkin,T., Dierich,A., Rochette-Egly,C., Decimo,D., Dolle,P., Mark,M., Durand,B. and Chambon, P. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 9032–9036.
25 Shen,P. and Huang,H.V. (1986) *Genetics*, **112**, 441–457.