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Bacteriophage Gene Targeting Vectors Generated by Transplacement

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ABSTRACT

A rate-determining step in gene targeting is the generation of the targeting vector. We have developed bacteriophage gene targeting vectorology, which shortens the timeline of targeting vector construction. Using retro-recombination screening, we can rapidly isolate targeting vectors from an embryonic stem cell genomic library via integrative and excisive recombination. We have demonstrated that recombination can be used to introduce specific point mutations or unique restriction sites into gene targeting vectors via transplacement. Using the choline/ethanolamine kinase α and β genes as models, we demonstrate that transplacement can also be used to introduce specifically a neo resistance cassette into a gene targeting phase. In our experience, the λ TK gene targeting system offers considerable flexibility and efficiency in TV construction, which makes generating multiple vectors in one week's time possible.

INTRODUCTION

With the estimated discovery of more than 35 000 genes via the genome projects, there is considerable interest in determining the function of these genes in the emerging era of functional genomics. Here there is a need to speed up the process of targeting vector generation and to develop technologies for the generation of subtle mutations beyond the gene knockout. We used bacteriophage λ as an efficient vector for scaffolding and transferring genomic alterations to embryonic stem cells via gene targeting. We have previously developed phage-plasmid recombination to direct modification cassettes to specific sites within phage targeting vectors via double crossover (5). Small regions of homology are used to direct modification cassettes to specific sites within the recombination-proficient gene targeting phages (5,7). A suppressor tRNA gene *supF*, positioned within the homologous region, acts as a positive selectable marker for recombination by suppressing amber mutations in λ TK. A negative marker gene, *gam*, is placed outside the region of homology to enrich for double-crossover recombinants by selectively killing single-crossover recombinants via spi selection (2,4).

We have also established new methods that use the sequential integrative and excisive recombination of plasmids. Retro-recombination screening allows the rapid purification of phage targeting vectors from an embryonic stem cell targeting vector library in λ TK (7), following the homologous integration of a *supF*-bearing recombination plasmid. As the region of homology is duplicated on integration and can revert under relaxed conditions, we incorporated *gam* into the recombination plasmid to spi select phages that have reverted to their original configuration (6,7). Using retro-recombination screening, we isolated specific targeting vector phages in 2–3 days to serve as templates for the rapid completion of targeting vector construction. An alternative method, called transplacement mutagenesis, uses an integrative and excisive recombination strategy to transfer point mutations to target phages using a recombination plasmid interface (6,8). Following the excision

of the plasmid, a mutation is transferred precisely in approximately 50% of condensants. We routinely introduce unique restriction sites into λ TK phages for the subsequent introduction of modification cassettes.

In theory, transplacement can also be used to introduce larger changes, including sequence additions, provided that sufficient regions of homology are included. Accordingly, we have investigated whether transplacement could be used as a strategy for introducing *neo^R* selection cassettes using integrative and excisive recombination (Figure 1). Using the mouse choline/ethanolamine kinase genes α and β (CK/EK α and β) (1) as a template, we demonstrate that retro-recombination screening and transplacement can be combined to insert unique restriction sites as targeting vectors are plaque-purified. We also show how modification cassettes can be introduced into targeting vectors via transplacement.

MATERIALS AND METHODS

Bacterial Strains, Recombination Plasmid Constructs, and Bacteriophages

The *E. coli* strains, λ TK embryonic stem cell library, and recombination plasmid π AN γ have been described previously (6,7). Recombination plasmid α sx π AN γ (Figure 2A) was created by ligating two genomic PCR fragments that surround the seventh exon of CK/EK α into π AN γ to introduce two novel restriction sites (*SalI* and *XhoI*) into the recombination probe sequence. A 247-bp (+19 non-homologous region) 5' *EcoRI*-*XhoI* fragment was generated by genomic PCR using forward (5'-AAAGAATTCCAGATAGTAGCTGAG-3') and reverse (5'-AAACTCGAGTCGACCTACAAGAGATTGG-3') primers. Similarly, a 213-bp (+16) 3' *XhoI*-*BamHI* fragment was generated by genomic PCR using forward (5'-AAACTCGAGAATATCTTATTGTTGGAAG-3') and reverse (5'-AAAGGATCCTCAGGGACAAACA-3') primers. Its derivative, α sxneo π AN γ , was generated by inserting a *XhoI* cassette of *pol2:neo^R* in opposite transcriptional orientation to the CK/EK α gene. For β sx π AN γ , a 194-bp (+18) 5' *EcoRI*-

XhoI fragment was generated by genomic PCR using forward (5'-AAAG-AATTCCCAGGACTGGAGCCACC-3') and reverse (5'-AAACTCGAGTC-GACTACCCCTGTCCCGT-3') primers. Similarly, a 198-bp (+15) 3' *XhoI*-*Bam*HI fragment was generated by genomic PCR using forward (5'-AACTCGAGGAGGGGCTGTC-3') and reverse (5'-AAAGGATCCTCAC-GGGACAAACGCTC-3') primers. Its derivative, β sxneo π AN γ (Figure 2A) was generated by inserting the pol2:neo^R cassette downstream of the CK/EK β initiation codon, in opposite transcriptional orientation to the gene.

Retro-Recombination Screening and Transplacement Mutagenesis

Retro-recombination screening was performed as previously described (8). Following retro-recombination screening, isolated plaques from the α sx π AN γ and β sx π AN γ screen were analyzed by PCR and restriction endonuclease diges-

tion to identify clones that underwent transplacement and retained the engineered *XhoI* and *Sall* restriction sites. Both CK/EK α and β phage eluates were screened by PCR using primers lying outside the original regions of homology that had been used in the recombination plasmids (α forward, 5'-TTCTTCACCATGGAGCCG-3', and α reverse, 5'-GAGTTCCAGAAGAGCTAA-3'; β forward, 5'-CTCAGTCAGTAAGAGCA-GA-3', and β reverse, 5'-GGAGGAGATCAAGGTCTTA-3'). Positive phages were also screened for the presence of a novel *Sall* site using digestion of the corresponding PCR products. Positive clones were subsequently restriction mapped to confirm the position of the introduced *Sall/XhoI* restriction sites.

Phage-plasmid recombination using α sx π AN γ - and β sx π AN γ -positive phage isolates and their corresponding neo^R recombination plasmids were used to investigate whether the neo^R cassette could be transplaced into a specific phage using integrative and excisive re-

Figure 1. Transplacement of selectable marker genes via phage plasmid recombination. A two-step procedure is used to transfer selectable markers from split homology recombination plasmids to targeting vectors in λ TK. (A) A recombination plasmid, bearing *supF* and *gam* markers and a split homologous region with a centered neo^R cassette, is integrated into λ TK by a single-crossover recombination event and selected using *supF*. (B) Following the relaxation of *supF* selection, phages that underwent condensation are enriched for by spi selection against *gam*. When excisional recombination occurs contra-laterally to the integration site (approximately 50% of the time), the neo^R cassette is left behind in the target phage. Note that the plasmid and phage are not drawn to scale.

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combination. The λ TKCK/EK α or β phages (1×10^4 PFU) were passaged over MC1061[p3] bearing α sxneo π -AN γ and β sxneo π AN γ , respectively. Supernatant phages were collected the next day and used to infect LG75 for the selection of single-crossover recombinants, which were then cored and eluted in SM buffer. Phages having undergone condensation were isolated by spi selection on P2392. Plaques were screened by hybridization using a *neo* probe to identify phage clones that retained *neo*. DNA from such clones was isolated in mini-scale (3) and digested to confirm the transplacement of *neo*^R and the identity of the resulting λ TKCK/EK α or β targeting vectors.

RESULTS AND DISCUSSION

Both retro-recombination screening and transplacement take advantage of a

cycle of integrative and excisive recombination, through which a recombination plasmid π AN γ that bears a short region that is homologous to sequences in a target phage is integrated and excised by a single-crossover recombination event (5,7,8). Based on their similarity, it is theoretically possible to combine both processes to isolate specific λ TK phage-bearing unique restriction sites. To test whether restriction sites could be transplaced into λ TK gene-targeting phages via retro-recombination screening, two recombination plasmids were constructed using homology derived from genomic sequences of the CK/EK α or β genes (Figure 2). The probe sequences within α sx π AN γ and β sx π AN γ contained 460 and 392 bp of homology surrounding exon 7 and exon 1 of CK/EK α or β , respectively. Each plasmid contained *Sal*I and *Xho*I sites that were approximately central within the disrupted probe homology region

(α , 247 and 213 bp, and β , 194 and 198 bp). These plasmids and their corresponding controls, α π AN γ and β π AN γ (data not shown), which did not contain the introduced restriction sites were used to screen 4×10^7 PFU of the λ TK embryonic stem cell library via retro-recombination screening.

Because centered mutations within transplacement vectors disrupt the homology, we determined that larger regions of homology were needed. Table 1 shows that the homology break introduced in α sx π AN γ and β sx π AN γ significantly reduced the frequency of plasmid-phage recombination. The control probe plasmids gave a robust recovery of clones. As expected, considerably fewer clones (approximately 2-fold) were recovered for β CK/EK, based on the smaller region of homology that was used. However, in both cases, a marked difference was observed using the transplacement plasmids. For

Table 1. Phage-Plasmid Recombination Screening Frequencies of CK/EK α and β Probe Plasmids with and without Centered *SalI* and *XhoI* Restriction Sites

Vector	Phage Titer ^a	No. Blue Plaques	Recombination Screening Frequency ^b
$\alpha\pi\text{AN}\gamma$	6×10^9	2×10^3	3.3×10^{-7}
$\alpha\text{sx}\pi\text{AN}\gamma$	2.4×10^{10}	9×10^2	3.8×10^{-8}
$\beta\pi\text{AN}\gamma$	2.5×10^9	2.3×10^2	9.2×10^{-8}
$\beta\text{sx}\pi\text{AN}\gamma$	3.3×10^9	2×10^2	6.1×10^{-9}

Each plasmid harbored in MC1061[p3] was used to screen the λ TK phage library (approximately 4×10^8 PFU/mL). Processed lysates were titrated on LE392, after which 10^9 – 10^{10} PFU were used to screen for co-integrants on LG75 (blue plaques).

^aTiter on LE392 expressed as PFU/mL.

^bRecombination screening frequencies were determined for each individual data set using the formula of number of blue plaques/phage titer.

the larger $\alpha\text{sxCK/EK}$ probe, a 9-fold reduction in the recovery of recombinants was observed over wild-type $\alpha\text{CK/EK}$, whereas a 15-fold reduction was ob-

served for the smaller $\beta\text{sxCK/EK}$ probe over the wild-type $\beta\text{CK/EK}$. Nonetheless, a significant number of recombinants (at least 20) were observed for

each of these probe plasmids, which indicates the possibility that considerably smaller regions of balanced homology might be used for successful retro-recombination screening using trans-placement probe plasmids.

Following isolation, 10 individual recombinant clones were passaged sequentially over the *supF*⁺ host LE392 and its P2 lysogen, P2392, to allow both condensation and spi selection. In agreement with our previous data (7), the condensation frequency was very high (10%–30%) for each recombinant because of the long lengths of homology used (data not shown). In contrast, when oligonucleotide-based regions of homology (approximately 50 bp) are used, this frequency drops by one order of magnitude (6). Whereas it might be expected that condensation frequency would occur less frequently in clones bearing split homology regions, this was not observed because of the short-

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ening of contiguous homology. Instead, comparable frequencies or greater frequencies were observed for the split homology plasmid over the control. These results suggest that when one is using larger homology regions, *cis* recombination frequencies are less responsive to contiguous homology than *trans*.

After condensation of the α and β clones, DNA from individual plaques was examined for the transplacement of *SalI* and *XhoI* sites. Table 2 indicates that, among the small number of clones that were examined, 50% or more transplaced these sites. Surprisingly, despite the unbalanced homology in the α probe (247 and 213 bp), the transplacement frequency remained at 50%. This is in sharp contrast to our previous experiments using oligonucleotide-based homologies, where subtle differences in length of homology or GC content can have a tremendous effect on the transplacement frequency (unpublished results). This result reiterates the idea that a threshold of homology is sufficient for *cis* recombination and that the choice of sides for excise recombination may be stochastic.

For both the CK/EK α and β genes, multiple, distinct λ TK clones were isolated that represented different regions of their respective loci. Figure 2B displays the restriction maps of the contigs isolated using retro-recombination transplacement. In each clone, both *SalI* and *XhoI* sites were transferred to their respective region of homology. The introduction of these rare restriction sites helped to determine rapidly the boundaries of each clone isolated from the library, facilitating the selection of a centralized clone for the completion of a centralized clone for the completion of the targeting vector and flanking clones for the isolation of a flanking probe.

For both the CK/EK α and β clones, the introduced *XhoI* site was not unique and confounded the completion of the targeting vectors via the introduction of an *XhoI* cassette of *neo^R*. Accordingly, we investigated whether a *neo^R* selection cassette could also be introduced using transplacement. The *neo^R*-containing homology plasmids, α and β , were used as a host to shuttle *neo^R* into λ TK α and β CK/EK

phages. Single phages from the α and β series were selected as substrates, based on the criteria that they were relatively small (approximately 12 kb) and centered within the contig (Figure 2B).

In a total of nine analyses, the

transplacement of *neo^R* occurred approximately 50% of the time (Table 2). As expected, restriction analysis of representative clones demonstrated that the *neo^R* gene was introduced into the seventh and first exons of CK/EK α and β ,

Figure 2. CK/EK recombination plasmids and λ TK clones following transplacement. (A) α and β clones and their derivatives were generated by subcloning genomic PCR fragments derived from the α and β CK/EK genes into π ANy. α and β clones were created by ligating two genomic PCR fragments to introduce centered *SalI/XhoI* sites into these sequences. Accordingly, the 5' and 3' regions of homology used for transplacement are denoted by divergent arrows. Their derivatives, α and β , were generated by the insertion of a *XhoI* *neo^R* cassette in the opposite transcriptional orientation. (B) Contigs of CK/EK α and β clones were generated from the λ TK clones that were isolated by retro-recombination screening transplacement. The bold line of each represents the gene structure. The dash beneath displays the probe homology region used. Following the gene structure diagram are diagnostic restriction maps of each locus. The individual λ TK clones that were isolated are portrayed. In these clones, *SalI* and *XhoI* sites were deposited into the middle of the region of homology. The asterisks represent clones that were used to generate targeting vectors using *neo^R* transplacement. For these clones, their respective *neo* insertions are indicated at the bottom of both the CK/EK α and β panels.

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Table 2. Transplacement Frequencies of Restriction Sites or *neo^R* Selectable Marker after Integrative and Excisive Recombination Using CK/EK α and β Probe Plasmids

Vector	Total Screened	Positive Clones	Frequency
α SK π AN γ	8	4	0.5
β SK π AN γ	12	7	0.58
α SK π eo π AN γ	87	64	0.54
	93	40	
	101	49	
	33	15	
	90	54	
β SK π eo π AN γ	236	161	0.6
	115	101	
	46	15	
	142	69	

Single-crossover recombinants were grown under relaxed conditions in LE392. Phages from this liquid lysis culture were passaged on P2392 to isolate phages that had undergone a condensation event. Resulting phages were analyzed for the presence of novel *SalI/XhoI* sites by restriction digestion and *neo^R* by hybridization.

respectively (Figure 2B). Like the retro-recombination screening transplacement experiments, the transfer of *neo^R* was as efficient as transferring restriction sites. Unlike the β experiments in which the regions of homology were balanced, no significant difference was detected with the α experiments when the regions of homology regions were slightly unbalanced, which again indicated that a certain low threshold of homology is sufficient for efficient recombination in *cis*.

In summary, we have demonstrated that transplacement provides a powerful, genetic approach to isolate and modify rapidly targeting vectors from a λ TK gene targeting library. Unlike double-crossover techniques previously described in which deleterious bacterial selection markers are deposited into targeting vectors (5,9) using transplacement, it is now possible to keep the extraneous selectable marker outside the region of homology. In addition, selection markers are recycled in transplacement, thus allowing an indefinite number of rapid sequential modifications such as the introduction of loxP sites after *neo^R* transplacement. Using this genetic approach, targeting vector construction is not labor intensive and allows multiple vectors to be constructed at one time. Thus, in the next generation of structure/function gene targeting

studies, batteries of targeting vectors with different subtle mutations may be generated in synchrony. No doubt, in the post-genome era where mouse genomic sequences will be readily available, complicated targeting vectors will be constructed in a week using transplacement.

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