

In vivo evaluation of PhiC31 recombinase activity using a self-excision cassette

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ABSTRACT

Gene targeting allows precise tailoring of the mouse genome such that desired modifications can be introduced under precise temporal and spatial control. This can be achieved through the use of site-specific recombinases, which mediate deletion or inversion of genomic DNA flanked by recombinase-specific recognition sites, coupled with gene targeting to introduce the recombinase recognition sites at the desired genomic locations within the mouse genome. The introduction of multiple modifications at the same locus often requires use of multiple recombination systems. The most commonly used recombination system is Cre/lox. We here evaluated *in vivo* the ability of PhiC31 phage integrase to induce a genomic deletion in mouse. We engineered a self-excision cassette, modeled after one previously designed for Cre, containing a positive selection marker and PhiC31 driven by a testis-specific promoter, all flanked by PhiC31 specific attP/B sites. We found *in vivo* PhiC31 mediated self-excision in 38% of transmitted alleles, although 18% of these showed evidence of imprecise deletion. Furthermore, in the 69% of un-recombined cassettes, sequence analysis revealed that PhiC31 mediated an intra-molecular deletion of the attB site preventing any subsequent recombination. This study demonstrates that PhiC31 can be used to automatically remove Neo, in the male chimera germline, although it is not as efficient or as accurate as Cre.

INTRODUCTION

Conditional mutagenesis requires precise engineering of the locus under investigation. This generally involves positioning two loxP sites surrounding the region to be deleted. Cre recombinase, under specific temporal and spatial control, then mediates site-specific recombination

between the two loxP sites, removing the intervening sequence. The Cre/loxP system is the main player in manipulating the mouse genome *in vivo*, because it is more efficient than the Flpe/Frt system (1). Since the introduction of this protocol, hundreds of loxP conditional mouse lines and Cre-driver lines have been created.

There are conditions in which it is desirable to introduce more than one modification into the same locus controlled by different temporal and tissue-specific drivers. For example, since the introduction of BAC clones for the construction of gene targeting vectors (2,3) it is possible to introduce multiple modifications, often associated with multiple positive selection cassettes. For this reason, efficient and diverse site-specific recombinases are often needed.

The *Streptomyces* phage, PhiC31, integrates its genome into the bacterial host using a site-specific integrase, which catalyzes an integration reaction between the attP/attB sites (attachment site Phage/attachment site Bacteria) (4); the sites generated after recombination are called attL and attR (Figure 1A). PhiC31 integrase belongs to the family of serine recombinases along with $\gamma\delta$ and Hin resolvase (5). Cre and Flpe are members of the tyrosine recombinase group and their mechanisms of action have been well reported. The crossover between the DNA-protein complexes proceeds through a Holliday junction intermediate that is resolved through a concerted rejoining of the opposite strands. In contrast to tyrosine recombinases, the mechanism of action of the serine recombinases is less well-characterized: the recombinase binds the recognition sites and generates a staggered double-strand break; then catalytic subunits rotate and rejoin and repair the broken ends. However, the details of these reactions are still not well-defined (5).

Because the reaction of PhiC31 involves two different sites and the resulting recombination products, attL and attR, are not a substrate for the opposite reaction in the absence of bacterial cofactors, the reaction in mammalian cells is unidirectional (4). The minimal size of the two sites in bacterial cells is 39 bp for the attP and 34 bp for the attB (6). Both sites share an identical 3-bp long central core 'TTG', where the crossover occurs. The arms, unlike

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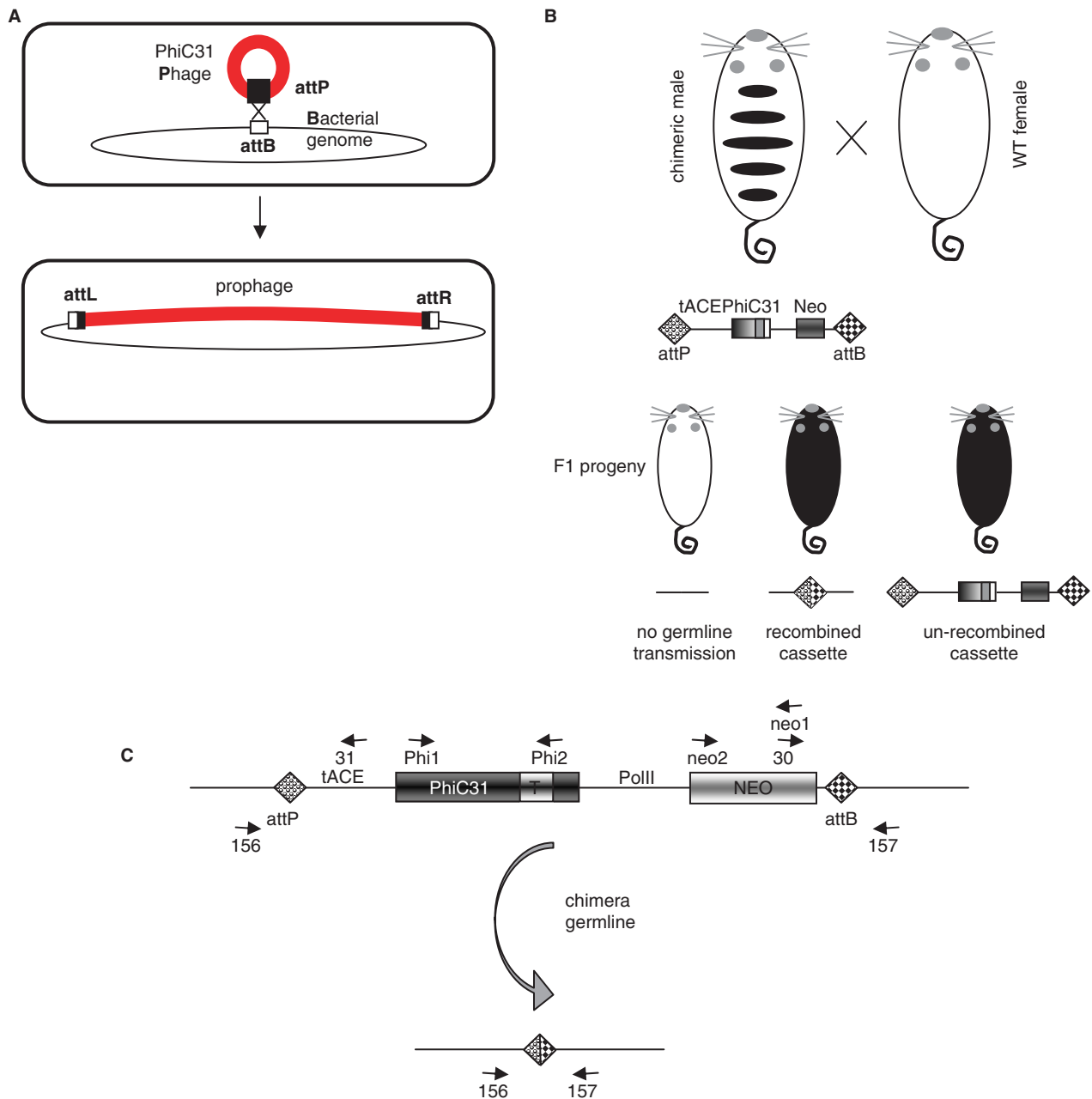


Figure 1. Schematic representation of PhiC31 self-excision. **(A)** Mechanism of integration of the PhiC31 phage into the bacterial genome. PhiC31 integrase mediates the integration reaction between the attachment site on the phage (attP) and the one in the bacterial genome (attB). The two sites are different, upon integration, the excision reaction is mediated exclusively by an excision factor produced by the bacterial cell, for this reason the reaction in mammalian cells is unidirectional. Upon recombination the newly formed hybrid sites are called attL and attR. **(B)** Mechanism of the SEC. The targeted allele contains a positive selection cassette (neomycin phospho-transferase), flanked by two attB/P sites. PhiC31 expressed in the chimeric male testes will mediate the automatic removal of the cassette during the passage through the male germline. In the F1 generation, three different outcomes are possible: no germline transmission of the targeted allele, the targeted allele will be transmitted without Neo and an attL site 'scar' will be present or the cassette will be transmitted unrecombined. The black color in the mouse body indicates the presence of the targeted allele. **(C)** Schematic representation of the PhiC31 SEC. The oligonucleotides used for the screening of the construct are represented above and below the SEC. Oligonucleotides 156–157 amplify the wild-type allele and the recombined allele containing the fused attP/B site.

the arms of the loxP/Frt sites, are imperfect inverted repeats, only partially conserved. PhiC31 has already been extensively tested *in vitro* as well as *in vivo* in mammalian cells (7). Its biochemical properties led many groups to use it to insert single copy transgenes in the

mouse genome as a nonviral approach for gene therapy (8). In this way, PhiC31 has been successfully used to achieve therapeutic levels of Factor IX in mouse *in vivo* (8) or to target single copy transgenes in mouse ES cells (9). However, this integrase is also able to drive an

intra-molecular recombination between its sites when oriented in the same direction in *cis*, inducing the deletion of the intervening sequence (10).

Introducing a modification in the mouse genome through gene targeting requires a positive selection cassette (usually a Neomycin phospho-transferase gene or Neo^r) to identify the correctly targeted ES cells clones. Several reports have found that the presence of Neo^r cassette often generates unwanted and unanticipated results (11–14). For this reason, following its use as a selection cassette, it is important to remove Neo^r, from the targeted locus, using a site-specific recombinase. To reduce manipulation of ES cells, the Flpe/Frt system is often used *in vivo* for this purpose. The Flpe ‘deleter’ mouse (15) removes the Neo^r selection cassette, flanked by two Frt sites. Nonetheless, several months of breeding are usually required to remove the selection cassette and then to breed out Flpe recombinase. In our laboratory, we have previously generated a self-excising selection cassette using the Cre/loxP system that is eliminated during passage through the male germline (16). This cassette cannot be used to make a conditional allele carrying two other loxP sites and when a cassette was engineered using Flpe/Frt system it was largely inefficient (Bunting, M., Wu, S. and M.R.C., unpublished data).

To test PhiC31 recombinase *in vivo*, a self-excision cassette (SEC) was generated. This assay allows evaluation of the efficiency of PhiC31 in removing the selection marker, Neo^r, while passing through the chimera germline. It is important to observe that it captures single recombination events through the germline, thus allowing a better evaluation of its activity. At the same time, if it works, it can be used to craft new targeted conditional alleles in conjunction with the Cre/loxP system.

We found that PhiC31 recombinase excised the selection cassette in 38% of analyzed mice. We were able to show that unlike Flpe or Cre, PhiC31 is not 100% precise in rejoining the attP/B sites, leading to variable deletions of the newly generated hybrid site. Furthermore, in 69% of unrecombined cassettes, PhiC31 induced an intra-molecular deletion of the attB site that prevented further recombination.

MATERIALS AND METHODS

The SEC was engineered following standard cloning procedures. A plasmid containing the PhiC31 wild-type sequence was a kind gift of Margaret Smith. The original sequence was PCR-modified to add a nuclear leading signal (NLS) to the C-terminal end of the protein (1). To prevent functional expression of the PhiC31 plasmid in bacteria, an intron, the large T intron from SV40, was introduced into the PhiC31 coding sequence. Large T intron was PCR-amplified, using Cre SEC as a template, with the following oligonucleotides (24a 5'GTAAGTGC TTCTTCCTTAAT3' and 20 5'CTAAAATACACAAAC AATTAG3') that allowed subcloning into the PhiC31 Eco47III restriction site. The entire modified PhiC31 was sequenced and found to be correct except for a missense mutation (c.90C>T). Because this change did not modify

the corresponding amino acid residue, it was left unchanged. The attB and attP sites 85-bp long were generated by re-annealing oligonucleotides. PhiC31 modified sequence and its sites have been subcloned, replacing Cre and loxP sites in the original Cre SEC. This cassette was inserted in a targeting vector containing 10 kb of homology region to the *Bmi1* locus, which we previously successfully targeted in mouse ES cells (17). Chimeras generated by blastocysts injection were bred with C57Bl/6J females and 173 mice derived from these crosses were analyzed to check for the recombined and unrecombined events. Oligonucleotides 156–157 (5'TGACACACACAATGGGAC3', 5'CGTGTGTTTATCAGCCATCAG3') amplified the wild-type and the recombined allele, while they were unable to amplify the unrecombined allele. Oligonucleotides Neo1-2 (5' GCAGGCATCGCCATGGTGC3', 5'GGACTGGCTGCTATTGGGCG3') confirmed the presence of the SEC in the unrecombined mice. To check for random deletions, we used the following oligonucleotides: 31 5'AGGTCTCTGTGAGGCTGGTAA3', Phi1 5'ATGACACAAGGGGTTG TGAC3', Phi2 5'TCTAAACCTTCCTCTTCTTC3', 30 5' CCTTC TTGACGAGTTCTTCTGA3'.

Each PCR product was purified with EXO-SAP (USB) using manufacturer recommendations and then sequenced by the Sequencing Core Facility at the University of Utah. For the recombined allele, the recombined band was gel purified and then sent directly for sequencing.

RESULTS

PhiC31 was tested *in vivo* by modifying an assay already established in our lab: two loxP sites flank an ES cell positive selection cassette containing a Neo^r under control of a general promoter derived from the large subunit DNA–RNA polymerase II, and Cre recombinase under control of a testis-specific promoter (tACE) (16). This SEC has been successfully used in many different laboratories to automatically remove Neo^r through the chimera germline, leaving only a residual loxP site. As a result, the final modification introduced into the genome is left ‘clean’ without any negative effect of the Neo^r gene or of its promoter on the locus under investigation. The same strategy was used to test PhiC31, replacing Cre/loxP sites with PhiC31 and its attB/P sites (Figure 1B–C). The wild-type PhiC31 sequence was modified to add to the C-terminal region, a NLS that has been shown in mammalian cells to improve efficiency of recombination (1) compared to the wild-type sequence. To prevent cassette self-excision during cloning procedures the large T intron from SV40 virus was introduced into the PhiC31 coding sequence.

Although minimal sizes of attP/B sites were established in bacterial cells as 39 and 34 bp, respectively, we decided to use 85-bp long sites that have already been proven to efficiently mediate recombination in mammalian cells (1).

This cassette was inserted into a targeting vector and successfully targeted into the *Bmi1* locus (Figure 2).

Male chimeras from one ES cell line (ES cell line 1h25) were bred to wild-type females and progeny assayed by

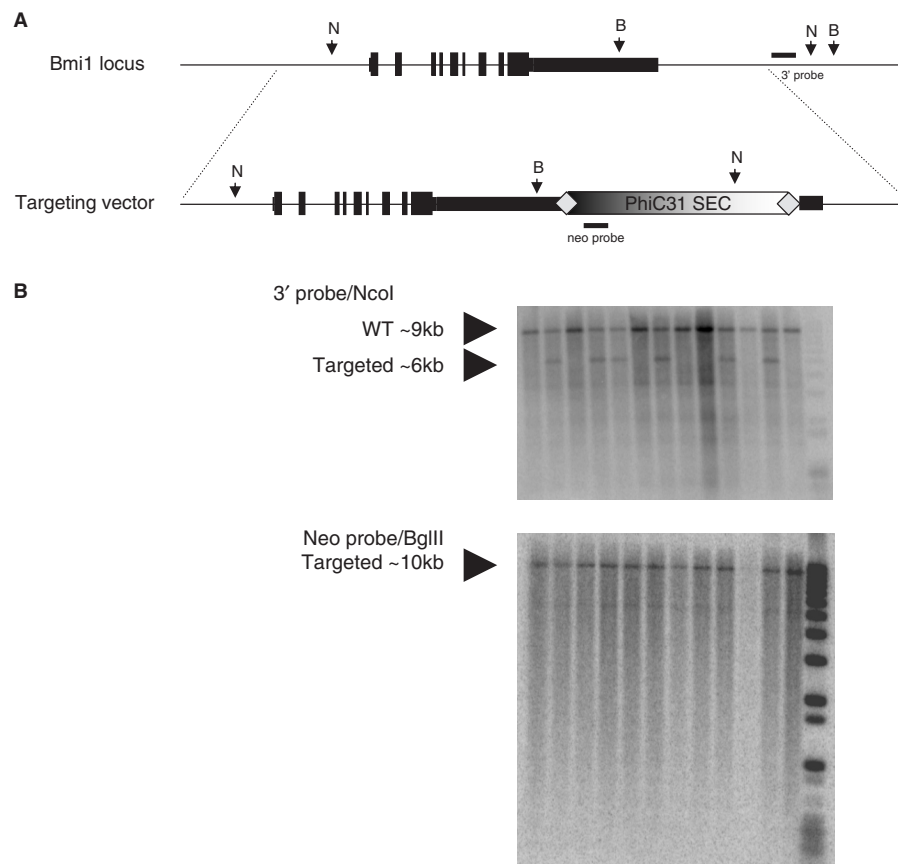


Figure 2. Targeting strategy. (A) Schematic representation of the *Bmi1* locus and the targeting vector containing the PhiC31 SEC. We indicated the positions of the restriction enzymes and the probes used (N, NcoI; B, BglII). (B) Southern blot analysis of the electroporated ES clones, showing the results of the 3' and Neo probe. The arrows indicate the wild-type and the targeted alleles.

PCR for transmission of the targeted allele (Table 1). To evaluate the efficiency of recombination, a PCR-based screening was used to identify in each F1 generation mouse, whether the cassette was transmitted recombined or unrecombined, as well as the wild-type allele. Two oligonucleotides (156–157) flanking the insertion site of the SEC, amplify the wild-type sequence and the recombined attP/B site. The unrecombined allele is not amplified because of its large size. Another pair of oligonucleotides inside the Neo gene was designed to confirm the presence of the cassette if unrecombined (Figure 1C). We screened 173 progeny from 14 chimeras. The SEC was transmitted intact to 58/173 mice in the F1 progeny (Table 1). In ~62% (36/58) of mice, the SEC was transmitted intact not recombined in 22/58 (38%) it was deleted. However, in 4/22 recombined cases, the recombined band was slightly smaller than expected. Sequence analysis revealed that in these four samples the recombination product contained an intra-molecular deletion of 13, 17, 38 and 64 bp of the newly created attL site spanning the TTG recombination site (Figure 3A and B). These four mice were generated from four different chimeras. In 18/22 mice, the size of the PCR product confirmed that recombination in the chimera germline generated the expected product among the attP and attB sites. We analyzed by sequencing 7 of the 18 mice with a normal PCR product, confirming

Table 1. SEC recombination analysis in the F1 progeny

Chimeras	F1 progeny				No germline transmission
	Unrecombined		Recombined		
	Deleted attB	WT attB/P	Precise	Deleted	
7001	–	–	–	–	7
7002	–	–	1	–	7
7003	2	1	1	1	5
7008	–	–	–	–	8
7009	–	–	4	–	4
7010	–	–	–	–	6
7012	4	–	2	1	10
7013	–	–	–	–	9
7014	1	2	1	1	4
7015	5	3	4	1	17
7016	–	–	–	–	16
7017	6	1	1	–	8
7018	4	2	1	–	8
7021	3	2	3	–	6
		36		22	
Total		58			115

In the left column are indicated the chimeric males crossed with C57Bl/6 females to generate 173 mice in the F1 progeny. In detail, from left to right are indicated per each chimera the number of mice with the SEC unrecombined with a deleted attB or WT attB and attP, and the number of mice with a WT recombination product or deleted. In the right column are indicated the number of mice who did not inherit the targeted allele.

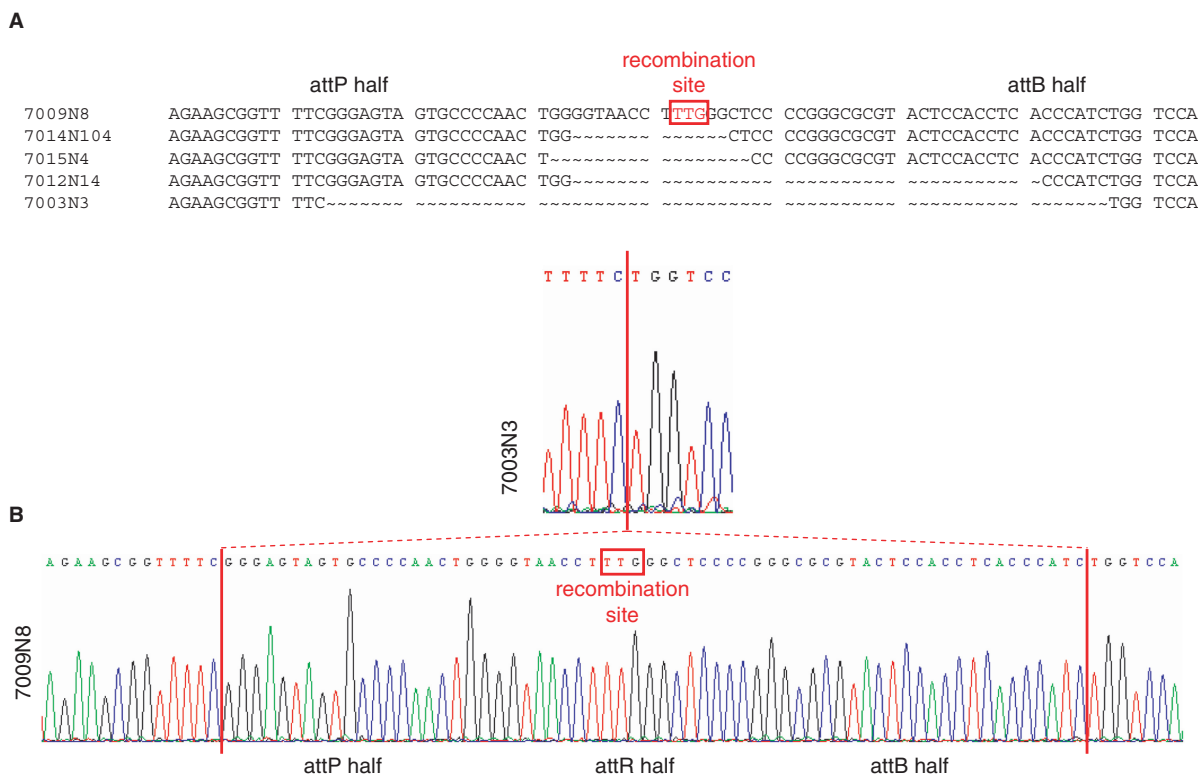


Figure 3. Deletion products of PhiC31 self-excision. (A) Sequence alignment of the predicted fusion product upon recombination between an attP/B site compared with four deletions. The deletions were 13, 17, 38 and 64 bp and were recovered in four mice born from four different chimeras. (B) Electropherogram of one deleted allele (7003N3) compared to the correct recombination product (7009N8).

that the recombination product was precise to the single base pair.

To rule out the presence of deletions or rearrangements in the cassette, all the unrecombined alleles were PCR amplified, using overlapping oligonucleotides to cover the whole cassette (Figure 1C). The PCR product across the attB site showed an unexpected variety in size (Figure 4A). All the attB sites from the unrecombined mice were sequenced and found to be deleted in 25/36 mice. Deletions ranged from 5 to 111 bp, always including the TTG recombination site and some including part of the polyadenylation signal of Neo located at the 3'-end of the attB site (Figure 4B and C). In three mice, the deletion was associated with the presence of 1, 4 and 10 extra nucleotides. The attP sites were also sequenced and found normal in all 36 unrecombined mice.

No other apparent deletion was present in the PCR products covering the rest of the cassette.

To investigate when the attB deletions occurred, the targeting vector plasmid that was electroporated in ES cells, the two correctly targeted ES clones (one of them was injected into blastocysts) and the tail DNA of two chimeras were PCR-amplified and sequenced. In all cases, the PCR products and the corresponding sequences were wild-type (Figure 4C).

To evaluate whether male mice with an intact SEC, and without an attB deletion, were able to recombine in the next-generation, one mouse was crossed with C57/Bl6 females. In 8/17 mice, the allele was recombined (three

alleles showed a slightly smaller band than the control), while in 9/17 the cassette remained intact, even though four alleles showed a smaller band for the attB site (Table 2). These results confirmed that the intact SEC is still able to recombine in the F2 generation although with the same outcome we observed in the F1 generation.

DISCUSSION

We have demonstrated that PhiC31 can be successfully used *in vivo* in the mouse to remove the Neo^r selection cassette. This cassette can be used along with the Cre/loxP system to generate a conditional allele carrying the desired modification minimizing the possibility of negative effects of Neo^r. At the same time, using this assay we were able to demonstrate that the PhiC31 had an efficiency of ~38% in recombining out the SEC sequence, indicating that the integrase is not as efficient as Cre *in vivo* in the same assay (Cre was always 100% efficient, in different constructs generated in our lab as well as in other labs). It is interesting to observe that when the Flp/Frt SEC was generated, using the same general configuration, it did not work (Bunting and Wu, unpublished data). After several changes and modifications a successful Flp/Frt SEC was engineered (Wu, manuscript in preparation), indicating that the overall recombination efficiency of the Flp/Frt system is lower than Cre and PhiC31 recombinases.

An unexpected finding was the imprecise recombination between the attP/B sites, leading in 4/22 cases to four

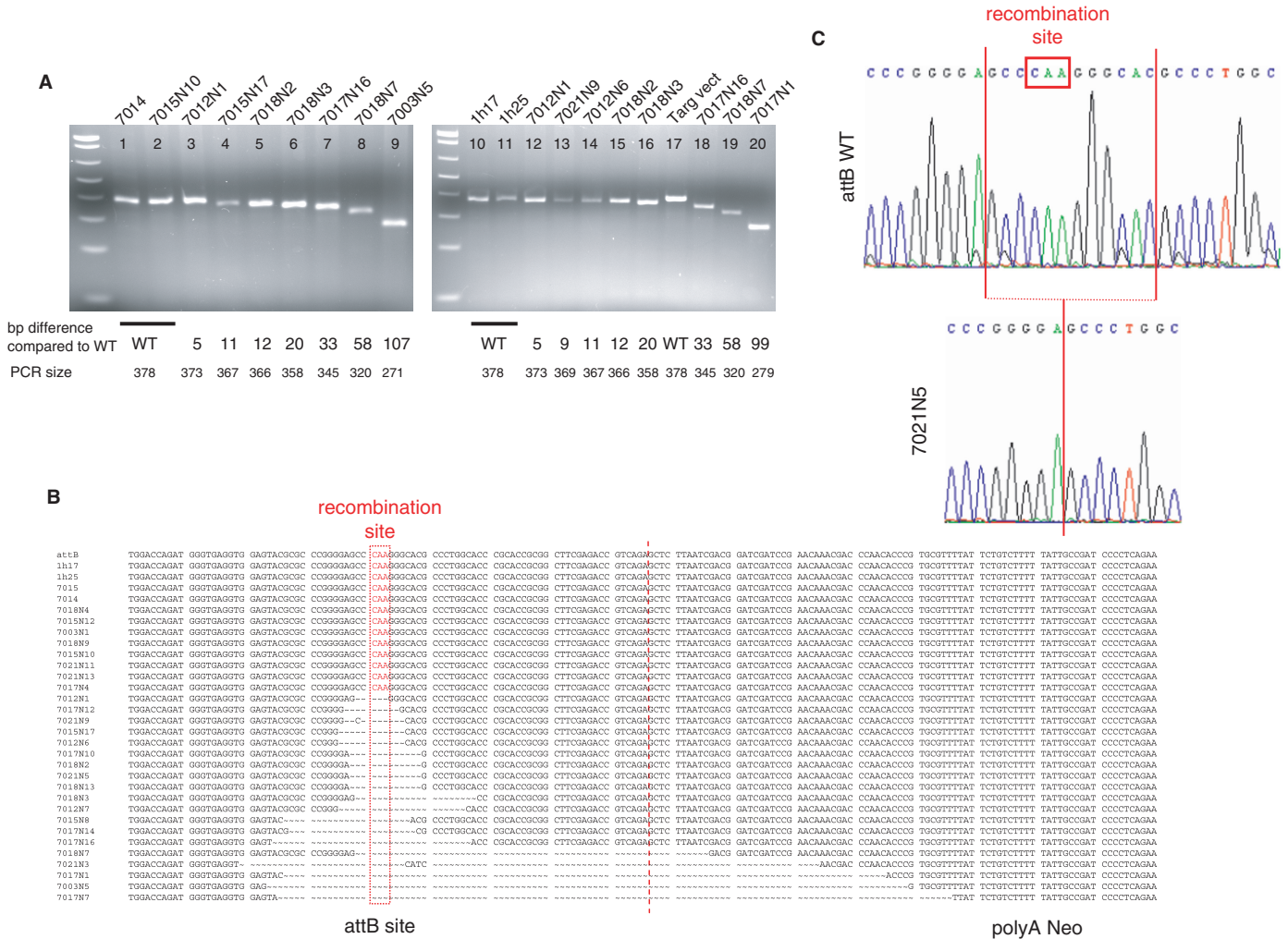


Figure 4. PCR analysis of the attB sites. (A) Representative PCR products of the attB sites from 15 different DNAs containing the cassette unrecombined, oligos used were 157–30. Lanes 1, 2, 10, 11, 17 represent the wild-type product obtained from one of the chimeras (7014), one F1 progeny mouse where the cassette was still intact and no deletion was found, two targeted ES clones and the targeting vector plasmid. Lanes 3–9, 12–16, 18–20 are F1 progeny mice containing different deletions of the attB site. Some of the PCR product from the same mice was loaded on both gels for comparison. Beneath each lane is the size of the deletion and the size of the PCR product. (B) Sequence comparison of the original targeting vector sequence (attB), two correctly targeted ES cell lines (1h17–1h25), two chimeras (7014, 7015), eight intact sequences from F1 progeny mice (from 7018N4 to 7017N4), 19 deleted sequences from F1 progeny mice (from 7012N1 to 7017N7). 7021N9 and 7021N3 have both a deletion and 1 and 4 extra base pairs that are not matching any sequence at the junctions. (C) Electropherogram of one (7021N5) deletion compared to the correct attB site, sequenced from the targeting vector plasmid.

Table 2. SEC recombination analysis in the F2 progeny

F1 male	F2 progeny				
	Unrecombined		Recombined		No transmission
	Deleted attB	WT attB/P	Precise	Deleted	
7014N12	4	5	5	3	13
Total		17			

In the left column is indicated the male (derived from the chimera 7014) crossed with C57Bl/6 females to generate 30 mice in the F2 progeny. In detail, from left to right are indicated the number of mice with the SEC unrecombined with a deleted attB or WT attB and attP, and the number of mice with a WT recombination product or deleted. In the right column are indicated the number of mice who did not inherit the targeted allele.

different deletions in the resulting attL hybrid site. Previous studies have documented such imprecision when PhiC31 was used to insert a transgene in pseudo-attP sites in mouse or in human cells (7). While those results could be easily explained by substitutions present in the pseudo-attP sites, our experiments clearly demonstrate the imprecise nature of recombination with the original sites in the context of mammalian cells. Moreover, in 25/36 unrecombined events, the attB site showed a series of deletions encompassing the TTG recombination site that prevented any further rearrangements with the attP site. To identify the source of the deletions, the original targeting vector plasmid, the attB sites from the two targeted ES cell clones and the DNA from two tail chimeras were sequenced. All sequences were wild-type.

Therefore, we ruled out the possibility that the original targeting vector contained a 'population' of deleted attB fragments. The correct sequences of the two correctly targeted ES cell clones (including the one injected into blastocysts) and the DNA extracted from the tail chimeras confirmed that deletions in the attB site do not represent a mitotic error. Because the only tissue with abnormal attB sites was the chimera's testes, where PhiC31 is transcribed, this provides strong evidence that PhiC31 integrase has spurious activity in the mammalian genome. It is noteworthy to observe that the same chimera gave rise to all different recombination products and the same attB deletions were present in mice coming from different chimeras, indicating that every deletion represents a unique event (Table 1). AttP/B deletions have been reported before in an experiment rearranging the centromere of the human Y chromosome (18). The probable cause for these events is in the intrinsic mechanism of the serine recombinases where the presence of the double-strand breaks recruits the cellular DNA-repair system leading to the repair of intermediate products of recombination.

Remarkably, only the attB site was mutagenized, as we found virtually no mutations in the attP site or anywhere in the rest of the cassette. This could provide the explanation for the well-known asymmetry in the PhiC31-attP/B system (9,19): every time PhiC31 integrase was used to insert a transgene in mammalian cells and the attB was the docking site with an incoming plasmid containing an attP site, the frequency of integration was equal to the frequency of random integration. On the other hand, the frequency of the opposite configuration, attP site as docking site with an incoming attB site, was 30–40% higher than random integration (9). It has been shown (20) that the PhiC31 integrase binds the attB site adopting a specific conformation that allows synapse formation with attP to recombine the two sites. We can speculate that attB binding in the context of the mammalian genome can be 'disturbed' or can be constrained by chromatin configuration or the presence, for example, of methylated CpG, which would prevent a correct synapse formation with the attP site leading to a 'premature' recombination with consequent internal deletion of the attB itself.

Several reports have raised concerns about the safety of using PhiC31 *in vivo*. In one experiment (21), stable expression of PhiC31 integrase in human fibroblasts was associated with chromosomal aberrations, while another report (22) showed that transient expression in the mouse liver was associated with the appearance of dysplastic cells. In a different experiment, we attempted to generate a PhiC31 general 'deleter' targeted to the large subunit of the RNA polymerase II (Polr2a) locus that has a very high targeting efficiency (~60%) (Thomas, K. and M.R.C., unpublished data). We used a very strong promoter to drive the expression of PhiC31 that has already been shown to be able to generate inter-chromosomal translocations using Cre (23); however, in three different electroporation experiments, we were not able to recover a single positive targeted clone out of 432 clones. This suggests that PhiC31 when highly expressed could have a 'toxic' effect in the context of the mammalian genome.

In spite of these results, we did not detect any negative effect in mice harboring the recombined or unrecombined allele in homozygous configuration. Homozygous mice were fertile when kept alive for up to 1 year, and did not show any evident sign of disease. We can not rule out that random deletions are introduced into the genome, but at least in the region surrounding the SEC we analyzed, there are no other evident abnormalities. In our experiment, PhiC31 is expressed only in testis and in the case of the recombined alleles the expression itself is self-limiting; moreover, in the presence of imbalanced chromosomal translocations it is conceivable that the sperm maturation itself would be compromised or those sperm would not be available for fertilization. This also implies that the frequency of the unrecombined events could be theoretically higher, because gametes carrying an imbalanced rearrangement could be selectively eliminated.

If PhiC31 is being used as a standard driver (transgenic or targeted) the possibility still remains that continuous expression in a specific domain, which usually is the domain under investigation, could lead to double-strand breaks with consequent deleterious effects. Another concern is the recombination efficiency observed is only ~38% compared to Cre, suggesting that if PhiC31 is used as a tissue-specific driver to delete a genomic region, a higher level of mosaicism has to be expected. For this reason, in order to improve PhiC31 efficiency *in vivo* and especially to prevent attB deletions, it is important to identify alternative attB sequence variants to improve its efficiency compared to the wild-type attB sequence in the mammalian genome. This report provides a useful tool for mouse conditional mutagenesis and highlights the less desirable properties of PhiC31 that need further improvement. One possibility readily available to test is the codon-optimized PhiC31o sequence (24) that demonstrated increased levels of recombination compared to the wild-type sequence. This new PhiC31 sequence along with the newly generated steroid-inducible PhiC31 (25) will be under scrutiny in the near future, in order to test whether their efficiency and fidelity will provide to the scientific community a better tool for a more sophisticated conditional mutagenesis approach.

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