Research

Phage integrases for the construction and manipulation of transgenic mammals

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Abstract

Phage integrases catalyze site-specific, unidirectional recombination between two short *att* recognition sites. Recombination results in integration when the *att* sites are present on two different DNA molecules and deletion or inversion when the *att* sites are on the same molecule. Here we demonstrate the ability of the φC31 integrase to integrate DNA into endogenous sequences in the mouse genome following microinjection of donor plasmid and integrase mRNA into mouse single-cell embryos. Transgenic early embryos and a mid-gestation mouse are reported. We also demonstrate the ability of the φC31, R4, and TP901-1 phage integrases to recombine two introduced *att* sites on the same chromosome in human cells, resulting in deletion of the intervening material. We compare the frequencies of mammalian chromosomal deletion catalyzed by these three integrases in different chromosomal locations. The results reviewed here introduce these bacteriophage integrases as tools for site-specific modification of the genome for the creation and manipulation of transgenic mammals.

Background

The ability to manipulate the mammalian genome *in vivo* is important in many areas of research. There is a growing need for site-specific recombinases that function in the mammalian environment to catalyze genomic integration and excision, for example in the construction of transgenic organisms and the study of gene function *in vivo*. The site-specific integrases of bacteriophages φC31, R4, and TP901-1 have been shown to function in human and mouse cells to catalyze excision on extrachromosomal vectors and chromosomal integration [1–4]. Phage φC31 integrase has been applied to achieve chromosomal inte-

gration in gene therapy studies [5–9]. These results suggested that these site-specific integrases would likely function in the mammalian embryonic environment and could have utility in the creation of transgenic animals and manipulation of the mammalian genome *in vivo*. The use of bacteriophage integrases in mammalian transgenesis is a new field. This article will discuss some of the potential applications of site-specific integrases in this area and the preliminary results our lab has obtained to date.

Site-Specific Integration for the Construction of Transgenic Mammals

Transgenic mammals are an essential component of bio-technology, having a wide variety of applications ranging from gene knockout studies to serving as living bioreactors. A common method of transgenic mammal production relies on microinjection of DNA directly into the pronucleus of a single-cell embryo, with genomic insertion generally resulting by random integration. Alternatively, mouse embryonic stem (ES) cells can be modified by homologous recombination and placed into a developing embryo. The latter approach results in a mosaic animal that may or may not have germ cells modified. Random integration and homologous recombination both occur at low frequencies (10^{-4} and 10^{-6}, respectively). Other methods have been developed for DNA delivery and maintenance in ES cells including mammalian artificial chromosomes [10,11], retroviruses [12–14], and the Sleeping Beauty transposon [15]. Nuclear transfer from more mature cells can also be used to introduce a new genome for a developing embryo, and this nuclear DNA can be first modified by any of the above methods [16,17].

The available technologies are hindered in that gene insertion is often random. In the resultant transgenic animals, the expression levels of the genes of interest are influenced by context factors that are difficult to control. For example, some influencing factors include the regulatory and bacterial elements in the insertion construct itself [18], the position of the insertion in the genome [19], and the number of integrated constructs [19,20]. Production of a desired clone often takes many rounds of screening. Following screening there may be additional problems caused by the transgene insertion resulting in sterility or pre-adolescent lethality, preventing preservation of the newly developed strain. Efficient, targeted single copy integrations would be helpful for the improvement of transgenic animal production. Site-specific integration systems could potentially be useful in this context. A bacteriophage integrase, such as that of phage φC31, can be used to integrate a donor plasmid into the mammalian genome, and integrants can be screened by a simple PCR reaction to identify integrations into known and desirable targets. A quantitative PCR approach can be used to ensure that only single integration events are selected. In the creation of multiple transgenic animal lines, candidate clones can be screened and selected so that the genomic integration event in each animal line is in the identical genomic location and orientation, thereby eliminating positional effect differences between the different transgenic lines. Any genomic location is likely to exert context effects that affect the expression of an integrated transgene, but the use of a site-specific integration system will help to ensure that these effects are not varied among different lines. To date, site-specific recombinases have not been used for the creation of transgenic mammals, though some, such as Cre, have been valuable for genomic manipulations in transgenic animals, as discussed below.

Phage Integrase Biology in the Mammalian Environment

Members of the serine family of site-specific recombinases [21], including integrases from phages φC31 [1,2,5,6,9], R4 [3], and TP901-1 [4], are good candidates for mammalian genomic modification. In nature, each integrase recognizes a sequence in the phage genome, the phage attachment site *attP*. Each integrase also recognizes a bacterial attachment site, *attB*, in the bacterial genome. Integrases of the serine family of recombinases are capable of recombining these short *attP* and *attB* attachment sites, whose minimal sizes are 35–50 bp. The products, two hybrid sites, *attL* and *attR*, are no longer substrates for recombination by the integrase, so the reactions are unidirectional [22]. While the integrase alone can only catalyze a recombination between an *attP* and an *attB* site, the result of the recombination can vary based on the location and orientation of these two *att* sites. When located on different DNA molecules, recombination between an *attP* and an *attB* results in an integration event. However, if the two sites are located on the same DNA molecule (plasmid or chromosome), two outcomes are possible: if the *attP* and the *attB* are in the same orientation on the DNA, the net result will be a deletion event; if the two *att* sites are in opposite orientations, the net result will be an inversion. Thus, by knowing the locations and orientations of compatible *att* sites, specific recombination events can be created and selected for.

We have demonstrated that the φC31 integrase is able to mediate efficient integration of *attB*-bearing plasmids into the human and mouse genomes, at inserted wild-type *attP* sites and at endogenous "pseudo" *attP* sites that have partial identity to *attP* [2]. We previously identified 26 pseudo *attP* sites in the human genome, and 57 pseudo *attP* sites in the mouse genome [2]. We have not detected the presence of pseudo *attB* sites in mammalian genomes, which should reduce the likelihood that endogenous genomic sequences are inadvertently deleted during an integration attempt. There may not be pseudo *attB*s that could be recognized and recombined with a pseudo *attP* by the integrase.

Site-Specific Chromosomal Deletions

In addition to chromosomal integration events, often times a chromosomal deletion is desired, such as in the generation of knockout transgenic animals. One way to accomplish deletion of a sequence of interest is through the use of site-specific recombinases, such as the Cre recombinase of coliphage P1 and its *loxP* recognition sites [23]. The deletion can be precisely confined to a specific

developmental time or tissue, based on control of recombinase expression. This outcome is most commonly achieved by creating a transgenic mouse that contains the gene of interest flanked by *loxP* sites, and then crossing that mouse with a Cre-expressing mouse [24]. The Cre gene may, for example, be under a tissue-specific promoter. Once crossed, the gene of interest will be deleted in all cells in which Cre is expressed, thereby generating a tissue-specific knockout [24–30]. The same approach can be used to activate a gene of interest, by inserting a stop cassette between the promoter and the gene of interest and flanking the stop cassette with *loxP* sites [31]. If the gene knockout is desired in the entire animal, the "floxed"-transgene mouse oocytes can be transfected with a Cre-expression plasmid or Cre mRNA, so that expression is transient, but sufficient to delete the gene of interest at an early developmental stage, thereby producing an animal devoid of the gene of interest [32,33].

Alternatively, excision can be controlled in a temporal-specific manner by expressing Cre from an inducible promoter. A fusion protein of Cre recombinase and a mutated hormone-binding domain of the murine estrogen receptor showed temporal-specific activity in transgenic mice that was controlled by the presence of tamoxifen [34]. Temporal-specific recombination has also been accomplished by driving Cre expression from inducible promoters, such as a tetracycline-inducible promoter [35]. Simultaneous temporal- and tissue-specific Cre expression has been demonstrated using tissue-specific promoters to drive expression of the Cre ORF fused to a hormone receptor ligand-binding domain [29,36].
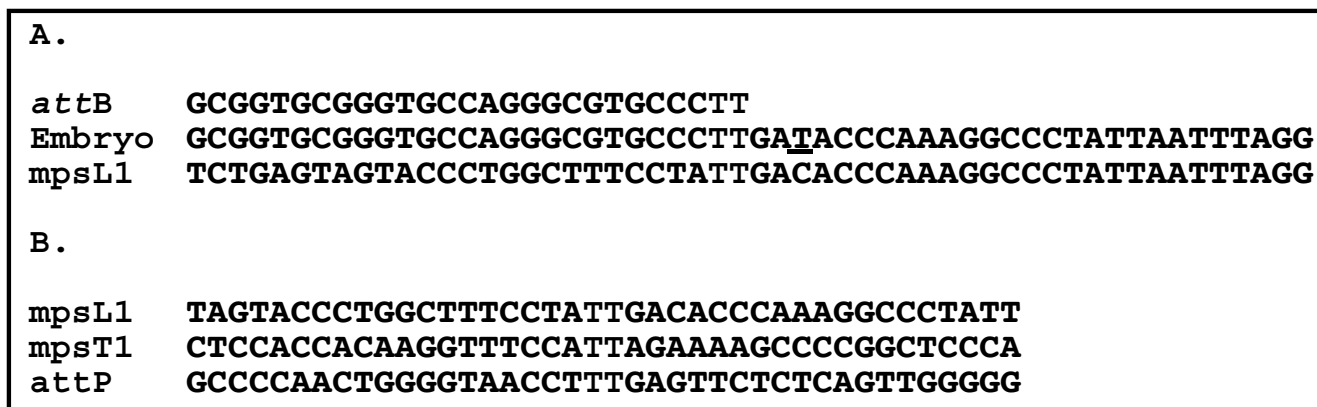
Some circumstances require more complicated deletion/insertion events, such as sequential gene inactivation and exon swapping [37]. Additionally, it may be desirable to remove extraneous sequences in an integrated vector, such as bacterial or mammalian drug selection markers [18]. The FLP recombinase from the 2 μm plasmid of *S. cerevisiae* has been used in transgenic mice as an alternative to Cre [38,39] and in combination with Cre [40], although FLP functions at only ~10–25% efficiency compared to Cre in generating chromosomal deletions [41]. The Cre and FLP recombinases are the only two enzymes currently available for genomic manipulation experiments, making complicated experimental schemes difficult to perform [25,40]. The increasing desire to perform complex genomic manipulations creates a need for additional site-specific recombinase tools that function efficiently in the mammalian chromosomal environment.

Results

Phage φC31 Integrase Functions in the Mouse Embryonic Environment and Mediates Genomic Integration

To demonstrate that the φC31 integrase would be functional in the embryonic environment, single-cell embryos of the FVB/NacfBR mouse strain were injected with 3 ng/μl nucleic acid solutions directly into the pronucleus. Two groups of injected embryos were studied. One group received pCMVInt plasmid DNA [1] as a source of integrase (n = 31), and the other received φC31 integrase mRNA (n = 32). Both groups were co-injected with the intramolecular-integration assay vector pBCPB [1] carrying *attB* and *attP* sites. The embryos were frozen at the two-cell stage, total DNA was extracted [42], and the expected wild-type *attL* junction was PCR amplified from recombined plasmid. A product of correct size was observed in both groups of embryos. This product is specific for the recombination event and is not present unless φC31 integrase is expressed [1]. Embryos co-injected with integrase mRNA produced a band of correct size with just ~25% of the template that was required to detect the same band from the pCMVInt/pBCPB injections. This result suggested that integrase mRNA was more efficient in the embryonic environment than DNA, presumably due to the elimination of the requirement for transcription. This result was consistent with observations that injection of Cre mRNA into mouse oocytes provided efficient Cre activity *in vivo* [33]. The use of mRNA instead of DNA also precluded undesirable integration of the integrase gene by random integration.

Phage φC31 integrase can efficiently catalyze a site-specific cassette exchange reaction using introduced wild-type *att* sites in mouse ES cells [43] and can recognize and insert *attB*-containing plasmids into pseudo *attP* sites in the murine genome [2,6]. A hotspot for φC31 integrase-mediated integration was identified in hepatocytes on chromosome 2 (GenBank: AC 079573) and named mpsL1 (mouse pseudo *attP* site in liver) [6]. Integration at mpsL1 was also detected in mouse NIH3T3 cells [2], indicating that mpsL1 may be a preferred integration site in the mouse genome. A GFP vector, pEGFPB2, carrying an *attB* site was injected into one-cell embryos either with (n = 35) or without (n = 27) φC31 integrase mRNA. GFP expression was used to indicate visually the number of embryos with integrated DNA, because non-integrated DNA should be gradually lost during a 5 day period of rapid cell division to the multi-cell or blastocyst stage. By fluorescent microscopic analysis, the two groups had similar numbers of detectibly GFP-positive embryos, 6/35 with integrase and 5/27 without integrase. Embryos were positive for GFP expression in all cells; however, the group that received the integrase mRNA contained embryos with an overall more intense green fluorescence.

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A.

attB      GCGGTGCGGGTGCCAGGGCGTGCCCTT
Embryo    GCGGTGCGGGTGCCAGGGCGTGCCCTTGATACCCAAAGGCCCTATTAATTTAGG
mpsL1     TCTGAGTAGTACCCTGGCTTTCCTATTGACACCCAAAGGCCCTATTAATTTAGG


B.

mpsL1     TAGTACCCTGGCTTTCCTATTGACACCCAAAGGCCCTATT
mpsT1     CTCCACCACAAGGTTTCCATTAGAAAAGCCCCGGCTCCCA
attP      GCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGG
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Figure 1
Integration at mouse genomic sequences. (A) Sequence from an embryo-derived PCR clone displays the characteristic site-specific crossover between the *attB* arm, shown in gray, and the murine pseudo *attP* site, mpsL1, with the TT core (in bold) located at the point of crossover. A one-base mismatch (underlined) located downstream of the crossover event may be a polymorphism between the mouse FVB/NacfBR strain and the C57Bl/6 strain that was sequenced in the database. (B) Comparison of the sequences of embryo-derived pseudo *attP* sites mpsL1 and mpsT1 with the wild-type φC31 *attP*. The common TT core is shown in bold. Both of these embryo-derived pseudo *attP* sites are ~30% identical to the wild-type φC31 *attP*. The matching base pairs with a 40 base *attP* are shown in gray.

Table 1: FVB/NacfBR transgenics obtained following injection with *attB*hFIX-containing vector with or without φC31 integrase mRNA.

ng/μl *attB* DNA/ Integrase mRNA	Embryos injected	Embryos implanted	Pups harvested	hFIX positive	φC31-mediated
3 DNA + 0 mRNA	68	48	16	0	0
3 DNA + 3 mRNA	144	102	39	3	1
3 DNA + 6 mRNA	415	284	66	1	0

DNA extracted from the embryos injected with pEGFPB2 with and without φC31 integrase mRNA was used as a template in a PCR assay designed to amplify the *attL* junction produced when φC31 integrase mediated site-specific integration at mpsL1. The group that received φC31 integrase mRNA produced a band of predicted size that was subsequently cloned and sequenced. The DNA sequence from the PCR product, shown in Figure 1A, demonstrated φC31 integrase-mediated site-specific integration at mpsL1 and proved that site-specific integration occurred within the embryonic genome. No integration into mpsL1 was detected in the GFP-positive embryos that did not receive integrase.

Construction Of Site-Specific Integrant Transgenic Animals
FVB/NacfBR single-cell embryos were injected with an *attB*-bearing donor plasmid, pBChFIXattB [6], with or without φC31 integrase mRNA. Injected embryos were

implanted into surrogate mothers at ~20 embryos per animal. Pups were harvested mid-gestation at approximately 2 weeks post-implantation, and genomic DNA was isolated and screened for integrated donor plasmids by PCR. To determine whether an animal contained an integrated donor vector, PCR was employed to amplify a 406-bp internal hFIX fragment of the donor plasmid. To determine whether donor vector was integrated site-specifically by a φC31-mediated event, PCR primers were designed to distinguish an intact *attB*, signifying random integration, from an *attL*, signifying φC31-mediated integration. Animals whose DNA produced a 406-bp donor band but not an intact *attB* band were likely to have site-specifically disrupted the *attB* sequence, indicating an integrase-mediated event (Table 1).

A mid-gestation mouse found to have a plasmid inserted site-specifically in its genome was further studied by PCR and sequence analysis. Using a nested PCR rescue tech-

nique [2], it was determined that this animal contained the donor plasmid site-specifically integrated into a sequence on chromosome 4 termed mpsT1 (mouse pseudo *attP* site in Transgenic 1), shown in Figure 1B. This integration site is located in the last intron of a putative gene detected in a 16-day neonate (Genbank: AK052928). The integration event did not prevent embryogenesis in this experiment, indicating that an integration event at mpsT1 was unlikely to disrupt development and this location was potentially a suitable target site for future transgene integrations. Like the mpsL1 pseudo site, mpsT1 has limited identity to the wild type *attP* sequence (Figure 1B). Chromatin structure may influence which pseudo sites are used [6]. Repeatedly targeting an identical genomic locus such as mpsT1 could allow for the creation of multiple transgenic lines with transgenes integrated at the same, predictable genomic locus. Creation, by directed evolution [44], of integrases with tighter site-specificity may allow for such a possibility.

The use of an engineered mouse strain containing an inserted wild-type *attP* site could help resolve position effects by providing a method to preferentially target transgenes to a predetermined location. The use of an *attP* transgenic mouse strain might also increase the integration frequency, because the integrase might work more efficiently at a perfect *attP* site, compared to pseudo *attP* sites. The detection of site-specific integrants would also be simplified because the sequence of the target site was known. Therefore, similar experiments were performed on a strain of transgenic mice we prepared that contained a wild-type φC31 *attP* site. Injections into this *attP*-bearing mouse strain were performed with φC31 mRNA and the *attB* donor plasmid pBChFIXattB. Of 7 injected pups that carried the wild-type *attP* site, none were found to have an insertion of the *attB* donor plasmid anywhere in their genomes. Although the number of pups examined was low, this negative result may indicate a poor genomic location of the φC31 *attP* site in this transgenic strain, with respect to chromatin access for the φC31 integrase. The creation of a strain with a more accessible *attP* site might result in a higher percentage of φC31-mediated integrations at the *attP* site.

Chromosomal Deletions Catalyzed by Phage Integrases
The integrases of phages φC31, R4, and TP901-1 function efficiently in mammalian cells to complete precise deletions on extrachromosomal introduced DNA [1–4]. It was recently shown that the φC31 integrase could perform intrachromosomal deletions at a frequency ∼10% that of Cre [41]. Addition of a nuclear localization signal (NLS) to φC31 integrase increased this frequency to ∼80% of that of Cre [41]. The ability of R4 and TP901-1 integrases to catalyze chromosomal deletions was unknown. To facilitate their use as genomic tools, it would be useful to have

a direct comparison of chromosomal deletion activity among these site-specific integrases. To determine the frequency of chromosomal deletions catalyzed by these integrases, we established human cell lines with an integrated copy of a vector containing the φC31, R4, and TP901-1 *attB* and *attP* recognition sites (Figure 2). Transfection of these cells lines with integrase expression plasmids, followed by quantitative PCR and Southern analysis, allowed us to calculate the chromosomal deletion frequency of each integrase. Because all three recognition site pairs were in the same vector, and thus in the same chromosomal context in each cell line, we were able to obtain a directly comparable measure of the chromosomal deletion frequency mediated by the φC31, R4, and TP901-1 integrases.

Mammalian expression vectors were created for each of the three integrases (Figure 2), in which the cytomegalovirus immediate early (CMV) promoter drove expression of the integrase, followed by an internal ribosome entry site (IRES), and the gene for green fluorescent protein (GFP). Cell lines were transfected with a pInt-I-hrGFP vector, and GFP-positive cells were sorted 72 h post-transfection, expanded, and genomic DNA was prepared. Separately for each sample, the total integrated vector copy number was determined by quantitative amplification of a region of the hygromycin resistance gene. The number of deletion products was determined by quantitative amplification of *attR* junctions. Deletion frequency was calculated as the ratio of *attR* junctions to total vector copies. The results are shown in Figure 3A.

Quantitative PCR analysis of TP901-1 integrase-catalyzed deletion was unsuccessful. The TP901-1 *attR* is highly A+T-rich, making Taqman primer/probe design difficult. Because we could not accurately determine TP901-1 deletion frequency by qPCR, Southern blot analysis was performed. This analysis was done for φC31 and R4 as well, in order to directly compare with the TP901-1 frequency and to independently verify the deletion frequency determined by qPCR. Genomic DNA from integrase-transfected cell lines was analyzed by Southern blot, and deletion frequency was quantified. Chromosomal recombination resulted in reduction of the genomic vector by deletion of 4.8 kb (φC31), 3.7 kb (R4), or 4.2 kb (TP901-1) of intervening vector sequence. Representative Southern blots of φC31-, R4-, and TP901-1-transfected cell line genomic DNA are shown in Figure 3B. Quantification of band intensity indicated that Southern analysis of chromosomal deletion frequency was in agreement with that calculated by qPCR analysis for the φC31 and R4-transfected cell lines.

The φC31 integrase consistently performed more efficiently than the R4 and TP901-1 integrases. In cell line
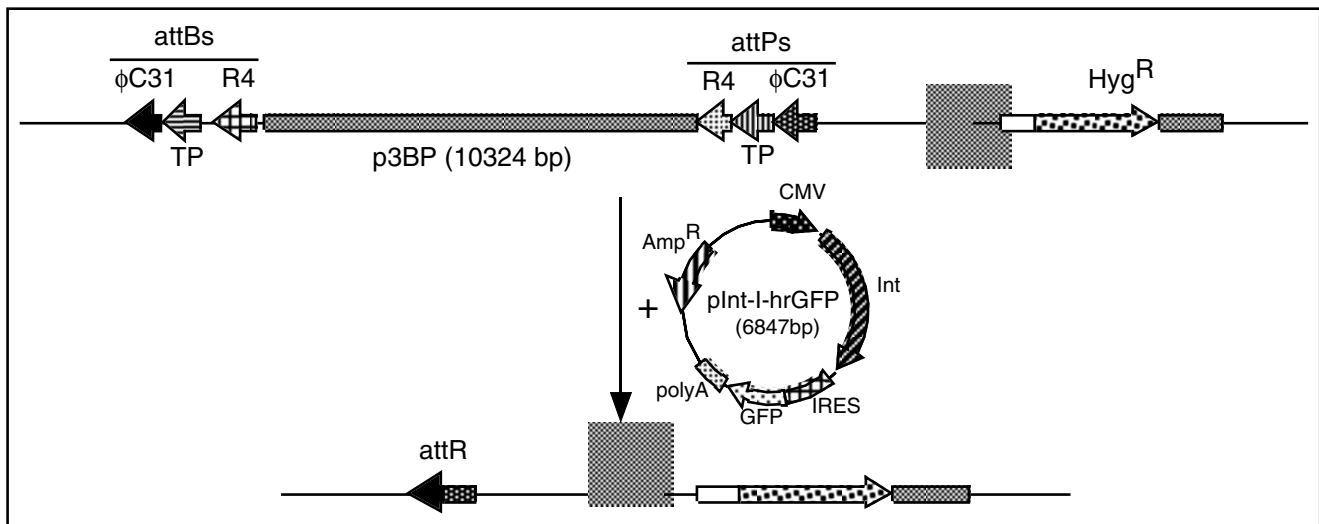
Figure 2
Chromosomal deletion analysis system. Plasmid p3BP was linearized and integrated into the genome of 293 cells. *AttB* and *attP* sites for φC31, R4, and TP901-1 integrases are indicated. pInt-I-hrGFP is an integrase expression plasmid, in which the CMV promoter drives simultaneous expression of integrase and GFP, through the action of an IRES. Independent expression vectors were cloned for the φC31, R4, and TP901-1 integrases. HygR, hygromycin resistance; AmpR, ampicillin resistance; IRES, internal ribosome entry site; CMV, cytomegalovirus immediate early promoter; Int, Integrase gene; GFP, green fluorescent protein; polyA, poly(A) addition site from simian virus 40.

293-3BP3, chromosomal deletion was catalyzed by the φC31 integrase with an average frequency of 51%, by the R4 integrase at an average frequency of 37%, and by the TP901-1 integrase at a frequency of 39%. The R4 and TP901-1 integrases performed equally well, though at a lower frequency than the φC31 integrase, in cell line 293-3BP6. The frequency of deletion catalyzed by the φC31 and R4 integrases was lower in all other cell lines to varying degrees, while TP901-1 integrase showed no measurable activity in these other cell lines.

The chromosomal deletion frequency of the φC31 integrase ranged from 51% in cell line 293-3BP3 to only 1.7% in the 293-3BP8 cell line. Similar ranges were observed for the R4 integrase. These variations in activity most likely represented a chromosomal position effect of the integrated vectors in different regions of the genome, which may affect the ability of the integrase to efficiently access the *att* sites. We have hypothesized that the φC31 integrase preferentially integrates into pseudo *attP* sites that are in actively expressed regions; an open chromatin configuration may be required for efficient targeting and recombination by the integrase [6]. This hypothesis was supported by the apparent chromosomal position effects on excisive recombination observed here. The TP901-1 integrase appeared to be even more susceptible to chromosomal position effects, as evidenced by its ability to perform

deletions at ~35% efficiency in two of the cell lines, the two with the highest deletion frequencies by φC31 and R4 integrases, but its lack of deletion formation in the other cell lines analyzed.

Discussion
The experiments reported here suggest that the φC31 integrase can be used to create transgenic mice carrying site-specific transgene insertions. The numbers of φC31-mediated transgenic animals generated were low, with approximately 2.6% of harvested pups representing φC31 integrase-mediated transgenic animals, in the best experiment. Our sequence data demonstrated that the φC31 integrase functioned in the embryonic nuclear environment to integrate plasmid DNA site-specifically into the genome. Additional experiments with greater numbers of animals will be required to optimize the procedure and determine the efficiency of such an approach for generating transgenic mammals. Such a tool would be beneficial because the genomic location of the integration site, either at an inserted wild-type *attP* site or at a known endogenous pseudo *attP* site, can be specifically detected by PCR amplification, potentially allowing the creation of multiple transgenic lines with different integrated transgenes in the same genomic context. This feature would permit creation and comparison of multiple transgenic animal strains without confounding chromosomal posi-

Figure 3
Integrase-mediated chromosomal deletion. (A) Chromosomal deletion frequencies in 293-3BP cell lines calculated by quantitative PCR. The values represent the average of two experiments, with standard deviation bars. Cell lines were transfected with pφC31-IRES-hrGFP (dotted bars) or pR4-IRES-hrGFP (dashed bars), GFP-positive cells were sorted and expanded, and genomic DNA was harvested and analyzed by qPCR. Deletion frequency was calculated as the ratio of *attR* junctions amplified to total vectors present (hygromycin resistance gene amplified), multiplied by 100. (B) Representative Southern blot of deletion reaction in cell line 293-3BP6. Untransfected and Int-transfected cell lines are shown, with the expected intact (unex.) and Int-deleted bands for φC31 (φex), R4 (R4ex), and TP901-1 (TPex) indicated. Twenty μg of genomic DNA were probed with a common 1.6 kb plasmid backbone fragment.

tion effects. The φC31 integrase has also been shown to function at efficiencies of ~50% for creation of transgenic *Drosophila* at inserted *attP* sites [Groth, A.C., Fish, M., Nusse, R., and Calos, M.P., submitted]. This integrase is therefore likely to be of use in construction of a wide range of transgenic species.

We also demonstrated that the integrases of bacteriophages φC31, R4, and TP901-1 performed efficiently in creating intrachromosomal deletions and should be of great value for manipulating mammalian genomes, both *in vitro* and *in vivo*. The maximum observed deletion frequencies presented here were in the range of 30–50%, but this range probably underestimates the activity of these integrases. The criteria used for sorting GFP-positive, integrase-transfected cells may have included untransfected cells. Following expansion of these cells, quantitative analysis of chromosomal deletion frequency would be depressed by the presence of cells that were never exposed to integrase. However, even at the observed frequencies of 30–50%, the φC31, R4, and TP901-1 integrases constitute efficient tools for the manipulation of mammalian chromosomes. In the experiments reported here, the φC31, R4, and TP901-1 integrases used were in their native prokaryotic form, with no addition of an NLS. A φC31-NLS integrase [41] may function significantly better, nearing 100%, in this chromosomal deletion assay, and addition of an NLS may have a similar positive effect on the efficiencies of the R4 and TP901-1 integrases. Such chromosomal deletion frequencies would make the φC31, R4, and TP901-1 integrases equivalent to Cre recombinase and valuable as molecular tools for mammalian chromosome engineering. Additional experiments will need to be conducted in order to determine the efficiency of these integrases in catalyzing chromosomal deletions *in vivo*. These serine family site-specific phage integrases clearly constitute new and valuable tools for genome engineering.

Materials and Methods
Transgenic Animal Experiments
Vector construction
The φC31 integrase gene was amplified to include the *Nde*I restriction site from pTA-Int [1] by using the primers "Native 5' NdeI (+)" (CGA CTA GTC ATA TGG ACA CGT ACG CGG GTG CT) and "3' BamHI φC31" (AGC CGG ATC CGG GTG TCT CGC TA). The pET11 vector (Novagen, Madison, WI) received the amplified φC31 integrase gene directionally inserted into *Nde*I and *Bam*HI sites so that the T7 promoter drove φC31 integrase mRNA production (pET11φC31). The oligonucleotides *Bam*HI-polyA-top (GAT CGC GCC AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA CCG) and *Bam*HI-polyA-bottom (GAT CCG GTT TTT TTT TTT TTT TTT TTT TTT TTT TGG CGC) were annealed, digested with *Bam*HI, and inserted into the *Bam*HI site in pET11φC31 to create pET11φC31polyA. The assay construct pEGFPB2 is based on the vector pEGFP-N2 (Clontech, Palo Alto, CA). A *lac* promoter to drive GFP expression in bacteria and codons for the first 26 amino acids of β-galactosidase were cloned into the *Nhe*I and *Hind*III sites within the multicloning site of pEGFP-N2, placing it in frame with eGFP to create

pCMV*lac*GFP. The *attB* fragment was released from pTA-*attB* [1] with *Eco*RI, and the ends of the fragment were blunted. The blunt *attB* fragment was inserted into pCMVlacGFP vector blunted at the *Dra*III restriction site. This cloning of the *attB* with the TT core pointing towards the GFP terminator sequence resulted in creation of the assay vector pEGFPB2.

Nucleic acid preparations for microinjection
The φC31 integrase mRNA was transcribed from the vector pET11φC31polyA using the mMessage mMachine (Ambion, Austin, TX). φC31 RNA and DNA were purified and diluted in microinjection TE (miTE) buffer (10 mM Tris and 0.1 mM EDTA, pH 7.4) to reach a final concentration of 3 ng/μl. For the injections 3 or 6 ng/μl solutions of each nucleic acid were used.

PCR screening for integration
Standard embryo DNA retrieval technique for PCR was employed [42]. The primers attBF2 (ATG TAG GTC ACG GTC TCG AAG C) and attP1+ (TGG CGG CCG CTC TAG AAC TA) were used to specifically amplify the wild type *attL* junction in integrase-reacted pBCPB. A 175 bp product will be amplified. For detection of mpsL1 integration in embryos, a primary PCR using the primers attBF3 (CGA AGC CGC GGT GCG) and mpsL1-R2 (GTA AAT GTT ATT GCG GCT CT) was purified and used as a template in a secondary PCR using attBF3 and the nested primer mpsL1-R1 (TGA GGA GGA GCC TTA GCA AC). The secondary PCR amplifies a nested product of 208 bp. For detection of the GFP transgene, the primers GFPfor (CTG GAC GGC GAC GTA AAC GGC CAC) and GFPrev (GGC GGA TCT TGA AGT TCA CCT TC) were used to specifically amplify a 453 bp segment of the GFP transgene. The hFIX positive cells were identified using the primers hFIXend (CAC TTC TGG AGG CCA AGG TGG G) and *attB*RF2-com (GCT TCG AGA CCG TGA CCT ACA TC), resulting in a 406 bp band being amplified. To identify φC31-mediated transgenics, the primers hFIXend and attBR2 (TCA ACT ACC GCC ACC TCG AC) were used, which provide a 508 bp product only if an intact *attB* is present. To detect the presence of an *attP* segment in transgenic mice, the primers For4TRI (AAC TCA GTT GGG CTC AAT TCC) and the Revr4TRI (CCA GTA GCT GAC ATT CAT CCG) were used to amplify a specific 1559 bp product.

PCR detection of integration into pseudo attP sites
To detect integration at mpsL1 in pups, attBF3 and mpsL1-R2 were used in a PCR reaction. Positive integrants at mpsL1 resulted in a 513 bp product. To discover unidentified pseudo *attP* sites, genomic DNA was digested using: *Avr*II, *Nhe*I, *Spe*I and *Xba*I. The genomic digests were ligated to produce circularized substrate for inverse PCR. The *attL* junctions were amplified using attBF2 and hFIXinvR1 (CAT CAA ATA CAG GGT GAC TGA TTC). A

nested reaction was then applied to increase specificity using the primers attBF3 and hFIXinvR2 (CAA AGG GAA ACA TAC GTC AG). Resulting bands were cloned and sequenced.

Wild-type attP transgenic mice
The plasmid pTA-attP333 [4] carries the full length TP901-1 *attP* site. The φC31 *attP* site was isolated as a blunted *Spe*I fragment from pBCPB [1] and ligated into the blunted *Ava*I site of pTA-attP333, creating the plasmid pBI(-). The R4 *attP* site was isolated from the plasmid pBCRattP64 [3] by digestion with *Pvu*II and *Sna*BI and blunt-ending with T4 polymerase. It was then ligated into the *Dra*III pf pBI(-), creating the plasmid pTRI(+). pTRI(+) was digested with *Bgl*II and *Hin*dIII to liberate a 1.9-kb fragment containing the three *attP* sites. This triple-*attP* fragment was gel isolated and purified using the QIAexII gel extraction kit (Qiagen, Valencia, CA) and eluted with miTE (10 mM Tris-HCl, 0.1 mM EDTA, pH7.4). DNA was prepared to a concentration of 25 ng/μL. To eliminate debris from the prep, the DNA was passed through a small 0.45 μm Millipore spin filter (Millipore, Billerica, MA). DNA was transferred to the Stanford Transgenic Research Facility for standard transgenic animal production by microinjection.

Chromosomal Deletion Experiments
Vector construction
The assay plasmid for detecting chromosomal deletions was generated as follows. Plasmid pBB-B304-P333 has been described [4] and will be referred to as pBB-TP(BP). This plasmid contains the full-length TP901-1 *attP* and *attB* recognition sites flanking the 3.5 kb *lacZ* gene. The 64 bp R4 *attP* site was generated by kinasing and annealing the oligonucleotides
5'-CCGGGCATGTTCCCCAAAGCGATACCACTTGAAGCAGTG GTACTGCTTGTGGGTACACTCTGCGGGTGTAC-3' and
5'-CCGGGTACACCCGCAGAGTGTACCCACAAGCAGTACCAC TGCTTCAAGTGGTATCGCTTTGGGGAACATGC-3', followed by ligation into the *Xma*I site of pBB-TP(BP), creating the plasmid pBB-TP(BP)-R4(P). Into the *Sac*II site of this plasmid, the 224 bp φC31 *attP* site was cloned as an *Xba*I-*Sca*I fragment from plasmid pBCPB+ [1], resulting in the plasmid pBB-TP(BP)-R4(P)-φC31(P). Into the *Xho*I site of this plasmid, the 313 bp φC31 *attB* site was cloned as an *Xho*I fragment released from pBCPB+, generating plasmid pBB-TP(BP)-R4(P)-φC31(BP). Into the *Bam*HI site of this plasmid, the 295 bp R4 *attB* site was ligated as an *Xho*I fragment released from the plasmid pBC-R4PB [3], creating the plasmid pBB-TP(BP)-R4(BP)-Phi(BP). Lastly, a 2.0 kb *Sal*I-*Nru*I fragment from pEF [4], encoding the gene for hygromycin resistance driven by the TK promoter, was cloned into the *Age*I site of pBB-TP(BP)-R4(BP)-φC31(BP), generating the assay plasmid pBB-TP(BP)-R4(BP-φC31(BP)-Hyg, referred to as plasmid p3BP.

Each integrase was independently cloned into the expression plasmid pIRES-hrGFP-1a (Stratagene, La Jolla, CA).

The TP901-1 integrase was cloned into the *Eco*RI-*Xho*I sites of pIRES-hrGFP-1a as a 1.5 kb *Eco*RI-*Xho*I fragment from pCS-TPInt [4], generating the plasmid pTP-I-hrGFP. A 1.4 kb *Eco*RI fragment from pTA-*sre* [3], containing the R4 integrase, was cloned into the *Eco*RI site of pIRES-hrGFP-1a, creating the expression plasmid pR4-I-hrGFP. The φC31 integrase was cloned from pCSI [1] as a 1.9 kb blunted *Spe*I-*Bam*HI fragment and ligated into the *Sma*I-*Bam*HI sites of pIRES-hrGFP-1a, to generate the vector pφ-I-hrGFP. Each of these vectors expresses integrase under the control of the CMV promoter, followed by an internal ribosome entry site (IRES) and the humanized *Renilla reniformis* GFP (hrGFP) open reading frame.

As a standard control for qPCR, the *att*R junction for each integrase and a hygromycin gene region were cloned into a single plasmid. Vector p3BP was transformed into *E. coli* strain DH-Int, which expresses the φC31 integrase [1], in order to generate a φC31-excised plasmid. The *attR* was PCR amplified and TOPO-cloned into vector pCR2.1 (Invitrogen, Carlsbad, CA), creating the plasmid pTA-φex. Next, the R4 *attR* was generated by intramolecular integration reaction in 293 cells co-transfected with p3BP and pCMV-sre [3], creating p3BP-R4ex. The R4 *attR* junction was PCR amplified from p3BP-R4ex with primers that added *Spe*I sites to each end of the *attR*. The PCR product was digested with *Spe*I and ligated into the *Spe*I site of pTA-φex, creating plasmid pTA-φex-R4ex. The TP901-1 *attR* junction was generated by transforming p3BP into DH-TPInts [4], generating plasmid p3BP-TPex. The TP901-1 *attR* was then PCR amplified, with addition of *Bam*HI sites to each end. The PCR product was digested with *Bam*HI and ligated into the *Bam*HI site of pTA-φex-R4ex, creating the plasmid pTA-φex-R4ex-TPex. Next, a region of human *Rad52* gene was PCR amplified from 293 genomic DNA with primers that added *Cla*I sites to each end. The PCR product was digested with *Cla*I and ligated into the *Cla*I site of pTA-φex-R4ex-TPex, creating the plasmid pTA-3attR-Rad52. Finally, a portion of the hygromycin gene encompassing the qPCR amplification region was PCR amplified from p3BP, digested with *Xba*I, and ligated into the *Xba*I site of pTA-3attR-Rad52, generating the standard vector p3attR-Rad52-Hyg. This vector was linearized with *Xcm*I, purified, and serially diluted for use in qPCR to generate a standard curve.

Cell lines
Plasmid p3BP was digested with *Xmn*I to generate a linear molecule. Five μg of linearized p3BP plasmid was electroporated into 293 cells using a Bio-Rad Gene Pulser according to the manufacturer's recommendations. The cells were then allowed to recover in nonselective Dulbecco's modified eagle medium, supplemented with 9% FBS and 1% penicillin-streptomycin. After 24 h, the cells were placed under selection using medium containing 200 μg/ml hygromycin B (Calbiochem, La Jolla, CA). After 14–21 d under selection, single, well-isolated colonies were picked and expanded. All cell lines were screened for the presence of intact triple-*attP* and triple-*attB* sites by PCR and by Southern analysis. Six 293-3BP cell lines were selected for further analysis.

Chromosomal deletion assay
293-3BP cell lines were transfected with 5 μg of pInt-I-hrGFP plasmid using Fugene 6 (Roche Applied Scientific, Indianapolis, IN), according to manufacturer's protocol. At 72 h post-transfection, GFP-positive cells were sorted and collected using a MoFLoPs FACS machine, thus sorting for cells that received integrase expression plasmid. Approximately 15 d after sorting and expansion, cells were harvested and genomic DNA was prepared using the Blood and Cell Culture DNA Maxi Kit (Qiagen, Valencia, CA).

Quantitative PCR analysis
Quantitative PCR on genomic DNA was performed using the following primer/probe combinations: TP901 attR: fwdTPex-qPCR 5'-TGATGTTACTGCTGATAATGTAGATATCATAT-3', revTPex-qPCR 5'-ATTAAAATTCACGGAAGAAAGCTTT-3', TPEx-attR-probe 5'-CGAGTTTTTATTTCGTTTATTTCAATCAAGGTAAATGC-3'; Φ C31 attR: fwdPhiEx-qPCR 5'-GGCTTCACGTTTTCCCAGGT-3', revPhiEx-qPCR 5'-CCAGATGGGTGAGGTGGAGT-3', PhiEx-attR-probe 5'-CTGGGGTAACCTTTGGGCTCCCCG-3'; R4 attR: fwdR4ex-qPCR 5'-TCTCATGCATAGAAGGCCCG-3', revR4ex-qPCR 5'-GGCTACACGGAGCAGGACC-3', R4ex-attR-probe 5'-CGATACCACTTGAAGCAGTGGTAGAAGGGCAC-3'. The hygromycin resistance gene was also amplified as an internal control for integrated p3BP vector, using the following primer/probe combination: fwdHyg-qPCR 5'-CTCGGAGGGCGAAGAATCTC-3', revHyg-qPCR 5'-GCAGCTATTTACCCGCAGGA-3', Hyg-probe 5'-TCAGCTTCGATGTAGGAGGGCGTGG-3'. Each of these probes was 5' labeled with the fluorophore 6-FAM and 3' labeled with the quencher TAMRA.

In addition to the primers and probes described above, PCR reagents used were from the TaqMan PCR Core Reagent Kit (Applied Biosystems, Foster City, CA). Amplification was performed in an ABI 7700 machine and the results were analyzed using SDS v1.7 software (Applied Biosystems). Each sample and standard was analyzed in triplicate.

Southern analysis
Genomic DNA was digested overnight with *Hin*dIII. Twenty μg of digested genomic DNA was loaded onto a

0.6% TBE agarose gel and separated. The gel was depurinated in 0.25 M HCl, denatured in 0.5 M NaOH, neutralized in 0.5 M Tris-HCl (pH7.0), and transferred to an S&S Nytran blotting membrane (Schleicher & Shuell, Keene, NH) in 20X SSC transfer buffer. The membrane was probed with a 1.6 kb fragment from the hygromycin resistance gene or with a 1.6 kb genomic fragment from 8p22 encompassing ϕC31 human pseudo *attP* site A (hpsA) [2], both labeled with ^{32}P by random primer extension, using the Ready-To-Go DNA Labeling Kit (-dCTP) (Amersham Pharmacia Biotech). Hybridization occurred at 65°C for ~20 h in Church buffer (1% BSA, 1 mM EDTA, 0.5 M NaHPO$_4$ pH 7.2, 7% SDS). Membranes were washed in 2x - 0.2x SSC at 65°C. Membranes were exposed to a phosphor screen and analyzed using a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Southern blot quantification was performed using Kodak 1D software.

Acknowledgments

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