IV (1 mg/ml; Invitrogen, Carlsbad, CA) for 7 min, washed with medium, and resuspended in 0.5 ml culture medium ($1.5–3.0 \times 10^7$ cells). Just before electroporation, 0.3 ml PBS (Invitrogen) containing 40 μg linearized targeting vector DNA was added. Cells were then exposed to a single 320 V, 200 μF pulse at room temperature using the BioRad Gene Pulser II (0.4 cm gap cuvette; BioRad, Hercules, CA). Cells were incubated for 10 min at room temperature and were plated at high density on one 10 cm culture dish coated with Matrigel. G418 selection (50 μg/ml, Invitrogen) was started 48 h after electroporation. After one week, G418 concentration was doubled and 6-TG selection (1 mM; Sigma, St. Louis, MO) was started. After three weeks, surviving colonies were analyzed individually by PCR using primers specific for the *neo* cassette and for the *HRPT1* gene just upstream of the 5′ homologous region, respectively. PCR-positive clones were rescreened by Southern blot analysis using *Pst*I-digested DNA and a probe on the 3′ side of the *neo* cassette.

***POU5F1* knock-in.** The gene-targeting vector was constructed by insertion of an IRES-*EGFP*, an IRES-*neo*, and an SV40 polyadenylation sequence (approximately 3.2 kb) into the 3′ untranslated region of the fifth exon of the human *POU5F1* gene. This cassette is flanked in the 5′ direction by a 6.3 kb homologous arm and in the 3′ direction by a 1.6 kb (6.5 kb in an alternative targeting vector) homologous arm. Isogenic homologous DNA was obtained by long-distance genomic PCR and subcloned. H1.1 human ES cells were cultured as described[5]. When an alternative targeting vector with a longer (6.5 kb) 3′ homologous arm was used, the rate of homologous recombination increased to almost 40% (22 homologous clones out of 56 stable clones).

Flow cytometry. Before flow cytometry, ES cell differentiation was induced by incubating the cells for 5 d in unconditioned medium on Matrigel. ES cells were treated with trypsin-EDTA and washed with PBS (both from Invitrogen). Dead cells were excluded from analysis by forward- and side-scatter gating. Samples were analyzed using a FACScan (Becton Dickinson) flow cytometer and Cellquest software (Becton Dickinson). A minimum of 50,000 events was acquired for each sample.

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Site-specific cassette exchange and germline transmission with mouse ES cells expressing ϕC31 integrase

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Currently two site-specific recombinases are available for engineering the mouse genome: Cre from P1 phage[1,2] and Flp from yeast[3,4]. Both enzymes catalyze recombination between two 34–base pair recognition sites, *lox* and *FRT*, respectively, resulting in excision, inversion, or translocation of DNA sequences depending upon the location and the orientation of the recognition sites[5,6]. Furthermore, strategies have been designed to achieve site-specific insertion or cassette exchange[7–10]. The problem with both recombinase systems is that when they insert a circular DNA into the genome (*trans* event), two *cis*-positioned recognition sites are created, which are immediate substrates for excision. To stabilize the *trans* event, functional mutant recognition sites had to be identified[8–12]. None of the systems, however, allowed efficient selection-free identification of insertion or cassette exchange. Recently, an integrase from *Streptomyces* phage ϕC31 has been shown to function in *Schizosaccharomyces pombe*[13] and mammalian[14,15] cells. This enzyme recombines between two heterotypic sites: *attB* and *attP*. The product sites of the recombination event (*attL* and *attR*) are not substrates for the integrase[16]. Therefore, the ϕC31 integrase is ideal to facilitate site-specific insertions into the mammalian genome.

Here we demonstrate that the ϕC31 integrase system is compatible with embryonic stem (ES) cell–mediated genomic alterations in the mouse and is particularly useful to achieve site-specific transgene insertions or

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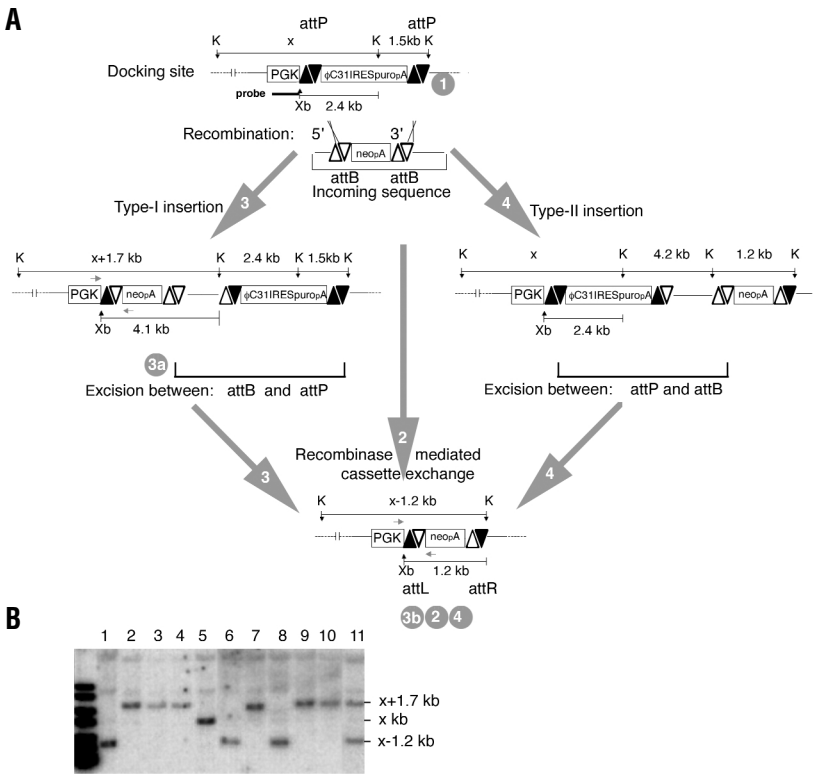

Figure 1. ϕC31 integrase–mediated insertion and cassette exchange strategy. (A) Structure of the docking site placed into the genome of ES cells and the incoming construct. The numbered gray arrows show the different scenarios for ϕC31 integrase–mediated insertions and the corresponding sequence of events. Critical restriction sites and the probe used for diagnostic Southern blots are also indicated. The numbered gray circles indicate the possible endpoints of docking-site structures after selecting for *neo* resistance subsequent to the electroporation of the incoming sequence. The different scenarios and resulting possible endpoints are detailed in the text. Paired triangles represent the *attP* and *attB* sites, in black and white, respectively. Mixed black and white paired triangles indicate chimeric *att* sites (*attL* and *attR*) resulting from ϕC31-mediated recombinations. (B) Diagnostic Southern blot to detect the different endpoints presented in (A) using the 212A ES cell line with a P-docking site and *Pgk-1* promoter probe. Lanes 2–4, 7, 9, and 10 show ES cell lines with type I insertion (gray circle 3a); lanes 1, 6, and 8 show complete cassette exchange (gray circle 2), or either type I or II insertion followed by an excision between *attB* and *attP* or *attP* and *attB* sites (gray circles 3b and 4); and lane 11 shows mosaic type I insertion and cassette exchange (gray circles 3a and 3b). Lane 5 shows ES cell lines with an intact docking site, a possible promoter trap event.

cassette exchanges. First we placed a sequence flanked by either *attP* or *attB* sites into the genome of ES cells (P- or B-docking site, respectively). Subsequently, a circular or linearized plasmid containing a sequence flanked either by *attP* or *attB* sites (P- or B-incoming sequence, respectively) was introduced in these ES cells. We placed the mouse phosphoglycerate kinase-1 (*Pgk-1*) promoter–driven *ϕC31* integrase gene into the docking site (Fig. 1A), which was followed by an internal ribosome entry site (IRES) sequence and a puromycin acyltransferase (*puro*) gene with a polyadenylation signal. This entire sequence, excluding the promoter, was flanked by either a 52– or 51–base pair functional[15] core (Fig. 2) of *attP* (P-docking site, Fig. 1A) or *attB* (B-docking site) sites, respectively. After electroporation of the plasmids containing B- and P-docking sites into R1 ES cells[17], the clones were screened for single-copy integration of intact inserts by Southern analysis (data not shown). The B- and P-incoming constructs contained an *attB*- or an *attP*-flanked promoterless neomycin phosphotransferase (*neo*) gene with a polyadenylation signal, respectively (Fig. 1A).

We evaluated all the paired combinations of ES cells containing B- or P-docking sites and B- or P-incoming constructs (electroporated in either linear or circular forms) for integration into the docking sites and for the structure of the integrants. A substantial increase in the number of *neo*-resistant colonies was consistently observed when circular B-incoming constructs were introduced into P-docking site–containing ES cells in comparison with the other combinations (Table 1).

Table 1. Number of G418-resistant colonies per electroporation of the incoming constructs into cell lines containing docking sites[a]

		Incoming sequence			
Cell line	**Docking site**	**B linear**	**B circular**	**P linear**	**P circular**
R1	No	2	10	36	7
19B	B	24	43	43	6
19C	B	8	12	50	7
110A	P	33	>200	>300	9
110H	P	27	>200	67	3
212A	P	21	140	11	7

[a]The shaded areas show the results where there was a heterotypic relation between the docking site and incoming sequence.

We hypothesized four different scenarios for the generation of *neo*-resistant colonies: (1) random integration accompanied by a promoter trap event; (2) cassette exchange, when recombinations occur simultaneously between the two pairs of heterotypic recognition sites; (3) recombination at the 5′ recognition site (type I insertion), which may or may not be followed by an integrase-mediated deletion event between the intact *attB* and *attP* sites; and (4) recombination at the 3′ recognition site (type II insertion) followed by a deletion event between the intact *attP* and *attB* sites (Fig. 1A).

Site-specific integration, which occurs in scenarios 2–4, alters the docking-site structure (Fig. 1A, B). Southern analysis of the *neo*-resistant colonies revealed that only the P-docking site with a B-incoming sequence resulted in stable site-specific integrations. Stable recombination into the P-docking site occurred in 77% ($n = 22$) and 89% ($n = 173$) of the ES cell clones with the linear or circular incoming plasmids, respectively.

Like scenario 2 (Fig. 1A), scenario 4 (type II insertion) results in cassette exchange. The same is true for scenario 3 only if the insertion is followed by an excision between *cis*-generated *attB* and *attP* sites. Therefore, the identification of cassette exchange end products does not discriminate between these three different scenarios. However, a type I insertion (scenario 3) could result in G418-resistant colonies without subsequent deletion of the sequence between *attB* and *attP* sites. In this case, the inserted *neo* employs the *Pgk-1* promoter, and the ϕC31 integrase is no longer being expressed. It is also possible that the decreasing amount of ϕC31 integrase creates mosaicism in the type I insertion clone, which can be stabilized after the integrase levels become depleted (Fig. 1B).

The proportions of the cassette exchange events, type I insertions, mosaicisms, and promoter trap events were similar among the *neo*-resistant colonies in independent experiments with linear or circular B-incoming sequences in the three independent ES cell lines with P-docking sites. Complete cassette exchange occurred in 33% of the colonies and type I insertions in 53%, whereas 8.5% were mosaic for these two events ($n = 188$). The frequency of random promoter trap insertions was only 11% ($n = 188$).

Combinations other than a P-docking site with a B-incoming sequence resulted in random promoter trap events only, with frequencies not substantially different from controls (R1 ES cell; Table 1). There was only one situation with a substantially higher number of *neo*-resistant colonies: when P-docking site ES cells received linearized P-incoming sequences. Because no docking site–specific integration was detected among the 36 clones examined by Southern blot and the high number of *neo*-resistant colonies was not reproducible in the other two experiments, no additional studies were done on this combination. Interestingly, we did not observe site-specific integration when a B-docking site was used with a P-incoming sequence. This intriguing lack of symmetry is consistent with the finding of Thyagarajan *et al*[14]. Using mammalian cell lines, they found considerably less efficient site-specific integration when an *attB* site was integrated into the genome. This phenomenon may indicate that the *attB* site in the genomic (chromatin) environment is less permissive for ϕC31 integrase–mediated integration than the *attP* site. Additional experiments are required to define the nature of this phenomenon.

The high efficiency of site-specific integration found in our experiments is, however, relative, because our selection system is one of the most stringent ones designed. The promoterless *neo*-containing incoming vector insertion detects only promoter trap events representing <1% of the random integrations[18]. In this context, the expectation of an absolute efficiency, which perhaps could reflect the efficiency in a completely selection-free condition, is not very high. To obtain experimental confirmation, we conducted an experiment in which no direct selection was used. We co-introduced a selectable marker–free incoming vector and a *Pgk-1* promoter–driven *neo* expression vector (30 μg and 5 μg, respectively, of DNA per electroporation) into our P-docking site–containing ES cell lines. A total of 275 *neo*-resistant colonies were picked and analyzed by Southern blotting (data not shown). None of the clones showed alteration of the docking site, indicating that the absolute efficiency of ϕC31 integrase–mediated integration was low if we only selected for cells that had received DNA and subsequently integrated the independent selectable marker vector. Therefore, as with the other recombinase systems, ϕC31 integrase–mediated cassette exchange requires selection to identify the desired integration event. If positive selection is used, the marker remains after integration, a situation that might not be ideal for some applications. If cassette exchange free of a selection system is needed, a negative selectable marker should be used, as is necessary in strategies using Cre or Flp recombinases[8,12]. One such solution is to place an *HSV-tk* or *hygromycin-tk*[8,12] fusion gene into the *attP*-flanked docking site.

To demonstrate the accuracy of ϕC31 integrase–mediated recombination, we carried out PCR amplification of the presumptive hybrid *attR* and *attL* sites with junction-specific primers. Subsequent sequencing proved that an *attL* hybrid site had been created with a breakpoint of invariable accuracy within the TTG core sequence of the *att* sites, as was shown by others[13,15,16] (Fig. 2).

Before recommending the use of ϕC31 integrase in ES cell–mediated genomic alterations in the mouse, it is necessary to determine whether its expression is compatible with germline competence. To address this question we used two of our cell lines expressing ϕC31 integrase (110H and 212A) to generate aggregation chimeras. We obtained germline transmission from both lines and could conclude

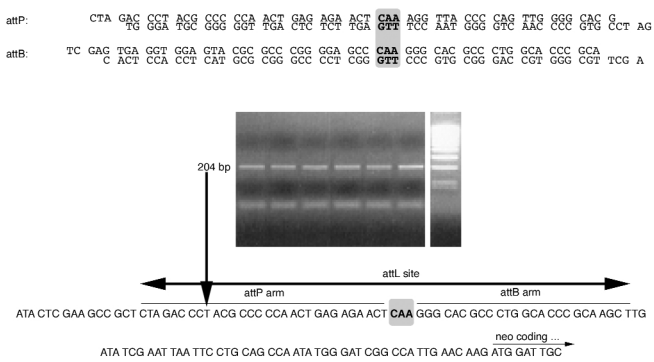

Figure 2. Sequence of the *attP* and *attB* sites used. The PCR product specific to ϕC31-mediated cassette exchange or type I insertion gave the expected 204 bp product from all the candidate cell lines diagnosed by the Southern blot. The sequence of this PCR product contained the predicted chimeric *att* site, comprising a P arm and a B arm around the core TTG (or inverted complement: CAA) triplet where the ϕC31-mediated recombination occurs (gray box).

that ES cell expression of the ϕC31 integrase is compatible with normal mouse development and adult functions including fertility.

To determine whether fertilized zygotes and ES cells offer different conditions for selection-free cassette exchange, we injected different B-incoming sequences into the pronucleus of zygotes from parents heterozygous for the P-docking site transgene. PCR testing revealed that none of the 119 embryos dissected at 9.5 d.p.c. were even mosaic for ϕC31 integrase–mediated integration into the docking site. This finding was not surprising in light of the low absolute efficiency of specific integration observed in ES cells.

The purpose of the experiments presented here was to provide proof-of-principle that the ϕC31 integrase can be used in ES cell–mediated strategies for tailoring the mouse genome, and that its expression does not interfere with germline compatibility. The proof-of-principle was obtained when *attP* sites were placed into the docking site and the incoming sequence contained *attB* sites. Our results demonstrate that, in addition to the Cre and Flp recombinases, the ϕC31 integrase can be applied to manipulate the mouse genome. Of the three enzymes, ϕC31 integrase is the best choice for site-specific transgene insertion or cassette exchange. As this new integrase becomes available for ES cell–mediated genome manipulations, the Cre and Flp systems can be deployed towards other goals, such as tissue- and cell type–specific alterations, allowing for more complex strategies than have been achieved before.

Experimental protocol

Constructs. The 52 bp and 51 bp central region of the full-length *attB* and *attP* sites[15], respectively, were generated by PCR (Fig. 2). To obtain *pBSattB_attB* or *pBSattP_attP* plasmids, two *attB* or *attP* sequences in the same orientation were inserted between the *Hin*dIII and *Xho*I as well as the *Xba*I and *Bam*HI sites, or between the *Xho*I and *Hin*dIII as well as the *Bam*HI and *Xba*I sites of pBluescript II KS(–) (Stratagene, La Jolla, CA). Then the coding sequence of ϕC31 integrase was excised from pFY6 (ref. 13) by *Nhe*I and *Bam*HI and ligated into pBluescript II KS(–) at the *Xba*I and *Bam*HI sites to create *pBSϕC31*. An *Eco*RI-*Bam*HI fragment containing an IRES joined to the puromycin acyltransferase gene and the bovine growth hormone polyadenylation signal was excised from *pCAAGSFlpe*IRES*puro* (a gift from Francis Stewart[19]) and blunt-ligated into the *Bam*HI site of *pBSϕC31* to create *pBSϕC31IP*. A *Not*I-*Eco*RV fragment containing the*ϕC31*-IRES-*puro-bpA* segment was excised from *pBSϕC31IP*, filled in, and subcloned into *pBSattB_attB* and *pBSattP_attP* at a blunted *Eco*RI site. The resulting plasmids were opened at its *Not*I site and blunt-ligated with the *Eco*RI-*Xho*I fragment of a *pPGKpuro* plasmid[20] containing the promoter of the *Pgk-1* gene. These final products were named *PGK-ϕC31IP* and *PGK-PP-ϕC31IP*. To generate the incoming constructs, an insert containing the *neo* coding sequence

and the bovine growth hormone polyadenylation signal was blunt-end ligated into *pBSattB_attB* and *pBSattP_attP* at the unique *Eco*RI site to make *pBBneo* and *pPPneo*, respectively.

ES cell culture and genetic alterations and generating transgenic mice. We used standard procedures[21] for all the ES cell and embryo work. All experiments including mice were done in accordance with the regulations of the local animal committee. Plasmid DNA (10 μg; *PGK-BB-ϕC31IP* or *PGK-PP-ϕC31IP*) was linearized with *Sca*I and electroporated to generate docking site–containing cells after puromycin selection (1.1 μg/ml). To introduce the incoming constructs, we used wild-type R1 ES cells or cell lines containing single-copy integrants of the docking site for electroporation. In all experiments, 20 μg of plasmid DNA (*pBBneo* or *pPPneo*) was electroporated into 10^7 cells either in uncut form or after linearization with *Sca*I. Cells were plated onto two plates, 100 mm in diameter. G418 selection (170 μg/ml) was started 24 h after electroporation. To characterize the ES cell lines, we carried out Southern blot analysis out on *Kpn*I-digested genomic DNA by following standard procedures[22].

PCR. The 25 μl reaction mixture contained 100 ng genomic DNA, 10 pmol of each primer (PGK-BG, 5′-CTTTCGACCTGCATCCATCT-3′ and NEO-BG1, 5′-TGTCTGTTGTGCCCAGTCAT-3′), 0.2 mM of each dNTP, 10 mM Tris, pH 8.8, 50 mM KCl, 0.08% Nonidet P-40, 1 mM $MgCl_2$, and 0.1 U *Taq* polymerase (Fermentas, Hanover, MD). The PCR program consisted of 4 min denaturation at 94 °C, then 35 cycles of 1 min at 94 °C, 45 s at 60 °C, 1 min at 72 °C, and finally 5 min at 72 °C. PCR products were analyzed on a 1.5% agarose gel, purified by the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA), and sequenced on an ABI Prism 377 DNA Sequencer (PerkinElmer, Boston, MA) using PGK-BG and NEO-BG1 primers.

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Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing

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RNA interference (RNAi) is a powerful method for specifically silencing gene expression in diverse cell types[1–3]. RNAi is mediated by ~21-nucleotide small interfering RNAs (siRNAs)[4–8], which are produced from larger double-stranded RNAs (dsRNAs) *in vivo* through the action of Dicer, an RNase III–family enzyme[9–11]. Transfecting cells with siRNAs rather than larger dsRNAs avoids the nonspecific gene silencing of the interferon response[12], underscoring the importance of developing efficient methods for producing reliable siRNAs. Here we show that pools of 20- to 21-base pair (bp) siRNAs can be produced enzymatically *in vitro* using active recombinant Dicer. Yields of ≤ 70% are obtained, and the siRNAs can be easily separated from any residual large dsRNA by a series of spin columns or gel purification. Dicer-generated siRNAs (d-siRNAs) are effective in silencing transiently transfected reporter genes and endogenous genes, making *in vitro* dicing a useful, practical alternative for the production of siRNAs.

Currently, siRNAs are produced by chemical synthesis[6,7], by transcription *in vitro* from short DNA templates[13], or by transcription *in vivo* from transfected DNA constructs[14–17]. All of these approaches are suitable for gene silencing experiments, but they all also have limitations. Synthetic siRNAs are expensive, and several may need to be tried before a particular gene is successfully silenced. DNA constructs for production of siRNAs *in vitro* or *in vivo* are less expensive, but still multiple sequences may need to be selected and evaluated. None of the methods are easily scaled up for screens, because for each member of a library, one or more oligonucleotides must be individually designed and synthesized.

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