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Site-Specific Transformation of Drosophila via ϕC31 Integrase-Mediated Cassette Exchange

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ABSTRACT

Position effects can complicate transgene analyses. This is especially true when comparing transgenes that have inserted randomly into different genomic positions and are therefore subject to varying position effects. Here, we introduce a method for the precise targeting of transgenic constructs to predetermined genomic sites in Drosophila using the ϕC31 integrase system in conjunction with recombinase-mediated cassette exchange (RMCE). We demonstrate the feasibility of this system using two donor cassettes, one carrying the *yellow* gene and the other carrying *GFP*. At all four genomic sites tested, we observed exchange of donor cassettes with an integrated target cassette carrying the *mini-white* gene. Furthermore, because RMCE-mediated integration of the donor cassette is necessarily accompanied by loss of the target cassette, we were able to identify integrants simply by the loss of *mini-white* eye color. Importantly, this feature of the technology will permit integration of unmarked constructs into Drosophila, even those lacking functional genes. Thus, ϕC31 integrase-mediated RMCE should greatly facilitate transgene analysis as well as permit new experimental designs.

BIOLOGICAL research is greatly facilitated by our ability to manipulate DNA sequences *in vivo*. In the model organism *Drosophila melanogaster*, exogenous sequences are routinely incorporated into the genome using the *P*-element transposon system developed by Rubin and Spradling (Rubin and Spradling 1982; Spradling and Rubin 1982). The harnessing of this technology afforded a new ability to alter the genome, allowing the construction of transgenic animals by the relatively simple method of embryonic injection. Furthermore, *P* elements have been indispensable as a platform for developing new technologies for Drosophila research including the GAL4/UAS system (Brand and Perrimon 1993), targeted deletions (Ryder *et al.* 2004), and high-resolution mapping of mutations (Zhai *et al.* 2003).

Recently, strategies have been developed to repeatedly incorporate transgenes into a single position in the genome. This provides a significant advantage over conventional *P*-element transformation, which occurs in an untargeted fashion and therefore subjects transgenes to varying position effects. In general these new strategies make use of site-specific recombinases, which catalyze crossovers between defined target sequences (Branda and Dymecki 2004). The most popular of these enzymes are FLP and Cre, which are widely used for many applications in Drosophila, including clonal analysis, targeted deletions, and tissue-specific excision (Golic and Lindquist 1989; Golic 1991; Siegal and Hartl 1996, 2000; Heidmann and Lehner 2001; Ryder *et al.* 2004). A simple form of transgene targeting using these enzymes involves *P* elements carrying two transgenes, one flanked by loxP target sites for the Cre recombinase and the other flanked by FRT target sites for the FLP recombinase. Once such a *P* element integrates, subsequent treatment with Cre or FLP excises one or the other transgene, effectively co-placing two different sequences at the same site (Siegal and Hartl 1996).

Other strategies have been developed to use recombinase recognition sequences as docking sites for the integration of exogenous sequences. An early application of this approach used the FLP recombinase to mobilize an FRT-bearing transgene and target it to a second FRT in the genome, but the efficiency of this method was limited by the reversible nature of FLP-mediated recombination (Golic *et al.* 1997). Another strategy was recently developed using the integrase from the phage ϕC31 (Thorpe and Smith 1998; Groth *et al.* 2004), which catalyzes recombination between two nonidentical recognition sites, attP and attB. Recombination in this system occurs at a core TTG common to both sites and produces two new sequences, attL and attR (Kuhstoss and Rao 1991; Rausch and Lehmann 1991). As these new sequences are not recognized by the integrase, ϕC31-mediated recombination generates stable integrants that cannot be excised or further exchanged (Thorpe *et al.* 2000). In this way,

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ϕC31-mediated recombination is directional and differs from that catalyzed by Cre or FLP, although recent advances have conferred directionality upon Cre and FLP through the development of heterotypic recognition sequences (Baer and Bode 2001). ϕC31 technology has proven to be highly efficient in Drosophila when targeting a plasmid carrying attB to a genomic attP (Groth *et al.* 2004), affording a powerful new means for transformation.

Recently, an integration strategy called recombinase-mediated cassette exchange (RMCE) has been developed in which the donor and target sequences are each flanked by recognition sites for site-specific recombinases. In this system, crossover events occurring on both sides of the donor and target sequences result in a clean exchange of the target sequence cassette with the donor cassette (Figure 1) (Schlake and Bode 1994; Bethke and Sauer 1997; Bouhassira *et al.* 1997; Seibler and Bode 1997; Seibler *et al.* 1998; Feng *et al.* 1999; Baer and Bode 2001). To ensure the stability of the integrant, cassettes can be flanked by either heterotypic or inverted recognition sequences, with both strategies allowing exchange between the donor and target but preventing excision of either cassette. Importantly, both of these variations on cassette design ensure the integration of the donor cassette itself, rather than its plasmid backbone. In addition, the use of inverted recognition sequences to flank the cassette can generate integrants in either of two orientations, which can subsequently be distinguished by PCR (Feng *et al.* 1999). RMCE was first developed for use in cell culture, but has since been demonstrated at the organismal level for several species, including fission yeast and mice (Thomason *et al.* 2001; Shmerling *et al.* 2005). Indeed, recent reports have demonstrated successful RMCE in Drosophila using both the loxP/Cre and FRT/FLP recombinase systems (Horn and Handler 2005; Oberstein *et al.* 2005).

Because RMCE involves the exchange of sequences rather than the simple insertion of a transgene, it affords two distinct advantages. First, RMCE integrates only the sequences within the cassette, in contrast to strategies that use a single recombinase recognition site and therefore integrate the entire donor plasmid, including antibiotic resistance genes and bacterial sequences. Second, RMCE has the potential to integrate sequences that do not on their own produce a phenotype, as a successful exchange can be detected simply by the loss of a marker carried by the target cassette (Seibler *et al.* 1998; Feng *et al.* 1999). Both of these advantages reduce the amount of extraneous sequence required for integration, simplifying cloning steps and precluding any requirement for nearby transcriptional units that may influence gene expression (Eszterhas *et al.* 2002). Thus, RMCE affords an efficient strategy for site-specific integration with minimal constraints on the sequence to be integrated.

Here we demonstrate a new system for RMCE in Drosophila using the attP and attB target sequences of the integrase ϕC31. Importantly, this system permits researchers to use RMCE in situations where the loxP/Cre or FRT/FLP recombinase systems are required for other manipulations. We observed efficient RMCE events at all four genomic target sites tested, implying that ϕC31-mediated RMCE is generally permissible in the Drosophila genome. Furthermore, we show that a transgene lacking a visible marker can be integrated with high efficiency by selecting only for loss of a phenotypic marker in the genomic target. Finally, we suggest a strategy to combine RMCE with existing methods of homologous recombination to systematically alter a gene at its natural location in the genome.

MATERIALS AND METHODS

Plasmid construction: The pUASTP2 plasmid, which contains the RMCE target cassette, was constructed by flanking the *mini-white* gene of the *P*-element vector pUAST (Brand and Perrimon 1993) with inverted attP sites. Briefly, pTA-attP (Groth *et al.* 2000) was digested with *Eco*RI, and the attP fragment was cloned into pUAST to make pUASTP1. Next, PCR was performed on pTA-attP with the attP1-P and attP2-P primers (see list of primer sequences below), and the resulting fragment was subcloned into pCR2.1-TOPO (Invitrogen). This fragment was then liberated with *Pst*I and ligated into the *Nsi*I site of pUASTP1 to make pUASTP2. Note that the UAS binding sites and *hsp70* promoter of pUAST lie outside of the target cassette.

The pCiB-y^{in} RMCE target plasmid was constructed by flanking an intronless version of the *yellow* gene with inverted attB sites in the vector pCAR4 (Rubin and Spradling 1982). First, PCR was performed on pTA-attB (Groth *et al.* 2000) with the attB1-P and attB2-P primers, and the resulting fragment was cloned into the pCR2.1-TOPO vector. This plasmid was digested with *Eco*RI and the attB fragment was cloned into pCAR4 to make pCAR4B1. Next, the pBS-*yellow*^{in} plasmid, which contains the *yellow* gene including upstream regulatory sequences but lacking the single intron (Geyer and Corces 1987), was digested with *Xba*I, and the fragment containing *yellow* was cloned into pCAR4B1 to make pCAR4B*y*. Finally, the attB fragment from pTA-attB was liberated with *Sal*I and cloned into the *Xho*I site of CAR4B*y* to make pCiB-y^{in}.

The piB-GFP plasmid contains the enhanced *GFP* gene linked to a minimal *hsp70* promoter and an SV40 tail, all of which are flanked by inverted attB sites in the vector pBluescript (pBS; Stratagene). Its construction was similar to that of pCiB-y^{in}; briefly, a pTA-attB *Sal*I fragment (containing attB) was ligated into the *Xho*I site of pBS to make pBS-B. Next, PCR was performed on the RINheXho-GFP plasmid [a gift from Sean Carroll (Wittkopp *et al.* 2002)] using the ry1 and RNXG-1 primers, and the resulting fragment was cloned into the pCR2.1-TOPO vector. This plasmid was digested with *Bam*HI, and the fragment containing *GFP* was cloned into pBS-B to make pBS-BGFP. For the final step, PCR was performed on the pTA-attB plasmid with the longattB3 and longattB4 primers, and the resulting fragment was cloned into the pCR2.1-TOPO vector. This plasmid was digested with *Not*I, and the attB fragment was cloned into pBS-BGFP to make piB-GFP. The fragment containing the *hsp70* promoter, *GFP*, and the SV40 tail can be excised from piB-GFP using *Bam*HI, *Sal*I or *Hin*dIII. Therefore, it can be replaced with a DNA fragment of interest,

which may then be used for RMCE. In addition, a unique *Cla*I site located upstream of the *GFP* promoter allows for use of the construct in enhancer studies.

Fly culture: Flies were cultured at 25° ± 1° on standard Drosophila cornmeal, yeast, sugar, and agar medium with *p*-hydroxybenzoic acid methyl ester as a mold inhibitor (Morris *et al.* 1998).

***P*-element mediated transformation:** *P*-element mediated germ-line transformation was performed to integrate the pUASTP2 target cassette into a y^- w^- background [$Df(1)y^-ac^-w^{1118}$, where y^- and w^{1118} are mutations in *yellow* and *white*, and ac^- is a mutation in the *achaete* gene with no relevance to the experiments described here] as previously described (Spradling and Rubin 1982; Morris *et al.* 1998). Transgenes were mapped to chromosomes by segregation analysis. For fine-scale mapping, transgenes were subjected to inverse PCR as described by E. J. Rehm of the Berkeley Drosophila Genome Project (http://www.fruitfly.org), and the resulting fragments were sequenced and assigned to a cytological band according to Release 4.0 of the Drosophila genome sequence.

ϕC31 integrase RNA preparation: ϕC31 integrase RNA was prepared as previously described (Groth *et al.* 2004). Briefly, the pET11ϕC31-polyA plasmid, in which a T7 promoter drives ϕC31 integrase RNA transcription, was linearized with *Bam*HI, precipitated, and resuspended in RNAse-free water. A total of 1 μg of linear DNA was used to transcribe integrase RNA using the mMESSAGE mMACHINE T7 transcription system (Ambion). RNA was subsequently precipitated using lithium chloride, resuspended in injection buffer (Morris *et al.* 1998), and mixed with donor plasmid. This mix was either used immediately or stored at −80° for up to 3 months prior to injection.

RMCE: RMCE with concurrent negative selection (loss of the *mini-white* phenotype) and positive selection (gain of the *yellow* phenotype) was performed by injecting embryos of a parental line that was homozygous for the RMCE target [in a $Df(1)y^-ac^-w^{1118}$ background] with pCiB-y^{in} (400 ng/μl) and ϕC31 integrase RNA (650 ng/μl). Adults derived from these embryos were mated individually to two to three $Df(1)y^-ac^-w^{1118}$ males or virgin females, and the resulting progeny were screened for w^- and/or y^+ phenotypes. RMCE with negative selection alone was performed by a similar protocol; single adults from embryos injected with piB-GFP (400 ng/μl) and ϕC31 integrase RNA (950 ng/μl) were mated to two to three virgin females of the genotype $Df(1)y^-ac^-w^{1118}$; *Sp Br* L^m/*CyO* or $Df(1)y^-ac^-w^{1118}$; *Sco/CyO* (*Sp, Br,* L^m, and *Sco* are dominant markers of the second chromosome, and *CyO* is a second chromosome balancer). The resulting progeny were screened for a w^- phenotype.

Molecular analysis: PCR was performed on genomic DNA from all lines containing putative RMCE events with primer pairs that flank the junctions at both ends of the integrated cassette: yatt3/lac4 for the 5′ end of the transgene and yatt3/ry1 for the 3′ end (lac4 and ry1 are specific to the 5′ and 3′ *P*-element ends, respectively, while yatt3 is specific to the attB-derived portion of attR; see primer sequences listed below). In addition, the presence of the GFP cassette was verified using a primer pair internal to the transgene, RNXG-6/RNXG-8. For Southern analysis, genomic DNA digested with the indicated restriction enzymes was separated on a 1% agarose gel, blotted to Hybond-N+ membrane (Amersham), and immobilized by UV irradiation. Probes were radiolabeled with [^{32}P]-dCTP using a Rediprime II kit (Amersham) and hybridized to membranes in 5× SSC/5× Denhardt's/1% SDS at 63°. Membranes were then washed sequentially in 2× SSC/0.1% SDS at room temperature for 10 min, 0.2× SSC/0.1% SDS at room temperature for 10 min, 0.1× SSC/0.1% SDS at 37° for 10 min, and 0.1× SSC/0.1% SDS at 63° for 10 min. Membranes were exposed on Kodak X-Omat film at −70°. Recognition sites for restriction enzymes in genomic DNA flanking the target insertions were as predicted by Release 4.0 of the Drosophila Genome Project, with the exception of an *Nde*I polymorphism detected near the parental 25C insertion that was confirmed by PCR and sequencing.

Primers: Primers used in this study are indicated below in the 5′ to 3′ orientation.

attP1-P: CTGCAGTACTGACGGACACACCGAA
attP2-P: CTGCAGTCGCGCTCGCGCGACTGACG
attB1-P: CTGCAGGATGTAGGTCACGGTC
attB2-P: CTGCAGATGCCCGCCGTGACCG
longattB3: CATTCGGCCGTGATGTAGGTCACGGTC
longattB4: CATGCGGCCGCATGCCCGCCGTGACCG
lac4: ACTGTGCGTTAGGTCCTGTTCATTGTT
ry1: CCTTAGCATGTCCGTGGGGTTTGAAT
yatt3: GATGGGTGAGGTGGAGTACG
RNXG-1: CAAACAGCGCTGACTTTGAG
RNXG-6: ATGGCATGGACGAGCTGTA
RNXG-8: GCAGTGCAGCTTTTTCCTTT

RESULTS

RMCE in Drosophila using ϕC31 integrase: Our strategy to adapt RMCE to Drosophila using the ϕC31 integrase system began with the design of a genomic target cassette and an appropriate donor cassette. We chose the ϕC31 integrase system for its reported efficiency and directionality, which we predicted would provide high rates of stable integration. In addition, we chose to flank the target and donor cassettes with inverted recombination sites to ensure integration of the cassette itself rather than the plasmid backbone (Feng *et al.* 1999). Finally, as detailed below, we chose the *mini-white* (w^+) gene to mark our genomic target cassettes and an intronless *yellow* (y^+) gene to mark our donor cassettes. Use of these markers in a $yellow^-$ $white^-$ (y^- w^-) background allows the status of any integration event to be assessed by simply scoring the eye color and cuticle pigmentation of adult flies for *mini-white* and *yellow* gene expression, respectively. Accordingly, all of our crosses were done in a y^- w^- background.

First, an RMCE target cassette was constructed in a *P*-element vector by flanking the *mini-white* gene with inverted attP sites (Figure 1). This target was introduced into a y^- w^- background using standard *P*-element mediated transformation, and flies carrying an integrated target cassette were identified by the eye color produced by *mini-white*. Thus far, we have used targets at four genomic sites; inverse-PCR and sequence analysis placed three on chromosome II at polytene positions 25C1, 42A13, and 52D9 and one on chromosome III at polytene position 82F7. For convenience, we call these target sites 25C, 42A, 52D, and 82F, and the lines of flies carrying these targets the parental target lines.

Next, a donor plasmid was created by flanking an intronless *yellow* gene with inverted attB sites (Figure 1). We predicted that recombination between the two

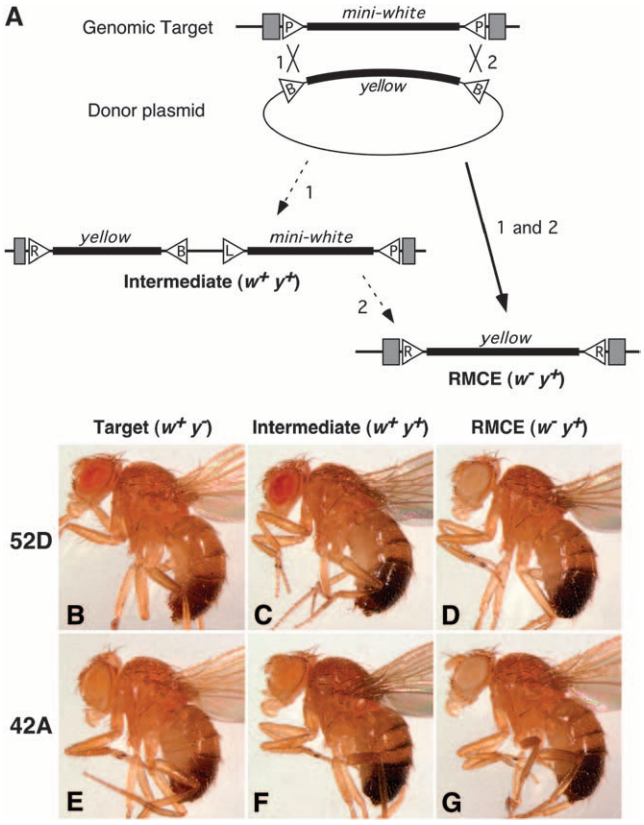

FIGURE 1.—Site-specific integration via ϕC31-mediated RMCE. (A) The target *mini-white* cassette is flanked by inverted attP sites within a genomic *P* element, and the donor *yellow* cassette is flanked by inverted attB sites on a plasmid. Thick solid line, transcription unit; shaded rectangles, *P*-element ends (not to scale); open triangles, att sites (not to scale). In this study, we use a 285-bp fragment from *Streptomyces lividans* for attB and a 221-bp fragment of ϕC31 for attP (GROTH *et al.* 2000). Crossovers at both ends of the two aligned cassettes result in an exchange of *mini-white* for *yellow*. Alternatively, a single crossover at one end of the aligned cassettes creates an intermediate that integrates the entire donor plasmid and thus carries both *yellow* and *mini-white*. The single crossover can also occur at the other attP/attB pair, resulting in a related structure in which *mini-white* is to the left of *yellow* (not shown). Either intermediate may then resolve into an RMCE event by a subsequent crossover between the remaining attP/attB pair. (B and E) Representative flies from parental lines 42A and 52D carrying the target *mini-white* cassette. (C and F) Flies resulting from a single crossover (intermediate event). The pigmentation of the wings, abdominal stripes, and bristles of these flies is darker compared with that of the parental lines (C *vs.* B and F *vs.* E). In addition, note the increased eye pigmentation relative to that of the parental lines. (D and G) Flies representing RMCE events.

attP/attB pairs on either side of a target cassette aligned with a donor cassette would result in a clean exchange of the *mini-white* and *yellow* cassettes, converting the w^+ y^- parental phenotype to w^- y^+ (Figure 1). In addition, we noted that a single crossover event between only one of the attP/attB pairs could integrate the entire donor plasmid, resulting in a w^+ y^+ "intermediate" fly, with the potential to resolve further into an RMCE event (Figure 1).

Embryos from the four parental lines carrying a *mini-white* target cassette were co-injected with the y^+ donor plasmid and RNA encoding the ϕC31 integrase (MATERIALS AND METHODS). All emerging adults were mated singly in vials, and their progeny were scored for putative RMCE events. Combining our data for all four genomic target sites, we obtained a total of 22 vials that contained w^- y^+ flies, as expected for successful RMCE events (Table 1 and Figure 1). Seventeen of the vials contained multiple w^- y^+ individuals (up to 78), which likely resulted from a common RMCE event. The frequencies with which we recovered vials harboring w^- y^+ flies ranged from 3 to 9% of total vials scored, depending on the parental line injected. By singly backcrossing one to three w^- y^+ flies from each of these vials to a balancer stock, we successfully generated 43 lines of flies representing 19 of the original 22 vials for subsequent molecular analysis.

To confirm that our candidates resulted from RMCE, we performed PCR and Southern analyses. Primers were designed for the *P*-element ends of the genomic target and for the attB-specific portion of attR, such that PCR products would be obtained only if ϕC31-mediated recombination had taken place (Figure 2). Consistent with the occurrence of RMCE, we detected PCR products of the expected sizes for both the 5′ and 3′ ends of the exchange cassette in all 43 lines tested. These products were verified for 10 lines by sequence analysis, which confirmed the expected conversion of attP to attR at both ends of the cassette (Figure 2). Finally, we performed Southern blots on 11 lines harboring potential RMCE events at 25C and 42A (Figure 3). Importantly, we observed no structural changes either in the cassette itself or in flanking genomic DNA, indicating that recombination was limited to the attP and attB sequences as expected. Southern blots also showed that the donor cassette can integrate in either orientation, as predicted by the inverted orientation of the attP and attB sites in the target and donor sequences.

In addition to RMCE events, we obtained seven vials with w^+ y^+ flies, as expected for a single crossover event that integrates the entire donor plasmid (Figure 1). Of these vials, three also contained RMCE events (Table 1), suggesting that the product of a single crossover can serve as an intermediate for a final RMCE product. To determine whether w^+ y^+ flies indeed represent a single crossover intermediate, we injected ϕC31 integrase RNA into embryos from one of the w^+ y^+ lines derived from the parental 42A target line. As predicted, we recovered w^- y^+ progeny that were identical in phenotype to flies with confirmed RMCE events at 42A; furthermore, PCR analysis of these w^- y^+ progeny generated the bands expected for an RMCE event (data not shown), verifying that w^+ y^+ inserts can resolve to an RMCE event.

During our analysis of w^+ y^+ intermediates, we noticed that the w^+ phenotype of these flies is enhanced relative to that of the parental target lines (Figure 1; compare B

TABLE 1

RMCE in Drosophila using ϕC31 integrase

Genomic position	Donor cassette	Embryos injected	Larvae hatched	Fertile adults	w^- y^+ (RMCE)[a]	w^+ y^+ (intermediate)[b]	Vials with RMCE (%)
25C	*yellow*	380	193	69	5	0	7.2
42A	*yellow*	520	333	106	3	1	2.8
52D	*yellow*	480	254	90	8	1	8.8
82F	*yellow*	440	204	94	6	5	6.4

Parental lines each contain a single *P*-element insertion carrying the *mini-white* gene. RMCE events were detected by screening for flies with w^- eyes and y^+ pigmented cuticle, indicating loss of *mini-white* and gain of *yellow*, respectively. Single crossover events retained *mini-white* and gained *yellow*. In parental line 42A, we see evidence of variegation in *mini-white* expression.

[a] Number of vials containing flies resulting from RMCE.

[b] Number of vials containing flies resulting from single crossovers. Three of these seven vials also produced flies resulting from RMCE.

vs. C, E *vs.* F). Although subtle, this effect was consistently observed at all three genomic sites where w^+ y^+ flies were generated. As these intermediates carry the entire donor plasmid, the increased expression of *mini-white* is likely attributable to the proximity of *yellow* or other sequences on the plasmid backbone. This observation highlights the complications that can arise from position effects and underscores the advantage of RMCE over insertion schemes that necessarily integrate donor plasmids in their entirety.

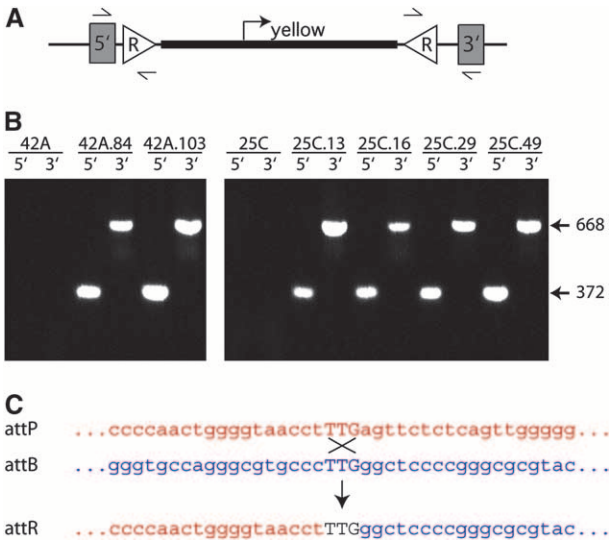

FIGURE 2.—Molecular analysis of RMCE candidates. (A) The structure of a *yellow* transgene resulting from an RMCE event. Each primer pair (arrows) used for PCR analyses included a primer that was unique to one end of the *P* element and a second primer in attR. Symbols are as defined for Figure 1. (B) Representative 5′ and 3′ PCR products from independent lines derived by RMCE from parental lines 25C and 42A. The expected 372-bp and 668-bp products were observed for all 43 lines tested. (C) Recombination between the attP (red sequence) and attB (blue sequence) sites occurs at a shared TTG to produce an attR (shown) and attL (not shown) site. Only sequences immediately flanking the core TTG are shown. PCR products, covering both recombination sites and representing 10 RMCE-derived lines from the 25C and 42A parental lines, were sequenced and found to contain the expected crossover.

In addition to flies resulting from RMCE and from intermediate single crossovers, we obtained one other class of exceptions. These flies were y^- with an eye color significantly darker than that of any parental line. Flies of this type were represented in only 2 of 359 vials scored (data not shown); because these events were of low frequency and easily distinguished from the desired flies carrying RMCE events, they were not analyzed further. Importantly, loss of the *mini-white* gene was always accompanied by its replacement with a *yellow* gene, indicating that all candidate RMCE events were *bona fide*.

RMCE by negative selection: In theory, our protocol should permit the identification of exchange events solely on the basis of loss of the w^+ phenotype, as RMCE-mediated loss of *mini-white* is necessarily accompanied by incorporation of the donor cassette. This feature should allow the recovery of integrated donor cassettes that do not on their own produce a visible phenotype. Based on our experience with RMCE using the *yellow* donor cassette, the potential to select for integrated unmarked cassettes seemed particularly plausible for two reasons. First, all w^- flies resulted from a clean RMCE event, with no evidence for aberrant loss of the target cassette. Second, the rate of RMCE was significantly greater than the rate of single crossovers, and therefore the majority of flies carrying an insertion could in fact have been identified simply by loss of the *mini-white* eye color.

To test whether we could recover RMCE integration events without incorporating a visible marker, we constructed a new donor plasmid with inverted attB sites flanking a *GFP* gene. This plasmid was co-injected with ϕC31 integrase RNA into embryos from the parental 25C and 42A target lines. For these experiments, we increased the concentration of integrase RNA ~1.5-fold in an effort to improve the efficiency of transformation. All emerging adults were mated singly in vials, and putative RMCE events were identified among the progeny solely by loss of the *mini-white* eye color.

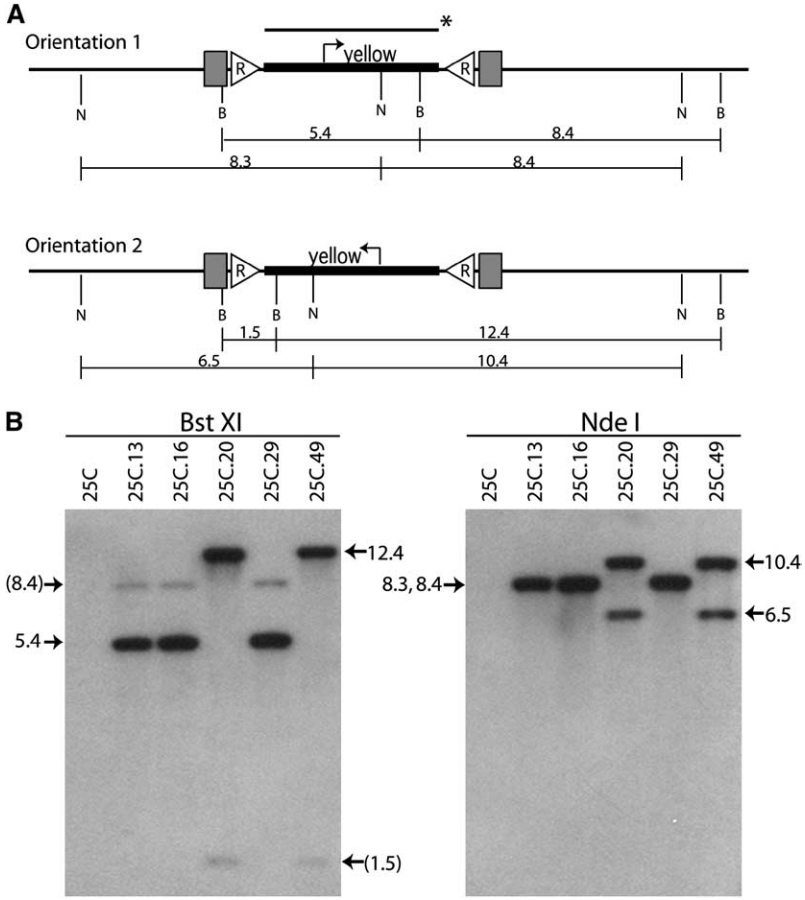

FIGURE 3.—Southern analysis of RMCE-derived insertions of the *yellow* gene. (A) The two possible orientations of *yellow* following RMCE-mediated insertion into the genome. Restriction sites used for two separate genomic DNA digests and the expected fragments resulting from these digests are shown (B, *Bst*XI; N, *Nde*I). * indicates the region (bar on top) homologous to the probe; all other symbols are as defined for Figure 1. (B) Southern analyses of five independent RMCE-derived lines from the 25C parental line. The 8.4- and 1.5-kbp bands from the *Bst*XI digestion are weakly labeled by the probe. Both orientations of *yellow* were detected.

Consistent with the higher levels of integrase, we recovered vials with w^- flies at an increased rate, up to 24% (Table 2). Using these w^- flies, we established 54 lines representing 36 of the vials for molecular analysis.

The 54 lines resulting from putative RMCE events were analyzed by PCR using primer pairs flanking the recombination junctions at the 5′ and 3′ ends of the integrated cassette and an additional primer pair within the *GFP* gene itself. All 54 were shown to carry an exchange of the *mini-white* target cassette for the GFP donor cassette (data not shown). This result was confirmed by Southern analysis on 10 lines, which indicated that the cassette itself and the flanking DNA remained intact (Figure 4). Thus, selection against the phenotype of the genomic target marker is an effective means for detecting integrants of the donor cassette.

DISCUSSION

Site-specific integration in Drosophila advanced significantly with the introduction of ϕC31 technology (GROTH *et al.* 2004). The protocol developed by GROTH *et al.* (2004) allowed high-efficiency targeting of multiple transgenes to one site in the genome for the first time. Our strategy for RMCE advances this technology in two ways: first, the exchange of cassettes allows researchers

TABLE 2

Detecting RMCE using negative selection

Genomic position	Donor cassette	Embryos injected	Larvae hatched	Fertile adults	Vials with RMCE[a]	Vials with RMCE (%)
25C	*GFP*	500	272	174	19	10.9
42A	*GFP*	330	147	92	22	23.9

Putative RMCE events were detected only by the loss of the *mini-white* marker phenotype.

[a] In addition to vials producing flies resulting from RMCE, 10 vials produced flies with increased eye pigmentation, which were not analyzed further.

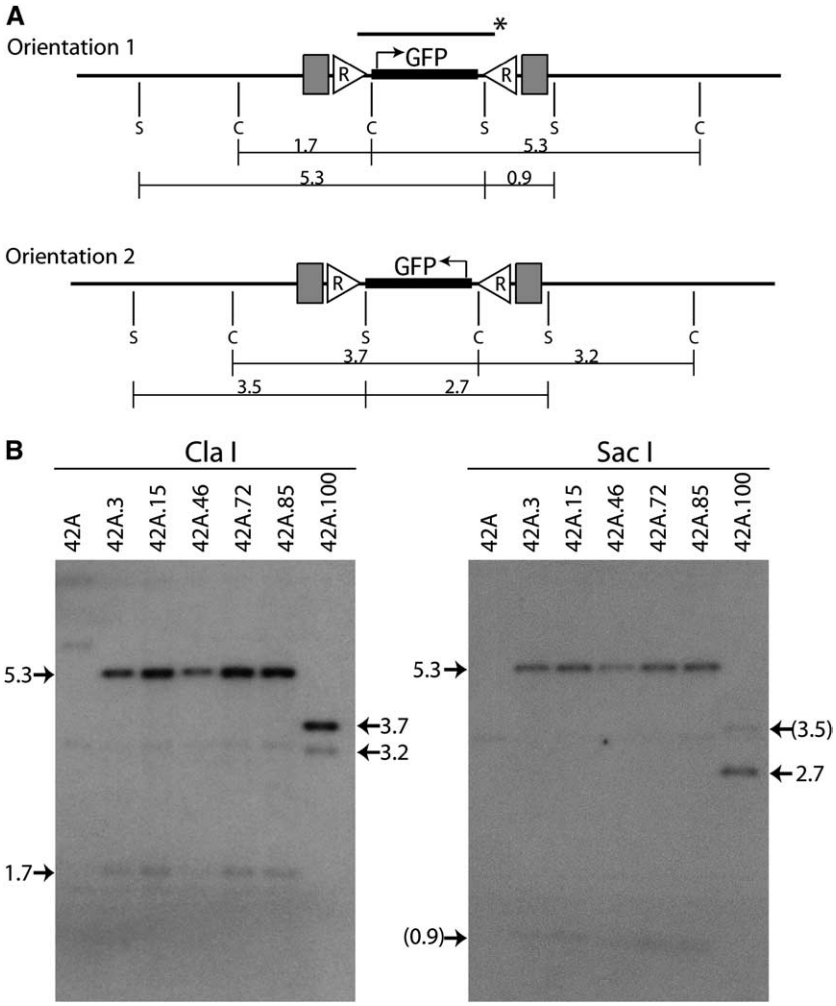

FIGURE 4.—Southern analysis of RMCE-derived insertions of GFP. (A) The two possible orientations of GFP following RMCE-mediated insertion into the genome. Restriction sites used for two separate genomic DNA digests and the expected fragments resulting from these digests are shown (S, *Sac*I; C, *Cla*I). * indicates the region (bar on top) homologous to the probe; all other symbols are as defined for Figure 1. (B) Southern analyses of six unique RMCE-derived lines from the parental 42A target line. The 0.9- and 3.5-kbp bands resulting from the *Sac*I digest are only weakly labeled by the probe. Both orientations of GFP were detected.

to incorporate a sequence of interest without the plasmid backbone. Second, our experiments demonstrate that an integration event can be identified solely by selection against the marker phenotype of the target cassette, negating the need for a visible marker in the incoming cassette. These aspects of the protocol are particularly advantageous for studies of gene regulation, as transcriptional units in close proximity can influence each other's expression (ESZTERHAS *et al.* 2002). Indeed, we observed evidence for this effect in our experiments; expression of *mini-white* is consistently higher in intermediate insertions carrying the entire donor plasmid including the *yellow* gene. Thus, RMCE affords a more precise level of control in the study of gene expression.

In our first set of injections using *yellow* as a donor, we achieved an RMCE efficiency of up to 9%, which is roughly comparable to rates of *P*-element transformation in our laboratory (data not shown). We were able to obtain efficiencies up to 24% in our second set of injections, which we attribute to the use of a higher concentration of integrase, but which may also reflect the smaller size of the 2-kbp *GFP* cassette as compared to the 5-kbp *yellow* cassette. Importantly, the *GFP* donor plasmid shared no significant homology with the genomic target, indicating that the rate of integration was driven solely by the integrase. Nevertheless, our rates are below the reported 55% transformation efficiency previously achieved using single attP and attB sites (GROTH *et al.* 2004). These different efficiencies likely reflect laboratory-to-laboratory variation rather than inherent differences between the two approaches as, in our hands, transformations using single attP and attB sites yielded efficiencies (5–19%; J. BATEMAN and A. LEE, unpublished observations) comparable to those of our RMCE results.

The use of ϕC31 integrase for RMCE has been previously reported for mouse cell culture (BELTEKI *et al.* 2003) and *Schizosaccharomyces pombe* (THOMASON *et al.* 2001). In these cases, the attP and attB sites flanking the cassettes were used in direct rather than inverted orientation, and selectable markers on the donor cassettes were used to enrich for putative integrants. An additional RMCE-like method using ϕC31 attP and attB sites in direct orientation was recently described for cultured silkworm cells (NAKAYAMA *et al.* 2006); although cassettes were not exchanged in this

in vitro system, the method permitted the integration of a fluorescent marker into the genome. RMCE using attP and attB sequences in direct orientation is also possible in Drosophila (A. Lee and J. Bateman, unpublished observations), but this strategy can result in the incorporation of the plasmid backbone rather than the donor cassette when a visible marker is not used. In contrast, RMCE via inverted attP and attB sites can allow only the incorporation of the cassette itself (Feng *et al.* 1999).

Recently, methodologies for RMCE using the FRT/FLP (Horn and Handler 2005) and loxP/Cre (Oberstein *et al.* 2005) recombinase systems were reported for Drosophila. These studies used heterotypic recognition sequences in direct orientation around the cassettes, which favors exchange with the donor vector over excision of the genomic target. While both methodologies produce rates of RMCE comparable to those obtained in this study, ϕC31-mediated RMCE may offer specific advantages. For example, it permits researchers the full benefit of RMCE while reserving the FLP/FRT and loxP/Cre recombinase systems for applications other than transformation. In addition, because the integrase destroys attP and attB during recombination, ϕC31-mediated RMCE can be reapplied in the presence of previous integrants without concern for undesired interactions between different cassettes. Finally, flanking cassettes with identical and inverted recognition sites permits the recovery of integrants in both orientations, providing a convenient control for potential position effects at a given chromosomal location (Feng *et al.* 1999).

We have shown that RMCE using ϕC31 integrase is an effective tool for targeting transgenes to a single genomic position. It is noteworthy that, while our experiments have relied on *P* elements to incorporate target cassettes at random sites in the genome, methods for placing targets at specific chromosomal locations via homologous recombination exist (Gloor *et al.* 1991; Rong and Golic 2000; Gong and Golic 2003). This capacity to place targets at designated positions suggests a highly efficient means for studying genes at their natural genomic location. Specifically, the "ends-out" and "ends-in" gene replacement technologies (Rong and Golic 2000; Gong and Golic 2003) could be used to replace part or all of a gene with a marked target cassette, and then RMCE could be applied to exchange this target with different versions of the gene constructed *in vitro*. This approach would circumvent the inefficiency inherent to the homologous recombination machinery of Drosophila, greatly facilitating all conversions subsequent to the initial insertion of the RMCE target. In addition to permitting genes to be studied at their natural chromosomal location, this approach could also aid the study of particularly large genes, which can be recalcitrant to conventional transgene studies. We are currently determining whether attP and attB sites smaller than the 300-bp sequences used in this study can support RMCE in Drosophila to reduce the amount of heterologous sequence that would be incorporated into the genome via an exchange. Previous studies have shown that attP and attB sequences <40 bp in size can support recombination by ϕC31 integrase (Groth *et al.* 2000; Nakayama *et al.* 2006), making it likely that the att sites used in RMCE can be significantly reduced in the future. Thus, combining the simplicity of RMCE with methodologies for homologous recombination could greatly facilitate the manipulation of genes in their natural location.

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