Construction of Transgenic Drosophila by Using the Site-Specific Integrase From Phage ϕC31

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ABSTRACT

The ϕC31 integrase functions efficiently *in vitro* and in *Escherichia coli*, yeast, and mammalian cells, mediating unidirectional site-specific recombination between its *attB* and *attP* recognition sites. Here we show that this site-specific integration system also functions efficiently in *Drosophila melanogaster* in cultured cells and in embryos. Intramolecular recombination in S2 cells on transfected plasmid DNA carrying the *attB* and *attP* recognition sites occurred at a frequency of 47%. In addition, several endogenous pseudo *attP* sites were identified in the fly genome that were recognized by the integrase and used as substrates for integration in S2 cells. Two lines of Drosophila were created by integrating an *attP* site into the genome with a *P* element. ϕC31 integrase injected into embryos as mRNA functioned to promote integration of an *attB*-containing plasmid into the *attP* site, resulting in up to 55% of fertile adults producing transgenic offspring. A total of 100% of these progeny carried a precise integration event at the genomic *attP* site. These experiments demonstrate the potential for precise genetic engineering of the Drosophila genome with the ϕC31 integrase system and will likely benefit research in Drosophila and other insects.

DROSOPHILA melanogaster is an excellent model organism for genetic studies due to its short generation time, ease of screening, polytene chromosomes, and sequenced genome. A very useful tool for the study of gene function in Drosophila is the *P*-element transposition system (Rubin and Spradling 1982). *P* elements have been utilized to knock out genes as well as to insert genes into the Drosophila genome. However, the insertion of genes occurs randomly. An efficient site-specific integration method would address one drawback of fly research.

While random *P*-element integration is useful for studies of gene function (O'Kane and Gehring 1987; Spradling *et al.* 1999), position effects can strongly influence gene expression, complicating the phenotypic analysis (Levis *et al.* 1985). It is therefore desirable to be able to insert genes at the same chromosomal location. A system involving Cre and FLP that allows researchers to study the function of two genes at identical places in the genome has been developed (Siegal and Hartl 1996, 2000). In that system, a fly line is created by *P*-element insertion that contains the two transgenes of interest flanked by either *loxP* or FRT sequences. Under Cre expression, one transgene is removed, while under FLP expression, the other transgene is removed. Each remaining transgene is then left in the same chromosomal context. Disadvantages of this approach are that only two genes can be directly compared, and for each set of two transgenes analyzed, a new *P*-element insertion must be made, which will be at a different location.

Another approach to the site-specific integration problem is the use of homologous recombination. The frequency of homologous recombination has been too low to be of practical use in Drosophila. However, the frequency of homologous recombination can be boosted by using *P*-element transformation to insert a construct containing the gene to be targeted, engineered with an I-*Sce*I cutting site and flanked by two FRT sites. This construct can then be mobilized as a circular DNA molecule by expression of FLP and made linear by the expression of I-*Sce*I, increasing the targeted recombination frequency (Rong and Golic 2000, 2001; Rong *et al.* 2002). In this system, a separate *P*-element insertion carrying the homologous DNA engineered with I-*Sce*I and FLP sites is required for each gene to be targeted. By this method, a targeted event could be obtained at a frequency of ~1 in 500–30,000 gametes from the female germline. Ideally, one could target an insertion to any position in the genome. However, even this increased frequency of homologous recombination is not high enough to allow researchers to target many different genes in an efficient way.

In lieu of the ability to insert genes into any desired place in the genome, a system that allows a researcher to insert any gene efficiently into one specified place would be useful. Such experiments have been conducted with the FLP/FRT system in Drosophila. An integration frequency of up to 5% into a FRT site in the Drosophila genome can be obtained when the target DNA is mobi-

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lized from elsewhere in the genome by FLP excision (Golic *et al.* 1997). However, this system still requires the creation of a new *P*-element line for each transgene.

The site-specific integrase from phage ϕC31 (Thorpe and Smith 1998) has been shown to function at high frequency in human and mouse tissue culture cells and *in vivo* in mice (Groth *et al.* 2000; Thyagarajan *et al.* 2001; Olivares *et al.* 2002; Ortiz-Urda *et al.* 2002, 2003a,b). The ϕC31 integrase requires no cofactors and mediates recombination between two sequences, the *attB* and *attP* sites, to create stable recombinants (Thorpe and Smith 1998). Both intra- and intermolecular recombination occur at high frequencies, and essentially no reversion of the reaction occurs. It has been demonstrated that the integrase can recognize and integrate into endogenous pseudo *attP* sites in the human and mouse genomes that have partial identity to *attP* (Thyagarajan *et al.* 2001; Olivares *et al.* 2002). Mouse and human pseudo *attP* sites are typically 30–45% identical to the wild-type *attP*. It seemed likely that the ϕC31 integrase system would also function in Drosophila and could benefit research in this organism by providing an efficient, site-specific integration tool. Experiments conducted in this study demonstrate that the ϕC31 integrase can mediate intra- and intermolecular site-specific recombination at high frequency in Drosophila S2 cells and that pseudo *attP* sites exist in the fly genome. In addition, transgenic flies were created in *attP*-containing fly lines at an average frequency of 47% of fertile crosses, by integrating an *attB*-containing plasmid injected along with integrase mRNA into Drosophila embryos.

MATERIALS AND METHODS

Plasmids: A plasmid, pMKInt (Figure 1A), which expresses ϕC31 integrase under the inducible control of the metallothionein promoter, was constructed as follows. The ϕC31 integrase gene was removed from the plasmid pCMVInt (Groth *et al.* 2000) by digestion with *Bam*HI and *Spe*I. This fragment was blunted with T4 DNA polymerase and ligated into the unique *Eco*RV site of the plasmid pMK33 (Bhanot *et al.* 1996).

The donor plasmid pDrBB2 (Figure 1C) was created as follows. The hygromycin gene driven by the copia promoter was removed from pMK33 by *Ssp*I digestion. The hygromycin gene was removed from pBB1 (Thyagarajan *et al.* 2000) by *Pvu*II and *Pfl*MI digestion and replaced with the copia-hygromycin cassette. To create the plasmid pMKInt-hyg (Figure 1D), the hygromycin gene was removed from the plasmid pMKInt by *Bgl*II and *Sna*BI digestion.

The ϕC31 integrase gene was amplified to include the *Nde*I restriction site from the pTA-int plasmid (Groth *et al.* 2000), using the primers Native 5′ *Nde*I (+) (CGA CTA GTC ATA TGG ACA CGT ACG CGG GTG CT) and 3′ *Bam*HI ϕC31 (AGC CGG ATC CGG GTG TCT CGC TA). The pET11 vector (Novagen, Madison, WI) was modified to contain the previously amplified ϕC31 integrase gene directionally inserted into *Nde*I and *Bam*HI sites so that the T7 promoter drives ϕC31 integrase RNA production (pET11ϕC31). The oligonucleotides *Bam*HI-poly(A)-top (GAT CGC GCC AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA CCG) and *Bam*HI-poly(A)-bottom (GAT CCG GTT TTT 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 pET11ϕC31poly(A).

The plasmid pCaryP (Figure 1E) was created by removing *attP* from pTAattP (Groth *et al.* 2000) by *Eco*RI digestion, filling in with T4 DNA polymerase (New England Biolabs, Beverly, MA), and cloning it into the *Sma*I site of pCary, a plasmid containing the *yellow* body color gene (gift of the Bruce Baker lab).

The plasmid pUASTB (Figure 1F) containing the white gene and ϕC31 *attB* was created as follows. The *attB* was removed from pTAattB, blunted with T4 DNA polymerase, and cloned into the *Bam*HI sites of pUAST (Brand and Perrimon 1993).

Transient intramolecular recombination assay: The plasmid pBCPB+ (Figure 1B), containing a *lacZ* gene flanked by wild-type *attB* and *attP* sites, was used to assay recombination (Groth *et al.* 2000). *D. melanogaster* S2 cells were maintained at 25° in Schneider medium supplemented with 9% fetal bovine serum, 1% penicillin/streptomycin. Cells were transfected with FuGENE 6 (Roche Diagnostics, Indianapolis) as follows. The DNA (salmon sperm carrier, pBCPB+ and carrier, or pBCPB+ and pMKInt) was added to an Eppendorf tube. Amounts of DNA were as follows: 50 ng pBCPB+, 2 μg pMKInt, and carrier to a total of 2.05 μg. A total of 100 μl Opti-Mem was added to each tube, followed by 12.3 μl FuGENE 6. After a 15- to 30-min incubation, the mixture was added to a 60-mm dish of S2 cells at ~80% confluency. At 24 hr, 50 units/ml DNaseI was added to destroy untransfected DNA, and 1/100 culture volume of $CuSO_4$ was added to induce integrase expression. At 72 hr, the DNA was harvested for Hirt extractions (Hirt 1967; Smith and Calos 1995). Extracted DNA was then transformed into electrocompetent DH10B *Escherichia coli*, spread on agar plates containing 25 μg/ml chloramphenicol and 50 μg/ml 5-bromo-4-chloro-3-indolyl β-d-galactoside (Xgal), and white and blue colonies were counted. The percentage of recombination was calculated as (number of white colonies)/(total number of colonies) × 100.

Pseudo *att* site identification: The plasmid pDrBB2 (Figure 1C) was cotransfected with pMKInt-hyg (Figure 1D) into S2 cells. Cells were transfected with 20 μg of the integrase plasmid and/or 400 ng of the donor plasmid. All transfections were made up to 20.4 μg with salmon sperm carrier DNA. The integrase was induced at 24 hr by the addition of $CuSO_4$. Cells were selected with 125 μg/ml hygromycin for ~5–6 weeks, at which time the cells were harvested and the genomic DNA was recovered with the QIAGEN (Valencia, CA) blood and cell culture Maxi kit. Genomic DNA was digested with two enzymes, for example, *Eco*RI and *Hin*dIII, which cut pDrBB2 on either side of *attB*. The DNA was then ligated at low concentration and subjected to nested PCR across the junction. The external primers were attLF1 (ATGCCGATATACTATGCC GATG) and attLR1 (GGTCCGGGACGACGTGA). The internal primers were attLF2 (GGATCAATTCGGCTTCAGG) and attLR2 (GCTGTACGCCGAGTGGT). The resulting PCR bands were gel isolated using the QIAGEN QiaQuick kit and Topo cloned using the Topo cloning kit (Invitrogen, Carlsbad, CA). DNA was prepared from resulting colonies with the QIAGEN Miniprep spin kit and sequenced using the T7 or M13 reverse primers. Sequences were examined with the Fly BLAST program (http://www.fruitfly.org/blast/index.html) to identify Drosophila genomic sequence.

Transient excision assay in whole flies: The ϕC31 mRNA was produced as follows. The plasmid pET11ϕC31poly(A) was digested with *Bam*HI to linearize it directly after the poly(A) of the integrase gene. A total of 1 μg of digested DNA was

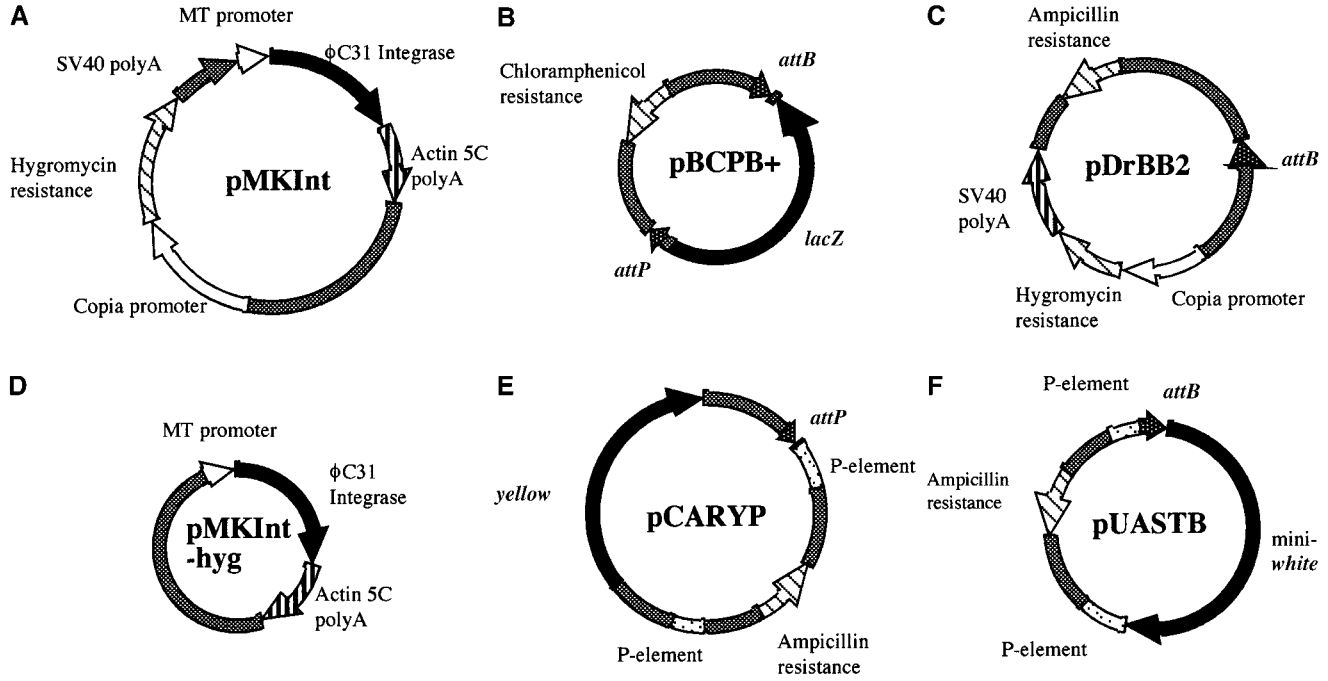

FIGURE 1.—Schematic of six plasmids used in demonstrating the utility of the ϕC31 integrase in Drosophila. (A) pMKInt, a plasmid expressing integrase under the control of the metallothionein promoter. (B) pBCPB+, a plasmid containing *lacZ* flanked by *attB* and *attP*. (C) pDrBB2, a plasmid containing *attB* and the hygromycin resistance gene driven by the copia promoter. (D) pMKInt-hyg, a plasmid derived from pMKInt that does not express the hygromycin resistance gene. (E) pCaryP, a plasmid containing *attP* and the *yellow* gene within a *P*-element cassette. (F) pUASTB, a plasmid containing *attB* and the mini-*white* gene.

used as template for the RNA production protocol of the mMessage mMachine kit (Ambion, Austin, TX). A total of 600 ng/μl of either pMKInt (Figure 1A) or ϕC31 RNA was co-injected into fly embryos with 200 ng/μl of pBCPB+ (Figure 1B). At 48 hr, embryos were harvested, crushed, and incubated with Proteinase K (Invitrogen) at 65°. Resulting DNA was subjected to PCR (AttB F_2, ATGTAGGTCACGGTCTCGAAGC; AttP1+, TGGCGGCCGCTCTAGAACTA), transformed into bacteria, and screened as above.

***AttP* fly lines:** A total of 600 ng/μl of pCaryP (Figure 1E) was co-injected with 125 ng/μl of transposase-expressing plasmid into w^-, y^- embryos according to a standard protocol. Flies that grew to adulthood were crossed to w^-, y^- flies. Eight y^+ fly lines were isolated, of which two could be made homozygous. The insertions were localized by GenomeWalker (BD Biosciences, Palo Alto, CA) to chromosomes 2R (attP1) and 3L (attP2). Primers were designed to determine if a fly line was transgenic attP1 or attP2. AttP1 was identified using DrGSP2 (CGAAATTTATGAGTGACTCTGCGACGTA) and A1-2R for (GCCGCTCAGAGACCGTTTGTGTATGTGC). AttP2 was identified using PCP for (GTCGCCGACATGACACAAG) and A2-3L rev (CTCTTTGCAAGGCATTACATCTG).

Integration into *attP* fly lines: A total of 60–100 pl of between 800 and 1000 ng/μl ϕC31 RNA was co-injected into *attP* fly embryos with 150–200 ng/μl pUASTB DNA. Flies that grew to adulthood were crossed with w^-, y^- flies. Fly DNA from red-eyed offspring was prepared according to the DNEasy protocol (QIAGEN). Integration was analyzed by PCR, by using primers specific for the pUASTB (GCTCCGCTGTCA CCCTG) and pCaryP (GGCTTCACGTTTTCCCAGGT; Figure 1E) plasmids, or by Southern blot.

Southern blots were performed as follows. A total of 10–15 μg of genomic DNA was digested overnight with *Xmn*I, subjected to gel electrophoresis, and transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH). Blots were prepared by a standard protocol and hybridized overnight to an *attP* probe. The probe was prepared by removing the *attP* site from pTA-attP by *Eco*RI digestion and radiolabeling using Ready-To-Go DNA labeling beads (Amersham Biosciences, Piscataway, NJ).

RESULTS

Intramolecular recombination in S2 cells: To test whether the ϕC31 integrase functions in Drosophila cells, a plasmid that expressed the integrase gene under the control of the metallothionein promoter was constructed. This plasmid was cotransfected with the assay plasmid pBCPB+ (Figure 1B) into Drosophila S2 cells. Integrase was induced with $CuSO_4$ at 24 hr, and the cells were harvested at 72 hr. The low molecular weight DNA was harvested by Hirt extraction and transformed into bacteria to assay for recombination. The plasmid pBCPB+ contained the *lacZ* gene flanked by the ϕC31 *attB* and *attP* attachment sites. If a recombination event occurred in the Drosophila cells, the resulting plasmid produced a white bacterial colony on plates containing Xgal. If no recombination event occurred, *lacZ* was expressed and the plasmid resulted in a blue colony. The percentage of recombinants was then calculated by counting the white colonies, dividing by the total number of colonies, and multiplying by 100.

The experiment was repeated in triplicate. Each experiment included carrier-only, integrase-only, and pBCPB+-only transfections, and three plates transfected with integ-

TABLE 1

Transient intramolecular recombination in S2 cells

DNA transfected (no. independent transfections)	White colonies	Total colonies	% recombination
Carrier only (3)	0	0	NA
pMKInt only (3)	0	0	NA
pBCPB+ only (3)	16	1000	2.0
pMKInt and pBCPB+ (9)	4009	8509	47.1

NA, not applicable.

rase and assay plasmid. The amounts of DNA and the transfection method used make it unlikely that cells would receive pBCPB+ and not the integrase plasmid. Recombination occurred at a frequency of 47%, while only 2% of the pBCPB+-only controls were white (Table 1). Forty-seven percent was likely an underestimate, due to untransfected donor plasmid that was never in contact with integrase protein. DNA from 53 of 56 white colonies from the integrase and pBCPB+ cotransfections analyzed by PCR had the expected product (95%). Four of these were sequenced and had the perfect crossover event. The 2% white colonies from the donor-only transfection were likely caused by transfection-related mutation (Lebkowski *et al.* 1984), as has been seen before (Groth *et al.* 2000; Olivares *et al.* 2001; Stoll *et al.* 2002). DNA from all white colonies that resulted from a control transfection was analyzed by PCR for the recombination junction, and none had the expected band. To ensure that the recombination occurred in the Drosophila cells and not in the bacteria, the integrase and assay plasmids were cotransformed directly into bacteria. No white colonies were found out of 1698 total colonies, indicating that pMKInt did not express integrase in bacteria and that the recombination occurred in the S2 cells. These experiments demonstrated that the ϕC31 integrase functioned efficiently in the Drosophila cell environment.

Integration *in vivo*: To verify that the ϕC31 integrase functioned *in vivo* in flies, a simple excision assay was performed. Embryos were co-injected with pBCPB+ (Figure 1B) plasmid DNA and either ϕC31 integrase mRNA or pMKInt (Figure 1A) DNA as sources of integrase. At 24 hr, the embryos were harvested, treated with Proteinase K, and homogenized. DNA was transformed into bacteria and colonies were assessed for white and blue colony number. The pMKInt injection yielded 7 white colonies and 1 blue colony (87.5% recombination), while the mRNA injection yielded 132 white colonies and no blue colonies (100% recombination). DNA from both injections showed a recombinant band by PCR analysis. On the basis of this experiment, the decision was made to use integrase mRNA rather than DNA in the remaining *in vivo* experiments, because of its higher efficiency and to remove the possibility of genomic integration of the ϕC31 integrase gene.

Drosophila pseudo *attP* sites: Once it was established that the ϕC31 integrase functioned well in Drosophila, experiments were conducted to determine whether the Drosophila genome contained endogenous sequences that could support ϕC31-mediated integration. S2 cells were cotransfected with pDrBB2 (Figure 1C) and pMKInthyg (Figure 1D), induced for integrase expression, and selected for ~6 weeks. Genomic DNA was then recovered and analyzed for integration events. To recover only integration events and not unintegrated plasmid, a PCR rescue technique was utilized. The genomic DNA was digested with two enzymes, for example, *Hin*dIII and *Eco*RI, that cut on either side of the *attB* in pDrBB2. The digested DNA was ligated overnight under dilute conditions (20 ng/μl). The small pieces of DNA that resulted from unintegrated plasmid should not be able to ligate to themselves, due to incompatible ends. However, if an integration event occurred, the plasmid may have integrated near an *Eco*RI site. If an *Eco*RI site was found in the genome before a *Hin*dIII site, then the *Eco*RI sites could ligate to each other, forming a minicircle. The ligations were subjected to nested PCR across the ligation junction. Bands of different sizes were gel isolated, Topo cloned, sequenced, and subjected to BLAST analysis. Sequences that were identified as Drosophila sequences were subjected to further analysis. Oligos were designed to the Drosophila genome flanking the crossover region. The hybrid *attL* and *attR* junctions were then amplified by PCR from the selected genomic DNA, Topo cloned, and sequenced. Perfect crossovers were found at several genomic sequences. Three pseudo *attP* sites from the Drosophila genome are listed in Table 2. These pseudo sites were 23–41% identical to the minimal 39-bp wild-type *attP*, similar to the level of identity in mammalian pseudo *attP* sites that have been isolated (Thyagarajan *et al.* 2001; Olivares *et al.* 2002). Dps1 was located on the X chromosome in an intron of the Tre1 gene. Dps2 was located in the repeated copia element, while Dps3 was located on chromosome 2L, upstream of the *mdg1* transposon.

An experiment was conducted to try to achieve integration at pseudo *attP* sites *in vivo*. A total of 700 ng/μl of ϕC31 mRNA and 150 ng/μl pUASTB (Figure 1F) DNA were co-injected into fly embryos. Injection of 564 injected embryos gave 414 larvae, which yielded 249 adults. Of these, 123 were fertile, but yielded no transgenics. While this was only one experiment and does not rule out the possibility of integration at pseudo *attP* sites *in vivo* in Drosophila, we chose not to pursue this line of experiments further, on the basis of the lack of utility of such an inefficient system. Higher integration efficiency at a chromosomally inserted wild-type *attP* site in the Drosophila genome was likely to be more useful to researchers than a low level of pseudo site integration.

***In vivo* integration into *attP* fly lines:** Two fly lines containing an *attP* site were created by *P*-element transposition. The GenomeWalker kit (BD Biosciences) was

TABLE 2

Drosophila pseudo *attP* 39-bp integration sequences

Site	Sequence[a]	% identity to *attP*
attP	CCCCAACTGG GGTAACCTTT GAGTTCTCTC AGTTGGGGG	100
Dps1	GCTCGGTTGA TGAGGAAATT GGTGAAAAAC TCGAACTTT	23
Dps2	TCCTTTCTTA ACACAAAATT GTCTCATCTC ATTTGACAA	41
Dps3	AAATATGGTT GTGAGATGCT GTAGTTAATT AGAATTCCA	23

[a] Underlined bases are identical to the wild-type site at that position.

used to localize the position of the *P*-element insertion. The first line, attP1, contained an insertion in chromosome 2R, band 55C–D, between genes *GM04742* and *jockey*. The second line, attP2, contained the *P*-element insertion in chromosome 3L, band 681A–B2, between genes *CG6310* and *Mocs1*. In the first *in vivo* experiment, embryos were obtained from stocks heterozygous for the attP1 insertion. These embryos were co-injected with ϕC31 Int RNA and pUASTB (Figure 1F), a plasmid containing *attB* and the *white* gene. A total of 339 embryos were injected with 1 μg/μl mRNA and 200 ng/μl DNA (Table 3). The 339 injected embryos yielded 95 larvae, which gave rise to 63 adults. Of 32 fertile y^+ crosses, 17 yielded w^+ progeny, a frequency of 53%. Nine crosses that yielded y^- flies, indicating that no *attP* was available for insertion, were all negative for the white gene. Two crosses that were y^+/balancer yielded red-eyed offspring. In total, of the 34 crosses that had an available *attP*, 19 had offspring with pigmented eyes, indicating that integration occurred at a frequency of 56%.

In the second set of injections, heterozygous embryos from both attP1 and attP2 lines were co-injected with 800 ng/μl Int mRNA and 150 ng/μl pUASTB DNA. A total of 280 injected embryos yielded 96 larvae, which resulted in 53 adults. Of these, 25 fertile y^+ crosses resulted in 11 w^+ progeny (44%) and 11 fertile y^- crosses yielded 0 w^+ progeny. These first two experiments had lower than expected larval survival rates. It was hypothesized that the water used to resuspend the nucleic acid was not at the proper pH. A third experiment was conducted on a fully homozygous population of the attP2 line. To increase the survival of the larvae, the DNA and mRNA were resuspended in nonautoclaved water. A total of 900 ng/μl mRNA and 150 ng/μl pUASTB DNA were injected. In this experiment, 68% of the flies survived to the larvae stage (186/273), which is in the normal range. A total of 104 of the flies grew to adults, with 22 of 51 fertile crosses yielding flies with pigmented eyes (43%). In total for the three experiments, 52 of 110 fertile y^+ crosses resulted in transgenic flies (47%; Table 3).

The flies with pigmented eyes were expanded as individual lines, and DNA was prepared for molecular analysis. PCR was done for the recombinant junction to verify site-specific integration into the genomic *attP*. Primers used in the PCR detected the recombinant *attR* junction, by hybridizing to the P arm of *attP* and plasmid sequences from the pUASTB (Figure 1F) plasmid downstream of the B′ arm of *attB*. All lines tested (48 of 52) yielded the appropriately sized band by PCR. Four lines were not tested due to low numbers. In addition, the fly DNA was subjected to PCR to determine whether the transgenic lines were derived from attP1 or attP2. In each case, a primer specific for the genomic DNA at the insertion site and a primer specific for the *P*-element insertion were used. All 18 lines tested from the first experiment yielded the band for the *attP* insertion in chromosome 2R. From the second experiment, in which embryos from both lines were injected, half of the transgenic fly lines were derived from attP1, while the other half were derived from attP2. One line was not tested due to low fly numbers. All 20 transgenics that were

TABLE 3

Transgenic frequencies

	Injected embryos	Larvae resulting	Adults resulting	Fertile crosses[a]	Crosses that yielded red-eyed offspring	% red-eyed offspring[b]
Expt. 1	339	95	63	34	19	56
Expt. 2	280	96	53	25	11	44
Expt. 3	273	186	104	51	22	43
Average	297	126	73	37	17	47

[a] Only flies with a *yellow* phenotype (carrying at least one copy of the *attP*) were included.
[b] The average numbers for the three experiments.

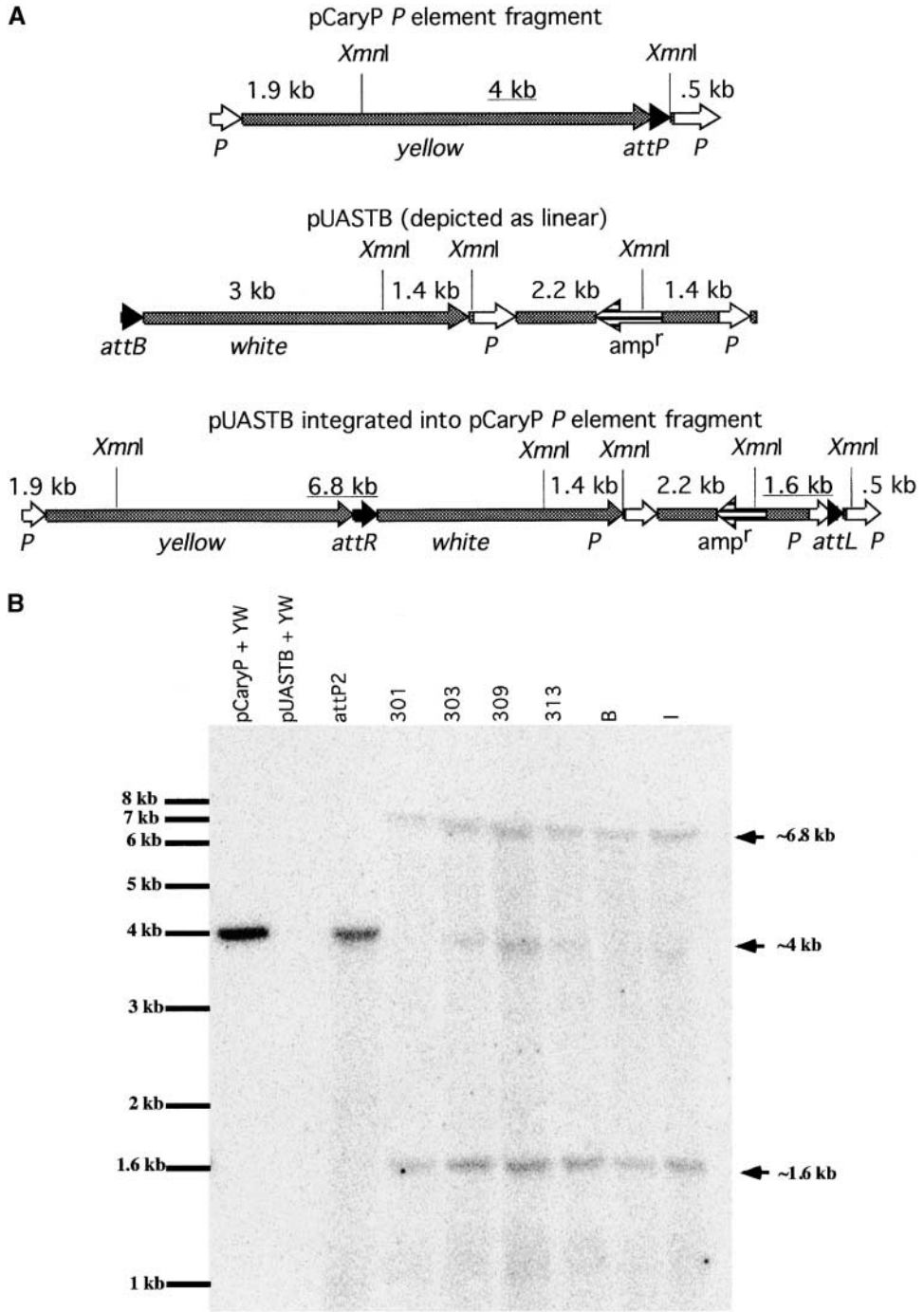

FIGURE 2.—Southern blot analysis of integration events. (A) Schematic of the pCaryP *P*-element insertion, the pUASTB plasmid, and the pUASTB plasmid integrated into the pCaryP *P*-element insertion is shown. The *Xmn*I sites are depicted, and the sizes of the various digestion fragments are shown in kilobases (kb). The bands that the *attP* probe hybridizes to are underlined. *P*, *P* element. (B) A Southern blot of *Xmn*I-digested DNA. Size markers are shown on the left. Expected bands are shown on the right. The plasmids pCaryP and pUASTB were digested and spiked with digested y^-/w^- genomic fly DNA. Lines 301, 303, 309, and 313 are independent transgenic lines from the third experiment. B and I are independent transgenic lines from the second experiment.

analyzed from the third experiment tested positive for the attP2 insertion. It appeared that both lines functioned equally well as targets for ϕC31 integrase-mediated integration.

Individual integrant lines were further analyzed by Southern blot (Figure 2). A total of 10–15 μg of genomic DNA was digested overnight with *Xmn*I, separated by gel electrophoresis, and transferred to a Nytran membrane. The wild-type *attP* was used as a probe. The parent fly lines should yield single bands of 4.0 kb. Upon integration, the *attP* is split into *attL* and *attR* flanking the integrated pUASTB (Figure 1F) plasmid. Both lines should yield bands of 1.6 and 6.8 kb upon integration. A total of 18 transgenic lines (7 from the first experiment, 5 from the second experiment, 6 from the third experiment) were analyzed by Southern blot, and all showed the expected banding pattern. In addition to the 1.6- and 6.8-kb integrant bands, some showed a band at 4 kb, indicating heterozygosity of the insertion. This is possible because each test cross was scored for red-eyed offspring and then all of the flies were left in the bottle to produce enough red-eyed flies for Southern analysis. This allowed both unintegrated and integrated *attP* sites to be carried in the population. Only flies with

pigmented eyes were analyzed by Southern blot, but these flies may have carried integrations at both *attP* sites or just one.

DISCUSSION

The ability of the ϕC31 integrase to perform unidirectional site-specific recombination in cell types from *E. coli* to human cells led us to hypothesize that the system would likely function in Drosophila. The integrase has indeed shown efficient recombination in Drosophila S2 cells, as well as *in vivo* in Drosophila embryos. Intramolecular recombination occurred in S2 tissue culture cells at a frequency of ~50% and in embryos at frequencies of 88–100%.

We have shown that the ϕC31 integrase can reproducibly produce transgenic Drosophila in the range of 43–56% of fertile adults at previously inserted *attP* sites. This frequency is higher than the intermolecular integration frequency that has been demonstrated with the ϕC31 integrase in mammalian cells (Groth *et al.* 2000). It is also approximately fivefold higher than the transgenic rate obtained by *P*-element insertion in our study. In the final optimized experiment, a transgenic frequency of 8% of total injected flies was obtained, whereas we have obtained ~2% using conventional *P*-element-mediated methods. The ϕC31 integrase system should thus allow researchers to inject fewer embryos to obtain transgenic flies.

The ϕC31 integrase is an improvement over the current methods available for the engineering of the fly genome. *P*-element insertions occur at approximately fivefold lower frequency at random genomic positions. Other site-specific recombinases, such as FLP, have a much lower targeting frequency than ϕC31, probably because integrants are substrates for excision, while the ϕC31 system is unidirectional. One study demonstrated that up to 5% of test crosses yielded an insertion at a genomic FLP site (Golic *et al.* 1997), while the ϕC31 system yielded ~50% of crosses having an insertion at *attP*. Protocols for using the FLP recombinase currently involve the creation of *P*-element insertions for each new gene of interest, making its use even more problematic.

Homologous recombination occurs at low frequency, but can be useful if it is critical to make an exact genomic change. However, the frequency of transgenics with the integrase system is much higher than the frequency with homologous recombination. In a representative study of homologous recombination targeted to five different genes, an average of 1.5% of test crosses from the male and female germlines yielded targeted events (Rong *et al.* 2002). Our system yielded 50% of test crosses with targeted events, which was 33-fold higher. Most of these had 30–50% red-eyed progeny.

Both the FLP-mediated recombination and the homologous recombination systems require the creation of a new *P*-element line per transgene, so the *P*-element transgenic frequency must also be taken into account. Once an *attP*-containing line has been made, no further *P*-element transformation is required with the ϕC31 system. The ϕC31 integrase system allows researchers to place any number of genes easily into the same chromosomal context, which can be essential for studies in which small changes in expression will affect the results.

This system will benefit researchers who rely on *P*-element transformation in flies by decreasing the number of injections that are required, allowing multiple genes to be integrated into the same chromosomal context and providing an easy PCR screen for insertions. The ability of ϕC31 integrase to mediate recombination in *D. melanogaster* makes it likely that the enzyme will work well in other insects. This development should help insect research as a whole, including the fight against crop pests and insect-borne diseases. The ϕC31 integrase system continues to prove itself as a versatile site-specific integration tool for the genetic engineering of a wide variety of organisms.

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