Generating customized transgene landing sites and multi-transgene arrays in Drosophila using phiC31 integrase

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ABSTRACT

Transgenesis in numerous eukaryotes has been facilitated by the use of site-specific integrases to stably insert transgenes at pre-defined genomic positions (landing sites). However, the utility of integrase-mediated transgenesis in any system is constrained by the limited number and variable expression properties of available landing sites. By exploiting the non-standard recombination activity exhibited by a phiC31 integrase mutant, we developed a rapid and inexpensive method for isolating landing sites that exhibit desired expression properties. Additionally, we devised a simple technique for constructing arrays of transgenes at a single landing site, thereby extending the utility of previously characterized landing sites. Using the fruit fly Drosophila melanogaster, we demonstrate the feasibility of these approaches by isolating new landing sites optimized to express transgenes in the nervous system and by building fluorescent reporter arrays at several landing sites. Because these strategies require the activity of only a single exogenous protein, we anticipate that they will be portable to species in which genetic manipulation is more challenging, such as non-model organisms, expediting the development of genetic resources in these systems.

INTRODUCTION

The ability to introduce exogenous DNA sequences (transgenes) into the genome of an organism or cell line (transgenesis) underpins much of modern molecular genetics and experimental biology. Transgenes are used to label cells, to manipulate physiology and behavior, and to test hypotheses about gene function. Hence, it is essential that the DNA sequences carried within a transgene be expressed at functional levels and with high spatial and temporal specificity. Yet endogenous regulatory elements frequently exert a strong influence over the expression of a locally inserted transgene, and it is common for two insertions of the same transgene to behave differently due to their distinct local chromatin environments (Lewis 1950; Spradling and Rubin 1983; Levis et al. 1985; Akhtar et al. 2013). Such “position effects” (PE) may lead to spatial and temporal mis-regulation or over-expression/silencing of transgenes. PE can seriously confound the interpretation of experimental results, so care must be taken to control for them.

Transgenesis strategies that allow researchers to control the genomic position of transgenes present an opportunity to standardize PE across transgenes and experiments. Among these, site-specific transgene integration mediated by a bacteriophage-derived integrase is especially popular technique due to its high rate of transgenesis and ability to function in a broad range of species (Smith et al. 2010; Geisinger and Calos 2013). For example, phiC31 integrase has been deployed in multiple eukaryotes, including fruit flies (Groth et al. 2004), zebrafish (Mosiman et al. 2013), tobacco (Lutz et al. 2004), and cultured human cells (Groth et al. 2000). Three components are required to integrate a transgene. The first component, the integrase protein (Int), mediates recombination between two specific DNA sequences called attP and attB (Kuhstoss and Rao 1991). The second component is the transgene to be integrated, which carries the attB sequence. The third component consists of a stable, molecularly mapped transposon insertion that carries the attP sequence; this genomic “landing site” serves as the platform into which the transgene is integrated. This organization gives Int-mediated transgenesis incredible flexibility: any transgene with attB can be integrated, and the genomic position of a transgene can be controlled based on landing site selection. Yet despite its considerable advantages, int-mediated transgenesis is constrained in many systems by a lack of well-characterized landing sites.

The primary measure of landing site quality is whether integrated transgenes express faithfully and at experimentally relevant levels. In the fruit fly Drosophila melanogaster, a number of groups have constructed collections of landing sites that (Groth et al. 2004; Venken et al. 2006; Bischof et al. 2007; Markstein et al. 2008; Ni et al. 2009), and two studies have explored the prevalence of PE by comparing the expression of reporter transgenes integrated at multiple landing sites across the genome (Markstein et al. 2008; Pfeiffer et al. 2010). Using a Gal4-inducible luciferase transgene integrated at 20 different landing sites, Markstein et al. (2008) observed both variable Gal4-induced expression and “leaky” expression of luciferase in the absence of Gal4. Furthermore, for a given landing site, the authors observed that the degree of luciferase induction in one tissue or cell type was not predictive of expression in other tissues or cell types (Markstein et al. 2008). Given that many landing sites (perhaps most) are subject to undesirable PE (Markstein et al. 2008; Pfeiffer et al. 2010), it is clear that landing sites should be tested for deleterious PE in the cell types or developmental stages of interest before embarking on an experiment. However, assessing large numbers of landing sites is
time- and resource-intensive (Markstein et al. 2008; Pfeiffer et al. 2010) and imposes a substantial burden on the research community. Thus, a strategy that streamlines the screening of landing site candidates would expedite the generation of transgenic resources tailored to specific experimental needs.

Our efforts to devise such a strategy led us to consider alternate enzymatic activities that might complement the normal integration function supplied by Int. Several integrase mutants that exhibit atypical recombination properties have been reported previously; these mutants can catalyze the excision of an integrated sequence and promiscuous recombination in vitro between nonstandard att site pairs (Rowley et al. 2008). We reasoned that the unique properties of these mutants might support the development of novel Int-mediated transgenesis techniques. For example, a mutant Int capable of regenerating a functional landing site by excising a previously integrated reporter would obviate several of the steps required to characterize new landings sites. Likewise, a hyperactive mutant Int capable of catalyzing promiscuous recombination between attB and one of the hybrid att sequences that flank an integrated transgene might be useful for inserting an additional transgene at an occupied landing site. Constructing transgene arrays in this fashion would mitigate the need for a large number of landing sites, while simultaneously simplifying complex genetic schemes. These considerations prompted us to investigate the capabilities of several integrase mutants in vivo using Drosophila melanogaster.

From a panel of eight integrase mutants (collectively referred to as Int*), we identified several that exhibited high levels of excision and promiscuous recombination. Int* allowed us to develop new techniques and genetic resources in the fly: Using a novel approach that leverages the excisionase activity of Int*, we generated a set of seven new landing sites that are optimized to express transgenes in the central nervous system of adult Drosophila. Notably, we were able to characterize the expression properties of potential landing sites in half as many generations and at a fraction of the cost compared to if we had used traditional methodology. Moreover, this method can be used to isolate landing sites optimized to any desired criteria. We also devised a protocol that employs Int* (as excisionase) and wild-type Int (as integrase) to relocate a previously integrated transgene to a different landing site on another chromosome. Finally, we exploited the relaxed recombination specificity of the Int* integrase activity to construct two-component transgene arrays at several landing sites. These techniques have the potential to significantly extend the integrase-mediated transgenesis platform.

**RESULTS**

Site-specific transgenesis permits position effects to be standardized across transgenes and obviates the need to map new insertions. One popular method uses the integrase from phiC31 bacteriophage to integrate transgenes at defined genomic positions that contain a phage-derived DNA sequence called attP (Figure 1A; Groth et al. 2004), which typically has been inserted into the genome randomly via a transposon. Such insertions, called landing sites, serve as platforms into which any transgene bearing the compatible attB sequence can be integrated. To integrate a transgene, Integrase (Int) recombines attP (in the landing site) with the corresponding attB sequence on the plasmid (Figure 1A). Following integration, the transgene is flanked by the hybrid sequences, attL and attR, which are each composed of a half-site from both attP and attB (Figure 1A).

Since Int cannot recombine attL and attR without an additional viral cofactor, the integrations reaction is unidirectional (Kuhstoss and Rao 1991; Khaled el et al. 2011). This feature is beneficial when integrating transgenes, but the ability to excise a previously integrated transgene and reconstitute a functional landing site could be advantageous in other circumstances. Previously, several Int mutants have been reported that can catalyze the excision reaction in vitro in the absence of the viral cofactor (Rowley et al. 2008). We characterized these and additional mutants in vivo for use in three novel techniques that expand the genetic toolkit available in Drosophila.

**Mutant integrase reconstitutes functional landing sites in vivo.**

No DNA sequence is gained or lost during the integration reaction (Kuhstoss and Rao 1991). Thus, reversing the reaction should reconstitute an intact attP sequence (i.e., a functional landing site) by excising an episome that corresponds to the originally integrated transgene (Figure 1B). To establish such an excisionase activity in vivo, we constructed a series of transgenic constructs that express different phiC31 integrase variants (collectively referred to as Int*) under the control of a heat shock promoter (Figure 1C). The Int* variants featured combinations of mutated glutamate residues at positions 449, 456, and 463 (Figure 1C), including three previously reported single mutants (Rowley et al. 2008) and five additional double or triple point mutants (Supp. Table 1A). Expression constructs were integrated into the same landing site so Int* variants could be compared without confounding PE. To prevent the Int* transgene from self-excising, we used a Cre-dependent strategy to delete the attR site from one end of the integrated transgene, rendering it immune to the activity of Int* (Figure S1A).

We determined the frequency with which Int* expression induced germline loss of a mini-white-marked transgene integrated into the attP18 landing site. Briefly, males bearing an Int* expression construct and the X-linked transgene were subjected to larval heat shock to induce Int*, then crossed to white virgins upon eclosion (Figure S1B). We inferred the excision frequency for each Int* variant by counting the number of female progeny lacking mini-
white. While some Int* variants had virtually undetectable excision, most exceeded 10% (Figure 1D and Table S1A). Two variants excised the tester transgene from more than half of chromosomes. We observed comparable rates of excision in the female germline (data not shown). For the mutants comprising our panel, the major determinant of excisionase activity seems to be the basic character of the residue at position 449. Hence, Int* variants with lysine at position 449 were generally more active than those with histidine, which were generally more active than those with glycine (Table S1A). These results mirror the in vitro activity trend that was obtained when E449 was systematically mutated (Rowley et al. 2008). We also asked whether a non-genetic source of Int* could drive transgene excision from two autosomal landing sites. Embryos were injected with a helper plasmid encoding the most active variant, Int*-KEE. Surviving adults were crossed to white flies, and progeny were assessed for mini-white. We observed appreciable excision, though at reduced rates relative to genetic sources of Int* (data not shown). Finally, to gauge the accessibility of landing sites in the genome generally, we assayed transgene excision frequency from six additional landing sites, including one on each major chromosome arm. Int* successfully excised the tester at each landing site (Table S1B), from which we infer that most sites in the genome are accessible to Int*.

Given our goal of reconstituting functional landing sites in vivo, it is essential that Int*-mediated excision leaves an intact attP site. To verify the integrity of reconstituted attP sites following Int*-mediated tester excision, we analyzed DNA sequences from individual progeny males in the immediate vicinity of the anticipated recombination region. Sequencing across this region revealed that each chromosome harbored a perfect attP (18/18 independent events, data not shown). These results predict that the integration efficiency of a reconstituted landing site should be equivalent to the corresponding native site that had never hosted a transgene. We tested this by integrating transgenes into reconstituted attP40 and reconstituted attP2, Color denotes Injections performed on the same day. Constructs: pJFRC2 (diamonds); pJFRC-10xUAS-tomoat-nls(w) (circles); pJFRC-10xUAS-mCD8-GFP(w) (triangles). Integration efficiency reflects the number of G0 germlines that gave rise to integrants divided by the number of germlines scored (Figure 1E). While there is significant spread in the data owing to the inherent variability of injections, trans-
genes were integrated into reconstituted landing sites as efficiently as native sites, consistent with our expectation that reconstituted landing sites are fully functional.

**Int** facilitates the identification of landing sites with desirable expression properties

The degree to which a given landing site can support robust and spatiotemporally appropriate expression of resident transgenes is a major consideration in experimental design. Indeed, there is clear evidence that expression properties are variable between landing sites and even vary for a single landing site examined in multiple tissues (Markstein et al. 2008; Pfeiffer et al. 2010). Currently, identifying landing sites with desirable expression properties can only be accomplished through individual empirical characterization of each landing site in a collection. This requires that a reporter transgene be separately integrated into each landing site, an investment of labor and time that is likely a disincentive to many researchers. A strategy that bypasses this requirement would facilitate screening large numbers of landing sites and enable researchers to isolate purpose-built landing sites.

Here we employ Int*-mediated transgene excision to facilitate the identification of landing sites with desirable expression properties. First, we mobilized a transposon-born landing site containing an integrated reporter transgene (referred to as attP[tester]) to new genomic positions to generate a panel of landing site candidates. Since these insertions are pre-loaded with a reporter transgene, they can be directly assayed for expression in any cell type of interest. Subsequently, we used Int* to excise the tester and reconstitute the attP sequence at desirable landing sites (Figure 2A). This strategy eliminates the need for separate tester integrations into each landing site and concomitantly reduces the number of fly generations between the isolation of new landing sites and their characterization. As such, this approach enjoys considerable advantages over the traditional method (compared in Figure S2A and S2B).

We undertook to isolate new landing sites that support robust expression in the Drosophila adult central nervous system and are homozygous viable without causing noticeable phenotypes. Specifically, in light of the large number of transgenes that have been characterized following integration into attP2 (e.g., Jenet et al. 2012; Kvon et al. 2014), we deemed that it would be useful to identify new sites that recapitulate the expression properties of this landing site. We selected a tester transgene with a sparse expression pattern to facilitate the primary screen, a simple visual screen using a fluorescence microscope. R11C05-LexA expresses in several regions of the Drosophila brain, including the ellipsoid body, the sub-esophageal zone, and the optic lobes (Figure 2B), but the limited number of cells expressing this driver ensures that deviations in expression can be easily spotted. Next, we mobilized the X-linked, transposon-born attP18[R11C05-LexA] using P transposase (Figure S2C), generating 172 new autosomal insertions. Fifty-five of these were homozygous lethal or sterile (Figure 2C), in line with P mutagenesis screens (Cooley et al., 1988); these insertions were discarded. The remaining landing site candidates (hereafter, “candidates”) were crossed to LexAop2-GFP in order to characterize the expression properties of R11C05-LexA.

Among the candidates that were visually screened, a large fraction (62%) expressed the R11C05-LexA tester incompletely, as they lacked expression in one or more of the scored brain regions (Figure 2B, panels i and ii). However, we judged ten candidates to support expression in the complete R11C05-LexA pattern, with four expressing GFP at levels similar to or higher than attP2[R11C05-LexA], which serves as a benchmark for comparison (Figure 2B, v-viii; Figure 2C). These results were corroborated when brains were stained for GFP and quantitatively imaged by confocal, although minor differences between several candidates and the benchmark were detectable following this higher resolution analysis (Figure S3A).

To refine the comparison between these ten candidates and the attP2 benchmark, we developed a proxy measure for GFP expression in different brain regions. Briefly, we quantitatively imaged native GFP fluorescence for each candidate, then computationally aligned image stacks to a reference brain using the neuropil marker N-Cadherin (Peng et al. 2011; Jenett et al. 2012). Samples that failed to achieve a minimum alignment score were excluded from subsequent consideration, as were candidates represented by fewer than four aligned samples (Figure S3B). Next, we measured the mean fluorescence intensity for three regions of the R11C05 pattern: the ellipsoid...
A. 

\[ \text{Tr} \rightarrow \text{tester} \rightarrow \text{Tr} \]

\[ + \text{ Transposase} \]

New attP[testing] insertions

Screen

Candidate landing sites

Excise tester

B. Benchmark: attP2[R11C05-LexA]

C. Percentage of new insertions

<table>
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<th>AttPsite</th>
<th>#</th>
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<tr>
<td>2</td>
<td>76</td>
</tr>
<tr>
<td>3</td>
<td>96</td>
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- Lethal / Sterile
- Incomplete Expression
- Full Pattern, Weak
- Full Pattern, Strong

n = 172

D. Mean Fluorescence Intensity

E. 

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ing indicates the region bounded by the attP2 samples. (E) Behavior of three additional driver transgenes in JK22C. To test whether JK22C is generally permissive for expressing driver transgenes, additional Gal4 or LexA constructs driven by different “enhancers” (listed along the top) were integrated. For comparison, the expression of each transgene in attP2 is shown. Brains were immunostained for GFP; images show maximum projections of representative confocal stacks. Scale bar, 50 μm.
body, the left optic lobe (primarily the lobula), and the sub-esophageal zone (Figure 2D, brain regions depicted in Figure S3C). Graphing these data in three dimensions gives a sense for how comparable different candidates are for expression levels (Figure 2D); candidates with similar values for all three regions cluster near each other. As shown, samples from candidates JK22C and JK73A exceed all other candidates as well as attP2[R11C05-LexA], in contrast, ellipsoid body expression in samples from candidate JK73A exceeds that of all other candidates as well as attP2[R11C05-LexA], but expression in the sub-esophageal zone is reduced. These data also support the qualitative assessment of the candidates from the visual screen.

The four strongly expressing candidates isolated above are promising landing sites. Yet, it may be that they are narrowly optimized to express R11C05-LexA, rather than being generally permissible for expression of various transgenes. To address this possibility, we reconstituted the landing sites in these candidates via Int*-mediated excision and integrated four additional transgenes in which the expression of Gal4 or LexA was under the control of different enhancer fragments. We assessed the ability of each of these transgenes to drive the appropriate GFP reporter and compared their expression to that observed when the corresponding Gal4 transgene (i.e., Gal4 driven by the corresponding enhancer fragment) was integrated in attP2 (Figure 2E, Figure S4). The results of these comparisons differentiated the JK22C landing site from the other three candidates: transgenes integrated into JK22C recapitulated attP2-like expression in each case, while each of the remaining three landing sites exhibited aberrant expression for at least one transgene (Figure S4; data not shown for the pan-neural driver R57C10-Gal4).

Finally, we investigated the chromosomal distribution of the new landing sites by mapping their genomic positions. Using splinkerette to amplify the genomic DNA flanking each P insertion (Potter and Luo 2010) and then mapping this sequence to the reference genome, we determined the positions of the four strongly expressing sites and the three weakly expressing lines that reproduce the complete R11C05-LexA pattern. These new landing sites were unevenly distributed on 2L and along both arms of the third chromosome, but no candidates mapped to 2R (Figure 3 and Figure S5). The locations of the new landing sites reflect the known bias of P elements to insert at promoters (Spradling 1999; Liao et al. 2000; Bellen et al. 2004), as four of the new landing sites inserted in the 5'UTR of a gene and the remaining three are within 500 base pairs of the nearest gene (Figure S5). Conveniently, the genetic map predicts that most of the new landing sites fall at least 5 cm distant from the nearest commonly used landing site, which should facilitate recombination between these sites.

**Int** enables transgenes to be shuffled between landing sites genetically

A transgene excised by Int* is released as an episome that bears a reconstituted attB site. This circular DNA element resembles the plasmid transgenic constructs that are injected into embryos and the intermediates of several genetic techniques for translocating DNA sequences from one position in the genome to another (Golic et al. 1997; Gohl et al. 2011). Therefore, we tested whether a transgene contained in an Int*-excised episome can be translocated from one landing site to another. Translocating a transgene (hereafter referred to as shuffling) would require two competing enzymatic activities: excisionase to release the transgene from the donor site and an integrase to re-integrate it at the receiver site (Figure 4A). Several Int* variants exhibit these opposing activities *in vitro* with distinct ratios of excisionase and integrase activities (Rowley et al. 2008). Therefore, we tested several variants for their ability to shuffle a transgene between landing sites on different chromosomes.

First, we assembled the donor and receiver landing sites in a stock, which we then crossed to flies carrying Int*. Crosses were heat shocked during the embryonic or larval stages to induce Int*, and the F1 progeny were crossed to *white* flies (Figure S6A). We screened the F2 progeny to identify individuals in which the transgene marker (mini-white) segregated from the donor site chromosome and co-segregated with the receiver site chromosome (distinguished by DsRed expression). Flies carrying candidate shuffle events were individually stocked to allow further characterization of the receiver locus. To calculate the efficiency of transgene shuffling for each Int* variant, we divided the number of crosses yielding a candidate shuffle...
event by the total screened.

For the landing site pair attP40 and ZH-86Fb, candidate shuffle events were recovered following heat shock at each of the developmental stages tested (Figure 4B), though the frequency declined sharply between heat shocks performed during the first and third larval instars. We confirmed proper re-integration for a subset of candidate shuffle events by using PCR to amplify the junction between the receiver landing site and the transgene. In all cases, the product matched the predicted size (data not shown). Besides correctly targeted shuffle events, we recovered a number of chromosomes that bore the transgene marker (mini-white) but lacked the landing site marker (DsRed). Molecular characterization revealed that a majority of these lines result from re-integration into the Int*-bearing chromosome, via recombination between attB and the attL lying downstream of the hs-Int* transgene (Figure S6B). Similar results were obtained from a second experiment in which the same transgene was shuffled between the landing sites attP2 and ZH-51C (Figure 4B).

Contrary to our expectation, the efficiency of transgene shuffling was uniformly low for each of the Int* variants tested (Figure 4C), possibly due to a failure of Int* to re-integrate the episome efficiently or because Int* vigorously re-excises the transgene once it has been integrated at the receiver landing site. Regardless, we speculated that the presence of wild-type Int during transgene shuffling would shift the balance of both reactions towards integration, favoring the recovery of shuffled transgenes. To test this hypothesis, we incorporated a germline source of wild-type Int (Figure S6A). With this regime (Int plus Int*), shuffle events were recovered at least three-fold more frequently for each Int* variant (Figure 4C). The presence of Int during transgene shuffling also yielded an unanticipated benefit. Wild-type Int improves the fidelity of transgene shuffling by curbing the hyperactive integrase activity of Int* (see below), possibly by forming heterodimers with Int*. This restricts re-integration events to those that result from recombination between attP and attB (Figure S6C).

**Int* can be used to assemble tandem arrays of transgenes via atypical recombination**

The hyperactive integrase activity of Int* can mediate recombination between non-standard att site pairs in vitro (Rowley et al. 2008) and in vivo (above, transgene shuffling). We wondered whether this activity might be harnessed to sequentially integrate multiple transgenes into a single landing site.

Linking transgenes through Int*-mediated atypical integration is predicted to result in a transgene array (hereafter, “array”) in which the constituents lie in a tandem tail-to-head orientation (Figure 5A). The integration site of each additional component (attL or attR) determines the order of the constituents. Integration into attL results in the primary transgene being upstream; attR yields the reverse order. To avoid ambiguity, we shall name constituents based on the temporal order in which they are integrated. Hence, the first transgene to be integrated is referred to as the primary transgene (Figure 5A, purple bar).

As a pilot experiment, we sought to assemble a two-component array in attP2 that consisted of LexAop2-nls-lacZ as the primary transgene and UAS-tdTomato-nls as the secondary transgene. We tested four Int* variants for the ability to integrate a secondary transgene. Embryos homozygous for the primary transgene were co-injected with a mixture of the secondary transgene and an Int*-expressing plasmid construct. G0 adults were crossed to the appropriate screening stock, and F1 progeny were screened for the presence of both transgenes (Figure S7A). To calculate the efficiency of array recovery for each Int* variant, we divided the number of G0 crosses that produced progeny carrying array candidates by the total number of G0 germ-lines screened. Two of the four Int* variants tested gave rise to array candidates from between 2% and 10% of fertile G0 crosses (Figure 5B). Repeating the experiment using only these two variants yielded similar results, with the Int*-KEE showing higher integration frequency.

To characterize array candidates, we confirmed the presence of each constituent transgene by crossing to Gal4 and LexA drivers. The order of primary and secondary transgenes in individual candidate arrays was determined by PCR (Figure S7B). Although Int*-KEE (Int-E449K) has been reported to catalyze recombination equally well between attB and attL or attR in vitro (Rowley et al. 2008), the arrays we characterized molecularly suggest that attL may be the preferred target in vivo (Figure S7B). Next, we sought to construct four additional arrays at attP2 using Int*-KEE. Two of these experiments failed to produce any candidates; the efficiency of the remaining two was comparable to what we observed during the pilot (Figure 5B).

The ability to construct arrays has a natural application in linking transgenes that must co-segregate, such as pairs of reporter transgenes for co-labeling experiments. However, it is essential that the transcriptional units within an array not interfere with one another. To investigate the prevalence of transcriptional crosstalk, we characterized the expression properties of the constituent transgenes within an array composed of LexAop2-nls-lacZ and UAS-tdTomato-nls (Figure S7B). Specifically, we assessed whether expressing one constituent would drive inappropriate expression or suppression of the other constituent.

To establish the baseline expression of the constituents, brains from heterozygous flies were immunostained for nls-LacZ and tdTomato-nls. Neither reporter was detectable in the absence of Gal4 and LexA, indicating that the mere presence of multiple transgenes at a landing site does not induce leaky expression (Figure S7C, top row). Next, we expressed either nls-lacZ or tdTomato-nls under the control of a strong, pan-neural driver to determine whether this would cause expression of the other reporter. In both cases, a small number of cells ectopically expressed the reporter that was not actively driven (Figure S7C, middle and bottom rows). Thus, the constituents of
The hyperactive integrase activity of Int* catalyzes recombination between one of the attB sequences flanking the primary transgene (purple) and the attR sequence associated with the secondary transgene (green). In the resulting array, the primary and secondary transgenes are separated by a reconstituted attB sequence, and the entire array is flanked by attR and attL sequences. The site where the secondary transgene integrates determined the order of transgenes in the array; the frequency at which we observed recombination at attL or attR is represented by the width of the arrow under each site (also see Figure S7B). (B) Int*-KEE is superior to other Int* mutants for constructing transgene arrays. The indicated variants were tested for the ability to produce transgene arrays using several primary/secondary transgene pairs. (For an overview of the genetics, please see Figure S7A) The efficiency of transgene shuffling more than the identity of Int*, pJFRC2 was shuffled from attP40 to ZH-86FB using the crossing scheme summarized in Figure S6A. For the indicated genotypes, wild-type integrase was expressed in the germline under the control of the nanos-promoter (P(nos-phiC31/Int-NLS); Bischof et al. 2007). Efficiency was calculated as in panel B. To derive a shuffling efficiency for each Int* variant, results were pooled from all variants; only Int*-KEE was used for the ZH-51C/attP2 experiment. Each point represents at least 11 germlines screened. (C) The presence of wild-type Int influences the efficiency of transgene shuffling more than the identity of Int*. pJFRC2 was shuffled from attP40 to ZH-86FB using the crossing scheme summarized in Figure S6A. 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Lastly, we asked whether the expression of one constituent of an array would suppress expression of the other. We selected Gal4- and LexA-expressing lines that were previously shown to have limited overlap in their expression and used these to drive expression of the array constituents. Specifically, \(fru^{P1\cdot LexA}\) (Mellert et al. 2010) and \(dsx^{Gal4,LexA}\) (Pan et al. 2011) each express in a limited number of cells in the posterior brain, with a subset of cells in males but not females co-expressing (Zhou et al. 2014). Consistent with our expectation, females possessed numerous cells in the posterior brain that expressed one or the other reporter, but no cells were labeled with both nls-LacZ and tdTomato-nls. In contrast, male displayed numerous co-labeled cells (Figure 5C), demonstrating that the constituents of the array do not suppress the expression of one another. We corroborated this result using a different pair of Gal4 and LexA drivers to probe the expression of a separate reporter array integrated into a different landing site (Figure 5D). Taken together, these results strongly imply that the constituents of a transgene array have minimal impact on the expression of one another.

**DISCUSSION**

We reasoned that the unusual recombination activities reported in vitro for several phiC31 integrase mutants could be applied in vivo to expand the available transgenesis toolkit. Using *D. melanogaster*, we tested integrase variants (Int*) for their ability to excise, integrate, and re-integrate transgenes. Using these activities, we developed methods that simplify the generation of landing sites with specific expression properties, enable the mobilization of an integrated transgene to a different landing site, and allow iterative insertion of multiple transgenes into a single landing site. After demonstrating that several Int* variants could excise transgenes from landing sites across the genome of *D. melanogaster*, we isolated a set of landing sites with expression properties optimized for the adult central nervous system. We also exploited this activity to genetically mobilize transgenes from one landing site to another. Fortuitously, we also observed that the most active integrase variants catalyze recombination between nonstandard att site pairs (e.g., attB and attL), which we exploited to build transgene arrays at several landing sites. Using Int* to perform any of these applications has distinct advantages over previous strategies.

**Int* facilitates isolation of landing sites with specific expression properties**

For any experimental system, the quality of available landing sites dictates the utility of site-specific integrases for transgenesis in that system. The Drosophila community benefits from having several landing sites that have been extensively characterized (Venken et al. 2006; Bischof et al. 2007; Markstein et al. 2008; Pfeiffer et al. 2010), allowing researchers to select sites to maximize transgene expression and minimize the impact of position effects (PE) that might confound the interpretation of results. Nonetheless, there is a limited number of landing sites that are viewed as having desirable expression properties, and several considerations suggest that it will be advantageous, if not necessary, to develop additional landing sites in the future. For example, the expansion of available transactivation systems (Lai and Lee 2006; Luan et al. 2006; Potter et al. 2010) and recombinases (Nern et al. 2011) has spurred the development of increasingly sophisticated genetic techniques that require many transgenes, straining the current set of commonly used landing sites (e.g., Awasaki et al. 2014). Furthermore, in *Drosophila* it has recently been appreciated that the potential for transvection, a phenomenon where transcriptional elements on one chromosome influence transcription from promoters located at the same position on the homologous chromosome, is widespread across the genome (Bateman et al. 2012; Mellert and Truman 2012). Thus, care must be taken when different transgenes occupy the same landing site on homologous chromosomes, if their independent expression is desired. The simple solution to transvection would be to locate transgenes at different landing sites, but this is complicated by the fact that the same transgene in different landing sites is frequently expressed at different levels due to PE (Pfeiffer et al. 2010). For sensitive phenotypes, this variation may not be acceptable.

The variability and unpredictability of PE make it unlikely that the current suite of commonly used landing sites will be sufficient to support robust transgene expression in all tissues or at every developmental stage. Ideally, for experiments involving any cell type at a given developmental stage, there would be the option to integrate transgenes at several landing sites that exhibit minimal PE. Developing a comprehensive landing site toolkit will require identifying additional landing sites specifically selected for these characteristics. Yet isolating landing sites customized to user-defined criteria has been impractical previously owing to the onerous and expensive process of characterizing the expression properties of landing site candidates. By alleviating these problems, Int*-facilitated landing site generation should empower researchers to isolate purpose-built landing sites, thereby expediting the develop-

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consistent with the published characterization of \(dsx^{Gal4,LexA}\) (Zhou et al. 2014). Cells expressing both reporters appear black. Scale bars are 100 μm (left panel) and 25 μm (right panel). (D) The components of a LexAop2-mCD8-GFP/UAS-mCD8-tdTomato reporter array retain transcriptional independence. In combination with R11C05-Gal4 and R32A11-LexA, the transgene array expresses both GFP and tdTomato in spatially distinct patterns, as shown in the single-channel gray-scale immunostaining images on the left. The merged image on the right reveals four cells in the sub-esophageal zone that co-express R11C05-Gal4 and R32A11-LexA. Scale bar indicates 100 μm.
ment of additional landing sites.

Using the method we describe, landing sites that are optimized to express transgenes in a particular tissue, cell-type, or during a specific developmental stage are regenerated from attP[tester] insertions. We demonstrated the efficacy of this method by generating landing sites that are optimized to express driver transgenes in the adult nervous system. Notably, we identified landing site candidates in half as many generations and at significantly reduced fly work compared to the traditional method for characterizing candidates. These savings, coupled with the fact that new candidates can be screened without additional injections, combine to make Int*-facilitated landing site identification orders of magnitude less expensive than the traditional, two-step methodology. Moreover, Int*-mediated landing site isolation has an advantage over other methods for introducing attP sites (e.g., by CRISPR/Cas9-induced recombination) because it allows an unbiased survey of sites in the genome.

The frequency at which landing site candidates were rejected from our screen underscores the advantage of having a minimally intensive strategy for assessing landing site candidates. Among the 172 new landing site candidates that we tested (which exceeds the combined total of landing sites previously reported in the literature), a scant eight expressed the full pattern of the tester in our visual screen. Of these, only half expressed at levels comparable to the tester transgene in attP2, which stood as the benchmark. The low frequency at which our screen identified landing sites with the desired expression properties (~4%) is likely a consequence of our stringent requirement that newly isolated candidates recapitulate the behavior of attP2.

The success of any landing site screen depends on the expression properties of the tester and the scale at which the screen is conducted. The sensitivity of the tester transgene to PE will dictate the number of candidates recovered from a landing site screen, as well as whether the expression properties of candidates extend to additional transgenes. Considering the extreme cases illustrates the point: A tester transgene that is highly sensitive to position effects may yield few or no landing site candidates. Conversely, screening with an unusually robust tester transgene may identify a larger number of candidates, but ultimately few of these may be suitable for expressing more typical transgenes. R111C05-LexA exhibited moderate sensitivity to position effects following integration at several commonly used landing sites (A. Jenett and C. McKellar, personal communication), making it ideal for our purposes. When such data are unavailable prior to initiating a screen for new landing sites, it may be prudent to generate candidates using several different tester transgenes. Another consideration when initiating a landing site screen is the mobility of the progenitor element used to generate new landing site candidates. Similar to other transposon insertions (Bellen et al. 2004; 2011), landing sites can exhibit vastly different transposition frequencies.

For example, attempts to mobilize su(Hw)attP8 and attP2 resulted in a lower frequency of new insertions than did attP18 (JMK, unpublished observations). Because lower mobility landing sites yield fewer new candidates for the same amount of fly handling, they should be avoided as progenitor elements for landing site screens.

Finally, we propose that Int*-facilitated landing site identification should be practical in any species or experimental system where transposition and site-specific integration have been demonstrated. Indeed, by reducing the number of separate transgenesis steps required to characterize landing sites, we anticipate this method can speed the development of genetic resources in these systems.

**Int* enables transgene shuffling between landing sites genetically**

Int*-mediated transgene shuffling offers an economical alternative to performing repeated injections. We envision it has several immediate applications, including placing one transgene at multiple landing sites. Likewise, transgene shuffling can facilitate moving an existing transgene collection into a new landing site, such as one that is better suited to planned experiments than the site in which the transgenes were initially integrated. Finally, for situations in which the plasmid construct containing a transgene of interest is unavailable, transgene shuffling offers the only viable strategy that does not require re-cloning the construct.

Our results suggest several points to bear in mind when shuffling transgenes. First, while Int* mutants possess both integrase and excisionase activities, excision (a unimolecular reaction) is favored over integration (a bimolecular reaction). Because shuffled transgenes are themselves flanked by attR and attL, they remain substrates for the Int* excisionase activity and can be excised from the receiver landing site following re-integration. Thus, including a source of wild-type Int is recommended to favor the recovery of shuffle events. Besides increasing the recovery of shuffled transgenes, we observed wild-type Int reduces the occurrence of undesired products that result from non-standard recombination reactions. Finally, although heat-shock during early development was sufficient to shuffle transgenes, the efficiency between a particular pair of sites may vary considerably between developmental stages. Thus, we recommend that users perform heat shocks at multiple time points when attempting to shuffle a transgene between two landing sites for the first time.

While the efficiency of recovering transgene shuffling events is within a practicable range, it might be improved further by reducing the likelihood of undesired integration events. In our experiments, the presence of the attL sequence downstream of hs-Int* appeared to reduce transgene shuffling by competing with the receiver landing site for the episome. Therefore, generating a source of Int* without this sequence (e.g., Int* inserted into the genome via a transposon) should result in increased shuffling...
Int* enables the construction of transgene arrays in single landing sites

The hyperactive integrase activity of Int* can sequentially integrate multiple transgenes into a single landing site to create an array of transgenes linked in tandem (hereafter, “array”). Int* incorporates successive units by recombining the attB sequence associated with the transgene to be added with attL or attR in the occupied landing site. Thus, Int* can be used to assemble transgene arrays at any landing site, and this technique is compatible with extant transgenes without requiring additional cloning or modification. To demonstrate the feasibility of constructing arrays with Int*, we built two-component arrays at landing sites on the X and third chromosomes.

Transgene arrays have several useful applications. Integrating multiple transgenes in an array can compensate for a shortage of landing sites in systems or species where landing sites are scarce and isolating new sites is impractical. Additionally, for experiments that rely upon co-segregation of transgenes, linking them in an array offers an ideal solution that simultaneously simplifies the experiment by reducing the number of loci that must be tracked. Furthermore, since arrays remain flanked by attL and attR sites following successive integrations, it is theoretically possible to link an arbitrary number of transgenes in an array. Although we have not attempted arrays with more than two components, there is no mechanistic reason to anticipate that integrating a third component will be more difficult than the previous step. Alternatively, wild-type Int could be used to integrate an attP-bearing transgene at the reconstituted attB site to yield a three-component array.

The competing integrase and excisionase activities of Int* result in a diverse mix of products for each iteration of array construction. Therefore, a robust strategy for identifying candidates possessing all components is imperative. Screening for arrays is straightforward when transgene components are differentially marked (e.g., by fluorescence and mini-white expression). Absent these, detecting transgene arrays can be challenging. Indeed, we speculate that our failed array construction attempts were unsuccessful because we could not detect candidates, rather than because no transgene arrays were produced. The development of additional transgene markers should facilitate the construction of transgene arrays using Int*, and may be essential when more than two transgenes are to be linked.

Concluding remarks

The experiments presented here offer a proof-of-concept demonstration of three new techniques that extend the phiC31 transgenesis platform. The efficiency of each technique likely can be further improved by optimizing the starting fly stocks, the timing or levels of Int* expression, and through the availability of additional genetic sources of Int*. We hope that these techniques will stimulate the development of genetic resources in Drosophila melanogaster and all the diverse species in which phiC31 integrase has been shown to function.

MATERIALS AND METHODS

Fly husbandry and Drosophila stocks. Flies were reared at 21°–25°C on standard cornmeal/molasses food. Crosses were heat-shocked in vials at 37°C for one hour in a circulating water bath. Injections were performed by Rainbow Transgenic Flies, Inc. (Camarillo, CA) using standard phiC31 transgenesis protocols, unless otherwise indicated.

The following insertions were generated for this study: the genetic sources of Int* ZH-51C[hs-Int*]; ZH-86FB[hs-Int*], P[attP][attP2][R57C10-Gal4] (w1), P[attP][attP2][pJFRC-13x-LexAop2-nls-LacZ], P[attP][attP2][pJFRC-10x-UAS-tdTomato-nls]. To make ZH-51C[hs-Int*] and ZH-86FB[hs-Int*], embryos carrying M[vas-int.Dm]ZH-2A; M{3xP3-RFP.attP}ZH-51C or M{vas-int.Dm}ZH-2A; M{3xP3-RFP.attP}ZH-86FB were injected with the Int* expression construct. G0 adults were crossed to w1, and integrants were detected among the F1 progeny by mini-white expression. To stabilize the hs-Int* transgene, males with the integrated transgene were crossed to virgins carrying hs-Cre on the homologous chromosome. Crosses were raised at 25°C without heat shock, and hs-Int*/hs-Cre progeny males were crossed to balancer virgins. In the F2 generation, flies carrying stabilized hs-Int* were identified by the absence of mini-white.

In addition, we used genotypes including the following substitutions, described elsewhere. From Bischof et al. (2007): M{3xP3-RFP.attP}ZH-51C and M{3xP3-RFP.attP}ZH-86FB and P{nos-phiC31-int.LNS.LS}. From Groth et al. (2004): P[attP][attP2]. From Markstein et al. (2008): P[attP][attP40]. From Pfeiffer et al. (2010): pJFRC19 integrated into P[attP]. P[attP2]; pJFRC12 integrated into P[attP][attP2]; pJFRC2 integrated into the landing sites P[attP][attP18], P[attP][attP40], P[attP][attP2], P[attP][attP6], P[attP][attP10], P[attP][attP30]; and P[attP][attP18], P[attP][attP40], P[attP][attP2] were a gift of A. Jenett and G. Rubin. Flies that were y1 w1; CyO, PBac{Delta2-3. Exel}2/amosTft were used to mobilize P element-based landing sites to generate new candidates. Flies that were y1 w1; CyO, PBac{Delta2-3. Exel}2/amosTft (Bloomington Stock #8201) were used to mobilize P element-based landing sites to generate new candidates. Flies that were y1 w1; y1 w1; CyO, PBac{Delta2-3. Exel}2/amosTft (Bloomington Stock #8201) were used to mobilize P element-based landing sites to generate new candidates. Flies that were y1 w1; y1 w1; CyO, PBac{Delta2-3. Exel}2/amosTft (Bloomington Stock #8201) were used to stabilize hs-Int* integrants.

Cloning and molecular biology. Int* Expression Constructs: Int* variants were synthesized by megaprimer PCR (Ke and Madison 1997) with slight modifications, using pET11-phiC31 (gift of Michele Calos) as the template. First, the Int coding sequence (CDS) was amplified in separate reactions as incomplete 5’ and 3’ fragments using primers that hybridized to the ends of the Int CDS pair with internal primers designed to introduce the desired point mutation(s). A 1:1 mixture of these two fragments, which overlap in the region to be mutated, served as the template for a second round of amplification using the 5’ and 3’ terminal primers to extend the full-length Int* sequence. The CDS for each Int* variant was subcloned, sequence verified, then cloned into pMGX-loxP-hsP or pMGXw-yR-hsP. These vectors were derived by replacing the UAS sites and minimal promoter of JFRC2 with the hsp70Bb promoter (Ingolia et al. 1980) and the mCD8-GFP sequence with the Int* CDS. Additionally, pMGXw-yR-hsP contains a DsRed transgene marker between the loxP sites and the hsp70Bb promoter. New Reporter Constructs: The CDS for β-galactosidase fused C-terminally to the SV40 nuclear localization signal was amplified from pNLZ (gift of Y.-L. Huang), sequence verified, and cloned into pJFRC19 (Pfeiffer et al., 2010) in place of myr-GFP to yield pJFRC-13x-LexAop2-nls-LacZ. To clone pJFRC-13x-LexAop2-GFP-nls, the CDS for Stinger GFP was amplified from pStinger (Barolo et al. 2000), sequence verified, and then cloned into pJFRC19 (Pfeiffer et al. 2010) in place of myr-GFP. To construct pJFRC-10xU-
AS-ttdTomato-nls, the CDS for tdTomato was amplified minus the stop codon from genomic DNA of flies carrying a codon-optimized tdTomato reporter (gift of B. Pfeiffer). Separately, the tra nuclear localization signal (Barolo et al. 2000; nls) was amplified from \( w^{118}\) genomic DNA, adding 5’ homology to tdTomato that placed the nls in-frame. These fragments were mixed at a 1:1 ratio and used as a template for overlap PCR. The resulting full-length tdTomato-nls CDS was sequence verified, then cloned into pJFRC2 (Pfeiffer et al. 2010) in place of mCD8-GFP. Re-integration assays using a non-genetic source of Int* were performed by crossing to a pan-neural Gal4. The re-integration site was molecularly verified by PCR with a pair of primers that hybridized within the landing site and the shuffled transgene, respectively.

**Two-component array construction.** Embryos homozygous for the primary transgene were co-injected with the plasmid bearing the secondary transgene and a helper plasmid encoding Int* in a 3:1 ratio, for a total of 16 μg DNA per injection. \( F_{a} \) progeny were screened for the presence of both the primary and secondary transgenes. For injections presented in Figure 5B, the primary and secondary transgenes and screening stock were as follows (primary 1 secondary screening): Pair 1 - attP2[13xLex- exAop-nls-lacZ] | pJFRC-10x-UAS-tdTomato[w]; Pair 2 - attP2[13xLexAop-nls-lacZ] | pJFRC-10x-UAS-tdTomato[w]. The reporter array characterized in Figure 5C was obtained from the Pair 1 injections, and these flies had the genotype \( w^{1118}; P\{CaryP\}attP2[13xLexAop2-nls-LacZ] \rightarrow P\{CaryP\}attP2[13xLexAop2-nls-LacZ] \rightarrow P\{CaryP\}attP2[13xLexAop2-nls-LacZ] | pJFRC-10xUAS-mCD8::tdTomato[w]. The reporter array presented in Figure 5D was constructed in the \( su(Hw)\) attP8 landing site (Ni et al. 2009), using pJFRC15 as the primary transgene (Pfeiffer et al. 2010) and pJFRC-10xUAS-mCD8::tdTomato as the secondary transgene. The genotype of these flies was \( y^{w}\ P\{CaryP\}su(Hw)\).
projections from the optic lobes using a qualitative scale. Candidates were discarded for weak or aberrant expression.

**Brain Alignment and Quantification of GFP expression.** Brains were dissected from 4-7 day-old R11C05-LexA>LexAop2-myRGFP females, fixed, stained with anti-DN-Cad to reveal the neuropil, and then quantitatively imaged for native GFP and neuropil using a confocal microscope. Stacks were aligned to a standard reference brain (Jenett et al. 2012) by neuropil signal, using the JBA algorithm (Peng et al. 2011). Maximum projections were prepared from aligned stacks; a custom FIJI script was used to collect mean fluorescence intensity from the GFP channel for five regions of the image. These included two blank regions to permit normalization of background fluorescence and three regions overlapping the ellipsoid body, the left sub-esophageal zone, and the left optic lobe.

**LITERATURE CITED**


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