INTRAMOLECULAR INTEGRATION ASSAY VALIDATES INTEGRASE PHI C31 AND R4 POTENTIAL IN A VARIETY OF INSECT CELLS

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Abstract. Phage ϕC31 and R4 integrases are site-specific and unidirectional serine recombinases. We have analyzed the ability of these integrases to mediate intramolecular integration between their *attB* and *attP* sites in 7 important insect cell lines as a means of predicting their relative mobility in the corresponding insect species. Both integrases exhibit significantly higher frequencies in *Drosophila* S2 cells than in the other insect cell lines examined, but do work well in all of the species tested. Our results, coupled with previous results of the activity of ϕC31 integrase in *D. melanogaster* and *Aedes aegypti*, suggest the family of serine catalyzed integrases will be useful site-specific integration tools for functional genome analysis and genetic engineering in a wide range of insect species.

INTRODUCTION

Practical genetic engineering of insects for potential utility as moderators of native populations requires new tools for efficient site-specific integration of genes to allow reliable prediction of expression stability and fitness costs to the transgenic insects. The most common method currently employed for engineering eukaryotic chromosomes is random integration facilitated by mobile genetic elements in which foreign or manipulated DNA is introduced into the chromosomes of an organism without control over the ultimate position of the insertion, resulting in unpredictable gene expression and possible insertional mutagenesis leading to fitness costs.

In contrast, phage integration mechanisms can provide high specificity and have been recognized as a powerful genetic tool in a variety of prokaryotic and eukaryotic cells. While site-specific recombinases are structurally and functionally diverse (Smith and Thrope, 2002), most can be classified into either the tyrosine or serine family based on amino acid sequence homologies and catalytic residues.

Recombinases such as Cre and FLP use a catalytic tyrosine to mediate bidirectional recombination between two identical sites (Stark *et al*, 1992). These recombinases rec-

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ognize DNA sequences that are typically 30-40 bp in length. They require no host-specific co-factors and have successfully functioned in mammalian cells, providing important and widely used tools for genome manipulation (O'Gorman *et al*, 1991; Sauer, 1994; Sorrell and Kolb, 2005). Although Cre and FLP recombinase-mediated integrations can be efficiently performed in mammalian cells, the net integration frequency is low (Sauer and Henderson, 1990) because of the reversibility of the reaction.

In contrast, phage integrases mediate unidirectional recombination between two different DNA sequences, the bacterial attachment site, *attB,* and the phage attachment site, *attP* (Campbell, 1992). These two sequences generally share a short stretch of identical bases where crossing-over occurs. After recombination, an integrated phage genome is flanked by two hybrid sites, *attL* and *attR*, each consisting of half *attP* sequence and half *attB* sequence. Only a subset of phage integrases, including those from phages ϕC31, R4, and TP-901-1, belong to the serine recombinase family (Christiansen *et al*, 1996; Matsuura *et al,* 1996; Thorpe and Smith, 1998; Brondsted and Hammer, 1999; Olivares *et al*, 2001; Hollis *et al*, 2003). Because these integrases cannot carry out the reverse excision reaction without additional co-factors, these enzymes are especially helpful for catalyzing stable integration reactions.

ϕC31 integrase is isolated from a *Streptomyces* phage (Kuhstoss and Rao, 1991; Rausch and Lehmann, 1991) and is reported to mediate intramolecular recombination of plasmids in *Escherichia coli* and *in vitro* with no requirement of host-specific co-factors (Thorpe and Smith, 1998). The 605-amino acid ϕC31 integrase can perform recombination between minimal 34-bp *attB* and 39-bp *attP* sites surrounding a core cleavage and ligation sequence of TTG in human cells (Groth *et al*, 2000). This integrase mediates stable, site-specific integration of plasmids bearing *attB* into *attP* sites randomly integrated into the genomes of cultured human cells (Thyagarajan *et al*, 2001). It also functions in *Rhodococcus equi* (Hong and Hondalus, 2008), *Schizosaccharomyces pombe* (Thomason *et al*, 2001), the silkworm cell line, BmN4 (Nakayama *et al*, 2006), wheat plants (Rubtsova *et al*, 2008), *Methanosarcina* species (Guss *et al*, 2008) and human cells (Thyagarajan *et al*, 2008). ϕC31 integrase has been utilized to efficiently create transgenic *Xenopus laevis* (Allen and Weeks, 2005, 2009), and mice (Belteki *et al*, 2003; Hollis *et al*, 2003).

The ϕC31 integrase may also be used to effectively target naturally occurring chromosomal sequences with partial sequence identity to *attP,* called pseudo *attP* sites (Thyagarajan *et al*, 2001). Site-specific integration of foreign genes at pseudo *attP* sites of *Drosophila* (Groth *et al*, 2004), bovine cells (Ma *et al*, 2006; Ou *et al*, 2009), and mammalian cells (Thyagarajan *et al*, 2001) has been detected. The genomic integrations mediated by ϕC31 were also detected by long-term expression of luciferase in human and mouse cells (Thyagarajan *et al*, 2001; Thyagarajan and Calos, 2005). This ability of ϕC31 integrase to find and utilize pseudo *attP* sites has been successfully used in gene therapy experiments in mouse liver, mouse muscle-derived stem cells, mouse muscle, rat retina, human myoblasts and human keratinocytes (Olivares *et al*, 2002; Ortiz-Urda *et al*, 2002, 2003; Quenneville *et al*, 2004; Chalberg *et al*, 2005; Held *et al*, 2005; Bertoni *et al*, 2006). The ϕC31 integrase system also produces stable transgene expression in adult mouse neural progenitor cells (mNPCs) and their progeny that may be useful in strategies for combating neurodegenerative disorders (Keravala *et al*, 2008).

The ability of ϕC31 integrase to efficiently target transgenes to specific chromosomal locations and the potential to integrate

very large transgenes has broad applicability for genetic manipulation of many medically and economically important insect species. ϕC31 integrase has demonstrated effectiveness to promote integration of *attB*-bearing plasmid into *P*-element vectored *attP* sites of two transformed *Drosophila* lines (Groth *et al*, 2004). The ϕC31 integrase system was used in conjunction with recombinase-mediated cassette exchange (RMCE) for precise targeting of transgenic construct to predetermined genomic sites in *Drosophila* (Bateman *et al*, 2006). This integrase system has also been used in the construction and manipulation of transgenic *Aedes aegypti* mosquitoes, increasing integration efficiencies by up to 7.9-fold (Nimmo *et al*, 2006) over alternative transposon-mediated protocols. Most recently, endogenous expression of ϕC31 integrase has been used to generate a collection of *Drosophila* lines having many different predetermined intergenic *attP* sites distributed throughout the fly genome (Bischof *et al*, 2007).

R4 integrase is a similar unidirectional site-specific recombinase derived from the *Streptomyces parvulus* phage R4. This streptomyces phage encodes a 469 amino acid protein termed *sre* for ***s****ite-specific recombinase* (Matsuura *et al*, 1996). *sre* mediates recombination between *attP* and *attB* sequences sharing a 12-bp common core region (Shirai *et al*, 1991). This integrase has demonstrated intramolecular integration capability in human cells (Olivares *et al*, 2001), and has been used for recombining two introduced *att* sites on the same chromosome resulting in deletion of the intervening sequences (Hollis *et al*, 2003).

Using extrachromosomal plasmid excision and integration assays, we investigated the ability of both ϕC31 and R4 integrases to mediate intramolecular recombination reactions between compact ϕC31 *attB* and *attP* or R4 *attB* and *attP* sites in a variety of cultured insect cells. We find that both ϕC31 and R4 integrases are capable of serving as effective tools for efficient recombination in a wild range of insects.

MATERIALS AND METHODS

Plasmids

ϕC31 integrase-expressing plasmids were constructed using two promoters. *Drosophila*-derived heat shock protein 70 (hsp70) promoter (Di Nocera and Dawid, 1983; Lis *et at*, 1983; Steller and Pirrotta, 1984; Spradling, 1986; Linsquist and Craig, 1988) was amplified by the PCR from the plasmid phsp-pBac with the primers 5'-ACTAGT CCCCCAGAGTTCTCTTCTTGTATTCAATAA and 5'-GGTACCATTCCCATCCCCCTAG AATCCCA. The *D. melanogaster* actin 5C promoter was removed from pAct5C-Int by digestion with the restriction enzymes *Acc65*I and *Spe*I, and was replaced by the heat-shock promoter, creating the plasmid phsp-Int. The *sre* gene was amplified by the PCR from the plasmids pCMV-sre (kindly provided by Dr MP Calos) with the primers 5'-GGATCCTCAAACCTTCCTCTTCTT CTTAGGCTCGGCCACGTCTCGCCACT and 5'-ACTAGTACCATGGGTATGAATC GAGGGGGGCCCACT. ϕC31 integrase was removed from phsp-Int by digestion with the restriction enzymes *BamH*I and *Spe*I, and was replaced by the *sre* gene, creating the plasmid phsp-sre.

Plasmid pBCPB+ (kindly provided by Dr MP Calos) containing ϕC31 *attP* and ϕC31 *attB* and plasmid pBC-P64-B295 (kindly provided by Dr MP Calos) containing R4 *attP* and R4 *attB* were used to detect intramolecular recombination in insect cells.

Cell line cultures

Seven insect cell lines used in this study were presenting 2 orders of insect, Diptera and Lepidoptera. Diptera, *Ae. aegypti* Aag2 cells, *Anopheles gambiae* Sua5B and

D. melanogaster S2 cells were maintained at 28ºC in Schneider's Drosophila medium (Gibco Laboratories) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 10% tryptose phosphate broth (SIGMA-ALDRICH) and 1% antibiotic/antimycotic solution (SIGMA-ALDRICH). *Ae. aegypti* ATC-10 cells and *Ae. albopictus* C6/36 cells were maintained at 28ºC in Leibovitz L-15 medium (Atlanta Biologicals) supplemented with 10% FBS, 10% tryptose phosphate broth and 1% antibiotic/antimycotic solution. Lepidoptera, silkworm *Bombyx mori* BmN4 and Fall armyworm *Spodoptera frugiperda* Sf9 cells were maintained at 28ºC in TNM-FH insect culture medium supplemented with 5% FBS but no FBS was supplemented in Sf9 cell culture, 10% tryptose phosphate broth and 1% antibiotic/antimycotic solution.

Transfection efficiencies measured by firefly luciferase expression

Insect cells, Aag2, ATC-10, C6/36, S2, Sf9 and Sua5B were transfected with either 1 µg of the firefly luciferase-expressing plasmid, pAct5C-Fluc or 1 µg of enhanced yellow fluorescent protein expressing plasmid, pAct5C-EYFP but BMN4 was transfected with either 1 µg of pAct3C-Fluc or pXLH-MASP1-Act3C-EYFP using either TransFectin Lipid Reagent (Bio-Rad Laboratories) or DOTAP Liposomal Transfection Reagent (Roche Applied Science). At 24 hours post-transfection, only S2 cells were harvested for firefly luciferase assay because actin 5C promoter (Act5C) originated from *D. melanogaster* (Fyrberg *et al*, 1981, 1983; Bond and Davidson, 1986), and it is strong promoter in these cells. If these cells were harvested at 96 hours post-transfection, the luciferase protein would be oversaturated and could not be assayed. For other insect cells, they were harvested at 96 hours post-transfection.

To assay firefly luciferase expression, the harvested cells were washed twice with 1x cold phosphate-buffered saline, pH 7.4 (Gibco laboratories). These washed cells were resuspended in 500 µl of 1x cold cell culture lysis reagent (Promega Corporation) and then incubated at room temperature for 5-10 minutes. The lysed cell suspension was transferred to microcentrifuge tubes and stored at -70ºC overnight. After overnight storage, this lysed cell suspension was thawed and centrifuged at 10,000 rpm for 30 seconds to remove cell debris. Luciferase activity in crude protein extracts was determined using Luciferase Assay Reagent (Promega Corporation) and the results were analyzed with SoftMax Pro 4.8 software. The luciferase activity in crude protein extracts was normalized by subtracting the measurement of the EYFP activity from that of the firefly luciferase activity.

Intramolecular reaction mediated by either ϕC31 or R4 integrase

Plasmid pBCPB+ or pBC-P64-B295 was used to detect intramolecular recombination, containing a *lacZ* gene flanked by their wild type *attB* and *attP* sequences. Each insect cell line was plated each well of a 6-well plate at 1x10^6 cells/well. Cells were co-transfected with either 2.5 µg of pBCPB+ and 7.5 µg of phsp-Int or 2.5 µg of pBC-P64-B295 and 7.5 µg of phsp-sre using TransFectin Lipid Reagent but using DOTAP Liposomal Transfection Reagent for C6/36 cells. Control transfection was performed using either pBCPB+-only, phsp-Int-only, pBC-P64-B295-only, phsp-sre-only or no DNA. At 24 hours post-transfection, cells were heat shocked at 37ºC for 1.15 hours and changed to complete media. The second heat shock was performed at 48 hours post-transfection and cells were allowed to grow. At 72 hours post-transfection, low molecular weight DNA was recovered as described by Hirt (1967). A half portion of this DNA was *BamH*I digested for reaction mediated by

ϕC31 integrase or *Mlu*I digested for reaction mediated by R4 integrase to reduce the background of unreacted DNA. DNA was then electroporated into competent DH10B *E. coli* cells (Invitrogen, Carlsbad, CA) and spread on LB plates containing 25 μg/ml of chloramphenicol and 50 μg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) to select for white recombinant plasmids reacted by ϕC31 integrase and screened by primers 5'-GGCGAACGTGGCGAGAAAGG and 5'-GGAAACCTGTCGTGCCAGCTG and the white recombinant plasmids reacted by R4 integrase are screened by primers 5'-CAGCTGGCACGACAGGTTTCC and 5'-CCTTTCTCGCCACGTTCGCC. PCRs on white bacterial colonies contained 200 nM forward and reverse primers, 2.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 mM dNTPs and 200 pg of either *in vitro*-reacted pBCPB+ or *in vitro*-reacted pBC-P64-B295 plasmid DNA as a positive control. PCR was conducted as follows: 95ºC for 2 minutes, followed by 40 cycles of 95ºC for 30 seconds, 60.3ºC for 30 seconds, 72ºC for 30 seconds. A final extension period of 72ºC for 7 minutes then was performed. PCR products were analyzed on 1.5% agarose (SIGMA-ALDRICH). The intramolecular integration frequency was determined as the PCR-screened positive white colonies divided by the total number of colonies X 100.

RESULTS

Transfection efficiencies of each cell line measured by firefly luciferase expression

We determined the relative transfection efficiencies for each insect cell line using either TransFectin or DOTAP and a firefly luciferase reporter plasmid (Table 1). Luciferase counts were 70 to 100-fold greater for S2 cells compared to the other insect cell lines transfected with the same reagent. DOTAP yielded 9-fold higher transfection efficiency for C6/36 cells than TransFectin based on relative luciferase counts, but displayed decreased transfection efficiency in Aag2 relative to TransFectin. The transfection efficiency results demonstrated TransFectin was most efficient for Aag2, ATC-10, BmN_4, S2, Sf9 and Sua5B cell lines, while DOTAP was most effective for C6/36 cells.

Intramolecular recombination assay for ϕC31 integrase activity

We developed an intramolecular recombination assay to assess the activities of ϕC31 and R4 integrases in a variety of insect cells.

Table 1
Transfection efficiency measured by firefly luciferase assay.

Cells	TransFectin Mean ± SE	DOTAP Mean ± SE
S2	1.10×10^5 ± 1,605.66	5.7×10^4 ± 1,908.89
Sf9	1.10×10^3 ± 13.22	7.34×10^2 ± 3.61
Sua5B	2.44×10^2 ± 10.85	2.37×10^2 ± 6.62
ATC-10	7.65 ± 0.24	1.12 ± 0.04
Aag2	5.46 ± 0.31	3.2×10^{-3} ± 0.0002
C6/36	4.68 ± 0.06	4.2×10^1 ± 0.50
BmN_4	6.8×10^{-1} ± 0.07	6.6×10^{-1} ± 0.03

Each value is the average of the three independent experiments, with the standard error.

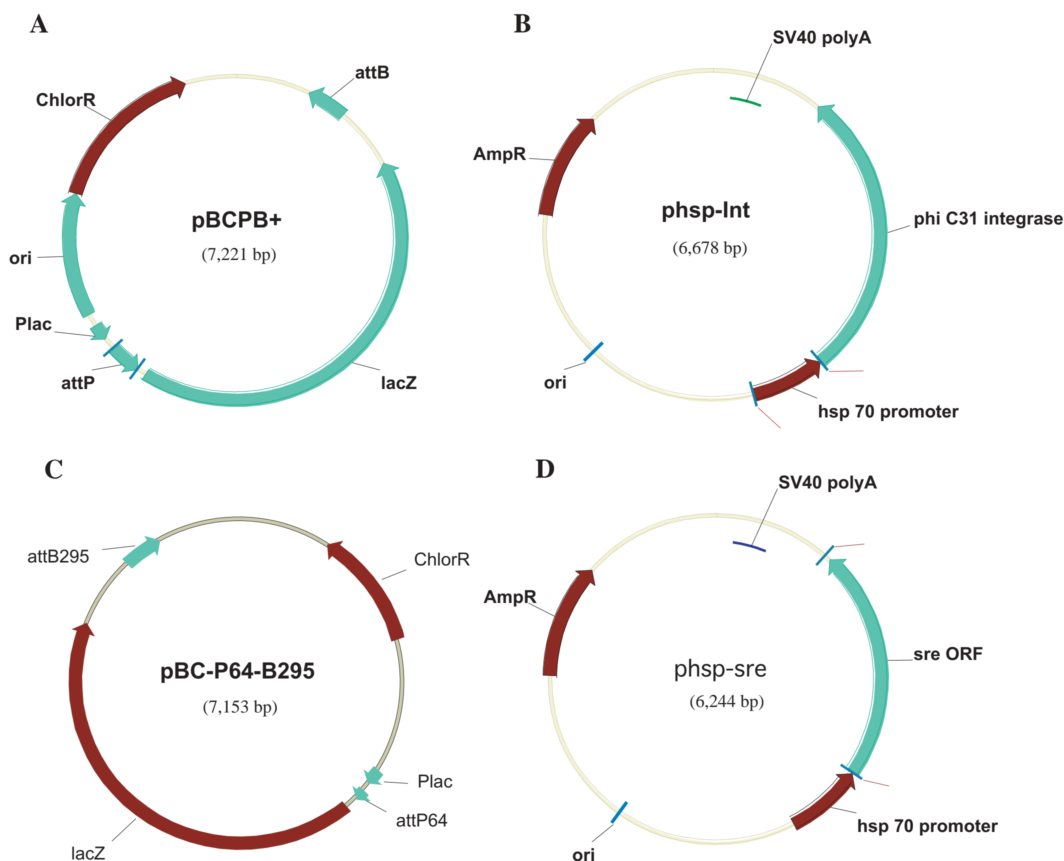

Fig 1–Plasmids used to monitor the ϕC31 and R4-mediated site-specific recombination in 7 insect cells. (A) pBCPB+, intramolecular integration assay vector used in reaction mediated by ϕC31 . (B) phsp-Int, a plasmid for expression of ϕC31 integrase. (C) pBC-P64-B295, intramolecular integration assay vector used in reaction mediated by R4. (D) phsp-sre, a plasmid for expression of R4 integrase.

For the ϕC31, we used the recombination assay plasmid, pBCPB+, containing ϕC31 *attP* and *attB* sites in direct orientation that flanked a *lacZ* gene driven by the native *lacZ* promoter on a chloramphenicol-resistant ColE1 derivative (Fig 1A). The helper plasmid for the assay expressed the ϕC31 integrase under the control of a minimal heat shock promoter (Lis *et al*, 1983), phsp-Int (Fig 1B). These plasmids were co-transfected into each of the 7 insect cell lines being examined and were subjected to heat shock for 1.15 hours at 37ºC at 24 and 48 hours post-transfection to induce integrase expression. Low molecular weight DNA was harvested at 72 hours post-transfection and a half portion of the recovered DNA was *BamH*I digested to reduce the background of unreacted plasmids prior to bacterial transformation.

Site-specific recombination mediated by ϕC31 integrase resulted in deletion of the *lacZ* gene from plasmid pBCPB+ (Fig 2B), yielding plasmids that produced white colonies on chloramphenicol/X-Gal plates upon

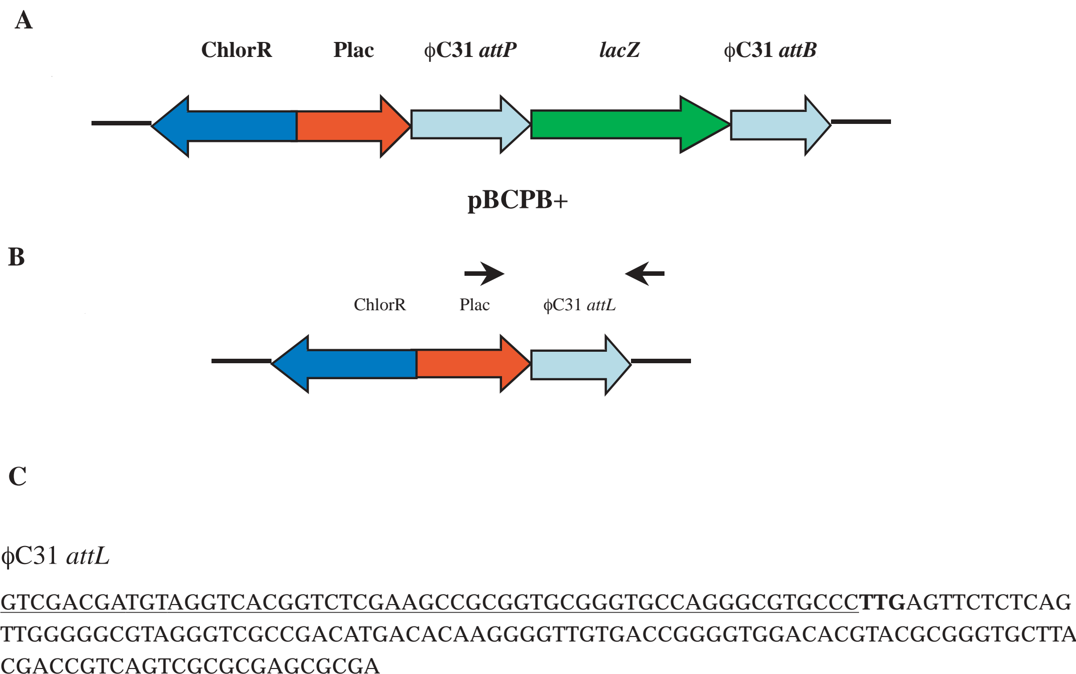

Fig 2-Schematic diagram of ϕC31-mediated site-specific recombination and DNA sequence. (A) plasmid pBCPB+ was used to assay ϕC31 integrase-mediated intramolecular integration in a variety of insect cells. DNA fragments of 285-bp ϕC31 *attB* and 221-bp ϕC31 *attP* flank *LacZ* gene driven by *lac* Z promoter (Plac). (B) ϕC31-mediated site-specific recombination resulted in deletion of a *LacZ* gene in pBCPB+. The ϕC31 *attL* junction is derived from ϕC31-mediated recombination between ϕC31 *attB* and ϕC31 *attP* sites. Arrows show the location of PCR primers that amplify 600 bp fragment specific for ϕC31 *attL*. (C) ϕC31 *attB*-derived flanking sequences are underlined and 3-bp common core region between ϕC31 *attB* and ϕC31 *attP* in which crossovers occur is shown in bold.

transformation of DH10B *E. coli* cells. However, the simple digestion and plating assay resulted in a high background of white colonies due to unexpectedly high transfection-associated mutation of the plasmid that was not specific for the *att* site. We therefore verified the site-specific recombination by colony PCR with specific primers that amplified a 600-bp product from the recombinant (Fig 3). DNA sequences of these PCR products were determined to confirm ϕC31 site-specific integration events (Fig 2C).

ϕC31 integrase catalyzed intramolecular recombination between ϕC31*attB* and *attP* sites in all 7 insect cells (Table 2). The highest recombination frequency was obtained in S2 cells which exhibited a 2-18-fold greater frequency compared to that of other insect cells. The increased frequencies of recombination events recovered in our assays did not necessarily correspond with the relative transfection efficiencies for each of the cell lines, suggesting these data do reflect relative activities to some extent recombination.

Intramolecular recombination assay for R4 integrase activity

We used the plasmid pBC-P64-B295, bearing R4 *attP* and *attB* sites flanking a *lacZ* gene (Fig 1C) as the reporter plasmid for R4 integrase activity, with a helper plasmid ex-

pressing the R4 integrase under the control of minimal heat shock promoter (Fig 1D). These plasmids were co-transfected into each of the cell lines followed by heat shock at 24 and 48 hours post-transfection to induce integrase expression. Low molecular weight DNA was harvested at 72 hours post-transfection and a half portion of the recovered DNA was *Mlu*I digested to reduce the background of unreacted plasmids, and putative recombination positive white colonies were recovered following transformation of DH10B *E. coli* cells by plating on chloramphenicol/X-Gal plates. As with the previous recombination assay, R4 integrase-mediated site-specific recombination resulted in deletion of the *lacZ* gene (Fig 4B), but the assay resulted in a high background of white colonies, necessitating PCR screening with specific primers that amplified a 678-bp product from the recombinant (Fig 5) and sequencing confirmation to recover R4 site-specific integration events (Fig 4C).

R4 integrase catalyzed recombination between its *attB* and *attP* sites in all 7 insect cells. The S2 cells displayed recombination frequency 2-9-fold greater than that observed for all other insect cells. Once again, the relative recombination frequencies for

Fig 3–PCR analysis of ϕC31-mediated site-specific recombination in a variety of insect cells. A 600-bp product is shown for the site-specific recombination. Lane 1, positive control, *in vitro*-reacted pBCPB+; lane 2, *in vitro*-reacted pBCPB+ from Aag2 cells; lane 3, *in vitro*-reacted pBCPB+ from ATC-10 cells; lane 4, *in vitro*-reacted pBCPB+ from BmN_4 cells; lane 5, *in vitro*-reacted pBCPB+ from C6/36 cells; lane 6, *in vitro*-reacted pBCPB+ from S2 cells; lane 7, *in vitro*-reacted pBCPB+ from Sf9 cells; lane 8, *in vitro*-reacted pBCPB+ from Sua5B cells; lane 9, negative control, *in vitro*-transfected pBCPB+; lane 10, negative control, *in vitro*- transfected phsp-Int; lane M, size markers.

Table 2
Integration frequency in a variety of insect cells.

Cells	% integration frequency mediated by ϕC31 integrase	% integration frequency mediated by R4 integrase
S2	$1.65^{a} \pm 0.2$	$1.16^{a} \pm 0.19$
C6/36	$0.98^{b} \pm 0.16$	$0.72^{b} \pm 0.11$
Sua5B	$0.24^{c} \pm 0.06$	$0.73^{b} \pm 0.19$
ATC-10	$0.39^{c} \pm 0.11$	$0.64^{b} \pm 0.12$
BmN_4	$0.40^{c} \pm 0.08$	$0.19^{c} \pm 0.06$
Sf9	$0.36^{c} \pm 0.05$	$0.13^{c} \pm 0.04$
Aag2	$0.09^{c} \pm 0.03$	$0.19^{c} \pm 0.09$

Each value is the average of the three independent experiments, with the standard error. Average followed by the different letter is statistically significant ($p < 0.001$).

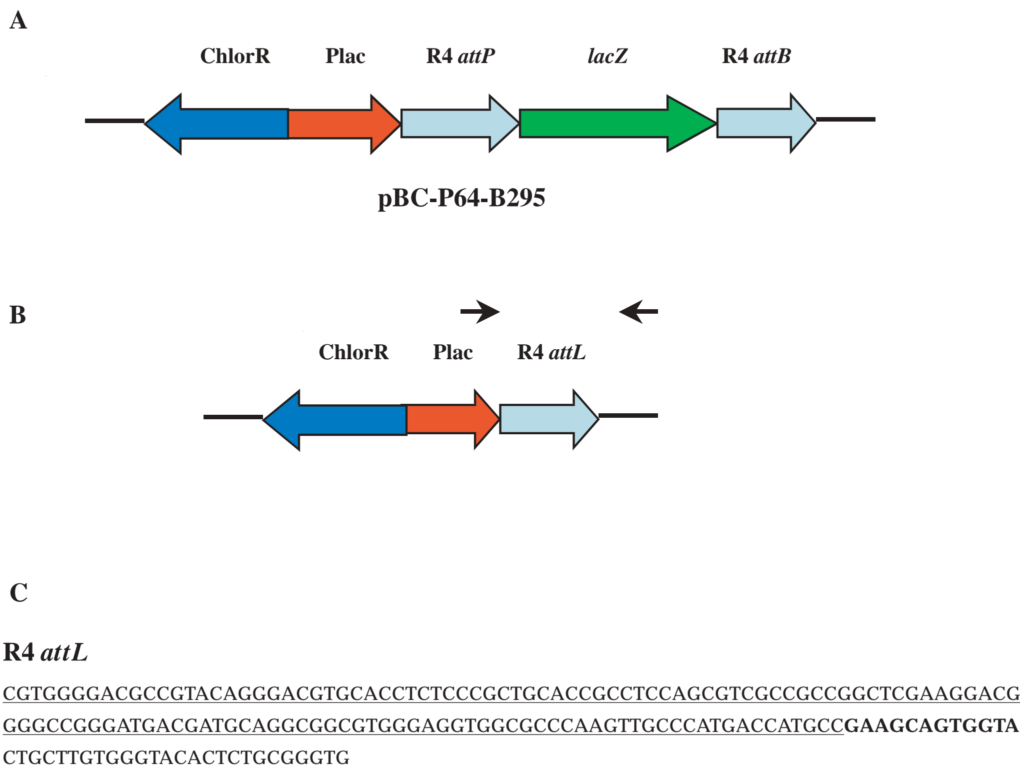

Fig 4–Schematic diagram of R4-mediated site-specific recombination and DNA sequence. (A) plasmid pBC-P64-B295 was used to assay R4 integrase-mediated intramolecular integration in a variety of insect cells. DNA fragment of 295-bp R4 *attB* and 64-bp R4 *attP* flank *LacZ* gene driven by *lac* Z promoter (Plac). (B) R4-mediated site-specific recombination resulted in deletion of a *LacZ* gene in pBC-P64-B295. The R4 *attL* junction is derived from R4-mediated recombination between R4 *attB* and R4 *attP* sites. Arrows show the location of PCR primers that amplify 678 bp-fragment specific for R4 *attL*. (C) R4 *attB*-derived flanking sequences are underlined and 12-bp common core region between R4 *attB* and R4 *attP* in which crossovers occur is shown in bold.

this integrase in each of the cell lines did not necessarily correspond with the relative transfection efficiencies, suggesting these frequencies were at least partly related to the activity of R4 integrase within the given cell line.

Control assay for recombination in transformed bacterial cells

While both ϕC31 and R4 integrases are active in bacterial cells, neither should be expressed within bacteria from our heat shock promoter helper plasmids. To assure that the ϕC31 integrase-mediated and R4 integrase-mediated site-specific integration events that we were detecting actually resulted from recombinations within the transfected insect cells and not upon transformation of the bacteria, we coelectroporated the integrase and assay plasmids directly into DH10B *E. coli* and examined the resulting colonies for evidence of recombination events. No white colonies were present on ampicillin/chloramphenicol/X-Gal plates out of total colonies recovered confirming that the site-specific recombination events

recovered in our assays could not have arisen from recombination in bacterial cells following harvesting plasmids from the transfected insect cells.

DISCUSSION

Integrase activity assays are greatly influenced by the ability to effectively transfect the plasmid DNA into target cells. A number of diverse methods have been explored for introducing DNA into eukaryotic cells including the use of calcium phosphate or other divalent cations, polycations, liposomes, retroviruses, microinjection and electroporation. An important addition to repertoire of DNA-transfection methodologies is cationic liposome-mediated transfection (lipofection) such as TransFectin Lipid Reagent and DOTAP liposomal Transfection Reagent. For this study, we tested the abilities of these liposome reagents to mediate transfection of firefly luciferase-expressing plasmids into seven of the most widely used insect cell lines.

The promoters chosen for the firefly luciferase (Fluc) reporter plasmids were based upon previous observations of promoter activity in a variety of cell lines (Burn *et al*, 1989; Han *et al*, 1989; Chung and Keller, 1990; Huynh and Zieler, 1999; Zhao and Eggleston, 1999). For most cell lines in this study the *Drosophila* actin 5C promoter proved adequate in reporting firefly luciferase activity. However, this promoter was inadequate for expressing Fluc in *Bombyx mori* cells (data not shown) and we chose instead to utilize the *B. mori* actin 3C promoter previously characterized (Zhang *et al*, 2008).

The transfection frequency obtained with TransFectin was greater for most insect cell lines with the notable exception of the mosquito C6/36 cell line. There are several possibilities that make the difference of

Fig 5–PCR analysis of R4-mediated site-specific recombination in a variety of insect cells. A 678-bp product is shown for the site-specific recombination. Lane 1, positive control, *in vitro*-reacted pBC-P64-B295; lane 2, *in vitro*-reacted pBC-P64-B295 from Aag2 cells; lane 3, *in vitro*-reacted pBC-P64-B295 from ATC-10 cells; lane 4, *in vitro*-reacted pBC-P64-B295 from BmN$_4$ cells; lane 5, *in vitro*-reacted pBC-P64-B295 from C6/36 cells; lane 6, *in vitro*-reacted pBC-P64-B295 from S2 cells; lane 7, *in vitro*-reacted pBC-P64-B295 from Sf9 cells; lane 8, *in vitro*-reacted pBC-P64-B295from Sua5B cells; lane 9, negative control, in *vitro*-transfected pBC-P64-B295; lane 10, negative control, *in vitro*- transfected phsp-sre; lane M, size markers.

both transfection frequencies. It might have to optimize transfection conditions of both transfection methods and cell types including cell density, duration of transfection, volume of medium during transfection and ratio of lipid reagent to DNA that are key factors for transfection efficiency. For TransFectin, it is a mixture of a proprietary cationic lipid and colipid DOPE (1,2-dioleoyl-phosphatidylethanolamine). When cationic lipids are brought into contact with aqueous solutions under special conditions, they form positively charged micelles or liposomes. These micelles associate with the negatively charged phosphates of nucleic

acids and form spontaneous complexes with DNA or RNA. The DNA-liposome complexes then fuse with cell membrane via hydrophobic and electrostatic interactions, and the complex is then internalized (Remy *et al*, 1994; Zhou and Huang, 1994). For DOTAP, it is aqueous dispersion (liposomes) in MBS (MES-buffered saline) that there might be some differences from TransFectin in formulation like colipid. In various reported studies, cationic liposomes function most efficiently when cationic lipid is mixed with a helper lipid or colipid like DOPE that is most commonly used in applications (Legendre and Szoka, 1993; Felgner *et al*, 1994; Farhood *et al*, 1995). DOPE is generally believed to rest on its propensity to form nonbilayer structures that are akin to membrane fusion intermediates (Hui *et al*, 1981). It is thought to help facilitate the fusion of cationic liposome in DNA-cationic liposome complexes to cell membrane. Therefore, the fusion between DNA-cationic liposomes and the cell membrane appeared from the use of TransFectin might play better role in gene delivery, expression including in determining transfection efficiency for most insect cells except C6/36 than that occurred from DOTAP. For C6/36 cell transfection, DOTAP works better than TransFectin. It might be because of cell membrane constituents and properties of C6/36 cells to be the critical barriers of cationic liposome-mediated gene delivery. DOTAP formulation might provide the DNA-cationic liposome complexes to better fuse with C6/36 cell type than other cell types resulting in being more taken by cells. Based on the transfection efficiency results, TransFectin was chosen for DNA transfection of Aag2, ATC-10, BmN_4, S2, Sf9 and Sua5B cell lines while DOTAP was used only for C6/36 transfection.

Previous work had shown that the serine-catalyzed ϕC31 integrase could occasionally integrate *attB* into endogenous sequences with partial sequence identity to *attP*, pseudo-*attP* sites of *Drosophila* (Groth *et al*, 2004), bovine cells (Ma *et al*, 2006; Ou *et al*, 2009) and mammalian cells (Thyagarajan *et al*, 2001). The genomic integration mediated by ϕC31 integtrase was also detected by long-term expression of luciferase over the 4-week time course (Thyagarajan *et al*, 2001; Thyagarajan and Calos, 2005). This ability of ϕC31 integrase to integrate into pseudo *attP* sites has been used in gene therapy experiments in mouse, human keratinocytes (Olivares *et al*, 2002; Ortiz-Urda *et al*, 2002, 2003; Quenneville *et al*, 2004; Held *et al*, 2005; Bertoni *et al*, 2006), and rat retina (Chalberg *et al*, 2005). The ϕC31 integrase system also produces stable transgene expression in adult mouse neural progenitor cells (mNPCs) and their progeny that may be useful in strategies for combating neurodegenerative disorders (Keravala *et al*, 2008).

Previous studies have demonstrated the potential for ϕC31 integrase to mediate site-specific recombination in some insect species. This integrase mediated site-specific integration of two ϕC31 *attP* flanking DsRed gene into the cultured silkworm cell line, BmN4, in which ϕC31 *attB* sites inserted between a baculovirus IE2 promoter and a polyadenylation signal are present in one chromosome (Nakayama *et al*, 2006). ϕC31 integrase was employed to target the *P* element-predetermined *attP* sites in transgenic *Drosophila* by injecting integrase mRNA together with ϕC31 *attB*, resulting in up to 55% of fertile adults producing transgenic offspring (Groth *et al*, 2004). The ϕC31 integrase system was used in conjunction with recombinase-mediated cassette exchange (RMCE) for precise targeting of transgenic construct to predetermined genomic sites in *Drosophila* that the efficient RMCE events were observed at all four genomic target sites tested and the transgene lacking a visible

marker can be integrated with high efficiency by selecting only for a phenotypic marker in genomic target (Bateman *et al*, 2006, Bateman and Wu, 2008). It has also been shown as improved current method to reproducibly produce transgenic *Ae. aegypti* in range of 17-32% (Nimmo *et al*, 2006).

Actually, transgenesis has been performed through many techniques. In *Drosophila*, transgenesis mostly relies on *P* element transposon. This transposon was introduced and has been one of the most important breakthroughs in germ line transgenesis in *Drosophila*. *P* element belongs to a group of transposable elements (designated class II, Finnegan, 1992). It contains two terminal repeats, including inverted repeat sequences and other internally located sequences motifs absolutely required for their mobilization and transposition (Beall and Rio, 1997).

Attempts to transform *Aedes* mosquitoes began in the late 1980s using the *P* element from *D. melanogaster* (Morris *et al*, 1989). This element would mobilize and integrate into the mosquito genome. The result of transformed lines containing exogenous constructs was obtained after microinjecting embryos with plasmid DNA. However, transformed lines were obtained at frequencies of less than 0.1% of the embryos injected and transformation appeared not be *P*-element mediated, but to result from illegitimate recombination. The low frequency of integration precludes the use of this approach for the routine transformation of mosquitoes.

Because of the failure of *P* element, many other methods of transformation have been developed. Transformation of *Ae. aegypti* and other mosquitoes was made possible by the isolation and characterization of additional class II transposable elements such as *Hermes*, *Mariner*, *Minos* and *piggyBac* (Franz and Savakis, 1991; Medhora *et al*, 1991; Atkinson *et al*, 1993; Fraser *et al*, 1996). These transposons were first discovered in insects, all except *piggyBac* belong to large families of elements that appear widespread throughout eukaryotes. Having learned a lesson from the many failed experiments with *P* element, researchers first tested these new elements for mobility with *in vivo* transposition assays. These experiments involve microinjecting embryos with three different plasmids, a donor that contains a marker gene flanked by the ITRs (inverted terminal repeats) of the transposable element, a target that carries a number of marker genes distinct from the donor, and a helper that contains the appropriate transposase under the control of an inducible promoter (O'Brochta and Handler, 1988). Induction of the transposase mobilizes the marker gene construct from the donor to the target. Successful transposition assays in *Ae. aegypti* embryos were reported for *Hermes* (Sarkar *et al*, 1997) and *Mariner* (Coates *et al*, 1998).

The *piggyBac* element is the only identified mobile member of a larger family of what are now called TTAA-specific elements (Fraser, 2000). This short inverted terminal repeat element is 2.5 kb long, having a paired inverted terminal repeat configuration, a 2.1 kb ORF and specificity for the target sequence TTAA which it duplicates upon insertion and precisely regenerates upon excision (Cary *et al*, 1989; Elick *et al*, 1996; Fraser *et al*, 1996). Both excision and interplasmid transposition assays indicate that this element is mobile in a wide variety of insects (Lobo *et al*, 1999; Lobo N, unpublished data) suggesting a ubiquitous mechanism of mobilization.

piggyBac has an experimentally demonstrated capability to mediate germ-line transformation in a myriad of insects including the Mediterranean fruit fly, *Ceratitis capitata* (Handler *et al*, 1998), the pink bollworm, *Pectinophora gossypiella* (Peloquin *et*

al, 2000), *D. melanogaster* (Handler and Harrell, 1999), the silkworm, *B. mori* (Tamura *et al*, 2000), the Caribbean fruit fly, *Anastrepha suspense* (Handler and Harrell, 2001), the Oriental fruit fly, *Bactrocera dorsalis* (Handler and McCombs, 2000), the housefly, *Musca domestica* (Hediger *et al*, 2001) and the mosquitoes, *Ae. aegypti* (Lobo *et al*, 1999, 2001, 2002; Kokoza *et al*, 2001; Nimmo *et al*, 2006; Sethuraman *et al*, 2007), *Ae. albopictus* (Lobo *et al*, 2001), *Ae. triseriatus* (Lobo *et al*, 2001), *An. gambiae*, (Grossman *et al*, 2001), *An. albimanus* (Perera *et al*, 2002) and *An. stephensi* (Nolan *et al*, 2002).

In this study, we demonstrated the ability of ϕC31 integrase to mediate unidirectional site specific recombination in 5 Dipteran cell lines *Ae. aegypti* Aag2, *Ae. aegypti* ATC-10, *Ae. albopictus* C6/36 , *An. gambiae* Sua5B, as well as *D. melanogaster* S2. We also demonstrated the effectiveness of this integrase in two Lepidopteran cell lines *B. mori* BMN_4, and *S. frugiperda* Sf9. Based upon these and previous analyses, there is ample evidence that this integrase can provide for effective integration in virtually any insect species.

This study also establishes that the R4 integrase derived from *S. parvulus* phage R4, like the ϕC31 integrase, mediates site-specific recombination in the 7 assayed insect cell lines. As with ϕC31, the R4 integrase has wide effective host range (Olivares *et al*, 2001). The ability of these integrases to function in such a wide range of species is directly related to their lack of cofactor requirement (Thrope and Smith, 1998).

Because the transfection abilities varied greatly among the cells examined, it is impossible to directly compare the relative effectiveness of ϕC31 integrase among these cells. However, there may be a correlation between the relative recombination frequencies and expression of the integrase from the hsp70 promoter. According to our results these integrases seem to perform best in *Drosophila* S2 cells, exhibiting a statistically significant higher integration frequency. This may be due to the relative hsp70 promoter activity in each of these cell lines. The hsp70 promoter was first discovered as a heat-induced puff on *Drosophila* polytene chromosomes (Ritossa, 1962). Since hsp70 promoter originated from *Drosophila* as a strong promoter, it is most commonly used promoter for transgenic work in *D. melanogaster* and with *Drosophila* cell lines (Cobreros *et al*, 2008; Siddique *et al*, 2008; Singh *et al*, 2008). It is heat-inducible and functions at all stages of development and in all *Drosophila* tissues (Di Nocera and Dawid, 1983; Steller and Pirrotta, 1984; Spradling, 1986; Lindquist and Craig, 1988). The hsp70 promoter has been widely used in mosquito cells (Lycett and Crampton, 1993; Shotkoski *et al*, 1996; Zhao and Eggleston, 1999), transgenic *Ae. aegypti* (Pinkerton *et al*, 2000; Nimmo *et al*, 2006; Sethuraman *et al*, 2007), *An. gambiae* cells and embryos (Miller *et al*, 1987; Windbichler *et al*, 2007) and lepidopteran cell lines and larvae (Helgen and Fallon, 1990; Morris and Miller, 1992; Plymale *et al*, 2008). Also, it was employed in driving the gene expression in *S. frugiperda* Sf9 cells (Sah *et al*, 1999) and transgenic silkworm, *B. mori*, (Suzuki *et al*, 2005; Dai *et al*, 2007). Because of its applicability in many insects, we chose hsp70 promoter to drive the expression of integrase genes for mediating the site-specific integration in a variety of insect cell lines. Phage ϕC31 and R4 integrases may come to represent a powerful site-specific integration tools for genetic manipulation of medically and economically important organisms.

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