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L.C. Thomason · R. Calendar · D.W. Ow

Gene insertion and replacement in *Schizosaccharomyces pombe* mediated by the *Streptomyces* bacteriophage ϕC31 site-specific recombination system

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Abstract The site-specific recombination system used by the *Streptomyces* bacteriophage ϕC31 was tested in the fission yeast *Schizosaccharomyces pombe*. A target strain with the phage attachment site *attP* inserted at the *leu1* locus was co-transformed with one plasmid containing the bacterial attachment site *attB* linked to a *ura4*$^+$ marker, and a second plasmid expressing the ϕC31 integrase gene. High-efficiency transformation to the Ura$^+$ phenotype occurred when the integrase gene was expressed. Southern analysis revealed that the *attB-ura4*$^+$ plasmid integrated into the chromosomal *attP* site. Sequence analysis showed that the *attB*×*attP* recombination was precise. In another approach, DNA with a *ura4*$^+$ marker flanked by two *attB* sites in direct orientation was used to transform *S. pombe* cells bearing an *attP* duplication. The ϕC31 integrase catalyzed two reciprocal cross-overs, resulting in a precise gene replacement. The site-specific insertions are stable, as no excision (the reverse reaction) was observed on maintenance of the integrase gene in the integrant lines. The irreversibility of the ϕC31 site-specific recombination system sets it apart from other systems currently used in eukaryotic cells, which reverse readily. Deployment of the ϕC31 recombination provides new opportunities for directing transgene and chromosome rearrangements in eukaryotic systems.

Keywords Site-specific integration · Gene targeting · Transgene · Transformation · Phage phiC31

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L.C. Thomason[1] · R. Calendar
Department of Molecular and Cell Biology,
University of California, Berkeley, CA 94720-3202, USA

D.W. Ow (✉)
Plant Gene Expression Center,
U.S. Department of Agriculture,
and Department of Plant and Microbial Biology,
University of California, 800 Buchanan St.,
Albany, CA 94710, USA
E-mail: ow@pgec.ars.usda.gov
Tel.: +1-510-5595909
Fax: +1-510-5595678

Present address:
[1]Molecular Sciences Institute Inc.,
2168 Shattuck Ave. (2nd Floor), Berkeley, CA 94704, USA

Introduction

Many bacteriophages and integrative plasmids encode site-specific recombination systems. In these systems, the minimal requirements for the reaction are two recombination sites and a recombinase or integrase that catalyzes the recombination event (Sadowski 1993). For phage integration systems, the sites are referred to as attachment (*att*) sites, with an *attP* element from phage DNA and an *attB* element from the bacterial genome. The two attachment sites can share as little sequence identity as a few base pairs. The integrase protein binds to both *att* sites and catalyzes a conservative and reciprocal exchange of DNA strands resulting in integration of the circular phage DNA into host DNA. Additional host factors, such as the DNA bending protein IHF (*i*ntegration *h*ost *f*actor), may be required for an efficient reaction (Friedman 1988; Finkel and Johnson 1992). The reverse excision reaction may require an additional phage factor, such as the *xis* gene product of phage λ (Weisberg and Landy 1983; Landy 1989).

The recombinases have been categorized into two groups, the λ integrase (Argos et al. 1996; Voziyanov et al. 1999) and the resolvase/invertase (Hatfull et al. 1988) families. These vary in the structure of the integrase enzymes and the molecular details of their mode of catalysis (Stark et al. 1992). The temperate *Streptomyces* phage ϕC31 encodes a 68-kDa recombinase of the latter class (Kuhstoss and Rao 1991; Rausch and Lehmann 1991). The efficacy of the ϕC31 integrase enzyme in recombining its cognate attachment sites was demonstrated both in vitro and in vivo in *recA* mutant *Escherichia coli* (Thorpe and Smith 1998). More

recently, ϕC31-mediated site-specific recombination has been reported between introduced plasmid DNA and episomal vectors in human cells (Groth et al. 2000). The ϕC31 integration reaction does not require host factors and appears irreversible, probably because an additional phage protein is required for excision. The phage and bacterial *att* sites share 3 bp of identity at the point of cross-over, and a total of only 16 bp of identity within a 50-bp span. For both attachment sites, the cross-over point is flanked by an inverted repeat that presumably provides two binding sites for the integrase protein. The extent and exact positions of the palindromes are unique to each site.

A number of site-specific recombination systems operate in eukaryotic cells. The Cre-*lox* system of bacteriophage P1, and the FLP-*FRT* system of *Saccharomyces cerevisiae* are widely used for transgene and chromosome engineering in animals and plants (Sauer 1994; Ow 1996). Other systems that operate in animal or plant cells include (1) the R-*Rs* system from *Zygosaccharomyces rouxii* (Onouchi et al. 1995); (2) the Gin-*gix* system from bacteriophage Mu (Maeser and Kahmann 1991); and (3) the β recombinase-*six* system from the bacterial plasmid pSM19035 (Diaz et al. 1999). These five systems have the common property that a single polypeptide recombinase catalyzes the recombination between two sites that are identical or nearly identical in sequence. The product sites generated by recombination are themselves substrates for subsequent recombination. Consequently, recombination reactions are readily reversible. Since intramolecular interactions are kinetically favored over intermolecular interactions, these recombination systems are efficient for deleting rather than integrating DNA.

In contrast, the ϕC31 recombinase has been reported to catalyze only the *attB*×*attP* reaction, and not the reverse reaction (Thorpe et al. 2000). The property of irreversibility would be most useful for integration, inversion and translocation reactions. Our ultimate aim is to develop this system for use in higher plants. As a first step, we tested different integration strategies in a simpler eukaryotic model system. Here, we present results on ϕC31 integrase-mediated delivery of circular or linear DNA into a chromosomal target in the fission yeast *Schizosaccharomyces pombe*. Moreover, we used a genetic test that is capable of detecting the reverse reaction, but did not find any evidence that it occurs. In combination with recent results from human cells (Groth et al. 2000), this demonstration opens up the prospect of deploying the ϕC31 system for the manipulation of transgenes and chromosomes of plants and animals.

Materials and methods

Recombinant DNA

Standard methods for DNA manipulation were used throughout. The *E. coli* strain XL2-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q} *Z*Δ*M15* Tn*10* (Tet^r) Amy Cam^r]) (Stratagene) served as the cloning host for DNA constructs.

Media

Fission yeast strains were grown on minimal medium (EMM-low glucose, obtained from Bio101) supplemented as needed with 225 mg/l adenine, histidine, leucine or uracil. Minimal plates containing 5-FOA (5-fluoro-orotic acid, obtained from Zymo Research) were prepared as described (Grimm et al. 1988) and were supplemented with adenine, histidine, and leucine. When required, thiamine was added to a final concentration of 5 μg/ml.

S. pombe ϕC31 *attP* target strains

The 84-bp ϕC31 *attP* site (abbreviated as PP'), isolated as an *Apa*I-*Sac*I fragment from pHS282 (Thorpe and Smith 1998), was cloned into the corresponding sites in the *S. pombe* integrating vector pJK148 (Keeney and Boeke 1994), to yield pLT44 (Fig. 1A). This plasmid was targeted to the *S. pombe leu1-32* allele by lithium acetate-mediated transformation with *Nde*I-cleaved DNA. The recipient strain FY527 (*h*$^-$ *ade6-M216 his3-D1 leu1-32 ura4-D18*, obtained from S. Forsburg), converted to Leu$^+$ by homologous recombination with pLT44, was examined by Southern analysis. One Leu$^+$ transformant, designated FY527attP (Fig. 1B), was found to contain a single copy of pLT44. Another transformant, designated FY527attPx2 (see below), harbors a tandem plasmid insertion.

Integrative *ura4*$^+$ vectors with single or dual ϕC31 *attB* sites

The plasmid pLT45 (Fig. 1B) was constructed as follows. The *S. pombe ura4*$^+$ gene, excised from pTZ*ura4* (S. Forsburg) on a 1.8-kb *Eco*RI-*Bam*HI fragment, was inserted into pJK148 cut with the same enzymes, to create pLT40. The ϕC31 *attB* site (abbreviated as BB'), isolated from pHS21 as a 500-bp *Bam*HI-*Xba*I fragment, was ligated into pLT40 cut with those enzymes, creating pLT42. Most of the *leu1* gene was removed from pLT42 by deleting an *Xho*I fragment, to create pLT45 (Fig. 1B). This left only 229 bp of *leu1* in pLT45 and reduced its transformation efficiency to that of a plasmid without any *leu1* homology.

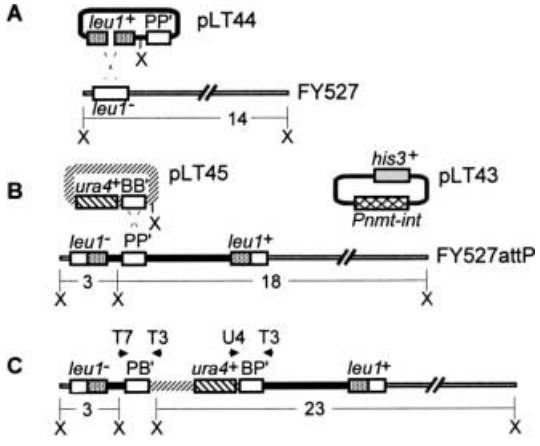

Fig. 1A–C Schematic (not to scale) representation of the chromosome structure at the *S. pombe leu1* locus. Homologous insertion of pLT44 into the chromosome (**A**) places a ϕC31 *attP* target between *leu1* alleles as shown in **B**. pLT43 promoted site-specific integration of pLT45 into the chromosomal *attP* target leads to the structure shown in **C**. The *arrowheads* indicate PCR primers corresponding to the T7 promoter (T7), T3 promoter (T3) and *ura4*$^+$ coding region (U4). Predicted sizes of *Xba*I (X) cleavage products are indicated

The plasmid pLT50 was generated in the following manner. A *Bam*HI-*Sac*I *attB* fragment from pLT42 was inserted into pUC19, retrieved as an *EcoR*I-*Sal*I fragment, and inserted into pLT45 cut with *EcoR*I and *Xho*I. The second *attB* site in the final construct was sequenced once on each strand and found to be identical to the first *attB* site.

Transformation with linear DNA

The *attB-ura4*$^+$*-attB* linear DNA was prepared as an *Att*II-*Alw*NI fragment purified from pLT50, or as a PCR product using pLT50 as template. PCR was conducted using standard conditions with a T3 primer and a second primer (5′-GGCCCTGAAATTGTTGC TTCTGCC-3′) corresponding to the plasmid backbone of pJK148.

Repressible synthesis of ϕC31 integrase

The *S. pombe Pnmt* promoter, which is repressible with vitamin B1, was excised as a 1.2-kb *Pst*I-*Sac*I fragment frompMO147 (from S. Forsburg) and inserted into the *his3*$^+$ *ars1* vector pBG2 (Ohi et al. 1996) cut with the same enzymes, creating pLT41. A 2.0-kb *Sac*I fragment containing the ϕC31 *int* coding region was transferred from pHS33 (Thorpe and Smith 1998) to the *Sac*I site of pLT41. A clone carrying the integrase ORF under the control of *Pnmt* was designated pLT43 (Fig. 1B).

Molecular analyses

Southern analysis was performed using the Genius system from Boehringer Mannheim. A 998-bp internal *Eco*RV fragment of *leu1*, a 1.8-kb fragment of *ura4*, and the 2.0-kb ϕC31 *int* gene were labeled with digoxigenin by the random primer method and used as probes. PCR was performed on a Perkin Elmer Cetus Gene Amp PCR 9600 using Stratagene Turbo PFU enzyme or VENT polymerase. The standard T3 and T7 primers were used where possible. The *ura4* primer (5′-GTCAAAAAGTTTCGTCAATATCAC-3′) and the pJK148 primers were purchased from Operon Technologies. For all PCRs, an annealing temperature of 51°C and a 30-s extension time were used. DNA sequence analysis was conducted by the U.C. Berkeley Sequencing Facility.

Results and discussion

Inserting a target site into the *S. pombe* genome

To create a host strain with a target site for ϕC31-mediated integration, the ϕC31 *attP* site (PP') was inserted by homologous recombination into the *leu1* locus of the fission yeast genome, to form the Leu$^+$ strain FY527attP (Fig. 1A). Previous studies (Keeney and Boeke 1994) had shown that when *S. pombe* DNA is cleaved with *Xba*I and probed with an internal 1-kb fragment of the *leu1*$^+$ gene, the probe detects a 14-kb band. When a Leu$^+$ plasmid, pJK148, is inserted at the *leu1-32* locus, the same probe detects 3-kb and 18-kb bands (Fig. 1B). Since pLT44 differs from pJK148 only by the inclusion of an 84-bp ϕC31 *attP* sequence, integration of pLT44 at *leu1-32* yields the same 3-kb and 18-kb hybridizing bands for FY527attP (Fig. 2A, panel P). The absence of other hybridizing fragments indicates that the pLT44 DNA is present as a single integrated copy.

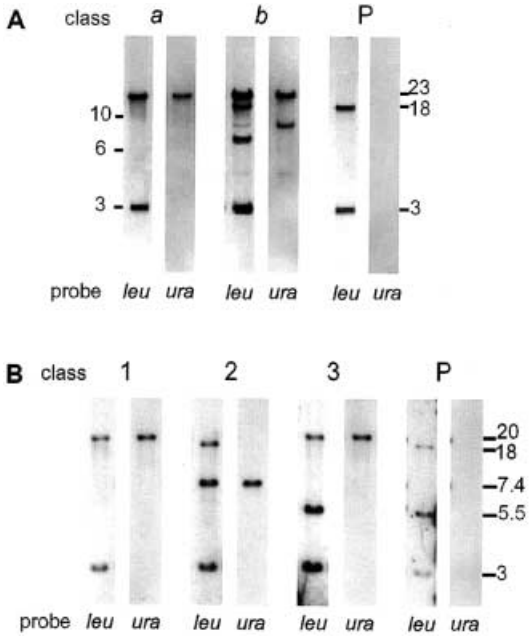

Fig. 2A, B Representative Southern blots of DNA cleaved with *Xba*I and probed with *leu1* or *ura4*. **A** FY527attP co-transformed with pLT45 (and pLT43) and inserting precisely as depicted in Fig. 1C (class a), or precisely but with additional copies or rearrangements at the locus (class b). Panel P shows the parental configuration in FY527attP. **B** FY527attPx2 transformed with linear *attB-ura4*$^+$*-attB* DNA (together with pLT43). Class 1, 2, and 3 structures are depicted in Fig. 3C, E, and F, respectively

ϕC31-integase-mediated transformation

FY527attP was transformed with pLT45, which harbors *ura4*$^+$ and an *attB* sequence (BB'), but lacks an origin of replication for *S. pombe*. This construct was introduced by itself or together with pLT43, a replicating *his3*$^+$ vector that produces ϕC31 integrase (Fig. 1B). The inclusion of pLT43 increased the number of Ura$^+$ transformants by 17-fold on average (Table 1). This enhancement cannot be attributed to recombination between pLT45 and the replication-proficient pLT43, as the effect is dependent on integrase gene expression. The integrase gene is transcribed from *Pnmt*, a promoter that is repressible by high levels of vitamin B1 (Maundrell 1993). The repression is not absolute (Forsburg 1993) but should reduce the production of integrase protein. When thiamine was added to the growth medium, the number of Ura$^+$ transformants decreased to near background level. The frequency of Ura$^+$ transformants did not change significantly whether or not the integrase plasmid was co-selected by omission of histidine from the medium. The transformation competency of FY527attP was estimated from the number of His$^+$ transformants obtained with pLT43 or its progenitor plasmid pBG2. Compared to the frequency of either replicating plasmid, the pLT43-dependent transformation of FY527attP averaged about 15%. Thiamine did not affect the transformation efficiency of the replicating plasmids (data not shown).

Table 1 Integrase-dependent site-specific insertion in *S. pombe* FY527attP

DNA (1 μg)	Selection	Vitamin B1 (5 μg)	Number of transformants per 10^7 cells (±SD)[a]	Relative value[b]	Class a	Class b	Others
pLT43	His^+	–	7200 (±2200)	100	–	–	–
pLT45	Ura^+	–	63 (±10)	0.88	0%[c]	0%[c]	100%[c]
pLT45 + pLT43	Ura^+	–	1100 (±120)	15	88%[d]	6%[d]	6%[d]
pLT45 + pLT43	Ura^+	+	120 (±16)	1.7	0%[c]	25%[c]	75%[c]

[a]Based on three independent experiments
[b]Expressed as (transformation efficiency of the DNA of interest/transformation efficiency of pLT43) × 100
[c]Number analyzed = 8
[d]Number analyzed = 16

φC31-integrase promoted *attP*×*attB* recombination

Recombination between the pLT45-encoded φC31 *attB* element and the chromosomal *attP* sequence (Fig. 1B) would incorporate the circular DNA into the *leu1* locus (Fig. 1C). If this reaction occurs, when *Xba*I-cleaved genomic DNA from the Ura^+ transformants is probed with *leu1* DNA, the 3-kb band will remain unchanged, while the 18-kb band should increase in size to 23 kb. Randomly selected Ura^+ colonies from each of the transformation experiments were examined by hybridization analysis.

In the pLT45 + pLT43 transformations, seven out of eight isolates derived from plates without thiamine showed the presence of this 23-kb band (Fig. 2A, class a). This same size band hybridized to the *ura4* probe. This contrasts with the lack of *ura4* hybridization with the parental strain (Fig. 2A, panel P), as expected from the fact that it carries the *ura4-D18* deletion allele. One of these seven isolates showed additional bands hybridizing to both probes (Fig. 2A, class b). This candidate appears to have a DNA rearrangement at the *leu1* locus in addition to a site-specific recombination event. The *leu1* rearrangement was probably catalyzed by the *S. pombe* homologous recombination system. The remaining isolate had not undergone a site-specific recombination event and appeared to have become prototrophic for uracil by recombination between pLT45 and pLT43. As these clones were selected as Ura^+, they could harbor pLT43 in the genome. Hybridization of the blot with an integrase gene probe showed that five of the eight isolates retained the His^+ plasmid.

Since the integrase gene is not needed after DNA integration, and the His^+ plasmid can be segregated away, we tested a second set of integrants that had lost the integrase plasmid. Ura^+ clones were grown non-selectively for a number of generations and screened for loss of pLT43, scored as a His^- phenotype. The analysis of eight representative Ura^+ His^- clones showed that all had a single copy of pLT45 precisely integrated at the chromosomal *attP* site (as in Fig. 2A, class a), and genomic DNA from these integrants did not hybridize with the integrase probe.

In pLT45 + pLT43 transformation experiments where vitamin B1 was included, the thiamine-repressible *Pnmt* promoter is expected to limit integrase production, and thereby hinder site-specific integration. Out of eight Ura^+ transformants examined by DNA hybridization, two Ura^+ candidates showed a band of 23 kb hybridizing to *leu1* and *ura4* probes (not shown). However, since both probes detected an additional band, these transformants have not undergone correct integration events, and we grouped them as class b integrants. In the other six isolates, the hybridization patterns are difficult to interpret (not shown). In some of them, the 3-kb band was not detected by the *leu1* probe, suggesting that the locus had been subjected to some rearrangement. In many of them, the weak hybridization to *ura4* suggested that the Ura^+ phenotype might not be due to the stable maintenance of pLT45 in the genome.

In pLT45-only transformations, site-specific recombination was not expected. Indeed, the Ura^+ clones gave the parental configuration of hybridizing bands at the *leu1* locus and additional faint bands at 5 kb and 7 kb (not shown). These observations are consistent with either integration of pLT45 elsewhere in the genome, or maintenance of the plasmid in some cells despite its lack of a *S. pombe* replication origin.

Conservative site-specific recombination

PCR was used to retrieve the *attP*/*attB* recombinant junctions from three representative Ura^+ candidates. One of the hybrid sites, *attR* (PB'), should be flanked by T3 and T7 promoters; the other site, *attL* (BP'), by the T3 promoter and *ura4* DNA (Fig. 1C). In each case, primer pairs directed to these sequences amplified a band of the expected size, while the original *attP* (PP') was no longer found (not shown). This contrasts with the parental strain FY527attP, in which *attP*, but neither *attL* nor *attR*, was detected. The nucleotide sequence of three representative *attL* and *attR* PCR products showed the absence of accompanying mutations (not shown). Hence, as in bacteria and mammalian cells, φC31-mediated site-specific recombination in *S. pombe* is a conservative recombination reaction.

Gene replacement via linear DNA

Certain gene transfer techniques, such the biolistic delivery of DNA into plant cells, are invariably associated with the insertion of a large number of linked DNA molecules. Such multicopy insertions are thought to be due to the concatemerization of DNA substrates before they interact with the host chromosome. The resulting substrate macromolecule may not necessarily be circular. In such a situation, few of the transformation substrates would be capable of inserting into atarget site as an intact single-copy circular molecule. On the other hand, a gene replacement-type of insertion might be less affected by concatemeric DNA. A unit copy of the transforming substrate within a large concatemer can recombine into a host site through recombination at sites that flank the unit copy.

A replacement strategy could also be used to deliver linear DNAs into a target cell. This should be possible if identical attachment sites (for example, two *attB* sequences) are provided, in direct orientation, at each end of a linear DNA substrate, and this molecule is transformed into a strain bearing a directly oriented tandem pair of chromosomally located target *attP* sites. This should result in replacement of the DNA between the chromosomal sites with the transforming DNA.

To test whether such a gene-replacement reaction is efficient, we isolated an FY527 derivative bearing a tandem insertion of pLT44. This strain, designated FY527attPx2, has two *attP* sites in direct orientation at the *leu1* locus, separated by a *leu1* gene and vector sequences (Fig. 3B). FY527attPx2 was transformed with linear DNA containing *ura4*$^+$ flanked by *attB* sites. The linear substrate was obtained either as a gel-purified fragment from pLT50 (Fig. 3A) or amplified as a PCR product from this plasmid. The plasmid pLT50, derived from pLT45, has a second directly oriented *attB* site on the other side of the *ura4*$^+$ gene. Both linear substrates gave approximately the same transformation efficiency when co-transformed with pLT43, which increased the number of Ura$^+$ transformants obtained (Table 2). In some experiments, the frequency was nearly as high as that for the replicating plasmid control.

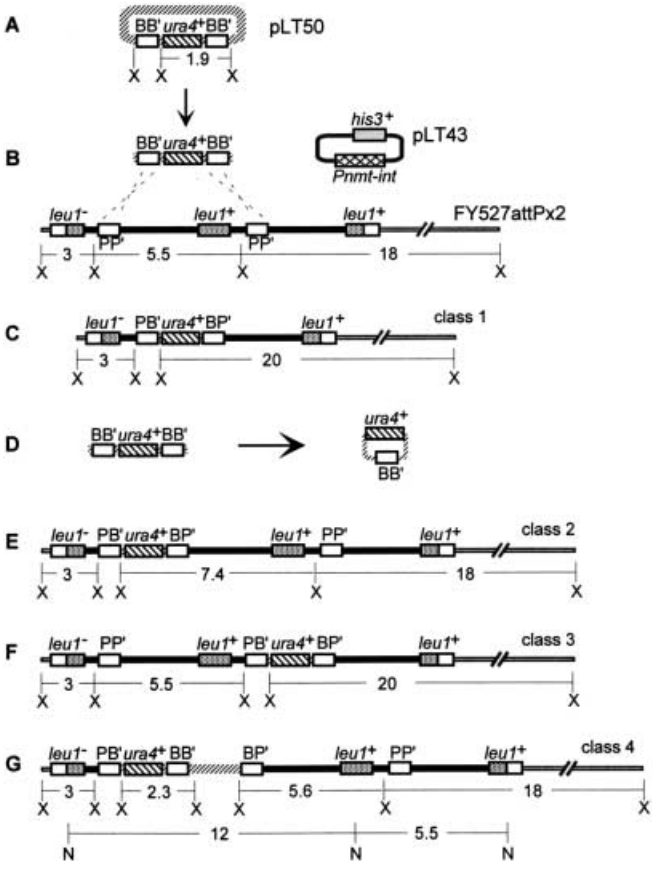

Fig. 3A–G Schematic (not to scale) representation of the dual-site recombination strategy at the *S. pombe leu1* locus. The linear *attB-ura4*$^+$*-attB* DNA (**B**), derived from pLT50 (**A**) recombines at both ends, resulting in a precise gene replacement (**C**, class 1). Alternatively, it forms a circular intermediate (**D**) prior to insertion into either the 5'*attP* (**E**, class 2) or the 3'*attP* site (**F**, class 3) of the target locus. In one clone, a single recombination between the 5'*attB* site of pLT50 and the 5'*attP* site of *leu1* locus produces the structure shown in **G** (class 4). Predicted sizes of *Xba*I (X) or *Nde*I (N) cleavage products are shown

Table 2 Integrase-dependent gene replacement in *S. pombe* FY527attPx2

DNA (1 μg)	Selection	Number of transformants per 10^7 cells (±sd)[a]	Relative value[b]	Class 1	Class 2	Class 3	Other
pLT43	His$^+$	4106 (±331)	100	–	–	–	–
Linear fragment	Ura$^+$	19 (±27)	0.46	–	–	–	–
Linear fragment + pLT43	Ura$^+$	1568 (±495)	38	38%[c]	12%[c]	38%[c]	12%[c]
pLT50	Ura$^+$	63 (±52)	1.5	–	–	–	–
pLT50 + pLT43	Ura$^+$	2560 (±919)	62	38%[c]	12%[c]	25%[c]	25%[c]
pLT45	Ura$^+$	66 (±46)	1.6	–	–	–	–
pLT45 + pLT43	Ura$^+$	683 (±298)	17	–	–	–	–

[a]Based on three independent experiments
[b]Expressed as (transformation efficiency of the DNA of interest)/(transformation efficiency of pLT43) × 100
[c]Number analyzed = 8

The intended gene replacement event is diagrammed in Fig. 3B, with recombination occurring between two 5′ sites and two 3′ sites (leftmost and rightmost, respectively, in Fig. 3B). Although the two cross-overs may occur sequentially rather than concurrently, the end product is the same (Fig. 3C, class 1). When the *Xba*I restriction pattern of eight representative Ura$^+$ His$^-$ clones was examined, seven showed patterns falling into three classes. Three of them had the class 1 pattern, in which the *leu1* probe hybridized to bands of 3 kb and 20 kb, and the *ura4* probe hybridized to a 20-kb band (Fig. 2B, class 1). The second and third classes represent events that appear to result from prior circularization of the linear fragment before site-specific insertion into an *attP* target. Figure 2D depicts the circularization reaction that would result from recombination between the duplicated *attB* sites. Integration of the circle into the 5′ *attP* site increases the size of the 5.5-kb plasmid band to 7.4 kb (Fig. 3E); this band would hybridize with both the *ura4* and *leu1* probes (Fig. 2B, class 2). This pattern was found in one transformant. Integration into the 3′ *attP* site increases the size of the 18-kb band to 20 kb (Fig. 3F), and allows its detection by both probes (Fig. 2F, class 3). This pattern was found in three transformants. The remaining clone had two copies of *ura4* and an additional copy of *leu1*, suggesting gene amplification at the *leu1* locus. It was not analyzed further.

Gene replacement *via* circular DNA

The class 2 and class 3 structures recovered following transformation with linear DNA suggest that a circular intermediate is formed, yet the linear fragment does not have complementary single-stranded ends that could anneal. The molecular structure is consistent with either intramolecular recombination between the *attB* sites, or some sort of ligation between the two ends. One possibility is that the high rate of circularization may be promoted by linear DNA ends. Linear ends may be more proficient at strand invasion or end joining, since double-strand breaks stimulate recombination in yeast (Szostak et al. 1983). If this were true, the incidence of class 2 and class 3 integrants might be minimized by the use of circular DNA.

This was tested by transforming FY527attPx2 with pLT50. The integration structures of eight representative Ura$^+$ His$^-$ clones from this transformation were analyzed. Six of the eight clones fell into the same three classes: three are in class 1, one in class 2, and two in class 3. The prevalence of class 2 and 3 integrants demonstrates that recombination between the duplicated *attB* sites does not require a linear substrate. It remains to be determined whether this type of event is promoted by *S. pombe* or by the ϕC31 integrase. One possibility is that the integrase interacts with *attB* even in the absence of an *attP* site. In vitro, no *attB*×*attB* recombination was detected (Thorpe et al. 2000). However, the ϕC31 recombinase is a member of the invertase-resolvase class of enzymes that catalyzes recombination by making double-strand breaks in each DNA substrate. If a break occurs at the *attB* site, it is possible that its presence may then recruit the generalized homologous recombination system. Reducing the degree of homology between the direct repeats on the plasmid to a minimum, 34 bp for *attB* and 39 bp for *attP* (Groth et al. 2000), may reduce the frequency of this unwanted side reaction.

In addition to these three classes of integration structures, there is the possibility of integration patterns resulting from incomplete recombination of *attB* x *attP* sites. This could occur if the amount of integrase protein is limiting, as it could be if pLT43 were lost from the cell. If the His$^+$ phenotype is not selected for, His$^-$ colonies are readily found. Four possible structures could arise from a single recombination event between the four sites: 5'*attB*×5'*attP*, 3'*attB*×3'*attB*, 3'*attB*×5'*attP* and 5'*attB*×3'*attP*. If followed by a second *attB*×*attP* reaction, the 5'*attB*×5'*attP* and the 3'*attB*×3'*attB* integrants would be converted to the class 1 structure, and the 3'*attB*×5'*attP* and 5'*attB*×3'*attP* integrants would not be found, as the *ura4*$^+$ marker would be deleted. One of the eight isolates gave a pattern consistent with the incorporation of intact pLT50 through a 5'*attB*×5'*attP* reaction. This class 4 structure is shown in Fig. 3G. The *ura4* probe detected a single 2.3-kb band, and the *leu1* probe detected bands of 3 kb, 5.6 kb and 18 kb (data not shown). Cleavage with *Nde*I gave a 12-kb band that hybridized to both the *leu1* and *ura4* probes, which is consistent with physical linkage of the two markers. The remaining isolate had also incorporated the entire plasmid but had gained additional bands hybridizing to both *leu1* and *ura4*. This represents a more complex event, perhaps indicating gene amplification at the locus.

Integration into FY527attPx2 was also examined using intact pLT45, which can insert into the chromosome at either the 5'*attP* or the 3'*attP* site. The additional *attP* target in the chromosome did not significantly change the transformation efficiency. When normalized to the number of His$^+$ transformants obtained with pLT43, the efficiency of ϕC31 integrase mediated transformation of FY527attPx2 is comparable with that found in the transformation of FY527attP with pLT45 (Tables 1 and 2). Thus, duplicated sites in both the target and donor molecules appear necessary for the increased transformation frequency observed with the gene replacement strategy.

Optimal integrase concentration

Transformations of FY527attP with pLT45 and FY527attPx2 with pLT50 were performed with varying amounts (0, 0.1, 1, 5, and 10 μ g) of pLT43 DNA. The DNA concentration should correlate with integrase abundance. The results in Fig. 4 show that both sets of transformations yielded a maximum number of Ura$^+$

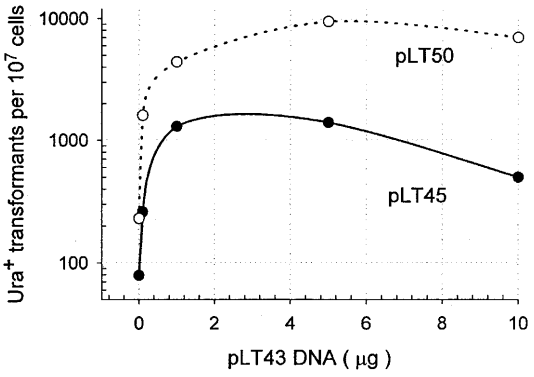

Fig. 4 Transformation efficiency as a function of integrase concentration. FY529attP or FY529attPx2 was transformed with 1 μg of pLT45 or pLT50 DNA, respectively, together with various amounts of pLT43 DNA

colonies with 5 μg of pLT43 DNA. The pLT50/FY527attPx2 transformation produced a 4- to 14-fold higher number of transformants compared to the pLT45/FY527attP transformation. This observation is consistent with the results shown in Tables 1 and 2. A similar stimulation of dual-site targeting over single-site targeting was also reported for the Cre-*lox* system (Bethke and Sauer 1997). However, our observed higher transformation frequency is offset by the lower frequency of precise events, 38% for pLT50 compared to 88% for pLT45.

ϕC31 integrase does not excise integrated molecules

It was previously reported that reversal of the ϕC31 integrase reaction could not be detected by analysis of gel-fractionated DNA fragments (Thorpe and Smith 1998). We examined the possibility of a reverse reaction using a genetic selection strategy**.** The precise integration of pLT45 into FY527attP was confirmed for three clones by Southern analysis; these strains were then re-transformed with pLT43. Excision of pLT45 would result in loss of the *ura4*$^+$ marker and the Ura$^-$ phenotype can be scored on plates with 5-FOA. The frequencies of Ura$^-$ segregants from cultures of the three Ura$^+$ His$^-$ progenitors were 5.7×10^{-4}, 7.1×10^{-4} and 5.6×10^{-4}. In contrast, the frequencies of Ura$^-$ colonies obtained from the three Ura$^+$ His$^+$ derivatives (from re-transformation of pLT43) were somewhat higher: 1.1×10^{-2}, 3.8×10^{-3} and 2.3×10^{-3}, representing 19-, 5- and 4-fold increases, respectively. When a control vector lacking the integrase gene, pBG2, was used for re-transformation, increased rates of 5-FOA resistance were also found: 1.0×10^{-2}, 1.0×10^{-2}, and 8.0×10^{-3}. Therefore, the re-transformation process itself appears to be mutagenic.

One Ura$^-$ His$^+$ clone from each of the three cultures that had been re-transformed by pLT43 was analyzed by Southern blotting. One isolate had a DNA pattern consistent with stable integration of pLT45 into FY527attP. Therefore, in this clone, the Ura$^-$ phenotype must be caused by a mutation that did not appreciably alter the nuclease cleavage pattern. The second clone showed a hybridization pattern characteristic of FY527attP (lacking a pLT45 insertion, as in Fig. 2A, panel P). The third clone had a pattern consistent with a mixture of two cell types, those like FY527attP (without a pLT45 insertion), and those like FY527. The FY527 structure could arise from intrachromosomal homologous recombination between the *leu* repeats, deleting the pLT44 and pLT45 DNA. If site-specific excision of the integrated plasmid DNA occurred in the candidates with the FY527attP structure, the *attP* site would be regenerated and this could be detected by PCR. This was not the case, as the size of the PCR product was that expected for an intact hybrid site. The presence of the hybrid site was confirmed by sequencing the PCR product. These observations are consistent with the idea that deletion of the *ura4* gene occurred by some mechanism other than ϕC31-mediated excision.

Conclusions and prospects

Like other recombination systems, the ϕC31 system can be used with selective placement of *attB* and *attP* sites to delete, invert, or insert DNA. In contrast to some other systems, the *attB*×*attP* reaction is irreversible in the absence of an excision-specific protein. This makes the ϕC31 recombination reaction particularly attractive for site-specific integration. In this study, we show that the integration of a circular molecule at a singletarget site is an efficient process, yielding precise insertions in nearly all transformants. The few aberrant events observed are probably largely attributable to the *S. pombe* recombination system acting on the *leu1* repeats. When integrase production was limited through the repression of its promoter, the number of transformants was reduced to near background level. Under these conditions, few of the recovered transformants had undergone ϕC31-mediated recombination.

The dual-site recombination reaction is even more efficient. The frequency of precise gene replacement events is about 14–24% of the transformation efficiency of a replicating plasmid vector (Table 2). The data in Fig. 4 show that at optimal integrase concentration, as inferred from the amount of co-introduced integrase DNA, the transformation efficiency increases further to a level approaching that of a replicating plasmid. One possible explanation for this higher transformation efficiency may be related to the effect of concatemeric substrates on transformation. Whereas the formation of concatemers would decrease substrate availability for a single-copy insertion event, it may have less effect on a replacement event.

The current data do not rule out the possibility that host-mediated factors, including the host homologous recombination system, contribute to the efficiency of integration. However, this seems unlikely given that purified components of the ϕC31 integration system appear fully operational in vitro (Thorpe and Smith 1998;

Thorpe et al. 2000). Other site-specific recombination systems, such as Cre-*lox,* operate efficiently and independently of host recombination systems, and in host cells that lack efficient homologous recombination. The ϕC31 system will probably function in a similar manner.

The recombination events that we monitor occur in the nucleus. Therefore, the ϕC31 integrase must have entered this organelle. However, we have not yet determined what percentage of the protein molecules are directed to this location. Although site-specific recombination was observed, it does not necessarily indicate optimal activity. It remains to be determined whether the inclusion of nuclear localization signals, or changes in codon usage preferences would be desirable or necessary in this and other eukaryotic systems.

In bacteria and yeasts, the high transformation efficiency of replicating plasmids has made direct cloning by functional selection possible. Similarly, the high transformation efficiency shown in Fig. 4 suggests that shotgun cloning by direct selection may be possible with the dual-site ϕC31 recombination system. In principle, a library of linear DNA molecules, such as a collection of cDNAs, need not be passed through a cloning vector system. It can be ligated to flanking *att* sites and introduced directly into a genomic *att-att* target in animal or plant cells. Although we observed a competing side reaction consisting of integration of circular molecules derived from the linear DNA, these undesired events might be minimized by use of the smallest functional attachment sites. In addition, if the target site DNA between two *attP* sites encoded a marker for which a negative selection exists, then only the full replacement of the marker would be detected. In light of the current need for functional genomic analysis, the development of procedures for high-efficiency delivery of selected cDNA libraries would represent a critical step in this direction.

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