

Insertional mutagenesis based on illegitimate recombination in *Schizosaccharomyces pombe*

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ABSTRACT

An efficient insertional mutagenesis system has been developed for *Schizosaccharomyces pombe* based on linear PCR-generated cassettes containing selectable markers. It depends upon illegitimate recombination for integration into the genome. Various selectable markers of different sizes can be used to obtain sufficiently high transformation and integration frequencies. Based on Southern blotting, a single insertion is found in each strain and integration sites are broadly distributed in the genome. Sequence analysis of the insert junctions frequently reveals small regions of homology (4–10 bp) between the ends of the integrated cassette and the disrupted gene. The system has been used for simple genetic screens of various types and as a promoter trap for in-frame GFP fusions.

INTRODUCTION

In *Saccharomyces cerevisiae* there is efficient integration of homologous DNA into the genome (1). Linear DNA with free ends within the region of homology gives the maximum frequency of homologous integration (2), enabling targeted gene disruptions and tagging (1,3). Although the one-step gene disruption method (4) is routinely used to generate deletion strains, a faster, cloning-free alternative is now available. This uses PCR-amplified constructs based on amplifying selectable markers using primers terminating in genomic sequences. These primers may have homologous regions as short as 40 bp (5,6). Extensions of this technology include constructs carrying several types of protein tags, such as Myc or GFP (green fluorescent protein), that can be precisely fused with the target gene (7,8). In *Schizosaccharomyces pombe*, similar tools are available, but longer stretches (60–80 bp) of homology are required for targeted integration (9,10).

In the absence of homologous regions, illegitimate recombination is thought to occur at a much higher rate in *S.pombe* than in *S.cerevisiae* (1,11,12). Non-homologous DNA end joining, an event of illegitimate recombination, has been shown to occur more efficiently in *S.pombe* than in *S.cerevisiae* (11,12). In this paper, we show that this frequency in *S.pombe* is sufficiently high to be readily exploited as an insertional mutagenesis technique. Mutants selected or screened based on this method allow for the retrieval of the gene without the need

for cloning by functional complementation. Since the *S.pombe* genome sequencing project is virtually completed (www.sanger.ac.uk), mutants isolated by this technique can be quickly identified. We show that the technique has general applicability using various screens, selectable markers and tagged constructs.

MATERIALS AND METHODS

Strains and media

Schizosaccharomyces pombe strains used in this study are listed in Table 1. Media preparation and manipulations of cells were performed as described previously (13–16).

Table 1. Strain list

Strain	Genotype	Source
Q252	<i>ade6-704 h⁺</i>	Laboratory collection
Q1411	<i>ura4-D18 h⁻</i>	Laboratory collection
Q1412	<i>ade1-D25 h⁺</i>	Laboratory collection
Q1620	<i>ade1-D25 ura4-D18 h⁹⁰</i>	This study

Molecular techniques

Standard molecular biology protocols were used (17,18). Table 2 shows the sequences of the oligo primers used to amplify the cassettes and for inverse PCR (19). The structures of the PCR cassettes used for insertional mutagenesis are presented in Figure 1.

Amplification of the *ura4⁺*, *ade1⁺* and *sup3-5* PCR cassettes was performed in 100 µl reactions containing 60 pmol of each primer, a final MgCl₂ concentration of 1.5 mM and 2.5 U of *Taq* polymerase (Promega or Pharmacia). The amplification conditions were as follows: an initial heat denaturation step of 95°C for 2 min, addition of *Taq* polymerase, followed by 35 cycles of denaturation (95°C for 60 s), primer annealing (55°C for 30 s) and extension (72°C for 90 s).

The amplification conditions of the larger *GFP* PCR cassette varied slightly from above. Specifically, 100 pmol of each primer, a final concentration of 1.5 mM MgCl₂ and 2.8 U of high fidelity *Taq* polymerase (Roche Molecular Biochemicals) were used in 50 µl reactions. The PCR reactions were subjected to an initial heat denaturation step of 94°C for 4 min, followed by 35 cycles of amplification (five cycles of 94°C for

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Table 2. Oligo primers used for amplification of cassettes and inverse PCR

Cassette	Sequences	
	Forward	Reverse
<i>ade1</i> ⁺	5'AAGAGTGTTCGTCGCTG3'	5'ATGTTTAAGGCGAGTACAGTAA3'
<i>ura4</i> ⁺ ^a	5'AGCTTAGCTACAAATCCCCTGGC3'	5'TGTGATATTGACGAAACTTTTGACAT3'
<i>sup3-5</i>	5'TAGGAGTTTACTTTGATAATAAAAAA3'	5'CGTCCCTATCTACATATTTATATAAGA3'
GFP	5'AAAACCTGGAGTTGGTTTTCAGGGTGTCTGAG3'	5'AGCTTAGCTACAAATCCCCTGGC3'
Inverse PCR		
<i>ura4</i> ⁺ ^b	5'CACCATGCCAAAAATTACACAAGATAGAAT3'	5'TTTGGTGGTTATTGAAAAAGTCGATGCCT3'
<i>ura4</i> ⁺ ^c	5'CAAAGTGCAAACATTATCATGAAAAAGAAC3'	5'TGGAGTCTATGGAGCTGGTCTGTAATCC3'
GFP	5'ATTGGCACAACTCCAGTG3'	5'ACATTGAAGATGGAAGCG3'

^a18 bp random extensions were added to the 5'-ends to generate the random tailed construct.

^{b,c}Nested primers that produce a 180 bp band shift of the inverse PCR product.

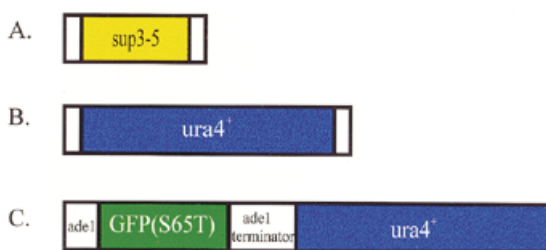


Figure 1. PCR cassettes used for insertional mutagenesis. (A) The *sup3-5* gene and (B) the *ura4*⁺ gene (including coding and flanking sequences) were used as selectable markers. The 18 bp random extensions are indicated. (C) The GFP-containing cassette used to generate gene fusion mutants. The *GFP* gene is preceded by 150 bp of the *ade1*⁺ gene to protect the *GFP* ORF during integration and is followed by the *ade1*⁺ terminator. This construct was used in an *ade1*⁻ *ura4*⁻ deletion strain.

45 s, 50°C for 30 s, 68°C for 4 min; 30 cycles of 94°C for 45 s, 55°C for 30 s, 68°C for 4 min) and a final extension of 68°C for 7 min.

For inverse PCR (IPCR) (19), genomic DNA was isolated (16) and digested with restriction enzymes such as *Hha*I (Promega) and/or *Eco*RI (Promega) that do not cut within the *ura4*⁺ or *GFP* cassettes. The reaction was then heat inactivated at 65°C for 20 min, treated with phenol/chloroform and ethanol precipitated for at least 1 h at -20°C. The digested DNA from the *ura4*⁺ insertional strains and GFP fusion mutants was ligated at concentrations of 5 µg/ml (100 ng in 20 µl final volume) and 0.5 µg/ml (100 ng in 200 µl final volume), respectively. Three units of T4 DNA ligase (Promega) was added to each reaction, which was allowed to proceed for 16 h at 12°C. The ligation reaction was then heat inactivated at 65°C for 20 min. For the 20 µl reactions, the entire ligation was used directly in the IPCR reaction with no additional MgCl₂. The 200 µl ligation reactions were treated with phenol/chloroform and ethanol precipitation prior to IPCR. The amplification conditions were as above except that a 5 min hot start and a longer extension time of 4 min were used for the *ura4*⁺ insertional experiments. A re-amplification using 2 µl of the reaction as template with the same temperature and time profiles was

performed if no PCR product was present after the first round of amplification.

Transformation and selection of stable integrants

All transformations were by the lithium acetate method (20). Specifically, a 100 ml cell culture was grown to a density of 0.5–1.0 × 10⁷ cells/ml in EMM low glucose (5 g/l) plus supplements at 30°C. The cells were then harvested by centrifugation (3000 r.p.m., 5 min), washed twice with 20 ml of 0.1 M lithium acetate (pH 4.9) to a final density of 1 × 10⁹ cells/ml and 100 µl aliquots were dispensed into microcentrifuge tubes. The cells were incubated at 25°C for 1 h. Transforming DNA (1/10 volume of a PCR reaction, 0.3–1.0 µg DNA) was mixed with 5 µl of 10 mg/ml sonicated salmon sperm DNA and added to each aliquot of cells. After a further 1 h incubation at 25°C, 290 µl of 50% PEG3350 (dissolved in 0.1 M lithium acetate, pH 4.9) was added and mixed by gentle pipetting. The cells were then incubated at 25°C for 1 h, heat shocked at 43°C for 15 min and allowed to recover at room temperature for 10 min. The cells were harvested by centrifugation (3000 r.p.m., 5 min) and the pellet resuspended in 10 ml of 0.5× YEA medium. This was followed by shaking for 1 h at 30°C. The cells were then harvested, resuspended in sterile water and plated onto selective media.

After 3–5 days at 30°C, transformants were maintained on non-selective medium for 1 week by replica plating once every 2 days on YEA. The transformants were then replica plated onto selective medium (EMM) and the stable integrants isolated as surviving colonies. Strains transformed with the *ura4*⁺ gene were checked for integration by demonstrating their sensitivity to 5-FOA. Routinely, 80–85% of the strains were integrants after 3 days growth on non-selective medium.

RESULTS AND DISCUSSION

High integration efficiencies are obtained by transformation of a variety of selectable markers of different sizes

An insertional mutagenesis system requires high transformation and integration efficiency of a non-homologous selectable marker. To determine whether this was achievable in *S.pombe*, we tested PCR products of various lengths (469–4000 bp)

containing different selectable markers (*ura4⁺* and *sup3-5*). Using the lithium acetate method (20), transformation efficiencies achieved with the *ura4⁺* cassette were similar to a larger *ura4⁺* cassette containing additional sequences of the *GFP* and *ade1⁺* gene, altogether ranging from 882 to 7400 stable integrants/ μ g DNA in various experiments (mean \pm SE, 3410 \pm 79, $n = 21$). In contrast, the smaller *sup3-5* cassette produced a mean transformation efficiency of 268 \pm 34 stable integrants/ μ g DNA ($n = 6$). The low frequency of stable transformants exhibited by the *sup3-5* cassette is presumably due to the lethal effects associated with expression of this nonsense suppressor tRNA (21). Although cells with single copies of *sup3-5* grow well, it is known that multicopy expression is lethal (21). Any cell taking up multiple copies of the *sup3-5* cassette prior to integration would display growth suppression and be lost. Similarly, it is known that unstable transformants contain multiple extra-chromosomal copies of the selectable marker (22), which may account for the low numbers of unstable transformants with the *sup3-5* cassette. This might explain the observed unstable transformant frequencies that appeared construct specific, ranging from ~85% of total transformants with the *ura4⁺* cassettes and 15% of total transformants with the *sup3-5* cassette. However, the unstable transformants do not interfere with insertional mutagenesis since they can be eliminated by maintaining the transformants on non-selective media for a few days (see Materials and Methods).

To determine whether another selectable marker could produce similar results, particularly with respect to the proportion of stable integrants, we also tried a cassette based on the *ade1⁺* gene in an *ade1-D25* background. The transformation efficiency of this cassette seemed comparable to the *ura4⁺* cassette, as was the proportion of stable integrants, which was determined directly to be 15%. The *ade1⁺* gene did not provide any particular advantage over the *ura4⁺* gene and was not further pursued.

Two conclusions can be reached from these results. First, any type of selectable marker can be used to produce large numbers of stable illegitimate recombinants when transformed into a suitable *S.pombe* strain. Selectable markers that show multicopy toxicity, such as the *sup3-5* gene, decrease the overall frequency of integrants. Second, the addition of other markers or tags that increase the size of the construct by >2-fold does not affect the transformation efficiency of this construct. This enables other types of selection schemes or sequence recovery mechanisms to be readily used.

Cassettes used for insertional mutagenesis must not have any homology to the genome

To ensure that all integration is illegitimate, there must be no homology to genomic sequences. The *ura4⁺* cassette was transformed into the *ura4-D18* strain, which is devoid of all cassette sequences (23). All integrants produced thus arose from illegitimate recombination events. However, the *ade6-704* strain used for *sup3-5* transformations contains a wild-type genomic copy of the *sup3-5* gene. Genetic analysis of 50 stable transformants indicated that 2% of the integrations had occurred at or close to the *sup3⁺* locus. The *sup3-5* portion of the cassette showed >90% homology with other tRNA^{Ser}-encoded genes such as *sup9⁺* and *sup12⁺*, suggesting that integrations might also occur at those sites. Linkage of integrants to the latter two loci was not tested.

In addition, as part of the GFP experiments described below, we initially placed an upstream linker containing 164 bp of homology to the upstream region of the small heat shock protein gene *hsp16*, and devoid of stop codons, to protect the *GFP* open reading frame from degradation. The *hsp16* sequence was chosen out of convenience since it was readily available in a plasmid. This cassette turned out to be unsuitable for insertional mutagenesis since homology of this length resulted in homologous integration with a frequency of 45% at the *hsp16* locus. Sequence analysis of several insert junctions (discussed later) indicated that the construct had circularized in the cell and integrated by a single homologous recombination event. It is conceivable that circularization prior to integration may be a common feature of all introduced linear sequences. Overall, our data indicate that any selectable marker can be used for mutagenesis and should work equally well provided no strong sequence homology exists within the genome.

Integration occurs at broadly distributed points in the genome

Random or near random integration of the PCR cassette would be optimal for insertional mutagenesis. Apart from illegitimate recombination, it is possible that random integrations can also be promoted by short homologous degenerate sequences at the ends of the cassette. Since studies have reported that sequences as short as 29 and 40 bp of homology are sufficient to drive homologous recombination in *S.cerevisiae* and *S.pombe*, respectively (9,24), shorter regions may target at lower frequency. To investigate this, primers were synthesized to generate a PCR product containing the *ura4⁺* gene flanked by 18 bp fully degenerate tails. This construct was then transformed and the efficiency compared to a tail-free *ura4⁺* cassette. The 18 bp extension length was chosen since, theoretically, every possible 18 bp sequence would be represented in the 200 nmol of primer that was synthesized. Similar numbers of stable integrants were produced by transformation of the *ura4⁺* cassette with (4197 \pm 126 per μ g DNA, $n = 2$) or without the random tails (3478 \pm 573 per μ g DNA, $n = 2$), indicating that the presence of these degenerate sequences did not affect the total number of integrants.

We next assayed the distribution of insertions in the genome amongst a population of stable integrants generated by the random tailed *ura4⁺* cassette. Southern blot analysis of 28 stable integrants, pooled in samples of five or six strains and using the *ura4⁺* construct as a probe, revealed 28 distinct *EcoRI* fragments (Fig. 2A), indicating that the integrations occurred at different positions in the genome. We also showed that each of the six strains in one of the pools contained a single insertion event (Fig. 2B). Based on the low probability of insertion events, the frequency of double integrants is expected to be low. In addition, Southern blots of three other mutant strains detected only one insertion (data not shown). Lastly, all strains subjected to detailed genetic analysis showed only one integration event.

Integration events are broadly distributed in the genome. Several lines of evidence support this. First, intercrosses between three individual *sup3-5* stable integrants (in an *ade6-704* background) and 10 randomly chosen ones all produced adenine auxotrophs, indicating that for each group of 10 crosses none of the strains had integrated the cassette at the same locus. We were also able to retrieve insertional mutants from various screens (see later) and, furthermore, examination

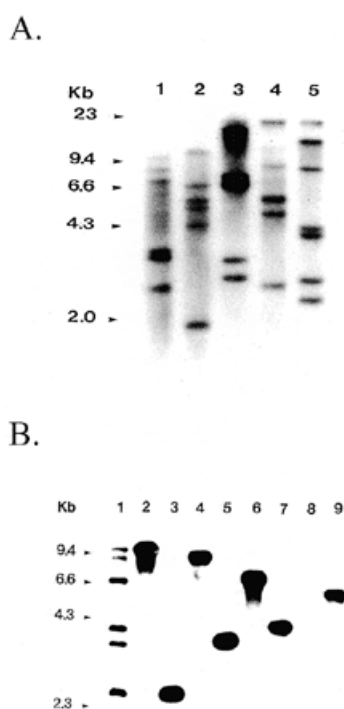


Figure 2. Southern analysis of stable *ura4⁺* integrants. (A) Pools of either five (lanes 3 and 4) or six (lanes 1, 2 and 5) individual stable integrant strains ($n = 28$ in total) were grown and total genomic DNA was prepared, digested with *EcoRI* and analyzed by Southern blotting using *ura4⁺* as a probe. Differences in band intensity within a lane reflect differing cell densities of individual strains in the cell cultures. The uppermost band in lanes 3–5 is undigested genomic DNA. (B) The six pooled integrant strains shown in lane 1 of (A) were analyzed individually. Southern blotting was performed as above except that total genomic DNA was prepared from individual strains. Lane 1, pooled DNA from all six strains; lanes 2–7, six individual integrant strains; lane 8, *ura4-D18* strain; lane 9, wild type.

of the insert locations in the genome for some of the mutants where flanking genomic sequence was retrieved revealed a broad distribution on all three chromosomes (Fig. 3).

We have not performed an exhaustive analysis of multiple integration sites within a single gene following mutagenesis with constructs with or without the degenerate tails to assess whether the tailed constructs have a broader range of integration sites. This would require the collection of a large number of integrations within a single gene using each construct followed by sequencing of the insertion sites. For practical purposes of insertional mutagenesis our data suggest that there is no difference between the tailed and non-tailed constructs in terms of larger scale distribution or utility.

Isolation of *cdr⁻* mutants by insertional mutagenesis

The insertional mutagenesis system was applied in a screen for changed division response (*cdr⁻*) mutants. These are characterized by their inability to properly down-regulate their cell size upon nitrogen starvation (25,26). This particular mutant class was chosen for two reasons. First, the distinct *cdr⁻* phenotype is easily screened and we have extensive experience with it.

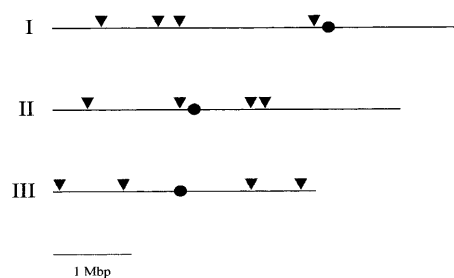


Figure 3. Location of a number of integrants in the genome (arrowheads). Sequences retrieved from insertional mutant strains were localized by comparison to the physical maps at www.sanger.ac.uk. Centromeres are indicated by filled circles.

Second, previous results from a chemical mutagenesis screen for *cdr⁻* mutants (26; unpublished data), where six major complementation groups were represented in a collection of 106 mutants, provided a comparison to determine whether there was bias in the types of mutants generated.

A PCR-generated construct containing the *ura4⁺* gene flanked by degenerate tails was transformed into a *ura4-D18* strain and the transformants were screened for the *cdr⁻* phenotype on minimal medium minus nitrogen plates (26). We recovered seven mutants representing alleles of four different *cdr⁻* genes in a total of 84 000 integrants for a frequency of ~ 1 in 12 000 overall. For the individual genes it ranged from one to three alleles, depending on the gene. The proportion of mutants isolated was comparable to the chemical mutagenesis screen (1.4×10^{-5}). Although the number of insertional mutants recovered was small, three of the four *cdr⁻* genes were identical to complementation groups previously identified by chemical mutagenesis as determined by genetic analysis (16). This suggests that the integration events were well distributed in the genome. Mutants recovered included three insertional alleles of the protein kinase *cdr2* (26–28), allowing for an assessment of the distributed sites of integration within a single gene (see below).

In addition, two other insertional mutagenesis screens were undertaken with similar success. A search for amiloride-resistant mutants recovered an insertional mutant allele of the *car1⁺* gene amongst 21 780 integrants (29) and a screen for extragenic suppressors of the *cdr1/nim1* kinase yielded an allele of *wee1⁺* (26,30,31) and an allele of *rad24⁺* (32) in a population of 25 000 stable integrants. These latter two loci were expected and isolated with a frequency similar to that seen in the *cdr⁻* insertional screen.

Examination of insertion sites from several of these mutants showed that two-thirds of the integrations occurred within the open reading frame while the remaining one-third was found proximal to the promoter sequences. This pattern of insertion is different from certain transposons, such as *Ty1* and *Tn916*, which exhibit a high insertion preference for intergenic DNA (33,34). Our ability to retrieve several insertional mutants at a reasonable frequency and an expected distribution of known linkage groups in three separate screens demonstrates the general applicability of this technique.

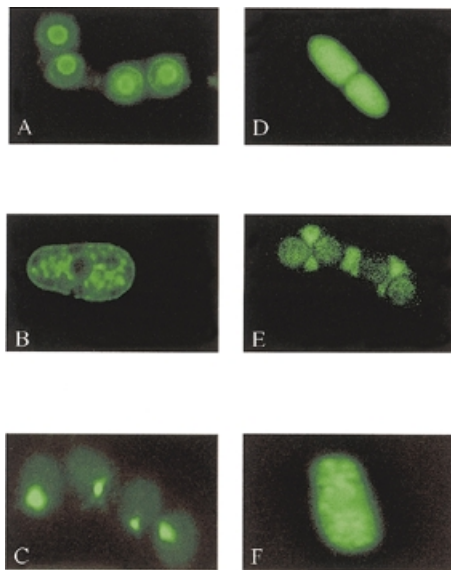


Figure 4. GFP fusion proteins displaying different cellular localizations. Living cells expressing GFP fusion proteins as genomic integrants were viewed by fluorescence microscopy. (A) *lt13-26* (predominant staining in nuclear envelope and spore cytoplasm); (B) *lt14-20* (nuclear envelope and ER); (C) *lt1-4* (punctate staining proximal to nucleus in spores); (D) *lt2-20* (cytoplasmic in septum-defective mutant); (E) *lt3-2* (cytoplasmic in forming ascus); (F) *lt2-3* (vacuolar). Images were taken with a Leica fluorescence microscope equipped with a high performance CCD camera (Sensicam) and Slidebook software (Intelligent Imaging Systems).

Insertional mutagenesis using a GFP-containing cassette can be used as a promoter trap and a screen for protein targeting signals

The insertional mutagenesis technique can be extended to include the use of more complex cassettes. These can carry additional selectable markers for subsequent cloning or tags for identifying the protein product at the site of integration. We have designed and tested a cassette which generates a GFP fusion protein upon integration, thus allowing for selection of mutants based, first, on expression levels of the protein and, second, cellular localization. The cassette consists of the *GFP* gene (35,36) preceded by an upstream sequence devoid of genomic sequences and without stop codons in any frame. This upstream sequence was used to protect the *GFP* open reading frame during integration. The *GFP* gene was followed by a spacer and the *ura4⁺* selectable marker.

Upon transformation into a *ura4-D18 ade1-D25* strain, integrants were illuminated with 450–480 nm light to excite the GFP and monitored for colony fluorescence using a 500 nm long pass filter. Fluorescent strains were expected to be low in frequency since they were a result of a 1 in 6 chance of the cassette integrating in the correct frame and orientation as well as having it falling under control of a promoter that possessed sufficient strength for detection. In haploid cells, our detection system was able to recover fluorescent strains at ~0.5% of total stable integrants (233 GFP-expressing strains in a population of 46 027 stable integrants). This population included GFP fluorescence localized in the nucleus, nuclear periphery, cytoplasm and meiotic spores (Fig. 4). We have also integrated the *GFP* cassette into

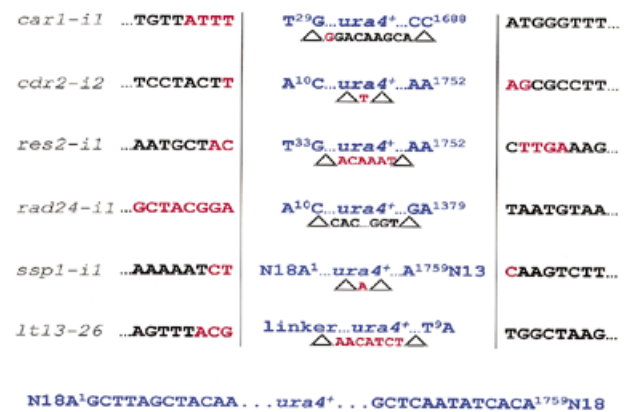


Figure 5. Sequence analysis of insert junctions in several mutants. Insertion of the *ura4⁺* cassette (blue text) into genomic DNA (red and black text) is shown. (Left) Genomic sequences at upstream junction; (right) genomic sequences at downstream junction; (center) inserted *ura4⁺* cassette sequences with terminal nucleotides and their positions (superscript) after integration. The red and black text flanked by deltas below the blue insert sequences are the genomic sequences deleted in each event. Nucleotide identity between the integrated construct and genomic DNA is displayed in red. Bottom line, the *ura4⁺* cassette (blue) showing the terminal 13 nt at each end and the random tails (N18) upstream (left) and downstream (right). The top five mutants were generated by the *ura4⁺* cassette flanked by degenerate tails while the bottom strain was mutagenized by the *GFP* cassette. Linker, upstream linker of the *GFP* cassette. The *rad24-11* strain contained a 1589 bp genomic deletion which included half of the 3'-end of the *rad24⁺* gene (nucleotide positions +568 to +1283) and sequences downstream.

diploid cells to isolate fusion mutants of essential genes. A collection of these mutants remains to be analyzed. These results indicate that protein fusion mutants can be isolated at a reasonable frequency by insertional mutagenesis and that the use of a more sensitive detector, such as a cooled CCD camera or FACS, would increase the overall number of fluorescently tagged integrants recovered.

Insert junctions show small deletions of the gene and marker and no sequence specificity

We recovered and sequenced a number of insert junctions in order to examine the nature of any chromosomal rearrangements and possible sequence preferences for integration. Examination of the sequences at the insert junctions showed that the majority of integration events resulted in small deletions within the gene and at both termini of the cassette (Fig. 5). These deletions appeared to be variable and sequence non-specific (Fig. 5). Only one exception was seen, where in this case a large fragment (1.5 kb) of the chromosome was deleted (Fig. 5). This suggests that if the selectable marker(s) is sensitive to loss of a few base pairs (such as the case of GFP), then spacers are required to flank the selectable marker within the cassette for protection during integration.

Small regions of homology are observed between the genomic DNA and the ends of the integrated cassette

Truncation of both ends of the integrating fragment in almost all cases hindered determination of whether the integration was actually dependent on any short (a few base pairs) homology

found within the random tails. However, sequence analysis of an intact random tail present in the *ssp1-il* integrant showed that the four terminal nucleotides at the upstream end were homologous to the disrupted gene at the insertion junction (Fig. 5). The occurrence of microhomology in illegitimate recombination has been reported in *S.cerevisiae* and mammalian cells (37–39). Interestingly, there were also small regions of homology (4–10 bp) between the ends of the integrated cassette and the disrupted gene in almost all of the insertional mutants analyzed (Fig. 5). A portion of the homologous region was usually deleted in the disrupted gene (Fig. 5). The region of homology found in the genomic sequence of one of the insertional mutants (*lt13-26*) contained both termini of the integrated cassette. These observations suggest that in some cases circularization of the cassette results in small deletions of the termini (12) and that the short sequences remaining at or proximal to the end-joining promote integration at genomic sites containing microhomology to these sequences. The insertional mutant (*rad24-il*) possessing a large genomic deletion is likely caused by integration of a linear cassette.

Our data suggest that the sites of genomic integration may be dependent upon microhomology at the upstream and downstream regions of the *ura4⁺* gene in the cassette after truncation, since in most cases the entire random tails were deleted. However, integration of the cassette may still be broadly distributed in the genome because the sequence homologies seen in illegitimate recombination were very short and the degree of truncation of the *ura4⁺* cassette was variable. Although only two *cdr2⁻* alleles were analyzed at the nucleotide level, they represent different insertion points in unrelated sequences in the gene (amino acid positions 369 and 426). While we have no proof that use of the degenerate tailed primers enhances the distribution of inserts, they are unlikely to detract. The broad applicability of this method provides a powerful tool for functional gene analysis in *S.pombe*.

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