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Positive Selection of FLP-Mediated Unequal Sister Chromatid Exchange Products in Mammalian Cells

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Site-specific recombination provides a powerful tool for studying gene function at predetermined chromosomal sites. Here we describe the use of a blasticidin resistance system to select for recombination in mammalian cells using the yeast enzyme FLP. The vector is designed so that site-specific recombination reconstructs the antibiotic resistance marker within the sequences flanked by the FLP target sites. This approach allows the detection of DNA excised by FLP-mediated recombination and facilitates the recovery of recombination products that would not be detected by available screening strategies. We used this system to show that the molecules excised by intrachromosomal recombination between tandem FLP recombinase target sites do not reintegrate into the host genome at detectable frequencies. We further applied the direct selection approach to recover a rare FLP-mediated recombination event displaying the characteristics of an unequal sister chromatid exchange between FLP target sites. Implications of this approach for the generation of duplications to assess their effect on gene dosage and chromosome stability are discussed.

Site-specific recombinases, such as FLP and Cre, catalyze the precise recombination of short and typically asymmetric DNA sequences (FLP recombination targets [FRTs] and Cre recombination targets [LoxP sites], respectively). When two target sequences are tandemly arrayed on a mammalian chromosome, the predominant recombination event is the excision of the intervening DNA as a circular molecule containing a single target site. The reciprocal product is a deleted chromosomal locus which also contains a target site (Fig. 1). It has generally been assumed that the excised DNA is degraded or diluted by subsequent cell division. However, previous experimental strategies were not designed to recover other types of recombination events or to determine if the excised molecules reintegrated at random chromosomal positions.

We have developed a strategy in which a functional antibiotic resistance marker is created de novo by FLP-mediated recombination on the circular molecule excised from a chromosomal substrate. This system is similar to others reported previously that used site-specific recombination to create novel functions, such as β-galactosidase (β-Gal) enzymatic activity (9), a functional hypoxanthine phosphoribosyltransferase locus (13), or antibiotic resistance (2, 7). However, the system described here differs from those reported previously in that the selective marker is regenerated within the recombined FRT-flanked insert and not in the deleted chromosomal excision site. Therefore, when the FRT-flanked DNA was excised by FLP-mediated recombination, this approach allowed the detection of the excised DNA. In principle, integration of the excised molecule at a site different from the original insertion would confer antibiotic resistance on the cell in which the event occurred, and the frequency of such reintegration events could be estimated from the number of resistant colonies that demonstrated the excision phenotype. Moreover, the configuration of the chromosomal substrate was such that other, presumably rare recombination events would also generate a functional resistance cassette.

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Blasticidin (6) was chosen as the selective agent for these experiments because selection is efficient, rapid (3 days for complete lethality and 7 to 10 days for the appearance of colonies), and inexpensive. Moreover, blasticidin resistance has not been used widely in other vectors, allowing the introduction of multiple vectors without recycling genetic markers. Our data demonstrate that this strategy can be used to select cells that harbor rare duplication products of FLP-mediated recombination, such as sister chromatid exchanges of the FRT-flanked sequences. Our results estimate the frequency of such duplications and establish that excision of the recombined DNA is not the only outcome of FLP-mediated recombination in mammalian cells.

MATERIALS AND METHODS

Cell lines and growth conditions. All the clones used are derivatives of the monkey kidney cell line CV-1. E25B4 and E25B4-10 have been described previously (9). Clones produced in the course of this work are described in Table 1. Cells were grown in Dulbecco's modified Eagle's medium with 5% calf serum. The medium was supplemented, when necessary, with G418 (Geneticin; Gibco-BRL; 400 μg/ml), hygromycin (Calbiochem; 120 U/ml), or blasticidin (ICN; 10 μg/ml).

Plasmid construction and transfections. pOG44, pOG45, and pNeoβ-Gal were described previously (9). pYN323, generously provided by F. Hanaoka (6), contains the blasticidin resistance coding sequence (BSR) linked to the SRα promoter. pBF was constructed by insertion of the FRT-containing oligonucleotide CGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGG AACTTCAAGCT into the *Sac*I site of pYN323, between the SRα promoter and the BSR open reading frame. pBF-GLO contains an 8-kb fragment from the human β-globin region (map units 59.9 to 68.8 of GenBank sequence J00179) within a *Not*I site 3′ to the BSR open reading frame in pBF. pOG45-GLO contains the same globin sequence within the *Hin*dIII site of pOG45. Transfection of linearized plasmids was performed by electroporation as described previously (9). For FLP-mediated excision, cells were transfected with 20 μg of the FLP expression vector pOG44 by the calcium phosphate method as described previously (9). For FLP-mediated insertion, cells were cotransfected with 2 μg of the circular targeting plasmid and 20 μg of pOG44. Colonies harboring the introduced plasmid DNA were selected on the basis of antibiotic resistance (G418 or hygromycin) and then screened for integration into the FRT by β-Gal histochemical staining (9) or by testing for sensitivity to 10 μg of blasticidin per ml. DNA isolation and Southern blot analyses were performed as described previously (11).

Flow cytometry. For isolation of β-Gal-positive and β-Gal-negative populations, flow cytometry was performed as described previously (8), with minor modifications (6a).

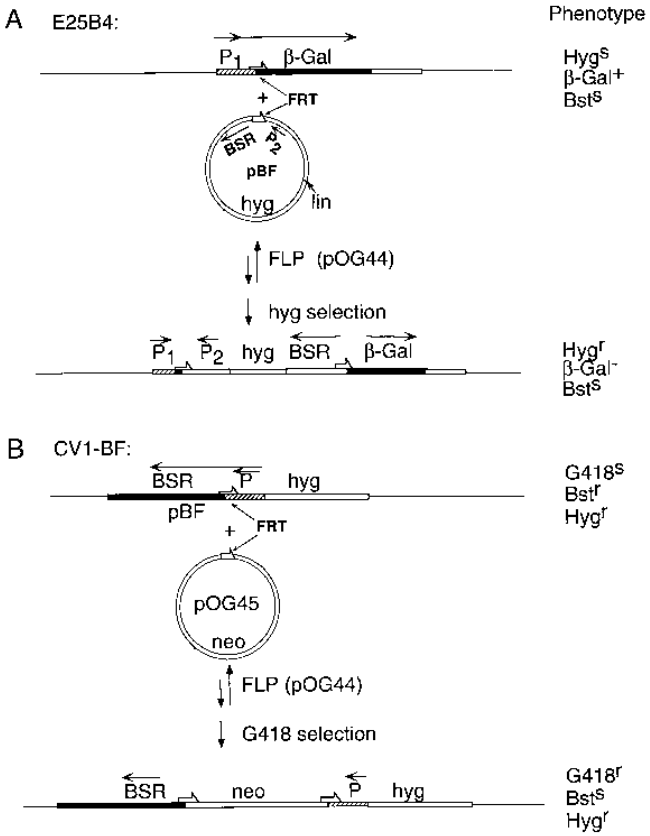

FIG. 1. Plasmids and cell lines. (A) The E25B4 cell line (9) contains an FRT inserted between the promoter (P_1) and the coding sequence but still allows expression from the β-Gal cassette. pBF contains an FRT (arrow) inserted between the SRα promoter (P_2) and the BSR, a functional hygromycin resistance gene (hyg) on an Okayama-Berg backbone plasmid (6), and two unique restriction sites, *Bam*HI and *Eco*RV, that can be used for linearization (lin). Recombinants in E25B4 (9) were created by transfecting circular pBF or derivatives thereof in the presence of excess FLP expression vector (pOG44). Integration of the plasmid resulted in the disruption of the β-Gal and blasticidin resistance coding sequences, generating integrants that are hygromycin resistant, blasticidin sensitive, and β-Gal negative. (B) Cell line CV1-BF carries a single copy of linearized pBF; therefore, these cells are resistant to both hygromycin and blasticidin. pOG45 (9) and derivatives thereof contain a single FRT and a neomycin resistance cassette (neo). Site-specific integration of pOG45 and its derivatives resulted in clones that are G418 resistant and blasticidin sensitive. Retransfection with pOG44 and selection for blasticidin resistance will select for colonies that have undergone excision. Although excision is expected to be more frequent than integration, integration is selected for by G418 resistance.

RESULTS

Two chromosomal targets for FLP-mediated recombination were employed in these studies. One was the β-Gal-positive cell line E25B4, containing a single chromosomally integrated copy of pNeoβ-Gal with an FRT site positioned between the simian virus 40 early promoter and a β-Gal coding sequence (9). FLP-mediated, site-specific insertion of a plasmid with a single FRT into this target should abolish β-Gal expression, and subsequent FLP-mediated excision of the inserted plasmid should restore β-Gal function (Fig. 1A). The second target was CV1-BF, containing a single chromosomally integrated copy of linearized pBF (Fig. 1B) with an FRT between an SRα promoter and the BSR (Fig. 1) (see Materials and Methods for details). Site-specific integration of FRT-bearing plasmids into the chromosomal pBF FRT should disrupt expression of the blasticidin resistance cassette and render the cells blasticidin sensitive (Bst^s) (Fig. 1B). Blasticidin resistance should be restored upon subsequent FLP-mediated excision.

Screening for FLP-mediated excision and ectopic integration. We initially employed the target cell line E25B4 (9) and a strategy that combined β-Gal screening and neomycin selection to estimate the frequency at which the excision products of FLP-mediated recombination reintegrated at random chromosomal sites. A single copy of the plasmid pOG45 was inserted into a single chromosomal FRT of E25B4 by cotransfection of pOG45 and an FLP-expressing plasmid (pOG44). This created the clone E25B4-10 (9). FLP-mediated excision of the pOG45 sequences should produce a circular molecule containing a neomycin resistance cassette and a single FRT (Fig. 1) and should activate β-Gal expression from the recombined chromosomal locus. In principle, the excised circular molecule could persist in the cell, reintegrate into its original position by FLP-mediated recombination, or integrate at other genomic sites to confer resistance to G418. It is unlikely that nonreplicating extrachromosomal molecules would be detected because they would be diluted by cell division, while site-specific reintegrations would recreate an insertion indistinguishable from the original E25B4-10 clones. In contrast, cells that harbor random integrations of the excision product should be distinguished by being both resistant to G418 and positive for β-Gal activity.

We attempted to determine the rate of random integration by measuring the frequency of G418-resistant ($G418^r$) cells that were also β-Gal positive. Cells that contained pOG45 at the E25B4 FRT were transiently transfected with the pOG44 FLP expression vector, maintained in G418, and screened for those cells that express β-Gal. Table 1 shows that no G418-resistant clones were β-Gal positive. We therefore attempted to enrich for β-Gal-positive cells immediately after transfection by using the fluorescence-activated cell sorter (8) to fractionate the population into high-level and low-level β-Gal expressers. G418 selection was applied to high- and low-level expressers after periods of nonselective growth that ranged from 1 week to 2 months. The ratio between β-Gal-positive and β-Gal-negative cells did not change appreciably during 4 weeks of nonselective growth. However, when selection was applied, none of the G418-resistant clones that were recovered in multiple experiments expressed β-Gal (Fig. 2). These results strongly suggest that the excised sequences did not integrate at random chromosomal positions at measurable frequencies.

Blasticidin resistance as a marker for FLP-mediated recombination. The attempts to identify reintegration events in the experiments described above may have been compromised by the background of G418-resistant cells in which FLP-mediated recombination had not occurred. We therefore implemented a strategy in which recombination resulted in the creation of a functional blasticidin resistance cassette on the excised molecule.

An initial set of experiments was designed to test whether the pBF blasticidin resistance cassette would be useful as an acceptor target for FLP-mediated recombination. If so, blasticidin resistance encoded by the linear pBF would be inactivated by FLP-mediated insertion and reactivated by FLP-mediated excision. CV1-BF cells, which were blasticidin resistant (Bst^r), were cotransfected with pOG45 (9) and the pOG44 FLP expression vector (Fig. 1B). This transfection should produce G418-resistant colonies through random integration of pOG45 or site-specific recombination of pOG45 into the chromosomal FRT. Approximately half of the G418-resistant colonies obtained in these experiments were blasticidin sensitive, suggesting that they represented site-specific integrations. When five such $G418^r$ Bst^s clones were transiently transfected with pOG44, between 0.4 and 2.7% of the cells became Bst^r and $G418^s$ (Table 1). These experiments demonstrated that a functional blasticidin resistance cassette could be inactivated and reactivated by successive rounds of FLP-mediated recom-

TABLE 1. FLP-mediated integration and excision

Target clone	Inserted plasmid sequence in target	Antibiotic marker	Clone phenotype	Outcome of FLP recombination	Expected phenotype of recombinant	Screening or selection method used to detect recombinants	% Recombined
E25B4	pOG45/pOG45-GLO	G418	$G418^r$ β-Gal^-	Excision	$G418^s$ β-Gal^+	β-Gal staining	20–50
E25B4	pOG45/pOG45-GLO	G418	$G418^r$ β-Gal^-	Excision and ectopic reintegration	$G418^r$ β-Gal^+	β-Gal staining of $G418^r$ cells	0 ($<10^{-6}$)
CV1-BF	pOG45/pOG45-GLO	G418	$G418^r$ Bst^s	Excision	$G418^s$ Bst^r	Bst^r colonies	0.4–2.7
E25B4	pBF/pBF-GLO	Hygromycin	Hyg^r Bst^s β-Gal^-	Excision	Hyg^s Bst^s β-Gal^+	β-Gal cell staining	18–30
E25B4	pBF/pBF-GLO	Hygromycin	Hyg^r Bst^s β-Gal^-	Sister chromatid exchange	Hyg^r Bst^r β-Gal^-	Bst^r colonies	0.05–0.07
E25B4	pBF/pBF-GLO	Hygromycin	Hyg^r Bst^s β-Gal^-	Excision and ectopic reintegration	Bst^r β-Gal^+	β-Gal staining of Bst^r colonies	0 ($<10^{-6}$)

bination. Similar frequencies of site-specific integration and excision were obtained with pOG45-GLO (containing an 8-kb sequence from the human β-globin locus), indicating that the recombination frequency is independent of size over the range examined.

We next constructed cell lines from which the pBF and pBF-GLO plasmids (see Materials and Methods and Fig. 1) could be excised as circular molecules. pBF or pBF-GLO was cotransfected with the FLP expression vector pOG44 into E25B4 cells. As predicted, the transfection yielded β-Gal-negative, hygromycin-resistant clones that were also blasticidin sensitive (Fig. 1). After FLP-mediated recombination, β-Gal-positive cells were generated at efficiencies ranging from 11 to 50% (Table 1). Consistent with previous studies (9), no cells positive for β-Gal staining were detected after mock transfection.

FLP-mediated excision of the pBF cassette from the chromosomal site should regenerate a functional blasticidin resistance cassette within the excised DNA (Fig. 1A). The frequency of β-Gal-positive, blasticidin-resistant clones should indicate the rate of random integration. Blasticidin-resistant colonies arose at a frequency of 5×10^{-4} to 7×10^{-4} in multiple transfection events from two different E25B4-BF clones (Table 1). Again, similar frequencies were obtained with pBF, a 6.2-kb molecule, and pBF-GLO, a 14.2-kb molecule, indicating that the range of insert size did not influence recombination frequency. Neither blasticidin-resistant colonies nor cells staining for β-Gal expression were detected after mock transfection.

FLP-mediated sister chromatid exchange. If the blasticidin-resistant colonies resulted from excision and random integration of the insert, the resistant cells would be expected to express β-Gal. Unexpectedly, we found that all the blasticidin-resistant clones were β-Gal negative, suggesting that blasticidin resistance was not created by a simple excision-integration event. One explanation of the blasticidin-resistant, β-Gal-negative phenotype is that it was created by a sister chromatid exchange (Fig. 3). If the blasticidin resistance was indeed reconstructed through a tandem duplication, the resulting structure could still be a substrate for further FLP-mediated excision of the intervening DNA. Indeed, transient transfection with pOG44 and subsequent histochemical staining for β-Gal indicated that all the blasticidin-resistant clones generated β-Gal-positive cells after FLP expression. The frequency of excision as measured by the appearance of β-Gal-positive cells was 30 to 50%, similar to the rate observed in the parental E25B4-BF cells.

Southern blot analyses were used to confirm that blasticidin resistance resulted from a tandem duplication. The restriction

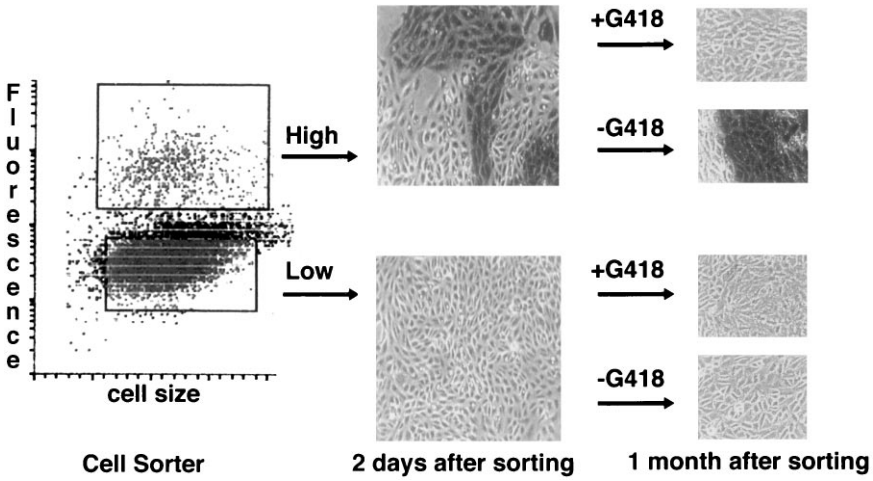

FIG. 2. Test for ectopic integration of excised DNA. The cells used in this experiment contained pOG45 sequences inserted into a single FRT in the coding sequence of the β-Gal expression cassette of E25B4. This insertion disrupted the cassette, resulting in cells that were β-Gal negative and resistant to G418. (Left panel) Transfection with the FLP expression plasmid pOG44. Two days posttransfection, the cells were sorted by a fluorescence-activated cell sorter according to their β-Gal expression levels (8). Two populations, those exhibiting high and low degrees of fluorescence, were collected and plated with and without G418. (Middle panels) The collected populations were histochemically stained to detect β-Gal expression. In the high-level-expresser population, about 50% of the cells stained positive for β-Gal activity, while all the cells in the low-level-expresser population were negative. (Right panels) Staining of sorted cells growing in the presence or absence of G418 for 1 month after the sorting experiment.

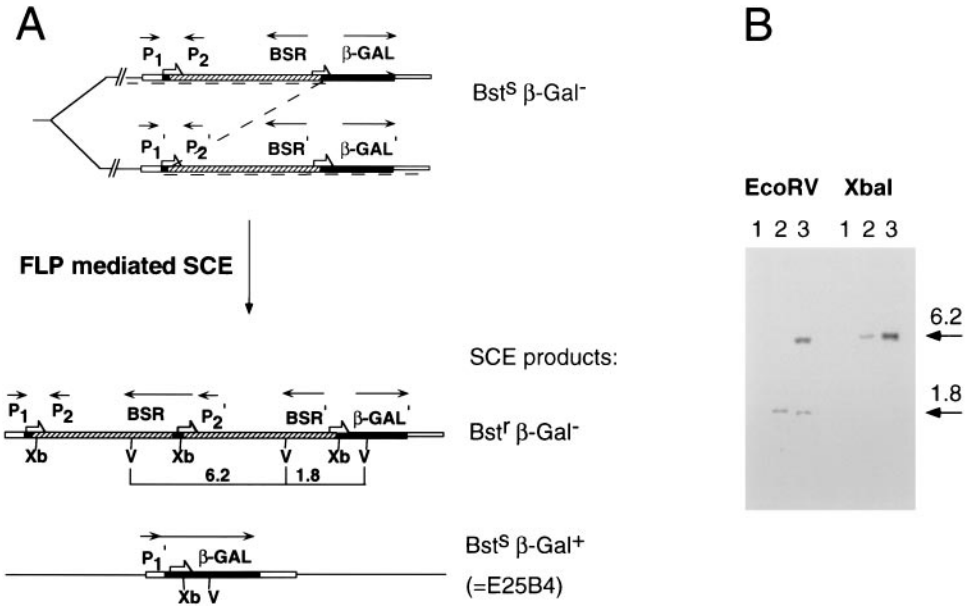

FIG. 3. FLP-mediated sister chromatid exchange. (A) Site-specific recombination between the 3′ FRT of one chromatid and the 5′ FRT of the other chromatid leads to sister chromatid exchange (SCE). SCE results in the formation of one product indistinguishable from E25B4 and another, blasticidin-resistant product carrying a duplication of the insert. This duplication results in the juxtaposition of the BSR from one chromatid and the SRα promoter (P_2') from the other chromatid. This duplication creates a new *Eco*RV fragment of unit insert length (6.2 kb) without changing the size of the junction fragment (1.8 kb). The restriction enzyme *Xba*I digests once within each FRT, yielding a fragment of unit insert length that does not change upon duplication. Abbreviations: Xb, *Xba*I; V, *Eco*RV. (B) Southern blot analysis of E25B4 (lanes 1), E25B4-BF1 (lanes 2), and an E25B4-BF1 clone containing the duplication of the insert (lanes 3). Since each FRT contains an *Xba*I site, probing an *Xba*I digest of genomic DNA from E25B4-BF and derivatives with a BSR probe yielded a single band the size of the integrated molecule (6.2 kb). An *Eco*RV digest of genomic DNA from E25B4-BF cells as well as from the recombined clone yielded a 1.8-kb fragment hybridizing with the BSR probe. In the blasticidin-resistant clone, the *Eco*RV digest of the duplicated sequence created a new restriction fragment the size of the inserted molecule.

enzymes *Eco*RV and *Xba*I were used to digest genomic DNA of three clones: (i) E25B4, containing no BSRs; (ii) E25B4-BF1, harboring pBF within the two FRTs in E25B4; and (iii) a blasticidin-resistant clone isolated following FLP-mediated recombination of E25B4-BF1. There is one *Eco*RV site within pBF, and there is an *Xba*I site within each of the FRTs of pBF. As shown in Fig. 3B, E25B4 DNA did not hybridize with a BSR probe. E25B4-BF1 digested with *Eco*RV showed a 1.8-kb hybridization fragment that is derived from the junction of the 3′ end of the targeted pBF plasmid and the chromosomal β-Gal coding sequence. Finally, *Eco*RV digests of the blasticidin-resistant clone DNA showed two hybridizing fragments of 1.8 and 6.2 kb. The 1.8-kb band again represented the 3′ junction between plasmid and β-Gal sequences. The 6.2-kb fragment is the same size as the pBF plasmid insert, as demonstrated by the *Xba*I digestions. A fragment of this size would be expected if a second copy of pBF had inserted into either of the FRTs. These results were reproduced in analyses of eight clones from two independent transfections (data not shown) and verified with *Not*I, which does not digest within the duplicated sequence. These results are consistent with the hypothesis that these clones arose from FLP-mediated duplication of the inserted sequence.

DISCUSSION

We used the reconstruction of an antibiotic resistance marker by FLP-mediated recombination to show that FLP-excised DNA did not integrate at ectopic sites at detectable frequencies. We further demonstrated that sister chromatid exchange of FRT-flanked sequences is an infrequent but reproducible product of FLP-mediated recombination. The ability to generate duplications in a reproducible manner could be useful for addressing gene dosage questions, as well as for studying the stability of tandem repeats in mammalian cells.

Our results show that the SRα-FRT-BSR cassette (as in CV1-BF cells) was useful as an accepter target for FLP-mediated recombination. The frequencies of excision of pOG45 or pOG45-GLO plasmids from the CV1-BF sites ranged from 0.4 to 2.7%, considerably lower than the frequencies at which plasmids can be excised from the E25B4 chromosomal FRT. While this may suggest that the rates of excision from chromosomal FRTs are position dependent, the rates cannot be compared directly because they were scored by two very different assays. It may well be that the accumulation of enough protein to allow blasticidin resistance requires more time than the accumulation of enough β-Gal protein to be detected histochemically. Indeed, we found that allowing more time between transfection and the application of selection (e.g., 48 or 72 h rather than the 24 h used here) led to the recovery of 5- to 10-fold more blasticidin-resistant colonies (data not shown). Regardless of the excision frequencies, these experiments show that the blasticidin resistance marker in pBF can be inactivated or activated by FLP-mediated recombination.

Our results suggest that pBF, pOG45, and their larger derivatives could be targeted to chromosomal FRTs at similar frequencies. Subsequent site-specific excision of the integrated DNAs resulted in the reconstruction of the marker abolished during the original integration. This established that the product of an insertion reaction still contained two functional recombination target sites. It has been suggested (1) that some FLP-mediated integrations of plasmids into chromosomal targets render one or both of the product FRTs nonfunctional, thereby trapping the integrated plasmid. While such a mechanism remains a formal possibility in mammalian cells, the fact that we were able to excise integrated plasmids from each of the cell lines tested suggests that this is not the predominant mechanism for integration.

Our data demonstrate (Fig. 2; Table 1) that the excised DNA did not usually reintegrate into the genome at ectopic sites. Rather, this DNA may have reintegrated at the remaining chromosomal FRT, creating a β-Gal-negative cell indistinguishable from the parent (Fig. 1), or it could have been eliminated through dilution by cell division, degradation, or micronucleation (12). The system described here may therefore be useful for identifying genetic elements, such as replication origins and kinetochore components, or other structural elements that enable retention and minimize loss of extrachromosomal sequences.

The functional blasticidin resistance cassette formed on the excised molecule by FLP-mediated recombination allowed us to monitor the fate of the excised DNA. In contrast with the experiments using a screen for integrated DNA, in which we were not able to recover any cells that had recombined and retained the antibiotic resistance marker, the selective advantage provided by the reconstruction of the blasticidin resistance cassette allowed the recovery of such clones. In these cells, a rare sister chromatid exchange event occurred between the FRT-flanked sequences. Sister chromatid events, as well as other forms of mitotic recombination, were shown to be mediated by site-specific recombinases in *Drosophila melanogaster* (3, 4) and in murine embryonic stem cells (13). The sister chromatid exchanges observed could have occurred following DNA replication, when two copies of the FRT-flanked insert are present on sister chromatids as shown in Fig. 3. An alternative pathway for creating such a duplication, also occurring following DNA replication, is the excision of the pBF sequences from one sister chromatid and the subsequent insertion of these sequences into the FRT of the other chromatid (in which excision has not occurred). Our data do not allow us to distinguish between these two pathways, since they will form identical products. Direct recombination between sister chromatids requires a single intermolecular recombination event, and the excision-integration pathway requires two steps (one intramolecular and one intermolecular). While this could indicate that the former is the more likely pathway, the highly efficient production of circular molecules upon recombination suggests that the intermolecular recombination event would be rate limiting for both mechanisms.

In the experiments described above, fewer than 1% of the clones that underwent excision (positive histochemical staining for β-Gal) yielded duplications (blasticidin-resistant colonies) (Table 1). Similarly, sister chromatid exchange occurs in the minority of flies undergoing recombination upon heat shock activation of FLP in *Drosophila melanogaster* (4). Although these recombination rates cannot be compared directly because they were scored by very different assays, this suggests that sister chromatid exchanges are minority products of FLP-mediated recombination between tandem FRTs, for otherwise each β-Gal-positive cell would have a blasticidin-resistant sister cell carrying a duplication (Fig. 3).

It is important to note that our assay clearly underestimates the frequency of duplication events because the duplications are themselves substrates for further FLP-mediated recombination. Recombination of the duplicated substrate could excise one or both of the repeats; the resulting products would resemble the original parent or the result of a simple excision of the original parent, respectively. Whatever the exact frequency, our model system demonstrates that duplication can be an end product of FLP-mediated recombination in mammalian cells, especially when FLP is expressed transiently. Given the mechanistic similarities between FLP and Cre, we anticipate that similar systems could be developed for the Cre recombinase and that the sister chromatid exchange events observed following FLP-mediated recombination could also be recovered after Cre-mediated recombination. Indeed, Cre-mediated chromosomal rearrangements have been demonstrated in mammalian cells following Cre-mediated recombination (10, 13).

Our data suggest both caution with regard to and additional applications of site-specific recombinases in multicellular eukaryotes. The generation of chromosomal duplications by FLP may be a concern for researchers who are trying to express this and other recombinases to generate homogeneous cell populations. For example, attempts to delete specific genetic markers in entire cell lineages during embryonic development (5) may result in a reciprocal cell population containing the duplicated marker. On the other hand, selecting for the duplication of a single-copy marker provides a system in which to study the effects of gene dosage in a constant chromosomal context. This can provide an advantage in studies of gene function since duplication of sequences in this manner does not necessitate the separate introduction of two different genetic markers. The ability to generate sister chromatid exchanges may also be valuable for analyzing sequences or structures that modulate chromosomal recombination or the activities of the site-specific recombinases themselves.

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