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Multiplex Cre/*lox* recombination permits selective site-specific DNA targeting to both a natural and an engineered site in the yeast genome

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ABSTRACT

Variant *lox* sites having an altered spacer region (heterospecific *lox* sites) are not proficient for Cre-mediated recombination with the canonical 34 bp *loxP* site, but can recombine with each other. By placing different heterospecific *lox* sites at different genomic locations, Cre can catalyze independent DNA recombination events at multiple loci in the same cell without concern that unwanted inter-locus recombination events will be generated. Such heterospecific *lox* sites also allow Cre to specifically target efficient integration of exogenous DNA to endogenous *lox*-like sequences that naturally occur in the genome. Specific targeting occurs only with a DNA vector carrying a heterospecific *lox* site in which the spacer region has been redesigned to match the 'spacer' region of the targeted chromosomal element. Moreover, in cells expressing a catalytically active Cre recombinase, naturally occurring *lox*-like sequences can exhibit almost 20% mitotic recombination. Thus, in the same cell, heterospecific *lox* sites can be used independently at multiple loci for integration, for deletion and for enhanced mitotic recombination, thereby increasing the repertoire of genomic manipulations catalyzed by the Cre recombinase.

INTRODUCTION

Site-specific DNA recombinases have become important tools for *in vivo* manipulation of eukaryotic genomes (1–6). Cre recombinase and its DNA recognition site *loxP* have been used both for activation and for elimination of genes in a variety of transgenic and gene-modified mice (7–9). In these genome manipulation strategies *loxP* sites are introduced into the genome using either traditional zygote injection techniques or by homologous recombination in pluripotent embryonic stem cells such that they flank the segment of DNA that is to be deleted from the genome. Upon expression of the Cre protein, efficient site-specific conservative recombination occurs at the *loxP* sites to excise the intervening segment of DNA from the genome. Because recombination occurs only in cells expressing Cre, tissue-specific deletion or activation of a gene can be made to occur by controlling the expression of the *cre* gene. Prior placement of Cre's DNA recognition site *loxP* into the genome also permits Cre-mediated integration of a *loxP*-containing plasmid to that chromosomal site, facilitating construction of isogenic cell lines and mice (10,11). Precise genome manipulation is possible not only because Cre is an efficient recombinase in eukaryotic cells, but also because the *loxP* size is sufficiently large (34 bp) that it is unlikely to occur naturally in any eukaryotic genome. Rare recombination events have been detected, however, with cryptic *lox* sites in both the *Escherichia coli* and the yeast genomes (12,13).

The *loxP* site consists of two 13 bp inverted repeats to which Cre binds (14,15) and an intervening 8 bp core. Only pairs of sites having identity in the central 6 bp of the core region are proficient for recombination; sites having non-identical core sequences (heterospecific *lox* sites) do not efficiently recombine with each other (16). Thus, the core region of the *lox* site determines the specificity of recombination with a *lox* partner. For example, a variant heterospecific *lox* site '*loxY*' would not be able to recombine with the canonical *loxP* site, but would be proficient for *loxY* × *loxY* recombination.

The existence of multiple heterospecific *lox* sequences suggests several novel recombinational strategies for genome manipulation. First, selective Cre-mediated targeting of different chromosomal *lox* sites may be achievable by placing heterospecific *lox* sites into the genome at different loci and then specifying the core sequence on the *lox* targeting plasmid to direct integration to the corresponding locus. Second, use of different pairs of heterospecific sites would allow Cre-mediated gene excision at different chromosomal locations without generating complicating chromosome inversions, translocations or large scale deletions. Third, because Cre requires as few as 8–10 bp of each 13 bp binding domain (17,18), eukaryotic genomes may naturally contain one or more functional *lox*-like sequences, but which differ from *loxP* in the core region. The feasibility of these strategies is demonstrated here using the genome of *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Plasmids

The yeast integrating vector pRB19 contains the *LEU2* gene for selection in yeast, but no *lox* site (10,19). Removal of a single *loxP* site from the *lox*^{2} *LEU2* construct pBS30 (20) generated the *loxP LEU2* integrating vector pBS222. The *mock loxFAS1* vector pBS214 was constructed by inserting the annealed oligos, 5′-AGC TTC GTA TAT ACC TTT CTA TAC GAA GTT GTG-3′ and 5′-GAT CCA CAA CTT CGT ATA GAA AGG TAT ATA

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CGA-3′, between the *Hin*dIII and *Bam*HI sites of pRB19. The *GAL1-cre* yeast expression *CEN* plasmids pBS49 (20) and pBS126 (21) respectively carry the selectable markers *URA3* and *ILV2-4-10* (sulfometuron methyl resistance) for selection in yeast (22). Plasmid pBS394, *GAL1-creY324C*, was constructed by replacing the wild-type *cre* sequence of pBS126 with that of the '*creNA2*' mutant (23).

Cre reactions

Cre reactions (10 µl) were performed under standard conditions at 30°C for 1 h and contained 2% polyvinyl alcohol (24,25), 20–30 fmol of each *lox* site and 2 pmol Cre protein or as indicated. Reaction products were visualized after gel electrophoresis by ethidium bromide staining or by autoradiography when using ^{33}P-end-labeled *loxFAS1* fragments generated by polymerase chain reaction (PCR) amplification and treatment with T4 kinase. Ethidium bromide stained gels were digitally photographed using a Foto/Eclipse camera system (Fotodyne, Inc.) and quantitated using NIH Image software. Radiolabeled gels were quantitated using the Fuji BAS 1500 phosphorimager.

Yeast strains and genetic manipulation

Strains used in this work are listed in Table 1. Culture conditions (S medium: minimal defined medium; YEPD: yeast extract, peptone, dextrose) and selection procedures have been described (20). BSY659 is a *ura1::LEU2* insertion/deletion derivative of DBY745 made by homologous recombination (26). In the *ura1::LEU2* insertion the *LEU2* gene replaces a 328 bp segment between the *Eco*RV and *Nco*I sites of *URA1* (a 986 bp fragment amplified from genomic DNA with the oligos 5′-CAG GTC GAC TCT AGA GGA CCA AAC ATG ACA GCC AG-3′ and 5′-GTA ACC CGG GAT CCA AGC TTA AAT GCT GTT CAA CTT CC-3′). Plasmids pBS126 and pBS394 were introduced into and maintained in BSY695 by selection for resistance to sulfometuron methyl (a gift of S. C. Falco, DuPont Co.). DNA transformation was with the Frozen-EZ Yeast Transformation Kit (Zymo Research).

For determination of the efficiency of Cre-mediated targeting, yeast strains (± a resident chromosomal *loxP* target and containing the *cre* gene under *GAL1* control) were induced for *cre* expression by pregrowth in S + galactose medium, transformed with the indicated amount of the appropriate *LEU2* targeting vector, and selected for leucine prototrophy.

For analysis of mitotic recombination, overnight cultures of the heterozygote BSY695 (Fig. 3) carrying the indicated *cre* expression construct were grown in SD (S + dextrose), washed, and diluted (final concentration: 1×10^6 cells/ml) into S medium supplemented with adenine, leucine, uracil and either galactose or dextrose. Cells were grown for the indicated time and samples plated for individual colonies on YEPD plates at 30°C. Phenotypes were determined by replica plating to SD plates with the appropriate nutritional supplements.

Polymerase chain reaction

Analysis of the *loxFAS1* region and preparation of *loxFAS1* fragments for recombination *in vitro* (above) was with the following primers: a, 5′-GGT CCA GAA GCA AGT ATG TCT ATG G-3′; b, 5′-CGC TGT TGC GTA ATT ATG CTT GGC-5′; 97, 5′-ATC AAG ACC AGG AAC AAT ACC-3′; 98, 5′-GCA CCT CGT GTA TCG TGA TGC-3′; L2b, 5′-GAC GAT TGC TAA CCA CCT ATT GG-3′; U, 5′-CAG GGT TAT TGT CTC ATG AGC GG-3′. Amplification was by 30 cycles: 30 s at 94°C, 30 s at 65°C, 60 s at 71°C, in a Perkin Elmer 9600 thermocycler with *Taq* polymerase (Perkin Elmer), using ~50 ng yeast genomic DNA in a 50 µl reaction volume.

RESULTS

Recombination at candidate *lox*-like sequences

A minimum requirement for a functional *lox* site is two 13 bp inverted repeat elements (for Cre binding) separated by 8 nt. Although several cryptic *lox* sites in the yeast genome have been identified previously, none contain two 'good' 13 bp repeat elements (13). Do there exist in eukaryotic genomes *lox*-like sequences having functional Cre-binding inverted repeats and a correctly sized heterologous spacer? A search of the GenBank genome database (Release 89.0) for *lox*-like DNA sequences with strong homology to the two *loxP* 13 bp inverted repeats found no eukaryotic sequences with identity in the inverted repeat regions of ≥9 bp and separated by a non-specified 8 nt core region. However, a potential recombination site with 9 bp of identity adjacent to the *loxP* core on one side, and a single mismatch in the 10 bp adjacent to the core on the other, was found in *S.cerevisiae* on chromosome XI, just upsteam of the *FAS1* gene (Fig. 1A) which encodes the β subunit of fatty acid synthetase (27,28). The core region of this *loxFAS1* site bears almost no homology to the *loxP* core. To determine the recombinational competence of this site, a *mock loxFAS1* site was synthesized having two functional inverted repeats flanking a core region identical to that of the *loxFAS1* site. The synthetic site was cloned into a *LEU2* integrative yeast vector for comparison with a similar *loxP* vector (Fig. 1B).

Table 1. Strain genotypes

Strain	Genotype	Reference
2132	*Mat***a** *trp1 leu2 cdc16 mak11-1*	(40)
DBY745	*Matα ade1-100 leu2-3, 112 ura3-52*	(17)
DBY931	*Mat***a** *his4 leu2-3, 112 ura3 met8-1 can1-101*	(17)
BSY3	*Matα ade1-100 leu2-3, 112 ura3* (pBS49)	(18)
BSY103	*Matα ade1-100 leu2-3, 112 ura3* Ch VII::*loxP* (pBS49)	(10)
BSY659	*Matα ade1-100 leu2-3, 112 ura3-52 ura1::LEU2*	This work
BSY671	*Matα ade1 1eu2 ura3* + + + (pBS49)	
	Mata + *leu2 ura3 met8 his4 can1*	BSY3 × DBY931
BSY695	*Matα leu2 ura3 ura1::LEU2* + + + *ade1*	
	Mata leu2 + + *trp1 cdc16 mak11* +	BSY659 × 2132

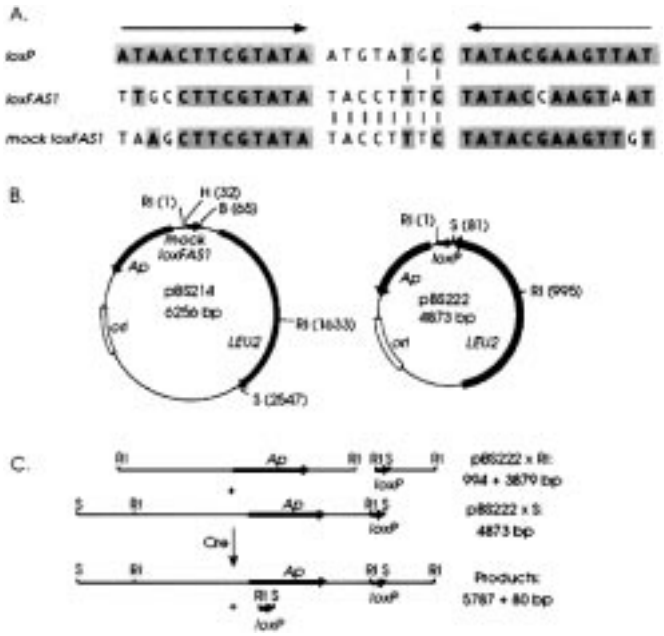

Figure 1. The *lox* recombination sites and vectors. (**A**) Alignment of *loxP* with the yeast *loxFAS1* site (accession no. X03977; nucleotides 505–538) and the synthetic *mock loxFAS1* site. Positions identical to those in *loxP* (41) are boxed in grey. The 13 bp inverted repeats are indicated by the arrows. Identity in the core region is emphasized by vertical lines. (**B**) *lox* integrating vectors. Both carry the *LEU2* gene for selection in yeast. The no *lox* control pRB19 is identical to pBS214 but carries no *lox* site. B, *Bam*HI; H, *Hin*dIII; RI, *Eco*RI; S, *Sal*I. (**C**) *In vitro* recombination assay. Linear *lox* recombination substrates were generated by restriction digest of either pBS222 (*loxP*) or pBS214 (*mock loxFAS1*). Production of new longer or shorter DNA fragments is indicative of Cre-mediated recombination, as sketched here for *loxP* site recombination using pBS222. Similar strategies were used to provide distinctly-sized *loxP* and *mock loxFAS1* substrates.

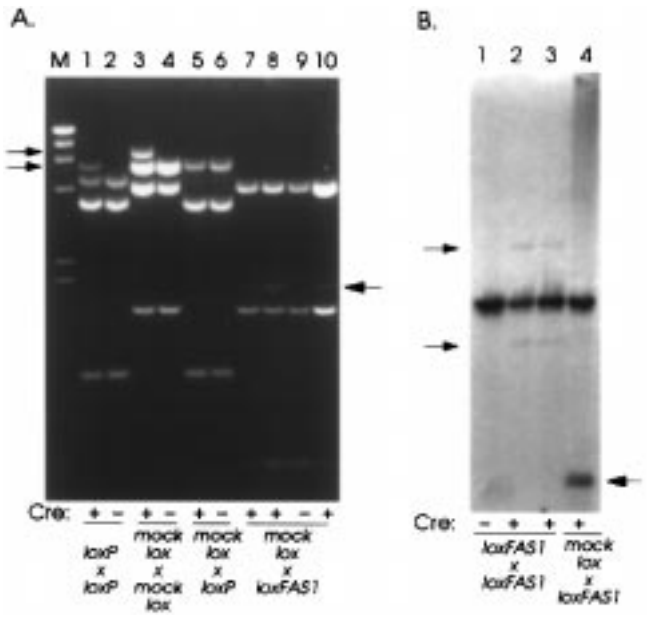

Figure 2. Recombination *in vitro*. (**A**) Linear *lox* containing substrates (Fig. 1) were mixed and treated with Cre as described in Materials and Methods. Reaction products were visualized by ethidium bromide staining. Arrows mark the position of predicted recombination products in '+ Cre' lanes. Lanes 1 and 2: pBS222 × *Eco*RI + pBS222 × *Sal*I; lanes 3 and 4: pBS214 × *Eco*RI + pBS214 × *Bam*HI; lanes 5 and 6: pBS214 × *Sal*I + pBS222 × *Eco*RI; lanes 7–10: pBS214 × *Eco*RI + 528 bp *loxFAS1* PCR fragment (primers 97 + 98; Fig. 3). Lanes 8 and 9 contain a 3-fold molar excess of the *loxFAS1* fragment; lane 10 contains a 3-fold molar excess of the *mock lox* plasmid substrate. M: size markers, a *Hin*dIII digest of phage λ. (**B**) A ^{33}P-end-labeled 440 bp *loxFAS1* PCR fragment (primers a + b; Fig. 3) was tested for recombination as in (A) with either a 2-fold molar excess (lanes 1 and 2) or equal molar (lane 3) unlabeled 528 bp *loxFAS1* fragment and with an equal molar amount of the *mock lox* plasmid pBS214 linearized with *Eco*RI (lane 4).

Intermolecular recombination *in vitro* with purified Cre recombinase (using the strategy shown in Fig. 1C) showed that the synthetic *mock loxFAS1* site was as proficient for Cre-mediated site-specific recombination as an authentic *loxP* site (Fig. 2A: compare lanes 1 and 2 with 3 and 4). Thus, this altered spacer itself does not markedly affect self by self recombination. As expected, recombination between heterospecific sites (*loxP* × *mock loxFAS1*) was not detectable and Cre did not generate a novel band diagnostic of *loxP* × *mock loxFAS1* recombination (lanes 5 and 6). The natural *loxFAS1* site was also proficient in DNA recombination with the synthetic *mock loxFAS1* site (lanes 7–10); the 3-fold reduced efficiency probably reflects a non-optimal interaction of Cre with the natural *loxFAS1* site that results in reduced recombinational proficiency. Consistent with this idea, a 3-fold molar increase of the *loxFAS1* site enhanced recombination whereas a 3-fold increase in the *mock loxFAS1* substrate had little effect. Intermolecular recombination between substrates carrying the natural *loxFAS1* sites is barely detectable *in vitro* (Fig. 2B), and is far less efficient than *loxFAS1* × *mock loxFAS1* recombination. These results suggested that the natural *loxFAS1* site would be targetable *in vivo* using the *mock loxFAS1* site, i.e. Cre may be able to target integration of an exogenous DNA carrying the synthetic *lox* site specifically to the endogenous yeast sequence upstream of the *FAS1* gene.

Genomic targeting

Both the efficiency and independence of genomic targeting at the *loxFAS1* site *in vivo* were examined using the *loxP* and *mock loxFAS1* yeast integration vectors carrying the *LEU2* marker (Fig. 1B). Such circular DNA vectors integrate at low efficiency by homologous recombination into the yeast genome at the chromosomal *LEU2* locus (29). However, Cre directs integration of the *loxP* vector into a chromosomal *loxP* site at high efficiency (10). The *mock loxFAS1* plasmid pBS214 was used to test for targeting in both haploid (BSY3 and BSY103) and diploid (BSY671) *leu2* auxotrophs carrying a *GAL1-cre* expression plasmid. Test strains were induced for recombinase expression by growth on galactose and then transformed with either the *loxP* or the *mock lox* plasmid. Leu$^+$ transformants were selected on plates containing glucose (to repress expression of *cre*), thereby trapping any *LEU2* plasmids integrated into the genome by Cre-mediated recombination (Table 2). As shown previously, in strain BSY103 (which has been engineered to have a *loxP* site near *PDR1* on chromosome VII), Cre-dependent targeting integrates the pBS222 *loxP* plasmid 50-fold more efficiently than a plasmid having no *lox* site (pRB19). In contrast, integration occurs at the same frequency with both pBS222 and pRB19 in strain BSY3, which lacks a chromosomal *loxP* site. Cre-mediated targeting of the *mock loxFAS1* plasmid pBS214 results in a 25-fold stimulation of Leu$^+$ transformants in all three strains compared with the no *lox*

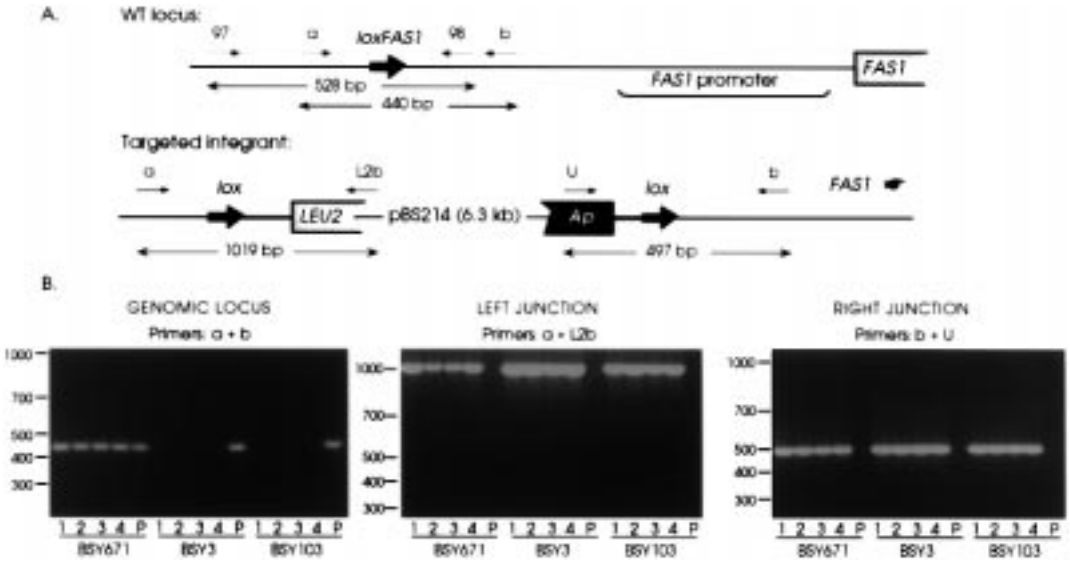

Figure 3. Analysis of pBS214 integrants. (**A**) Map of the wild-type and targeted *loxFAS1* region. PCR primers diagnostic for the undisrupted genomic locus and for the predicted junction fragments after pBS214 integation at the *loxFAS1* site are shown. The *lox* sites are represented by stubby black arrows. (**B**) PCR analysis. For each of the three strains targeted with pBS214 in Table 2 genomic DNA was prepared from four independent Leu$^+$ transformants and from the Leu$^-$ parent (P) and then amplified with one of the three sets of primers diagnostic for the endogenous *loxFAS1* locus, for the left junction or for the right junction, as predicted after site-specific integration. Analysis of an additional 26 Leu$^+$ transformants showed identical specificity in *loxFAS1* targeting by pBS214.

control pRB19, regardless of the presence or absence of a chromosomal *loxP* site. This result suggests (i) that Cre is directing integration of the *mock loxFAS1* site into an endogenous yeast locus, presumably the *loxFAS1* site, and (ii) that integration does not disrupt an essential yeast gene.

Table 2. Selective targeting of chromosomal *lox* sites by Cre

Strain	µg DNA	Leu$^+$ transformants		
		pBS222 (*loxP*)	pBS214 (*mock lox*)	pRB19 (no *lox* site)
BSY103	1	65	24	0
(*loxP* target)	4	119	47	2
BSY3	1	3	84	3
(no *loxP* target)	4	6	324	13
BSY671	1	–	–	–
(no *loxP* target)	4	1	149	–

Inspection of the Cre-mediated Leu$^+$ transformants obtained with the *mock loxFAS1* vector showed that integration was specific to the genomic *loxFAS1* site. Importantly, the presence of the *loxP* site in strain BSY103 did not misdirect integration of the *mock loxFAS1* plasmid. PCR analysis on four pBS214 Leu$^+$ yeast transformants from each of the three strains targeted in Table 2 confirmed that all had integrated a copy of the *mock loxFAS1* integration plasmid at the *loxFAS1* chromosomal target (Fig. 3). As expected, diploid strains retained, in addition, one intact copy of the *loxFAS1* locus on the untargeted chromosome homologue.

Stimulation of mitotic crossover events

The *in vitro* results indicated that the natural *loxFAS1* site is proficient for a low level of self × self recombination. Such Cre catalyzed site-specific recombination *in vivo* should be manifest as a hotspot for mitotic recombination, but only under conditions of Cre induction. Figure 4 shows a test of this prediction schematically using *leu2/leu2* diploid cells heterozygous at the *URA1* and *CDC16* loci. Crossover events centromere proximal to the *URA1/ura1::LEU2* heteroalleles predict the production of Ura$^-$ and Leu$^-$ segregants to a maximum of 50% if crossovers occur in every cell. To test Cre's ability to generate such mitotic recombinants an appropriately marked diploid carrying the *cre* gene under *GAL1* control was grown in either glucose- (non-inducing) or galactose-containing media and the generation of auxotrophs was monitored. After 18–24 h of Cre induction (+ galactose) there is a remarkable rise in the incidence of mitotic recombination to a level of almost 20% with equal numbers of Ura$^-$ and Leu$^-$ segregants being produced (Table 3). These auxotrophs are not generated by a chromosome loss event: such Leu$^-$ segregants would necessarily be temperature-sensitive since the *URA1* chromosome carries the centromere proximal *cdc16* marker, yet none of the auxotrophs obtained was temperature-sensitive. The lack of temperature-sensitive auxotrophs also indicates that the crossover events must have taken place centromere distal to *cdc16*, consistent with recombination having occurred at the *loxFAS1* site. Note that although BSY695 is heterozygous at the *ADE1* locus, no Ade$^-$ auxotrophs were induced by Cre, indicating that Cre does not result in a general stimulation of mitotic crossing-over. These results clearly show that Cre provokes a dramatic elevation in the incidence of mitotic crossover events on chromosome XI, as expected for site-specific recombination at the *loxFAS1* site.

The crossover events detected after Cre induction are most likely catalyzed directly by Cre recombinase although the frequency observed is surprisingly high given the low frequency of recombination between *loxFAS1* sites *in vitro*. To rule out the possibility that Cre binding and ensuing synapsis alone are sufficient for increased crossing over, the assay was repeated using a mutant Cre protein (Y324C) specifically lacking Cre catalytic activity due to mutation of the catalytic tyrosine (23). As

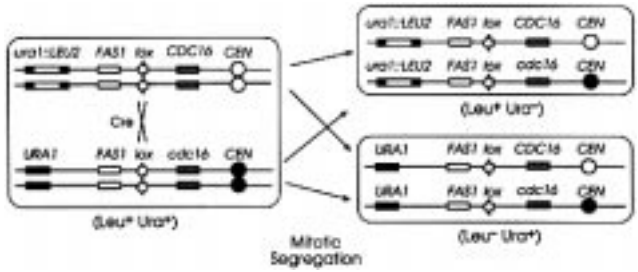

Figure 4. Generation of auxotrophs by Cre-mediated DNA crossover and mitotic segregation. In a diploid *leu2/leu2* strain heterozygous for a *LEU2* insertion at *URA1* (*URA1/ura1::LEU2*), mitotic recombination at the centromere proximal *loxFAS1* site followed by mitotic segregation predicts the induction of Ura$^-$ and Leu$^-$ segregants. Cre-mediated recombination at the *loxFAS1* site is shown occurring just after chromosome XI replication but before centromere (CEN) segregation in a diploid heterozygote. After mitotic crossover, 50% of the centromere segregation events result in equal numbers of Leu$^-$ and Ura$^-$ singly auxotrophic segregants (as shown) and half regenerate Leu$^+$ Ura$^+$ heterozygous progeny (not shown). This gives the frequency of mitotic recombination as twice the incidence of auxotrophy. Inclusion of the recessive temperature-sensitive *cdc16* marker distinguishes mitotic crossover events from chromosome loss events.

shown in Table 3, expression of the mutant *cre* gene did not stimulate mitotic recombination. Thus, Cre catalysis is required for these crossover events at the endogenous *loxFAS1* site.

DISCUSSION

I have shown here that a naturally occurring sequence near the *FAS1* gene of yeast is proficient for Cre-mediated DNA recombination. By respecifying the core region of a synthetic *lox* site to match that of the yeast genomic sequence, Cre specifically targets exogenous DNA bearing the synthetic site to that chromosomal locus, and does so efficiently: targeting of the endogenous *FAS1* locus with the *mock lox* vector is only 2-fold less efficient than *loxP* vector targeting of an authentic *loxP* site previously engineered into the yeast genome.

How many heterospecific *lox* sites are proficient for recombination? If the central six positions of the core could accept any base then there would be $4^6 = 4096$ possible sites. However, not all of these sites are proficient for self × self recombination. Hoess *et al.* (16) showed that mutation of the central TpA dinucleotide in the *loxP* core to TpG abolishes recombination and proposed that this region is required for unwinding of the *lox* site during strand exchange. The necessity for a central TpA dinucleotide in *loxP* suggests that proficient sites require a region within the core that facillitates DNA unwinding. Since the central dinucleotide of the *loxFAS1* and the synthetic *mock loxFAS1* sites is CpT, these sites must bypass an absolute TpA requirement by a compensating feature in these sites' core region. The trinucleotide TpTpT may play this role in the *loxFAS1* site.

One other striking feature of the *loxP* site is the unusual alternating pattern of purine and pyrimidine bases throughout the entire spacer region. In contrast, both the *mock loxFAS1* and the natural *loxFAS1* sites contain a 4 nt homopyrimidine stretch in the core region. Although at times functional significance has been ascribed to unusual sequence patterns, the work here clearly indicates that the alternating purine–pyrimidine character of the *loxP* spacer is not critical for efficient Cre-mediated recombination either *in vitro* or *in vivo*.

Use of heterospecific *lox* sites allows multiplexing of Cre-mediated recombination and thus has important practical implications for the use of Cre in genetically manipulating the genomes of all eukaryotes. Placement of multiple independently acting *lox* sites into the genome at defined locations by homologous recombination allows subsequent high efficiency Cre-mediated targeting of a transgene construct to different chromosomal locations in the same cell by simply specifying the corresponding *lox* site on the targeting vector. Such a strategy will be of particular utility in mammalian systems which show considerable variability of transgene expression depending on the site of transgene integration.

Genetic manipulation of endogenous genes in eukaryotes by homologous recombination with an incoming exogenous DNA (gene targeting) necessarily involves the use of a selectable marker gene as only a small percentage of cells incorporate DNA after transfection. In some cases, however, it would be highly desirable to remove that selectable marker. For example there is only a small number of selectable markers available for genetic manipulation and often it would be advantageous to re-use the same marker gene in subsequent rounds of gene targeting. An additional consideration supporting removal of the selectable marker is that the promoter and enhancer elements used to drive expression of the marker gene have the potential to interfere with correct expression of neighboring endogenous genes (30,31). Cre recombinase has become a useful tool for the removal of *loxP*-flanked selectable marker genes and other unwanted DNA both in yeast (20,21) and in higher eukaryotes (25,32–35), allowing selectable markers to be recycled for subsequent re-use.

Table 3. Cre-meditated mitotic crossover on chromosome XI[a]

Cre	h post-shift	Carbon source	No. colonies	Auxotrophs	Leu$^-$ Ura$^+$	Leu$^+$ Ura$^-$	Leu$^-$ Ura$^-$	ts auxotrophs	% Mitotic crossover
WT	0	Dex	298	0	–	–	–	–	0
	18	Dex	344	1	0	1	0	0	0.58
	24	Dex	419	0	–	–	–	–	0
	18	Gal	377	31	14	17	0	0	16
	24	Gal	316	27	12	15	0	0	17
Y324C	0	Dex	485	0	–	–	–	–	0
	18	Dex	408	0	–	–	–	–	0
	24	Dex	409	0	–	–	–	–	0
	18	Gal	425	2	2	0	0	0	0.94
	24	Gal	815	0	–	–	–	–	0

[a]Strain BSY695 contained either pBS126 (wild-type Cre) or pBS394 (CreY324C) as indicated.

Multiple rounds of selectable marker removal will, however, leave multiple *loxP* sites in the genome. Recombination between these chromosomal *loxP* sites by Cre recombinase will generate complicating and unwanted chromosomal translocations, deletions and/or inversions. A second situation in which this problem can potentially occur is when Cre is used to make a tissue-specific or conditional knockout of two or more genes in the same cell. Certainly in yeast interchromosomal recombination is efficiently catayzed by Cre both between homologues, as shown here, and between heterologous chromosomes (unpublished data), similar to results obtained with the R recombinase of pSR1 (36). Indeed, in *Drosophila melanogaster* interhomologue FLP-mediated recombination occurs at sufficiently high efficiency that it has been used to routinely generate genetic mosaics (37,38). Although Cre-mediated interchromosomal recombination has not always occurred at high frequency in mouse embryonic stem cells (35,39,40), at least when the sites are very far apart, it is unlikely that *lox*-tagged adjacent genes would likewise be refractory to efficient Cre-mediated inter-locus recombination. Simply employing non-interacting heterospecific *lox* sites such as those described here at each chromosomal locus at which a Cre-mediated recombination event is desired would circumvent such potential complications.

Lastly it is likely that, as in yeast, potentially functional *lox* sequences exist in the much more complex mammalian genome. Such mammalian sites, especially if they occur at a nonessential chromosomal locus, present a unique strategy for Cre-mediated gene targeting of an endogenous chromosomal site using a targeting vector carrying an appropriately designed *mock lox* sequence. If sufficiently efficient, Cre-mediated targeting of such natural sites in the genome would facilitate the generation of transgenic animals by eliminating position and copy number effects on transgene expression. Efficient Cre-mediated targeting of an endogenous *lox*-like site in the human genome could lead to alternative strategies in gene therapy that eliminate problems due to random DNA integration by permitting precise stable integration of exogenous DNA at a defined genomic locus.

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