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Influence of DNA Sequence Identity on Efficiency of Targeted Gene Replacement

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We have developed a system for analyzing recombination between a DNA fragment released in the nucleus from a single-copy plasmid and a genomic target in order to determine the influence of DNA sequence mismatches on the frequency of gene replacement in *Saccharomyces cerevisiae*. Mismatching was shown to be a potent barrier to efficient gene replacement, but its effect was considerably ameliorated by the presence of DNA sequences that are identical to the genomic target at one end of a chimeric DNA fragment. Disruption of the mismatch repair gene *MSH2* greatly reduces but does not eliminate the barrier to recombination between mismatched DNA fragment and genomic target sequences, indicating that the inhibition of gene replacement with mismatched sequences is at least partially under the control of mismatch repair. We also found that mismatched sequences inhibited recombination between a DNA fragment and the genome only when they were close to the edge of the fragment. Together these data indicate that while mismatches can destabilize the relationship between a DNA fragment and a genomic target sequence, they will only do so if they are likely to be in the heteroduplex formed between the recombining molecules.

DNA sequences engineered in vitro can be readily introduced into the genome of *Saccharomyces cerevisiae* cells by homologous recombination, facilitating the creation of duplications, insertions, and deletions of anything from single genes to large chromosomal fragments (33, 38). In contrast, early experiments with mammalian cells showed that recombinant DNA molecules are most often inserted randomly into the genome by a mechanism that does not require extensive identity between the DNA fragment and the genomic sequences (32, 40, 47). More recently, it was shown that the efficiency of homologous gene replacement in mammalian cells can be greatly enhanced relative to random integration by using DNA sequences from a source that is isogenic to the recipient cells, suggesting that the presence of mismatches between the DNA fragment and the genome strongly inhibits homologous recombination (10, 45).

DNA sequence mismatching presents a considerable barrier to homologous recombination in a wide variety of systems (5, 9, 10, 14, 21, 31, 34–36, 41, 45, 49, 50). Several laboratories have observed that defects in the mismatch repair machinery in bacterial species greatly lower the barrier against recombination between mismatched sequences (12, 18, 25, 29, 36, 54). Other investigators have shown that mutations in mismatch repair genes in yeast (9, 34) and mammal (11) cells similarly reduce the inhibitory effect of mismatches, indicating that this genetic mechanism is evolutionarily conserved. It has been suggested that nonidentical sequences are prevented from recombining because the mispairing that occurs when heteroduplex DNA is formed is recognized by the mismatch repair machinery, after which the heteroduplex is unwound (9) or multiply nicked (27). The mismatch repair machinery in yeast corrects mismatches in the heteroduplex formed during recombination (1, 19, 28, 51), suggesting that it could have a role in dissolving mismatched heteroduplexes. Alternatively, mismatch repair may inhibit the creation of mismatched heteroduplexes. In vitro studies of RecA-mediated strand transfer in *Escherichia coli* support this hypothesis, because the mismatch repair protein MutS can bind mismatches (23) and inhibit strand transfer between mismatched sequences (52). A yeast homolog of this protein, Msh2p, also binds mismatches in vitro (2, 22, 26), suggesting that a similar mechanism could obtain in yeast.

We have developed a novel assay for studying homologous gene replacement in yeast cells in which a DNA fragment, released in vivo from a single-copy plasmid, recombines with a genomic target. This approach was taken because introduction of DNA fragments by transformation presents different numbers of DNA molecules to each cell in the culture; therefore, the yield of gene replacement events reflects interactions between different numbers of fragments and the genomic target in different cells. We used our method to determine the effect of DNA sequence mismatching on the frequency of gene replacement and found that by using fragments that were 17% mismatched with the genomic target reduced the frequency at least 1,000-fold relative to recombination between identical sequences. The mismatch repair pathway was implicated in this inhibition, because a mutation in the *MSH2* gene stimulated the frequency of recombination between mismatched sequences over 25-fold relative to identical sequences. Interestingly, chimeric DNA fragments made up of half mismatched and half identical sequences recombined with the genomic target at a frequency that was intermediate to that of completely mismatched and identical fragments. This indicates that the similarity of the sequences on both sides of the fragment to the target determines the frequency of gene replacement. We also observed that a DNA fragment containing a mismatched sequence bordered on both sides by sequences that are identical to the target recombines at the same frequency as a completely identical sequence. Failure to inhibit recombination suggests that the mismatches on this DNA fragment were not detected, possibly because they were not incorporated into a heteroduplex during recombination. This final observation

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TABLE 1. Plasmids constructed for this study

Plasmid	Description
pLAY98	117-bp fragment of the *MAT***a** locus containing the 22-bp recognition sequence for HO-endonuclease from pRK113 cloned at either end of the polylinker sequence in pRS416.
pLAY99	117-bp fragment of the *MAT***a** locus containing the 22-bp recognition sequence for HO-endonuclease from pRK113 cloned at either end of the polylinker sequence in pRS413.
pLAY108	842-bp *Eco*RV/*Hin*dIII fragment of the *SAM2* locus containing the 3′ end of the *SAM2* coding sequence up to the termination codon inserted into the polylinker of pLAY98. The first and third nucleotides downstream from the *SAM2* termination codon (Fig. 2) were modified by site-directed mutagenesis to create a *Hin*dIII site immediately adjacent to the termination codon.
pLAY109	842-bp *Eco*RV/*Hin*dIII fragment of the *SAM1* locus containing the 3′ end of the *SAM1* coding sequence up to the termination codon inserted into the polylinker of pLAY98. The *Hin*dIII site is immediately adjacent to the termination codon at the *SAM1* locus (Fig. 2).
pLAY111	453-bp *Eco*RV/*Sal*I fragment of the *SAM2* coding sequence from pLAY108 replacing the corresponding *SAM1* information in pLAY109.
pLAY112	453-bp *Eco*RV/*Sal*I fragment of the *SAM1* coding sequence from pLAY109 replacing the corresponding *SAM2* information in pLAY108.
pLAY114	1.3-kb DNA fragment containing the *HIS3* coding sequence inserted into the *Sal*I site in the *SAM2* coding sequence on pLAY108.
pLAY135	1.4-kb DNA fragment containing the *CYH2* gene and a 1.2-kb DNA fragment containing the *URA3* gene inserted into the *Sal*I site in the *SAM2* coding sequence on pLAY108.
pLAY164	1.5-kb *Bgl*II/*Xho*I fragment containing the entire *SAM2* locus inserted into the polylinker of pLAY99.
pLAY165	1.5-kb *Eco*RV/*Eco*RI fragment containing the 3′ end of the *SAM2* gene and 3′ flanking material inserted into the polylinker of pLAY99.
pLAY168	453-bp *Eco*RV/*Sal*I fragment of the *SAM1* coding sequence from pLAY109 replacing the corresponding *SAM2* information in pLAY164.
pLAY172	453-bp *Eco*RV/*Sal*I fragment of the *SAM1* coding sequence from pLAY109 replacing the corresponding *SAM2* information in pLAY165.

suggests strategies for DNA fragment design that can be used to increase the efficiency of homologous gene replacement.

MATERIALS AND METHODS

Strains. The following yeast strains were used: ABX94-13B (*MAT***a**::*LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 sam1*-Δ*Bgl*II *sam2*-Δ*Sal*I::*HIS3*), ABX110-28A (*MAT***a**::*LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 sam1*::*LEU2 sam2*-Δ*Sal*I::*HIS3*), ABM54 (*MAT***a**::*LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 sam1*-Δ*Bgl*II *sam2*-Δ*Sal*I::*HIS3 msh2*::*hisG*), and ABT160 (*MAT***a**::*LEU2 ade2-1 can1-100 his3-11,15 leu3-2,112 trp1-1 ura3-1 sam2*-Δ*Sal*I::*CYH2*::*URA3 cyh2*^{r}). The strains were isogenic and were constructed for this study. Standard methods for the genetic manipulation of yeast were employed (37). Cycloheximide-resistant (*cyh2*^{r}) yeast strains were selected from 10^8 cells plated on YPD plates containing cycloheximide at 10 μg/ml and incubated at 30°C for 5 days (39). *E. coli* DH5α [F′/*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA argF*) *U169* (ϕ*80dlac*Δ(*lacZ*)*M15*] was used for all plasmid DNA amplification according to standard protocols for bacterial cell growth and transformation (17).

Media. All *S. cerevisiae* strains were maintained and grown on the appropriate dropout medium (37) in either the presence or the absence of *S*-adenosylmethionine (AdoMet [Sigma]) at a concentration of 0.1 mg/ml. Sporulation medium was made as described previously (37). Sufficient 5-fluoroorotic acid (5-FOA [PCR Inc.]) for a final concentration of 75 μg/ml was added to molten medium before pouring, and cycloheximide (Sigma), for a final concentration of 10 μg/ml, was added to solid medium before plating when required for selection (39).

Plasmids. All of the plasmids constructed for this study are described in Table 1 and were constructed by established molecular techniques (17). The *SAM1* and *SAM2* sequences were derived from genomic clones contained on plasmids pWJ259 and pWJ260 as described previously (5). A plasmid, pKC3 (K. Chapman and J. Boeke), containing a 1.4-kb complete cDNA clone of the *CYH2* gene was used in the construction of plasmid pLAY135 (Table 1). This plasmid also contained a 1.2-kb genomic clone of the *URA3* gene from pUC-URA3 (33). Similarly, a 1.3-kb genomic clone of the *HIS3* gene from pUC-HIS3 (33) was used in the construction of the plasmid pLAY114 (Table 1). A DNA fragment containing the *msh2*::*hisG*::*URA3*::*hisG* allele of the *MSH2* mismatch repair gene from *S. cerevisiae* (30) was obtained from the plasmid pEAI98 (E. Alani and R. Kolodner) and used to create mismatch repair-defective mutants by one-step gene disruption (33). The 117-bp fragment of the *MAT***a** gene containing the recognition sequence for HO-endonuclease (24) used in the construction of pLAY98 and pLAY99 (Table 1) was removed from plasmid pRK113 (R. Kostricken). The backbones for pLAY98 and pLAY99 were the single-copy yeast plasmids pRS416 and pRS413, respectively (7). The plasmid pGHOT (Rob Jensen and Ira Herskowitz) is a single-copy plasmid containing the *GAL1*::*HO* fusion gene, permitting galactose-regulated expression of HO-endonuclease (16).

Mutant *SAM* gene constructions. The *sam1*-Δ*Bgl*II frameshift and *sam1*::*LEU2* deletion/disruption mutations were constructed and used as previously described (3–5). The *sam2*-Δ*Sal*I::*HIS3* and *sam2*-Δ*Sal*I::*CYH2*::*URA3* gene disruption constructions described above were transplaced into the yeast genome by single-step gene disruption (33). The presence of the mutant genes was detected both by the loss of *SAM* gene function, which cosegregated with the dominant selectable marker in crosses, and by alterations in the structure of the *SAM* loci as detected by genomic Southern blot analysis (53).

Recombination assays. (i) Spontaneous gene replacement. Single colonies of yeast strains containing a *SAM* substrate plasmid (Fig. 1), but not pGHOT, were inoculated into 1 to 10 ml of 2% glucose liquid medium lacking uracil for selection of the plasmid, but supplemented with AdoMet, and grown to a density of 1×10^7 to 3×10^7 cells/ml at 30°C. Appropriate dilutions of these cultures were plated onto plates containing medium lacking Ura (−Ura) supplemented with AdoMet, and the cells were grown at 30°C for 5 to 7 days to determine the number of viable cells in the culture. An appropriate number of cells were also plated onto 10 −Ura plates and incubated at 30°C for 5 to 7 days, selecting for the growth of AdoMet prototrophs. AdoMet prototrophs can arise as the result of several different events. In addition to recombination between the *SAM* substrate fragment on the plasmid and the *SAM2* locus, gene conversion between the *SAM1* and *SAM2* loci, as well as spontaneous reversion of the mutations at either of the *SAM* loci, can result in AdoMet prototrophy (3–5). The opposite orientations of the *SAM1* and *SAM2* genes relative to their centromeres preclude the isolation of reciprocal recombinants (5). In order to determine the fraction of AdoMet prototrophs that were due to events at the *SAM2* locus (the intended target of gene replacement), AdoMet prototrophs were replica plated to medium lacking histidine, scoring for the loss of the *HIS3* marker inserted into the *SAM2* coding sequence. As previously observed (3–5), the majority of the events (greater than 70%) occurred at *SAM2*. The fraction of these events that were attributable to gene conversion between *SAM1* and *SAM2* was determined in strains lacking a *SAM* substrate fragment on a plasmid (Fig. 1). No AdoMet$^+$ prototrophs were obtained with a strain lacking *SAM1* sequences, indicating that spontaneous loss of the insertion at *SAM2* cannot account for the formation of AdoMet prototrophs. Recombination frequencies at *SAM2* were determined by dividing the number of His$^-$ AdoMet$^+$ colonies by the total number of viable cells plated.

(ii) HO-stimulated gene replacement. Single yeast colonies of cells containing both a *SAM* substrate plasmid (Fig. 1) and pGHOT (16) were used to inoculate 1 to 10 ml of 2% glucose liquid medium lacking both uracil and tryptophan to select for both plasmids and containing AdoMet, and the cultures were grown to a density of 1×10^7 to 3×10^7 cells/ml. Dilutions of these cultures were plated onto −Trp −Ura medium containing AdoMet and incubated at 30°C for 5 to 7 days to determine the number of viable cells in the culture. Appropriate volumes

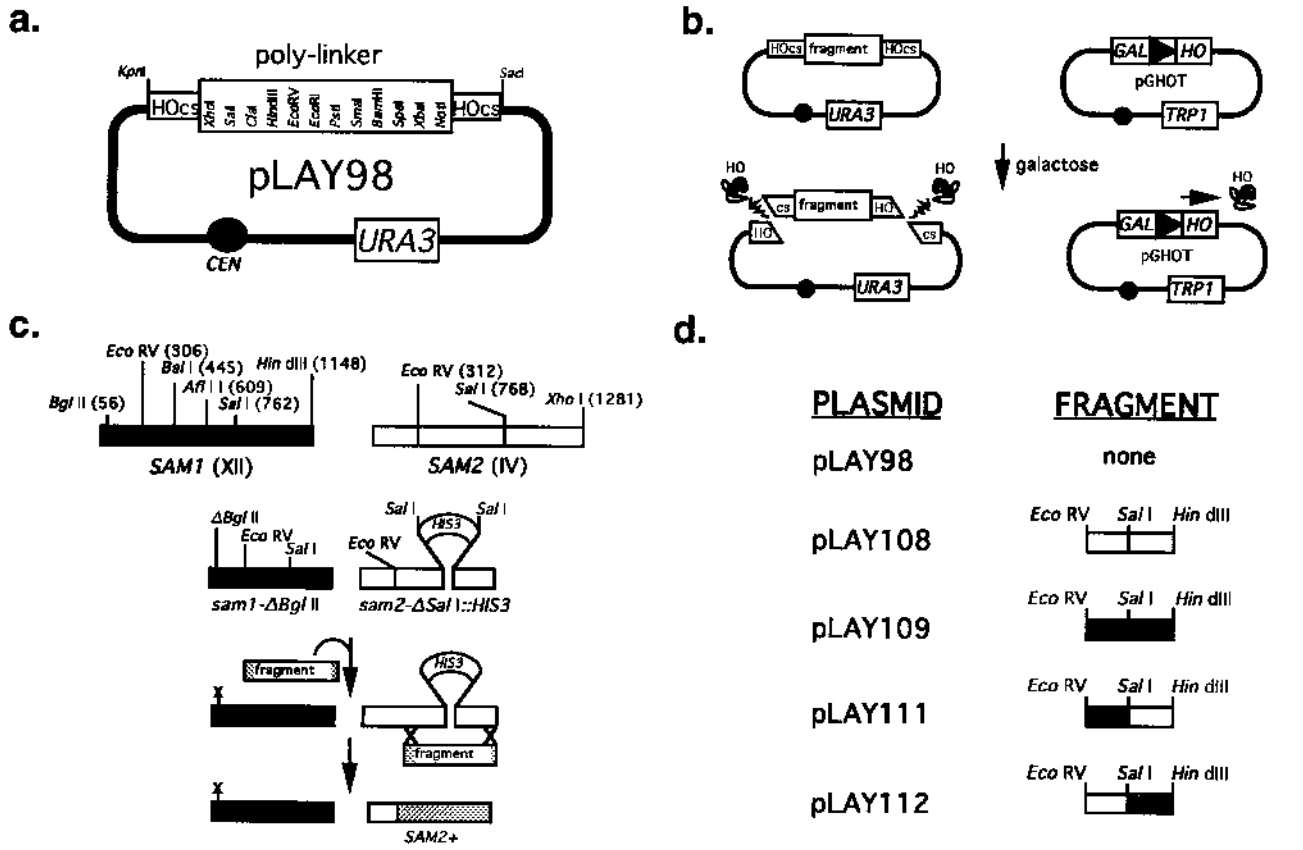

FIG. 1. Homologous gene replacement assay with DNA fragments released in the nucleus from single-copy plasmids. (a) Plasmid pLAY98. The single-copy yeast-*E. coli* shuttle vector pLAY98 was constructed from the plasmid pRS416 (7) by insertion of a 117-bp fragment of the yeast mating-type locus carrying the recognition sequence for HO-endonuclease (24) on either side of the polylinker. This allows for the insertion of any DNA sequence between the HO-cut-sites (HOcs). (b) Fragment release. A single copy of pLAY98 containing the DNA fragment is stably maintained in a host yeast strain by selection for the *URA3* genetic marker. The DNA fragment can be released from the plasmid if a second single-copy plasmid (pGHOT) carrying a galactose-inducible HO-endonuclease gene is present and galactose is added to the growth medium. HO cutting at the HOcs releases the DNA fragment. (c) Homologous gene replacement. The DNA fragment (842 bp) released from pLAY98 can recombine with homologous genomic sequences in the experimental strain ABX94-13B. Recombination between the fragment and the genomic target at the *SAM2* locus simultaneously restores the ability to grow without AdoMet and results in an auxotrophy for histidine. Recombination between the fragment and the *SAM1* locus cannot restore AdoMet prototrophy, because the fragment and *SAM1* sequences do not overlap at the site of the mutation in *SAM1*. (d) Plasmids used in this analysis. Fragments of the *SAM1* and *SAM2* coding sequences were inserted into the polylinker of pLAY98, creating plasmids pLAY108, pLAY109, pLAY111, and pLAY112 as described in Table 1 and the text.

of cell culture were plated onto −Trp medium containing 2% galactose (Sigma) to induce the expression of the *HO* gene and were incubated at 30°C for 5 to 7 days, selecting for AdoMet prototrophs that contain pGHOT. Uracil is included in the medium because plasmids cut by HO-endonuclease in vivo are efficiently lost (4). Consistent with these observations, none of the AdoMet prototrophs that we obtained were able to grow on −Ura medium (data not shown). The AdoMet prototrophs were replica plated to −His medium to determine which were due to events at the *SAM2* locus. Recombination frequencies at *SAM2* were determined by dividing the number of His^- $AdoMet^+$ colonies by the number of viable cells plated. Genomic DNA from a single $AdoMet^+$ recombinant colony from each plating was analyzed as described below.

(iii) Mismatch context shift. Single colonies of yeast cells containing both a *SAM* substrate plasmid (see Fig. 3) and pGHOT were inoculated into 1 to 10 ml of liquid medium containing 3% glycerol and 3% lactate but lacking both histidine and tryptophan in order to select for both plasmids. The cultures were grown to a density of 5×10^6 to 1×10^7 cells/ml at 30°C as determined by hemacytometer counting, and appropriate dilutions of cells were plated on −His −Trp medium to determine the number of viable cells in the culture. The appropriate volume of 20% galactose was then added to bring the concentration to 2%. The addition of galactose to the medium induced the expression of HO-endonuclease, and 2 h of incubation at 30°C was sufficient to obtain 90 to 95% release of the *SAM* substrate from the plasmids in wild-type cells (data not shown). After this induction, an appropriate dilution of these cells was plated onto −Trp 2% glucose medium to assess viability. We observed that greater than 70% of the cells survived induction, but none of the survivors that we tested were able to grow on −His medium, indicating that the plasmid was efficiently lost from the cells after it was cut.

The induced cells were plated on synthetic medium containing both cycloheximide (10 μg/ml) and 5-FOA (75 μg/ml) to select for loss of the *CYH2* and *URA3* sequences at the *SAM2* locus by recombination between the released *SAM* fragment and the target at *SAM2*. The plates were incubated for 5 to 7 days at 30°C. It was necessary to induce the expression of HO-endonuclease before plating on cycloheximide-containing medium because the induction requires protein synthesis, and this would not occur in a strain containing the wild-type *CYH2* sequence. Because of the differences in how the *GAL*::*HO* gene is induced, the frequencies of recombination obtained in this assay are not directly comparable with those in the gene replacement assays described in the legend to Fig. 1. Loss of the wild-type *CYH2* and *URA3* sequences from *SAM2* uncovered the presence of recessive alleles at the *CYH2* and *URA3* loci that confer resistance to cycloheximide and 5-FOA. The recombination frequency was determined by counting the number of cycloheximide- and 5-FOA-resistant colonies that arise and dividing by the number of viable cells (determined after induction) that were plated. The spontaneous appearance of cycloheximide- and 5-FOA-resistant colonies was very rare (Table 2). Genomic DNA from a single Cyh^r $5\text{-}FOA^r$ recombinant colony from each plating was analyzed as described below.

(iv) Statistical analysis. The median frequency from a minimum of 10 trials was used to compare recombination frequencies in different strains. We tested for statistically significant differences between the frequencies obtained with different pairs of strains by determining the number of trials with each strain that gave recombination frequencies that were above and below the median frequency obtained from both strains and then comparing those numbers by contingency χ^2 analysis and with Yate's correction for continuity (8).

Analysis of genomic DNA from recombinants. DNA was prepared from 1-ml saturated YPD cultures of selected recombinant colonies by the method of Hoffmann and Winston (15). The DNA was digested with either *Bal*I and *Xho*I or *Afl*II and *Xho*I restriction endonucleases (New England Biolabs) before elec-

TABLE 2. Frequencies of spontaneous and HO-stimulated gene replacement at the *SAM2* locus in wild-type and mismatch repair mutant cells[a]

Plasmid[b]	Insert	Replacement frequency in[c]:		
		MSH2		*msh2*::*hisG* HO stimulated
		Spontaneous	HO stimulated	
Group 1				
pLAY98	None	2.0×10^{-9}		
pLAY108	*sam2*	1.4×10^{-7}	2.1×10^{-6}	8.9×10^{-5}
pLAY109	*sam1*	2.0×10^{-9}	2.1×10^{-9}	2.4×10^{-6}
pLAY111	*5′-sam1-sam2-3′*	1.2×10^{-8}	3.5×10^{-7}	2.9×10^{-5}
pLAY112	*5′-sam2-sam1-3′*	1.7×10^{-8}	1.8×10^{-7}	3.2×10^{-5}
Group 2				
pLAY99	None		4.0×10^{-10}	
pLAY164	*sam2*		1.3×10^{-7}	
pLAY165	*sam2*		8.0×10^{-8}	
pLAY168	*5′-sam2-sam1-sam2-3′*		5.6×10^{-8}	
pLAY172	*5′-sam1-sam2-3′*		3.4×10^{-9}	

[a] Gene replacement assays were conducted as described in the text and in the legends to Fig. 1 and 3. Frequencies are expressed as the number of AdoMet$^+$ His$^-$ recombinants per viable cell in group 1 and as the number of 5-FOA- and cycloheximide-resistant recombinants per viable cell in group 2. The median frequency from a minimum of 10 separate determinations is reported. Assays were conducted at 30°C.

[b] Replacement substrates either remained in the plasmids during spontaneous recombination assays or were released from the plasmids in vivo by HO-endonuclease as described in the text. Replacement substrates are pictured in Fig. 1 and 3 and described in Table 1.

[c] Replacement frequencies were determined with isogenic wild-type (*MSH2*) and *msh2*::*hisG* mutant cells.

trophoresis and blotting to nylon membranes (Amersham). Blots were hybridized to a radioactively labeled 514-bp *Sal*I/*Xho*I fragment of the *SAM2* gene (Fig. 3) and autoradiographed under established conditions (5).

RESULTS

DNA sequence identity is an important determinant of the frequency of gene replacement. We have developed an assay to examine the replacement, by homologous recombination, of a genomic sequence with DNA fragments released from a single-copy plasmid (Fig. 1). The fragments consisted of 842 bp of coding sequence from the 3′ ends of the *SAM1* gene, the *SAM2* gene (Fig. 2), or chimeras containing half of each (Fig. 1d). The *SAM1* and *SAM2* genes encode AdoMet synthetase isozymes, are unlinked, and are 83% identical at the DNA sequence level (46). The *SAM1* gene in the experimental strain contains a 4-bp insertion at the 5′ end of the gene, while the *SAM2* gene contains a 1.3-kb insertion of a wild-type copy of the *HIS3* gene at the *Sal*I site in the 3′ end of the coding sequence (Fig. 1c). Since both genes are nonfunctional, the strain requires AdoMet for growth.

We conducted our recombination assays by growing cells nonselectively in liquid cultures that contain AdoMet and then plating them on solid medium that lacks AdoMet, selecting for the growth of cells that have a wild-type *SAM* gene. Because we were interested in recombination events that replaced the *HIS3* insertion sequences at the *SAM2* locus with *SAM* information, only AdoMet$^+$ His$^-$ recombinants were used in our calculations of the frequency of gene replacement.

Two different types of experiment were conducted with this system: (i) spontaneous recombination between the plasmid sequences and the genome and (ii) recombination stimulated by the release of the *SAM* sequences from the plasmid by HO-endonuclease (Table 2). Both spontaneous recombination and HO-stimulated recombination between the plasmid and genomic sequences were sensitive to mismatches (Table 2). While the frequency of spontaneous recombination to produce AdoMet$^+$ His$^-$ cells was no higher with the *SAM1* sequences in the plasmid (pLAY109 [2.0×10^{-9}]) than it was when no *SAM* sequences were present (pLAY98 [2.0×10^{-9}]), the frequency of AdoMet$^+$ His$^-$ recombinants with the *SAM2* fragment in the plasmid (pLAY108 [1.4×10^{-7}]) was increased 70-fold. The plasmids carrying the chimeric *SAM* sequences recombined with the *SAM2* locus at frequencies (pLAY111, 1.2×10^{-8}; pLAY112, 1.7×10^{-8}) that were intermediate to the *SAM1* and *SAM2* frequencies. These results suggest that the frequencies of spontaneous gene replacement are determined by the affinity of both halves of the chimeric sequences for the genomic target.

Releasing the *SAM* sequences from the plasmids with HO-endonuclease increased the frequency of gene replacement at the *SAM2* locus at least 10-fold with all of the plasmids except the one carrying *SAM1* sequences exclusively (pLAY109). The failure to see an increase in the frequency of recombination with the *SAM1* sequences in pLAY109 cannot be due to a nonfunctional fusion of *SAM1* and *SAM2* sequences, because the *SAM1* sequences in pLAY111 and pLAY112 can recreate a functional *SAM2* locus. Interestingly, the *SAM1* sequences on the chimeric fragments in pLAY111 and pLAY112 replace sequences at the *SAM2* locus 86- to 167-fold more frequently when tethered to *SAM2* sequences than when tethered to *SAM1* sequences on pLAY109 (Table 2). However, these frequencies were still significantly lower (1.8×10^{-7}, $\chi^2 = 7.2$, $P = 0.007$; 3.5×10^{-7}, $\chi^2 = 4.2$, $P = 0.027$) than when the complete *SAM2* fragment was present (2.1×10^{-6}), demonstrating that the *SAM1* sequences on the chimeric fragments still inhibited recombination with the *SAM2* target.

One explanation for the residual inhibition is that a significant number of the chimeric fragments were sequestered by interactions with the *SAM1* locus, reducing the likelihood of recombination with the *SAM2* locus. Alternatively, interactions between the mismatched fragment and target sequences could be aborted by the cell. When we repeated the experiments described above (Fig. 1) in a strain lacking *SAM1* genomic sequences (ABX110-28A), we found that the frequencies of recombination (data not shown) were not significantly different from those observed in the strain with a *sam1* insertion allele (ABX94-13B). This demonstrates that the presence of an alternative genomic target does not affect the efficiency of re-

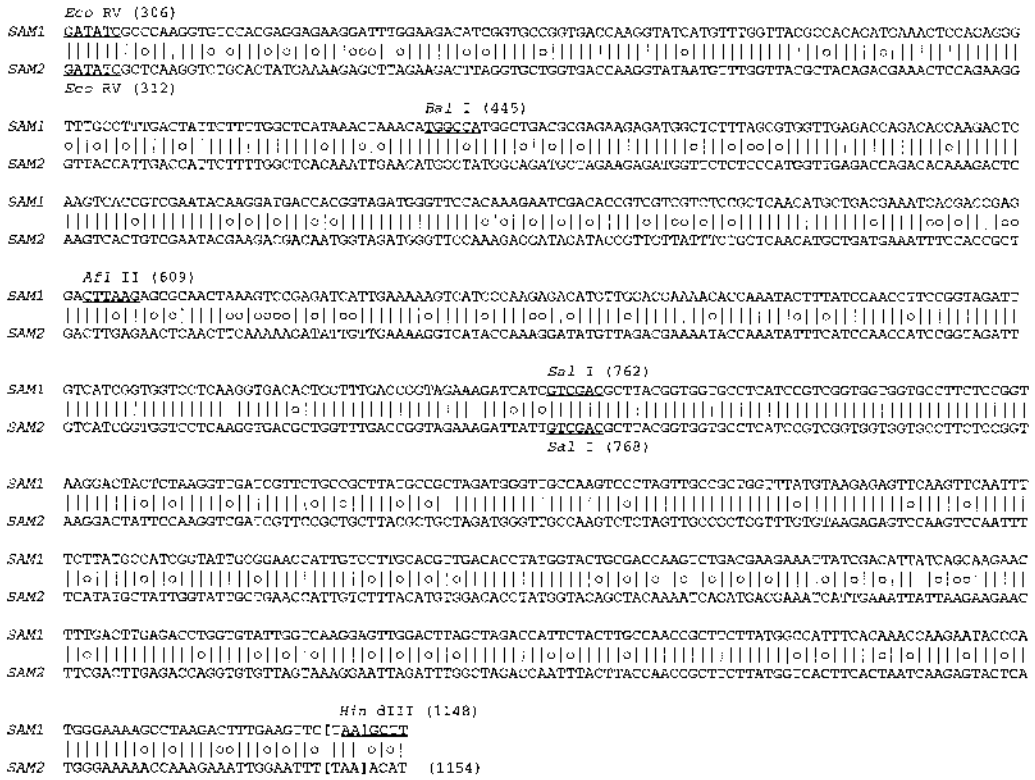

FIG. 2. DNA sequence of *SAM1* and *SAM2* fragments. The 842-bp DNA sequence from the 3′ ends of the *SAM1* and *SAM2* genes used in the gene replacement experiment depicted in Fig. 1 is shown. These sequences run from the *Eco*RV sites in the coding sequences to just beyond the termination codons (in brackets) of both *SAM1* and *SAM2* and are displayed in alignment. There are 139 mismatches in the 842 bases of sequence for an overall level of mismatching of 16.5%. The mismatches are evenly distributed (i.e., mismatching between the 5′ halves of the fragments, from the *Eco*RV to *Sal*I sites, is 17.5%, while mismatching between the 3′ halves of the fragments, from the *Sal*I sites to just beyond the termination codons, is 15.3%). Similarly, the first and last 50 bp of these sequences are equivalently mismatched, with 12 mismatches in the first 50 bp and 13 mismatches in the last 50 bp.

combination between the *SAM* fragments and the *SAM2* locus. Furthermore, this suggests that mismatched and identical sequences are equally likely to encounter each other, but that when mismatched sequences interact, they are often prevented from recombining.

A mutation in the mismatch repair gene *MSH2* reduces the barrier against gene replacement with mismatched sequences. A null allele of the yeast mismatch repair gene *MSH2* has previously been shown to increase recombination between mismatched sequences to a greater extent than with identical sequences (9, 34). Similarly, we have found that while the frequency of HO-stimulated gene replacement at the *SAM2* locus with all of the *SAM* fragments was higher in the *msh2* mutant strain than in the wild-type strain (Table 3), the frequency of replacement with the mismatched *SAM1* fragment (pLAY109) was increased over 1,000-fold (2.1×10^{-9} to 2.4×10^{-6}), while the frequency with the identical *SAM2* fragment (pLAY108) was increased only about 40-fold (2.1×10^{-6} to 8.9×10^{-5}). Also, the frequencies of recombination with the chimeric fragments (pLAY111 and pLAY112) were increased from 80- to 180-fold (3.5×10^{-7} to 2.9×10^{-5} and 1.8×10^{-7} to 3.2×10^{-5}) in the *msh2* mutant cells, so that the frequencies of gene replacement at the *SAM2* locus with the chimeric and identical fragments were no longer significantly different from one another ($\chi^2 = 1.8, P = 0.18$). These results indicate that an element of the mismatch repair system opposes gene replacement with mismatched sequences. The increase in the frequency of gene replacement with all of the fragments in the *msh2* mutant cells suggests that Msh2p, to some extent, suppresses gene replacement with all of the experimental sequences. It should also be noted that the frequency of HO-stimulated gene replacement at the *SAM2* locus with the mismatched *SAM1* fragment from pLAY109 (2.4×10^{-6}) is 16-fold higher than the frequency of spontaneous gene replacement in the *msh2* mutant cells (1.5×10^{-7}). This indicates that the failure of HO cutting to stimulate gene replace-

TABLE 3. Distribution of recombinant types

Plasmid[a]	Genotype[b]	No. of recombinant types with characteristic/no. sampled[c]		
		SAM2$^+$ (0)	*SAM2*$^+$ (Δ*B*)	*SAM2*$^+$ (Δ*BA*)
pLAY111	*MSH2*	1/16	4/16	11/16
	msh2::*hisG*	7/17	3/17	7/17
pLAY164	*MSH2*	16/16	0/16	0/16
pLAY172	*MSH2*	3/10	3/10	4/10

[a] Replacement substrates were released from the plasmids in vivo by HO-endonuclease as described in the text and the legends to Fig. 1 and 3. Replacement substrates are pictured in Fig. 1 and 3.

[b] Recombinants obtained from isogenic wild-type (*MSH2*) and *msh2*::*hisG* mutant cells were analyzed.

[c] The presence or absence of *Bal*I or *Afl*II restriction endonuclease sites in the *SAM2* coding sequence in cycloheximide- and 5-FOA-resistant colonies was scored as described in the text. The (0) class contains both sites, the (Δ*B*) class is missing the *Bal*I site, while the (Δ*BA*) class is missing both sites. Recombinants missing only the *Afl*II site were not observed.

ment with pLAY109 above spontaneous levels in wild-type cells (Table 2) is due to the inhibitory action of the mismatch repair system and is not due to a defect in the assay.

In recent studies, it has been shown that mismatch repair proteins from *E. coli* can inhibit RecA-catalyzed branch migration of Holliday junctions through mismatched sequences in vitro (52). It has been proposed that yeast mismatch repair proteins might play a similar role in inhibiting recombination between mismatched sequences (1, 52). We studied this possibility by exploiting the differences between *SAM1* and *SAM2* restriction endonuclease susceptibility (3–5, 46) in an attempt to determine the extent of information exchange during recombination in wild-type and mismatch repair-defective cells (Table 3). We used *Bal*I and *Afl*II sites that are unique to the *SAM1* gene (Fig. 2) to indicate how much of the 453-bp *SAM1* segment of the DNA fragment on pLAY111 was introduced into the *SAM2* locus during gene replacement. We found three classes of recombinant in both cell types: one in which both sites still remained (0), one in which the *Bal*I site was missing (Δ*B*), and one in which both the *Bal*I and *Afl*II sites were missing (Δ*BA*). No recombinants lacking only the *Afl*II site were observed.

Significant differences ($\chi^2 = 5.5$, $P = 0.06$) in the distribution of recombinant types were observed in the wild-type and *msh2* mutant cells (Table 3). We found that there were more recombinants missing both restriction sites (Δ*BA*) in the wild-type cells than in the *msh2* mutants (11 of 16 versus 7 of 17), while there were more recombinants retaining both restriction sites (0) in the *msh2* mutants than in the wild-type cells (7 of 17 versus 1 of 16). These results strongly suggest that mismatch repair has a role in determining the extent of heteroduplex formation and/or mismatch repair during recombination in yeast and that it is at least one determinant of the extent of information exchange during gene replacement. These results are also consistent with the previous suggestion that mismatch repair restricts recombination between mismatched sequences by limiting branch migration (1, 52).

The inhibitory effect of mismatched sequences on gene replacement is dependent on their proximity to the end of the DNA fragment. Strathern and colleagues found that the further a mismatch is from a double-strand break at the *MAT* locus, the less likely it will be coconverted during the gene conversion event that repairs the break (20). This indicates that the further a sequence is from the edge of the recombination substrate, the less likely it will be subjected to mismatch repair during recombination. We designed an experiment to test whether mismatched sequences that are distal to the edge of the DNA fragment suppress replacement of the genomic target as well as sequences that are immediately adjacent to the end of the fragment (Fig. 3). Similar to our previous experiments (Fig. 1 and Table 2), a 453-bp segment of the *SAM1* gene significantly reduced the frequency of gene replacement (3.4×10^{-9} versus 8.0×10^{-8}, $\chi^2 = 16.2$, $P = 0.001$) when it was substituted for the analogous *SAM2* sequences at the end of a *SAM2* DNA fragment (Fig. 3 [pLAY172 versus pLAY165]). However, when the same *SAM1* segment replaced a *SAM2* sequence that was equidistant (~500 bp) from the ends of a *SAM2* fragment (Fig. 3 [pLAY168 versus pLAY164]), it had no significant effect on the gene replacement frequency (5.6×10^{-8} versus 1.3×10^{-7}, $\chi^2 = 0.18$, $P = 0.67$). Therefore, the inhibition of gene replacement by mismatching is dependent on the distance between the mismatches and the end of the DNA fragment, and 500 bp is too long for the inhibition in yeast.

Previously, we observed a correlation between low frequencies of gene replacement (Table 2) and a high incidence of restriction site loss (Table 3) during recombination between mismatched DNA fragment and genomic target sequences. Similarly, we found that loss of the *Bal*I and *Afl*II sites (Table 3) from the *SAM1* portion of the low-efficiency gene replacement substrate from pLAY172 often accompanied recombination (7 of 10 recombinants), while no restriction site loss was observed in the recombinants obtained with the high-efficiency pLAY168 substrate (0 of 16 recombinants). In combination with the previous results that implicated the mismatch repair system as a factor in restriction site loss, these results suggest that the *Bal*I and *Afl*II sites in the pLAY172 fragment are often involved in a heteroduplex with the genomic target and that they are subjected to mismatch repair. Conversely, the presence of the *Bal*I and *Afl*II sites in all of the pLAY168 recombinants suggests that they are not involved in a heteroduplex or included in a mismatch repair tract, possibly because these sequences lie too far from the edge of the fragment. It is possible that a failure of the mismatch repair system to detect the *SAM1* sequences in the DNA fragment from pLAY168 explains why they fail to inhibit recombination at the *SAM2* locus (Table 2).

DISCUSSION

We have designed a new assay for determining the frequency of recombination between a single DNA fragment released in vivo and a genomic sequence and have used it to show that DNA sequence identity is an important determinant of the efficiency of gene replacement in yeast. We have also shown that the mismatch repair gene *MSH2* is responsible, in part, for the inhibition of gene replacement with mismatched sequences. In addition, we have shown that there is a relationship between the position of mismatched sequences in a DNA fragment and the efficiency of gene replacement that could be determined by the accessibility of the mismatches to mismatch repair.

One peculiarity of the new assay system is that the fragments released from the plasmids by HO-endonuclease have 45- and 72-bp fragments of the HO-cut-site sequence left on their ends after cleavage (24). In experiments in which *SAM* DNA fragments with and without 70- and 40-bp heterologous DNA fragments added onto the ends were transformed into yeast cells, we found that the heterologous ends reduced the efficiency of gene replacement at the *SAM2* locus by less than threefold (53). This result suggests that the presence of the HO-cut-site sequences on the ends of the *SAM* fragments released in vivo does not greatly affect the efficiency of gene replacement. Previous experiments indicated that the nuclease encoded by the *RAD1* and *RAD10* genes (42, 48) is required to cleave away nonhomologous HO-cut-site sequences before HO-catalyzed double-strand breaks in genomic sequences can be repaired by recombination (6, 13). In recent experiments with *rad1*-null mutant cells, we have found that the frequency of HO-stimulated gene replacement at the *SAM2* locus by a *SAM2* fragment released from pLAY108 (Fig. 1) is not significantly different from the frequency of spontaneous gene replacement (53). This result suggests that the Rad1/Rad10 nuclease is also required to cleave the HO-cut-site sequences from the *SAM* fragments before they can efficiently recombine with the *SAM2* genomic target.

While the frequency of recombination between mismatched DNA fragment and genomic target sequences was increased relative to the frequency of recombination between identical sequences in the mismatch repair-defective *msh2* cells, we were surprised to find that even the frequency of recombination between identical DNA fragment and genomic sequences

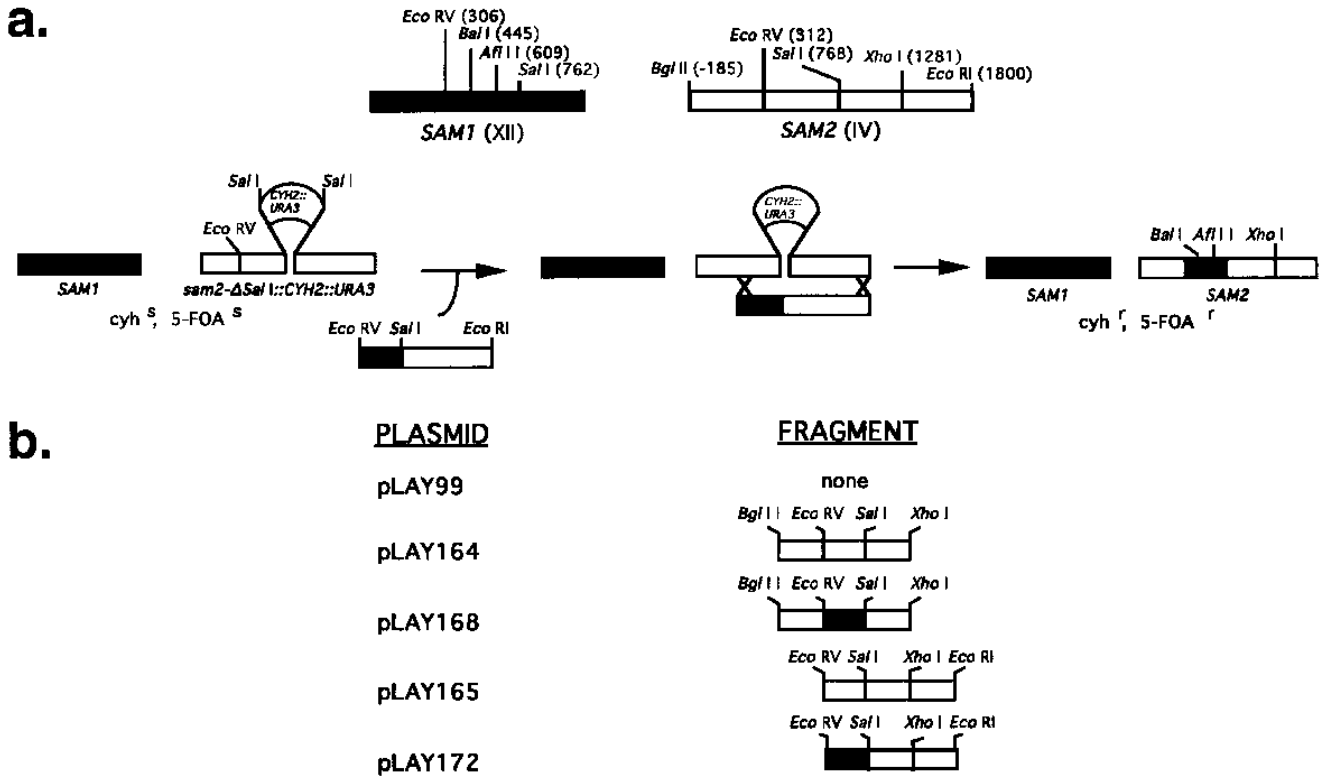

FIG. 3. Mismatch context shift experiment. (a) This assay is a modified version of the homologous gene replacement assay described in the legend to Fig. 1. The *SAM2* locus (chromosome IV) has been modified by insertion of a 1.4-kb DNA fragment carrying the wild-type *CYH2* gene and a 1.2-kb DNA fragment containing the wild-type *URA3* gene at the unique *Sal*I site in the *SAM2* coding sequence. Along with a disruption of the *MAT* locus, this strain (ABT160) contains a recessive cycloheximide resistance allele at the *CYH2* locus and the recessive *ura3-1* allele at the *URA3* locus that confers resistance to 5-FOA. Loss of the wild-type *CYH2* and *URA3* genes by insertion of a DNA fragment into the *SAM2* locus by homologous recombination uncovers the recessive alleles at the *CYH2* and *URA3* loci and changes the cycloheximide- and 5-FOA-sensitive strain into a strain that is resistant to both cycloheximide and 5-FOA. (b) Each assay strain contains a plasmid that is comprised of a *SAM* DNA fragment inserted into the plasmid pLAY99. pLAY99 is identical to the plasmid pLAY98, described in the text and the legend to Fig. 1, except that the selectable marker is *HIS3* instead of *URA3*. Each *SAM* DNA fragment is 1.5 kb in size and consists either completely of *SAM2* sequences (open boxes) or of both *SAM2* and the 453 bp of *SAM1* sequence between the *Eco*RV and *Sal*I sites (solid boxes). The plasmid pGHOT, which contains the galactose-inducible HO-endonuclease gene, is also present in the assay strains. HO-endonuclease expressed from this plasmid releases the *SAM* fragment from pLAY99. Recombination between the released fragment and the *SAM2* locus replaces the *CYH2*::*URA3* insertion with *SAM* sequences and creates a cycloheximide- and 5-FOA-resistant cell.

was increased over 40-fold (Table 3). While increases in the frequency of recombination between identical sequences have been observed previously in mismatch repair-defective cells (5, 9, 34, 51), the increases reported here exceed the previously reported increases by as much as 10-fold. One explanation is that the DNA fragments released from the plasmid by HO-endonuclease may be more stable in *msh2* mutant cells than in wild-type cells, suggesting that Msh2p could have a role in the degradation of broken DNA molecules. We found, however, that plasmid DNA molecules were no more stable after digestion by HO-endonuclease in *msh2* mutant cells than in wild-type cells (53), arguing against a role for Msh2p in the exonucleolytic processing of DSBs.

Alternatively, the *msh2* mutation might stimulate recombination between the *SAM2* fragment and the *SAM2* locus, because there could be small differences in the DNA sequences of the cloned *SAM2* gene used to construct our recombination substrates and the *SAM2* genomic target in our yeast strains. To test this hypothesis, we reconstructed plasmid pLAY108 (Fig. 1) with *SAM2* sequences cloned from one of our yeast strains and repeated the replacement experiments described above (Fig. 1) with wild-type and *msh2* mutant cells (53). We obtained HO-stimulated replacement frequencies in wild-type (3.0×10^{-6}) cells and *msh2* (1.4×10^{-4}) mutant cells that were not statistically different from those reported above (Table 2), indicating that the *msh2* mutation did not increase gene replacement with the original *SAM2* DNA fragment because of mismatches between the DNA fragment and genomic target.

Finally, because the design of our gene replacement assay requires that crossovers occur between both sides of the DNA fragment and the genomic target, Msh2p has two chances to abort recombination by degrading the heteroduplex at either end of the fragment. In contrast, the intrachromosomal recombination events that were previously found to be stimulated in *msh2* mutant cells can result from single crossovers (9, 34). Therefore, loss of Msh2p might have a more stimulatory effect on the frequency of recombination in our assays, because crossing over at both ends of the DNA fragment is exponentially more likely to occur.

We found that chimeric DNA fragments containing both *SAM1* and *SAM2* information (Fig. 1) recombined with the *SAM2* target at frequencies that were intermediate to the frequencies observed when only *SAM1* or *SAM2* sequences were used (Table 2). This indicates that the frequency of gene replacement is dependent upon the ability of both ends of the DNA fragment to recombine with the genomic target. Interestingly, the frequencies of recombination with the chimeric fragments (pLAY111 and pLAY112) are three- to fivefold higher than expected if those frequencies reflected the probability of the *SAM1* and *SAM2* ends independently recombining

with the *SAM2* target (obtained by multiplying the square root of the frequencies obtained with the *sam1* fragment from pLAY109 and the *sam2* fragment from pLAY108). Therefore, it appears that by tethering the *SAM1* segments to *SAM2* segments, they have a higher probability of recombining with the mismatched *SAM2* genomic target than if they are tethered to the equivalent *SAM1* segments. One potential explanation for this observation is that because the *SAM2* segments have a high probability of recombining with the genomic target, a linked *SAM1* segment that is initially prevented from recombining with the genomic target is likely to have a second chance to recombine because it can not diffuse away from the target. Interestingly, the difference between the observed and expected values for the frequencies of gene replacement obtained with the chimeric fragments is reduced to twofold in *msh2* mutant cells (Table 3), indicating that there is less facilitation in mismatch repair-defective cells.

We also discovered that removal of *SAM1* sequences from the genome did not affect the frequency of DNA fragment integration at the *SAM2* locus. This suggests that gene replacement with the *SAM1*/*SAM2* chimeric fragments (pLAY111 and pLAY112) is less efficient than replacement with the *SAM2* fragment (pLAY108), because the *SAM1* ends are blocked from recombining with the *SAM2* locus, not because they recombine more readily with an alternative genomic target at the *SAM1* locus. If similar forces govern gene replacement in mammalian cells, then the frequency with which a DNA fragment recombines with a genomic target may be determined solely by its ability to interact with that target, not by its ability to recombine homologously or illegitimately with other genomic sequences.

The effect of mismatched sequences on the frequency of gene replacement was found to be dependent upon context (Table 2). DNA fragment insertion into the *SAM2* locus occurred 18-fold less frequently when *SAM1* sequences were at the end of the fragment than when they were surrounded on both sides by *SAM2* sequences. Interestingly, the inhibition of recombination observed when the *SAM1* sequences were at the end of the fragment correlated with evidence that these sequences formed a heteroduplex with the genomic target and underwent mismatch repair (Table 3). These observations suggest that mismatched sequences must form a heteroduplex and possibly undergo limited mismatch repair before they are blocked from recombining. Our failure to observe evidence of heteroduplex formation and mismatch repair of the internal *SAM1* sequences suggests that the heteroduplex and/or mismatch repair tracts were usually shorter than ~650 bp, which is the distance from the end of the fragment to the *Bal*I site in the internal *sam1* sequence (Fig. 3). Perhaps the junction between identical sequences and mismatched sequences presents a barrier to the extension of the heteroduplex that favors resolution of recombination before a mismatched heteroduplex is created, a view supported by previous work in other laboratories (43, 49). Alternatively, the mismatch repair machinery may unravel a heteroduplex that forms between mismatched sequences, leaving only a heteroduplex between identical sequences. Finally, a heteroduplex between the mismatched fragment and target sequences may not form because the average length of a heteroduplex is below 500 bp, although this seems unlikely when lengths of heteroduplex in mitotic cells can be thousands of base pairs long (44).

The observations reported above could lead to improved strategies for gene replacement in mammalian cells. Because a small amount of sequence divergence can lead to a dramatic decrease in the ability of a DNA fragment to recombine with homologous genomic sequences in wild-type mouse cells (10, 11, 45, 47), it is clear that subtle differences between the genetic backgrounds of the cells that donate the DNA fragment and the cells that receive it can profoundly affect the likelihood of successful gene replacement. Perhaps by obtaining sequences that are identical to the genomic target by PCR and substituting them for nonidentical sequences at one or both ends of the DNA fragment, the efficiency of recombination between the DNA fragment and the genomic target can be increased. In addition, one or the other allele can be targeted if the ends of the DNA fragment contain polymorphisms that are unique to one but not the other allele.

In conclusion, we have found that the rejection of recombination between mismatched sequences in yeast is spatially limited and that this is probably a property of heteroduplex formation and mismatch repair. If these observations also hold true in mammalian cells, then the strategies discussed above could be used to overcome one of the barriers to efficient gene replacement.

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