Reciprocal Translocations in *Saccharomyces cerevisiae* Formed by Nonhomologous End Joining

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ABSTRACT

Reciprocal translocations are common in cancer cells, but their creation is poorly understood. We have developed an assay system in *Saccharomyces cerevisiae* to study reciprocal translocation formation in the absence of homology. We induce two specific double-strand breaks (DSBs) simultaneously on separate chromosomes with HO endonuclease and analyze the subsequent chromosomal rearrangements among surviving cells. Under these conditions, reciprocal translocations via nonhomologous end joining (NHEJ) occur at frequencies of $\sim 2-7 \times 10^{-5}$/cell exposed to the DSBs. Yku80p is a component of the cell's NHEJ machinery. In its absence, reciprocal translocations still occur, but the junctions are associated with deletions and extended overlapping sequences. After induction of a single DSB, translocations and inversions are recovered in wild-type and *rad52* strains. In these rearrangements, a nonrandom assortment of sites have fused to the DSB, and their junctions show typical signs of NHEJ. The sites tend to be between open reading frames or within Ty1 LTRs. In some cases the translocation partner is formed by a break at a cryptic HO recognition site. Our results demonstrate that NHEJ-mediated reciprocal translocations can form in *S. cerevisiae* as a consequence of DSB repair.

BALANCED translocations are common findings in leukemias, lymphomas, and sarcomas and in many cases are thought to be the causative event in tumorigenesis. Translocations can cause the misjoining of segments of oncogenes, leading to their improper expression (Barr 1998; Rowley 2001). The junction sequences sometimes fall within dispersed repeats such as Alu or L1 elements (So *et al.* 1997; Strout *et al.* 1998). More commonly, the junctions show little or no homology and appear to have arisen by nonhomologous end joining (NHEJ) of broken DNA ends (Bodrug *et al.* 1991; Wiemels and Greaves 1999). This idea is supported by the observation of breakpoints at sites of V(D)J rearrangements (Boehm *et al.* 1989; Hiom *et al.* 1998), as well as the characteristic translocations seen in leukemias arising after use of topoisomerase II inhibitors. These drugs may generate chromosomal breaks directly (Domer *et al.* 1995) or act indirectly by causing specific apoptotic cleavage events (Betti *et al.* 2001, 2003).

Recombination events have been studied extensively in *Saccharomyces cerevisiae* (Paques and Haber 1999), although this organism's marked propensity for homologous recombination (HR) has meant that NHEJ has been relatively less well examined. Assay systems have been developed in which reciprocal translocations are generated between dispersed repeated sequences or between heteroalleles by homologous recombination (Potier *et al.* 1982; Jinks-Robertson and Petes 1986; Fasullo and Davis 1988), sometimes with the assistance of an induced double-strand break (DSB; Haber and Leung 1996; Fasullo *et al.* 1999). Speciation as well as ongoing selection may occur by reciprocal or nonreciprocal translocations and duplications at sites of Ty1 retrotransposons, their LTRs, or other repeated sequences (Casaregola *et al.* 1998; Ryu *et al.* 1998; Rachidi *et al.* 1999; Fischer *et al.* 2000; Dunham *et al.* 2002). Nonreciprocal translocations joined by microhomologies, a hallmark of NHEJ, have been examined in diploid yeast cells, as well as in haploid cells in which rearrangements occur distal to the last essential gene on a chromosome (Chen and Kolodner 1999; Myung *et al.* 2001; Tennyson *et al.* 2002). These events occur at very low rates, but can be stimulated either by mutations in genes involved in genome stability or by induced DSBs. In contrast, no experimental system has been developed to study reciprocal translocations resulting from NHEJ, and only one report of a naturally occurring event of this type has appeared (Perez-Ortin *et al.* 2002).

We recently developed a system to study NHEJ-mediated chromosomal rearrangements occurring after a unique DSB in haploid yeast cells with different repair mutations (Yu and Gabriel 1999, 2003). Using this assay, we identified multiple repair events that can occur after a break to ensure survival, including insertions, inversions, and translocations in wild-type and *rad52*

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cells, gene conversions in wild-type and *yku80* cells, and large and small deletions in all backgrounds. Here we characterize the nature of the translocations and inversions. Further, we expand the system by generating two simultaneous DSBs on separate chromosomes. Under these circumstances, reciprocal translocations are readily generated and detected. By analyzing the frequency and junction sequences of reciprocal translocations in cells with or without defects in Rad52p and/or Yku80p (key components of HR and NHEJ, respectively) we can study the formation of reciprocal translocation events and the factors that regulate their occurrence.

MATERIALS AND METHODS

Yeast strains and assay conditions: The *S. cerevisiae* strains used in this work are listed in Table 1. Generation of the Δ*yku80::KanMX4* mutation and Δ*rad52::hisG* mutation has been described (Yu and Gabriel 1999, 2003). Construction of the *URA3*::actin intron cassette and the *URA3*::actin intron::HOcs (−) orientation cassette (previously referred to as the *URA3*::actin intron::HOcs cassette) was detailed in previous work (Yu and Gabriel 1999). During creation of the *URA3*::actin intron::HOcs cassette plasmid, the HO cut-site region was inserted at the *Xho*I site within the actin intron. This occurred in both orientations of the HO cut site relative to the intron. This is noteworthy because the HO recognition sequence is not palindromic and cleavage of double-strand DNA (dsDNA) by HO endonuclease results in an asymmetric 4-base 3′ overhang of sequence AACA-3′OH [designated (+) orientation] or TGTT-3′ OH [designated (−) orientation]. In the two-cut-site strains used in this work, the orientation of the HO cut site is (−) relative to the coding strand of *URA3* and (+) relative to the coding strand of *LEU2*. In the one-cut-site strains, experiments were done using both orientations of the HO cut site relative to the coding strand of *URA3*. Translocations and inversions were identified after DSB formation using either orientation HO cut site.

Previous studies (Yu and Gabriel 1999, 2003) have detailed the use of the plasmid containing the *URA3::*actin intron::HOcs (−) orientation cassette (AGE1662). In addition, a plasmid containing the *URA3::*actin intron::HOcs (+) orientation cassette (AGE1658) was made. The *URA3::*actin intron cassette was introduced into previously described strains containing *leu2*::HOcs to create the single cut site at *LEU2* strains AGY245, AGY262, AGY601, and AGY622. The *URA3*::actin intron::HOcs (−) orientation cassette was introduced into previously described strains containing *leu2*::HOcs to create the double-cut-site strains AGY113, AGY121, AGY285, and AGY289. In addition, the *URA3*::actin intron::HOcs (+) orientation cassette was introduced into previously described strains containing *LEU2* to create the single-cut-site strains AGY116 and AGY126. All strains were confirmed by PCR of the genomic DNA and by Southern blots. Media and growth conditions, induction conditions, determinations of frequency of cell survival, and fluoroorotic acid (FOA) resistance have all been described (Yu and Gabriel 1999, 2003).

Analysis of surviving and FOA-resistant colonies by PCR and sequencing: Genomic DNA was extracted from independent FOA-sensitive (FOA^S) or FOA-resistant (FOA^R) colonies (Hoffman and Winston 1987). Portions of the *URA3*::actin intron::HOcs region and/or *leu2*::HOcs, centered on the HO endonuclease recognition sequence, were PCR amplified using *Taq* polymerase. The oligonucleotides used are available upon request. Genomic DNA from clones that repeatedly failed to amplify using locus-specific primers, but that did amplify using primers at other loci, were subjected to inverse PCR as previously described to identify junction sequences (Yu and Gabriel 2003). On the basis of these results, appropriate primers were generated for further PCR and sequencing reactions to clarify both reciprocal junctions.

PCR products were sequenced according to the dsDNA cycle sequencing technique provided by GIBCO BRL (Gaithersburg, MD). Sequences obtained were identified using BLAST searches of the *Saccharomyces* Genome Database (http://genomewww.stanford.edu /Saccharomyces/).

Southern blot analysis: Genomic DNA was digested with *Stu*I, separated by agarose gel electrophoresis, transferred to nitrocellulose membranes, and hybridized with a ^{32}P-labeled full-length *URA3* gene probe. Pulsed-field gel electrophoresis, using a CHEF-DR III pulsed field electrophoresis system (Bio-Rad, Richmond, CA), was performed as per the instrument's instruction manual and application guide, using a program to maximize 400–600 kb separation. After transfer to nitrocellulose, the filter was hybridized with a full-length *URA3* probe, as above.

Recombination test: Starting plasmids and strains were the kind gift of Jac Nickoloff (University of New Mexico). pUHAC contains a nonfunctional allele of *ura3* with a linker sequence at base 432 in the *URA3* open reading frame (ORF). pUHAC-HO432 is identical, except for the presence of 30 bases of *MAT***a** sequence from the Y/Z junction (5′-TTT ATG GGA CTA CTT CGC GCA ACA GTA TAA-3′), inserted in the unique *Eco*RI site of the linker. A total of 39–61 bases centered on the observed breakpoints of eight different translocations/inversions were inserted at the *Eco*RI site of pUHAC (Nickoloff *et al.* 1990; Sweetser *et al.* 1994): base pair 291,216–291,254 on chromosome V, GAG CTA CAT CCT TAC TG/ GTG CGA ATA AAT GAC ATA ATC T; base pair 555,782–555,820 on chromosome II, GTT ACA CTC GCA AAA TTT TTT T/G CTG GGC AAA ATA AAG C; base pair 521,623–521,668 on chromosome IV, TTA GTC ACA TCG GAA AGT ATG CTT G/C TGC ATT AAA TAC ATG CAT GT; base pair 909,487–909,533 on chromosome IV, GTG CCG TGA TAC TCT TTT CCG CAA C/A AAA GCA ATA TTT CCC TCG GCC; base pair 151,077–151,137 on chromosome XI, GTG TTG TTC AAA ATA ATC TGT TTG /CT GCG TTT AAT AAC TAC AAC AGT TAA CGG AAA CAG TG; base pair 484,398–484,446 on chromosome V, ACT TTC TAC GTT GAC AGA AAA TTT TCG TGA TTT GCC GTT CCC ATC GCT C; base pair 502,979–503,030 on chromosome V, ACT TAA CCG CAT TTT TCC ATT TTC CG/ CCG CAC TAT ATA GAT GGT AGA AAA GC; and base pair 258,866–258,915 on chromosome II, CAC CGC ATG TGA TAT AAT AGT AAC ATG AG/ TGC TGC GAA ATA GAT GAT AT. The resulting plasmids were transformed into yeast strain DY3026 (*MAT***a**-inc, *lys2-Δ1:LYS2 GALHO*, *ura3*-X764, *ade2-101*, *his3-200*, *leu2-Δ1*, and *trp1-Δ1*) by standard procedures. DY3026 is notable for containing a nonfunctional allele of *ura3* on chromosome V with a nonrevertible frameshift mutation at base 764. The assay was performed as follows: patches grown on SC-his, glucose plates at 30° for 2 days were replica plated to SC-his plates containing either glucose or galactose and incubated at 30° for 2 days. Patches were then scraped, resuspended, sonicated, and plated onto SC-his, glucose plates, and SC-his-ura, glucose plates. Recombination frequencies were calculated as the ratio of Ura+His+ colonies per milliliter to total His+ colonies per milliliter. The induced recombination frequency was calculated as the ratio of recombination frequency from the cells exposed to galactose *vs.* the recombination frequency from cells exposed to glucose. A total of 8–12 trials were done for each sequence tested.

Statistical analysis: Comparisons of recombination frequen-

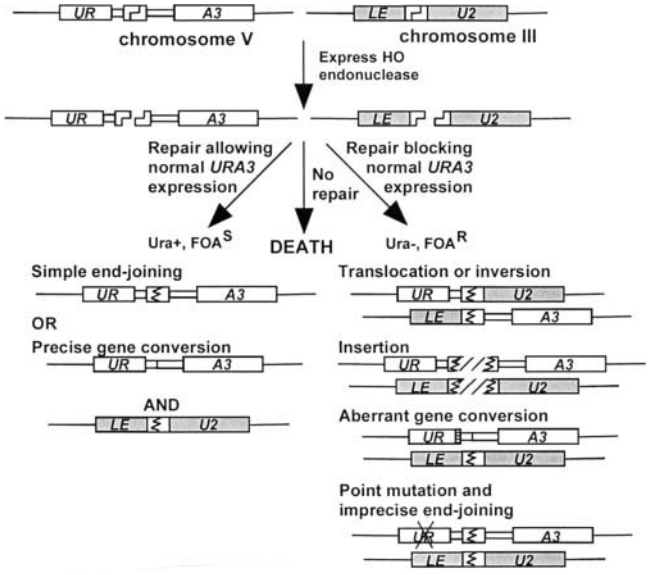


FIGURE 1.—The assay system. The *ACT1* intron placed into the *URA3* gene is normally spliced, resulting in uracil prototrophy (Ura^+) and sensitivity to the drug 5-fluoroorotic acid (FOA^S). After creating a persistent DSB with HO endonuclease, cells die, are repaired in a way that does not reconstitute the cut site but allows normal splicing (imprecise end joining or precise gene conversion), or are repaired in ways that result in complex chromosomal rearrangements, which directly or indirectly prevent splicing. The latter situation leads to uracil auxotrophy (Ura^-) and resistance to 5-FOA (FOA^R). A second copy of the HO cut site within the *LEU2* coding region is simultaneously cleaved and must also be repaired for the cell to survive.

cies in the recombination test to identify potential HO recognition sequences were made using the nonparametric Mann-Whitney rank test. Analysis of intergenic *vs.* intragenic breakpoints was done using the χ^2 test.

RESULTS

DSB repair in two-cut-site strains: The basis for our repair and rearrangement assay system (YU and GABRIEL 1999, 2003) is shown in Figure 1. The *S. cerevisiae* actin intron, containing a 117-bp HO recognition sequence from *MAT***a**, was engineered into the *URA3* allele on chromosome V of a strain containing a galactose-inducible HO endonuclease gene (see Table 1). By plating cells on galactose-containing media, endonuclease is expressed, the engineered HO cut site is cleaved, and a cell's ability to survive to form a colony depends on its successful repair of the resulting DSB(s). To compare repair events in the presence or absence of key proteins in the HR or NHEJ pathways, we made a set of isogenic wild-type, *rad52*, *yku80*, and *rad52 yku80* strains. A parallel set of strains contained an identical *MAT***a** recognition sequence within the *LEU2* gene on chromosome III, while a third set of strains contained DSB sites at both *LEU2* and *URA3*.

To examine the frequency and type of repair events generated in these strains, we plated them under either noninducing or inducing conditions and determined survival relative to induction (Figure 2). We then replica plated the colonies to 5-FOA to select for survivors that had lost *URA3* expression due to mutational events around the HO cut site (YU and GABRIEL 1999, 2003). Independent isolates were then analyzed by PCR, sequencing, and/or Southern blot analysis to determine the basis for FOA resistance.

Analysis of survival after two cuts: We first compared cell survival after two simultaneous DSBs in strains capable of HR or NHEJ, both, or neither (Figure 2 and Table 2). In wild-type cells, 0.15% of cells survived two simultaneous cuts, while in *rad52* cells, survival was 5-fold lower. For *yku80* cells, survival after two DSBs decreased ~40-fold relative to wild type and, in *rad52 yku80* cells, survival decreased >60-fold relative to wild type. Therefore, the presence of *YKU80* was the more important factor in maintaining survival after two DSBs.

To understand the events that allowed cells to survive after two simultaneous cuts (and remain Ura+), we PCR amplified and sequenced the regions around the two HO cut sites in multiple independent survivors (Figure 3). In wild-type cells, 6 of 7 clones had imprecise end joining at the breaks in the *URA3* and *LEU2* loci, while one showed imprecise end joining at *LEU2* and complete removal of the *MAT***a** segment at *URA3* via gene conversion of the actin intron::HOcs by the endogenous actin intron (see YU and GABRIEL 2003 and Figure 1 for explanation). Thus, in wild-type survivors, repair of the breaks occurred primarily via NHEJ events. In some cases, the repair events at the two cut sites were clearly independent (y956, y1584, y1586, and y1588 in Figure 3), while in other cases, repair at one cut site could have templated repair at the second site by homologous gene conversion (y954, y955, and y957). Among 10 clones in the *rad52* background, 3 had imprecise end joining at both sites, 6 had no apparent changes at either site, and 1 had imprecise end joining at *LEU2* and no apparent change at *URA3*. For *yku80* and *rad52 yku80* survivors, not one clone checked (7 and 9, respectively) had sequence changes at either cut site. In each case in which no change in the cut site was observed, we verified that the clones were indeed resistant to galactose induction (data not shown). These results suggest that for strains lacking *YKU80*, DSBs cannot be repaired by NHEJ and usually result in lethality. In these strains, survival most commonly results from mutations that somehow block a cell's ability to cleave DNA with HO endonuclease.

We next compared survival after single DSBs at either *URA3* or *LEU2* with survival after two DSBs at *URA3* and *LEU2* (Figure 2). For all four backgrounds, survival frequencies after a single cut at either of the two loci were similar. After two breaks, survival exceeded the product of individual survival frequencies by factors ranging from 12 (wild type) to 350 (*rad52 yku80*). The basis for

TABLE 1

Yeast strains used in this study

Yeast strains	Relevant genotype	Common name used in this work	Reference
YFP17	Δ*hml::ADE1* Δ*mat***a***::hisG* Δ*hmr::ADE1* *ade3::GAL-HO leu2*::HOcs[a] *ura3-52*		Paques *et al.* (1998)
AGY117	YFP17, *LEU2 URA3*::ai[b]::HOcs (−) orientation	WT, one cut site at *URA3*	Yu and Gabriel (1999)
AGY116	YFP17, *LEU2 URA3*::ai::HOcs (+) orientation		This work
AGY245	YFP17, *URA3*::ai	WT, one cut site at *LEU2*	This work
AGY113	YFP17, *URA3*::ai::HOcs (−) orientation	WT, two cut sites[c]	This work
AGY127	YFP17, *LEU2 URA3*::ai::HOcs (−) orientation Δ*rad52::hisG*	*rad52*, one cut site at *URA3*	Yu and Gabriel (1999)
AGY126	YFP17, *LEU2 URA3*::ai::HOcs (+) orientation Δ*rad52::hisG*		This work
AGY262	YFP17, *URA3::ai* Δ*rad52::hisG*	*rad52*, one cut site at *LEU2*	This work
AGY121	YFP17, *URA3*::ai::HOcs (−) orientation Δ*rad52::hisG*	*rad52*, two cut sites	This work
AGY287	YFP17, *LEU2 URA3*::ai::HOcs (−) orientation Δ*yku80::KanMX4*	*yku80*, one cut site at *URA3*	Yu and Gabriel (2003)
AGY601	YFP17, *URA3*::ai Δ*yku80::KanMX4*	*yku80*, one cut site at *LEU2*	This work
AGY285	YFP17, *URA3*::ai::HOcs (−) orientation Δ*yku80::KanMX4*	*yku80*, two cut sites	This work
AGY481	YFP17, *LEU2 URA3*::ai::HOcs (−) orientation Δ*yku80::KanMX4* Δ*rad52::hisG*	*rad52 yku80*, one cut site at *URA3*	Yu and Gabriel (2003)
AGY622	YFP17, *URA3*::ai Δ*yku80::KanMX4* Δ*rad52::hisG*	*rad52 yku80*, one cut site at *LEU2*	This work
AGY289	YFP17, *URA3*::ai::HOcs (−) orientation Δ*yku80::KanMX4* Δ*rad52::hisG*	*rad52 yku80*, two cut sites[c]	This work

WT, wild type.
[a] HOcs, HO cut site.
[b] ai, actin intron.
[c] Two cut sites, HO cut sites present at both *URA3* and *LEU2*.

this differential cooperative effect is unclear, although factors that simultaneously affect both cut sites, *e.g.*, nonfunctional HO endonuclease or nonfunctional galactose induction, make a relatively greater contribution to survival when the underlying mechanisms of repair are lost by elimination of Rad52p and/or Yku80p.

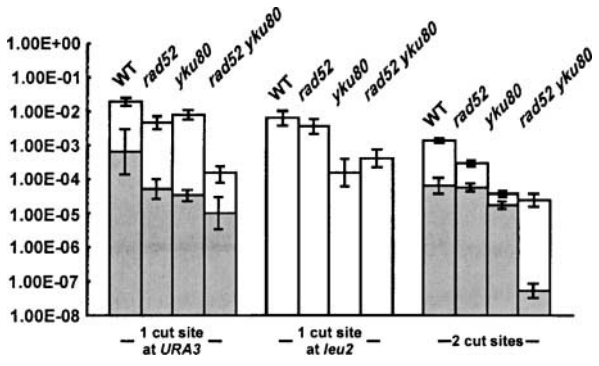


Figure 2.—Comparison of survival frequencies per plated cell after single cleavages at the *URA3*::actin intron::HOcs, at the *leu2*::HOcs, or after simultaneous double cutting (open bars). Shaded bars represent frequencies of FOA resistance among plated cells. Frequencies are plotted on a log scale. Standard deviations are shown as error bars. Each mean survival frequency was based on between 10 and 21 independent trials involving a minimum of three separate experiments.

Survivors that have lost *URA3* function: We previously showed that loss of uracil function (*i.e.*, FOA resistance) was relatively rare among survivors of a single HO cut at the *URA3* locus (Yu and Gabriel 2003; Figures 1 and 2). Among survivors of two DSBs, the frequency of FOA resistance per survivor depended greatly on the strain background and did not correlate with the single cut results. This lack of correlation between single- and double-cut strains reflects not just the greater damage from two breaks, but the different repair pathways available to cells in the different backgrounds as well. Differences in the terminal sequences of the joining ends (*e.g.*, noncohesive ends in double-cut strains *vs.* cohesive ends in single-cut strains) could also have subtle but important effects on repair and survival.

A reciprocal translocation joining the broken ends of chromosomes V and III will split the *URA3* gene between two chromosomes and result in FOA resistance (see Figure 1). We analyzed FOA^R two-cut-site colonies using appropriate reciprocal *URA3* and *LEU2* PCR primers and obtained discrete PCR products in 79% of the examined wild-type clones (34/43), 100% of the examined

TABLE 2

Repair and rearrangements at the *URA3*::actin intron::HOcs locus after two simultaneous DSBs

Strain	Frequency of survival per plated cell	Frequency of FOA resistance per plated cell	Chromosomal rearrangements resulting in FOA resistance			
			Translocations[a] (%)	Insertions (%)	Other[b] (%)	N^c
WT, two cuts	1.46×10^{-3}	6.89×10^{-5}	79[d]	9	12	43
rad52, two cuts	3.01×10^{-4}	6.62×10^{-5}	100			54
yku80, two cuts	3.66×10^{-5}	2.01×10^{-5}	100			52
rad52 yku80, two cuts	2.44×10^{-5}	5.42×10^{-8}			100	18

WT, wild type.

[a] This designation is based on PCR results, as well as sequencing or Southern blot analysis in selected cases.

[b] The nature of "other" varies by strain as described in the text.

[c] *N*, the number of independent 5-FOA-resistant colonies from which genomic DNA was obtained and analyzed.

[d] Translocations were all simple, except for one in which PCR products were longer than expected (y1532). This clone, described in text, contained Ty1 cDNA insertions at both translocation junction sites.

rad52 clones (54/54), 100% of the examined *yku80* clones (52/52), but 0% (0/18) of the examined *rad52 yku80* clones (Figure 2 and Table 2). This indicates that, in the presence of Yku80p and/or Rad52p, translocation formation is a major source of FOA resistance in cells surviving two simultaneous DSBs.

A subset of PCR products was sequenced to determine the breakpoint junctions of the reciprocal translocations (Figure 3). In wild-type cells, the positions of the translocation junctions were heterogeneous. The junctions contained short overlaps of zero to three nucleotides, resulting in the addition or deletion of several bases. The two junctions for the translocated chromosomes appeared to have been generated independently of one another. For example, in clone y1536, a 16-bp deletion on the 5′ side of *LEU2* joined a 1-base deletion on the 3′ side of *URA3*, whereas a 2-bp deletion on the 5′ side of *URA3* joined a 1-bp, nontemplated base addition on the 3′ side of *LEU2*. Little sequence was lost from the ends of any of the breaks. Among 36 ends from 18 wild-type translocation junctions, the median deletion length was 1.5 bp. These features of the junctions are typical of Ku-mediated NHEJ, as has previously been described in yeast (Kramer *et al.* 1994; Boulton and Jackson 1996; Wilson and Lieber 1999; Yu and Gabriel 2003). In one unusual reciprocal translocation (y1532), the junctions each contained independent Ty1 insertion events, rather than simple end-joins. This event appears to be a more complex version of a phenomenon that we and others previously characterized after a single HO cut, *i.e.*, insertion of segments of Ty1 cDNA at a DSB site (Moore and Haber 1996; Teng *et al.* 1996; Yu and Gabriel 1999).

In *rad52* cells, the absolute frequency of translocation events was similar to the wild-type strain (5.4 *vs.* 6.6×10^{-5}/cell plated). The structure around the junctions was also similar to wild-type cells, but showed more extensive sequence loss (median deletion length of 6 bp) and longer overlaps at the junctions (0–7 bp; Figure 3). In both of the strains in which *YKU80* was present, the translocation junctions were heterogeneous.

Cells lacking Yku80p showed a very different pattern among FOA^R survivors. Fifty-five percent of all survivors were FOA^R, and all 29 of those examined contained reciprocal translocations. Since none of the FOA-sensitive survivors had changes at either cut site and are presumed to be defective in their ability to generate a DSB, rejoining leading to translocation appears to be the only mechanism still available to *yku80* cells to repair these two DSBs. Equally striking was the loss of sequence heterogeneity at the translocation junctions derived from *yku80* cells. The junction of the 5′-end of *URA3* with the 3′-end of *LEU2* always consisted of a 13- to 15-bp deletion from the *URA3* end and a 78- to 80-bp deletion from the *LEU2* end. The junctions all contained a 6-bp precise overlap, which could be extended to three related sequences with 11/12, 11/11, or 9/11 bp of identity, allowing for C/T mismatches (Figure 3). For the reciprocal 5′ *LEU2*-3′ *URA3* junction, all sequences were identical, with an ~20-bp deletion on the *LEU2* side, an ~90-bp deletion from the *URA3* side, and a junction with a 7-bp precise overlap that could be expanded to 10/11 bp with one mismatch. These results suggest that Yku80p functions to maintain the integrity of the DSB ends, allowing the broken chromosome arms to join in a variety of overlaps.

When both *RAD52* and *YKU80* were deleted, the overall survival frequency was not significantly lower than that of the *yku80* strain by itself. The frequency of FOA resistance among survivors, however, was only 0.26% *vs.* the ~50% when *RAD52* was present in the cells. We obtained only 18 FOA^R clones in this strain. By PCR and sequencing, none were translocations and the HO cut sites were intact in each case. The same was true for 7 FOA^S clones in this background. The absolute frequency of FOA resistance per cultured cell in this background is

Repair in FOA sensitive survival cells

HO cut site

GTTTATAAAATTATACTGTTGCGGAAAGCTGAAACTAAAAG (@*URA3*) tttcagctttccgcaacagtataatttt (@*leu2*)

WT after 2 cuts

y954	GTTTATAAAATTATACTG**G**TTGCGGAAAGCTGAAACTAAAAG	+1/0	tttcagctttccgcaac**c**agtataatttt +1/0
y955	GTTTATAAAATTATACTG**G**TTGCGGAAAGCTGAAACTAAAAG	+1/0	tttcagctttccgcaac**c**agtataatttt +1/0
y957	GTTTATAAAATTATACTG**G**TTGCGGAAAGCTGAAACTAAAAG	+1/0	tttcagctttccgcaac**c**agtataatttt +1/0
y956	GTTTATAAAATTATAC**TG**TGTTGCGGAAAGCTGAAACTAAAAG	+2/0	tttcagctttccgcaaca**aca**gtataatttt +3/0
y1586	GTTTATAAAATTATAC**TG**TGTTGCGGAAAGCTGAAACTAAAAG	+2/0	tttcagctttccgcaaca**aca**gtataatttt +3/0
y1588	GTTTATAAAATTATACTG----------AAACTAAAAG	-2/-11	tttcagctttccgcaaca**ca**gtataatttt +2/0
y1584	ATTGCTCGAGAGATTTCTCTTT	gene conversion	tttca----------gtataatttt -13/0

rad52 after 2 cuts

1600	GTTTATAAAATTATACTGTTGCGGAAAGCTGAAACTAAAAG	0/0	tttcagctttccgcaaca**ca**gtataatttt +2/0
y964	GTTTATAAAATTATACTG**G**TTGCGGAAAGCTGAAACTAAAAG	+1/0	tttcagctttccgcaaca---taatttt 0/-3
y1604	GTTTATAAAATTATACTGTT**GTT**GCGGAAAGCTGAAACTAAAAG	+3/0	tttcagctttccgcaaca**aca**gtataatttt +3/0
y965	GTT----------CT**A**AAAAG	-17/-14/+1	tttcagctttccgcaaca**caca**gtataatttt +4/0

Repair in FOA resistant survival cells

A) Inter-chromosomal reciprocal translocations:

HO cut site "after translocation":

GTTTATAAAATTATACTGTT gtataattttataaaccc (U/L) tagtttcagctttccgcaaca GCGGAAAGCTG (L/U)

CAAATATTTTAATATG ttgtcatattaaaatatttgggg atcaaagtcgaaaggcg ACAACGCCTTTCGAC

WT after 2 cuts

y801	GTTTATAAAATTATACTGT-**aga**---agtataatttttataaaccc-	1/+3/-3	tagtttcagctttccgcaacaTGTTGCGGAAAGCTG 0/0
y1535	GTTTATAAAATTATACTGTT**G**--------aattttataaaccc	0/+1/-8	tagtttcagct----------TGTTGCGGAAAGCTG -10/0
y1527	GTTTATAAAATTATACTGTT--------aattttataaaccc	0/-8	tagtttcagctttccgcaaca-GTTGCGGAAAGCTG 0/-1
y807	GTTTATAAAATTATACTGT-aacagtataatttttataaaccc	-1/0	tagtttcagctttcc------TGTTGCGGAAAGCTG -6/0
y1514	GTTTATAAAATTATACTG----cagtataatttttataaaccc	-2/-2	tagtttcagctttccgcaaca-GTTGCGGAAAGCTG 0/-1
y1536	GTTTATAAAATTATACTG--**c**aacagtataatttttataaaccc	-2/+1/0	ta----------GTTGCGGAAAGCTG -19/-1
y1522	GTTTATAAAATTATACT----------aaaccc	-3/-16	tagtttcagctttc----------CGGAAAGCTG -7/-5
y1533	GTTTATAAAATTATAC-------agtataatttttataaaccc	-4/-3	tagtttcagct----------TGTTGCGGAAAGCTG -10/0
y803	GTTTATAAAAT----------aattttataaaccc	-9/-8	tagtttcagctttccgcaacaTGTTGCGGAAAGCTG 0/0

y1532 GTTTATAAAATTATACTGTT**gggat...caagg**ataattttataaa tagtttcagctttccgcaaca**ttgata...tttctcatg**GTTGCGGAAAGCTG

(Ty1 insertion, 5891c-5819c) +67 (Ty1 insertion, T+5066-5918+TG) +856

rad52 after 2 cuts

y208	GTTTATAAAATTATACTGTT----------ttataaaccc	0/-12	tagtttcagcttt---------GTTGCGGAAAGCTG -8/-1
y209	GTTTATAAAATTATACTGT-------ataatttttataaaccc	-1/-6	tag----------CTG -18/-12
y1463	GTTTATAAAATTATACTGT-------ataatttttataaaccc	-1/-6	tagtttcagctttccgcaaca-GTTGCGGAAAGCTG 0/-1
y1469	GTTTATAAAATTATACTGT-------ataatttttataaaccc	-1/-6	tagtttcagctttccgcaaca-GTTGCGGAAAGCTG 0/-1
y810	GTTTATAAAATTATACTG--aacagtataatttttataaaccc	-2/0	tagtttcagctttccgcaaca-GTTGCGGAAAGCTG 0/-1
y811	GTTTATAAAATTATACTG----cagtataatttttataaaccc	-2/-2	tagtttcagctttccgcaaca-GTTGCGGAAAGCTG 0/-1
y1450	GTTTATAAAATTATACT----------ttataaaccc	-3/-12	tagtttcagctttccgcaaca-GTTGCGGAAAGCTG 0/-1
y1457	GTTTATAAAATTATA----------aaccc	-5/-17	tagtttcagctttccgca-------GCGGAAAGCTG -3/-4
y207	GTTTATAAAATT----------c	-8/-21	tagtttcagct----------G -10/-14
y821	GTTTATAAAATT----------ttataaaccc	-8/-12	tagtttcagc----------TTGCGGAAAGCTG -11/-2
y1461	GTTTATAAAATT----------ttataaaccc	-8/-12	ggtgacca----------**AT**TGTTGCGGAAAGCTG -288/+2/0
y1477	GTTTATAAA----------ccc	-11/-19	gccaggtga----------AAGCTG -291/-9
y813	GTTTATAAA----------ccc	-11/-19	tagtttcagctttc----------CGGAAAGCTG -7/-5
y814	GTTTATAA----------**g**cc	-12/+1/-20	tagtttcagctttcc----------AAAGCTG -6/-8
y1475	GTTTATA----------attttataaaccc	-13/-9	tagtttcagctttccg----------GAAAGCTG -5/-7

yku80 after 2 cuts

ACCAGGGTTTATAAAATT.. ..caaggtttactaaaaatcc (U/L) tttttcttttagtt.. ..GAGATTTCTCTTTTACC (L/U)

(17)	ACCAGGGTTTA----------ctaaaaatcc	-15/-78	tttt----------TCTTTTACC -26/-91
(8)	ACCAGGGTTTA----------taaaaatcc	-15/-79	tttt----------TCTTTTACC -26/-91
(4)	ACCAGGGTTTATA----------aaaaatcc	-13/-80	tttt----------TCTTTTACC -26/-91

B) Intra-chromosomal insertions

HO cut site:

AAAATTATACTGTT GCGGAAA (@*URA3*) tagtttcagctttccgcaaca gtataat (@*leu2*)

TTTTAATATG ACAACGCCTTT atcaaagtcgaaaggcg ttgtcatatta

WT after 2 cuts

y798 AAAATTATACTG**T**aaatt...ttaac**T**GTTGCGGAAA -1/+54/0

(mtDNA insertion, 44638c->44583c, intergenic)

tagtttcagctttccgc**a**taattg...gtatt**a**cagtataat -3/+58/-1

(mtDNA insertion, 20523-20583, coding region of COXI, AI4 and AI5-alpha)

y799 AAAATTATACT**atttt.....attaa**TGTTGCGGAAA -3/+344/0

(mtDNA insertion, compound, a-21S-rRNA, 58590c-58542c; b-15S-rRNA, 7157c-7038c; c-tRNA-HIS, 64591-64685; d-intergenic, 65762c-65684c)

tagtttcagctttccgcaac**a**ttttt....**aaat**agtataat 0/+343/-3

(mtDNA insertion, compound, a-intergenic, 65684-65762; b-tRNA-HIS, 64685c-64591c; c-15S-rRNA, 7038-7157; d-21S-rRNA, 58542-58590)

y1512 AAAAT**TATAaaatg.....catgg**TGTTGCGGAAA -5/+167/0

(Ty1, 5751->5918+TGG)

tagtttcagctttccgcaac**a**ccatg.....ttttatataat 0/+169/-5

(Ty1, CCA+5918c->5751c)

y1525 AAAATTATACTG**T**aacct....tcatg**T**TGCGGAAA -1/+634/-1

(Ty1, 5286->5918+TG)

tagtttcagctttccgca-cagtataat -1/0

FIGURE 3.—Repair junctions of FOA-sensitive and FOA-resistant colonies from strains that survived two HO cuts. Actual sequences at the repair junctions for each genetic background are shown. Given the orientation of the *URA3* and *LEU2* genes relative to their respective centromeres, and the orientation of the HO cut sites relative to the two marker genes, only reciprocal translocations in which the joined ends are noncohesive (*i.e.*, AACA to AACA or TGTT to TGTT) are potentially viable, as they do not lead to dicentric and acentric chromosomes. Sequences from the *URA3* locus are in uppercase type and sequences from the *LEU2* locus are in lowercase type. Boldface letters with dots are insertions and dashed lines are deleted bases. Overlapping bases are underlined. The numbers following each sequence with +, −, or 0 represent the numbers of bases added or lost at the junction. For example, 0/+1/−8 for y1535 means no change at the left side of the junction, deletion of 8 bases at the right side of the junction, and one additional base added in between. For the yku80 strain, the numbers of events found with each specific joint are listed to the left of the sequence.

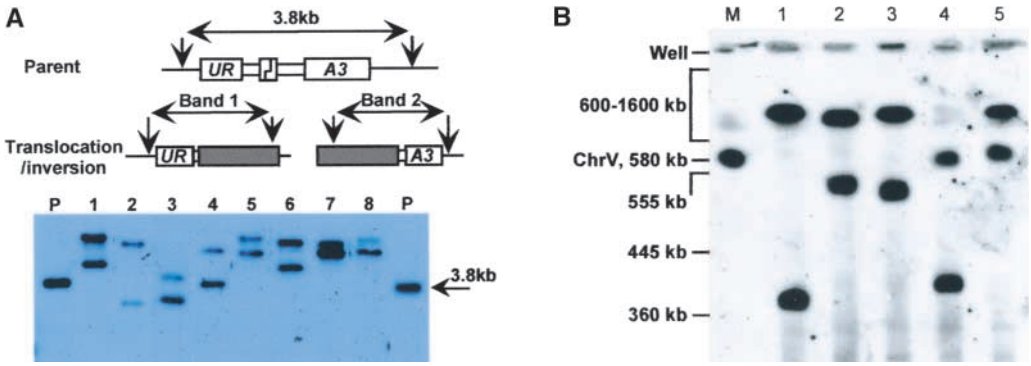


FIGURE 4.—Physical analysis of translocations or inversions after a single DSB. (A) Southern blot of FOA^R clones from one-cut strains that later were identified as translocations or inversions, as well as a schematic of expected products after a translocation or inversion. The filter was probed with a full-length *URA3* gene. "P" represents the parent genomic DNA from AGY117. For each lane the position of the second break was subsequently determined and corresponds to information in Table 3: 1, chromosome V-291232; 2, chromosome IV-521645; 3, chromosome IV-909511; 4, chromosome II-258897; 5, chromosome XI-74383; 6, chromosome XI-151100; 7, chromosome IX-135439; 8, chromosome X-117514. (B) Southern blot of chromosomes separated by pulsed-field gel electrophoresis of FOA^R clones from one-cut-site strains. The switching times and ramping used resolved chromosomes $< \sim$600 kb. The filter was probed with a full-length *URA3* gene. M refers to New England Biolab *S. cerevisiae* chromosome pulsed-field gel markers from strain YPH80 (no. 345). These chromosomes were used as size markers. Lanes: 1, chromosome II-258897; 2, chromosome XI-74383; 3, chromosome VII-79033; 4, chromosome IX-135439; 5, chromosome X-117514.

5.4×10^{-8}, which is similar to our observed spontaneous mutation frequency at *URA3* and suggests that no doubly cut cells remain viable.

These results demonstrate that after two simultaneous DSBs within unique sequences, survival is extremely rare in the absence of Yku80p. Survival is limited to either cells that have not experienced a cut or cells that have resolved the breaks by finding the extended complementarity between heterologous chromosomal arms and joining these ends. The absence of translocations in the double mutant suggests that the opportunity for even these rare events is lost in the absence of both Yku80p and Rad52p.

Nontranslocation events among FOA^R two-cut-site clones: Among the 43 FOA^R clones analyzed from the wild-type strain, we could not amplify several samples with reciprocal pairs of *URA3* and *LEU2* primers. Four were subsequently determined to be insertions with Ty1 cDNA or mitochondrial DNA at *URA3* and *LEU2* (Figure 3 and Table 2). For both y799 and y1512, the same insertion was present at both the *URA3* and *LEU2* cut sites. This strongly suggests that the two repair events were coupled, most likely by insertion at one site followed by gene conversion of the second site, with which it shares *MAT*-related sequences. In the third case (y798), two different mitochondrial insertions were seen at the two cut sites. The fourth event was a Ty1 insertion at *URA3* with imprecise end joining at *LEU2* (y1525). The insertions of mitochondrial fragments are similar to those that we and others have reported at natural or induced DSB sites (SCHIESTL and PETES 1991; RICCHETTI *et al.* 1999; YU and GABRIEL 1999). Other observed FOA^R colonies in wild-type cells included aberrant gene conversions at *URA3* with imprecise end joining at *LEU2* or combinations of intrachromosomal imprecise end joining plus presumed additional point mutation within *URA3* that result in FOA resistance (Figure 1 and data not shown).

Chromosomal rearrangements in one-cut-site strains: In previous experiments we used strains with a unique cut site at the *URA3*::actin intron::HOcs locus and examined FOA^R colonies occurring after a single induced DSB (YU and GABRIEL 2003). A fraction of samples in the wild-type and *rad52* backgrounds were not amplifiable using primers that flanked *URA3*. We further analyzed these clones by Southern blotting and found that *URA3* sequences were present on two different fragments consistent with translocations (Figure 4A). The physical phenomenon of translocation was confirmed in several cases by pulsed-field gel electrophoresis (Figure 4B). Using inverse PCR (OCHMAN *et al.* 1988), we identified several sequences joined to *URA3* and found numerous independent clones in which the junction sequences represented reciprocal translocations or inversions. The junction sequences were similar to those we had seen with two cut sites, *i.e.*, 0–11 bases of overlapping homology with little deletion of sequences around each breakpoint (see supplemental Figure 1 available at http://www.genetics.org/supplemental/). In total, we identified 14 different translocations or inversion sites, with several identified independently more than once (Table 3). Our data showed that translocation breakpoints were nonrandomly distributed. Of the 14 breakpoints, 11 were located between ORFs, including 2 within Ty1 LTRs, while only 3 were within nonessential ORFs (Table 3). Given an ORF density of almost 70% in the yeast genome, we would have expected only 4/14 translocation junctions to be intergenic. The finding that 11/14 are intergenic is significant to a P of <0.01.

It is not obvious why translocations or inversions should occur after a single DSB. One inversion occurred, independently, eight times. A plausible explanation is that

TABLE 3

Translocations observed in strains after one HO cut

Position of translocation partner	Times observed	Location in genome
Chromosome V-291232	8[a,b]	Intergenic between YER066W and YER067W
Chromosome V-484426	1[b]	Intergenic between YER156C and YER157W
Chromosome V-503008	1[b]	Intergenic between YER162C and YER163C
Chromosome IV-521647	4[a]	Intergenic between YDR034W-B and YDR035W
Chromosome IV-909511	3[a]	Intergenic between YDR221W and YDR222W
Chromosome IV-960105	1[b]	Within YDR250C (hypothetical ORF)
Chromosome II-258897	1[a]	Within YBRCΔ11 (Ty1 LTR)
Chromosome II-341565	1[a]	Intergenic between YBR053C and YBR054W
Chromosome II-555802	1[b]	Intergenic between YBR157C and YBR158W
Chromosome XI-74383	1[a]	Within YKLWΔ1 (Ty1 LTR)
Chromosome XI-151100	4[b]	Intergenic between YKL161C and YKL160W
Chromosome VII-79031	1[b]	Intergenic between YGL224C and YGL223C
Chromosome IX-135437	1[b]	Within YIL120W encoding MFS-MDR transporter
Chromosome X-117514	1[b]	Within YJL161W (hypothetical ORF)

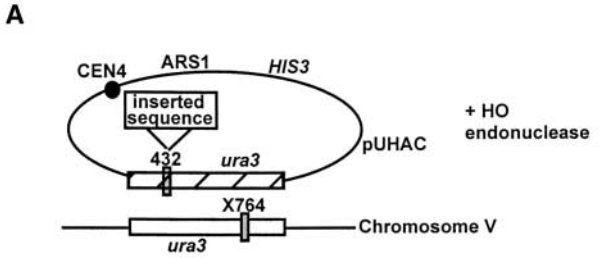
[a] Wild-type strain background.
[b] rad52 strain background.

the reciprocal sites joined to the cut site at *URA3* represent cryptic HO cut sites, analogous to translocations observed after overexpression of Cre-recombinase in yeast strains with a single recombinase recognition sequence (Sauer 1992). To examine this possibility, we adapted the recombination assay in Figure 5A (Nickoloff *et al.* 1990; Sweetser *et al.* 1994). Recombination between two *ura3* alleles, one on a plasmid and the other on a chromosome, can result in uracil prototrophy. This is an extremely rare event unless recombination is stimulated by the creation of a DSB. Under these conditions, induction of HO endonuclease in cells containing the 30 bp of *MAT***a** within the *ura3* allele on the plasmid stimulates uracil prototrophy by several thousandfold (Figure 5C). We placed 40–60 flanking bases from the presumed site of breakage for seven reciprocal translocations at the same position on the plasmid to determine whether they too stimulated recombination between *ura3* heteroalleles when HO endonuclease was either induced (galactose) or silent (glucose). After quantification of uracil prototrophy (Figure 5C) the most obvious finding was that the sequence around base 291,232 on chromosome V (obtained eight times) is a cryptic HO recognition site, causing an 11-fold increase in recombination on galactose (Figure 5C). This site shares all the most highly conserved bases with those in a derived HO recognition consensus sequence (Nickoloff *et al.* 1990; Figure 5B). The other tested sites ranged from background to at most 3-fold above background for the generation of uracil prototrophy on galactose. No site showed increased recombination in glucose. In four of seven cases, the differences in recombination frequencies, compared to no insertion control, reached statistical significance (Figure 5C). None of these sites contained all of the highly conserved bases in the consensus sequence, so it is not clear how these sites became targets for translocations.

DISCUSSION

We have created two simultaneous DSBs in yeast cells and shown that they can readily form reciprocal translocations by NHEJ. Previous studies in yeast have examined formation of nonreciprocal translocations derived by NHEJ or HR (Bosco and Haber 1998; Chen and Kolodner 1999; Tennyson *et al.* 2002), but none have examined the proposition that simultaneous DSBs in a cell will lead to the generation of reciprocal translocations, even in the absence of homology. A study in mammalian cells showed that the presence of two DSBs leads to reciprocal translocations, although in that study one of the two junctions (the selectable junction) arose by single-strand annealing, a form of HR, while the other junction formed by NHEJ (Richardson and Jasin 2000). Thus our data demonstrate for the first time that two DSBs are sufficient for NHEJ-mediated reciprocal translocation formation.

Reciprocal translocations were a major outcome among survivors after two DSBs. One of every ~30 wild-type survivors had switched arms, and the heterogeneous collection of junction sequences formed was similar to the intrachromosomal imprecise end-joining events that we observed for FOA^s survivors. In wild-type cells, it is likely that each pair of broken arms is stably held together and repaired. The four arms only occasionally become separated and subsequently recaptured, with the opportunity for interchromosomal rejoining. This conclusion is consistent with recent cytological data in *S. cerevisiae*. By marking both sides of a chromosomal DSB with fluorescent probes, Lisby *et al.* (2003a) showed



B

```
                          Y Z
                          87654321123456789101112
MATa sequence:            CAGCTTTCCGCAACAGTAT
ChrV-291232:              CATTTATTCGCACCAGTAA
ChrII-555802:             AATTTTTTTGCTGGGCAAA

Consensus sequence:       ...TNNNC/TGCA/GNC/AANT/G...
```

C

Construct	Induced recombination frequency (*)	Folds to (-) control
pUHAC (-)	1.1 ± 0.6	1
pUHAC-HO432 (+)	4134 ± 1385*	3758
pUHAC-ChrV-291232 (x8)	12.3 ± 8.5*	11
pUHAC-ChrII-555802 (x1)	1.0 ± 0.5	1
pUHAC-ChrIV-521647 (x4)	3.2 ± 1.9*	3
pUHAC-ChrIV-909511 (x3)	2.0 ± 1.0*	2
pUHAC-ChrXI-151100 (x4)	1.9 ± 1.6	2
pUHAC-ChrV-484426 (x1)	1.5 ± 0.5	1
pUHAC-ChrV-503008 (x1)	3.1 ± 1.6*	3
pUHAC-ChrII-258897 (x4)	2.2 ± 1.2*	2

Figure 5.—Assay for cryptic HO cut sites at translocation breakpoints. (A) pUHAC and chromosome V contain nonfunctional heteroalleles of *ura3*. Recombination between heteroalleles may result in a functional *URA3* gene, a process that is greatly enhanced by a DSB within *ura3*. Details of the assay are in materials and methods. (B) Comparison of the sequence surrounding the HO cut site in *MAT***a**, two candidate sequences found at translocation breakpoints, as well as the consensus sequence determined by Nickoloff *et al.* (1990). (C) Recombination test results for potential HO recognition sequences. Chromosome numbers followed by the position numbers of the second break sites are listed. The number of times each sequence was found at the junction of independent samples is shown in parentheses. *, a significant difference compared to the background frequency of uracil prototrophy.

that the two ends separated only in S phase, and even then separation was seen in <15% of cells. The strong intrachromosomal repair bias seen with wild-type cells was lost in *rad52* cells, where approximately half of all survivors of two breaks resolved the damage by reciprocal translocation. This result is consistent with Rad52p playing a direct or indirect role in maintaining the association of the two ends after a DSB, even in the absence of homology. In these cells, overall survival is decreased fivefold, perhaps because arm separation typically results in inviability rather than recapture and repair.

The intrachromosomal repair bias is reversed in *yku80* cells, in which all survivors of the two cuts have formed reciprocal translocations, utilizing a very specific extended sequence to form the junctions (Figure 3). This Yku80p-independent joint is similar to those we observed for large-scale deletions in our previous study of rearrangements occurring after a single DSB at *URA3* (Yu and Gabriel 2003). In that study, a specific 5.3-kb deletion with 11/12 bp overlap was the most common repair event in cells lacking Yku80p, and we postulated that one side of the break searches its partner sequence until it finds sufficient complementarity to anneal and allow repair events to proceed. Given that result, the absence of a class of survivors in our current study with two separate intrachromosomal deletion events on chromosomes V and III was surprising. *LEU2* is flanked on its telomeric side by an essential gene, but the first essential gene on its centromeric side is 26 kb away. Therefore, either there is no sequence within 26 kb that can efficiently anneal with the end of *LEU2* or the Ku-independent repair mechanism used at the *URA3*::actin intron::HOcs locus does not occur at the *LEU2* cut site. This is amenable to experimental dissection. Another surprising result was the lack of reciprocal translocations or any other repair event for the *rad52 yku80* strain. Perhaps the four broken ends are unable to stay together or find one another in the absence of both of these DNA end-binding proteins.

What do our results say about the process of translocation formation? In a study of wild-type cells containing two DSBs, repair by HR was equally likely to result in a reciprocal translocation or two intrachromosomal deletions (Haber and Leung 1996). A recent *in situ* fluorescence study demonstrates that the ends of two DSBs, as well as Rad52p, rapidly colocalize to a discrete subnuclear focus, possibly a repair site (Lisby *et al.* 2003b). Colocalization occurs despite a lack of homology at the break sites, indicating that Rad52p functions prior to the homology-dependent events. This is in agreement with our data.

Why is survival in our system so rare? Our assay places a severe restriction on survivors; *i.e.*, they must eliminate both cut sites to grow up into viable colonies, and this is a relatively unlikely outcome. After two DSBs, cells arrest, a permanent state unless resolution occurs (Lee *et al.* 1998; Galgoczy and Toczyski 2001; Vaze *et al.* 2002). In the case of *yku80* cells, resolution requires that the separate ends be rejoined by a Ku-independent mechanism. The process of rejoining is likely enhanced by the DNA ends being in close proximity to one another within a repair focus. Either Rad52p or Yku80p may be sufficient to bring the ends to a repair focus where arm exchange can occur. In the absence of both of these proteins, this recruitment may not be possible.

We also found translocations and inversions in strains in which we had intended to create only a single cut. This is most consistent with a concomitant second break elsewhere in the genome. Understanding how and where such second breaks arise could provide insights into fragile sites and the organization of different chromosomal regions. One striking result was the tendency for

second breaks to occur between genes or within Ty1 LTRs, rather than within nonessential ORFs. Intergenic regions, which contain promoter regions, may be more accessible to nucleases. The most obvious nuclease source is HO endonuclease. Although purported to be extremely site specific, weak binding might be sufficient to generate a break. This phenomenon is likely amplified by HO overexpression and our ability to detect rare events. The recombination assay we used to detect DSB formation confirmed that one site on chromosome V is indeed a cryptic HO cut site. For three of the other six candidate sequences, there was a slight but significant increase in recombination, and these showed partial matching to the consensus recognition sequence for HO endonuclease (Figure 5). Given the degeneracy of the consensus sequence, a large portion of the genome should be accessible to HO binding. Therefore, observed cleavage sites must reflect relative accessibility of certain genome regions to HO, a property that could be used to examine genome-wide chromatin accessibility.

Three of the seven sequences tested did not have significantly more recombination than a negative control, and these break sites require additional explanations. We examined genome-wide databases of meiotic recombination hotspots (Gerton *et al.* 2000) and locations of replication fork initiation and collision (Raghuraman *et al.* 2001). In neither case did we find a correlation between the regions surrounding our sites and these genomic landmarks. Continued acquisition of breakpoints and correlation with genomic landmarks may allow us to better understand these cases of translocation formation.

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