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A Genetic Screen For DNA Double-Strand Break Repair Mutations in Drosophila

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ABSTRACT

The study of DNA double-strand break (DSB) repair has been greatly facilitated by the use of rare-cutting endonucleases, which induce a break precisely at their cut sites that can be strategically placed in the genome. We previously established such a system in Drosophila and showed that the yeast I-SceI enzyme cuts efficiently in Drosophila cells and those breaks are effectively repaired by conserved mechanisms. In this study, we determined the genetic requirements for the repair of this I-SceI-induced DSB in the germline. We show that Drosophila Rad51 and Rad54 are both required for homologous repair by gene conversion, but are dispensable for single-strand annealing repair. We provided evidence suggesting that Rad51 is more stringently required than Rad54 for intersister gene conversion. We uncovered a significant role of DNA ligase IV in nonhomologous end joining. We conducted a screen for candidate mutations affecting DSB repair and discovered novel mutations in genes that include *mutagen sensitive 206*, *single-strand annealing reducer*, and others. In addition, we demonstrated an intricate balance among different repair pathways in which the cell differentially utilizes repair mechanisms in response to both changes in the genomic environment surrounding the break and deficiencies in one or the other repair pathways.

A eukaryotic cell employs a variety of conserved mechanisms to repair double-strand breaks (DSBs), which threaten the integrity of its genome. These mechanisms can be grossly grouped into two pathways: homologous recombinational (HR) repair and nonhomologous end joining (NHEJ). Gene conversion (GC) is a common outcome of HR both in mitotic and in meiotic cells (reviewed in Paques and Haber 1999). In GC, the DSB is repaired by DNA synthesis templated from a homologous segment. GC is generally conservative, resulting in no net loss of DNA sequences. If the template for GC is located on the sister chromatid, such repair precisely restores the original sequence at the break. Many factors play important roles in regulating GC, notably the Rad52 epistasis group in budding yeast and their homologs in other organisms (Symington 2002). These include Rad50, Rad51, Rad52, Rad54, Rad59, Mre11, and others. Single-strand annealing (SSA) repair is commonly used to repair DSBs that occur between direct repeats (Paques and Haber 1999). SSA is nonconservative, resulting in the loss of one of the repeats as well as the segment between the repeats. The budding yeast Rad52 and Rad59 proteins are essential for SSA (Ivanov *et al.* 1996; Sugawara *et al.* 2000; Davis and Symington 2001), but Drosophila homologs for neither protein can be identified by sequence homology searches. The identification of their functional homologs in flies would have important implications since a similar situation exists for both *Caenorhabditis elegans* and Arabidopsis.

In NHEJ, the two ends of the DSB are ligated with little or no homology requirement between them. NHEJ is intrinsically mutagenic in that it can lead to sequence alteration at the site of DSB. On the other hand, precise end joining can be a predominant pathway if the ends have complementary single-stranded overhangs (Boulton and Jackson 1996). Several conserved proteins have been shown to regulate NHEJ, which include the Ku70–Ku80 heterodimer and DNA ligase IV (reviewed in Daley *et al.* 2005). However, recent studies in Drosophila shed doubts on the importance of ligase IV in regulating end joining (Bi *et al.* 2004; McVey *et al.* 2004a; Romeijn *et al.* 2005).

Our understanding of repair mechanisms has been greatly enhanced by studies using site-specific endonucleases, especially rare cutters. The advantages of being able to induce site-specific DSBs on demand are manyfold. One can control the timing and severity of DSB generation by manipulating endonuclease production. One can control the number and genomic location of the DSB by strategically placing the enzyme cut site. Last, one can engineer a specific genomic environment

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surrounding the DSB site so that a particular mode(s) of repair can be studied in detail. These advantages have been best exemplified by the use of the HO endonuclease in the yeast *Saccharomyces cerevisiae* (reviewed in Paques and Haber 1999), which leads to our detailed understanding of important molecular events during DSB repair *in vivo*, such as 5′–3′ break resection (White and Haber 1990), sequential loading of repair proteins (Sugawara *et al.* 2003; Wolner *et al.* 2003), and *de novo* telomere formation (Diede and Gottschling 1999). Modeled after the success of the HO system, the yeast rare-cutting I-SceI enzyme was successfully introduced to both plant and mammalian cells to induce site-specific DSBs (Puchta *et al.* 1993; Rouet *et al.* 1994) and subsequently to conduct functional studies of repair factors, especially those that may be specific to higher eukaryotes (*e.g.*, Moynahan *et al.* 1999, 2001; Tauchi *et al.* 2002).

We and others have successfully introduced the I-SceI site-specific DSB system to Drosophila (Bellaiche *et al.* 1999; Rong and Golic 2000). We showed that I-SceI cuts very efficiently in the fly genome and the single DSB at its cut site can be effectively repaired by a variety of mechanisms, which include SSA, GC, and imprecise NHEJ (Rong and Golic 2003). Since a DSB can be repaired by either HR or NHEJ, this creates a potentially competitive situation. Competition between precise end joining and GC for the same DSBs has been demonstrated in yeast (Frank-Vaillant and Marcand 2002). In other yeast studies, competition between NHEJ and HR was not observed, casting doubts on the generality of interpathway competitions (Karathanasis and Wilson 2002; Zhang and Paull 2005). Within each pathway, either HR or NHEJ, a similar competitive situation could also exist. Recently, we showed that interhomolog GC competes effectively with SSA in the germline of male Drosophila (Rong and Golic 2003). More recently, differential usage of repair pathways during normal Drosophila development has also been demonstrated (Preston *et al.* 2006a,b). However, mutational studies have been scarce in metazoans in which one investigates whether defects in one repair mechanism can be compensated by a higher utilization of other mechanisms (*e.g.*, Johnson-Schlitz and Engels 2006; Johnson-Schlitz *et al.* 2007). In this study, we combine the use of several versatile I-SceI-based repair assays and the use of known repair-defective mutants to demonstrate an intricate balance among repair pathways.

Defects in DNA damage repair often lead to cellular sensitivity to DNA damaging agents (Game and Mortimer 1974). Mutagen-sensitive (*mus*) mutations in Drosophila were first reported >30 years ago (Smith 1973; Boyd and Setlow 1976; Graf and Wurgler 1978). Subsequent genetic screens, especially a recent one conducted by Laurencon *et al.* (2004), have led to a large collection of Drosophila mutants sensitive to DNA damaging agents. Over the years, some of these mutations were shown to affect both well-characterized DSB repair functions [*e.g.*, mus209 = PCNA (Henderson *et al.* 1994); mus309 = Bloom RecQ (Kusano *et al.* 2001)] as well as ones that were novel [*e.g.*, mus312 (Yildiz *et al.* 2002)]. Therefore, molecular and functional characterization of these mutations will continue to yield important insights into DNA repair mechanisms especially in areas that relate to the development of multicellular organisms. We screened a collection of existing *mus* mutations with the I-SceI-based repair assays and succeeded in identifying several mutations that have various defects in DSB repair. To our knowledge, this is the first of such screens conducted in a metazoan.

MATERIALS AND METHODS

Drosophila stocks: Description of stocks not provided here can be found in FlyBase (http://flybase.net; Drysdale *et al.* 2005). The *heat-shock protein 70* (*hsp70*) promoter-driven I-SceI transgene (*70I-SceI*) has been described previously (Rong and Golic 2000). The lines used in this study were *[70I-SceI]2B* on chromosome *2* and *[70I-SceI]1A* on chromosome *3*. The *wIw* reporter construct has been described (Rong and Golic 2003). The lines used were *[wIw]4A* on chromosome *2* and *[wIw]2* on chromosome *3*. The lines *[wIw]8z* and *[wIw]yellow* were derived from *[wIw]2* by imperfect NHEJ (Rong and Golic 2003) and used in combination with *[wIw]2* in the homozygous assays. An *X* chromosome *hsp70*-driven FLP line (*70FLP3F*) has been described previously (Golic *et al.* 1997). All the mutant lines except the following were obtained from the Bloomington Stock Center (Indiana): $mei\text{-}41^{29D}$ from Tin Tin Su at the University of Colorado (Laurencon *et al.* 2003); $mus209^{B1}$ and $mus209^{2735}$ from Darryl Henderson at SUNY of Stony Brook (Henderson *et al.* 1994); mio^{2} from Mary Lilly at NIH (Iida and Lilly 2004); okr^{WS}, okr^{RU}, and okr^{AA} from Trudi Schüpbach at Princeton University (Ghabrial *et al.* 1998); $sir2^{2A\text{-}7\text{-}11}$ from Kent Golic at the University of Utah (Xie and Golic 2004); and $mus309^{D2}$ and $mus309^{D3}$ from Jeff Sekelsky at the University of North Carolina (Adams *et al.* 2003). For deletion mapping of *ssar*, deficiencies from Bloomington's "Deficiency Kit" were used that cover the region from 85 to 91.

Our repair assays require male fertility, and we were unable to get enough fertile test males for the following mutagen-sensitive (*mus*) mutations: *mus106*, *mus108*, *mus111*, *mus115*, *mus302*, *mus310*, *mus315*, *mus318*, and *mus320*. Some of the chromosome *3 mus* mutants recovered from a recent screen were also excluded due to the loss of mutagen sensitivity (Laurencon *et al.* 2004; R. Hawley and K. Burtis, personal communication to FlyBase). In addition, our repair assays are based on eye pigmentation. This made it difficult to test mutations on a *cinnabar* (*cn*) and *brown* (*bw*) doubly mutant chromosome *2* since *cn bw* double homozygotes have white eyes regardless of the state of the *w* gene. These include *mus204*, *mus205*, and the entire chromosome *2* collection from the Laurencon screen.

Generation of a DNA ligase IV (*lig4*) mutant: Line EP(X)0385 has a w^{+}-marked *P* element inserted upstream of the *X* chromosome gene *CG12176*, which encodes the Drosophila lig4 homolog. EP0385 males with the *Δ2–3* P transposase gene on a *Stubble*(*Sb*)-marked chromosome *3* were singly mated to *C(1)DX, y f/Y* females. White-eyed and Sb^{+} males were recovered, from which 13 lines were established. PCR tests were performed to detect genomic alteration covering the *lig4* region. The sequences of the primers used are available upon request. In the $lig4^{11}$ mutation, nt13501164–nt13501714 were

deleted, which included the first 148 codons of lig4 (for nucleotide numbering see FlyBase).

Drosophila genetics for I-SceI assays: Test males for various repair assays were produced as shown in the crossing schemes (APPENDIX). The parents were transferred every 3 days and the progeny were immediately heat-shocked for 1 hr at either 38° or 32°. The test males were testcrossed as shown in the APPENDIX. To score the "recut" phenotype, white$^+$ (w^+) progeny were directly examined for eye mosaicism if they have inherited the *Scutoid*(*Sco*)-marked *[70I-SceI]2B* transgene, which has leaky somatic expression. Alternatively, w^+ progeny were crossed individually to flies with *[70I-SceI]2B* and scored for mosaicism in the next generation. Interhomolog GC events from the *[wIw]8z* homozygous assay were scored by allelic PCR as described (RONG and GOLIC 2003). Briefly, DNA from single w^+ recut$^-$ flies was PCR amplified with w7926u and 8z-minus to score interhomolog GC and with w7926u and w14178d as a control.

Testing the effect of *mus307^{D1}* on the heat-shock response: In *wIw*, the mini-*w* is flanked by direct repeats of FLP recombination targets, *FRT*s (for a more detailed description of *wIw*, see RONG and GOLIC 2003). FLP can excise a portion of w^+ from the chromosome leading to its inactivation. The *70FLP* transgene that we used to induce FLP production was identically constructed as *70I-SceI* except for the enzyme coding regions. Therefore, a mutation that reduces heat-shock-induced transcription ought to similarly decrease *70FLP* expression, which in turn would lead to a reduced rate of w^+ excision. We measured FLP-induced w^+ loss in the germline of both wild-type (WT) and *mus307^{D1}* males that had been heat-shocked at 38° during early development and obtained essentially identical frequencies for both genotypes.

Preliminary mapping of *ssar*: We constructed flies that were heterozygous for both *mus307^{D1}* and individual deficiencies that uncover overlapping segments of the region between 85 and 91. These flies were tested with the hemizygous assay to measure SSA repair. In no combination, did we observe a drop of SSA rate beyond the one for *mus307^{D1}* heterozygotes (data not shown), suggesting that the mutation that reduces SSA is not located in the tested interval. The map position of *mus307^{D1}* was 3-59 (BOYD *et al.* 1981), placing it close to the *Sb* (3-60) mutation. We attempted to separate our SSA-reducing mutation from *mus307^{D1}* by meiotic recombination. We placed *mu307^{D1}* over a *cu*- and *Sb*-marked chromosome and recovered recombinants that had a crossover between the two markers in the region between 86 and 89. Eight lines were established from these recombinants, representing four lines for each of the reciprocal classes. These lines were tested over the original *mus307^{D1}* chromosome for their ability to inhibit SSA repair. All of the *Sb*-marked lines but none of the *cu*-marked lines were able to reduce SSA to ~0.40, the rate recovered from original *mus307^{D1}* homozygotes (Table 1 and data not shown). This suggests that the SSA-reducing mutation is likely located to the left of *cu*, consistent with our earlier mapping results with overlapping deficiencies. BOYD *et al.* (1981) recovered a second *mus* mutation to the left of *cu* on the *mus307^{D1}* chromosome. It remains to be determined whether our SSA-reducing mutation is allelic to this *mus* mutation.

Characterization of NHEJ deletions in *lig4* mutant: DNA from independent w^+ recut$^-$ lines was PCR amplified using primers w7514u (5′-caactgaaggcggacattga) and w14178d (5′-tgtgtgtttggccgaagtat), which generates a 600- bp product. For negative samples, a control PCR was carried out with w7728d (5′-aaacacccatctgccgagca) and w11678u (5′-tcatcgcagatcagaa gcgg), which generates a 1-kb product. All negative samples were positive for the control PCR. All samples that were negative for PCR with w7514u and w14178d were amplified with w7514u and one of the following primers: w13623d (5′-cgtagttgctctttcgctgt), w12254d (5′-acaacggtgagtggttccag), or PE5′ (5′-gatagccgaagcttaccgaagt). PCR products, if any, were sequenced to localize the NHEJ junction.

Statistics: Under our experimental conditions, DSB repair induced by I-SceI cutting occurs in the premeiotic male germline (RONG and GOLIC 2003). A single DSB repair event could be amplified, leading to multiple progeny with that event. Therefore, individual progeny from a single test male cannot be considered as having independent events. We determined, for every test male, the percentage of progeny that harbor a particular class of repair event (*i.e.*, SSA, GC, or both). We used the permutation test developed by William Engels at the University of Wisconsin (PRESTON *et al.* 2006a) to compare the means of these ratios from WT *vs.* mutant males.

RESULTS

We set out to identify new factors that are important for DSB repair in Drosophila by screening a collection of existing *mus* mutations with several repair assays that are based on the I-SceI site-specific DSB system. In these assays, a single DSB has been induced in different genomic environments. Using these assays, we measured the effect of different mutant backgrounds on the repair of that single DSB in the Drosophila germline. As a proof of principle, we first tested a few known DSB repair mutations.

The repair assays: All repair assays were based on the *wIw P*-element construct described previously (RONG and GOLIC 2003) and diagramed in Figure 1. An I-SceI cut site was placed between two copies of the *w* gene. The *w* copy to the right of the cut site was functional, whereas the copy to the left of the cut site was not, containing only the 3′ portion of *w*. In other words, two direct *w* repeats, each ~3-kb in size, flanked the future DSB. Since all the genetic experiments were performed in the *w^{1118}* null background except otherwise noted, the integrity of this mini-*w* gene dictated eye pigmentation.

We termed the first assay "the hemizygous assay" on the basis of the chromosomal configuration of the *wIw* insertion. Males with a single copy of *wIw* and a heat-inducible *70I-SceI* transgene were generated by crossing and heat-shocked. These males displayed eye color mosaicism due to somatic w^+ loss induced by I-SceI cutting. They were mated individually to *w* females. By scoring their progeny, we estimated the contributions from different repair pathways in the male premeiotic germline.

As described previously (RONG and GOLIC 2003), we recovered three classes of phenotypically distinct progeny, which are attributable to different types of repair of the I-SceI-generated DSB (Figure 1). Molecular analyses confirmed that the white-eyed progeny were the result of SSA repair, which led to the loss of one copy of the *w* repeats as well as all the intervening sequences. The rest of the progeny had pigmented eyes (w^+), and they could be further divided into two classes on the basis of whether they had inherited an intact I-SceI cut site (recut$^+$) or a mutated one (recut$^-$). The recut$^+$ progeny displayed eye color mosaicism in the presence of I-SceI

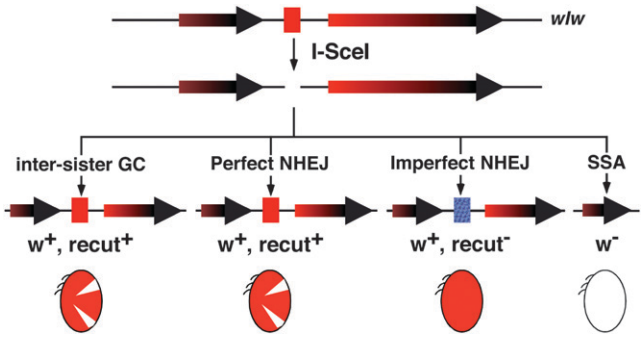

Figure 1.—The hemizygous assay. The *wIw* insertion has two *w* genes: the copy to the left of the I-SceI cut site (red box) is nonfunctional (shorter arrow), and the copy to the right is a functional mini-*w* (longer arrow). The shading helps illustrate the part of *w* that is repeated. Four possible repair mechanisms are given below the I-SceI-generated DSB (middle), with the names of the mechanism on top and the phenotypic classifications at the bottom of the diagrams that depict the molecular structures of the different repair products. The blue box represents a mutated I-SceI cut site due to imperfect NHEJ. The ovals represent eyes with different degrees of pigmentation. A mosaic eye has both white and red areas.

as described earlier, whereas the recut$^-$ offspring showed solid pigmented eyes even in the presence of I-SceI. These w$^+$ recut$^-$ progeny were the result of imperfect NHEJ repair. On the other hand, the w$^+$ recut$^+$ offspring could arise from perfect end joining since I-SceI generates a DSB with complementary 5′ overhangs or GC using an intact sister chromatid as the template, both restoring the cut site. Alternatively, they could be the result of I-SceI failing to cut.

We primarily used the hemizygous assay to screen different mutations. For certain mutations with a suspected effect on GC, we used two additional assays to estimate repair contributions from GC between the homologous chromosomes. We termed these two assays "the homozygous assay," since the *wIw* insertion was in a homozygous state. Only one homolog carries an I-SceI cleavable *wIw* (Figure 2), and the other carries a *[wIw]2* derivative with a mutated I-SceI cut site. We could correctly identify the I-SceI-cut chromosome in the progeny because (1) one of the *wIw* chromosomes was marked with the dominant *Sb* mutation, (2) Drosophila males do not have meiotic recombination, and (3) mitotic DSB repair under our experimental conditions seldom leads to crossing over (Rong and Golic 2003).

The *[wIw]8z* template has a small mutation at the I-SceI cut site. We recovered three classes of progeny similar to the ones from the hemizygous assay: w$^-$, w$^+$ recut$^+$, and w$^+$ recut$^-$ (Figure 2A). The w$^+$ recut$^-$ progeny could be further categorized into two subclasses: those that had inherited a mutated I-SceI cut site identical to the one in *[wIw]8z* due to interhomolog GC and those that had inherited a randomly mutated cut site due to imperfect NHEJ. By an allele-specific PCR method based on the known *[wIw]8z* sequences (Rong and Golic 2003), we identified the first subclass as w$^+$ recut$^-$ and PCR$^+$ and the second subclass as w$^+$ recut$^-$ PCR$^-$.

In the above *[wIw]8z* homozygous assay, the detection of interhomolog GC events relied on sampling by PCR. We developed a second homozygous assay in which interhomolog GC events could be visually identified (Figure 2B). During the course of studying imperfect NHEJ at the *[wIw]2* insertion, we recovered a derivative that we named *[wIw]yellow*, which had a 333-bp deletion to the right of the I-SceI cut site including the right half of the cut site. Flies with the original *[wIw]2* have orange eyes due to the hypomorphic nature of the mini-*w* gene. Flies with *[wIw]yellow* have a light yellow eye color. Presumably, the small deletion eliminated upstream regulatory elements of mini-*w*, further weakening its expression. In the new assay, the right end of the I-SceI-generated DSB does not have immediate homology to the *[wIw]yellow* homolog. We imagine two ways that interhomolog GC could still occur (Figure 3). First, nucleolytic degradation of double-stranded DNA to the right end would expose homology to allow GC to proceed by the traditional DSB repair model (Szostak *et al.* 1983). Second, interhomolog GC could occur by the synthesis-dependent strand-annealing (SDSA) mechanism (Nassif *et al.* 1994). In SDSA, the left end of the I-SceI-induced DSB invades *[wIw]yellow* to initiate repair synthesis. After synthesis has gone past the right side of the 333-bp deletion, the invading strand could detach from the *[wIw]yellow* template and anneal with the complementary single-stranded region from the right end of the DSB. This annealing would be followed by single-strand tail trimming and ligation. For both *[wIw]8z* and *[wIw]yellow* homozygous assays, SSA repair requires the same amount of end processing, yet more extensive end processing is needed for GC in the *[wIw]2/[wIw]yellow* setting. We therefore predicted a decrease in the GC/SSA ratio in the *[wIw]yellow* assay when compared to the *[wIw]8z* assay.

Drosophila Rad51 is essential for GC but dispensable for SSA: The *spindleA* (*spnA*) gene encodes the Drosophila homolog of bacterial RecA and eukaryotic Rad51 proteins (Staeva-Vieira *et al.* 2003), which is essential for GC repair. Interestingly, in cases where GC and SSA compete for the same set of DSBs, yeast *rad51* mutations lead to an increase of SSA repair at the expense of GC (Ivanov *et al.* 1996; Osman *et al.* 2000). A similarly competitive situation exists for our hemizygous assay (Figure 1). In the S/G2 phase of the cell cycle, the I-SceI-generated DSB can be repaired by either SSA or GC from an intact sister chromatid. Therefore, we predicted that *spnA* mutations would lead to an elevated SSA frequency due to their inhibiting effects on intersister GC. As shown in Table 1, a WT male, when heat-shocked at 38°, produced a germline SSA frequency of 0.847. For males either homozygous or hemizygous for

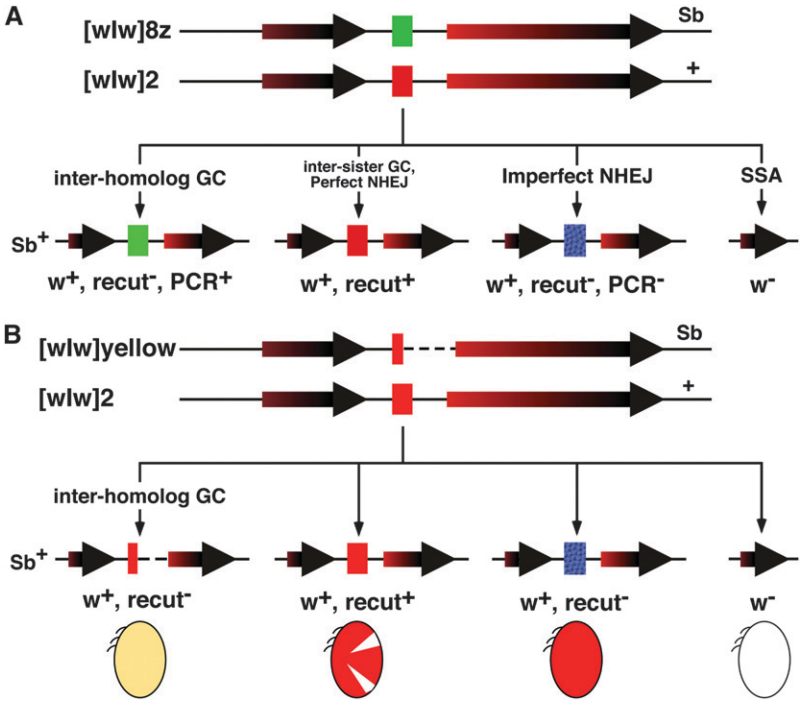

FIGURE 2.—The homozygous assays. (A) The assay with *[wIw]8z* as a template for interhomolog GC. The red box represents a normal I-SceI cut site. The green box represents the mutated cut site in *[wIw]8z*. The four possible outcomes of this assay are given at the bottom. The phenotypic classification of the progeny was done the same way as it was done for the hemizygous assay, except that the products of interhomolog GC were distinguished from those of imperfect NHEJ by an allelic-specific PCR reaction. (B) The assay with *[wIw]yellow* as the GC template. The *[wIw]yellow* chromosome has half of an I-SceI cut site remaining (the narrower red box), and a 333-bp deletion (dotted line), which includes part of the mini-*w* gene (see main text). The interhomolog GC products can be scored directly by the presence of yellow-colored eyes (yellow oval).

the *spnA*^{1} mutation, the SSA frequency was significantly elevated, approaching 0.95. We established six independent white-eyed lines from the *spnA*^{1}*/Df(3R)X3F* experiment. Southern blot analyses conducted as previously described confirmed that they arose from SSA repair (data not shown; RONG and GOLIC 2003). Taken collectively, our results suggest that Rad51 deficiency causes an increased usage of SSA repair at the expense of intersister GC.

Although the results from the hemizygous assay suggest that Rad51 is essential for GC, the evidence remains indirect, since intersister GC cannot be directly measured in this assay. To directly measure the frequency of GC, we used the *[wIw]8z* homozygous assay that permits efficient interhomolog GC (Figure 2A) (RONG and GOLIC 2003). In this assay, the I-SceI-induced DSB can be repaired to produce the same three classes of progeny as the hemizygous assay. An additional fourth class is expected to arise as a result of interhomolog GC. From WT males that had been heat-shocked at 38°, 43.4% of the progeny were the result of SSA repair and an equal portion was derived from interhomolog GC (Table 2A). In sharp contrast, *spnA* mutant combinations led to elevated SSA frequencies (≥0.919), which are virtually identical to the ones obtained from the hemizygous assay (Table 1). None of the w^{+} recut^{-} progeny was positive for allele-specific PCR for detecting interhomolog GC. These results suggest that both interhomolog and intersister GC are abolished in the absence of Rad51. For the *spnA*^{1}*/Df(3R)X3F1* combination (Table 2A), 95.5% of the progeny were *w*$^{-}$, the result of SSA. The rest (4.5%) of the progeny were w^{+}. Of those, 8 of 38 were also recut^{-}, which translates to an imperfect NHEJ frequency of 0.009. Taken together, these results suggest that cutting under this condition is very efficient, approaching 100%, and that imperfect NHEJ is very inefficient in the male germline when SSA repair is feasible.

Drosophila Rad54's role in GC and SSA repair: The *okra* (*okr*) gene encodes the fly homolog of Rad54 (GHABRIAL *et al.* 1998), which intimately interacts with Rad51 to promote its function in GC. In both budding and fission yeast, GC is also abolished by *rad54* mutations (reviewed in PAQUES and HABER 1999). We predicted that *okr* mutations, similar to *spnA*, would lead to an elevated SSA frequency due to their inhibiting effects on intersister GC. Surprisingly, we observed SSA frequencies similar to the WT level for all three *okr* mutations tested in a hemizygous configuration (Table 1). We considered two possible explanations for this result. First, the *okr* alleles that we tested may be weak so that they retain substantial ability to repair DSBs by GC. We considered this model unlikely. Mutations in *okr* also cause female sterility partly due to patterning defects in the egg (GHABRIAL *et al.* 1998). The *okr*^{AA} and *okr*^{RU} mutations are caused by premature stop codons and have been characterized as null or strong loss-of-function alleles in terms of their effects on oogenesis. Second, we hypothesized that Drosophila Rad54 might have a less significant role in intersister GC than Rad51. In the hemizygous assay, the majority of the DSBs were eventually repaired via SSA. A smaller enhancing effect of *okr* on SSA may not acquire statistical significance.

We then used the homozygous assays to measure the effect of *okr* mutations on GC in a different way. In the *[wIw]8z* homozygous assay (Figure 2A), *okr* mutant males

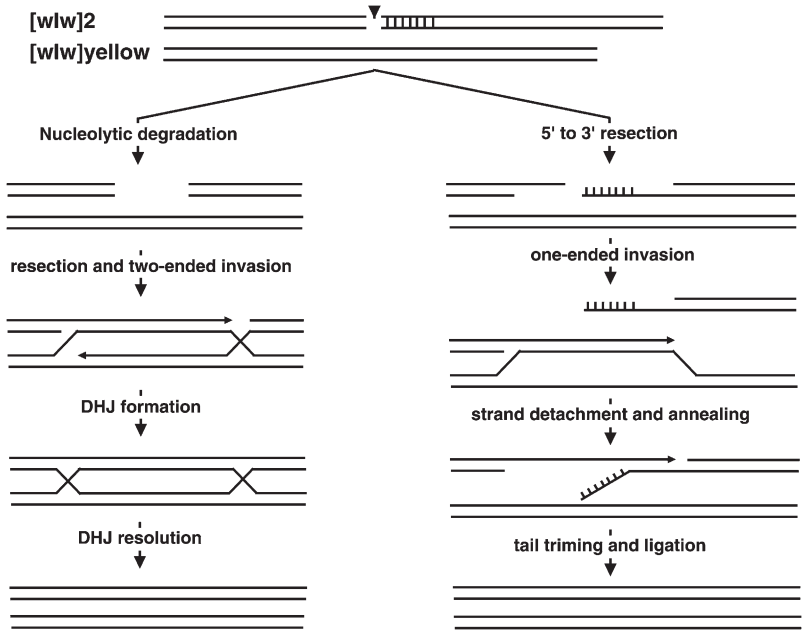

FIGURE 3.—Two possible mechanisms for interhomolog GC with *[wIw]yellow*. Horizontal lines represents DNA single strands. The region marked by vertical lines represents the 333-bp region that is not present on the *[wIw]yellow* chromosome. To the left is the traditional "Double Holliday Junction (DHJ)" model for GC. First, the DSB is enlarged to a double-strand gap removing heterology between the homologs (area marked by vertical lines). Both ends invade the template and initiate DNA synthesis with the direction indicated by the arrow. DHJ formation and resolution complete GC repair. To the right is the SDSA model for GC. Following single-strand resection, only the left end of the DSB invades the template and initiates DNA synthesis. Once synthesis has passed the heterologous region marked by vertical lines, the invading strand detaches from the template and anneals with the right end of the DSB. Other mechanisms might also be possible.

had SSA frequencies (0.728–0.869) similar to the combined frequency of SSA and GC from WT males (0.799). We infer from this result that interhomolog GC is inhibited by the *okr* mutations leading to the increase of SSA. However, these SSA frequencies from *okr* males do not differ from the ones observed for 38°-treated WT (0.867) or *okr* males (0.770–0.900) in the hemizygous assay, which suggests that intersister GC in the homozygous assay was not greatly affected by *okr*, similar to the situation in the hemizygous assay under the same heat-shock condition.

We also used the *[wIw]yellow* homozygous assay (Figure 2B) to directly measure the effect of the *okr* mutations on GC. When comparing the *[wIw]yellow* and the *[wIw]8z* assays, we discovered that WT males had a reduced interhomolog GC frequency [from 0.415 to 0.306 and a corresponding increase of SSA from 0.384 to 0.509 ($P = 0.0001$ for comparing SSA frequencies)]. This confirms our earlier hypothesis that the more extensive end processing needed for GC would favor SSA in this new homozygous assay. We examined 20 independent yellow-eyed progeny by PCR and sequencing and confirmed that they were the result of copying the small deletion onto the original *[wIw]2* chromosome (data not shown). For *okr* mutants, we obtained SSA rates that are similar to ones from the *[wIw]8z* homozygous assay (Table 2B). Interestingly, we also detected a small but significant frequency of interhomolog GC under this condition (0.017 for okr^{4A} and 0.009 for okr^{RU}). By PCR and sequencing, we verified the presence of the 333-bp deletion in 10 independent events. We believed that these were genuine GC repair products, but not the rare events caused by mitotic crossing over between the *Sb* marker and the *[wIw]yellow* insertion since we recovered 103 potential interhomolog GC events without recovering a single reciprocal product. Therefore, *okr* mutant germlines can support a very low level of GC. This may be attributed to maternal Rad54 contribution or Rad54 independent repair.

In summary, Drosophila Rad54 is essential for interhomolog GC, but may have a less important role in intersister GC than Drosophila Rad51.

Intersister GC can be better estimated with less I-SceI cutting: Intersister GC restores the cut site rendering the chromosome susceptible to a second round of cutting, and repeated I-SceI cutting would "select" repair products that have a mutated I-SceI cut site such as those from SSA repair. In addition, when the nuclease is abundant, both sisters can be cut, which prevents intersister GC. Therefore, intersister GC events are likely underrepresented in progeny after a 38° induction. To better estimate the contribution from intersister GC, we repeated the hemizygous assay with a less severe heat shock of 1 hr at 32°. With this condition, WT SSA frequency drops to ~0.30. As we expected, the SSA frequency was elevated from 0.319 to 0.548 in $spnA^{1}$ hemizygotes, an increase of >80% from the WT level. In addition, all three *okr* mutations led to an SSA frequency increase of as high as 100% of the WT level (Table 1). The similar effects of *spnA* and *okr* mutations on SSA repair under this condition suggest that Drosophila Rad54 is indeed important for intersister GC.

We also repeated the *[wIw]yellow* homozygous assay with a 32° heat induction. WT males gave rise to reduced SSA (0.149) and interhomolog GC (0.112) frequencies (Table 2). Interestingly under the lower I-SceI inducing condition, we recovered close to equal proportions of SSA and interhomolog GC whereas the more severe 38° heat shock led to almost twice as much SSA as interhomolog-GC repair products (0.509 *vs.* 0.306).

TABLE 1

Hemizygous assays on known mutants

Genotype	38°[a] N[b]	38°[a] SSA freq.[c] ± SEM	38°[a] P[d]	32°[a] N[b]	32°[a] SSA freq.[c] ± SEM	32°[a] P[d]
			Chromosome *X*			
+[e]	33 (1858, 2032)	0.893 ± 0.023		19 (771, 928)	0.241 ± 0.044	
lig4^{11}	36 (2062, 2409)	0.946 ± 0.012	0.0340	26 (1587, 1791)	0.284 ± 0.035	0.4522
			Chromosome *2*			
+/+[e]	29 (1456, 1376)	0.867 ± 0.015		27 (936, 895)	0.290 ± 0.041	
okr^{WS}/*Df*[f]	14 (736, 692)	0.770 ± 0.052	0.0530	22 (969, 911)	0.494 ± 0.044	0.0016
okr^{RU}/*Df*[f]	27 (1372, 1348)	0.900 ± 0.025	0.2648	19 (1193, 1143)	0.537 ± 0.028	<0.0001
okr^{AA}/*Df*[f]	35 (2218, 2174)	0.884 ± 0.017	0.4614	29 (1611, 1529)	0.602 ± 0.030	<0.0001
			Chromosome *3*			
+/+[e]	46 (2766, 2474)	0.847 ± 0.014		27 (2314, 1977)	0.318 ± 0.042	
spnA^{1}/*Df*[g]	29 (2148, 2032)	0.948 ± 0.010	<0.0001	40 (3167, 2929)	0.548 ± 0.027	<0.0001
spnA^{1}	10 (628, 641)	0.935 ± 0.030	0.0096		Not done	

[a] The heat-shock temperatures for I-SceI induction.
[b] *N*: sample size, the number of male parents tested followed by the number of progeny with and without the I-SceI cut chromosome scored in parentheses.
[c] SSA frequency, the proportion of white-eyed progeny. SEM: standard error of the mean.
[d] The two-tailed *P*-value calculated using the permutation test for the null hypothesis that the median SSA frequencies are the same between a particular mutant and the WT control. The underlined numbers indicate that the two samples were statistically different.
[e] w^{1118}.
[f] *Df=Df(2L)JS17.*
[g] *Df=Df(3R)X3F.*

This suggests that some of the 38° SSA repair events were the results of repeated cutting of a chromosome with a restored I-SceI cut site.

We tested the *okr* mutations with the *[wIw]yellow* homozygous assay at 32°. Consistent with previous 38° results, we observed a decrease of interhomolog GC frequency to ~0.02 and an increase of SSA repair up to 0.543 when compared to WT males (Table 2). In *okr* mutants, "visible" repair events (SSA plus interhomolog GC) account for up to 56.3% of the progeny *vs.* 26.1% in WT, suggesting that the "invisible" intersister GC accounts for at least 30% of the total repair events.

In summary, by lowering the severity of I-SceI induction, we have prevented some of the intersister GC events from being converted to SSA or interhomolog GC, thus providing a better way to estimate the contribution for this important mode of DSB repair.

DNA ligase IV facilitates end-joining repair: Having demonstrated that mutants defective in HR could be readily identified by our assays, we tested the feasibility of using the same assays to uncover mutants defective in NHEJ. For that purpose, we tested a Drosophila *ligase4* (*lig4*) mutation.

In our hemizygous assay with a 38° heat shock, the *lig4*^{11} deletion mutation led to an increase in SSA frequency from 0.893 to 0.946 (Table 1). Ten independent lines were set up from white-eyed progeny. Southern blot analyses confirmed that they were the result of SSA repair (data not shown; Rong and Golic 2003). The increase in SSA repair was also evident in *lig4*^{11} mutant soma. The loss of white by SSA leads to the appearance of white patches in the eye on an otherwise pigmented background. Eyes from *lig4*^{11} mutants with hemizygous *wIw* and *70I-SceI* were mostly or completely white, yet similar *lig4*$^{+}$ eyes had larger pigmented areas and were almost never completely white (data not shown).

To provide further support for our hypothesis that some DSBs in lig4-deficient cells were channeled to SSA and perhaps GC, we measured SSA and GC in the *lig4*^{11} germline using the *[wIw]yellow* homozygous assay with a 38° induction. We observed an overall increase of visible repair products from 80.0% in WT to 89.2% in *lig4*^{11}. Interestingly, the increase of visible repair events in the *lig4*^{11} mutant can be entirely accounted for by the increase in SSA repair, again suggesting that SSA is favored over interhomolog GC under this condition. Our results are consistent with those from a recent report (Johnson-Schlitz *et al.* 2007).

The most obvious reason for the increase in SSA repair in a lig4-deficient background would be that imperfect NHEJ is inhibited, resulting in a greater number of the I-SceI- generated DSBs being repaired by SSA. We investigated whether imperfect NHEJ was significantly impaired by the *lig4* mutation. In our assays, imperfect NHEJ events can be identified as w^{+} $recut^{-}$ (Figure 1). Since we were assaying repair events in mitotic germline cells, a single imperfect NHEJ event could be replicated, leading to multiple progeny with non-independent events. In addition, there were very few

TABLE 2

Homozygous assay

Genotype	N^a	SSA freq.^{b} ± SEM	P^c	GC freq.^{d}	P	N for recut^e	SSA + GC	N for PCR^f	P
				A. With *[wIw]8z*					
				Chromosome *2* at 38°^{g}					
+/+	30 (1819, 1981)	0.384 ± 0.029		0.415		Not done		141 (94)	
okr^{WS}/Df^h	26 (2112, 2050)	0.728 ± 0.045	<0.0001			Not done			
okr^{RU}/Df	24 (2064, 2110)	0.822 ± 0.026	<0.0001			Not done			
okr^{AA}/Df	26 (1946, 1950)	0.869 ± 0.018	<0.0001			Not done			
				Chromosome *3* at 38°					
+/+	27 (1445, 1700)	0.434 ± 0.047		0.426		411 (366)		100 (83)	
$spnA^1$/+	28 (1407, 1358)	0.463 ± 0.043	0.6147	0.399		372 (297)		100 (95)	
$spnA^1$	30 (1965, 1925)	0.919 ± 0.018	<0.0001			Not done			
$spnA^1/Df^i$	30 (1647, 1770)	0.955 ± 0.010	<0.0001	<0.001^j		38 (8)		8 (0)	
				B. With *[wIw]yellow*					
				Chromosome X at 38°^{g}					
+	30 (4739, 4618)	0.505 ± 0.035		0.295 ± 0.024			0.800 ± 0.029		
$lig4^{11}$	29 (4064, 4199)	0.663 ± 0.047	0.0094	0.229 ± 0.034	0.1177		0.892 ± 0.028		0.0251
				Chromosome *2* at 38°					
+/+	40 (4424, 4135)	0.509 ± 0.017		0.306 ± 0.017			0.815 ± 0.016		
$mus206^{A1}$	41 (3809, 3703)	0.585 ± 0.023	0.0093	0.295 ± 0.022	0.6953		0.878 ± 0.013		0.0028
okr^{AA}/Df^h	30 (3368, 3956)	0.868 ± 0.017	<0.0001	0.017 ± 0.007	<0.0001		0.885 ± 0.013		0.0016
okr^{RU}/Df	40 (5096, 5181)	0.829 ± 0.013	<0.0001	0.009 ± 0.004	<0.0001		0.838 ± 0.012		0.2453
				Chromosome *2* at 32°					
+/+	40 (3383, 2998)	0.149 ± 0.017		0.306 ± 0.017			0.261 ± 0.019		
okr^{AA}/Df	32 (2995, 3098)	0.543 ± 0.017	<0.0001	0.019 ± 0.006	<0.0001		0.562 ± 0.019		<0.0001
okr^{RU}/Df	30 (2608, 2470)	0.431 ± 0.026	<0.0001	0.020 ± 0.008	<0.0001		0.451 ± 0.027		<0.0001

[a] *N*: sample size, the number of male parents tested followed by the number of progeny with and without the I-SceI cut chromosome scored in parentheses.

[b] SSA frequency, the proportion of white-eyed progeny. SEM: standard error of the mean.

[c] The two-tailed *P*-value calculated using the permutation test for the null hypothesis that the median SSA frequencies are the same between a particular mutant and the WT control. The underlined numbers indicate that the two samples were statistically different.

[d] GC frequencies for the *[wIw]8z* assay are calculated as follows: $w^+/(w^+ + w^-) \times \text{recut}^-/(\text{recut}^+ + \text{recut}^-) \times \text{PCR}^+/(\text{PCR}^+ + \text{PCR}^-)$, for testing both the *lig4* and *spnA* mutations with the homozygous assay using the *[wIw]8z* template. GC frequencies for second chromosome mutations are calculated as follows: $w^+/(w^+ + w^-) \times \text{PCR}^+/(\text{PCR}^+ + \text{PCR}^-)$, since w^+ progeny were taken for allelic PCR directly without scoring for the recut phenotypes. Since these GC frequencies are derived from complex calculations, no *P*-values were given for these samples. GC events for the *[wIw]yellow* assay were scored directly as progeny with light yellow eyes (Figure 2B). GC frequencies are calculated as the proportion of these yellow-eyed flies in total progeny.

[e] The number of flies tested for eye mosaicism in the presence of I-SceI to assay the integrity of the I-SceI cut site. The numbers in parentheses are the number of flies producing nonmosaic progeny.

[f] The number of flies tested with allelic PCR to detect interhomolog GC with the *[wIw]8z* template. The numbers in parentheses are the number of flies producing a positive PCR product.

[g] The heat-shock temperatures for I-SceI induction.

[h] *Df=Df(2L)JS17.*

[i] *Df=Df(3R)X3F.*

[j] Since none of the eight w^+ $recut^-$ events was PCR^+, the highest possible GC rate was calculated as 0.001 ($0.045 \times \frac{1}{38}$).

progeny in the w^+ $recut^-$ class (<5% of the relevant progeny), which suggests that all imperfect NHEJ events from a single father were likely the result of a single-germline repair event. We thus considered only the number of WT or *lig4*^{11}males that gave rise to at least 1 imperfect NHEJ progeny. In the hemizygous assay, 14 of 17 WT males produced imperfect events, while 8 of 29 *lig4*^{11} males did so. The difference is highly significant ($P = 0.0004$ from a one-tailed Fisher's exact test). In a later experiment using the homozygous assay, we recovered 9 independent imperfect NHEJ events from 30 *lig4*^{11} males and 19 events from 30 WT males. The difference is again significant ($P = 0.0095$). By combining these two sets of results, which are not statistically different, we conclude that there are over twice as many WT males (33/47) as *lig4* males (17/59) producing imperfect NHEJ events. Therefore, imperfect NHEJ is significantly impaired but not eliminated by the

lig4 deficiency, which is consistent with earlier results (McVey *et al.* 2004a; Romeijn *et al.* 2005; Johnson-Schlitz *et al.* 2007).

We were interested in how an NHEJ-defective mutant would behave under moderate I-SceI induction, and we repeated the hemizygous assay with a milder 32° heat shock. We observed no increase of SSA frequency, suggesting that NHEJ was not a major repair pathway, possibly because intersister GC repair is highly efficient under this condition.

End degradation is more extensive in a *lig4*-mutant background: We imagine that inefficient end joining due to the loss of lig4 function may render DSBs a longer half-life, making them more susceptible to nucleolytic digestion. We obtained supporting evidence for this hypothesis from our molecular analyses of imperfect NHEJ events. From independent w^+ $recut^-$ lines obtained from the hemizygous and homozygous assays, we amplified DNA fragments with a pair of primers ~300 bp to either side of the I-SceI cut site. Interestingly, 5 of the 20 events from $lig4^{11}$ failed to yield a PCR product. Further analyses revealed that they all harbor a large deletion to the left of the I-SceI cut site. One deletion ends at the *P* element. The other four extend beyond the *P* element and into neighboring genomic regions (see materials and methods). Therefore, they are at least 4 kb in size. On the contrary, 31 of the 32 events recovered from WT males yielded a PCR product with the same pair of primers. The one exception had a ~500-bp deletion to the left of the I-SceI cut site. Therefore, large deletions were preferentially recovered in a *lig4* mutant background ($P = 0.0060$ for deletions >4 kb and $P = 0.0263$ for deletions >500 bp, both from one-tailed Fisher's exact tests). Our results are consistent with previous observations that larger deletions during NHEJ are preferentially recovered from yeast, worm, and fly *lig4* mutants (Wilson *et al.* 1997; Morton *et al.* 2006; Johnson-Schlitz *et al.* 2007). Although this would be consistent with our idea that *lig4* mutations render breaks more susceptible to degradation, more analyses are needed to establish the exact role of lig4 in preventing end degradation.

Novel mutations that affect DSB repair efficiency: Having established that a combination of our repair assays can be used to uncover mutations affecting both HR and NHEJ repair, we set out to screen the existing *mus* mutants (Table 3). We expected that a mutation affecting SSA would have a reduced proportion of white-eyed progeny when tested with the hemizygous assay at both 38°- and 32° heat shock; a mutation affecting GC would have an elevated proportion of white-eyed progeny at 38°, but more so at 32°; and a mutation affecting NHEJ would have an increased proportion of white-eyed progeny only at 38°.

***ssar* inhibits SSA repair:** We discovered that in the presence of the homozygous $mus307^{D1}$ chromosome, the frequency of SSA repair induced by a 38° heat shock was reduced from 0.847 to 0.423 (Table 3). When induced by 32°, SSA repair in the same mutant background was reduced by a similar degree from 0.318 to 0.139. Interesting, this SSA-inhibiting mutation acted in a semidominant way so that heterozygous mutant males had a 38°-induced SSA frequency intermediately reduced to 0.698. It is possible that the SSA-inhibiting effect of $mus307^{D1}$ was due to the indirect effect of a mutation that attenuated the heat-shock response. We ruled out this hypothesis by comparing the rates of site-specific recombination catalyzed by the FLP recombinase expressed from a *hsp70-FLP* transgene in WT *vs.* $mus307^{D1}$ backgrounds (100% for WT, $n = 13$ males; 99% for $mus307^{D1}$, $n = 14$ males; see materials and methods).

We were interested in whether a drop of SSA repair was accompanied by an increase in another repair mechanism in $mus307^{D1}$ males. We measured imperfect NHEJ by scoring the $recut^-$ phenotype in 331 randomly selected w^+ progeny from *mus307* males (see materials and methods). Fifty-six of them were w^+ $recut^-$, which translates to an overall imperfect NHEJ frequency of 0.10, which is similar to the normal one of 0.06. Therefore, $mus307^{D1}$ did not markedly increase imperfect NHEJ at the expense of SSA repair.

We mapped this SSA-inhibiting mutation outside of the region between the *curled* and *stripe* mutations (see materials and methods), which is where $mus307^{D1}$ was previously mapped (Boyd *et al.* 1981; Drysdale *et al.* 2005). Therefore, this mutation can be separated from $mus307^{D1}$ and likely represents a new repair mutation. We name the locus *single-strand annealing reducer* (*ssar*) and the allele $ssar^1$.

***mus206*^{A1} promotes *SSA*:** We identified the $mus206^{A1}$ mutation as having a similar effect on SSA as the *spnA* or *okr* mutations. With a 38° heat shock, *mus206* males produced a higher SSA frequency than WT males (0.935 *vs.* 0.867). The milder 32° heat shock also led to a greater SSA increase in *mus206* males (from 0.290 to 0.436). We performed the homozygous assay with the *[wIw]yellow* template to directly measure the effect of $mus206^{A1}$ on GC. Different from what was observed with *spnA* and *okr*, *mus206* males produced more SSA repair products (58.5%) than WT males (50.9%), but with no reduction in interhomolog GC products (Table 2B). The lack of an effect of $mus206^{A1}$ on interhomolog GC is consistent with the fact that $mus206^{A1}$ homozygous females are fertile, whereas strong mutations affecting GC inevitably lead to female sterility (*e.g.*, Ghabrial *et al.* 1998; Abdu *et al.* 2003; Staeva-Vieira *et al.* 2003).

mei-9 encodes the Drosophila Rad1 homolog (Sekelsky *et al.* 1995). In budding yeast, the Rad1–Rad10 endonuclease complex serves to cleave the 3′ protruding tails during SSA (Sugawara *et al.* 1997). Mutations in *rad1* or *rad10* reduce SSA efficiency in yeast. However, we did not observe any inhibition of SSA by the $mei\text{-}9^{A2}$ null mutation. Therefore, the Drosophila Rad1 is not required for the trimming of 3′ tails in our SSA assay.

TABLE 3

Mutant screen with the hemizygous assay

Genotype	38°[a] N[b]	38°[a] SSA freq.[c] ± SEM	38°[a] P[d]	32°[a] N[b]	32°[a] SSA freq.[c] ± SEM	32°[a] P[d]
		Chromosome *X*				
+[e]	33 (1858, 2032)	0.893 ± 0.023		19 (771, 928)	0.241 ± 0.044	
mus101^{D1}	28 (1712, 1725)	0.786 ± 0.056	0.0694	30 (1582, 1690)	0.150 ± 0.035	0.1033
mus101^{D2}	24 (1726, 2015)	0.811 ± 0.045	0.0896	18 (788, 889)	0.318 ± 0.066	0.3364
mus102^{D1}	23 (1592, 1824)	0.909 ± 0.019	0.6580	25 (1033, 1129)	0.325 ± 0.059	0.2931
mus105^{A1}	25 (1305, 1373)	0.919 ± 0.015	0.4433		Not done	
mus109^{D1}	25 (1797, 1955)	0.945 ± 0.014	0.0791	22 (997, 1114)	0.272 ± 0.039	0.6077
mus109^{D2}	30 (1153, 1158)	0.891 ± 0.024	0.9521	30 (773, 850)	0.246 ± 0.033	0.9360
mus112^{RT2}	30 (1024, 1063)	0.892 ± 0.017	0.9812	24 (497, 535)	0.201 ± 0.037	0.4793
mus114^{RT1}	26 (848, 917)	0.891 ± 0.023	0.9647	20 (729, 670)	0.182 ± 0.036	0.2943
mei-9^{A2}	21 (1415, 1649)	0.890 ± 0.047	0.9518	24 (1699, 1864)	0.301 ± 0.052	0.4099
mei-41^{2}	24 (798, 844)	0.769 ± 0.033	0.0020		Not done	
mei-41^{29D}	28 (1377, 1386)	0.871 ± 0.016	0.5035	31 (1683, 1883)	0.271 ± 0.050	0.6917
		Chromosome *2*				
+/+[e]	29 (1456, 1376)	0.867 ± 0.015		27 (936, 895)	0.290 ± 0.041	
mus201^{D1}	35 (1961, 1931)	0.808 ± 0.030	0.1024	30 (1848, 1764)	0.335 ± 0.048	0.4720
mus206^{A1}	30 (1033, 981)	0.935 ± 0.016	0.0027	22 (993, 921)	0.436 ± 0.042	0.0172
mus207^{A1}	23 (858, 864)	0.928 ± 0.018	0.0114	23 (1040, 976)	0.278 ± 0.033	0.8296
mus209$^{B1/2735}$	27 (1362, 1338)	0.830 ± 0.024	0.1746	25 (1561, 1585)	0.261 ± 0.035	0.6086
rad201^{1}	23 (1019, 935)	0.865 ± 0.027	0.9544	23 (948, 797)	0.300 ± 0.025	0.8431
sir2^{2A}	29 (1646, 1704)	0.840 ± 0.022	0.3060	26 (1402, 1411)	0.230 ± 0.026	0.2352
mio^{2}	26 (1295, 1323)	0.870 ± 0.021	0.8976	30 (1486, 1470)	0.177 ± 0.025	0.0193
		Chromosome *3*				
+/+[e]	46 (2766, 2474)	0.847 ± 0.014		27 (2314, 1977)	0.318 ± 0.042	
mus301$^{D1/D4}$	18 (1080, 1027)	0.840 ± 0.029	0.8188	17 (728, 634)	0.330 ± 0.060	0.8647
mus304$^{D1/D3}$	20 (973, 994)	0.804 ± 0.023	0.1092	14 (494, 418)	0.308 ± 0.051	0.8903
mus304^{D3}	13 (815, 715)	0.876 ± 0.030	0.3432		Not done	
mus305$^{D1/D2}$	26 (1551, 1507)	0.878 ± 0.032	0.3221	28 (1550, 1511)	0.265 ± 0.042	0.3849
mus306^{D1}	27 (1943, 1728)	0.776 ± 0.046	0.0135	24 (1784, 1637)	0.364 ± 0.028	0.4079
mus308^{D2}	22 (1870, 1693)	0.811 ± 0.051	0.3921	21 (1417, 1283)	0.367 ± 0.047	0.4424
mus309$^{D2/D3}$	23 (1350, 1245)	0.792 ± 0.041	0.1221	10 (650, 569)	0.232 ± 0.047	0.2654
mus311$^{D1/D2}$	22 (1033, 982)	0.902 ± 0.018	0.1220		Not done	
mus312$^{D1/D2}$	22 (986, 845)	0.793 ± 0.037	0.1039	27 (1224, 1025)	0.319 ± 0.042	0.9791
mus324$^{ZIII4325/ZIII5997}$	30 (4236, 4168)	0.884 ± 0.025	0.1735	25 (2653, 1843)	0.210 ± 0.025	0.0062
mus327^{ZIII5906}	29 (3623, 3479)	0.869 ± 0.031	0.4647	24 (2772, 2615)	0.238 ± 0.034	0.1575
mu2^{1}	23 (3069, 3073)	0.889 ± 0.012	0.0530	31 (3803, 3731)	0.462 ± 0.021	0.0040
mu2^{1}/*Df*[f]	26 (3678, 3467)	0.891 ± 0.017	0.0515	30 (3852, 3806)	0.425 ± 0.024	0.0328
spnB^{1}	13 (714, 661)	0.884 ± 0.038	0.2623		Not done	
spnD^{1}	14 (1048, 950)	0.790 ± 0.031	0.0706		Not done	
spnE^{1}/*Df*[g]	17 (684, 614)	0.846 ± 0.036	0.9905		Not done	
SSAR^{1} *mus307*^{D1}	26 (2257, 2226)	0.428 ± 0.040	<0.0001	30 (2210, 1809)	0.139 ± 0.030	0.0012
SSAR^{1} *mus307*^{D1}/+ +	42 (3041, 2667)	0.698 ± 0.026	<0.0001		Not done	
SSAR^{1} +/*SSAR*^{1} *mus307*^{D1}	22 (1605, 1388)	0.407 ± 0.043	<0.0001		Not done	

[a–e] See footnotes in Table 1.
[f] *Df=Df(3L)Aprt-1.*
[g] *Df=Df(3R)sbd105.*

mei-41 encodes the Drosophila homologs of mammalian ATR (Hari *et al.* 1995). We observed an allelic-specific inhibition of SSA (Table 1), which suggests that the effect was specific to that allele or to X-linked second-site mutations.

missing oocytes (*mio*), *mutator2* (*mu2*), and *mus324* behaved similarly in reducing SSA efficiency only with the milder 32° heat shock. *mio* encodes a protein that was implicated to function in meiotic DSB repair (Iida and Lilly 2004). *mu2* encodes a putative Drosophila homolog of mammalian MDC1 DNA damage checkpoint protein (Kasravi *et al.* 1999; J. Mason, personal communication). The effect of these mutations may be complex and will be the subject of future studies.

mus207^{A1} and *mus306*^{D1} behaved similarly to *lig4*^{11}; *i.e.*, we observed a significant increase of SSA repair with the

38° induction but not with the 32° induction, suggesting that both mutants might be defective in end joining. To investigate whether *mus207* behaves similarly to *lig4* under a different condition, we conducted the *[wIw]yellow* homozygous assay, but did not observe a significant effect.

DISCUSSION

In this study, we further develop our I-SceI-based germline assays in two important areas: (1) we showed that the important contribution of intersister GC can be better estimated with reduced I-SceI cutting and (2) we developed a versatile assay using the *[wIw]yellow* chromosome in which interhomolog GC events can be phenotypically recognized. Using a series of repair assays combined with different degrees of I-SceI induction, we determine some of the genetic requirements for DSB repair in the Drosophila germline.

We demonstrated that Drosophila Rad51 is essential for both intersister and interhomolog GC repair, but dispensable for SSA. Our results are consistent with those from earlier studies (McVey *et al.* 2004b; Johnson-Schlitz *et al.* 2007). Contrary to results from McVey *et al.* (2004b), we did not observe any effect of *spnA* heterozygosity on either intersister or interhomolog GC (Table 2A). This could be due to the fact that different assays were employed in the two studies: McVey *et al.* studied the repair of a large gap induced by P transposase while we create a simple break with I-SceI. The abundance of spnA may be more important for the repair of a large gap.

We also showed that Drosophila *rad54* (*okr*) mutations behaved very similarly to *spnA* in our repair assays suggesting that Rad54 is also essential for GC repair. We were first perplexed by the apparent lack of an effect of *okr* on what we inferred as intersister GC events with a 38° I-SceI induction, whereas *okr* mutations clearly inhibited both intersister and interhomolog GC at 32°. We suspected that the acute heat treatment might have been the cause. As we were preparing this article, Johnson-Schlitz *et al.* (2007) made several interesting observations on *okr* using I-SceI-based repair assays that are very similar to ours. A *ubiquitin* promoter-driven I-SceI source was used by Johnson-Schlitz *et al.*, which provides strong and ubiquitous I-SceI expression that might be similar to our 38° heat induction. In their "cross 1," similar to our hemizygous assay, *spnA*, but not *okr,* led to an increased usage of SSA. The results from the two studies suggest that the lack of an effect of *okr* on intersister GC is specific to the situation in which intersister GC is selected against due to excessive I-SceI cutting. We suggest that Drosophila Rad54 is less important than Rad51 in intersister GC. On the other hand, we showed that both Rad51 and Rad54 were essential for interhomolog GC. Perhaps Rad54 promotes pairing between the broken DNA and its repair template. This function may be more stringently required for interhomolog GC than for intersister GC since sister chromatids are held together by cohesins.

The important function of DNA ligase IV in genome maintenance was not as clearly defined in Drosophila as for HR repair factors. It was concluded that *lig4* mutants were generally not sensitive to DNA damaging agents (Gorski *et al.* 2003; McVey *et al.* 2004a) and that they were not grossly deficient for imperfect joining of broken ends (McVey *et al.* 2004a; Romeijn *et al.* 2005). In addition, our *lig4* mutation did not suppress end fusion between unprotected telomeres (Bi *et al.* 2004). Our current results are more consistent with those from a recent publication (Johnson-Schlitz *et al.* 2007). We provided several pieces of evidence suggesting that lig4 is important for end joining in Drosophila: (1) the number of test males producing progeny with imperfect end-joining repair was significantly reduced under the *lig4* mutant background in both the hemizygous and homozygous assays, (2) imperfect NHEJ with large deletions were preferentially recovered in a *lig4* mutant background, and (3) loss of lig4 function led to elevated frequencies of homology-based repair (SSA). This compensatory relationship between NHEJ and HR was also evident in an earlier study in which Romeijn *et al.* (2005) suggested that a *lig4* mutation caused an increased utilization of somatic interhomolog GC for the repair of a P transposase-induced DSB. However, the authors did not estimate the frequencies of NHEJ in WT or the lig4-deficient germline. In another study, McVey *et al.* (2004a) estimated the frequency of end joining in the germline but did not observe any effect from a *lig4* mutation. In that study, DSBs were induced by P transposase at a low frequency, making it difficult to uncover a significant but not essential role of lig4 in end joining. In addition, P transposase creates a large gap of a few kilobases in that study whereas I-SceI creates a simple break in the current study. Moreover, P transposase generates long (17-bp) and noncomplementary overhangs, while I-SceI generates short (4-bp) and complementary overhangs. These differences in break configuration may have altered the stringency for the requirement of lig4 function.

A recurring observation that we made during the course of this study was the competition between different repair mechanisms. We demonstrated an intricate balance among different repair mechanisms by manipulating the genetic control of these mechanisms, or the immediate genomic environment surrounding the DSB, or both simultaneously. We showed earlier that interhomolog GC effectively competes with SSA given that homology is provided immediately adjacent to the DSB on the homolog (Rong and Golic 2003). By inhibiting GC with a *spnA* or *okr* mutation or by limiting the immediate homology to only one side of the DSB with the *[wIw]yellow* GC template, we can tilt the balance back in favor of SSA.

GC using the sister chromatid is generally believed to be the most efficient way of DSB repair (Engels *et al.*

1990; Kadyk and Hartwell 1992; Johnson and Jasin 2000). However, the contribution from intersister GC is difficult to measure due to the lack of genetic consequence for these events. We were able to get around this problem by taking advantage of the competitive nature of DSB repair in the Drosophila germline so that these invisible events can be converted to visible ones by the use of mutations. With a 38° heat induction, ~85% of the progeny from a WT male experienced SSA, while ~95% of those from *spnA* males did so, suggesting that intersister GC accounts for at least 10% of the progeny from a WT male. This is an underestimation since intersister GC renders the chromosome susceptible to a second round of cutting. Support for the above reasoning came from experiments in which the same repair assay was carried out but with a milder 32° heat shock to produce less I-SceI. Under this new condition, SSA frequency was elevated from ~0.30 in WT to 0.55 in *spnA* and up to 0.60 in *okr*, suggesting that intersister GC accounts for at least 25–30% of the progeny in a WT male. From these data we suggest that lesser I-SceI induction is a better way to estimate the contributions from intersister GC repair. In addition, considering that SSA but not intersister GC is permitted throughout most of the cell cycle, the frequency ratio (close to 1:1) between SSA and intersister GC measured with a 32° heat shock suggests that there might be at least as many S/G2 cells as G1 cells in the male germline during the first 3 days of development. This extrapolation may be experimentally tested using cell-cycle-specific markers.

We screened a collection of existing mutations with the I-SceI-based repair assays and succeeded in identifying several mutations that had various defects in DSB repair. We discovered *ssar* as a locus necessary for normal SSA repair. There are a limited number of mutations identified in budding yeast that are defective for SSA (Ivanov and Haber 1995; Ivanov *et al.* 1996; Sugawara *et al.* 1997, 2000; Davis and Symington 2001). Rad52 and Rad59 have the most important function of promoting strand annealing, while the Rad1–Rad10 endonuclease and the Msh2–Msh3 mismatch repair proteins play important roles under certain conditions. However, what is known about yeast SSA-defective mutants was little help for us in identifying *ssar*. Homologs of Drosophila Rad52, Rad59, and Msh3 cannot be identified by sequence homology (Sekelsky *et al.* 2000). Our results suggest that Drosophila mei-9/Rad1 is dispensable for SSA repair in our assay. Drosophila homologs for Msh2 and Rad10 are both located on a different chromosome. Therefore, *ssar* likely encodes a novel repair protein important for SSA repair. Perhaps *ssar* encodes a Drosophila functional homolog of the fungal Rad52/Rad59 protein. This predicts that $ssar^{1}$ would also inhibit GC. However, we have not been able to recover recombinant chromosomes with both $ssar^{1}$ and *[wIw]2* or its derivatives, most likely due to their close genetic linkage. The effect of $ssar^{1}$ on GC repair awaits future investigation.

We identified $mus206^{A1}$ as a new mutation that might enhance SSA. By using the hemizygous assay, we showed that $mus206^{A1}$ causes an increase in SSA usage under both 38° and 32° heat-shock conditions. By using the homozygous assay, we showed that $mus206^{A1}$ did not affect interhomolog GC, suggesting that its effect on SSA might be direct. It is also possible that $mus206^{A1}$ weakens sister chromatid cohesion, specifically affecting intersister but not interhomolog GC. We have not excluded the possibility that the mutation affecting SSA is different from the one conferring mutagen sensitivity since second-site mutations could have accumulated over the years.

Johnson-Schlitz *et al.* (2007) tested many of the same mutations that we have tested in this study. The two studies led to consistent results for some genes (*i.e.*, *spnA*, *okr*, and *lig4*), but not others. We did not detect an effect of *mei-9*, *mus101*, and *mus301* mutations on SSA repair. We suspect that the different results were mainly caused by the difference in the repair assays employed. In our assays, the *white* duplication is ~3000 bp, which is significantly longer than the 147-bp repeat used by Johnson-Schlitz *et al.* In addition, the intervening sequence flanked by the repeats is ~1.5 kb in our assay, again significantly longer than the one used in the other study. Either one or both of these features may have rendered our assay insensitive to those mutations tested. Nevertheless, we uncovered the $ssar^{1}$ mutation, which causes the largest drop of SSA efficiency among all mutations tested in the two studies. The two studies also came to different conclusions regarding Drosophila ATR's role in SSA repair. Although we observed a drop in SSA with the $mei\text{-}41^{2}$ allele, which is similar to what was observed with the $mei\text{-}41^{D5}$ allele by Johnson-Schlitz *et al.*, we observed no effect from the $mei\text{-}41^{29D}$ allele. In addition, LaRocque *et al.* (2007) observed a drop of SSA using $mei\text{-}41^{29D}$ in our hemizygous assay. However, the decrease in SSA efficiency seen in the LaRocque study seems less severe than the one observed by Johnson-Schlitz *et al.*, even though $mei\text{-}41^{29D}$ was classified as a null (Laurencon *et al.* 2003) and that $mei\text{-}41^{D5}$ is likely a weaker allele evidenced by the fertility of $mei\text{-}41^{D5}$ females. A plausible explanation for these different results on the effect of mei-41 on SSA repair is that the $mei\text{-}41^{29D}$ chromosome may have an SSA-enhancing mutation masking the effect of $mei\text{-}41^{29D}$. The exact role of Drosophila ATR in SSA repair may require additional investigation.

The most significant factor that may have limited the success of our screen is that our assays require male fertility. Therefore, we would have missed genes whose products are required not for the viability of single cells but for the development of complex organisms. For examples, *mre11* and *nbs* null mutations are viable in yeast but lethal in flies (Bi *et al.* 2004, 2005). So are strong loss-of-function *atm* mutations in Drosophila, while a weak *atm* mutation is male sterile (Bi *et al.* 2004; Gong *et al.* 2005). In the future, somatic reporter constructs expressing

fluorescent proteins could be used to assay lethal or sterile mutations (Preston *et al.* 2006a).

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APPENDIX: CROSSING SCHEMES

Hemizygous assay:

1. Chromosome *X* mutants (*mut^X*):

   w mut^X; [wIw]2 Sb/+ males ⊗⊗ *C(1)M3, y/Y; [70I-SceI]1A/TM6, Ubx e* females

   ⇓⇓

   w mut^X; [wIw]2 Sb/[70I-SceI]1A male ⊗⊗ *w^{1118}* females

   ⇓⇓

   Score repair products in Sb progeny.

2. Chromosome *2* mutants (*mut^2*):

   w; mut^2/Sco; [70I-SceI]1A Sb/+ males ⊗⊗ *w; mut^2/CyO; [wIw]2 /+* females

   ⇓⇓

   w; mut^2/mut^2; [wIw]2 /[70I-SceI]1A Sb males ⊗⊗ *w^{1118}* females

   ⇓⇓

   Score repair products in Sb$^+$ progeny.

3. Chromosome *3* mutants (*mut^3*):

   w; [70I-SceI]2B Sco/+; mut^3/Sb males ⊗⊗ *w; [wIw]4A/+; mut^3/TM6* females

   ⇓⇓

   w; [wIw]4A/[70I-SceI]2B Sco; mut^3/mut^3 males ⊗⊗ *w^{1118}* females

   ⇓⇓

   Score repair products in Sco$^+$ progeny.

Homozygous assay:

1. lig4^{11}:

   w; [70I-SceI]2B Sco/+; [wIw]8z Sb/+ males ⊗⊗ *w lig4^{11}; [wIw]2/TM6* females

   ⇓⇓

   w lig4^{11}; [70I-SceI]2B Sco/+; [wIw]8z Sb/[wIw]2 males ⊗⊗ *w^{1118}* females

   ⇓⇓

   Score repair products in Sb$^+$ progeny.

2. Chromosome *2* mutants (*mut^2*: *okr*, *mus206*, *mus207*):

   w; mut^2/Sco; [wIw]8z [70I-SceI]1A Sb/+ males ⊗⊗ *w; mut^2/CyO; [wIw]2/+* females

   ⇓⇓

   w; mut^2/mut^2; [wIw]2 /[wIw]8z [70I-SceI]1A Sb males ⊗⊗ *w^{1118}* females

   ⇓⇓

   Score repair products in Sb$^+$ progeny.

3. *spnA^1* and *Df(3R)X3F (Df)*:

   w; [70I-SceI]2B Sco/+; [wIw]8z e spnA^1/+ males ⊗⊗ *w; [wIw]2 Sb e Df/TM6* females

   ⇓⇓

   w; [70I-SceI]2B Sco/+; [wIw]8z e spnA^1/[wIw]2 Sb e Df males ⊗⊗ *w^{1118}* females

   ⇓⇓

   Score repair products in *Sb* progeny.

Notes: We scored all the progeny from a cross regardless of whether the progeny have inherited a [*wIw*] chromosome. In general, progeny with or without the [*wIw*] chromosome are of equal proportion, which suggests that most of the DSBs were repaired. The control males for each chromosome were all derived from w^{1118}.

For the hemizygous assay, test males were generated by mass mating and heat-shocked early in development, but they were testcrossed to two to three w females individually. For *X* chromosome mutants, the [*70I-SceI*]*1A* transgene came from the mother so that there could be maternal contribution of the I-SceI enzyme. For mutations on a w^+ *X* chromosome, only male progeny, which inherited the w^{1118} chromosome from the mother, were scored for repair events.

For the homozygous assay, test males were generated by mass mating, but tested individually. For testing WT, $lig4^{11}$, $mus206^{A1}$, $mus207^{A1}$, and *okr* with the new homozygous assay, the crossing scheme was identical except that the [*wIw*]*yellow* insertion was used in place of [*wIw*]*8z*. *okr* mutations were tested over *Df(2L)JS17*.