

End-Joining Repair of Double-Strand Breaks in *Drosophila melanogaster* Is Largely DNA Ligase IV Independent

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ABSTRACT

Repair of DNA double-strand breaks can occur by either nonhomologous end joining or homologous recombination. Most nonhomologous end joining requires a specialized ligase, DNA ligase IV (Lig4). In *Drosophila melanogaster*, double-strand breaks created by excision of a *P* element are usually repaired by a homologous recombination pathway called synthesis-dependent strand annealing (SDSA). SDSA requires strand invasion mediated by DmRad51, the product of the *spn-A* gene. In *spn-A* mutants, repair proceeds through a nonconservative pathway involving the annealing of microhomologies found within the 17-nt overhangs produced by *P* excision. We report here that end joining of *P*-element breaks in the absence of DmRad51 does not require *Drosophila* LIG4. In wild-type flies, SDSA is sometimes incomplete, and repair is finished by an end-joining pathway that also appears to be independent of LIG4. Loss of LIG4 does not increase sensitivity to ionizing radiation in late-stage larvae, but *lig4 spn-A* double mutants do show heightened sensitivity relative to *spn-A* single mutants. Together, our results suggest that a LIG4-independent end-joining pathway is responsible for the majority of double-strand break repair in the absence of homologous recombination in flies.

THE accurate repair of DNA damage is crucial to the survival and genomic integrity of cells. Double-strand breaks (DSBs) are a particularly problematic type of DNA damage. Failure to repair DSBs leads to cell death and apoptosis (RICH *et al.* 2000), while inaccurate repair can cause genomic instability and cancer (KHANNA and JACKSON 2001). The importance of DSB repair is highlighted by the recent identification of a multitude of human diseases that result from mutations in DSB repair genes (JACKSON 2002).

Depending on the type of break, stage of the cell cycle in which the break occurs, and other factors, repair of a DSB can occur through one of two general sets of mechanisms. The first group, collectively called homologous recombination (HR), occurs when the broken DNA strand uses a homologous template to prime repair DNA synthesis (VAN DEN BOSCH *et al.* 2002; WEST 2003). In most cases of HR, the choice of an appropriate template results in conservation of the original DNA sequence at the break site.

Alternatively, the DSB can be repaired by nonhomologous end joining (NHEJ) when the broken chromosome is sealed without consulting external homologies (LIEBER *et al.* 2003). This often results in the loss or addition of nucleotides and is therefore considered to

be error prone. The extent to which each of these two types of repair mechanisms is used varies between different organisms, different cell types, and even different developmental stages within the same organism.

In *Drosophila melanogaster*, excision of a transposable *P* element creates a DSB that can be repaired by HR mechanisms. To explain the repair products observed after *P* excision, the synthesis-dependent strand-annealing (SDSA) model has been proposed (NASSIF *et al.* 1994). In SDSA, the broken strand is resected to create a long, 3' single-strand tail that can invade a homologous template, thereby priming repair DNA synthesis to copy the *P*-element sequence back into the break site. Unlike normal DNA synthesis observed during replication, repair DNA synthesis that occurs during SDSA does not appear to be processive (PÂQUES *et al.* 1998). In fact, DNA repair synthesis across a large gap may occur by sequential strand invasion and DNA synthesis steps, in which each round of repair DNA synthesis extends the broken end by several hundred base pairs (MCVEY *et al.* 2004).

Perhaps as a result of this low processivity of DNA repair synthesis, the generation of internally deleted *P* elements occurs at a significant frequency even in wild-type flies. The creation of internally deleted *P* elements is most easily explained by a model in which SDSA initiates but the DNA needed to fill the entire gap is not synthesized. In such cases of incomplete SDSA, repair of the broken chromosome must be completed by an end-joining mechanism.

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In the budding yeast *Saccharomyces cerevisiae*, there are at least three distinct forms of end joining. In one, DSBs with short overhangs containing complementary ends, such as those created by restriction endonucleases, are precisely joined. In the absence of complementary ends, an imprecise NHEJ pathway can take over, annealing the broken ends at sites of microhomology of as little as 1 bp and trimming or filling in missing nucleotides before ligation, thereby creating small insertions or deletions. Both of these forms of NHEJ require the Ku70/80 heterodimer (MILNE *et al.* 1996), the Rad50p-Mre11p-Xrs2p (MRX) complex (BOULTON and JACKSON 1998), and the DNA ligase IV protein, Dnl4p (TEO and JACKSON 1997; WILSON *et al.* 1997). In addition, a Ku-independent form of imprecise NHEJ, termed microhomology-mediated end joining (MMEJ), has recently been described (MA *et al.* 2003). MMEJ requires the MRX complex, but is only partially dependent upon Dnl4p.

In mice, DNA ligase IV operates with a partner protein, Xrcc4, that apparently increases the efficiency of the ligation reaction. Yeast has a functional ortholog of Xrcc4, called Lif1p, that is also required for NHEJ (TEO and JACKSON 2000). A candidate XRCC4 gene in *Drosophila*, *CG3448*, was identified on the basis of sequence homology (SIBANDA *et al.* 2001). Recently, it was shown that the protein product of *CG3448* interacts with *Drosophila* DNA ligase IV (LIG4) in a two-hybrid assay (GORSKI *et al.* 2003). Therefore, it seems that the ligase-IV/XRCC4 complex is widely conserved throughout eukaryotes.

However, the importance of the ligase IV-XRCC4 complex seems to vary between different organisms. Mice with null mutations in *Lig4* die before birth, with massive neuronal apoptosis in the brain (BARNES *et al.* 1998; FRANK *et al.* 2000). Human cells lacking detectible *LIG4* are extremely sensitive to ionizing radiation (IR; GRAWUNDER *et al.* 1998), as are Arabidopsis *Atlig4* mutants (VAN ATTIKUM *et al.* 2003). In contrast, *S. cerevisiae* *dnl4* mutants show almost no sensitivity to IR (TEO and JACKSON 1997). In fact, a requirement for Dnl4p in budding yeast becomes apparent only in *rad52* mutants, which are deficient in homologous recombination (BOULTON and JACKSON 1996). Recently, a *Drosophila* *lig4* mutant was described and characterized (GORSKI *et al.* 2003). Flies lacking the LIG4 protein are viable, but sensitive to IR during the first day of development. This sensitivity increases in a *Rad54*-mutant background, where HR is impaired, suggesting that both types of repair are important in flies.

To further investigate the role of LIG4 in flies, we have conducted experiments to determine the mechanisms by which *lig4* mutants repair DSBs in both the presence and the absence of functional HR repair. The human Rad51 protein is required for strand invasion *in vitro* (MCILWRAITH *et al.* 2000). In *Drosophila*, the Rad51 ortholog is encoded by the *spn-A* gene and func-

tions in both DNA repair and meiosis (STAEVA-VIEIRA *et al.* 2003). *spn-A* mutant flies are unable to repair *P*-element-induced DSBs by HR, presumably due to a defect in strand invasion (MCVEY *et al.* 2004). We report here that repair of gamma-ray-induced DSBs is only marginally impaired in late-stage *lig4* mutant larvae, although a synergistic sensitivity is observed in the absence of Rad51-mediated HR repair. Furthermore, *lig4* mutant flies are proficient in the repair of DSBs created by *P*-element excision, even in the absence of Rad51. In the case of *P*-element excision, repair appears to proceed through annealing and ligation at microhomologous sequences. Taken together, these data reveal that LIG4-dependent end joining is a minor DSB repair pathway in flies and argue for the existence of a LIG4-independent end-joining pathway that can operate in the absence of HR-mediated repair.

MATERIALS AND METHODS

***Drosophila* stocks and genetics:** Flies were raised on standard cornmeal agar medium at 25°. The *spn-A* alleles used in these experiments were a gift from R. Lehmann. *spn-A*⁹³ is a nonsense mutation with no detectable DmRad51 protein, and the *spn-A*⁵⁷ missense mutation behaves as a null allele in genetic assays (STAEVA-VIEIRA *et al.* 2003). Other genetic components, including balancer chromosomes, are described in FlyBase (2001).

The *P*{*w*⁰} transgene used in this study is described in KURKULOS *et al.* (1994) and in ADAMS *et al.* (2003). For *P*{*w*⁰} crosses involving the *lig4* single mutant, we used the *P*{*ry*⁺, Δ 2-3}(99B) transposase source. Because this transposase source maps very near the *spn-A* locus, the *H*{*w*⁺, Δ 2-3}Hop2.1 transposase source was used in all *spn-A* mutant crosses. For the *P*{*w*⁰} assays, single males of genotype *lig4* *P*{*w*⁰}/*Y*; *Sb* *P*{*ry*⁺, Δ 2-3}/*TM6B* or *lig4* *P*{*w*⁰}/*Y*; +/*CyO*, *H*{*w*⁺, Δ 2-3}; *ru st e ca* *spn-A*⁵⁷/*ru h st th cu e ca* *spn-A*⁹³ were crossed to four *y w* *P*{*w*⁰} virgin females in vials, and the eye colors of all *Sb*⁺ or *Cy*⁺ progeny were scored. A heteroallelic combination of *spn-A* alleles was used to avoid detrimental effects sometimes observed when using homozygous mutant chromosomes.

The *P*{*w*⁰} assay for DSB repair: *P*{*w*⁰} carries the *apricot* allele of the *white* (*w*) gene. In *w*⁰, a *copia* retrotransposon in an intron causes decreased expression of *w*, so that female flies with two copies of *w*⁰ have apricot-colored eyes and flies with one copy have yellow eyes. *P*{*w*⁰} is inserted in an intron of the essential X-linked *scalloped* (*sd*) gene. *P*{*w*⁰} is crossed into males harboring transposase, which excises *P*{*w*⁰}, creating a double-strand gap of ~14 kb relative to the sister chromatid containing an intact *P*{*w*⁰}. Individual males are crossed to four *P*{*w*⁰} females in vials and the eye color of female progeny lacking transposase is assayed to determine type of repair.

Complete repair of the DSB by SDSA regenerates *P*{*w*⁰} and apricot eye color in the female progeny. The *copia* element in *w*⁰ contains directly repeated 276-bp long terminal repeats (LTRs) at each end. During the DNA repair synthesis step of SDSA, these repeats can anneal to one another, producing *P*{*w*^{aLTR}}, which contains only a single LTR in the *white* intron. Expression of *white* in *P*{*w*^{aLTR}} approaches wild-type levels, generating a flat red eye color. Numbers of apricot- and red-eyed female progeny were totaled and assigned to the "complete SDSA or no excision" class. Female progeny with yellow eyes are primarily the result of repair events in which repair DNA synthesis during SDSA was incomplete or absent, al-

though a small number of flanking deletions are also recovered (ADAMS *et al.* 2003).

Molecular analysis of P-element-induced DSB repair events:

Individual repair events were isolated by crossing one yellow-eyed female from each vial to *FM7w* males. Genomic DNA was isolated from white-eyed male offspring of this cross according to GLOOR *et al.* (1993) and used for molecular analysis. Primers flanking *P{w^o}* were used to amplify DNA spanning the repair junction in PCR reactions containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 2.5 mM MgCl₂, 0.1% Triton X-100, 1.25 μM each primer, 250 μM dNTPs, 2 μl of genomic DNA prep, and Taq polymerase in a 20-μl volume. PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining, excised, and purified using Microcon columns (Millipore, Bedford, MA). DNA was sequenced and compared with the original *P{w^o}* sequence to determine the most likely mechanism of repair. For those repair events in which SDSA initiated, the lengths of repair synthesis tracts were determined as described previously (ADAMS *et al.* 2003).

Generation of *lig4* mutants: The *Drosophila EP(X)0385* insertion line *CG12176^{EP(X)0385} (w¹¹¹⁸ P{w^o+mc} EP{EP385})* was used in an excision screen to generate deletion mutants of *lig4*. Females homozygous for *EP(X)0385* were crossed to males of genotype *yw/y⁺ Y; Sb P{γ⁺, Δ2-3}99B/TM6*. Male progeny in which the EP element was mobilized had mosaic eyes. Single mosaic-eyed males were crossed to *FM7w* females and the resulting bar-eyed female progeny were screened for eye color. Only females with white eyes (putative deletion mutants), distinct from the pale-yellow eye color of the original *EP(X)0385* insertion line, were analyzed further. Individual females were crossed to *FM7w* males in vials. Bar-eyed female progeny and non-bar-eyed male progeny from each vial were interbred to obtain female progeny homozygous for the putative deletions. These females were screened by PCR, using primers -311 (5'-CAATAACTGTGGACCGAACC-3') and 2568a (5'-GATCC TGCAGCTCCTCCACG-3') to determine which possessed deletions of the *Lig4* gene.

Overall, 71 independent deletions were isolated; 24 of these had accompanying deletions into the adjacent *CG11164* open reading frame and were not analyzed further. The *lig4¹⁶⁹* deletion was chosen for further analysis. For the *P{w^o}* assay, the *lig4¹⁶⁹* allele was crossed onto a chromosome containing the *P{w^o}* element. PCR was used to verify the presence of the *lig4¹⁶⁹* mutation.

Assaying sensitivity to DNA-damaging agents: To test for IR sensitivity of *lig4* mutants, *lig4¹⁶⁹/FM7w* females were crossed to *lig4¹⁶⁹/Y* males. Females were allowed to lay eggs for 12 hr and the embryos underwent further development for 72–80 hr at 25°. The resultant third-instar larvae were irradiated with increasing doses of gamma rays, using a Gammator 50 irradiator with a dose rate of ~300 rad/min. Progeny were grown at 25° and scored after 10–12 days. Survival rates were measured by calculating the ratio of *lig4/lig4* to *lig4/FM7w* females or *lig4/Y* to *FM7w/Y* males. Relative survival rates were calculated by comparing the survival rates at each IR dose to that of the untreated control.

To test for methyl methanesulfonate (MMS) sensitivity, four *lig4¹⁶⁹/FM7w* females were crossed to two *lig4¹⁶⁹/Y* males in vials. After 3 days, parents were transferred to new vials and allowed to lay eggs for 2 days. These vials were aged for 1 day and 250 μl/vial of 0.05 or 0.075% freshly made MMS in ddH₂O was added. Survival rates were calculated relative to the untreated control vials. Each experiment consisted of 20 control vials and 20 experimental vials.

To ascertain the importance of *lig4* to DSB repair in the absence of SDSA, the IR experiments were repeated in a *spn-A* mutant background. Double-mutant flies were created by crossing *lig4¹⁶⁹/FM7; spn-A⁵⁷/TM3* females to *lig4¹⁶⁹/Y; spn-A⁹³/*

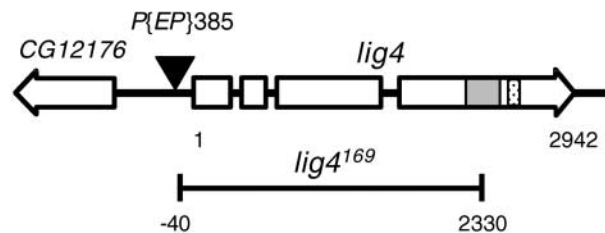


FIGURE 1.—The *Lig4* region and the *lig4¹⁶⁹* deletion. The four coding exons for LIG4 are shown as boxes; the shaded box represents the BRCT motif, and the stippled box corresponds to the region responsible for binding CG3448, the putative *Drosophila* XRC4 homolog. Transcription of the adjacent *CG12176* locus occurs in the opposite direction of *Lig4*. The *P{EP}385* element used to create the *lig4¹⁶⁹* deletion is located 40 bp upstream of *Lig4*. The *lig4¹⁶⁹* deletion extends from -40 to +2330 (+1 corresponds to the translation start site).

TM3 males. Third-instar larvae from this cross were exposed to increasing doses of gamma rays. Relative survival rates of single and double mutants were calculated relative to the survival rate of *lig4/FM7 spn-A/TM3* heterozygous females at each dose.

RESULTS

***Drosophila lig4* mutants are viable and fertile:** The *Drosophila Lig4* gene (*CG12176*) maps to 12B2 on the X chromosome. We generated a deletion of *Lig4* through excision of the *EP385* P element, inserted 40 bp upstream of the start of the coding region of *Lig4* (Figure 1; see MATERIALS AND METHODS). Excision of *EP385* was assayed by scoring for white eyes. Of 410 white-eyed flies isolated, 71 were accompanied by deletion into flanking sequences. Of these stocks, 24 were female sterile, most likely owing to an accompanying deletion of part or all of the adjacent *CG11164* gene (data not shown). Of the remaining 47, 4 had deletions of at least three-quarters of the *Lig4* coding sequence. All 4 of these isolates displayed viability and fertility rates similar to wild type (data not shown), indicating that the *Drosophila* LIG4 protein is not essential.

We chose one of the isolates, *lig4¹⁶⁹*, with which to perform further phenotypic analysis. The *lig4¹⁶⁹* isolate is a deletion of 2368 bp, including most of the coding region (Figure 1). This deletion removes the entirety of the highly conserved ATPase and adenylation domains, along with most of the conserved BRCA carboxy-terminal-related (BRCT) DNA-binding domain (GORSKI *et al.* 2003). No potential in-frame ATG start sites exist within the remaining *lig4¹⁶⁹* coding sequence. We therefore conclude that we have constructed a null allele of *lig4*.

LIG4 is not essential for double-strand break repair in *Drosophila*: Ionizing radiation creates DSBs, in addition to other types of DNA damage. We tested whether late-stage *lig4* mutant larvae were sensitive to ionizing radiation. Third-instar larvae resulting from a cross between a *lig4/+* female and a *lig4* male were exposed to

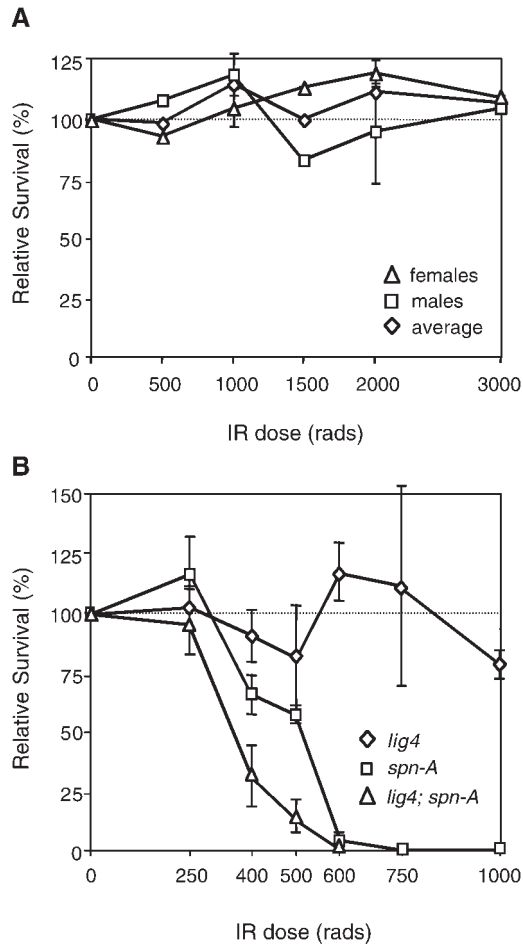


FIGURE 2.—*lig4¹⁶⁹* mutants are not sensitive to ionizing radiation. (A) Third-instar larvae from the cross *lig4¹⁶⁹/+* × *lig4¹⁶⁹/Y* were irradiated with indicated doses of gamma rays, and survival rates of *lig4¹⁶⁹/lig4¹⁶⁹* homozygous to *lig4¹⁶⁹/+* heterozygous female progeny, or *lig4¹⁶⁹/Y* to *+/Y* male progeny, were calculated. Survival rates are given relative to the ratios observed for the untreated controls. (Δ) females, (□) males, (◇) average. (B) Mutation of *lig4* and *spn-A* results in synergistic sensitivity to intermediate doses of X rays. Double-mutant flies were created by crossing *lig4¹⁶⁹/FM7; spn-A⁵⁷/TM3* females to *lig4¹⁶⁹/Y; spn-A⁹³/TM3* males. Third-instar larvae were exposed to indicated doses of gamma rays and relative survival rates were calculated relative to the survival rate of *lig4 spn-A* heterozygous females at each dose. (◇) *lig4*, (□) *spn-A*, (Δ) *lig4 spn-A*. Heightened sensitivity was observed in the double mutant at doses between 400 and 500 rad.

varying doses of gamma radiation, and the number of surviving *lig4* homozygotes was compared to the number of *lig4/+* heterozygotes. Neither female *lig4* homozygotes nor male *lig4* hemizygotes showed hypersensitivity to IR (Figure 2A). We conclude that, at late stages of larval development, repair of DSBs created by IR occurs mainly by a LIG4-independent mechanism. This conclusion is supported by data recently published by GORSKI *et al.* (2003). Similarly, *lig4* homozygous mutants were not hypersensitive to the alkylating agent MMS. At a dose of 0.075% MMS, 81% of the number of expected

lig4 mutants survived, and at a high dose of 0.1% MMS, 76% of the expected *lig4* mutants survived (data not shown).

In *S. cerevisiae*, loss of Dnl4p causes sensitivity to IR only when homologous recombination is impaired by mutation of *RAD52* (TEO and JACKSON 1997). To determine whether this is the case in *Drosophila*, we conducted IR sensitivity tests in *lig4 spn-A* mutants. These flies lack the DmRad51 protein and are unable to initiate DSB repair by HR (MCVEY *et al.* 2004). In the absence of IR, we recovered expected Mendelian ratios of both male and female *lig4¹⁶⁹ spn-A⁵⁷/spn-A⁹³* mutants (data not shown; $P > 0.1$ by chi-square test). Therefore, LIG4 is not required for viability in the absence of DmRad51.

When we exposed third-instar larvae to varying doses of IR, we observed a small but reproducible difference in the expected survival rate of the *lig4 spn-A* mutant relative to *spn-A* (Figure 2B). This difference could be observed only at doses of 400–500 rad, since higher doses were lethal to the *spn-A* single mutants. We conclude that most IR-induced DSBs are repaired by HR pathways. In the absence of homologous recombination, both LIG4-dependent and, to a greater extent, LIG4-independent end-joining mechanisms are used to fix DSBs.

DSB repair pathways are not affected by loss of LIG4:

Because IR and MMS can cause a variety of DNA damage, we wanted to more explicitly determine whether *lig4* mutants are fully competent to repair DSBs. To do this, we employed a recently described assay, in which a $P\{w^0\}$ transposable element is excised to create a double-strand break at a predetermined site (ADAMS *et al.* 2003). We have previously demonstrated that repair of this DSB occurs primarily by SDSA, using the sister chromatid as a template (ADAMS *et al.* 2003). Relative to the sister chromatid, the DSB is actually a 14-kb gap. Individual repair events that occur in premeiotic germline cells of males are recovered by crossing to females homozygous for $P\{w^0\}$.

SDSA involving complete resynthesis of $P\{w^0\}$ results in progeny with apricot eye color. However, most progeny with apricot eyes are probably derived from cells in which $P\{w^0\}$ did not excise. Red eye color is diagnostic of repair by SDSA; the repair product is likely formed by repair synthesis from both ends of the gap and annealing at 276-bp internal repeats (for full details, see MATERIALS AND METHODS). In some cases, SDSA aborts before the entire P element is copied back into the break site, and repair is completed through an unknown end-joining pathway, resulting in an internally deleted P element (incomplete SDSA). Progeny inheriting these incomplete SDSA repair events have yellow eyes, because they inherit one complete copy of $P\{w^0\}$ from their mothers.

To test whether incomplete SDSA requires LIG4, we executed the $P\{w^0\}$ excision assay in wild-type and *lig4*

TABLE 1
Repair of *P*-element-induced DSBs in *lig4*, *spn-A*, and *lig4 spn-A* mutants

Genotype of males	% complete SDSA or no excision			% end joining	N
	Apricot ^a	Red ^b	Total		
Wild type	93.6	4.3	97.9	2.1	1187
<i>lig4</i> (mothers <i>lig4</i> /+)	94.9	2.9	97.8	2.2	3388
<i>lig4</i> (mothers <i>lig4</i> / <i>lig4</i>)	93.1	3.1	96.2	3.8	883
<i>spn-A</i>	79.9	0	79.9	20.1	9502
<i>lig4 spn-A</i>	78.1	0	78.1	21.9	6228

^a Most apricot eyes result from inheritance of *P*{*w*^o} that has not been excised.

^b Red eyes result from SDSA repair with annealing of 276-bp repeated sequences within *P*{*w*^o}.

mutant males (Table 1). In the wild-type background, ~2.1% of the progeny had yellow eyes, indicating some type of end joining had occurred. When using the third chromosome transposase source, we estimate that ~14% of total progeny inherit the product of a repair event (ADAMS *et al.* 2003). Therefore, ~15% (2.1/14) of all repair events in wild-type flies involve end joining. If *lig4* mutant flies were defective in end joining, we would expect the number of yellow-eyed flies to decrease. However, we found that 2.2% of the progeny (16% of all repair events) derived from *lig4* males had yellow eyes, similar to the wild-type frequency.

We considered the possibility that maternal supplies could provide sufficient LIG4 to conduct end joining in these males. To test this, we repeated the assay using males whose mothers were homozygous for *lig4*¹⁶⁹. Interestingly, 3.8% of the progeny had yellow eyes, corresponding to ~27% of repair events. This slightly elevated frequency of incomplete SDSA repair events in males completely lacking LIG4 could be explained by the premeiotic nature of the repair events. However, 16 of 43 vials containing a single wild-type male produced at least one yellow-eyed fly, while 19 of 25 vials with an individual *lig4* mutant male gave rise to one or more yellow-eyed progeny. Therefore, *lig4* mutant males are not impaired in their ability to repair *P*-element-induced double-strand breaks through incomplete SDSA.

The above results show that *lig4* mutants are not impaired in their ability to do end joining after some SDSA synthesis. To ensure that loss of LIG4 does not affect strand invasion or SDSA synthesis, we quantified the lengths of synthesis tracts in the incomplete SDSA repair products. Yellow-eyed female progeny of *lig4* mutant males were crossed individually to *FM7w* males, and genomic DNA was isolated from their sons and analyzed in PCR reactions using primers internal to *P*{*w*^o}. Of 37 that were analyzed, 25 (68%) had synthesis of at least 2.4 kb from the right end of *P*{*w*^o} and 9 (24%) had synthesis of at least 4.6 kb. These percentages are quite similar to the values of 69 and 20% that we reported previously for incomplete SDSA events from wild-type males (ADAMS *et al.* 2003). In addition, we did not ob-

serve any defect in the ability of *lig4* mutants to complete SDSA repair, as assayed by the number of progeny with red eyes. We conclude that both complete SDSA, involving annealing of complementary sequences prior to ligation, and incomplete SDSA, involving end joining and ligation, proceed normally in the absence of LIG4.

End joining of DSBs resulting from *P*-element excision in the absence of DmRad51 occurs through a LIG4-independent pathway: In wild-type flies, almost all repair events recovered in the *P*{*w*^o} assay show evidence for some repair synthesis. This was also true in *lig4* mutants. Repair synthesis could potentially result in a structure that is refractory to LIG4-dependent NHEJ. For example, long single-strand DNA generated by SDSA synthesis or proteins involved in strand invasion and synthesis may prevent LIG4 or other NHEJ proteins from binding. To investigate this possibility, we asked whether LIG4 is required to repair a DSB in an HR-deficient background.

The *spn-A* gene encodes the Drosophila Rad51 ortholog, DmRad51 (STAEVA-VIEIRA *et al.* 2003). We have shown that all DSBs resulting from excision of *P*{*w*^o} in a *spn-A* mutant background are repaired by end joining, with little or no repair DNA synthesis (MCVEY *et al.* 2004). Because *spn-A* is very near the chromosome 3 transposase source, we used a source of transposase on chromosome 2. This source appears to have higher transposase activity; we estimate that when this transposase is used, ~20% of the recovered progeny are derived from premeiotic stem cells that have undergone excision and repair.

We previously recovered *P*{*w*^o} excision repair products from *spn-A* mutant males (MCVEY *et al.* 2004). We observed a large increase in the percentage of progeny with yellow eyes, corresponding to a decrease in the number of progeny with apricot or red eyes (Table 1). The results of PCR using primers flanking the *P*{*w*^o} insertion site were consistent with repair by end joining without synthesis. In a few cases, we obtained a product where repair DNA synthesis appeared to have initiated from one side of the break. Because the amount of synthesis was always small, we hypothesized that residual

TABLE 2
Repair junctions from *spn-A* mutants

Left end ^a		Right end
accagacCATGATGAAATAACATA		accagac
tggtctg		ATACAATAAAGTAGTACtggtctg
Junctions with microhomology ^b		
accagac	<u>CATGATGA</u>	accagac
accagacCATGATGAAATAAC	<u>AT</u>	GTTATTTTCATCATGaccagac
accagacCATGATGAAATAACA	<u>TA</u>	TGTTATTTTCATCATGaccagac
accagacCATGATGAAATAA	<u>CA</u>	CCTTATGTTATTTTCATCATGaccagac
accagacCATG	<u>ATG</u>	TTATTTTCATCATGaccagac
accagacCATGATGAAATAA	<u>CAT</u>	Gaccagac
accagac	<u>CATGA</u>	accagac
—	<u>TGACCCAGAC</u>	TGaccagac
accagac...(165 nt)...AAATT	<u>TG</u>	TTATTTTCATCATGaccagac
accagac...(150 nt)...AAATGA	<u>A</u>	TGTTATTTTCATCATGaccagac
Junctions with T-nucleotides ^c		
accagacCATGATGAAATAACATA	[a]	CATCATGaccagac
accagacCATGATGA	[g]	CATCATGaccagac
accagacCA	[ag]	ATGaccagac
accagacCATGATGA	[ac]	CATCATGaccagac
accagacCATGATGAAATAACATA	[ac]	ATGTTATTTTCATCATGaccagac
accagacCATGATGAAATAACAT	[ttaacataac]	ATGTTATTTTCATCATGaccagac
accagacCATGATGAAATAA	[taataataataa]	TATGTTATTTTCATCATGaccagac
accagacCATGATGAAATAACAT	[gtattacataac]	ATGTTATTTTCATCATGaccagac
accagacCATGATGAAATA	[tcatgaaatcata]	TCATCATGaccagac

^a For the left and right ends, the lowercase represents the 8-bp genomic target site that is duplicated in the *P{w}* insertion; the *P*-element sequence is given in uppercase. The overhangs remaining after *P*-element excision are shown at the top of the table.

^b Underlined sequences in the center of the table represent the apparent microhomology between the left and right ends. The first sequence was observed eight times, the second three times, and the third twice. All other sequences were unique. The numbers in parentheses in the last two sequences under "Junctions with microhomology" indicate the length of sequence from repair synthesis.

^c Inserted nucleotides are listed in lowercase in the center. Possible templates and templated insertions are underlined.

maternal DmRad51 allowed for one cycle of strand invasion and synthesis, after which the ends were joined.

To determine whether the end-joining repair that occurs in the absence of DmRad51 requires LIG4, we repeated the assay using *lig4 spn-A* double-mutant males. The frequency of end-joining repair products in the double mutant was similar to that observed in the *spn-A* single mutant (Table 1). PCR across the insertion site gave results identical to those of *spn-A* single mutants (data not shown). Thus, flies lacking LIG4 are not impaired in their ability to repair DSBs by end joining.

Junctions created by end joining are similar in the presence or absence of LIG4: To gain insight into end-joining pathways used in *Drosophila*, we sequenced repair junctions from *spn-A* mutants and *lig4 spn-A* double mutants. Approximately two-thirds of the junctions from *spn-A* mutants are consistent with annealing at sites of 1- to 4-bp microhomologies within the 17-nt overhangs remaining after *P*-element excision (Table 2). The remaining repair products had filler DNA (small insertions at the junction site). The filler DNA sequences often contained short, imperfect repeats. In

many of these repair products, we were able to identify a possible template for the inserted nucleotides in nearby sequences. We therefore refer to these insertions as T-nucleotides (templated nucleotides). We previously reported the apparent use of microhomologies and the existence of T-nucleotides in incomplete SDSA products recovered from wild-type flies (ADAMS *et al.* 2003).

End-joining repair products from *lig4 spn-A* double mutants were similar in structure (Table 3). About two-thirds appeared to involve annealing at short microhomologies, and the remainder had filler DNA sequences, often with apparent T-nucleotides. We conclude that LIG4 is not required for end-joining repair of *P*-element-induced DSBs, regardless of whether DNA synthesis (by SDSA or addition of filler DNA) was involved.

DISCUSSION

LIG4 is dispensable for most DSB repair in flies: In many organisms, DNA ligase IV-mediated end joining plays a major role in the repair of DSBs. Human pre-B

TABLE 3
Repair junctions from *lig4 spn-A* mutants

Left end		Right end
accagacCATGATGAAATAACATA tgggtctg		accagac ATACAATAAAGTAGTACtgggtctg
Junctions with microhomology ^a		
accagacCATGATGAAATAAC	AT	GTTATTTTCATCATGaccagac
accagac	CATGATGA	cccagac
accagacCATGATGAAATAACA	TA	TGTTATTTTCATCATGaccagac
accagacCATGATGAAATAACA	T	TATTTTCATCATGaccagac
accagacCATGATGAAA	TA	TTTCATCATGaccagac
accagacCATGATGAAATAACA	T	CATCATGaccagac
accagac	CATGA	cccagac
accagac	CAT	CATGaccagac
—	GACCCAGAC	—
(7-nt deletion)	TGA	(43-nt deletion)
accagacCATGATGAAATAA	CATA	(56 nt)...accagac
accagacCATGATGAAATAAC	ATA	(173 nt) accagac
Junctions with T-nucleotides		
accagacCATGATGAAATAACATA	[taataaattatggtatg]	TTATGTTATTTTCATCATGaccagac
accagacCATGATGAAATAACATA	[cataacatacataacataacataac]	ATGTTATTTTCATCATGaccagac
accagacCATGATGAAATAACATA	[tgttattatgttattatggtattattataataac]	ATGTTATTTTCATCATGaccagac
accagacCATGATGAAATAACAT	[ttataa]	TATGTTATTTTCATCATGaccagac
accagacCATGATGAAATAACATA	[ttca]	TATTTTCATCATGaccagac
accagacCATGATGAAATAACAT	[ca]	TTATTTTCATCATGaccagac
accagacCATGATGAAATAA	[taaataa]	ATGTTATTTTCATCATGaccagac
acc	[gtagcacaccttacctatgctagactatagactat accataccataaccatgccataccatactata ccataccataccatcatcaccctg]	CATCATGaccagac
accagacCATGATGAAATAACAT	[taacatac]	ATAC-(82 nt)-accagac
accagacCATGATGAAATAACA	[ataaataacaataacaataaataaataa]	AACT-(204 nt)-accagac

See Table 2 legend for explanation.

^aThe first sequence was observed eight times, the second four times, and the third twice. All other sequences were unique.

cells deficient for *LIG4* display a >90% reduction in their ability to perform end joining (SMITH *et al.* 2003), and Chinese hamster ovary cells lacking XRCC4 are unable to repair DSBs that contain partially complementary overhangs (LEE *et al.* 2003). Ligase IV-deficient mice die *in utero* with massive neuronal apoptosis (FRANK *et al.* 2000). Arabidopsis *Atlig4* mutants are hypersensitive to ionizing radiation (VAN ATTIKUM *et al.* 2003).

In contrast, *S. cerevisiae dnl4* mutants are not IR sensitive, although loss of Dnl4p does increase sensitivity in a *rad52* background. This implies that yeast cells repair most DSBs primarily by HR and that NHEJ serves as a backup pathway or repairs only certain types of DSBs. Consistent with the second model, *dnl4* mutants are severely impaired in their ability to repair DSBs that have complementary overhanging ends, such as those created by restriction endonucleases.

We find that the *Drosophila lig4* mutant phenotype more closely resembles the phenotype of yeast *dnl4* mutants than that of its mammalian and plant counterparts. Flies lacking LIG4 are viable and fertile and are not hypersensitive to either MMS or IR. GORSKI *et al.* (2003) recently reported that *Drosophila lig4* mutant embryos and larvae that are treated with γ rays at 4–48 hr after

egg laying are sensitive to the effects of IR. Although we did not test γ -ray sensitivity of our mutants at these early time points, our findings that late-stage larvae (72–96 hr) are not sensitive to IR are in agreement with their published results. We did observe a mild enhancement of *spn-A* IR sensitivity by the *lig4* mutation, suggesting that some γ -ray-induced DSBs are repaired using the LIG4 protein when homologous recombination is absent. This interpretation is further supported by the increased IR sensitivity of a *lig4 okr* mutant, which lacks the *Drosophila* Rad54 ortholog (GORSKI *et al.* 2003).

An alternative repair mechanism for P-element-induced DSBs: Analysis of repair products from DSBs created by P-element excision revealed no obvious defect in *lig4* mutants. In wild-type flies, DSBs resulting from P excision are usually repaired by homologous recombination by SDSA (Figure 3, left). In the system employed in these studies, SDSA occasionally terminates before repair DNA synthesis can proceed along the length of *P*{*w*^o}. The reason(s) for this early termination are presently not understood. One model is that repair DNA synthesis is nonprocessive and that synthesis of the entire *P*{*w*^o} element requires multiple rounds of strand invasion and priming of DNA synthesis (McVEY *et al.*

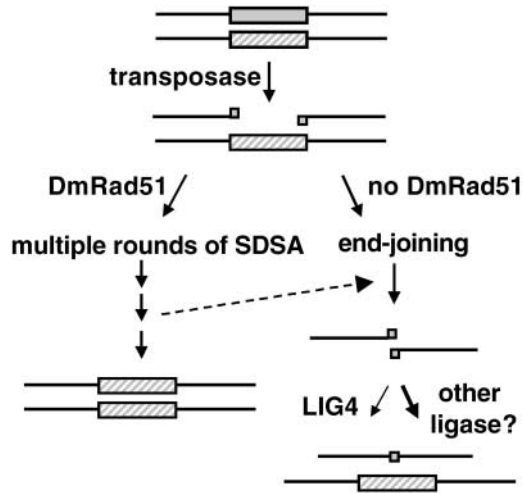


FIGURE 3.—Model for repair of *P*-element-induced DSBs in *Drosophila*. Transposase cuts at both ends of the *P* element, creating a double-strand gap with 3' overhanging ends that can invade into the unbroken sister chromatid (shaded) and initiate homologous repair by SDSA. Occasionally, for unknown reasons, SDSA aborts and repair of the broken chromosome occurs by end joining (dashed line). In the absence of the strand-exchange protein DmRad51, SDSA cannot initiate and end joining proceeds by alignment of small microhomologies, presumably followed by trimming of overhanging ends, filling in of gaps, and ligation. The majority of this end-joining repair occurs via a LIG4-independent pathway.

2004). At each successive round of strand invasion, changing cellular conditions or activation of checkpoint pathways may increase the probability that an alternative repair pathway is used.

In the instances of incomplete SDSA that we observed, repair appeared to proceed through an end-joining pathway in which the DNA ends were aligned at short microhomologies. Presumably, repair of the break was completed by trimming of ends, filling of gaps, and ligation. Our results demonstrate that LIG4 is not required for the ligation step. Surprisingly, we found that the *Lig4* gene is also not required for end joining in *spn-A* mutants, in which the initiation of SDSA is prevented (Figure 3, right). The DSB created by *P*{*w*^o} excision possesses 17 nucleotide noncomplementary 3' overhangs (BEALL and RIO 1997). After initial excision, it is likely that resection of the 5' strand occurs, as we observed several repair junctions that involved joining at microhomologies outside of the 17-nt overhangs. We hypothesize that structures remaining after *P*-element excision and initial processing are not preferred substrates for canonical NHEJ, and repair must therefore proceed by an alternative end-joining pathway. This pathway is likely the same one used during incomplete SDSA, as repair junction sequences of incomplete SDSA products from both wild-type and *lig4* mutants are similar (M. McVEY and J. J. SEKELSKY, unpublished data).

Our observation that DSBs can be efficiently repaired in the absence of LIG4 implies that a different ligase

must be involved in the final joining reaction. In this study, the nature of the breaks generated by *P* excision may result in repair substrates with single-strand nicks that are sufficiently displaced for LIG1 to recognize and repair them independently. In flies, homologs for mammalian LIG1 and LIG3 have been identified (FLY-BASE 2001). Currently, no mutations exist in either of these genes. Null mutants would presumably be inviable; however, the generation of hypomorphic alleles may allow testing of this hypothesis.

Considerable evidence exists for LIG4/XRCC4-independent end-joining repair in mammalian cells (KABO-TYANSKI *et al.* 1998; VERKAIK *et al.* 2002). Experiments done in a human pre-B cell line also provided evidence for residual rejoining of broken plasmids in a *LIG4* null background (SMITH *et al.* 2003). Interestingly, end-joining products similar to those observed in the LIG4-independent studies have been observed in the absence of the Ku heterodimer (WANG *et al.* 2003). It will be important to determine whether the LIG4-independent end joining that we observe is also independent of the Ku heterodimer. At present, no *Drosophila* stocks exist with mutations in the Ku70 or Ku80 genes.

In yeast, a Ku-independent end-joining pathway operates at DSBs that lack complementary end sequences (BOULTON and JACKSON 1996; MA *et al.* 2003; YU and GABRIEL 2003). This pathway, MMEJ, proceeds by annealing at 8- to 10-bp imperfect microhomologous sequences. MMEJ requires Mre11p, Rad50p, and Rad1p, but is only partially dependent upon Dnl4p. Repair by MMEJ is often accompanied by substantial deletions of up to 300 bp on either side of the break (MA *et al.* 2003; YU and GABRIEL 2003). Using the *P*{*w*^o} assay, the vast majority of repair products recovered from the *lig4 spn-A* mutants did not have flanking deletions outside of *P*{*w*^o}. A possible explanation for this might be that mutation of *spn-A* impairs HR, thereby preventing further resection of the broken strands. However, repair products isolated from yeast strains lacking both Ku and Rad52p still possessed deletions (although the deletions were smaller), suggesting that impairment of HR does not affect deletion formation in MMEJ (MA *et al.* 2003). Thus, an alternative hypothesis is that the LIG4-independent end-joining pathway that operates after *P*-element excision is distinct from MMEJ characterized in yeast.

LIG4-independent joining and T-nucleotides: Approximately one-third of repair products from *spn-A* single mutants and *lig4 spn-A* double mutants involved the insertion of filler DNA. The average number of inserted nucleotides may be larger in the double mutants, but our sample size was too small to determine whether this increase is significant. T-nucleotides were often apparent in the filler DNA. T-nucleotides have also been observed at the site of chromosomal translocations in follicular lymphomas (JAGER *et al.* 2000) and promyelocytic leukemias (YOSHIDA *et al.* 1995). There-

fore, it is of general interest to determine the molecular mechanisms that create these insertions.

Templates for the T-nucleotides that we observed could often be identified in adjacent sequences, although the insertions frequently contained repeats and mismatches relative to the templated sequence. This suggests the involvement of short-patch DNA synthesis involving an error-prone polymerase. We speculate that T-nucleotides are generated by the action of an error-prone polymerase in an attempt to create microhomologies that can then be used in end joining. In many organisms, members of the Pol X family of error-prone DNA polymerases, including mammalian Pol μ and *S. cerevisiae* Pol4p, are important for canonical end joining (WILSON and LIEBER 1999; MAHAJAN *et al.* 2002; TSENG and TOMKINSON 2002). DNA ligase IV associates with Pol μ in mammals (MAHAJAN *et al.* 2002), and yeast Pol4p interacts with Dnl4p-Lif1p (TSENG and TOMKINSON 2002). However, extensive homology searches have failed to identify *Drosophila* homologs for any of the family X DNA polymerases (SEKELSKY *et al.* 2000). Therefore, it will be interesting to determine which polymerase(s) might be involved in the generation of T-nucleotides in flies.

We conclude that the canonical ligase IV-dependent end-joining pathway plays only a minor role in DSB repair in *Drosophila*. Instead, an alternative, error-prone end-joining mechanism may take over whenever repair by HR is compromised or impractical. Currently, there is scant information concerning proteins that operate in this alternative end-joining mechanism. The use of the $P\{w^{\rho}\}$ assay to test candidate genes and in screens for components of this alternate end-joining pathway may provide further insight into DSB repair in *Drosophila* and could prove useful in identifying error-prone DNA repair pathways that are responsible for genomic instability and cancer in humans.

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